Viral infection-related gene upregulation in monocytes in children with signs of β-cell autoimmunity

Milla Valta, Masahito Yoshihara, Elisabet Einarsdottir, Sirpa Pahkuri, Sini Ezer, Shintaro Katayama, Mikael Knip, Riitta Veijola, Jorma Toppari, Jorma Ilonen, Juha Kere, Johanna Lempainen

1Immunogenetics Laboratory, Institute of Biomedicine, University of Turku, Turku, Finland
2Department of Biosciences and Nutrition, Karolinska Institutet, Huddinge, Sweden
3Science for Life Laboratory, Department of Gene Technology, KTH-Royal Institute of Technology, Solna, Sweden
4Stem Cells and Metabolism Research Program, University of Helsinki, and Folkhälsan Research Center, Helsinki, Finland
5Pediatric Research Center, Children's Hospital, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
6Research Program for Clinical and Molecular Metabolism, Faculty of Medicine, University of Helsinki, Helsinki, Finland
7Folkhälsan Research Center, Helsinki, Finland
8Department of Pediatrics, Tampere University Hospital, Tampere, Finland
9Department of Pediatrics, PEDEGO Research Unit, MRC Oulu, Oulu University Hospital and University of Oulu, Oulu, Finland
10Institute of Biomedicine, Research Centre for Integrative Physiology and Pharmacology, University of Turku, Turku, Finland
11Department of Pediatrics, University of Turku and Turku University Hospital, Turku, Finland
12Clinical Microbiology, Turku University Hospital, Turku, Finland

Abstract

Objective: The pathogenesis of type 1 diabetes (T1D) is associated with genetic predisposition and immunological changes during presymptomatic disease. Differences in immune cell subset numbers and phenotypes between T1D patients and healthy controls have been described; however, the role and function of these changes in the pathogenesis is still unclear. Here we aimed to analyze the transcriptomic landscapes of peripheral blood mononuclear cells (PBMCs) during presymptomatic disease.

Methods: Transcriptomic differences in PBMCs were compared between cases positive for islet autoantibodies and autoantibody negative controls (9 case–control pairs) and further in monocytes and lymphocytes separately in autoantibody positive subjects and control subjects (25 case–control pairs).

Results: No significant differential expression was found in either data set. However, when gene set enrichment analysis was performed, the gene sets “defense response to virus” (FDR <0.001, ranking 2), “response to virus” (FDR <0.001, ranking 3) and “response to type I interferon” (FDR = 0.002, ranking 12) were enriched in the upregulated genes among PBMCs in cases. Upon further analysis, this was also seen
in monocytes in cases (FDR = 0.01, ranking 2; FDR = 0.04, ranking 3 and FDR = 0.02, ranking 1, respectively) but not in lymphocytes.

**Conclusion:** Gene set enrichment analysis of children with T1D-associated autoimmunity revealed changes in pathways relevant for virus infection in PBMCs, particularly in monocytes. Virus infections have been repeatedly implicated in the pathogenesis of T1D. These results support the viral hypothesis by suggesting altered immune activation of viral immune pathways in monocytes during diabetes.

**KEYWORDS**
- monocytes
- type 1 diabetes
- viral response
- β-cell autoimmunity

1 | INTRODUCTION

In type 1 diabetes (T1D), functional pancreatic β-cells are lost due to an autoimmune reaction. The destruction of β-cells seems to happen in a T cell-mediated manner after self-antigen presentation, but several immune cell populations within both the adaptive and innate compartments are thought to take part in the process.

Activated cytotoxic CD8^+^ T cells and macrophages are the major contributors to active insulitis, in which they infiltrate the pancreatic islets of Langerhans. While β-cell specific CD8^+^ cells are found at similar frequencies in the peripheral circulation of healthy donors and patients with T1D, they display markers of antigen-driven expansion in patients with newly diagnosed T1D. Additionally, the cytotoxic reactivity against islet autoantigens from human samples has been demonstrated.

Specific subsets of CD4^+^ T helper cells have long been known to contribute to the differentiation of B cells into antibody-secreting plasma cells. Since the most prominent genetic risk for T1D is mediated by the HLA locus, encoding for the class II MHC molecules, and as CD4^+^ T helper cells are also found in insulitis, CD4^+^ cells are an attractive candidate for facilitating the emergence of humoral immunity in T1D. A potential model for follicular and peripheral CD4^+^ T helper cell involvement was recently suggested.

In addition, CD4^+^ T helper cells have been shown to play a critical role in autoreactive CD8^+^ T cell maintenance.

B cell derived plasma cells produce β-cell specific autoantibodies which to date are the most important biomarkers of islet autoimmunity before clinical diagnosis of diabetes. Monocytes are precursors to both macrophages and myeloid dendritic cells and have a role in antigen trafficking and presentation. Their subpopulation compartment sizes have been observed to be altered in T1D patients and the cytokine milieu of monocyte populations has been reported to favor more proinflammatory phenotypes.

Despite these discoveries, the exact mechanism underlying T1D development remains largely unknown and heterogeneity in disease pathogenesis is strongly suspected. In this study, to infer biological events during T1D development, we set out to analyze transcriptional differences in peripheral blood mononuclear cells (PBMCs) among subjects with HLA-conferred risk for childhood T1D and signs of advanced β-cell autoimmunity and autoantibody negative control subjects.

2 | MATERIALS AND METHODS

2.1 | Study subjects

The study subjects were participants in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study and carried HLA class II genotypes associated with an increased risk for the development of T1D.

Subjects with a disease-predispensing HLA genotype were invited to a prospective follow-up for signs of β-cell autoimmunity and dysglycemia. At study visits, the participants were screened for signs of humoral β-cell autoimmunity: during the early study, for islet cell antibodies (ICA), and if ICA were detected, for biochemical autoantibodies including insulin autoantibodies (IAA), antibodies against the 65 kD isofom of GAD (GADA), and antibodies against the protein tyrosine phosphatase-related IA-2 molecule (IA-2A), from all available samples, including those obtained before seroconversion to ICA positivity. At later stages of the study, all participating children were screened for all four antibodies in samples collected during visits. Diabetes was diagnosed according to WHO criteria. The study protocol was approved by the local ethical committees and an informed consent was obtained from the guardians of the study participants.

The current analysis consists of two parts: a pilot cohort with nine case–control pairs and a confirmation cohort with 25 case–control pairs (Table S1). In the pilot cohort, all case subjects were positive for ICA and at least one biochemical autoantibody (IAA, GADA and/or IA-2A) at the time of sample collection and had developed T1D during later follow-up. The PBMC-samples were stored frozen after sample collection. The first nine subjects for whom such a sample and a healthy control, matched for age at sampling, gender, HLA-DR/DQ genotype and length of freezing time, were available, were selected in the cohort.

The confirmation cohort comprised 25 case–control pairs. The case subjects tested positive for at least two of the autoantibodies except for five cases having one biochemical autoantibody and ICA. The controls were matched for age at sampling, gender, HLA-DR/DQ genotype and date of sample collection. In the confirmation cohort, fresh samples were used for cell separation and criteria meeting subjects and controls were selected among children taking part in regular follow-up visits.
2.2 | Autoantibody analysis and HLA genotyping

The analysis of the major HLA-DR-DQ haplotypes conferring T1D risk was performed using sequence-specific oligonucleotide probes as described earlier.\textsuperscript{17} The protocol for determining ICA, IAA, GADA and IA-2A in the DiPPI study has been described previously.\textsuperscript{14,18}

2.3 | PBMC isolation and sample handling

PBMCs were collected from lithium heparin blood using Ficoll-Paque Plus density gradient centrifugation and resuspended in RPMI 1640 medium. In the pilot cohort, the cells were stored frozen at $-150^\circ$C (cryopreserved in 10% DMSO) until analysis. Before RNA isolation, the cells were thawed and lysed in Buffer RLT Plus (Qiagen, Hilden, Germany).

2.4 | PBMC fractionation

Samples from the confirmation cohort were fractionated and lysed fresh and immediately after PBMC isolation fractionated into monocytes and the remaining PBMC fraction with EasySep Human CD14 positive selection kit II (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. The purity of the monocyte and remaining lymphocyte fractions was confirmed by flow cytometry (Table S2). Both cell fractions and unfractonation PBMC were immunostained with anti-CD3 PE (SK7, BD Biosciences, San Jose, CA, USA) and anti-CD19 APC (SJ25C1, BD Biosciences) for 30 min at $+4^\circ$C. The PBMC and remaining fraction were furthermore stained with anti-CD14 FITC (M5E2, BD Biosciences) for 30 min at $+4^\circ$C to assess the initial and remaining amounts of monocytes in the sample. After immunostaining the cells were washed twice with phosphate buffered saline (PBS) for 5 min at 2500 rpm with Sorvall MC 12 V (Thermo Fischer Scientific, USA). The cells were fixed with 0.1% formaldehyde in PBS. The samples were analyzed using an Accuri C6 flow cytometer (BD Biosciences). Fractionated cells were lysed in Buffer RLT Plus (Qiagen) and stored at $-80^\circ$C prior to RNA extraction.

2.5 | RNA isolation

RNA was extracted from the PBMCs using the RNaseasy Plus Mini Kit (Qiagen) in the pilot cohort and RNeasy Plus Micro Kit (Qiagen) in the confirmation cohort according to the manufacturer's instructions. RNA quality and quantity in these cohorts were assessed using the Agilent RNA 6000 Nano Kit (Agilent, Santa Clara, CA, USA) and Agilent RNA 6000 Pico Kit (Agilent), respectively, on a 2100 Bioanalyzer (Agilent). RNA integrity number (RIN) $\geq 8$ was used as RNA-quality cut-off for inclusion.

2.6 | RNA library preparation and sequencing

RNA libraries for the pilot cohort were made using a modified version of the single-cell tagged reverse transcription (STRT) method,\textsuperscript{19} described in detail in Reference 20 to prepare a 48-plex Illumina-compatible sequencing library from 10 ng of each RNA sample. Briefly, RNA samples were placed in a 48-well plate in which a universal primer, template-switching oligos, and a well-specific 6-bp barcode sequence (for sample identification) were added to each well.\textsuperscript{20,21} The synthesized cDNAs from the samples were then pooled into one library and amplified by single-primer PCR with the universal primer sequence. The library was sequenced on three Illumina HiSeq2000 (Illumina, San Diego, CA, USA) lanes, using the Illumina TruSeq v3 60-80 bp single-read protocol. Sequencing was performed at the Bioinformatics and Expression Analysis (BEA) core facility at Karolinska Institutet (Huddinge, Sweden).

RNA libraries for the confirmation cohort were made using 20 ng RNA as starting input and the libraries were sequenced on an Illumina NextSeq 500, High Output (75 cycles). Sequencing was done at Biomedicum Functional Genomics Unit (FuGU), University of Helsinki, Finland.

2.7 | Sequencing data analysis

For the pilot cohort, sequence data was converted to fastq files using Casava 1.8.2 (Illumina), and processed using the STRTPrep pipeline available at https://github.com/shka/STRTprep (also described in Reference 20).

For the confirmation cohort sequence data was processed as described previously.\textsuperscript{22} Briefly, raw base call (BCL) files were demultiplexed and converted to FASTQ files using Picard tools (v2.10.0; http://broadinstitute.github.io/picard/), and aligned to the human reference genome hg19, human ribosomal DNA unit (GenBank: U13369), and ERCC spike-ins (SRM 2374) with the GENCODE (v28) transcript annotation by HISAT2 (v2.1.0).\textsuperscript{23} The uniquely mapped reads within the 5'-UTR or 500 bp upstream of the protein-coding genes were counted using Subread featureCounts (v1.6.2).\textsuperscript{24}

After quality check, three controls and two cases were excluded from the PBMC dataset, and one control and one case were excluded from the lymphocyte dataset.

In all three datasets, differential expression analysis between the controls and cases was performed using the R (v3.6.2) package DESeq2 (v1.24.0),\textsuperscript{25} where gender was considered as a covariate. Gene set enrichment analysis (GSEA) was performed using GSEA (v4.0.3) using the GSEAPreranked tool,\textsuperscript{26} where genes were preranked based on their p-values and fold changes.

3 | RESULTS

To characterize the profiles of RNA expression in immune cell subsets in children with advanced autoimmunity and compare those to that of matched controls, RNA sequencing was performed from the whole PBMC compartment (pilot cohort) and later from monocytes and lymphocytes separately (confirmation cohort).

In the pilot cohort, case subjects with advanced $\beta$-cell autoimmunity that developed into T1D during later follow-up were compared
| Rank | Pilot Name | Size | ES  | NES  | NOM p-val | FDR q-val | Monocyte Name | Size | ES  | NES  | NOM p-val | FDR q-val | Lymphocyte Name | Size | ES  | NES  | NOM p-val | FDR q-val |
|------|------------|------|-----|------|-----------|-----------|---------------|------|-----|------|-----------|-----------|-----------------|------|-----|------|-----------|-----------|
| 1    | Regulation of response to biotic stimulus | 92   | 0.29| 3.22 | 0.00E+00 | 0.00E+00 | Response to type I interferon | 71   | 0.28| 2.88 | 0.00      | 0.02      | Electron transport chain | 145  | 0.29| 4.10 | 0.00      | 0.00E+00 |
| 2    | Defense response to virus | 162  | 0.21| 3.10 | 0.00E+00 | 0.00E+00 | Defense response to virus | 178  | 0.18| 2.81 | 0.00      | 0.01      | Cellular respiration | 162  | 0.28| 4.04 | 0.00      | 0.00E+00 |
| 3    | Response to virus | 215  | 0.19| 3.10 | 0.00E+00 | 0.00E+00 | Response to virus | 240  | 0.15| 2.60 | 0.00      | 0.04      | Small molecule catabolic process | 293  | 0.21| 4.01 | 0.00      | 0.00E+00 |
| 4    | Response to molecule of bacterial origin | 197  | 0.19| 3.09 | 0.00E+00 | 0.00E+00 | Vesicle organization | 250  | 0.14| 2.57 | 0.00      | 0.04      | Cofactor metabolic process | 351  | 0.18| 3.98 | 0.00      | 0.00E+00 |
| 5    | Positive regulation of defense response to virus by host | 20   | 0.57| 3.04 | 2.44E-04 | 0.00E+00 | Interferon gamma mediated signaling pathway | 71   | 0.26| 2.55 | 0.00      | 0.04      | Generation of precursor metabolites and energy | 389  | 0.17| 3.82 | 0.00      | 0.00E+00 |
| 6    | Defense response to other organism | 273  | 0.16| 2.99 | 2.04E-04 | 0.00E+00 | Response to interferon gamma | 141  | 0.18| 2.50 | 0.00      | 0.05      | Mitochondrial translational termination | 88   | 0.33| 3.70 | 0.00      | 0.00E+00 |
| 7    | Response to bacterium | 309  | 0.15| 2.97 | 5.30E-04 | 0.00E+00 | Ribonucleoprotein complex biogenesis | 402  | 0.11| 2.46 | 0.00      | 0.06      | ATP synthesis coupled electron transport | 79   | 0.36| 3.69 | 0.00      | 0.00E+00 |
| 8    | Lipopolysaccharide mediated signaling pathway | 39   | 0.38| 2.83 | 1.67E-03 | 0.00E+00 | Regulation of gene silencing | 111  | 0.19| 2.38 | 0.00      | 0.09      | Mitochondrial translation | 132  | 0.27| 3.68 | 0.00      | 0.00E+00 |
| 9    | Positive regulation of cytokine production | 282  | 0.15| 2.81 | 1.76E-03 | 0.00E+00 | Ribosome biogenesis | 261  | 0.12| 2.33 | 0.00      | 0.11      | Respiratory electron transport chain | 95   | 0.32| 3.65 | 0.00      | 0.00E+00 |
| 10   | Positive regulation of DNA binding transcription factor activity | 152  | 0.19| 2.78 | 2.08E-03 | 0.00E+00 | De novo protein folding | 35   | 0.32| 2.33 | 0.00      | 0.10      | Anaphase promoting complex dependent catabolic process | 78   | 0.35| 3.63 | 0.00      | 0.00E+00 |
| 11   | Negative regulation of viral genome replication | 38   | 0.39| 2.78 | 2.00E-03 | 0.00E+00 | Organic cyclic compound catabolic process | 465  | 0.09| 2.32 | 0.00      | 0.10      | Cellular amino acid metabolic process | 218  | 0.21| 3.59 | 0.00      | 0.00E+00 |
| 12   | Response to type I interferon | 64   | 0.29| 2.76 | 1.94E-03 | 0.00E+00 | ncRNA metabolic process | 403  | 0.10| 2.31 | 0.00      | 0.10      | Mitochondrial respiratory chain complex assembly | 89   | 0.33| 3.59 | 0.00      | 0.00E+00 |
| 13   | Cytokine production | 450  | 0.12| 2.71 | 3.02E-03 | 0.00E+00 | Multi organism localization | 67   | 0.24| 2.29 | 0.00      | 0.10      | Oxidative phosphorylation | 114  | 0.28| 3.50 | 0.00      | 0.00E+00 |
| 14   | Regulation of defense response to virus by host | 29   | 0.42| 2.68 | 4.12E-03 | 0.00E+00 | Response to interferon alpha | 17   | 0.45| 2.26 | 0.00      | 0.11      | Aerobic respiration | 75   | 0.34| 3.48 | 0.00      | 0.00E+00 |
| 15   | Response to interferon beta | 23   | 0.45| 2.68 | 3.84E-03 | 0.00E+00 | Organelle localization | 477  | 0.09| 2.24 | 0.00      | 0.13      | NADH dehydrogenase complex assembly | 57   | 0.38| 3.40 | 0.00      | 0.00E+00 |
| Rank | Pilot Name | Size (ES) | NES | NOM p-val | FDR q-val | Monocyte Name | Size (ES) | NES | NOM p-val | FDR q-val | Lymphocyte Name | Size (ES) | NES | NOM p-val | FDR q-val |
|------|------------|-----------|-----|-----------|------------|---------------|-----------|-----|-----------|------------|----------------|-----------|-----|-----------|------------|
| 16   | Leukocyte cell adhesion | 218 (0.16) | 2.68 | 0.00 | 3.60E-03 | Vesicle targeting | 82 (0.21) | 2.22 | 0.00 | 0.13 | Translational termination | 100 (0.29) | 3.40 | 0.00 | 0.00E+00 |
| 17   | Cellular response to biotic stimulus | 148 (0.18) | 2.66 | 0.00 | 4.04E-03 | ncRNA processing | 343 (0.11) | 2.21 | 0.00 | 0.13 | DNA dependent DNA replication | 134 (0.25) | 3.28 | 0.00 | 0.00E+00 |
| 18   | Cytokine mediated signaling pathway | 476 (0.11) | 2.62 | 0.00 | 5.59E-03 | tRNA transport | 35 (0.31) | 2.20 | 0.00 | 0.14 | Mitochondrial gene expression | 156 (0.23) | 3.24 | 0.00 | 0.00E+00 |
| 19   | Cell adhesion | 344 (0.12) | 2.58 | 0.00 | 7.55E-03 | COPII coated vesicle budding | 64 (0.24) | 2.20 | 0.00 | 0.13 | Energy derivation by oxidation of organic compounds | 219 (0.19) | 3.19 | 0.00 | 0.00E+00 |
| 20   | Regulation of defense response to virus | 55 (0.29) | 2.57 | 0.00 | 7.78E-03 | Nuclear transcribed MRNA catabolic process nonsense mediated decay | 114 (0.18) | 2.19 | 0.00 | 0.13 | Nucleobase containing small molecule metabolic process | 278 (0.16) | 3.15 | 0.00 | 0.00E+00 |
| 21   | Response to interferon gamma | 130 (0.19) | 2.54 | 0.00 | 8.98E-03 | ncRNA export from nucleus | 39 (0.30) | 2.19 | 0.00 | 0.12 | Antigen processing and presentation of exogenous peptide antigen via MHC class i | 75 (0.30) | 3.11 | 0.00 | 3.33E-05 |
| 22   | Inflammatory response | 370 (0.11) | 2.52 | 0.00 | 0.01 | rRNA metabolic process | 193 (0.13) | 2.14 | 0.01 | 0.16 | Organic acid catabolic process | 187 (0.19) | 3.05 | 0.00 | 9.43E-05 |
| 23   | Negative regulation of viral process | 65 (0.27) | 2.50 | 0.00 | 0.01 | Membrane fusion | 111 (0.17) | 2.13 | 0.00 | 0.16 | Regulation of cellular amino acid metabolic process | 53 (0.35) | 3.02 | 0.00 | 1.20E-04 |
| 24   | Regulation of multi organism process | 253 (0.13) | 2.50 | 0.00 | 0.01 | Regulation of nuclease activity | 20 (0.39) | 2.11 | 0.00 | 0.18 | Detoxification | 90 (0.27) | 2.99 | 0.00 | 1.44E-04 |
| 25   | Regulation of body fluid levels | 229 (0.14) | 2.47 | 0.00 | 0.01 | Endoplasmic reticulum to golgi vesicle mediated transport | 165 (0.14) | 2.11 | 0.00 | 0.17 | Mitochondrial electron transport NADH to ubiquinone | 44 (0.38) | 2.98 | 0.00 | 1.38E-04 |
| 26   | Regulation of cell adhesion | 227 (0.14) | 2.46 | 0.00 | 0.01 | Cotranslational protein targeting to membrane | 94 (0.18) | 2.09 | 0.00 | 0.20 | Nucleoside phosphate biosynthetic process | 216 (0.17) | 2.94 | 0.00 | 1.33E-04 |
| 27   | Negative regulation of multi organism process | 109 (0.20) | 2.45 | 0.00 | 0.02 | DNA recombination | 208 (0.12) | 2.09 | 0.00 | 0.19 | Regulation of cellular amine metabolic process | 66 (0.30) | 2.93 | 0.00 | 1.53E-04 |
| 28   | Adaptive immune response | 261 (0.13) | 2.43 | 0.00 | 0.02 | Apoptotic DNA fragmentation | 19 (0.39) | 2.06 | 0.01 | 0.22 | Drug metabolic process | 219 (0.17) | 2.91 | 0.00 | 1.48E-04 |
| 29   | Cellular response to interferon beta | 15 (0.51) | 2.40 | 0.00 | 0.02 | Glycosylation | 164 (0.14) | 2.06 | 0.00 | 0.21 | Cellular ketone metabolic process | 142 (0.21) | 2.88 | 0.00 | 1.67E-04 |

(Continues)
| Rank | Pilot Name                                      | Size | ES   | NES  | NOM p-val | FDR q-val | Monocyte Name                                      | Size | ES   | NES  | NOM p-val | FDR q-val | Lymphocyte Name                                      | Size | ES   | NES  | NOM p-val | FDR q-val |
|------|-----------------------------------------------|------|------|------|-----------|-----------|-----------------------------------------------|------|------|------|-----------|-----------|-----------------------------------------------|------|------|------|-----------|-----------|
| 30   | Regulation of immune effector process         | 245  | 0.13 | 2.36 | 0.00     | 0.03      | Response to topologically incorrect protein   | 162  | 0.14 | 2.05 | 0.01     | 0.22      | Regulation of cell cycle g2 m phase transition   | 197  | 0.17 | 2.67 | 0.00     | 2.08E – 04|
| 31   | Positive regulation of NF-KappaB transcription factor activity | 102  | 0.20 | 2.31 | 0.00     | 0.04      | Positive regulation of defense response       | 360  | 0.09 | 2.04 | 0.00     | 0.21      | Antigen processing and presentation of peptide antigen via MHC class i | 91   | 0.26 | 2.82 | 0.00     | 3.60E – 04|
| 32   | Negative regulation of immune system process | 252  | 0.13 | 2.30 | 0.00     | 0.04      | Vesicle budding from membrane                 | 90   | 0.18 | 2.03 | 0.00     | 0.23      | Cellular detoxification                          | 84   | 0.26 | 2.81 | 0.00     | 4.36E – 04|
| 33   | Regulation of response to external stimulus   | 376  | 0.10 | 2.29 | 0.00     | 0.04      | Transport of virus                            | 54   | 0.23 | 2.02 | 0.01     | 0.24      | Amine metabolic process                          | 102  | 0.23 | 2.75 | 0.00     | 8.24E – 04|
| 34   | Production of molecular mediator involved in inflammatory response | 41   | 0.30 | 2.29 | 0.00     | 0.04      | Synapse organization                          | 162  | 0.14 | 2.02 | 0.00     | 0.23      | Proteasomal ubiquitin independent protein catabolic process | 21   | 0.49 | 2.73 | 0.00     | 9.44E – 04|
| 35   | Interleukin 6 production                       | 86   | 0.21 | 2.28 | 0.00     | 0.04      | Vesicle localization                          | 192  | 0.13 | 2.01 | 0.01     | 0.23      | Purine containing compound biosynthetic process | 154  | 0.19 | 2.72 | 0.00     | 9.77E – 04|
| 36   | Positive regulation of protein kinase b signaling | 68   | 0.24 | 2.28 | 0.00     | 0.04      | Spliceosomal SNRNP assembly                    | 35   | 0.29 | 2.00 | 0.01     | 0.23      | Nuclear DNA replication                           | 50   | 0.32 | 2.70 | 0.00     | 1.11E – 03|
| 37   | Interleukin 6 secretion                        | 22   | 0.39 | 2.28 | 0.00     | 0.04      | DNA catabolic process endonucleolytic          | 23   | 0.35 | 2.00 | 0.00     | 0.24      | Meiotic cell cycle process                        | 122  | 0.21 | 2.69 | 0.00     | 1.13E – 03|
| 38   | T cell mediated immunity                       | 64   | 0.24 | 2.27 | 0.00     | 0.04      | Lymphocyte chemotaxis                         | 32   | 0.29 | 1.99 | 0.01     | 0.24      | tRNA metabolic process                            | 150  | 0.18 | 2.67 | 0.00     | 1.34E – 03|
| 39   | Immune response regulating signaling pathway   | 390  | 0.10 | 2.24 | 0.00     | 0.05      | RNA catabolic process                         | 336  | 0.10 | 1.99 | 0.00     | 0.24      | Antigen processing and presentation of peptide antigen | 170  | 0.18 | 2.66 | 0.00     | 1.43E – 03|
| 40   | Interferon gamma production                    | 68   | 0.23 | 2.22 | 0.00     | 0.05      | Golgi vesicle transport                       | 285  | 0.10 | 1.98 | 0.01     | 0.24      | Cellular protein complex disassembly              | 182  | 0.17 | 2.61 | 0.00     | 2.15E – 03|
| 41   | Cytokine metabolic process                     | 71   | 0.23 | 2.22 | 0.00     | 0.05      | Recombinational repair                        | 95   | 0.17 | 1.96 | 0.00     | 0.26      | Negative regulation of cell cycle g2 m phase transition | 94   | 0.23 | 2.60 | 0.00     | 2.29E – 03|
| 42   | Response to lipid                              | 457  | 0.09 | 2.19 | 0.00     | 0.06      | Nuclear transport                            | 279  | 0.10 | 1.96 | 0.00     | 0.26      | Branched chain amino acid catabolic process        | 19   | 0.48 | 2.59 | 0.00     | 2.45E – 03|
Table 1 (Continued)

| Rank | Name                                                  | Size | ES   | NES   | NOM p-val | FDR q-val | Name                                                  | Size | ES   | NES   | NOM p-val | FDR q-val | Name                                                  | Size | ES   | NES   | NOM p-val | FDR q-val |
|------|-------------------------------------------------------|------|------|-------|-----------|-----------|-------------------------------------------------------|------|------|-------|-----------|-----------|-------------------------------------------------------|------|------|-------|-----------|-----------|
| 43   | Positive regulation of cytokine secretion             | 68   | 0.22 | 2.19  | 0.00      | 0.06      | Telomere maintenance via semi conservative replication | 23   | 0.34 | 1.96  | 0.00      | 0.25      | Cofactor biosynthetic process                         | 179  | 0.17 | 2.58  | 0.00      | 2.44E - 03|
| 44   | Cytolysis                                             | 17   | 0.44 | 2.18  | 0.01      | 0.06      | Golgi vesicle budding                                  | 72   | 0.19 | 1.96  | 0.00      | 0.25      | Nucleobase containing small molecule catabolic process | 38   | 0.36 | 2.57  | 0.00      | 2.51E - 03|
| 45   | Cytokine production involved in inflammatory response | 24   | 0.37 | 2.17  | 0.00      | 0.07      | Regulation of posttranscriptional gene silencing      | 88   | 0.18 | 1.96  | 0.01      | 0.25      | Nucleoside monophosphate biosynthetic process          | 38   | 0.35 | 2.57  | 0.00      | 2.45E - 03|
| 46   | Regulation of lymphocyte migration                    | 37   | 0.29 | 2.14  | 0.00      | 0.08      | DNA repair                                            | 440  | 0.08 | 1.94  | 0.01      | 0.27      | Tricarboxylic acid cycle                               | 32   | 0.38 | 2.57  | 0.00      | 2.45E - 03|
| 47   | positive regulation of myeloid leukocyte mediated immunity | 21   | 0.38 | 2.13  | 0.00      | 0.09      | Vesicle targeting to from or within golgi             | 67   | 0.20 | 1.93  | 0.01      | 0.28      | Ribonucleoside catabolic process                       | 17   | 0.52 | 2.56  | 0.00      | 2.54E - 03|
| 48   | Cytokine production involved in immune response       | 64   | 0.23 | 2.11  | 0.01      | 0.09      | RNA export from nucleus                                | 129  | 0.14 | 1.93  | 0.01      | 0.27      | DNA conformation change                                | 199  | 0.16 | 2.56  | 0.00      | 2.53E - 03|
| 49   | Positive regulation of ERK1 and ERK2 cascade          | 85   | 0.20 | 2.11  | 0.00      | 0.09      | Establishment of protein localization to endoplasmic reticulum | 106  | 0.16 | 1.93  | 0.01      | 0.27      | Monosaccharide catabolic process                       | 32   | 0.38 | 2.56  | 0.00      | 2.54E - 03|
| 50   | Cytokine secretion                                    | 120  | 0.17 | 2.11  | 0.00      | 0.09      | Defense response to other organism                     | 310  | 0.09 | 1.93  | 0.00      | 0.26      | Fatty acid beta oxidation                              | 62   | 0.28 | 2.55  | 0.00      | 2.64E - 03|

Note: GSEA was performed with RNA sequencing data pre-ranked based on fold changes and significance of differential expression. The analysis revealed a monocyte specific upregulation of gene sets relating to viral response and response to type I interferon in autoantibody positive cases. The table details the enrichment score (ES), normalized enrichment score (NES), nominal p-value (NOM p-val) and false discovery rate corrected q-value (FDR q-val) for each term in pilot and main cohorts.
to matched control subjects. No significant differences in gene expression between the two groups were observed (data not shown). However, in a subsequent gene set enrichment analysis (GSEA), implemented on RNA sequencing data pre-ranked based on fold changes and significance of differential expression, differences linked to virus immunity were detected (Table 1). The upregulated genes included gene sets corresponding to the terms “defence response to virus” (FDR <0.001, ranking 2), “response to virus” (FDR <0.001, ranking 3) and “response to type I interferon” (FDR = 0.002, ranking 12; Table S3).

A confirmation cohort, comparing case subjects with advanced β-cell autoimmunity and matched control subjects, was then analyzed to further investigate these findings. In this cohort, fresh PBMC samples were separated into monocyte and lymphocyte compartments to study the role of monocytes in viral and type I interferon responses observed in the pilot. Both fractions were analyzed separately.

As in the pilot cohort transcription profiles, there were no significantly differentially expressed genes when comparing cases and controls (data not shown). However, as in the pilot cohort, the GSEA analysis suggested differences in virus-associated immune activation between case and control subjects in the monocyte compartment (Table 1). The GSEA confirmed the terms “defence response to virus” (FDR = 0.02, ranking 2), “response to virus” (FDR = 0.04, ranking 3) and “response to type I interferon” (FDR = 0.02, ranking 1) among upregulated genes (Table S3). In contrast, enrichment of these gene sets between cases and controls could not be observed in the lymphocyte compartment in the GSEA analysis.

4 | DISCUSSION

Various immune cell populations are implicated to play a role in the β-cell destruction leading to T1D. However, factors affecting altered immune activation are not fully described. Understanding the differences in the distinct immune cell compartment function might provide essential information about the pathogenesis of T1D. Here we explored transcriptional profiles in PBMC of children with advanced β-cell autoimmunity and compared them with those of autoantibody negative children matched for sex, age and HLA. The study was conducted in two parts, first a pilot cohort performed with frozen PBMC and second, a confirmation cohort with fresh PBMC that were fractionated into monocytes and remaining lymphocytes. While statistically significant gene expression differences could not be observed, three gene sets associated with the terms “defence response to virus,” “response to virus” and “response to type I interferon” were consistently upregulated in PBMCs and further in monocytes of case subjects.

Viral infections have long been linked with T1D pathogenesis. Especially enteroviral infections have been found to associate with increased risk for disease onset and this has also been seen in the DIPP cohort. Many strains are known to be able to cause chronic systematic infections as well as infect the pancreas. According to the current understanding, these conditions may drive strong inflammatory responses and autoimmunity. In our present study, the observed upregulation of genes essential in response to virus infections was detected in PBMCs but in the further analysis the finding was restricted to peripheral blood monocytes. Innate immunity is classically responsible for the acute response to viral threats, but the combination of a lack of detectable response from lymphocytes and our specific set of three significant GSEA terms also suggested that the monocytes themselves could be infected with a virus. Coxsackie virus B4, which belongs to the group of enteroviruses, has been shown to infect monocytes and monocyte-derived macrophages, with the potential to establish a persistent infection. Monocyte derived macrophages also produce a strong cytokine response, including IL-6 and TNFα, to Coxsackie virus B4. Another study by Alidjinou et al reported that enteroviral RNA could be detected in monocytes of some T1D patients, although viral loads in many cases seemed low and difficult to detect with RT-PCR. Furthermore, the presence of enteroviral RNA coincided with the presence of IFNα mRNA in most subjects. It is therefore possible that some of the cases in our study may have an ongoing enteroviral infection, reflected both by the upregulation of virus response genes and type I interferon response genes.

Innate immune function accompanied by a type I interferon signature, that is, detectable transiently starting shortly before seroconversion, has been reported in longitudinal studies investigating T1D pathogenesis. Kallionpää et al detected this signature in whole blood transcriptomics of autoantibody positive DIPP children, starting before seroconversion and persisting until diagnosis of clinical disease. Enterovirus-associated transcriptomic profiles were also observed in a subset of these children. Similar findings to Kallionpää et al were evident in the Environmental Determinants of Diabetes in the Young study (TEDDY) among the children whose first islet autoantibody was against insulin. Interestingly, the association of Coxsackie B1 enterovirus infections and islet autoimmunity was found specifically in children with insulin autoantibodies as the first sign of autoimmunity in the DIPP study. Enterovirus-associated transcriptomic profiles were observed in a subset of these children. Ferreira et al reported a transient type I interferon signature in genetically predisposed children before the autoantibodies were developed, but not in children with existing disease. Our observation, that a gene set corresponding to the term “response to type I interferon” is upregulated in peripheral blood immune cells, and particularly in monocytes, is in line with these previous observations.

Several studies have explored peripheral blood transcriptomic signatures in the context of T1D from various angles. Stechova et al compared the transcriptional profiles of pediatric T1D patients, their clinically healthy first-degree relatives and healthy, unrelated controls and found that the most significant difference was between first-degree relatives and unrelated controls. Accordingly, Eli et al did not observe differences in gene expression profiles of children positive for β-cell autoantibodies and children who had progressed to T1D. Similarly, in a study investigating monocytes of twins discordant for T1D, healthy twin pairs and healthy singleton controls, Beyan et al saw that most of the abnormally expressed genes observed in
T1D twins were also abnormal in their non-diabetic twins. It would therefore seem like gene expression differences already exist in genetically predisposed but healthy individuals. Additionally, many previous findings of differential gene expression in PBMCs in the context of T1D have been made with T1D patients or a combination of presymptomatic cases and those diagnosed with the disease compared to healthy controls. Observations concerning peripheral blood monocytes have similarly been made predominantly in patients with existing T1D and could be attributed to the metabolic crisis and ongoing stress triggered by disease onset, as the loss of glucose tolerance appears only shortly before it. As a consequence, it is possible that the changes our cases have, especially in monocytes, are difficult to distinguish due to some of the strengths of this study: close genetic matching of cases and controls and using samples predating the metabolic state caused by T1D itself. Therefore, the controls in our study may also have changes in their PBMCs because of the genetic T1D-risk they carry and immunological changes in the early phase of disease progression are likely to be relatively minute compared to those during disease onset.

Limitations of this study include the use of peripheral blood cells, limiting statistical power and in parts of the study, heterogenous populations. Additionally, there is a lack of a control group without HLA-conferred genetic risk to T1D. It is likely that all these factors contribute to the lack of statistically significant gene expression differences in this study. This could be addressed in future studies by more detailed cell fractionation and possible additional controls. A time series could help to pinpoint the timing of monocyte activation in T1D.

5 | CONCLUSION

Transcriptional profiles of children with advanced β-cell autoimmunity and those of their autoantibody negative controls matched for age, sex and genetic T1D-risk did not differ significantly in monocytes or monocyte-depleted PBMCs. However, gene sets essential in responses to virus were consistently upregulated in PBMCs and specifically in monocytes of subjects with advanced β-cell autoimmunity. This result supports earlier findings implicating the role of viral infections in T1D pathogenesis and the emergence of β-cell autoimmunity.

ACKNOWLEDGMENTS

The skilful technical assistance of Anne Suominen (University of Turku) and Auli Saarinen (Folkhålsan Research Centre) is gratefully acknowledged. The authors thank Biomedicum Functional Genomics Unit (FuGU), University of Helsinki, for STRT library sequencing services. The computations were performed on resources provided by SNIC through Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX) under Project b2014069.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Juha Kere, Jorma Ilonen, and Johanna Lempainen designed the study. Milla Valta, Masahito Yoshihara, Elisabet Einarsdottir, Sirpa Pahkuri, and Sini Ezer, conducted the laboratory analyses. Mikael Knip, Riitta Veijola, and Jorma Toppari, provided study material. Masahito Yoshihara and Shintaro Katayama, analysed the data and Milla Valta, Masahito Yoshihara, Juha Kere, Jorma Ilonen and Johanna Lempainen interpreted the results. Milla Valta drafted the manuscript, Masahito Yoshihara, Elisabet Einarsdottir, Mikael Knip, Jorma Toppari, Juha Kere, Jorma Ilonen and Johanna Lempainen reviewed the manuscript and contributed to the discussion.

DATA AVAILABILITY STATEMENT

Research data are not shared.

ETHICS STATEMENT

The study protocol has been approved by the local ethical committees. The guardians of the study subjects have given informed consent to study participation.

ORCID

Milla Valta https://orcid.org/0000-0001-6674-4182
Masahito Yoshihara https://orcid.org/0000-0002-8915-9282
Mikael Knip https://orcid.org/0000-0003-0474-0033
Riitta Veijola https://orcid.org/0000-0002-6557-270X

REFERENCES

1. Ilonen J, Lempainen J, Veijola R. The heterogeneous pathogenesis of type 1 diabetes mellitus. Nat Rev Endocrinol. 2019;15(11):635-650. doi:10.1038/s41574-019-0254-y
2. Willcox A, Richardson SJ, Bone AJ, Foulis AK, Morgan NG. Analysis of islet inflammation in human type 1 diabetes. Clin Exp Immunol. 2009;155(2):173-181. doi:10.1111/j.1365-2249.2008.03860.x
3. Skowera A, Ladell K, McLaren JE, et al. β-Cell-specific CD8 T cell phenotype in type 1 diabetes reflects chronic autoantigen exposure. Diabetes. 2015;64(9):916-925. doi:10.2337/db14-0332
4. Babon JAB, DeNicola ME, Blodgett DM, et al. Analysis of self-antigen specificity of islet-infiltrating T cells from human donors with type 1 diabetes. Nat Med. 2016;22(12):1482-1487. doi:10.1038/nm.4203
5. Vamdamme C, Kinnunen T, B cell helper T cells and type 1 diabetes. Scand J Immunol. 2020;92(4):12943. doi:10.1111/sji.12943
6. Espinosa-Carrasco G, Le Saout C, Fontanaud P, et al. CD4(+) T helper cells play a key role in maintaining diabetogenic CD8(+) T cell function in the pancreas. Front Immunol. 2018;8:2001. doi:10.3389/fimmu.2017.02001
7. Ziegler AG, Rewers M, Simell O, et al. Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. JAMA. 2013;309(23):2473-2479. doi:10.1001/jama.2013.6285
8. Anand V, Li Y, Liu B, et al. Islet autoimmunity and HLA markers of Presymptomatic and clinical type 1 diabetes: joint analyses of prospective cohort studies in Finland, Germany, Sweden, and the U.S. Diabetes Care. 2021;44(10):2269-2276. doi:10.2337/dc20-1836
9. Jakubzick CV, Randolph GJ, Henson PM. Monocyte differentiation and antigen-presenting functions. Nat Rev Immunol. 2017;17(6):349-362. doi:10.1038/nri.2017.28
10. Irvine KM, Gallego P, An X, et al. Peripheral blood monocyte gene expression profile clinically stratifies patients with recent-onset type 1 diabetes. Diabetes. 2012;61(5):1281-1290. doi:10.2337/db11-1549
46. Reynier F, Pachot A, Paye M, et al. Specific gene expression signature associated with development of autoimmune type-I diabetes using whole-blood microarray analysis. Genes Immun. 2010;11(3):269-278. doi:10.1038/gene.2009.112

47. Wang X, Jia S, Geoffrey R, Alemzadeh R, Ghosh S, Hessner MJ. Identification of a molecular signature in human type 1 diabetes mellitus using serum and functional genomics. J Immunol. 2008;180(3):1929-1937. doi:10.4049/jimmunol.180.3.1929

48. Helminen O, Aspholm S, Pokka T, et al. OGTT and random plasma glucose in the prediction of type 1 diabetes and time to diagnosis. Diabetologia. 2015;58(8):1787-1796. doi:10.1007/s00125-015-3621-9

SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Valta M, Yoshihara M, Einarsdottir E, et al. Viral infection-related gene upregulation in monocytes in children with signs of β-cell autoimmunity. Pediatr Diabetes. 2022;23(6):703-713. doi:10.1111/pedi.13346