



Gene Expression Profiles and Gene Interaction Networks Observed in Peripheral Lymphomononuclear Cell Populations of Recently Diagnosed Type 1 Diabetes Mellitus Patients: Differential Role of HLA Susceptibility Alleles

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Abstract

Type 1 diabetes mellitus (T1DM) is caused by selective destruction of pancreatic β cells, primarily mediated by CD4⁺T, CD8⁺T and CD14⁺ cells. We evaluated the transcript profiling of CD4⁺, CD8⁺ and CD14⁺ cells of recently diagnosed T1DM patients stratified according to the presence or not of high-risk HLA class II alleles, using microarrays. Twenty recently diagnosed patients (10 high-risk and 10 moderate/low-risk) and 10 controls were studied. The interaction between differentially expressed genes in each cell subset was evaluated throughout transcriptional networks. The expression signature of high-risk and moderate/low-risk patients was distinct from each other and distinct from controls. Compared to controls, several modulated genes were observed for patients, some ones shared by two or three of the CD4⁺, CD8⁺ and CD14⁺ cells, while others primarily observed in each cell population. Patients exhibiting high-risk or moderate/low-risk alleles presented shared (HLA-DRB1, PTPN11, DCLRE1C, FAS, GABRA1, INSR) and particular gene nodes. HLA-DRB1 modulated PTPN11, ZNF3, PFK2B, FAS, DCLRE1C, AGT genes, which differed according to cell population and HLA typing. These results indicate that HLA-DRB1 plays a differential role in T1DM according to the susceptibility allele.

Keywords: Type 1 Diabetes Mellitus; Gene Susceptibility Regions; HLA Alleles; Gene Expression; Gene Network

Introduction

The incidence of type 1 diabetes mellitus (T1DM) varies in worldwide populations, ranging from as low as 0.1/100,000 in Chinese and Venezuelan populations to approximately 37/100,000 in Sardinian and Finnish populations [1], and an intermediate cipher of 7.6/100,000 has been described for Brazilians [2]. Recently, it has been reported an increase of T1DM incidence ranging from 2 to 5% worldwide in the last years [3]. Despite frequency variation, the genetic background of populations seems to be particularly relevant.

Although more than 20 gene regions have been associated with T1DM susceptibility [4], approximately 50% of the genetic risk for the disease is conferred by the MHC region, which has been referred to as IDDM1 (insulin-dependent type 1 diabetes mellitus-region 1) [5]. The role of MHC class II genes has been extensively studied in several populations. Overall, the DRB1*03 and DRB1*04 allele group, and the DQA1*03:01/DQB1*03:02 and DQA1*03:01/DQB1*02:01 alleles have been primarily associated with high susceptibility risk to T1DM, whereas the DRB1*15:01, DQA1*01:02/DQB1*06:02, DRB1*07:01, DQA1*02:01/DQB1*03:03 alleles have been associated with protection against T1DM development [6]. Although the Brazilian population is considered to be highly genetically diverse, the DRB1*03 and *04 allele groups and DQB1*03:02 and *02:01 alleles have also been associated with T1DM susceptibility; however, no association was observed for the DQA1 alleles [7].

T1DM pathogenesis has not been completely elucidated; however, T and B lymphocytes, and

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Table 1: Demographic, clinical and laboratory features of type 1 diabetes mellitus patients. High-risk (in bold) and moderate/low-risk (in italics) DRB1 and DQB1 alleles, as classified by Atkinson & Eisenbarth [21], are also shown.

| Patient | Sex | Age | Time | Glucose | HLA-DRB1 | HLA-DQB1 |
|---------|-----|---------|----------|---------|-----------------------|-----------------------|
| | | (years) | (months) | (mg/dL) | | |
| 1 | M | 12 | 6 | 92.4 | '03:01, '04:05 | '02:01, '03:02 |
| 2 | M | 7 | 1 | 114.4 | <i>'01:01, '04:03</i> | <i>'05:01, '03:04</i> |
| 3 | M | 9 | 4 | 105.9 | <i>'04:03, '15:02</i> | <i>'03:04, '06:01</i> |
| 4 | F | 9 | 1 | 71.1 | '04:02, '12:01 | '03:01, '03:02 |
| 5 | M | 7 | 4 | 117.6 | <i>'07:01, '16:01</i> | <i>'02:02, '05:02</i> |
| 6 | M | 12 | 3 | 103.8 | <i>'01:01, '07:01</i> | <i>'02:02, '05:01</i> |
| 7 | M | 13 | 2 | 110 | <i>'07:01, '15:02</i> | <i>'02:02, '06:01</i> |
| 8 | M | 14 | 6 | 106.2 | '03:01, '04:03 | '02:01, '03:02 |
| 9 | M | 14 | 4 | 108.6 | '04:01, '13:02 | '06:03, '03:02 |
| 10 | F | 14 | 4 | 71.3 | '03:01, '07:01 | '02:01 '02:02 |
| 11 | F | 4 | 1 | 75.4 | <i>'13:01, '09:01</i> | <i>'05:01, '02:02</i> |
| 12 | M | 11 | 2 | 110 | '03:01 | '02:01 |
| 13 | F | 6 | 1 | 82.5 | '01:01, '04:01 | '05:01, '03:02 |
| 14 | F | 11 | 6 | 105 | '04:01, '07:01 | '02:02, '03:02 |
| 15 | M | 3 | 2 | 85.4 | '03:01, '04:01 | '02:01, '03:02 |
| 16 | M | 10 | 2 | 76.1 | <i>'11</i> | <i>'05, '03:01</i> |
| 17 | M | 9 | 3 | 83.8 | <i>'01:01, '08:01</i> | <i>'05:01, '04:02</i> |
| 18 | M | 11 | 2 | 94.1 | '04:02, '11 | '03:02, '05:01 |
| 19 | F | 6 | 2 | 87.9 | <i>'14:01, '07</i> | <i>'05:03, '02:02</i> |
| 20 | M | 13 | 4 | 114.9 | <i>'07:01, '08:01</i> | <i>'03:01</i> |

antigen-presenting cells are considered to be major actors. CD4⁺T and CD8⁺T cells are important for the destruction of pancreatic beta cells, acting in concert with macrophages, in the context of MHC class I and class II antigens, generating inflammatory mediators such as cytokines, chemokines, nitric oxide and oxygen free radicals [8,9]. Indeed, adoptive transfer of cells in animal models confirms that both CD4⁺ T and CD8⁺T cells play a relevant role on beta cell destruction [9]. In addition, after monocyte depletion, passively transferred diabetogenic T-cells failed to induce diabetes [10], indicating the role of antigen presentation to T cells. The role of monocytes committed to the Th17 polarization has also been described in T1DM pathogenesis [11].

The study of differentially expressed genes in T1DM has contributed to unveil many features of the disease, propitiating further approaches to T1DM pathogenesis [12,13]. In previous studies, evaluating the differential gene expression in lymphomononuclear cells of recently diagnosed T1DM patients, we reported that some of these genes were related to phosphate, protein, lipid, DNA and RNA metabolism in patients exhibiting normal glucose and normal glycated hemoglobin levels [14], indicating that even compensated several metabolism gene alterations still exist in T1DM patients. In addition, we reported that differentially expressed genes were located at chromosomal regions (IDDM1, IDDM2 and IDDM11), previously described in association with T1DM in linkage studies [15]. Finally, we also reported that the differential gene profile of T1DM patients may depend on the MHC class II profile [16]. Although there are few studies evaluating the differentially gene expression in total peripheral blood lymphomononuclear cells of T1DM patients [17], including CD4⁺ [18,19], CD8⁺ [19] and CD14⁺ [20], none have focused on HLA susceptibility alleles.

Considering that: i) Recently diagnosed T1DM patients represent the end stage of pancreatic β cell destruction by lymphomononuclear cell lines. ii) Peripheral lymphomononuclear cells may act as reporters of the elsewhere ongoing chronic tissue inflammation. iii) There are no studies evaluating gene transcriptome profiles in lymphomononuclear cell subsets of T1DM patients focusing on the role of HLA class II genes, in this study we evaluated the differential large scale gene expression profiling of CD4⁺, CD8⁺ and CD14⁺ cells obtained from T1DM patients (stratified according to the presence or not of high-risk HLA class II alleles) and controls, using microarrays. To evaluate the relationship between differentially expressed genes in each cell subset, we further performed transcriptional interaction gene networks.

Patients and Methods

Study samples

A total of 20 recently diagnosed patients with T1DM aged 3 to 14 years (median = 11 years), followed-up at the outpatient clinics of the Division of Endocrinology, Department of Medicine, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil, were submitted to complete anamnesis and physical examination. Ten patients exhibited at least one high-risk susceptibility alleles, including DRB1*04:01/*04:02/*04:05-DQB1*03:02, DRB1*03:01-DQB1*02:01, and 10 patients exhibited moderate/low-risk alleles, primarily represented by DRB1*08:01-DQB1*04:02, DRB1*01:01-DQB1*05:01, DRB1*09:01-DQB1*03:03, DRB1*04:01/DQB1*03:01, DRB1*04:03-DQB1*03:02, DRB1*07:01-DQB1*02:01, and DRB1*11:01-DQB1*03:01, according to Atkinson & Eisenbarth, 2001 [21]. All patients were studied only after control of clinical and laboratory variables, i.e., in the absence of ketoacidosis,

hyperglycemia, and increased glycosylated hemoglobin levels. (Table 1) shows demographic, clinical and laboratory features of patients.

Ten healthy individuals presenting no family history of T1DM or other autoimmune disorders and matched to the patients for sex and age were studied. Five individuals exhibited at least one high-risk susceptibility alleles for T1DM and 5 individuals exhibited moderate/low-risk alleles. The protocol of the study was approved by the local Ethics Committee (Protocol # 4017/2007).

Blood collection

A total of 20 mL of peripheral blood cells were collected and divided in two aliquots. The first, collected in Vacutainer tubes (Beckton & Dickinson, Plymouth, England), containing EDTA (0.054 mL/tube), was used for DNA extraction using a salting out procedure [22]. The second, collected in Vacutainer tubes, containing 72 units U.S.P of sodium heparin per tube, was used for isolation of lymphomononuclear cells and further RNA extraction. RNA concentrations and ratios were checked with the NanoDrop ND-1000 spectrophotometer (Wilmington, DE) and RNA quality was assessed using the 2100 Bioanalyzer (Agilent). All RNA samples exhibited high integrity numbers ≥ 8.0 .

Mononuclear cell separation

Total peripheral blood mononuclear cells were isolated by gradient density using Ficoll-Hypaque (1,077g/L, Sigma, Saint Louis, MO). Lymphocyte subsets were separated using columns containing electromagnetic beads conjugated with anti-CD14, anti-CD8 and anti-CD4 antibodies (Dyna, NY). Purity of cell populations was evaluated by flow cytometry (FACSVantage device Beckton Dickinson, San Jose, CA), using anti-CD3 antibody conjugated with fluorescein isothiocyanate (Beckton Dickinson, Heidelberg, Germany) and anti-CD14, anti-CD8 and anti-CD4 antibodies conjugated with phycoerythrin (Becton Dickinson). The monocyte population was CD3⁻ CD14⁺. Cell subset purity was more than 95% in all assays.

HLA-DRB1 and DQB1 typing

HLA class II typing at a low- or high-resolution level was performed using PCR-amplified DNA hybridized with sequence-specific primers, using commercially available kits (One-Lambda, Canoga Park, CA).

Microarray hybridization

Total RNA was extracted from each cell population using the Trizol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Since the amount of RNA obtained from each cell population was not sufficient for the microarray assays, we pooled each cell population (CD4⁺, CD8⁺ or CD14⁺ cell isolates) RNA, yielding several RNA pools: i) 3 (one of each cell type) from patients presenting at least one high-risk HLA susceptibility allele. ii) 3 from patients presenting at least one moderate/low-risk HLA allele. iii) 3 from healthy controls exhibiting at least one high-risk T1D susceptibility alleles and iv) 3 from healthy individuals presenting moderate/low-risk T1D susceptibility alleles.

Gene expression was assessed in duplicates by hybridization with glass slide microarrays prepared on silane-coated Ultra GAPS slides (Corning, New York, NY). All experimental conditions and expression data are available at ArrayExpress databank (<http://www.ebi.ac.uk/arrayexpress/>) under the accession number E-MEXP-3223.

The 4,500 cDNA sequences were retrieved from the human-expressed sequence tag (EST) cDNA library (IMAGE Consortium, <http://image.hudsonalpha.org>). Microarrays were prepared based on published protocols, using PCR products from the cDNA clones and a Generation III Array Spotter (Amersham Molecular Dynamics, Sunnyvale, CA).

The cDNA complex probes from patients or controls, and reference samples were prepared by reverse transcription using 10 μ g of total RNA labeled with the Cy3 fluorochrome, using the CyScribe post labeling kit (GE Health-Care Life Sciences, Sunnyvale, CA). A 15-hour period was required for hybridization, followed by washing using an automatic slide processor system (Amersham). Microarrays were scanned using a Generation III laser scanner (Amersham). As a reference for the hybridization procedure, we used equimolar quantities of cDNAs obtained from the total RNA of different human cell strains (Jurkat, HeLa, HEp-2, and U343). This approach allowed the estimation of the relative amount of cDNA target sequence in each microarray spot.

A complete file providing all genes and ESTs present in the microarray used in this study, as well as the quantitative data and experimental conditions is available on line at MIAME public database (<http://www.mged.org/Workgroups/MIAME/miame.html>).

Microarray data analysis

Microarray image quantification was performed using the Spotfinder software [23]. The quality control and normalization intra and inter-slides were carried out using the R platform, implemented by the limma (<http://www.bioconductor.org/packages/2.13/bioc/html/limma.html>) and aroma (<http://www.aroma-project.org>) packages.

Statistical analyses and clustering were performed using the Multiexperiment Viewer (MeV) software version 4.6 (<http://www.tm4.org/mev.html>) [23]. Two analyses were performed: i) two class analyses using T-test ($P \leq 0.01$) followed by SAM unpaired ($FDR \leq 0.05$) to identify differentially expressed genes between control and patients; ii) multiclass analyses by ANOVA ($P \leq 0.01$) and SAM ($FDR \leq 0.05$) to compare the control and patients stratified according to HLA susceptibility alleles.

Functional annotation

Data obtained using statistical analyses were used. Data mining of the significantly and differentially expressed genes was performed using the SOURCE database (<http://source.stanford.edu>).

Gene networks

The interaction between genes based on their transcriptional profile was evaluated throughout reconstruction of regulatory networks using the GeneNetwork tool [24], which compares the medians of different gene expression values, establishing interactions of selected genes. The Bayesian model was used as a parameter to compare patient and control samples, allowing the reconstruction of networks, permitting the visualization of gene nodes. Gene networks were edited using the Cytoscape software (<http://www.cytoscape.org>) [25]. Based on: i) the great number of comparisons between induced and repressed genes and great amount of information, ii) the idea that induced genes may actively target or be target for other genes, only induced genes were used to construct gene networks. Additionally, gene nodes obtained in each cell population were organized to construct Venn diagrams using gplots R package [26].

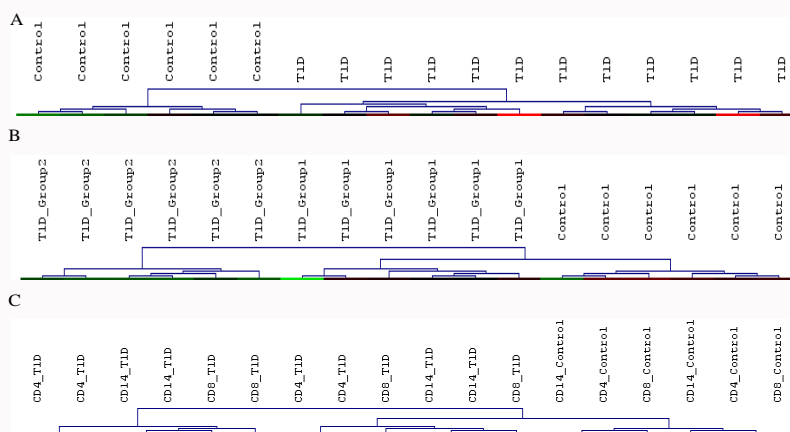


Figure 1: Hierarchical clustering of the sample genes observed for type 1 diabetes (T1DM) patients and controls, as identified by the SAM program. Characterization of gene signatures, as analyzed by the Cluster & TreeView program, was evaluated at three different ways. Panel A shows gene profiles of T1DM patients considered as a whole and controls. Hybridization signature observed for patients was distinct of that of controls. Panel B shows the hybridization signatures of patients and controls, stratifying patients according to the presence or not of high-risk HLA class II susceptibility alleles. Left patient cluster encompasses patients with high-risk alleles and right patient cluster, patients without these alleles. Panel C shows the hybridization signatures after stratification according to the cell subset (CD4⁺, CD8⁺ and CD14⁺ cells). Only patients exhibiting high-risk susceptibility class II alleles exhibited a distinct hybridization profile for each cell population (left cluster).

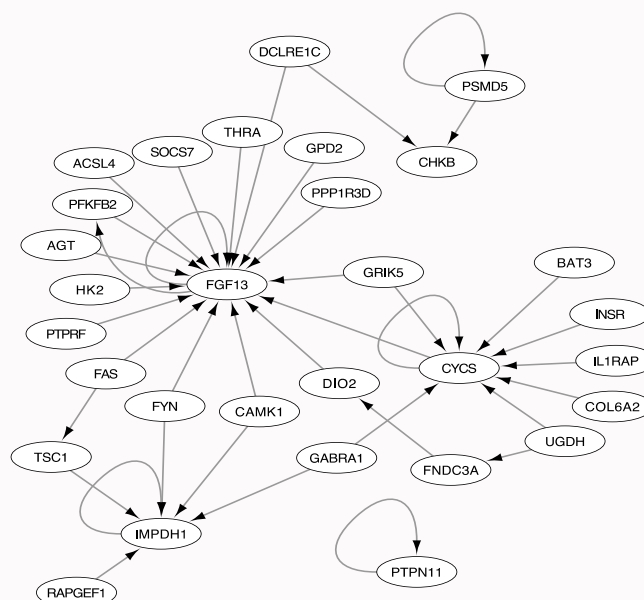


Figure 2A: Gene networks observed for CD4 cells of T1DM patients exhibiting high-risk HLA class II susceptibility alleles after comparing patients with controls. Major gene nodes are highlighted in gray and the arrows indicate the modulated genes. For instance, if a gene (g) is upregulated, it regulates one (h) or some downregulated genes (g→h).

Results

Differential gene expression analysis

The unsupervised hierarchical clustering of samples (SAM algorithm), disclosed 825 significantly and differentially expressed genes after comparing T1DM patients considered as a whole and controls (Figure 1A).

When patients and controls were stratified according to the presence or not of high-risk HLA-DRB1 and DQB1 alleles, patients and controls continued to exhibit distinct gene profiles, disclosing one cluster encompassing controls and moderate/low-risk patients and another cluster for high-risk T1DM patients (Figure 1B).

When patients and controls were stratified according to lymphomononuclear cell subset hybridization profiles, two clusters

were observed, one for moderate/low-risk patients and controls, and a second one encompassing only high-risk HLA class II alleles (Figure 1C).

Gene network analysis

Considering that major differences in terms of hybridization profiles observed for T1DM patients were related to the presence or not of high-risk alleles and cell subsets, we constructed gene networks according to these variables.

Figure 2A shows the transcriptional gene network analysis for CD4⁺ cells of T1DM patients exhibiting high-risk susceptibility alleles and (Figure 2B) shows the analysis of CD4⁺ cells of patients without high-risk alleles, when compared to controls. (Figure 3A) shows the transcriptional gene network analysis of the CD8⁺ cells

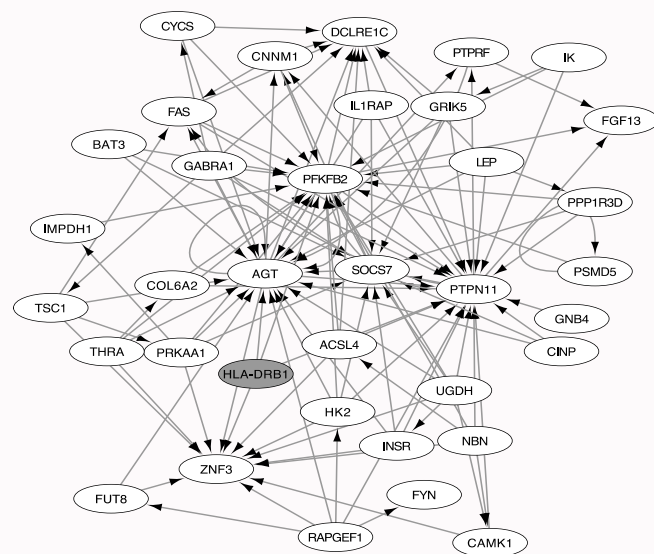


Figure 2B: Gene networks observed for CD4 cells of T1DM patients exhibiting moderate/low-risk HLA class II susceptibility alleles after comparing patients with controls. Major gene nodes are highlighted in gray and the arrows indicate the modulated genes. For instance, if a gene (g) is upregulated, it regulates one (h) or some downregulated genes (g→h).

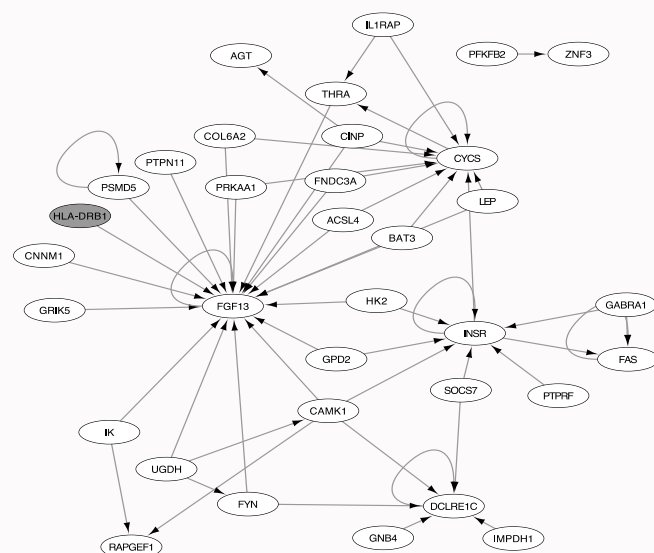


Figure 3A: Gene networks observed for CD8 cells of T1DM patients exhibiting high-risk HLA class II susceptibility alleles after comparing patients with controls. Major gene nodes are highlighted in gray and the arrows indicate the modulated genes. For instance, if a gene (g) is upregulated, it regulates one (h) or some downregulated genes (g→h).

of patients exhibiting high-risk susceptibility alleles and (Figure 3B) shows analysis of the CD8⁺ cells of patients without high-risk alleles, when compared to controls. (Figure 4A) shows the transcriptional gene network analysis of the CD14⁺ cells of patients exhibiting high-risk susceptibility alleles and (Figure 4B) shows analysis of the CD14⁺ cells of patients without high-risk alleles, when compared to controls. Major gene nodes, q-values, chromosomal location and biological processes are shown in (Table 2).

Shared and particular gene nodes in lymphomononuclear cell populations, stratified according to the HLA class II alleles

Major gene nodes obtained in gene networks were further used to construct Venn diagrams (Figures 5A and 5B). (Figure 5A) shows shared and particular genes observed in CD4⁺, CD8⁺ and CD14⁺ cells of T1DM patients exhibiting HLA high-risk alleles, and (Figure 5B)

those genes in patients exhibiting moderate/low-risk alleles.

Discussion

The major results of the present study included: i) the differential gene expression profile of T1DM patients is different from controls; ii) the differential gene expression profiles varies according to T1DM susceptibility alleles; iii) the differential expression of each lymphomononuclear cell subset is similar within patient group, iv) major gene nodes observed in each cell population show shared genes within the same patient subgroup.

The gene network analysis displayed differential results. For CD4⁺ cells obtained from patients presenting high-risk alleles, the FGF13, CYCS and IMPDH1 genes displayed major interactions with others genes. The protein encoded by the FGF13 (fibroblast growth factor 13) exhibits broad mitogenic and cell survival activities (Table 2).

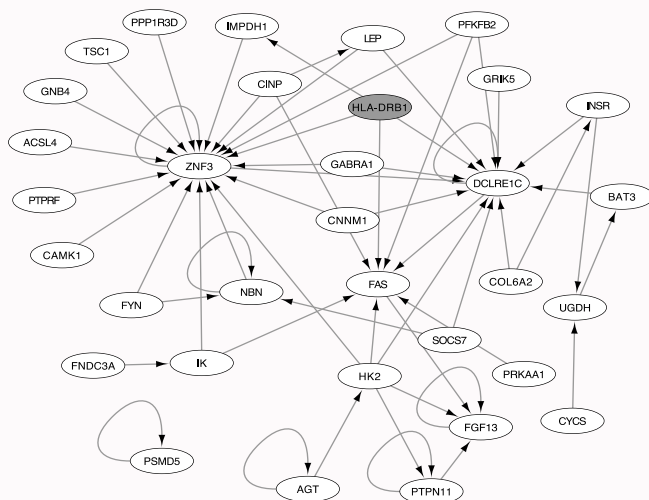


Figure 3B: Gene networks observed for CD8 cells of T1DM patients exhibiting moderate/low-risk HLA class II susceptibility alleles after comparing patients with controls. Major gene nodes are highlighted in gray and the arrows indicate the modulated. For instance, if a gene (g) is upregulated, it regulates one (h) or some downregulated genes (g→h).

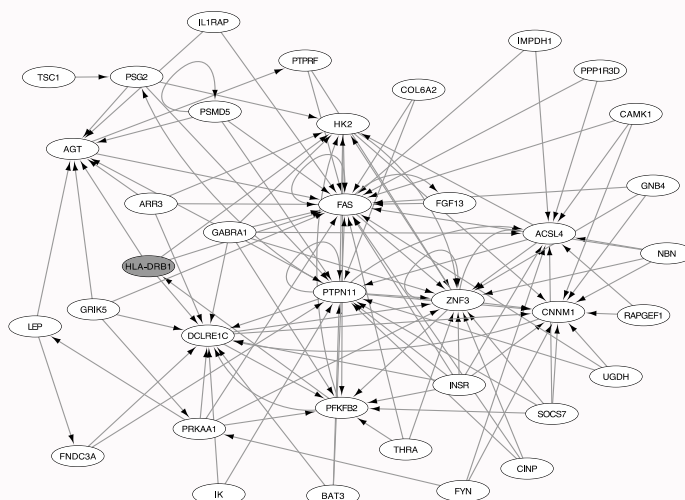


Figure 4A: Gene networks observed for CD14 cells of T1DM patients exhibiting high-risk HLA class II susceptibility alleles after comparing patients with controls. Major gene nodes are highlighted in gray and the arrows indicate the modulated genes. For instance, if a gene (g) is upregulated, it regulates one (h) or some downregulated genes (g→h).

The only literature study regarding the role of this gene in T1DM patients reports no significant differences in FGF13 serum levels in young diabetics in relation to controls [27]. The FGF13 gene was modulated by several other genes, highlighting the FAS gene that plays a central role on apoptosis. Other major node was headed by the cytochrome c (CYCS) gene that has also a central role in the initiation of mitochondrial-mediated apoptosis (Table 2), a cell death mechanism that is very important for beta cells in T1DM patients. Besides self-modulation, the CYS gene was modulated by other transcripts, including BAT3 (HLA-B associated transcript), INSR (insulin receptor), IL1RAP (interleukin 1 receptor accessory protein) and GABRA1 (gamma-aminobutyric acid-GABA A receptor, alpha 1). In addition to CYCS, GABRA1 also modulated another gene node headed by IMPDH1 [(inosine 5'-monophosphate) dehydrogenase 1 gene (Figure 2A). The protein encoded by IMPDH1 regulates cell growth, displaying a critical role for pancreatic beta-cell proliferation [28]. A much denser gene network was observed for CD4+ cells of T1DM patients exhibiting moderate/low alleles, and major nodes included PTPN11, AGT, PFKB2 and ZNF3, the last two genes were

modulated by INSR gene. It is interesting to note that HLA-DB1 gene modulated all these four nodes (PTPN11, AGT, PFKB2 and ZNF3). PTPN11 (protein tyrosine phosphatase, non-receptor type 11) is known to be a signaling molecules for the regulation of many cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation, and displaying a relevant role on autoimmune diseases (Table 2). Besides the well known association of autoimmune disease and PTPN22 gene, PTPN11 (also designated as SHP2) was identified as a potential molecular target for treatment of diabetes and obesity, since deletions of the PTPN11 protein in mice have been associated with insulin resistance, hyperglycemia and obesity [29]. Angiotensinogen (AGT) is involved in maintaining blood pressure and in the pathogenesis of hypertension and glomerular hyperfiltration, and the blockade of the renin-angiotensin system is commonly used to modify hyperfiltration and delay of progression of diabetic nephropathy [30]. Although T1DM patients evaluated in this study were recently diagnosed, the up-regulation of this gene even in the initial stage of the disease together with the myriad of genes that modulate AGT, particularly in patients without T1DM high-risk

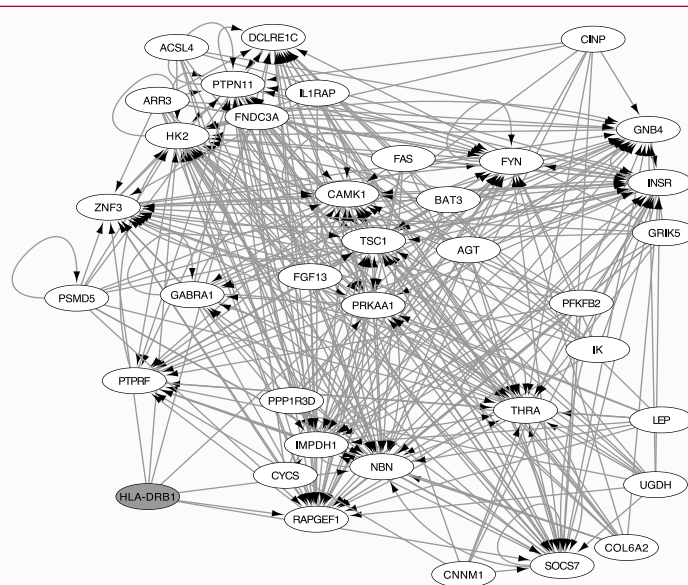


Figure 4B: Gene networks observed for CD14 cells of T1DM patients exhibiting moderate/low-risk HLA class II susceptibility alleles after comparing patients with controls. Major gene nodes are highlighted in gray and the arrows indicate the modulated genes. For instance, if a gene (g) is upregulated, it regulates one (h) or some downregulated genes (g→h).

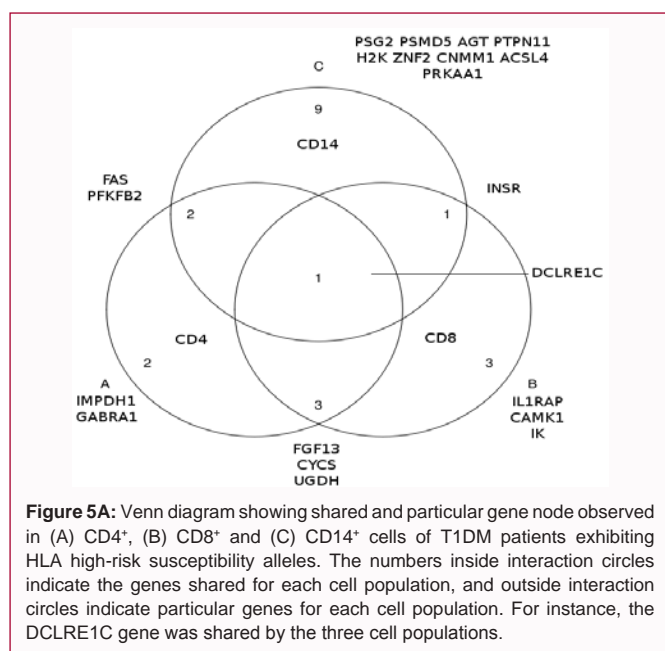


Figure 5A: Venn diagram showing shared and particular gene node observed in (A) CD4⁺, (B) CD8⁺ and (C) CD14⁺ cells of T1DM patients exhibiting HLA high-risk susceptibility alleles. The numbers inside interaction circles indicate the genes shared for each cell population, and outside interaction circles indicate particular genes for each cell population. For instance, the DCLRE1C gene was shared by the three cell populations.

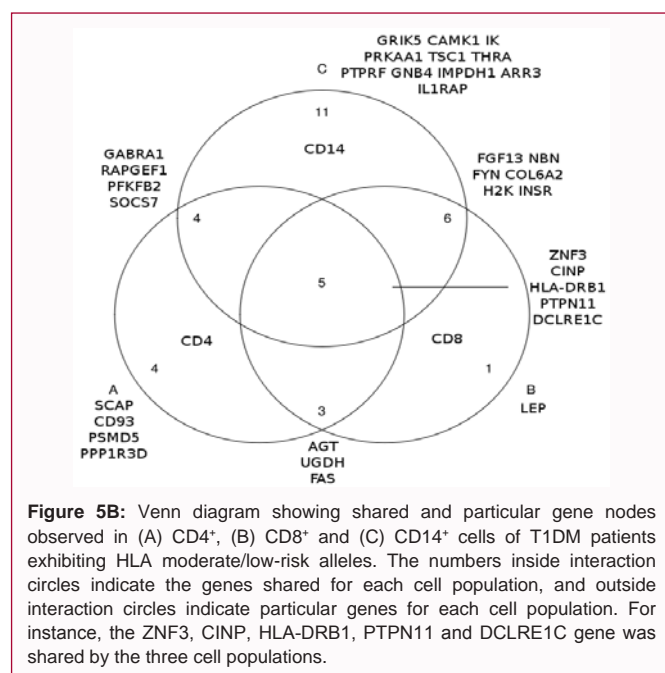


Figure 5B: Venn diagram showing shared and particular gene nodes observed in (A) CD4⁺, (B) CD8⁺ and (C) CD14⁺ cells of T1DM patients exhibiting HLA moderate/low-risk alleles. The numbers inside interaction circles indicate the genes shared for each cell population, and outside interaction circles indicate particular genes for each cell population. For instance, the ZNF3, CINP, HLA-DRB1, PTPN11 and DCLRE1C gene was shared by the three cell populations.

alleles, may indicate that these patients may be more susceptibility to chronic complications, deserving further attention. The PFKFB2 gene (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2) codes a regulatory molecule that controls glycolysis and its interaction with glucokinase activates glucose phosphorylation and glucose metabolism in insulin-producing cells [31]. Interestingly, this gene was modulated by several others, including FAS, GABRA1, HLA-DRB1, among many others (Figure 2B). Two gene nodes that emerged in CD4⁺ cells from patients with high-risk alleles also appeared in CD8 cells of the same patients, i.e., FGF13 and CYCS genes. Besides these genes, another important node was the INSR gene. Binding of insulin to the insulin receptor (INSR) stimulates glucose uptake, and normal cell expression of this receptor has been reported for T1DM patients [32]. Besides self-modulation, the INRS gene is modulated by GABRA1 and modulates FAS (Figure 3A), a mechanism that may

play a relevant role on the destruction of pancreatic beta cells. Major nodes observed for CD8⁺ cells of T1DM patients with moderate/low-risk alleles included the DCLRE1C (DNA cross-link repair) and ZNF13 (zinc finger protein 3) genes. Although these genes have no direct association with T1DM, they are modulated by several genes, highlighting HLA-DRB1 and GABRA1 (Figure 3B), both associated with T1DM pathogenesis.

In CD14⁺ cells from patients with high-risk alleles, PTPN11, GABRA1, FAS, INSR, ZNF3, PFKFB2 headed major nodes, as seen for CD4⁺ and CD8⁺ cells. In addition, SOCS7 and CNNM1 headed nodes that had not been observed in CD4⁺ and CD8⁺ cells. SOCS (suppressor of cytokine signaling) proteins act as negative regulators of cytokines, inhibiting JAK/STAT signaling, and are implicated in the negative regulation of insulin signaling, since SOCS7-deficient

Table 2: Major gene nodes, q-values, chromosomal location and biological processes are shown.

| Gene symbol | Gene Name | Cytoband | Biological Function | qvalue |
|-------------|--|-----------------|---|--------|
| ACSL4 | Acyl-CoA synthetase long-chain family member 4 | Xq22.3-q23 | immune system process;fatty acid metabolic process | *** |
| AGT | Angiotensinogen (serpin peptidase inhibitor, clade A, member 8) | 1q42.2 | proteolysis | *** |
| ARR3 | Arrestin 3, retinal (X-arrestin) | Xcen-q21 | G-protein coupled receptor protein signaling pathway | *** |
| BAT3 | BCL2-associated athanogene 6 | 6p21.3 | Regulation of cell proliferation; transport | *** |
| CAMK1 | Calcium/calmodulin-dependent protein kinase I | 3p25.3 | cell differentiation | *** |
| CD93 | CD93 molecule | 20p11.21 | macrophage activation; phagocytosis | *** |
| CHKB | choline kinase beta | 22q13.33 | Phospholipid biosynthetic process | ** |
| CINP | cyclin-dependent kinase 2 interacting protein | 14q32.31 | cell cycle; DNA repair | ** |
| CNNM1 | Cyclin M1 | 10q24.2 | signal transduction; cell-cell adhesion | *** |
| COL6A2 | Collagen, type VI, alpha 2 | 21q22.3 | oxidative phosphorylation; respiratory electron transport chain; apoptosis | ... |
| CYCS | Cytochrome c, somatic | 7p15.3 | DNA repair | *** |
| DCLRE1C | DNA cross-link repair 1C | 10p13 | cellular defense response, cellular amino acid metabolic process; fatty acid biosynthetic process | ... |
| DIO2 | deiodinase, iodothyronine, type II | 14q24.2-q24.3 | Small molecule metabolic process | ** |
| FAS | Transcribed locus | 10q24.1 | cell cycle; transmembrane receptor protein tyrosine kinase signaling pathway | ... |
| FGF13 | Fibroblast growth factor 13 | Xq26.3 | apoptosis | *** |
| FNDC3A | fibronectin type III domain containing 3A | 13q14.2 | Cell-cell adhesion | ** |
| FUT8 | fucosyltransferase 8 (alpha (1,6) fucosyltransferase) | 14q24.3 | Receptor metabolic process | ** |
| FYN | FYN oncogene related to SRC, FGR, YES | 6q21 | neurological system process; cation anion transport | *** |
| GABRA1 | Gamma-aminobutyric acid (GABA) A receptor, alpha 1 | 5q34 | G-protein coupled receptor protein signaling pathway | *** |
| GNB4 | Guanine nucleotide binding protein (G protein), beta polypeptide 4 | 3q26.33 | glycolysis | *** |
| GPD2 | glycerol-3-phosphate dehydrogenase 2 (mitochondrial) | 2q24.1 | Small molecule metabolic process | ** |
| GRIK5 | glutamate receptor, ionotropic, kainate 5 | 19q13.2 | Cellular response to glucose stimulus | ** |
| HK2 | Hexokinase 2 | 2p13 | antigen processing and presentation of peptide or polysaccharide antigen | ... |
| HLA-DRB1 | Major histocompatibility complex, class II, DR beta 1 | 6p21.3 | immune responses | *** |
| IK | IK cytokine, down-regulator of HLA II | 2p15-p14 5q31.3 | immune system process | *** |
| IL1RAP | Interleukin 1 receptor accessory protein | 3q28 | purine base metabolic process | *** |
| IMPDH1 | IMP (inosine 5'-monophosphate) dehydrogenase 1 | 7q31.3-q32 | transmembrane receptor protein tyrosine kinase signaling pathway | ... |
| INSR | Insulin receptor | 19p13.3-p13.2 | cytokine-mediated signaling pathway | *** |
| LEP | Leptin | 7q31.3 | DNA repair;DNA recombination | *** |
| NBN | Nibrin | 8q21 | glycogen metabolic process | *** |
| PFKB2 | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 | 1q31 | immune system process; calcium-mediated signaling | *** |
| PPP1R3D | Protein phosphatase 1, regulatory subunit 3D | 20q13.3 | regulation of glycogen biosynthetic process | ** |
| PRKAA1 | Protein kinase, AMP-activated, alpha 1 catalytic subunit | 5p12 | protein modification process | *** |
| PSG2 | pregnancy specific beta-1-glycoprotein 2 | 19q13.1-q13.2 | Cell migration | ** |
| PSMD5 | Proteasome (prosome, macropain) 26S subunit, non-ATPase, 5 | 9q33.2 | mitosis; transmembrane receptor protein tyrosine kinase signaling pathway | ... |
| PTPN11 | Protein tyrosine phosphatase, non-receptor type 11 | 12q24 | intracellular protein transport; exocytosis | *** |
| PTPRF | Protein tyrosine phosphatase, receptor type, F | 1p34 | transmembrane receptor protein tyrosine kinase ;cytokine-mediated signaling pathway | ... |
| RAPGEF1 | Rap guanine nucleotide exchange factor (GEF) 1 | 9q34.3 | intracellular signaling cascade;regulation of transcription | *** |
| SCAP | SREBF chaperone | 3p21.31 | cell cycle | *** |
| SOCS7 | Suppressor of cytokine signaling 7 | 17q12 | polysaccharide and lipid metabolic process | ** |
| THRA | Thyroid hormone receptor, alpha | 17q11.2 | spermatogenesis; regulation of transcription | *** |
| TSC1 | Tuberous sclerosis 1 | 9q34 | cell-matrix adhesion | *** |
| UGDH | UDP-glucose 6-dehydrogenase | 4p15.1 | small molecule metabolic process | *** |
| ZNF3 | Zinc finger protein 3 | 7q22.1 | regulation of transcription, DNA-dependent; leukocyte activation | ... |

** q-value <0.001 ***q-value <0.0001

mice exhibited lower glucose levels and prolonged hypoglycemia during an insulin tolerance test [33]. CNM1 (cyclin M1) is located at chromosome 10q24.2, a region that has been previously associated with susceptibility to T1DM, defined as IDDM17 [34]. Cyclin M1 exhibits a 36% normalized expression in pancreatic islets and was originally identified as a protein which levels increase during interphase and decrease rapidly to zero as cells entered the M phase [35]. Although there are different cyclins that act at different stages of the cell cycle, the D-type cyclins are associated with the regulation of pancreatic beta cell, preserving insulin secretion [36]. The CNM1 gene was modulated by other genes that headed other nodes, including ZNF3, PFKFB2, and SOCS7, and further modulated FAS. In a previous study, we reported that this gene was also upregulated in total lymphocytes of another series of recently diagnosed T1DM patients, irrespective of the HLA class II typing [14].

A much denser gene network was observed for CD14⁺ cells of T1DM patients with moderate/low-risk alleles, encompassing genes that headed gene nodes in CD14⁺ cells of patients exhibiting high-risk susceptibility alleles, such as GABRA1, INSR, ZNF3, SOCS7 and PTPN11 genes. In addition, several other nodes were observed including CAMK1, COL6A2, DCLRE1C, PRKAA1, TSC1, IMPDH1, HK2, GNB4, PTPRF, LEP, FYN, NBN, THRA, UGDH genes. Among these genes, it is worth mentioning that TSC1 (tuberous sclerosis 1) is involved in response to insulin stimulus and modulation of apoptosis, and exposure of proximal tubular epithelial to high glucose promotes apoptosis of these cells in diabetes [37]. The protein encoded by IMPDH1 (inosine 5'-monophosphate dehydrogenase 1) gene regulates cell growth and is involved with lymphocyte proliferation, and adequate IMPDH activity is a critical requirement for beta-cell proliferation [28]. HK2 (hexokinase 2) phosphorylates glucose to produce glucose-6-phosphate, the first step in most glucose metabolism pathways. Increased activity of hexokinase was observed in rat erythrocytes exhibiting diabetes [38]. PTPRF (protein tyrosine phosphatase, receptor type, F) regulates epithelial cell-cell contacts, and increased expression of the encoded protein was found in the insulin-responsive tissue of obese, insulin-resistant individuals, potentially contributing to insulin resistance. LEP (leptin) encodes a protein secreted by white adipocytes, playing a major role on the regulation of body weight, and is involved on the regulation of immune and inflammatory responses, hematopoiesis, angiogenesis and wound healing (Table 2). Leptin therapy reverses hyperglycemia in mice with streptozotocin-induced diabetes [39]. Studies have suggested that weight gain in early childhood may play a role in determining disease risk, with increased risk in children who have gained more weight [40]. CAMK1 (calcium/calmodulin-dependent protein kinase I) is activated by increased concentrations of intracellular Ca²⁺, a key intracellular mediator of glucose-stimulated insulin secretion [41,42], playing an important role on glucose-upregulated transcriptional activation of the insulin gene [43]. The gene modulates and is modulated by GABRA1 (Figure 4B).

Considering patients exhibiting high-risk alleles, only the DCLRE1C gene was shared by all three cell populations. Three genes were shared by CD4⁺ and CD8⁺ cells (FGF13, CYCS and UGDH), two genes were shared by CD4⁺ and CD14⁺ cells (FAS and PFKFB2), and only the INSR gene was shared by CD8⁺ and CD14⁺ cells. Among gene nodes observed exclusively for each cell population included: i) CD4⁺ cells: IMPDH1 and GABRA1; ii) CD8⁺ cells: IL1RAP, CAMK1 and IK; iii) CD14⁺ cells: PSG2, PSMD5, AGT, PTPN11, H2K, ZNF2, CNM1, ACSL4 and PRKAA1, as shown in the diagram of Figure 5A.

Considering patients exhibiting moderate/low-risk alleles, five genes (ZNF3, CINP, HLA-DRB1, PTPN11 and DCLRE1C) were shared by all three cell populations, three (AGT, UGDH and FAS) were shared by CD4⁺ and CD8⁺ cells, and four (GABRA1, RAPGEF1, PFKFB2 and SOCS7) were shared by CD4⁺ and CD14⁺ cells. Several genes were shared by CD8⁺ and CD14⁺ cells, including FGF13, NBN, FYN, COL6A2, H2K and INSR. Among gene nodes exclusively observed for each cell population included: i) CD4⁺ cells: SCAP, CD93, PSMD5 and PPP1R3D; ii) CD8⁺ cells: only LEP; iii) CD14⁺ cells: GRIK5, CAMK1, IK, PRKAA1, TSC1, THRA, PTPRF, GNB4, IMPDH1, ARR3 and IL1RAP (Figure 5B).

The only gene node observed for all patients irrespective of the HLA susceptibility allele was DCLRE1C, a gene that encodes a nuclear protein involved in V(D)J recombination and DNA repair. Since there are no studies of this gene in T1DM, it deserves further evaluation. Regarding the other genes shared by T1DM patients exhibiting moderate/low-risk alleles, two genes (HLA-DRB1 and PTPN11) deserve attention, which were also observed in patients with high-risk alleles, but did not head major gene nodes. Irrespective of the HLA allele, HLA-DRB1 modulated several genes in different cell population, including ZNF3, AGT, PTPN11 in CD4⁺ cells (Figures 2A and 2B), FGF13, ZNF3, DCLRE1C, FAS in CD8⁺ cells (Figures 3A and 3B), and many other genes in CD14⁺ cells, highlighting the modulation of the SOCS7, NBN, DCLRE1C and AGT genes (Figures 4A and 4B). All these genes deserve further attention. It is interesting to mention that HLA-DRB1 genes also modulated several genes in patients with other human autoimmune disorders [44] or in collagen-induced arthritis in mice [45]. Despite the role of HLA-DRB1 on the modulation of all these genes, it should be mentioned the role of HLA-DQB1 genes, which are also considered to be major susceptibility alleles in T1DM. First, unfortunately, this gene was not present among the transcripts of our microarray library, composed of approximately 4,500 genes. Second, in a previous study, evaluating the expression of HLA-DQ molecules on the cell surface of CD4⁺ cells of recently diagnosed T1DM patients, we observed decreased percentage of this cells in patients bearing the HLA-DQB1*02:01 allele [7].

Taken together, our results show the effect of the HLA profile and of the cell type. This is the first study evaluating uncultured cell subset transcriptional profiles in T1DM patients, stratified according to the HLA susceptibility alleles. HLA-DRB1 alleles played a differential role on the transcriptional profiles of T1DM patients, controlling a gene node or being controlled by other genes. Since little information about the role of HLA-DRB1 genes in T1DM is available, since much of our results were *in silico* studies, and since many genes influenced by HLA-DRB1 alleles have never been associated with T1DM, further studies are needed to clarify the role of HLA class II genes on the susceptibility to T1DM.

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