HLA Class II antigen processing and presentation pathway components demonstrated by transcriptome and protein analyses of islet β-cells from donors with type 1 diabetes

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Guarantors: NGM and DMH are guarantors of this work, and as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Keywords: autoimmunity, CIITA, DiViD, nPOD, Class II, transcriptome, islet cell, type 1 diabetes

Running title: **Class II β-cell expression in type 1 diabetes**

Diabetes Publish Ahead of Print, published online March 4, 2019

Abbreviations:

APC	Antigen presenting cell
CIITA	Class II major histocompatibility complex transactivator
Class I	HLA Class I
Class II	HLA Class II
CTSS	Cathepsin S
DiViD	Diabetes Virus Detection study
EADB	Exeter Archival Diabetes Biobank
ESAT	End sequence analysis tool
FACS	Fluorescence activated cell sorting
FFPE	Formalin-fixed paraffin embedded
FSC-H, FSC-A	Forward scatter-height, forward scatter-area
FMO	Fluorescence minus one
ICA	Independent component analysis
IEQ	Islet equivalent
IIAM	International Institute for the Advancement of Medicine
IIDP	Integrated Islet Distribution program
IRF8	Interferon Regulatory Factor 8
mRNA	messenger RNA
NDRI	National Disease Research Interchange
nPOD	Network for Pancreatic Organ Donors with Diabetes
PCA	Principal component analysis
RIN	RNA integrity number
SPI1	Spi-1 Proto-Oncogene
TMM	Trimmed mean of M values
TPM	Transcripts per kilobase million
t-SNE	T-distributed stochastic neighbor embedding
UMI	Unique molecular identifier

Total Word Count: 5,078

Number of Figures: 8

Number of Tables: 0

Number of Supplementary Figures: 6

Number of Supplementary Tables: 3

Online Supplementary Material: 1

Abstract

Type 1 diabetes studies consistently generate data showing islet β-cell dysfunction and T-cell mediated anti-β-cell specific autoimmunity. To explore the pathogenesis, we interrogated the β-cell transcriptomes from donors with and without type 1 diabetes using both bulk-sorted and single β-cells. Consistent with immunohistological studies, β-cells from donors with type 1 diabetes displayed increased Class I transcripts and associated mRNA species. These β-cells also expressed mRNA for Class II and Class II antigen presentation pathway components, but lacked macrophage marker, CD68. Immunohistological study of three independent recent-onset type 1 diabetic donor cohorts showed Class II protein and its transcriptional regulator Class II major histocompatibility complex trans-activator (CIITA) protein expressed by a subset of insulin$^+$ CD68$^-$ β-cells, specifically found in islets with lymphocytic infiltrates. β-cell surface expression of HLA Class II was detected on a portion of CD45$^-$insulin$^+$ β-cells from donors with type 1 diabetes by immunofluorescence and flow cytometry. Our data demonstrate that pancreatic β-cells from donors with type 1 diabetes express Class II molecules on selected cells with other key genes in those pathways and inflammation-associated genes. β-cell expression of Class II molecules suggests that β-cells may interact directly with islet-infiltrating CD4$^+$ T-cells, and may play an immunopathogenic role.

The immune system plays a critical role in human type 1 diabetes pathogenesis. Varying proportions of T-cell subsets ($CD8^+$ and $CD4^+$) and B cells infiltrate the pancreatic islets (1) and target β-cells by recognizing type 1 diabetes-associated autoantigens (2, 3). The immunological mechanism(s) recruiting these cells to the islets had remained incompletely understood because until recently, islets from type 1 diabetic donors were not available for study. Antigen presentation to T-cells is mediated by antigen presenting cells (APCs) via two classes of HLA molecules: HLA Class I, recognized by $CD8^+$-expressing T-cells, (Class I is present on nearly all nucleated cells), and HLA Class II, recognized by $CD4^+$-expressing T-cells. Class II has a more limited tissue expression pattern, being present under most circumstances only on "professional" APCs, such as dendritic cells and macrophages (4).

Immunohistochemical studies of type 1 diabetic donor pancreas samples obtained at autopsy or by surgical biopsies (2, 5, 6, 7, 8, 9), have consistently reported islet cell "hyper-expression" of Class I molecules relative to that observed in non-diabetic donor pancreas sections. Even so, it was surprising when Bottazzo (10), Foulis (11) and others suggested that some β-cells from patients with type 1 diabetes also expressed HLA-DR Class II molecules (11, 12, 13, 14, 15, 16). This pattern of expression by β-cells was termed "aberrant" as expression is absent from β-cells in persons without type 1 diabetes. These reports raised the intriguing possibility that in individuals with type 1 diabetes, β-cell HLA-DR expression might allow direct presentation of autoantigens to infiltrating $CD4^+$ T-cells (17).

Several questions have been raised about these observations, not least because the identity of the islet cell subtypes affected was equivocal (18, 19). Some suggested that the cellular co-

localization of insulin and Class II might reflect β-cells phagocytosed by scavenger immune cells (e.g. macrophages). Moreover, the functional significance of any expressed Class II was questioned since β-cells may not have the capacity to load these molecules with antigens. The immunological consequences of β-cell Class II expression have been debated (20, 21) and remain unclear (22).

Using isolated human islets from donors with and without type 1 diabetes, we performed RNA-Seq of bulk sorted cells, single cell RNA-Seq of islet cells, and immunostaining of pancreatic islet sections and flow cytometry of dispersed islets to examine the β-cell specific expression of Class I and II molecules and associated regulatory genes. We now confirm that a subset of β-cells from donors with type 1 diabetes express Class II mRNA and cell surface protein and co-expresses CIITA, many other genes in the Class II pathway, and inflammation-associated gene products. The results indicate that β-cells can, when in a pro-inflammatory environment, upregulate Class II molecules *in vivo*. Overall, these results raise the possibility that β-cells may become involved in autoantigen presentation to $CD4^+$ T-cells during the pathogenic process culminating in type 1 diabetes.

RESEARCH DESIGN AND METHODS

Islets and Tissue

Isolated human islets from anonymous organ donors (exempt from Institutional Review Board review) were obtained through the Network of Pancreatic Organ Donors (nPOD), the Integrated Islet Distribution program (IIDP), Prodo Laboratories, Inc., or through the efforts of Vanderbilt University via either the National Disease Research Interchange (NDRI) or the International Institute for the Advancement of Medicine (IIAM). **Supplementary Table 1** lists all donors with demographic information. Some type 1 diabetic donor samples were processed as described (3).

Islets were isolated for RNA-Seq from 13 donors without diabetes (nine males, four females, age range from 10-30 years old; 16.1 +/- 1.6, mean +/- SEM) and from 4 donors with type 1 diabetes (three males and one female, age range from 12-24 years old; 20.3 +/- 2.6) with disease durations of 0.42, 2, 3, and 7 years.

Formalin-fixed paraffin embedded (FPPE) pancreas sections from donors with and without type 1 diabetes were from the Exeter Archival Diabetes Biobank (EADB) (http://foulis.vub.ac.be/), the Diabetes Virus Detection (DiViD) study (8), and nPOD. The four EADB donors without diabetes included at least three males (the 4th's gender was not reported) ranging in age from 5-47 years (24.8 +/- 10.9). The 18 donors with type 1 diabetes (from all three cohorts) studied using immunohistology included eleven males and seven females ranging in age from 1- 42 years (19.7 +/- 2.8 years) and with a range of disease duration of <1 week to 6 years (0.61 +/- 0.34). Full ethical approval was available for all samples studied and all six DiViD study

participants provided written consent.

Dissociation, Fixation, Staining, and Fluorescent Activated Cell Sorting (FACS)

Dissociation, fixation, and staining were as described previously (23), using the following stains: Zombie Violet Fixable Viability dye (BioLegend), anti-insulin Alexa fluor 647 (C27C9, Cell Signaling Technology), anti-glucagon (K79bB10, Sigma-Aldrich) labeled with Zenon Alexa fluor 568 and anti-somatostatin (7G5) labeled with Zenon Alexa fluor 488 (Thermo Fisher). Islet cells subsets (α-cells and β-cells) were sorted using a BD Biosciences FACSAria with the following gating strategy: live cells were selected based upon Zombie Violet dye exclusion. These cells were then selected based upon the presence or absence of an insulin positive signal. Insulin positive cells were further gated for single cells (FSC-H v. FSC-A) prior to sorting. All insulin negative cells were first gated for single cells and then gated for glucagon and somatostatin positive signals prior to sorting.

Cryopreserved non-diabetic donor islets (14149, 14128, 14219, and 14223) and cryopreserved islets from donors with type 1 diabetes (nPOD donors 6414, 6480, and 6323 and T1D.13, T1D.18, and T1D.19) were thawed (100-300 IEQ/aliquot) dispersed with Accutase™ (Stemcell Technologies), stained for viability (Live/dead Aqua™, Thermo Fisher), blocked with 50% human sera, and surface stained for CD45 (2D1, BioLegend) and +/- anti-HLA-DR (L243, BioLegend). After washing, cells were fixed, permeabilized and stained for intracellular insulin as described above. For treatment of fresh islets with proinflammatory cytokines, islets were cultured with IFNγ, IL-1β, and TNFα (R&D Systems) for 48 hours, dispersed with enzyme, and stained for viability before staining with anti-CD45 and anti-HLA-DR. Cells were washed,

fixed/permeabilized and stained intracellularly for insulin and glucagon as described. FMO controls were used to determine gates for each stain. Cells were run on a BD Biosciences LSRII and analyzed with FlowJo® (version 10.3) software.

RNA-Seq library construction, sequencing, gene expression, and statistical analysis

Sorted insulin$^+$ and glucagon$^+$ cells were pelleted and resuspended in Digestion Buffer and treated with Proteinase K for 3 hours at 50°C (RecoverAll, Thermo Fisher). The RecoverAll protocol for RNA isolation was followed for samples with at least 75,000 cells. For samples with less than 75,000 cells, nucleic acids were purified using Agencourt RNAClean XP beads (Beckman Coulter), digested with TURBO DNase (Thermo Fisher), and intact RNA was isolated with Agencourt RNAClean XP beads. RNAs were quantified using Qubit HS RNA (Thermo Fisher) and the Agilent Bioanalyzer RNA Pico chip was used to assess purity. Based upon RNA concentration and RNA integrity number (RIN), libraries were constructed using either the Ovation Human FFPE RNA-Seq Library System (NuGen) or the SMARTer Stranded Total RNA-Seq Kit (Pico Input Mammalian, Takara) and sequenced as 42bp paired-end reads on a NextSeq 500 (Illumina). We processed sequence data essentially as described in (24) using FastQC 0.10.1 for quality control analysis and FASTX-Toolkit version 0.0.13 to trim the low-quality ends of each read. Bowtie2 version 2.1.0 removed rRNA and snRNA, Tophat2 version 2.0.14 mapped reads to the genome and transcriptome, RSEM version 1.2.28 and Bowtie version 0.12.9 quantified gene abundance. Harman (25) normalized batch effects in transcripts per kilobase million (TPM) count data to account for technical differences in library construction while maintaining biological variability. To normalize for gene length, TPM counts were converted to expected counts which we submitted to DESeq2 version 1.10.2 with

parametric fitting and row sum = 10 for differential gene expression analysis. For statistical analysis, the Wald test with Benjamini-Hochberg correction was used. All bulk and single cell RNA-Seq data is available in the Gene Expression Omnibus (GEO) repository (DataSet Identifier GSE121863).

inDrop™ single cell RNA-Seq gene expression and statistical analysis

Dissociated islets from selected preparations were droplet encapsulated and single cell RNA-Seq libraries were generated as described (26). Sequence output from NextSeq 500 was analyzed as described (27) except that an additional filtering step was added to the ESAT output, where the unique molecular identifier (UMI) for each 'singleton' (gene transcripts represented by only one read) was compared to the UMIs of all transcripts for that gene. If any transcript with at least two reads was only one base different from the singleton, the singleton was merged with the multi-read transcript.

The islet cell subsets (α-cells and β-cells) were identified using a multistage process. In the initial stage, transcript count data subjected to TMM normalization (*DESeq2*), and principal component analysis (PCA, *prcomp*) was performed on the normalized data to identify genes that were most responsible for the variance in the data. Independent component analysis (ICA, *fastICA*) was applied for dimensionality reduction, followed by t-distributed stochastic neighbor embedding (t-SNE, *Rtsne*) and density-based clustering. T-SNE groups cells into clusters based on the similarity of their entire transcriptomes, such that cells of a similar type form distinct clusters. Resulting clusters were examined to find the genes that were most differentially expressed in each cluster compared to all other cells. These differentially expressed genes were used to

identify and remove cell clusters that were *not* α- or β-cells: for instance, we identified acinar (where the majority of the cells in the cluster showed relatively high expression of *CPA1, PRSS1*, and *PNLIP*), ductal (high in *KRT19*) stellate (*PDGFRB, COL1A1, ACTA2*), vascular (*PECAM1, ESM1, FLT1*), professional APC (high *HLA-DR*) and δ or PP cells (respectively high expression of somatostatin and pancreatic polypeptide Y). The remaining cells were then re-normalized and clustered using the steps described above, resulting in 5 clusters. The cells in one of the clusters were mainly from the donor with type 1 diabetes, and showed high expression of both insulin and glucagon RNA. These rare events likely represent 'doublets', i.e. droplets containing more than one cell. The remaining cells formed four distinct clusters composed mainly of β-cells (high insulin mRNA expression) and α-cells (high glucagon mRNA expression) from the non-diabetic donor and the donor with type 1 diabetes. For differential expression analysis, we used the clusters to divide the cells into α- and β-cells, and used the sample label to distinguish between the donor without diabetes and the donor with type 1 diabetes. Genes with a Benjamini-Hochberg adjusted p-value <0.05 were selected as differentially expressed, regardless of the log2 fold-change difference.

Immunostaining

A standard immunoperoxidase approach was used to examine single antigens on FFPE tissue sections (28). To examine multiple antigens within the same tissue, an immunofluorescence approach was employed in which antisera were applied sequentially (**Supplementary Table 2**). For some antibodies, the fluorescence signal was enhanced by tyramide signal amplification according the manufacturer's instructions (Thermo Fisher). Images were captured using either a Leica AF6000 fluorescence microscope (Leica) or a Leica SP8 confocal instrument with a PL

APO 40X/1.25NA lens and 488 and 561 nm laser lines. Analysis of the images was performed using either LAS AF software (Leica) or Image J Version 1.50b Java 1.8.0-77. In some cases, Huygens deconvolution software was used to take high-resolution confocal images (SVI).

Statistical Analysis

When two groups were compared, Student's t-test or Wilcoxon Signed Rank test was used. Where more than two groups were compared, a one-way ANOVA was employed with Tukey post hoc test to determine the statistical significance. Results were considered statistically significant when $p<0.05$.

Results

Bulk sorted β-cells from type 1 diabetic donors express Class I and Class I associated mRNA transcripts

Human isolated islets (**Supplementary Table 1**) were dissociated and insulin$^+$ β-cells obtained by FACS were used to create RNA libraries. RNA-Seq was performed to determine β-cell gene expression profiles from donors without diabetes (N=12; donor 17181 was only analyzed using inDrop™ single cell RNA-Seq) or those diagnosed with type 1 diabetes (N=4). Using a fold change of > 2 and *p* value < 0.05 as a cutoff to define differential expression, we found 650 differentially expressed genes in β-cells isolated from donors with type 1 diabetes (**Fig. 1)**. 504 genes were upregulated (red) while 146 others were downregulated (green). Class I and Class II pathway genes and pro-inflammatory-associated genes upregulated in the type 1 diabetic donor β-cells are labeled in the figure. Bulk FACS sorted type 1 diabetic donor β-cells (relative to donors without diabetes) heterogeneously displayed upregulated mRNA expression of *HLA-A, B,*

C, and F Class I mRNA transcripts as well as the Class I transactivator mRNA *NLRC5* (29). Gene expression levels ranged from 1.9 to 5.6-fold higher in the cohort of β-cells with type 1 diabetes, with significant *p* values ranging from 0.02 to $6.3x10^{-8}$ (**Supplementary Fig. 1)**. These results were consistent with immunohistochemical studies on pancreata from donors with type 1 diabetes (5, 6, 9, 29). In addition, genes for pro-inflammatory cytokines and associated factors were also found to be differentially expressed by β-cells (*IRF8, TNFRSF1B, TNF, IL1*β) (**Fig. 1**).

Bulk sorted β-cells from donors with type 1 diabetes express Class II and Class II related genes

Class II genes *HLA-DPA1, DPB1, DRA,* and *DRB1* were also significantly upregulated in the type 1 diabetic donor β-cells. Gene expression levels ranged from 18 to 52.4-fold at statistically significant *p* values ranging from $2.8x10^{-3}$ to $6.3x10^{-8}$ (**Fig. 1 and 2A**), highlighting the large donor to donor variability in gene expression and heterogeneous nature of HLA Class II pathway upregulation. Type 1 diabetic donor β-cells differentially expressed additional genes in the Class II pathway including the Class II invariant chain *CD74* and *CIITA*, the transcriptional regulator controlling Class II gene expression (**Figs. 2A and 2B**). We were not able to correlate expression of these factors and disease duration due to the variability seen in expression in these genes from the four donors with type 1 diabetes. Genes for two upstream CIITA transcriptional transactivators, *IRF8* and *SPI1,* and the downstream *CTSS* (cathepsin S enzyme that cleaves CD74 to yield the CLIP fragment) were also expressed at higher levels in type 1 diabetic donor β-cells relative to donors without diabetes (gene expression levels were increased by 5.9 to 54.8-fold with *p* values between $5.8x10^{-3}$ and $6.7x10^{-14}$). These findings show that the mRNA for Class II HLA and other Class II molecules and associated factors are upregulated in the β-cells

from donors with type 1 diabetes.

Single cell RNA-Seq reveals β-cell gene expression differences between a donor with type 1 diabetes and a donor without diabetes.

Applying a complementary methodology to study the β-cell population, single cells from dissociated islets were encapsulated using the inDrop™ system and prepared for RNA sequencing. Single islet cells (1,167 in total) from a 16-year-old donor without diabetes (donor 17181) and from a 23-year-old diagnosed with type 1 diabetes five months before death (nPOD donor 6414; 1,235 cells in total) were collected and sequenced. α-cells and β-cells were identified based on the absence of endothelial, stellate, ductal, and other non-endocrine cell markers, and validated by the presence of glucagon and insulin hormone expression. tSNE plots clustered the cells into unique subpopulations based on their gene expression patterns. The α-cells and β-cells separated into two subpopulations, predominately based on the donor's diabetes status (**Fig. 3A)**. In the donor without diabetes, 481/1,167 of the cells identified were α-cells or β-cells and there were approximately 0.84 β-cells for every α-cell. From the donor with type 1 diabetes, 468/1,235 of the cells were α-cells or β-cells, with approximately 0.59 β-cells for every α-cell. Consistent with the bulk type 1 diabetic donor β-cell population observations, the type 1 diabetic donor's β-cells were more frequently positive for *Class II* and *CD74* transcripts (**Fig. 3B)** with 35.2% of the type 1 diabetic donor's β-cells expressing *Class II* and/or *CD74* genes versus 7.8% of the β-cells from the donor without diabetes, a 4.5-fold difference.

Some β-cells in type 1 diabetic donor islets co-express Class II and CIITA

Type 1 diabetic donor pancreas section immunostaining revealed that Class II was expressed on

some cells within a subset of islets (**Fig. 4 A-C**). Class II expression was never observed in the non-diabetic donor pancreatic islet cells (**Supplementary Table 3**). Since the tissue used in these studies was recovered at autopsy, we also examined pancreas tissue collected by biopsy from six DiViD study living donors to obviate *post mortem* artifacts. In 5 of 6 DiViD cases, islet cells expressed Class II although the proportion of Class II immunopositive islets was variable, ranging from <10% in two cases to ~70% in another. From 4 pancreata from donors without diabetes (each containing between 50-100 islets), we found no islet CIITA expression (a representative image from one pancreas from a donor without diabetes, **Fig. 4 D**). However, in 8/12 (67%) age-matched type 1 diabetic donor pancreata, strong CIITA immunostaining was detected in at least 1 islet on each section examined, (**Fig. 4 E, F, G, I, J, L, M, O** and **Supplementary Table 3**). Most sections from donors with type 1 diabetes contained multiple islets staining for CIITA and this was largely, but not exclusively restricted to β-cells (**Fig. 4 G-I**). Of 20 islets from 4 donors with type 1 diabetes, 74.9% of CIITA positive cells co-stained for insulin. Even though CIITA exerts its effect in the nucleus, the cytoplasmic staining pattern for CIITA was also seen in human tonsil tissue (**Supplementary Fig. 2**) which is similar to the cytoplasmic CIITA staining seen in islet β-cells. Pancreas sections from two donors with type 1 diabetes (nPOD 6414 and 6367) with Class II and Class II-associated mRNA species (like *CIITA* mRNA), also showed immunoreactive Class II and CIITA protein expression in insulin^{+} β-cells (nPOD 6414, **Fig. 4 M-O)** and in certain insulin-negative islet cells (nPOD 6367, **Supplementary Fig. 3A-D**), confirming the transcriptome data.

Surface expression of HLA Class II on insulin^{+} β-cells was detected by co-localization with upregulated HLA-F expression on pancreas sections from donor E560 with type 1 diabetes **(Fig.**

5 A-E, Supplementary Fig. 3 E-G, and *z*-stack staining shown in **Online Supplemental Material).** Dispersed islets were analyzed for surface expression of CD45 and HLA-DR and intracellular expression of insulin by flow cytometry. Examples of $CD45^-$ and $CD45^+$ cells from islets are shown (**Supplementary Fig. 3 H-J**). From donors with type 1 diabetes (N =6), 15.64% +/- 8.01 of $CD45^-insulin^+$ islet cells had detectable surface HLA-DR as compared to those cells from donors without diabetes (N = 4, 1.32% +/- 1.32) (**Fig. 5 F-H**). Islet cells ($CD45^-$ $insulin^+$) from a donor without diabetes, that were treated with pro-inflammatory cytokines, upregulated surface HLA-DR (**Supplementary Fig. 3 K and L** and (30)).

Islet CIITA expression correlates with Class II, but not Class I or enterovirus VP1

Using immunohistology to study a range of pancreata from donors without diabetes, we found no evidence of cells expressing CIITA or Class II in islets. Conversely, in all type 1 diabetic cases studied where CIITA positive islets were detected, islets expressing Class II were also present (**Supplementary Table 3**). We next employed co-immunofluorescence staining to localize CIITA and Class II within islets and found that all islet endocrine cells expressing Class II also co-expressed CIITA (**Fig. 4 J-O**) while occasional CIITA positive islet cells did not stain positively for Class II.

In contrast to *Class II* mRNA species, the transcription of *Class I* genes is regulated by NLRC5 in many cell types. Earlier studies did not find elevated NLRC5 by immunostaining or RNA array analysis in type 1 diabetic donor islets which hyperexpressed Class I (9, 31). Using methods with higher sensitivity, we observed clear evidence of upregulated *NLRC5* mRNA in purified β-cells from the donors with type 1 diabetes (**Supplementary Fig. 1**). We examined

whether some Class I expressing cells might have elevated CIITA expression. As expected, numerous insulin-containing type 1 diabetic donor islets hyper-expressed Class I in all endocrine cell subtypes (**Supplementary Fig. 4 A-C**). CIITA was not found in islets having normal levels of Class I expression, but was present in a few cells in some islets with upregulated Class I. Many islets with elevated Class I had no detectable CIITA. In common with Class II, the enteroviral capsid protein VP1 has also been detected in a small subset of type 1 diabetic donor β-cells (32). CIITA was occasionally expressed in islets that also contained VP1-positive cells but, among a total of 29 $VP1^+$ cells in 12 islets, the two antigens were never seen in the same cells (**Supplementary Fig. 4 D-F**). When pancreas sections were co-stained with antisera directed against Class I and Class II, all Class II^+ β-cells had Class I expression, but many Class I positive islet cells had no detectable Class II (**Supplementary Fig. 5**).

Islet CIITA expression correlates with the number of islet-associated $CD45^+$ cells.

Activated $CD8^+$ T-cells secrete pro-inflammatory cytokines like IFNγ, which is reported to mediate CIITA (and thereby Class II) expression in certain non-hematopoietic cells (33, 34). Our immunohistological analysis suggested that islets with infiltrating or peri-islet $CD45^+$ lymphocytes contained more CIITA positive islet cells. In general, islets with more than 25% of endocrine cells expressing CIITA also had large numbers of lymphocytes within, or in contact with, the islet boundary (**Fig. 6A-C**). Thus, CIITA expressing islets display greater numbers of infiltrating $CD45^+$ cells as shown within individual donor sections (**Fig. 6D**), or when all type 1 diabetic donor islet data were pooled (**Fig. 6E**). As expected, certain immune cells located either at the islet periphery or in other pancreas section areas, also stained for CIITA and Class II (**Fig. 6C** and **Supplementary Fig. 4 D and F**).

CIITA and insulin co-stained cells are not macrophages which have phagocytosed β-cells

Since Class II and insulin expression within the same cell could be due to a professional APC engulfing a dead or damaged β-cell, we co-stained for CIITA, CD68 (a macrophage marker) and insulin. We found no cells stained by all three antisera. In fact, we found no cells that were stained for both insulin and CD68, although multiple CD68 positive cells were found across the sections (**Fig. 7 A-E**). As expected, CD68 positive cells expressed CIITA, albeit at much lower levels than that seen in those islet endocrine cells where this transcription factor was detected.

Discussion

Several lines of evidence have suggested that β-cells are not simply passive victims of a T-cell mediated destructive process in type 1 diabetes. We now report data obtained using multiple methodologies confirming that Class II molecules and transcripts, along with other molecules in the Class II pathway, are expressed by a population of type 1 diabetic donor endocrine cells. The Class II expressing cells were predominantly β-cells, although islet cells that did not stain for insulin were also seen to express Class II. In addition to other islet endocrine cells, these cells might include de-granulated, dysfunctional and de-differentiating β-cells. Our conclusions are supported by immunohistological, flow cytometric, and mRNA expression assays, using both bulk sorted and single β-cells isolated from donors with type 1 diabetes. The transcriptomic analysis revealed that the β-cells display increased levels of genes associated with both Class II expression (including *CIITA*, *IRF8* and *SPI1*) and function (*CD74* and *CTSS*, gene for cathepsin S), supporting the conclusion that β-cell Class II expression is a *bona fide* phenomenon in human type 1 diabetes. Immunohistological analysis verified that the small complement of β-cells

expressing Class II also stain for CIITA, which was mainly located in the cytoplasm rather than in the nucleus. Nuclear CIITA has been visualized using GFP or FLAG-tagged CIITA (35, 36), or by using sufficient nuclear protein for Western blot detection. However, we were unable to detect CIITA protein in the nucleus of endocrine or immune cells in pancreas tissue sections, in human tonsillar tissue sections (**Supplementary Fig. 2**), or in cell lines with confirmed surface HLA-DR Class II expression, including human mature monocyte-derived dendritic cells, two B-LCL cell lines, and a cell line transduced to express CIITA, CIITA-SupT1 (37) (data not shown). This is likely due to the blocking of the anti-CIITA antibody's binding site in the N-terminal region of the protein which overlaps with domains that bind to basal transcriptional machinery (38). We were not able to detect CIITA by Western blot of the nuclear fraction of $CD45^{-}$Class II^{+} cells sorted from proinflammatory cytokine-treated islets from donors without diabetes, most likely due to the insufficient quantity of nuclear protein recovered from the small number of cells sorted from dissociated islets, though we detected nuclear CIITA from B-LCL (data not shown). Even so, detection of Class II transcripts and proteins strongly indicates CIITA is performing its Class II transcriptional transactivation function in the nucleus. We also show that Class II and CIITA are absent from islet endocrine cells in individuals who do not have diabetes. The β-cell Class II upregulation appears to be unique to type 1 diabetes since it is not seen in people with type 2 diabetes or in other diseases of the pancreas such as cystic fibrosis or autoimmune pancreatitis (11).

Autoreactive $CD8^{+}$ T-cells within islets of donors with type 1 diabetes (2, 3, 39), Class I expression and CD8-mediated β-cell killing, and *in vitro* hyperexpression of Class I on β-cells in *in vitro* models are well-established (40, 41). However, surface expression of Class II on β-cells

implies that β-cells may serve as APC and may directly play a role in the autoimmune response. As has been reported (30), *in vitro* treatment of islets with pro-inflammatory cytokines upregulated Class II expression on the surface of some β-cells, detected by flow cytometry (**Supplementary Fig. 3K and L**). Co-localized Class II and HLA-F (9), an upregulated gene product in the β-cells of type 1 diabetic donors (**Supplementary Fig. 1**), was detected on the surface of β-cells from a donor with type 1 diabetes by immunofluorescence (**Fig. 5**) in a pattern also seen on dendritic cells (42). In the dendritic cell study, 5% of total Class II expression was estimated to be on the cell surface. From cryopreserved islets of 6 donors with type 1 diabetes, as compared to those from 4 donors without diabetes, by flow cytometry, we detected HLA-DR expression, albeit at a low intensity, on the surface of a portion (15.64 +/- 8.01%) of $CD45^-$ $insulin^+$ cells despite known decreased insulin expression from cryopreserved human islets (43).

In order to address the possibility that the co-localization of Class II with insulin-immunopositivity in islets might reflect the phagocytosis of dead or dying β-cells by professional APCs (44) rather than by the β-cells *per se*, we stained sections with an anti-CD68 antibody to detect resident macrophages (45, 46). As expected, the islet macrophages expressed Class II but, interestingly, these were readily distinguished from Class II-expressing β-cells as the $CD68^+$ cells displayed less intense CIITA immunostaining. We found no human islet macrophages that stained positively for CD68 and Class II and that also stained for insulin.

We also show that CIITA expression is closely correlated with inflammation in the type 1 diabetic donor islets, as judged by the presence of immune cells close to, or within, the islet boundary. Various lines of evidence suggest that certain pro-inflammatory cytokines (principally

IFNγ) can promote the expression of CIITA and Class II in non-hematopoietic cells and this has also been shown in islet cells (14, 47, 48, 49, 50). Hence, it is plausible that the local IFNγ associated with islet inflammation may drive β-cell Class II expression in type 1 diabetes. While this conclusion conflicts with other reports (11, 15), one potential explanation is the improved sensitivity of contemporary methods.

$CD8^+$ T-cells represent the most abundant cell type in the inflammatory infiltrates in individuals with type 1 diabetes (51, 52, 53) and since they can secrete IFNγ, it seems likely that infiltrating $CD8^+$ T-cells may promote β-cell Class II expression. It is unclear why the response is restricted to only a few scattered β-cells within any given islet, since the islet infiltrating lymphocyte-secreted cytokines should bathe most islet cells. One possibility is that the differential response may reflect the known heterogeneity among β-cells *in vivo* (53, 54, 55). Interestingly, several inflammatory-associated genes were found to be upregulated in β-cells from donors with type 1 diabetes (*IRF8, TNFRSF1B, TNF, IL-1*β), some of which are downstream of signaling by CXCL10, reported to be pivotal in recruiting immune cells to islets (56, 57, 58).

The β-cell CIITA and Class II expression now reported contrasts markedly with that reported for Class I which appears to be independent of inflammation (59). In support of a differential response, Class I hyperexpression was readily detected in islet cells that did not stain for CIITA and where no Class II was seen. Class I expression is often driven by the *NLRC5* complex (**Supplementary Fig. 6**) and, in the present transcriptomic analysis, we found increased *NLRC5* mRNA in the type 1 diabetic donor β-cells. This result stands in contrast to earlier work where no increase in NLRC5 was detected either by RNA array analysis of laser capture micro-

dissected islets from nPOD donors with type 1 diabetes or by immunostaining of islets in pancreas sections (9). The present data reinforce the conclusion that Class I is hyperexpressed on type 1 diabetic donor β-cells, and now suggest a role for NLRC5 in addition to STAT1 as previously shown (9).

The role of Class II-expressing professional APCs is to take up and process exogenous proteins, then present peptides in the context of Class II to $CD4^+$ T-cells. In the Class II pathway (**Fig. 8**), we have depicted this as a 'question mark' as β-cells' ability to take up exogenous antigen in this manner or present endogenous peptides is not known. A key question arising from this work is whether the β-cell Class II expression enables the cells to act within islets as surrogate APCs to $CD4^+$ T-cells. Neither CD80 nor CD86 (costimulation required for effective presentation to naïve, but not memory T-cells) could be detected on the β-cells of patients with recent onset type 1 diabetes (60), and we found no such staining in the present study (not shown). Whether other proteins could fulfill this costimulatory role is unknown.

Taken as a whole, this study clarifies the much-debated suggestion that some β-cells from individuals with type 1 diabetes express Class II, as well as associated pathway genes and inflammatory pathway genes. Further work is needed to determine whether, and how, β-cell Class II expression contributes to type 1 diabetes pathogenesis.

Acknowledgements

For the University of Massachusetts efforts, we acknowledge support from the Diabetes Center of Excellence and in particular Susanne Pechhold from the UMass Flow Cytometry Flow Core Facility, Drs. Rita Bortell, Laura Alonso, and Dale Greiner for helpful comments during the

study progress and data analysis, Megan E. DeNicola and Juanita Campos Rivera for technical assistance, and Patricia Cannon and Lisa Hubacz for administrative support. We thank Lawrence Stern (University of Massachusetts Medical School) for the SupT1 and CIITA-SupT1 cell lines. We are also grateful to Drs. Alberto Pugliese, Francesco Vendrame and George Burke III for helpful advice and discussions. We also thank Professor Alan Foulis for helpful discussion and access to samples.

K.D.-J. is principal investigator of the DiViD study. The authors thank the surgeons Bjørn Edwin and Trond Buanes and specialist nurse Trine Roald, Oslo University Hospital, Norway, whose invaluable efforts were essential to the success of the DiViD study.

We are grateful to the Organ Procurement Organizations partnering with the International Institute for Advancement of Medicine (IIAM) and National Disease Research Interchange (NDRI).

This research was performed with the support of the Network for Pancreatic Organ donors with Diabetes (nPOD; RRID:SCR-014641), a collaborative type 1 diabetes research project sponsored by JDRF (nPOD: 5-SRA-2018-557-Q-R) and The Leona M. & Harry B. Helmsley Charitable Trust (Grant#2018PG-T1D053). Organ Procurement Organizations (OPO) partnering with nPOD to provide research resources are listed at http://www.jdrfnpod.org//for-partners/npod-partners/. We thank the families of the donors.

This research was performed using resources and/or funding provided by the NIDDK-supported

Human Islet Research Network (HIRN, RRID:SCR-014393; https://hirnetwork.org; UC4 DK104211, DK108120, DK104218, DK112232, and DK116284), by DK106755, DK72473, DK89572, DK97829, DK94199, the Vanderbilt Diabetes Research and Training Center (DK20593), and by grants from the JDRF, the Leona M. and Harry B. Helmsley Charitable Trust (to DMH and ACP), the JDRF, NIDDK RO1 DK105837, Diabetes UK (16/0005480), and the Department of Veterans Affairs and a JDRF Career Development Award (5-CDA-2014-221-A-N) to S.J.R. The DiViD study was funded by grants from South-Eastern Norway Regional Health Authority (to K.D.-J.); The Novo Nordisk Foundation (to K.D.-J.) and through the PEVNET Study Group funded by the European Union Seventh (8) Framework Programme (FP7/2007-2013) under grant agreement 261441 PEVNET (http://www.uta.fi/med/pevnet/publications.html). DMB received support from the Babson Faculty Research Fund.

Duality of Interest

The authors declare there is no duality of interest associated with this manuscript.

Contribution statement

MAR, SJR, NGM designed the immunohistology studies, and analyzed that data. MAR and PL collected data. For the RNA transcriptome work, the bulk sorted human islet cell studies were designed and performed by DMB, SDR, and DMH, and the single cell studies were performed by CY and SDR. AGD, AK, and MGG were primarily responsible for the RNA-Seq analysis with biological insight from DMB, SDR, CY, SCK, and DMH. SCK, SDR, and JABB performed experiments with cryopreserved and fresh islets for flow cytometry. ACP, MB, RH, RB, and JMS procured or analyzed some patient material and data. LK and KD-J provided patient material from the DiViD study. All authors helped draft, then reviewed and approved the manuscript. NGM and DMH are guarantors of the work and, as such, had full access to all data in the study and take full responsibility for the integrity of the data and the accuracy of data analysis.

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Figure legends

Figure 1 - Volcano plot shows differential gene expression from bulk β-cell populations and highlights upregulation of immune response specific genes in β-cells from donors with type 1 diabetes. β-cells were isolated using FACS based on insulin expression from donors without diabetes (N=12) and with type 1 diabetes (N=4). RNA was isolated and libraries were sequenced. The volcano plot shows the 650 genes that are differentially ($p<0.05$ and fold-change>2) upregulated (red circles, 504 genes) and downregulated (green circles, 146 genes) in the donors with type 1 diabetes. The lines point to individual circles and identify the $-\log_{10}p_{adjusted}$ and $\log_2$FoldChange for specific genes.

Figure 2- Differentially expressed RNA transcripts from sorted β-cell populations displaying the increased T1D donor gene expression of Class II, upstream regulatory genes for the Class II pathway, and downstream response element genes. Gene expression levels from donors without diabetes (N=12, open circles) and with T1D (N=4, filled shapes; circle: nPOD 6414, diamond: nPOD 6367, square: nPOD 6268, triangle: T1D.6) are shown on the ordinate (as expected counts of raw values calculated using RSEM) and the gene name, expression fold change, and adjusted *p* value are shown below the abscissa. Increased Class II associated gene expression is shown in (A), i.e. mRNA for 4 Class II chain proteins (*HLA-DP* alpha and beta chains, *HLA-DR* alpha and beta chains) and the Class II invariant chain *CD74* in T1D donor β-cells and (B) Class II transactivator (*CIITA*) and its positive regulators *IRF8* and *SPI1*, and CLIP cleavage protein CTSS which plays a critical role in antigen loading on Class II. Bars indicate the mean values +SEM.

Figure 3- Single cell transcriptome using inDrop™ single cell RNA-Seq analysis to separate and identify α-cells (triangles) and β-cells (circles) from two donors, a 16-year-old male without diabetes (open shapes; donor ID 17181) and a 23-year-old donor male with 0.42 years duration of T1D (filled shapes; nPOD 6414). Single α- and β-cells were identified as described in the methods and plotted by tSNE into the observed subpopulations. (A) α- and β-cells show two distinct subpopulations, depending on donor diabetes status. (B) Focusing only the β-cell subpopulation (indicated by the dotted arrows), CD74 and/or Class II positive β-cells (red circles) are 4.5 times more prevalent in the donor with T1D (filled circles) than the donor without diabetes (unfilled circles).

Figure 4- Class II and CIITA are expressed in discrete islet endocrine cells in donors with T1D. Representative images of islets from donors with T1D from the EADB (A-B, E-L; E560 or E124B), (C) DiViD (DiViD 4), or nPOD (M-O, 6414) cohorts. Sections were immunostained with an antiserum raised against Class II protein (A-C, K, L, N, O), CIITA (D-G, I, J, L, M, O), and/or insulin (H, I, K, L, N, O) using either an immunoperoxidase (A-F) or immunofluorescence (G-O) approach. Representative images of pancreas sections probed with an antiserum against CIITA from either a healthy control individual (D, PM65/71) or from donors with T1D (E, F; E560, E124B). Black arrows indicate cells stained positively for Class II (B, C) or CIITA (E, F). (G-I) Representative immunofluorescence images from a pancreas from a donor with T1D (E560) probed for CIITA (G; red) and insulin (H; light blue). A merged image is shown (I). The red arrow indicates a cell positive for both CIITA and insulin. (J-O)

Representative images of T1D donor pancreas sections from EADB (J-L; E560) and nPOD (M-O; 6414) were probed with CIITA (J, M; red), Class II protein (K, N; green) and insulin (K (inset), L, N (inset), O; light blue). Merged images are shown (L, O). Class $II^{+}insulin^{+}CIITA^{+}$ cells are denoted by the white arrow in (L, O). The nPOD donor examined was part of the cohort from which RNA transcriptome data is shown in Figures 2 and 3. Scale bar (A,C-F) 50μm, (B,J-O) 10μm, (G-I) 20μm.

Figure 5 - β-cell expression of HLA-Class II (HLA-DR) and on the surface of a portion of $CD45^{-}insulin^{+}$ cells from donors with T1D. (A-E) Representative image of a pancreas section from a single donor with T1D (E560) stained for Class II (A, C-E; green), HLA-F (B-E; red) and insulin (D; light blue), with merged images shown (C-E). An enlargement of the highlighted area in panel D (dotted white box) is presented in A-C and E. A high-resolution image of the same cells is shown in **Supplementary Fig. 3** and in **Online Supplementary Material**. Images were captured at x400 magnification unless otherwise stated; scale bar (A-E) 25μm. Cryopreserved, non-hand-picked islets (100-300 IEQ/stain) were thawed, washed, dispersed with enzyme, washed, stained for viability, washed and blocked with 50% human serum and then surface stained for CD45 and with and without anti-HLA-DR (L243 mAb). Cells were washed and stained for intracellular insulin as described in the methods. Single viable cells were analyzed for surface CD45 and intracellular insulin (left panels of each group). $CD45^{-}insulin^{+}$ cells were then analyzed for HLA-DR expression (middle and right panels of each group). Islets from donors without diabetes (14223, 14219, 14128. and 14149) are shown in (F). Islets from donors with type 1 diabetes (T1D.13, T1D.18, T1D.19, nPOD6323, nPOD6414, and nPOD6480; disease duration is indicated left of the sample identifier) are shown in (G). Samples from 14223 and

nPOD6480 were prepared and analyzed as a set, 14219 and nPOD6414 were prepared and analyzed as a set, and the remainder of the samples were prepared and analyzed as one set. Gates were set for FMO stains for each marker. For HLA-DR staining, this is shown for each sample. The percentage of $CD45^{-}insulin^{+}HLA\text{-}DR^{+}$ cells for each sample are shown in Q2 for each sample (far right columns in F and G). A comparison of the frequencies of $CD45^{-}insulin^{+}HLA\text{-}DR^{+}$ cells for donors without diabetes and donors with type 1 diabetes is shown in (H) by Wilcoxon Signed Rank test. Donor samples nPOD6414, nPOD6367, and 14223 were analyzed for RNA transcriptome (Figs.1 and 2 and Supplementary Fig. 1).

Figure 6- The number of CIITA positive islet cells in sections of pancreas from individuals with T1D correlates with the presence of infiltrating immune cells. (A-C) Representative images of pancreas sections from 3 donors with T1D stained for CD45 (red) or CIITA (green). Islets are highlighted with dashed lines and the images depict islets with either less than 25% of CIITA positive cells (A) or those containing more than 25% CIITA positive cells (B). An enlargement of the highlighted area (dotted white box) is also presented (C). Arrows indicate cells amongst the immune cell infiltrate positive for CIITA. Images were captured at x200 magnification. Multiple <25% and >25% CIITA+ islets were imaged in each of five pancreases from donors with T1D and the number of CD45+ cells within (or immediately adjacent to) each islet was counted. (D) Data are shown for each individual case studied and (E) for all cases combined. Bars indicate the mean values ±SEM. Scale bar, 20μm. **p<0.01, ***p<0.001.

Figure 7- Islet cells staining for CIITA and insulin are not CD68 immunopositive. Immunofluorescence staining of a pancreas section from a donor with T1D, probed for CIITA (A, B, D, E: red), CD68 (B, C, E: green) and insulin (B, E: light blue). Enlargement of the highlighted area (dotted white box) in the upper right panel is shown (C, D, E). The red arrow indicates an insulin positive cell which stained positively for CIITA but not CD68. The green arrow highlights a nearby CD68 immunopositive cell which is also positive for CIITA, but negative for insulin. The asterisks indicate areas of autofluorescence. Images captured at x400 magnification; scale bar, 10μm.

Figure 8- Upregulated protein and mRNA species in T1D donor β-cells from the Class II processing and presentation pathway. The observed upregulated proteins and mRNA species from the Class II processing and presentation pathway T1D donor β-cells are displayed according to their intracellular locations and with their functions. Depicted by symbols with black borders are upregulated proteins (red circles) and mRNA species (red rectangles), while putative upregulated proteins identified by upregulated mRNA species are shown with red ovals, diamonds or elongated ovals. Gray ovals depict mRNA species that were detected, but not upregulated in the β-cells from islets of donors with type 1 diabetes.

Fig. 1

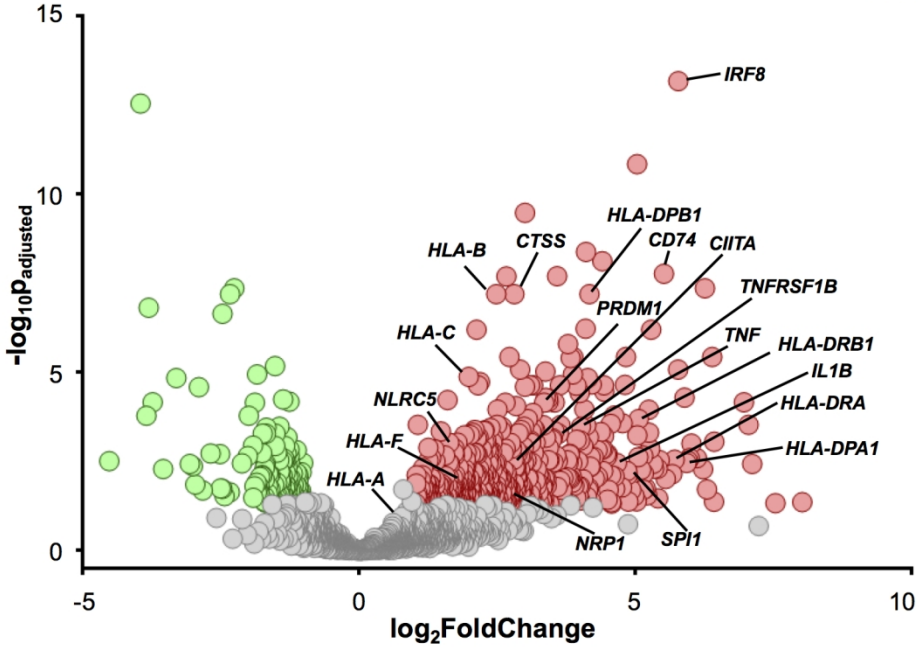

Figure 1 - Volcano plot shows differential gene expression from bulk β-cell populations and highlights upregulation of immune response specific genes in β-cells from donors with type 1 diabetes. β-cells were isolated using FACS based on insulin expression from donors without diabetes (N=12) and with type 1 diabetes (N=4). RNA was isolated and libraries were sequenced. The volcano plot shows the 650 genes that are differentially ($p<0.05$ and fold-change>2) upregulated (red circles, 504 genes) and downregulated (green circles, 146 genes) in the donors with type 1 diabetes. The lines point to individual circles and identify the -log10padjusted and log2FoldChange for specific genes.

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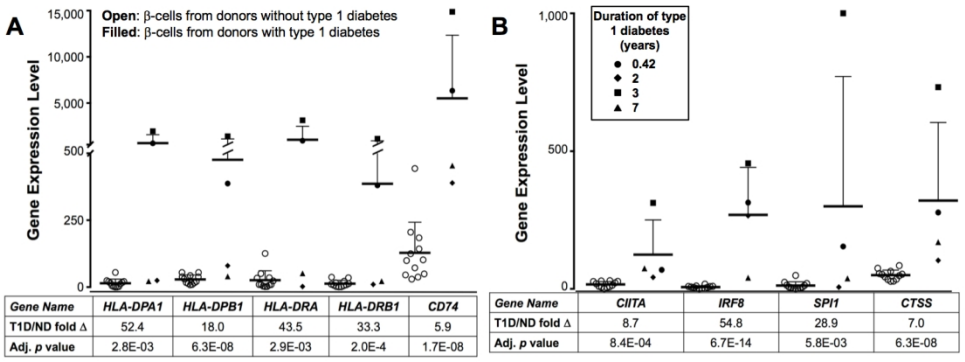

Gene Name	HLA-DPA1	HLA-DPB1	HLA-DRA	HLA-DRB1	CD74
T1D/ND fold Δ	52.4	18.0	43.5	33.3	5.9
Adj. *p* value	2.8E-03	6.3E-08	2.9E-03	2.0E-4	1.7E-08

Gene Name	CIITA	IRF8	SPI1	CTSS
T1D/ND fold Δ	8.7	54.8	28.9	7.0
Adj. *p* value	8.4E-04	6.7E-14	5.8E-03	6.3E-08

Fig. 2

Figure 2- Differentially expressed RNA transcripts from sorted β-cell populations displaying the increased T1D donor gene expression of Class II, upstream regulatory genes for the Class II pathway, and downstream response element genes. Gene expression levels from donors without diabetes (N=12, open circles) and with T1D (N=4, filled shapes; circle: nPOD 6414, diamond: nPOD 6367, square: nPOD 6268, triangle: T1D.6) are shown on the ordinate (as expected counts of raw values calculated using RSEM) and the gene name, expression fold change, and adjusted p value are shown below the abscissa. Increased Class II associated gene expression is shown in (A), i.e. mRNA for 4 Class II chain proteins (HLA-DP alpha and beta chains, HLA-DR alpha and beta chains) and the Class II invariant chain CD74 in T1D donor β-cells and (B) Class II transactivator (CIITA) and its positive regulators IRF8 and SPI1, and CLIP cleavage protein CTSS which plays a critical role in antigen loading on Class II. Bars indicate the mean values +SEM.

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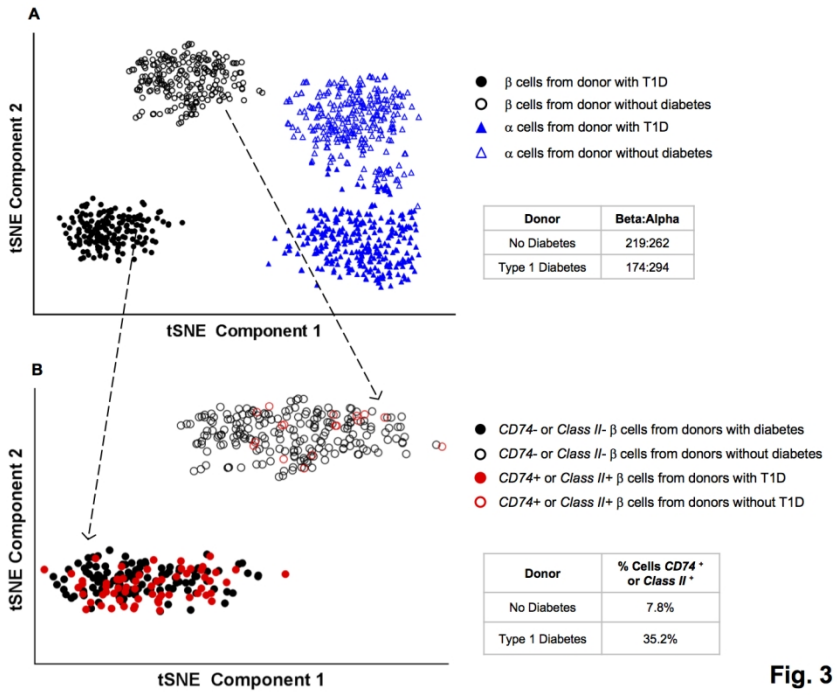

Fig. 3

Figure 3- Single cell transcriptome using inDrop™ single cell RNA-Seq analysis to separate and identify α-cells (triangles) and β-cells (circles) from two donors, a 16-year-old male without diabetes (open shapes; donor ID 17181) and a 23-year-old donor male with 0.42 years duration of T1D (filled shapes; nPOD 6414). Single α- and β-cells were identified as described in the methods and plotted by tSNE into the observed subpopulations. (A) α- and β-cells show two distinct subpopulations, depending on donor diabetes status. (B) Focusing only the β-cell subpopulation (indicated by the dotted arrows), CD74 and/or Class II positive β-cells (red circles) are 4.5 times more prevalent in the donor with T1D (filled circles) than the donor without diabetes (unfilled circles).

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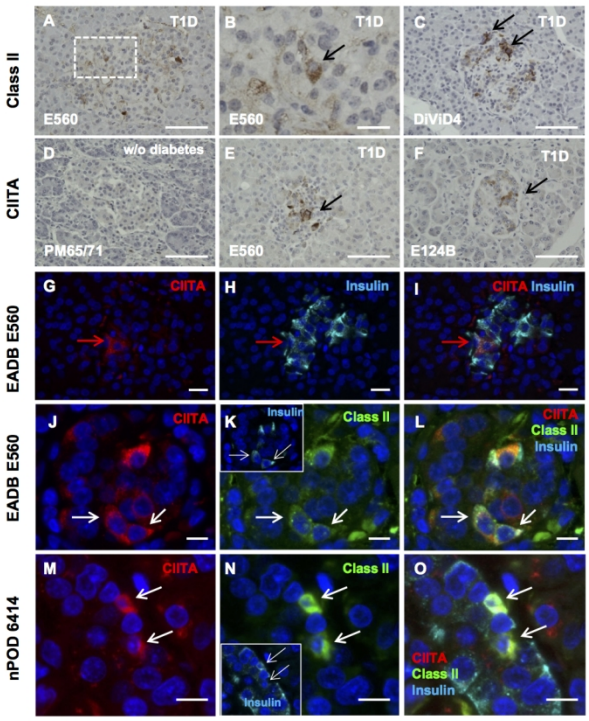

Fig. 4

Figure 4- Class II and CIITA are expressed in discrete islet endocrine cells in donors with T1D. Representative images of islets from donors with T1D from the EADB (A-B, E-L; E560 or E124B), (C) DiViD (DiViD 4), or nPOD (M-O, 6414) cohorts. Sections were immunostained with an antiserum raised against Class II protein (A-C, K, L, N, O), CIITA (D-G, I, J, L, M, O), and/or insulin (H, I, K, L, N, O) using either an immunoperoxidase (A-F) or immunofluorescence (G-O) approach. Representative images of pancreas sections probed with an antiserum against CIITA from either a healthy control individual (D, PM65/71) or from donors with T1D (E, F; E560, E124B). Black arrows indicate cells stained positively for Class II (B, C) or CIITA (E, F). (G-I) Representative immunofluorescence images from a pancreas from a donor with T1D (E560) probed for CIITA (G; red) and insulin (H; light blue). A merged image is shown (I). The red arrow indicates a cell positive for both CIITA and insulin. (J-O) Representative images of T1D donor pancreas sections from EADB (J-L; E560) and nPOD (M-O; 6414) were probed with CIITA (J, M; red), Class II protein (K, N; green) and insulin (K (inset), L, N (inset), O; light blue). Merged images are shown (L, O). Class II+insulin+CIITA+ cells are denoted by the white arrow in (L, O). The nPOD donor examined was part of the cohort from which RNA transcriptome data is shown in Figures 2 and 3. Scale bar (A,C-F) 50μm, (B,J-O) 10μm, (G-I) 20μm.

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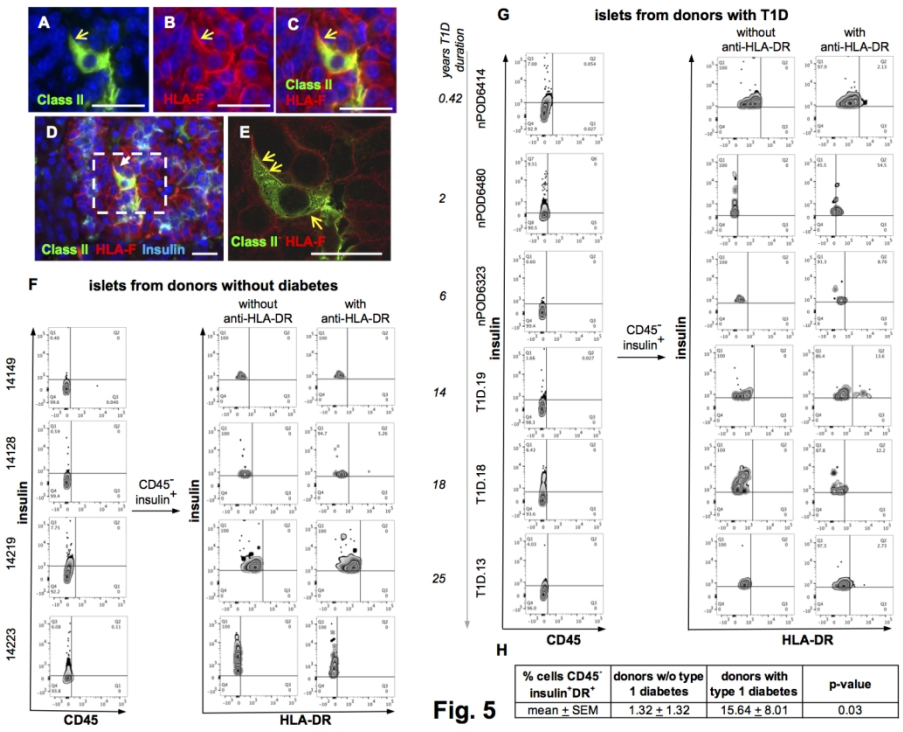

% cells CD45⁻ insulin⁺DR⁺	donors w/o type 1 diabetes	donors with type 1 diabetes	p-value
mean ± SEM	1.32 ± 1.32	15.64 ± 8.01	0.03

Figure 5 - β-cell expression of HLA-Class II (HLA-DR) and on the surface of a portion of CD45-insulin+ cells from donors with T1D. (A-E) Representative image of a pancreas section from a single donor with T1D (E560) stained for Class II (A, C-E; green), HLA-F (B-E; red) and insulin (D; light blue), with merged images shown (C-E). An enlargement of the highlighted area in panel D (dotted white box) is presented in A-C and E. A high-resolution image of the same cells is shown in Supplementary Fig. 3 and in Online Supplementary Material. Images were captured at x400 magnification unless otherwise stated; scale bar (A-E) 25μm. Cryopreserved, non-hand-picked islets (100-300 IEQ/stain) were thawed, washed, dispersed with enzyme, washed, stained for viability, washed and blocked with 50% human serum and then surface stained for CD45 and with and without anti-HLA-DR (L243 mAb). Cells were washed and stained for intracellular insulin as described in the methods. Single viable cells were analyzed for surface CD45 and intracellular insulin (left panels of each group). CD45-insulin+ cells were then analyzed for HLA-DR expression (middle and right panels of each group). Islets from donors without diabetes (14223, 14219, 14128. and 14149) are shown in (F). Islets from donors with type 1 diabetes (T1D.13, T1D.18, T1D.19, nPOD6323, nPOD6414, and nPOD6480; disease duration is indicated left of the sample identifier) are shown in (G). Samples from 14223 and nPOD6480 were prepared and analyzed as a set, 14219 and nPOD6414 were prepared and analyzed as a set, and the remainder of the samples were prepared and analyzed as one set. Gates were set for FMO stains for each marker. For HLA-DR staining, this is shown for each sample. The percentage of CD45-insulin+HLA-DR+ cells for each sample are shown in Q2 for each sample (far right columns in F and G). A comparison of the frequencies of CD45-insulin+HLA-DR+ cells for donors without diabetes and donors with type 1 diabetes is shown in (H) by Wilcoxon Signed Rank test. Donor samples nPOD6414, nPOD6367, and 14223 were analyzed for RNA transcriptome (Figs.1 and 2 and Supplementary Fig. 1).

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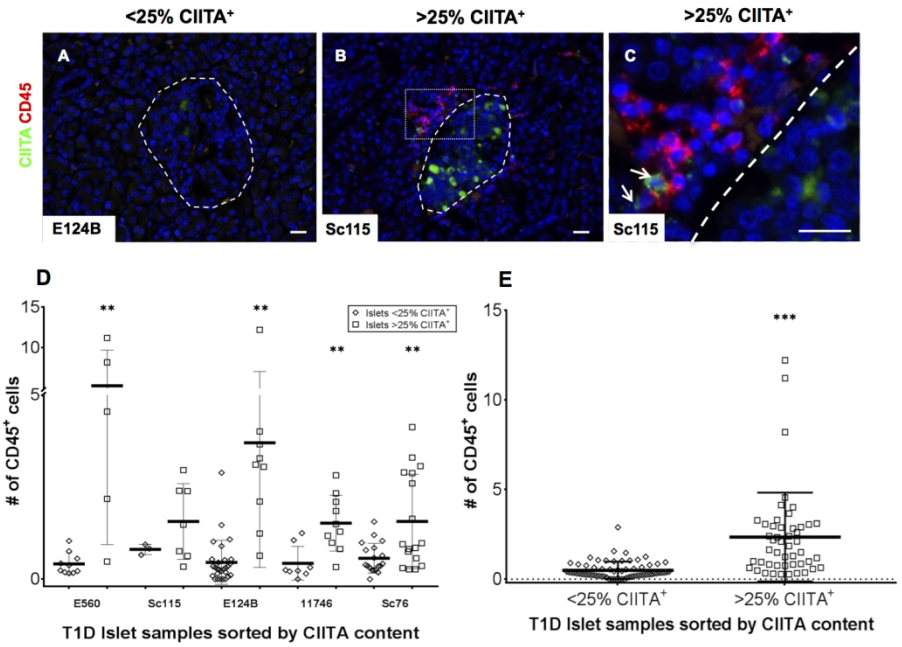

Fig. 6

Figure 6- The number of CIITA positive islet cells in sections of pancreas from individuals with T1D correlates with the presence of infiltrating immune cells. (A-C) Representative images of pancreas sections from 3 donors with T1D stained for CD45 (red) or CIITA (green). Islets are highlighted with dashed lines and the images depict islets with either less than 25% of CIITA positive cells (A) or those containing more than 25% CIITA positive cells (B). An enlargement of the highlighted area (dotted white box) is also presented (C). Arrows indicate cells amongst the immune cell infiltrate positive for CIITA. Images were captured at x200 magnification. Multiple <25% and >25% CIITA+ islets were imaged in each of five pancreases from donors with T1D and the number of CD45+ cells within (or immediately adjacent to) each islet was counted. (D) Data are shown for each individual case studied and (E) for all cases combined. Bars indicate the mean values ±SEM. Scale bar, 20μm. **p<0.01, ***p<0.001.

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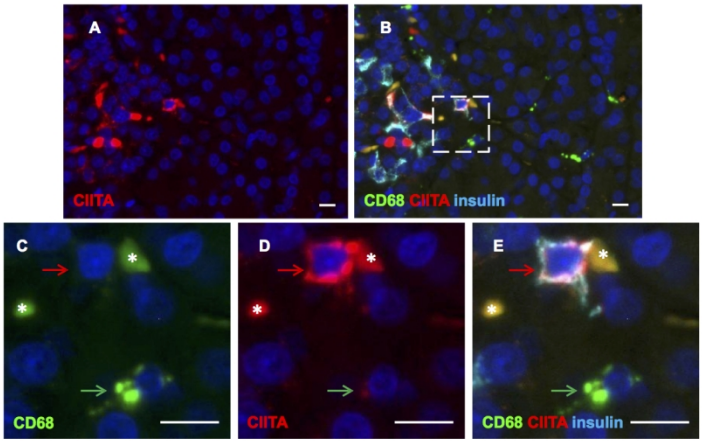

Fig. 7

Figure 7- Islet cells staining for CIITA and insulin are not CD68 immunopositive. Immunofluorescence staining of a pancreas section from a donor with T1D, probed for CIITA (A, B, D, E: red), CD68 (B, C, E: green) and insulin (B, E: light blue). Enlargement of the highlighted area (dotted white box) in the upper right panel is shown (C, D, E). The red arrow indicates an insulin positive cell which stained positively for CIITA but not CD68. The green arrow highlights a nearby CD68 immunopositive cell which is also positive for CIITA, but negative for insulin. The asterisks indicate areas of autofluorescence. Images captured at x400 magnification; scale bar, 10μm.

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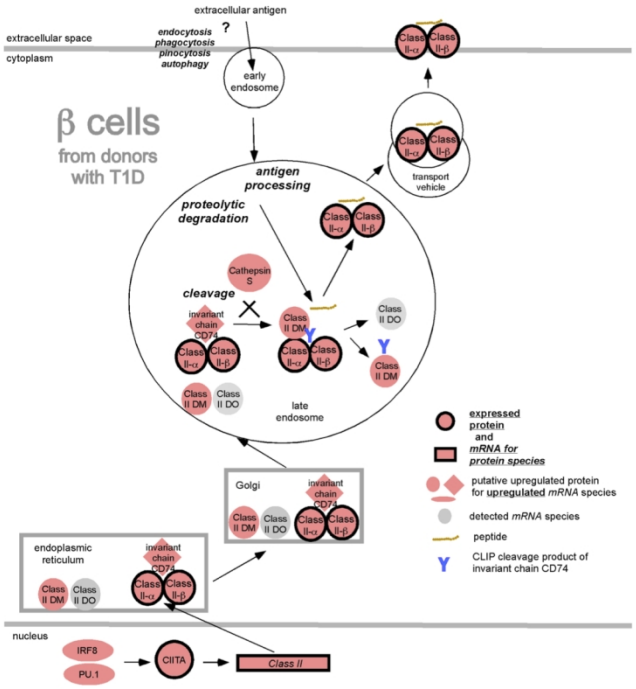

Fig. 8

Figure 8- Upregulated protein and mRNA species in T1D donor β-cells from the Class II processing and presentation pathway. The observed upregulated proteins and mRNA species from the Class II processing and presentation pathway T1D donor β-cells are displayed according to their intracellular locations and with their functions. Depicted by symbols with black borders are upregulated proteins (red circles) and mRNA species (red rectangles), while putative upregulated proteins identified by upregulated mRNA species are shown with red ovals, diamonds or elongated ovals. Gray ovals depict mRNA species that were detected, but not upregulated in the β-cells from islets of donors with type 1 diabetes.

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Supplementary Table and Figure Legends

Supplementary Table 1 - Demographics and identifiers of donor samples. Isolated β-cells from donors used for transcriptome analyses and tissue sections from donors without diabetes (w/o diabetes) and donors with type 1 diabetes used for immunohistochemical analyses (immunohistochemical and/or immunofluorescence, IHC/IF), RNA-Seq, or flow cytometry (FC) are listed from the EADB, DiViD, VU/UMass (VU/UMass cohort were obtained through a collaborative agreement with Vanderbilt University), or from UMass (from either Prodo Labs or IIDP, RRID codes) cohorts. Partial data previously described for the samples 13226 and 13255 in (23) and for T1D.6 in (3). N/A: not applicable. N/R: not reported. w, m, y: weeks, months, or years of disease duration.

Supplementary Table 2- Antibody details and immunocytochemistry conditions used for staining human pancreas specimens are detailed.
IHC; immunohistochemistry, IF; immunofluorescence.

Supplementary Table 3 - Case by case examination of CIITA and Class II staining. Green colour indicates cases in which CIITA was absent, whereas red colour demarks cases containing CIITA positive islets. ND: not determined.

Supplementary Figure 1 - Bulk β cell population, as sorted by intracellular insulin staining, isolated from donors with type 1 diabetes shows increased HLA Class I expression. β cell gene expression (shown as expected counts of raw values calculated using RSEM) in donors without diabetes (N=12, open circles) and with type 1 diabetes (N=4, filled shapes, circle: nPOD 6414, diamond: nPOD 6367, square: nPOD 6268, triangle: T1D.6) are shown on the ordinate for 4 HLA Class I mRNA species (*HLA-A, HLA-B, HLA-C, and HLA-F*) and the HLA Class I transactivator, *NLRC5*. The fold change increase in expression from donors with type 1 diabetes and the adjusted *p* value are shown below the abscissa. Bars indicate the mean values +SEM.

Supplementary Figure 2- CIITA is expressed in the tonsil, and has predominantly a cytosolic localisation. (A) Representative image of tonsil tissue from a single donor stained using an immunoperoxidase approach probed with an antibody raised against CIITA. An enlargement of the highlighted area (dotted white box) is presented (B). The white arrow indicates cells with high levels of cytoplasmic, but low nuclear CIITA. Images were captured at x400 magnification; scale bar (A) 50μm, (B) 20μm.

Supplementary Figure 3 – Surface expression of HLA Class II on β-cells. From a single donor with T1D (nPOD 6367) from which RNA transcriptome data is shown in Figs. 1 and 2 and Supplementary Fig. 1, pancreas tissue was stained with antibodies to HLA Class II (green, A, D), insulin (light blue, B, D), and CIITA (red, C, D). Merged images are shown (D). White arrows denote a Class $II^{+}insulin^{-}CIITA^{+}$ cell. Images captured at x400 magnification; scale bar, 10μm. A high-resolution image of the same cell shown in Fig. 5 (Fig. 5 A-D) was captured using deconvolution software at x630 magnification with optical zoom (E-G). The yellow arrows indicate areas in which Class II and HLA-F co-localise. An entire *z*-stack of this region is presented as **Online**

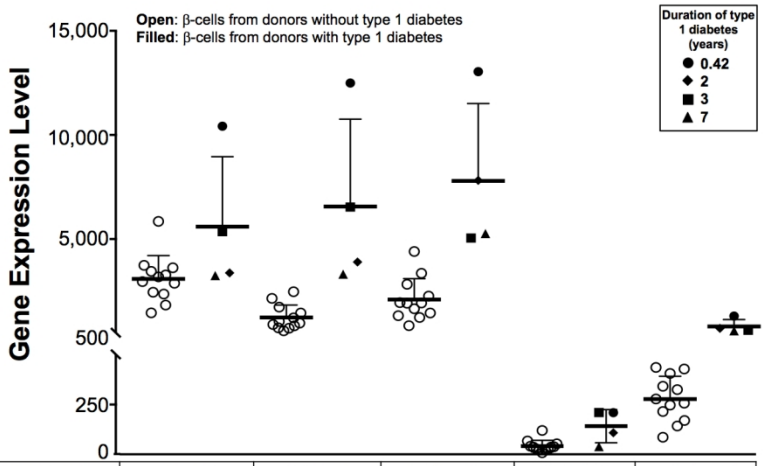
Supplementary Material. $CD45^+$ cells from islets are detected by flow cytometry. As described in Fig. 5, islets were dispersed and surface stained for CD45 and HLA-DR. Examples of the $CD45^-$ and $CD45^+$ populations within two islet samples [nPOD6367 (an islet sample analyzed for transcriptome, Figs. 1 and 2) (H) and T1D.19 (I)] are shown. A representative profile of surface $CD45^+$ cells for HLA-DR staining is shown for T1D.19 (J). $CD45^-$ cells were then analyzed for surface HLA-DR and intracellular insulin as shown in Fig. 5. Representative plots are shown for islets for Induction of surface HLA Class II expression on islet β-cells by pro-inflammatory cytokines (K and L). 2000 IEQ from a control, non-diabetic donor (18076) were treated +/- proinflammatory cytokines as indicated in the figure for 48 hours. Islets were collected, dispersed with enzyme, stained with viability dye, blocked, surface stained for CD45 and HLA-DR (L243), washed, fixed/permeabilized and stained intracellularly for glucagon and insulin as described. Approximately 50% of $insulin^+$ β-cells upregulated surface HLA-DR upon treatment with proinflammatory cytokines. Similar results were seen with 3 other islet isolations.

Supplementary Figure 4 - CIITA expression does not correlate with the presence of Class I or enterovirus VP1, in the islets of donors with type 1 diabetes. Representative images from pancreas sections of individuals with type 1 diabetes stained with antisera raised against CIITA (A, red), Class I (B, green), Class II (D, red), VP1 (E, green) and insulin (C, F, light blue). Merged images are presented (C, F). White arrows in the upper panel (A-C) point to examples of CIITA immunopositive cells. White arrows (D-F, lower panels) indicate Class II positive cells that are negative for VP1. Yellow arrows show a VP1 positive cell which is immunonegative for Class II. Red arrows indicate an example of an immune cell that stains positive for HLA Class II in the islet periphery. Images captured at x400 magnification; scale bar 20μm.

Supplementary Figure 5 - Class I and Class II expression do not correlate within islets. Representative immunofluorescence image of a pancreas section from a donor with type 1 diabetes stained for Class I (A, D: red), Class II (B, D: green) and insulin (C, D: light blue). The white arrow indicates an islet cell co-expressing Class I and Class II, whereas the yellow arrow marks a cell within the islet that is positive for Class I, but negative for Class II. Islet associated endothelial cells expressing Class II were also observed (green arrow). Images captured at x400 magnification; scale bars, 20μm.

Supplementary Figure 6 - Upregulated protein and mRNA species in β cells from donors with type 1 diabetes from the HLA Class I processing and presentation pathway. The observed upregulated proteins and mRNA species from the HLA Class I processing and presentation pathway in β cells from donors with type 1 diabetes are displayed according to their intracellular locations and with their functions. Depicted by symbols with black borders are upregulated proteins (red circles) and mRNA species (red squares), while putative upregulated proteins identified by upregulated mRNA species are shown with red ovals, diamonds or elongated ovals, and gray ovals depict mRNA species that were detected, but not upregulated in the islets of donors with type 1 diabetes. A detected, upregulated protein, STAT1 (9), but without mRNA detection, is indicated by an empty circle with a black border.

Additional Online Supplementary Material Figure 1 - *z*-stack of HLA Class II and HLA -F co-localise on the surface of an islet cell in a donor with type 1 diabetes. A high-resolution z-stack of the islet shown in Fig. 5 A-E and Supplementary Fig. 3A-D is presented. Sections from a donor with type 1 diabetes (E560) were stained with antisera against Class II (green) and HLA-F (red), with areas of co-localisation appearing yellow. This islet was imaged using deconvolution software at x630 magnification with additional optical zoom.



Gene Name	*HLA-A*	*HLA-B*	*HLA-C*	*HLA-F*	*NLRC5*
T1D/ND fold Δ	1.9	5.6	4.0	3.5	3.1
Adj. *p* value	0.09	6.3E-08	1.3E-05	0.02	9.4E-05

Supplementary Fig. 1

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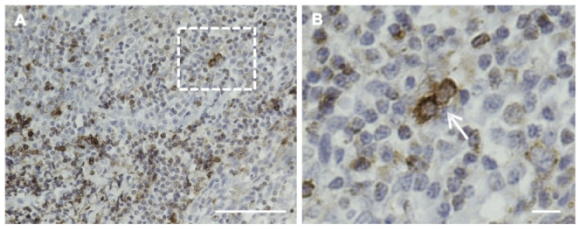

Supplementary Figure 2

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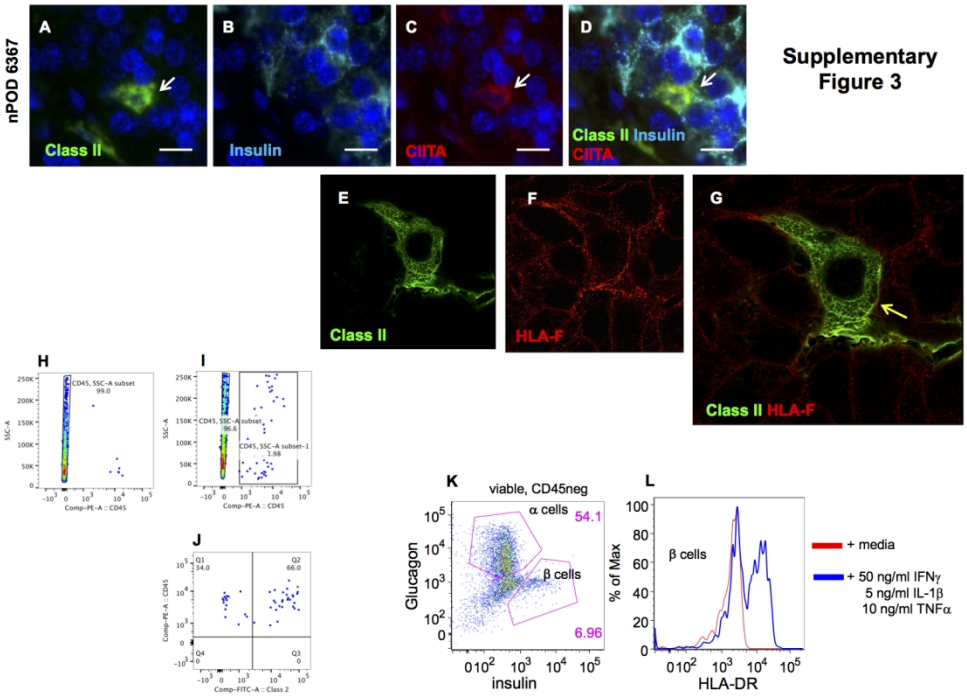

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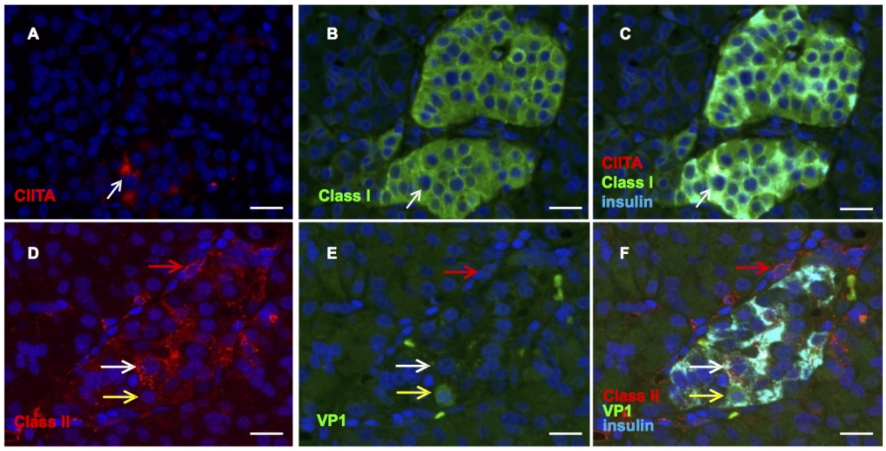

Supplementary Figure 4

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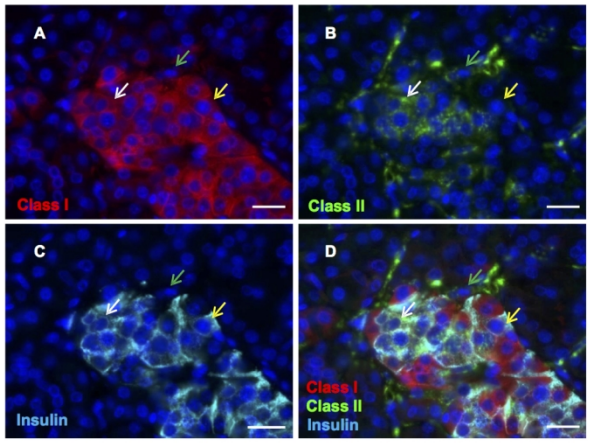

Supplementary Figure 5

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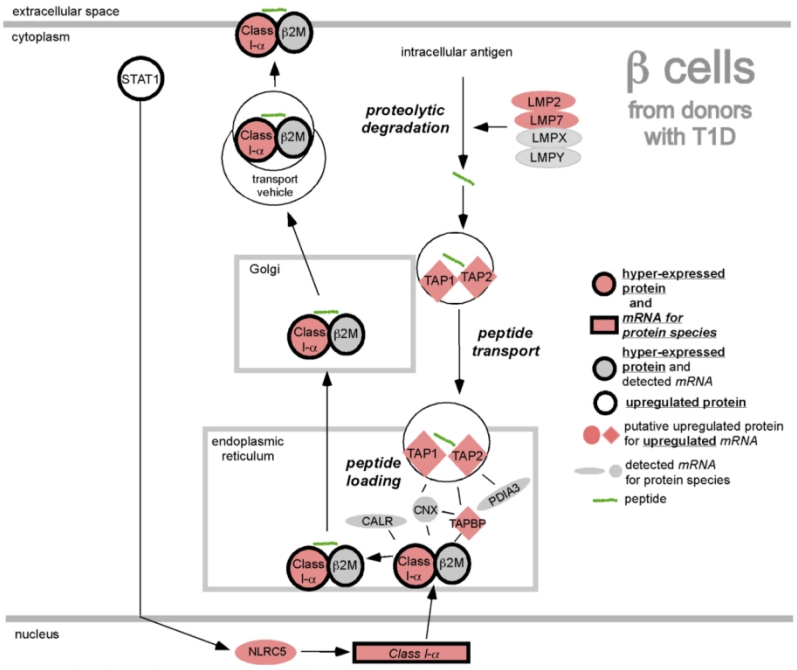

Supplementary Figure 6

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A- Donors without diabetes

Case Code	RRID Source: IIDP	Case Type	Cohort	Use of islets	Age (years)	Gender
PM65/71		w/o diabetes	EADB	IHC/IF	40	M
8579		w/o diabetes	EADB	IHC/IF	7	N/R
330/71		w/o diabetes	EADB	IHC/IF	47	M
184/90		w/o diabetes	EADB	IHC/IF	5	M
17188		w/o diabetes	VU/UMass	RNAseq	10	F
14223		w/o diabetes	VU/UMass	RNAseq, FC	11	M
16149		w/o diabetes	VU/UMass	RNAseq	11	M
16181		w/o diabetes	VU/UMass	RNAseq	12	F
16191		w/o diabetes	VU/UMass	RNAseq	12	F
16278	SAMN0876 9124	w/o diabetes	UMass	RNAseq	15	M
16029		w/o diabetes	VU/UMass	RNAseq	16	M
16338		w/o diabetes	VU/UMass	RNAseq	16	F
17181		w/o diabetes	VU/UMass	RNAseq	16	M
13226		w/o diabetes	UMass	RNAseq	18	M
17103		w/o diabetes	VU/UMass	RNAseq	19	M
15049		w/o diabetes	UMass	RNAseq	23	M
13255	SAMN0878 3913	w/o diabetes	UMass	RNAseq	30	M
18076		w/o diabetes	UMass	FC	33	F
14128	SAMN0877 5087	w/o diabetes	VU/UMass	FC	45	M
14149	SAMN0877 5077	w/o diabetes	VU/UMass	FC	49	F
14219	SAMN0877 5025	w/o diabetes	UMass	FC	52	F

B- Donors with type 1 diabetes

Case Code	Case Type	Cohort	Use of islets	Age (years)	Gender	Time from diagnosis
E560	T1D	EADB	IHC/IF	42	F	18m
E124B	T1D	EADB	IHC/IF	17	M	1w
E375	T1D	EADB	IHC/IF	11	F	1w
11746	T1D	EADB	IHC/IF	6	M	<1w
11713	T1D	EADB	IHC/IF	3	M	3m
Sc76	T1D	EADB	IHC/IF	21	M	3w
Sc115	T1D	EADB	IHC/IF	1	F	<1w
Sc109	T1D	EADB	IHC/IF	20	M	6y
9256	T1D	EADB	IHC/IF	2	M	2w
8566	T1D	EADB	IHC/IF	12	F	3w
DiViD1	T1D	DiViD	IHC/IF	25	F	4w
DiViD2	T1D	DiViD	IHC/IF	24	M	3w
DiViD3	T1D	DiViD	IHC/IF	34	F	9w
DiViD4	T1D	DiViD	IHC/IF	31	M	5w
DiViD5	T1D	DiViD	IHC/IF	24	F	5w
DiViD6	T1D	DiViD	IHC/IF	35	M	5w
nPOD6414	T1D	nPOD	RNAseq IHC/IF/FC	23	M	5m
nPOD6480	T1D	nPOD	FC	17	M	2y
nPOD6367	T1D	nPOD	RNAseq IHC/IF/FC	24	M	2y
nPOD6268	T1D	nPOD	RNAseq	12	F	3y
nPOD6323	T1D	nPOD	FC	22	F	6y
T1D.6	T1D	VU/UMass	RNAseq	20	M	7y
T1D.18	T1D	VU/UMass	FC	24	F	18y
T1D.19	T1D	VU/UMass	FC	54	F	14y
T1D.13	T1D	VU/UMass	FC	56	M	25y

Supplementary Table 1

279x215mm (150 x 150 DPI)

Primary Antibody	Manufacturer and clone	Antigen Retrieval	Antibody Dilution	Incubation Time with Primary Antibody	Secondary Detection System
CIITA	Santa Cruz C#sc-13556 Mouse monoclonal	10mM citrate pH6.0	1/100	IHC 1h RT	Immunohistochemistry using Dako REAL™ Envision™ Detection System or immunofluorescence using tyramine signal amplification kit (HRP conjugated secondary antibody: 1/100 for 1h, fluorescent tyramide substrate; 1/100 10mins)
				IF o/n 4°C	
HLA-DR	Santa Cruz C#sc-25614 Rabbit polyclonal	10mM citrate pH6.0	1/400	IHC 1h RT	Immunohistochemistry using Dako REAL™ Envision™ Detection System or immunofluorescence using tyramine signal amplification kit (HRP conjugated secondary antibody: 1/100 for 1h, fluorescent tyramide substrate; 1/100 10mins)
				IF o/n 4°C	
HLA-ABC	Abcam C#ab70328 Mouse monoclonal	10mM citrate pH6.0	1/1000	1h at RT	Immunofluorescence staining using anti-mouse IgG (H+L) Alexa Fluor™-conjugated secondary antibodies (1/400 for 1hr)
Insulin	Dako C#A0564 Guinea-pig polyclonal	10mM citrate pH6.0	1/700	1h at RT	Immunofluorescence staining using anti-guinea-pig IgG (H+L) Alexa Fluor™ -conjugated secondary antibodies (1/400 for 1hr)
CD45	Dako C#M0701 Mouse monoclonal	10mM citrate pH6.0	1/1000	1h at RT	Immunofluorescence using tyramine signal amplification kit (HRP conjugated secondary antibody: 1/100 for 1h, fluorescent tyramide substrate; 1/100 10mins)
CD68	Dako C#M0876 Mouse monoclonal	10mM citrate pH6.0	1/400	1h at RT	Immunofluorescence staining using anti-mouse IgG (H+L) Alexa Fluor™-conjugated secondary antibodies (1/400 for 1hr)
CD80	Abcam C#ab86473 Mouse monoclonal	10mM citrate pH6.0	1/500	o/n at 4°C	Immunohistochemistry using Dako REAL™ Envision™ Detection System or immunofluorescence staining using anti-mouse IgG (H+L) Alexa Fluor™-conjugated secondary antibodies (1/400 for 1hr)
CD86	Abcam C#ab53004 Rabbit monoclonal	10mM Tris, 1mM EDTA pH9.0	1/300	o/n at 4°C	Immunohistochemistry using Dako REAL™ Envision™ Detection System
VP1	Dako C#M7064 Mouse monoclonal	10mM citrate pH6.0	1/2000	o/n at 4°C	Immunofluorescence staining using anti-mouse IgG (H+L) Alexa Fluor™-conjugated secondary antibodies (1/400 for 1hr)

Supplementary Table 2

279x215mm (150 x 150 DPI)

Case Code	Case Type	Islets + or – for CIITA	Number of Class II+ islets
PM65/71	without diabetes	-	0
8579	without diabetes	-	0
330/71	without diabetes	-	0
184/90	without diabetes	-	0
Sc109	with type 1 diabetes	-	0
9256	with type 1 diabetes	-	0
8566	with type 1 diabetes	-	1
E375	with type 1 diabetes	-	ND
E560	with type 1 diabetes	+	16
E124B	with type 1 diabetes	+	84
Sc76	with type 1 diabetes	+	113
SC115	with type 1 diabetes	+	ND
11746	with type 1 diabetes	+	22
11713	with type 1 diabetes	+	16
DiViD3	with type 1 diabetes	+	26
DiViD6	with type 1 diabetes	+	2

Supplementary Table 3

279x215mm (150 x 150 DPI)