

The Extra-Islet Pancreas Supports Autoimmunity in Human Type 1 Diabetes

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eLife Assessment

This **valuable** study leverages innovative high-dimensional imaging strategies to interrogate pancreatic immune cell profiles and distributions throughout stages of type 1 diabetes (T1D). Despite a notable limitation in the number of donor samples analyzed, the authors identify a series of intriguing "immune signatures" and histopathological features that collectively constitute a **solid** foundation for future investigations into immunological processes underpinning the pathogenesis of T1D. Accordingly, the work will be of considerable interest to the community of T1D researchers and clinicians.

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Abstract

In autoimmune Type 1 diabetes (T1D), immune cells infiltrate and destroy the islets of Langerhans — islands of endocrine tissue dispersed throughout the pancreas. However, the contribution of cellular programs outside islets to insulinitis is unclear. Here, using CO-Detection by indEXing (CODEX) tissue imaging and cadaveric pancreas samples, we simultaneously examine islet and extra-islet inflammation in human T1D. We identify four sub-states of inflamed islets characterized by the activation profiles of CD8⁺T cells enriched in islets relative to the surrounding tissue. We further find that the extra-islet space of lobules with extensive islet-infiltration differs from the extra-islet space of less infiltrated areas within the same tissue section. Finally, we identify lymphoid structures away from islets enriched in CD45RA⁺ T cells — a population also enriched in one of the inflamed islet sub-

states. Together, these data help define the coordination between islets and the extra-islet pancreas in the pathogenesis of human T1D.

Introduction

In Type 1 diabetes (T1D), insulin-producing β -cells are killed by islet-infiltrating immune cells in a process called “insulinitis”. T1D results in a critical requirement for exogenous insulin and affects over eight million individuals world-wide with an estimated 0.5 million new diagnoses each year.¹

Recently, the first immunotherapy for delaying T1D onset, teplizumab (a human anti-CD3 monoclonal antibody) was approved by the US Food and Drug Administration.² However, this treatment and other immunotherapies help only a small fraction of patients and are significantly less effective after patients progress to overt T1D.^{3–9} A better understanding of T1D pathogenesis is essential to building on this progress.

One of the challenges of studying human T1D pathology is the availability of suitable tissue samples. Obtaining pancreatic biopsies raises the risk of surgical complications and the progressive nature of T1D would necessitate serial, longitudinal studies over time, which is prohibitive.¹⁰ Fortunately, the Juvenile Diabetes Research Foundation (JDRF) Network for Pancreatic Organ Donors with Diabetes (nPOD) have provided human pancreatic tissues from cadaveric donors for this study.^{11,12} nPOD has enabled substantial progress towards characterizing the pathology of human T1D.^{13–17}

Our understanding of key features of human T1D pathology remains limited. Although the cellular composition of insulinitis, inflammation specifically of the islets, has been studied extensively, this has been done in separate studies looking at different tissue sections, prohibiting an understanding of how the numerous cellular programs in insulinitis are coordinated throughout disease. This was recently addressed using Imaging Mass Cytometry (IMC), which uncovered alterations in β -cell phenotypes, immune composition, vascular density, and basement membrane that accompany T1D.^{18,19} However, these studies did not deeply phenotype islet-infiltrating CD8⁺T cells, believed to be major driver of β -cell elimination.

Recently, intriguing differences in the extra-islet spaces of T1D and healthy controls have been reported. First, the abundance of multiple types of immune cells outside islets are increased in T1D patients compared to non-T1D controls.^{20–22} Second, HLA-DR expression is increased on ductal cells in T1D tissue donors, hinting at a functional link with CD4⁺T cells.²³ Third, peri-insulinitis, the accumulation of immune cells outside islets, is observed in tissues from patients with T1D²⁴, indicating that not all T cells enter the pancreas directly via islet microvasculature.²⁵ Fourth, in human T1D, but less so in most animal models, islets in different regions of the pancreas are infiltrated at strikingly different rates for reasons that are unknown.²⁶ This suggests that the extra-islet compartment could be responsible by governing the targeting of islets. Finally, tertiary lymphoid structures (TLS) — dense aggregates of lymphoid cells indicative of local immune activation — are observed outside islets in T1D patients.¹⁷

In summary, analyzing both compartments simultaneously could help identify how these extra-islet factors influence islet pathogenesis. However, to date, multiplexed imaging studies have only examined islets. A comprehensive, spatially resolved cellular analysis of both compartments in T1D is lacking.

Results

Here, we investigated the islet and extra-islet pancreas together. We used CO-Detection by indexing (CODEX) with an antibody panel targeting 54 antigens to samples from a cohort of T1D patients with insulinitis as well as non-T1D individuals with and without islet-specific autoantibodies (AA- and AA+ respectively) obtained through the JDRF nPOD program. We analyzed approximately 2000 islets and broad swaths of the extra-islet tissue to evaluate local and distal spatial architecture. We then used pseudotime analysis to characterize insulinitis sub-states based on the activation states of islet-infiltrating CD8⁺T cells. We further investigated the cellular changes in niches and lobules beyond islets. Our results implicate both the islet microenvironment and inflammation at distal sites within the pancreas in the progression of insulinitis.

Cohort curation, image acquisition, and cell annotation

The JDRF nPOD is a national registry of cadaveric pancreases donated by T1D patients that has transformed the ability of researchers to investigate the pathways underlying the progression of human T1D.^{11,12} Insulinitis is present in only a small fraction of T1D cases, including those available from nPOD.^{27,28} Although nPOD has close to 200 T1D cases, at the time of our study, nPOD had only 17 with documented insulinitis. Of these, triple-immunohistochemistry for Insulin, Glucagon, and CD3 was performed. T1D and AA+ cases that had CD3+ staining in islet or peri-islet spaces and tissue still available were selected for our study. The final cohort included two AA+ cases, eight T1D cases, and three non-T1D controls. Given that insulinitis is not detected in non-T1D cases,²⁹ the blocks from controls were selected randomly. The T1D cases varied in the time between diagnosis and death from 0 years (diagnosed at death) to 6 years (**Figure 1.A**, left). The causes of death were mostly unrelated to T1D complications (Supplemental Table 1). Therefore, the time since diagnosis is not a reflection of the aggressiveness of the individual's disease.

Large regions averaging 55 mm² were imaged with CODEX as previously described^{30–32}. Regions were selected to capture islets and the surrounding region simultaneously (**Figure 1.A**, center and right). CellSeg was used to segment cell nuclei and quantify marker expression from CODEX images as previously described³³. In total, our dataset consisted of 7.0×10⁶ cells across all donors (ranging from 3.0×10⁵ to 9.8×10⁵ cells per donor). Twenty-one cell types were identified with Leiden clustering and manual merging and visualized using Uniform Manifold Approximation and Projection (UMAP) (**Figure 1.B**, Supplemental Table 2). Endocrine cells were manually gated from UMAP embeddings derived from Proinsulin, Glucagon, and Somatostatin to identify β-cells, α-cells, and δ-cells respectively. Immune cells were sub-clustered with the Leiden algorithm using immune-specific markers (**Figure 1.C**, Supplemental Table 2). To verify the accuracy of our annotations, we overlaid cell labels onto the original images (Supplemental Figure 1.A). Of note, we could not accurately identify macrophage subsets or distinguish dendritic cells from macrophages due to the panel design, complex combinations of co-expression, and the difficulty in segmenting and quantifying markers on myeloid populations due to their morphology. Therefore, we refer to this cluster as “macrophage/DCs” In addition, we identified a cell population that could not be definitively annotated, expressing high levels of CD45, CD69, Granzyme-B, and CD44, intermediate levels of CD16, S100A6, Galectin-3, and Hyaluronan, but not expressing CD3, CD20, CD56, CD57, CD15, or MPO. We confirmed from the raw images that CD3, CD4, and CD8 were not internalized, indicating activation, nor did these cells express other T cell activation markers CD45RA, CD45RO, PD-1, or LAG-3 (**Figure 1.C**, Supplemental Figure 1A, bottom right). This population could represent a type of innate lymphoid cell³⁴ and was labeled “Granzyme-B⁺/CD3⁻”.

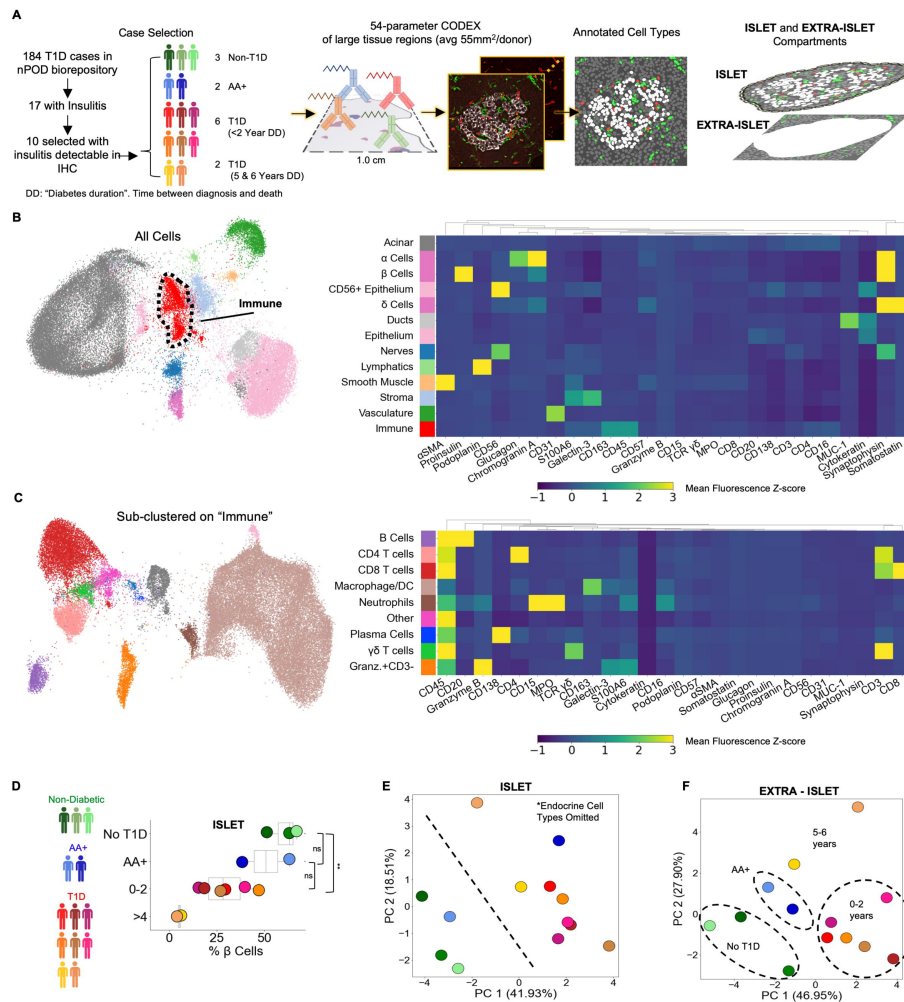


Figure 1

Profiling T1D pancreata with CODEX high-parameter imaging reveals alterations in the cellular composition of islet and extra-islet compartments.

Figure 1.A Left: Schematic of the workflow for selection of nPOD cases. Blues, greens, and reds indicate non-T1D, AA+, or T1D status, respectively. Center: Schematic for acquisition and processing of CODEX highly multiplexed imaging dataset. Right: Schematic of islet and extra-islet pancreatic regions.

Figure 1.B UMAP and Leiden clustering of major cell types. Colors match those in the heatmap shown to the right. Heatmap of mean z-normalized marker expression in each cell-type cluster. Only a subset of the markers used for the UMAP are included in the heatmap to facilitate visualization. A full description of the markers used for the clustering stages is available in Supplemental Table 2.

Figure 1.C UMAP of the immune population identified in Figure 1B further clustered using additional immune markers as described in Supplemental Table 2. Colors match those in heatmap shown in the heatmap to the right. The heatmap is generated in an identical manner as the heatmap in Figure 1B.

Figure 1.D Frequency of β -Cells per donor determined by dividing the number of β -cells by the total number of β -cells, α -cells, and δ -Cells. Blues, greens, and reds indicate non-T1D, AA+, or T1D status, respectively. Significance was determined using the t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Figure 1.E Principal component analysis of the islet compartment. The number of cells of each cell type (omitting α -, β -, and δ -cells) were divided by the number of endocrine cells to adjust for different islet areas. Blues, greens, and reds indicate non-T1D, AA+, or T1D status, respectively.

Figure 1.F Principal component analysis of the extra-islet compartment. The number of cells of each cell type (omitting α -, β -, and δ -cells) were divided by the number of acinar cells to adjust for different areas imaged. Blues, greens, and reds indicate non-T1D, AA+, or T1D status, respectively.

Islet- and extra-islet regions are altered in T1D

We first sought to identify cellular changes in T1D within islets specifically. Previous reports observed that insulin-containing islets are significantly more common in recent-onset T1D cases than cases with diabetes durations of greater than one year^{27,35,36}. Similarly, we found that samples from patients who had been diagnosed with T1D for 0-2 years had significantly reduced β -cell frequencies compared to non-T1D controls. Furthermore, samples from subjects with disease durations of 5-6 years had minimal remaining β -cell mass (**Figure 1.D**). Whereas one AA+ case had β -cell mass comparable to those of cases with disease duration of 0-2 years, the other AA+ case was comparable to non-T1D controls (**Figure 1.D**). The total islet area imaged was comparable across all donors (Supplemental Figure 1.B).

Next, we investigated how the abundances of non-endocrine cell types inside islets differed across donors. We performed Principal Component Analysis (PCA) on the donors using the frequencies of non-endocrine cell types located in islets. Donors were clearly separated into two groups by the first two principal components; one group included all T1D cases and one AA+ case and the second included all non-T1D cases and the other AA+ case (**Figure 1.E**). In this analysis, we did not consider β -cells, α -cells, and δ -cells. Thus, donors were stratified by disease duration strictly according to the abundances of immune and other pancreatic, non-endocrine cell types in the islets.

We next considered only cells located outside islets. Again, donors were clearly separable by the first two principal components (**Figure 1.F**). The first principal component separated cases with times since diagnosis between 0-2 years from non-T1D, AA+, and cases with diabetes durations of 5-6 years (**Figure 1.F**). The second principal component separated cases with diabetes durations of 5-6 years from the rest (**Figure 1.F**). Therefore, both the islet and extra-islet spaces of T1D and non-T1D cases were distinct.

Many cell types were increased in T1D cases with times since diagnosis of 0-2 years relative to non-T1D controls (Supplemental Figure 1C). In T1D cases with times since diagnosis of 5-6 years, the abundance of different cell types either remained higher than non-T1D controls or returned to baseline (Supplemental Figure 1C). This trend was present in both islet and extra-islet regions. These data demonstrate that the immune activity between the islet and extra-islet compartments are coordinated.

Pseudotemporal reconstruction of islet pathogenesis identifies a conserved trajectory of insulinitis

In human T1D, β -cell destruction does not occur simultaneously across all islets and even neighboring islets can be at different stages of destruction^{18,26,27}. We therefore used pseudotime analysis to infer the most likely progression of a single islet through disease space¹⁸. To develop a pseudotemporal map, we quantified the cellular composition of each islet, including cells within 20 μm of the islet's boundary, and applied the pseudotime algorithm PArTition-based Graph Abstraction (PAGA)³⁷ (**Figure 2.A**; **Figure 2.B**). PAGA was selected because it is a high-performing algorithm able to identify multiple trajectories, if they exist, while making minimal assumptions about the true structure³⁸.

Displayed in **Figure 2.B** is the PAGA force-directed layout where each point represents an islet. Each islet's color reflects the pseudotemporal distance from the centroid of non-T1D islets. As expected, the islets from different donor groups (no T1D, AA+, T1D) had different distributions across the PAGA map (**Figure 2.C**). In the PAGA map, a continuum is apparent from islets abundant in insulin-expressing β -cells on the left of the map to islets depleted in β -cells on the right (**Figure 2.D**, **Figure 2.E**, **Figure 2.F**, top row). PAGA uses Leiden clustering internally, enabling the following regions of the pseudotime map to be labelled objectively: 1) Islets with low

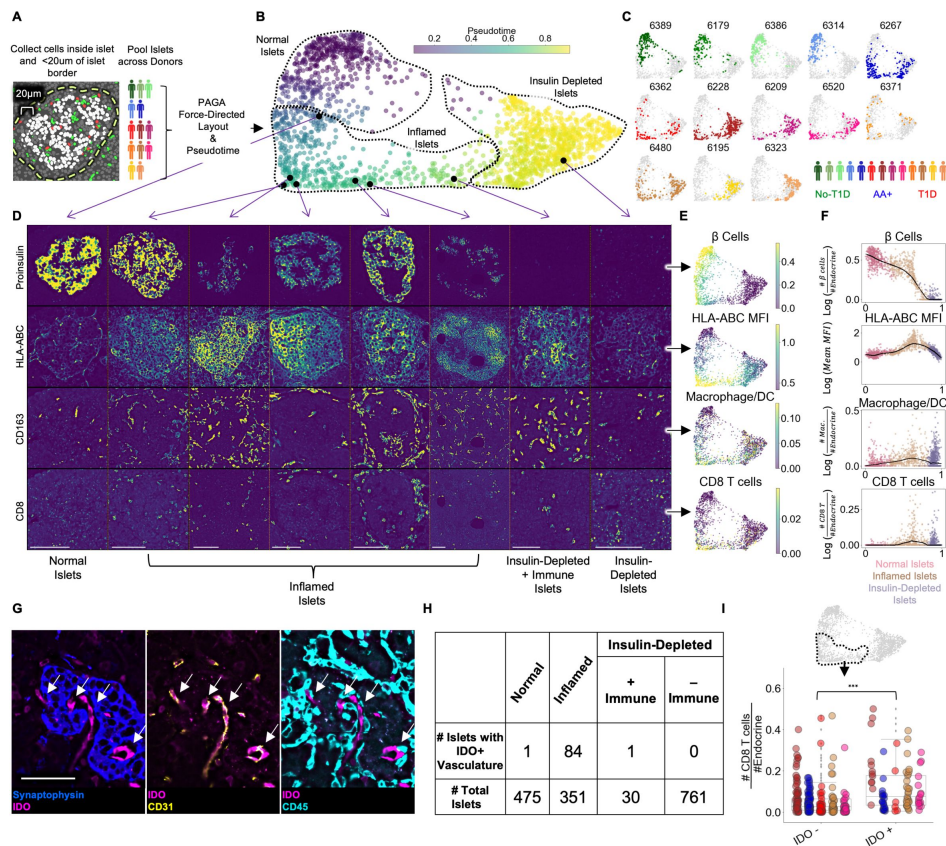


Figure 2

Pseudotemporal reconstruction of insulitis identifies IDO on islet vasculature.

Figure 2.A Schematic of islet segmentation and quantification of islet cellular composition.

Figure 2.B PAGA-force directed layout of islets colored by pseudotime. The start point of pseudotime was calculated as the centroid of the non-T1D islets. Representative islets from different stages of pseudotime are indicated with black points and their raw images are depicted in **Figure 2.D**. Normal, Inflamed, and Insulin-Depleted groups were obtained by merging the clusters output by the PAGA algorithm (Supplemental Figure 2.A).

Figure 2.C Islet distribution across pseudotime for each donor. The titles indicate nPOD case IDs as in Supplemental Table 1. The frequency of islets from each donor in each stage of islet pseudotime is reported in Supplemental Figure 2.C.

Figure 2.D Images of Proinsulin, HLA-ABC, CD163, and CD8 staining in islets representative of different points along pseudotime as indicated in B. Scale bars (lower left of each column) indicate 100µm.

Figure 2.E Quantification of selected features across pseudotime overlaid onto the PAGA force-directed layout. For β-cells, macrophage/DCs, and CD8⁺T cells, the values correspond to log(# cells/# endocrine cells). For HLA-ABC, the mean HLA-ABC expression for each cell in the islet was computed and log transformed.

Figure 2.F Quantification of selected features across pseudotime. For β-cells, macrophage/DCs, and CD8⁺T cells, the values correspond to log(# cells/# endocrine cells). For HLA-ABC, the mean HLA-ABC expression for each cell in the islet was computed and log transformed. Color legend: Normal islets: pink; Inflamed islets: brown; Insulin-Depleted islets: purple. Black points demarcate LOWESS regression.

Figure 2.G Representative image of an islet from the Inflamed group stained with IDO and, from left to right, Synaptophysin, CD31, and CD45. Arrows indicate IDO⁺/CD31⁺ vasculature. Right shows that IDO⁺ cells are negative for CD45 and therefore, are not immune cells associated with vasculature. Scale bar (bottom left image) indicates 50 µm.

Figure 2.H Distribution of IDO expression on islet vasculature across pseudotime.

Figure 2.I Association of IDO⁺ islet vasculature with islet CD8⁺T cell density. The y-axis corresponds to the number of CD8⁺T cells per endocrine cell per islet. CD8⁺T cell counts were normalized to adjust for islet size. The x-axis indicates whether islets contain IDO⁺ vasculature. Each color corresponds to a donor. All donors with detectable IDO⁺ islet vasculature are displayed: 6480, 6267, 6520, 6228, and 6362. Colors are same as in **Figure 2.C**. IDO⁺ vasculature was manually quantified. For combined donors, significance was determined with a mixed-effect linear model, $p = 1.5 \times 10^{-12}$ (Satterthwaites's method lmerTest R package). The complete breakdown per donor is reported in Supplemental Figure 2.F.

pseudotime values on the left of the map (PAGA-internal Leiden clusters 0 and 5 in Supplemental Figure 2.A) were labelled “Normal” even if they originated from T1D donors. 2) Islets in the middle of the map (PAGA-internal Leiden clusters 6, 2, and 8 in Supplemental Figure 2.A) were elevated in HLA-ABC (MHC Class I) expression, CD8⁺T cells, and macrophage/DCs (**Figure 2.D**, **Figure 2.E**, **Figure 2.F**, rows 2-4) and were labelled “Inflamed”. 3) Islets with late pseudotime values on the right of the map (PAGA-internal Leiden clusters 1, 3, 7, and 4 in Supplemental Figure 2.A) were devoid of β -cells and were labelled “Insulin-Depleted” (**Figure 2.D**, **Figure 2.E**, **Figure 2.F**, top row).

In addition, islets lacking β -cells occasionally contained CD8⁺T cells and were labelled “Insulin-Depleted + Immune Islets” (**Figure 2.D**, **Figure 2.E**, **Figure 2.F**, rows 2-4). The presence of these islets suggests that signals retaining CD8⁺T cells in islets linger after β -cells die. The distribution of all cell types across pseudotime is reported in Supplemental Figure 2.B.

Islets from non-T1D controls and one of the AA⁺ donors (6314) were primarily in the Normal group to the left of the map (**Figure 2.C**, Supplemental Figure 2.C). Islets from subjects who had had T1D for of 5-6 years (cases 6195 and 6323) were primarily in the Insulin-Depleted group to the right of the map (**Figure 2.C**, Supplemental Figure 2.C). Islets from the remaining T1D donors and the other AA⁺ donor were distributed broadly throughout the map (**Figure 2.C**, Supplemental Figure 2.C).

We quantified the fraction of each cell type in swaths at varying distances from the islet edge. We found that for B Cells, CD4⁺T cells, CD8⁺T cells, macrophage/DCs, neutrophils, and plasma cells, the fraction of the given cell type in the islet relative to outside the islets increased (Supplemental Figure 2.D), demonstrating that the inflammation in islets was distinct from the inflammation in the extra-islet tissue. Thus, immune cells were targeting islets specifically.

Together, these results illustrate a single, non-branching progression from Normal Islets to Insulin-Depleted Islets via Inflamed Islets, consistent with previous pseudotime analyses¹⁸.

IDO expression on islet vasculature is linked to T cell infiltration

We observed islets with vasculature staining positive for indoleamine 2, 3-dioxygenase 1 (IDO). In the tumor microenvironment, IDO is commonly expressed by myeloid cells and suppresses CD8⁺T cell activity through multiple mechanisms, including the induction of FOXP3⁺ regulatory T cells and the inhibition of CD8⁺T cell function³⁹. In islets, IDO co-stained with CD31⁺ vasculature but not CD45⁺ immune cells adjacent to vasculature (**Figure 2.G**). IDO was not expressed by endocrine or other cell types in islets or by vasculature or any cell type outside islets (Supplemental Figure 2.E). We manually quantified vascular expression of IDO on islets throughout pseudotime and found that all but two IDO⁺ islets were in the Inflamed group (**Figure 2.H**). Therefore, IDO expression by islet vasculature was tightly associated with insulinitis.

A potent inducer of IDO expression is Interferon- γ (IFN- γ), a cytokine highly expressed by activated T cells and macrophages³⁹. Therefore, we hypothesized that IDO expression was induced by infiltrating immune cells during insulinitis. We compared the frequency of CD8⁺T cells and macrophage/DCs in islets from the Inflamed group with and without IDO⁺ vasculature and found that CD8⁺T cells were significantly more abundant in islets with IDO⁺ vasculature than islets without IDO⁺ vasculature (**Figure 2.I**). In addition, the expression of HLA-ABC, another interferon-stimulated gene, was higher in β -cells in IDO⁺ islets than in β -cells in IDO⁻ islets (Supplemental Figure 2.F). However, the abundance of $\gamma\delta$ -T cells and CD4⁺T cells was less strongly associated with IDO⁺ vasculature and the abundance of macrophage/DCs was not significantly associated with IDO⁺ vasculature (Supplemental Figure 2.F).

In summary, IDO expression by islet vasculature is positively associated with T cell infiltration and may be an immunoregulatory checkpoint.

Insulitis has sub-states, defined by functional states of CD8⁺T cells

CD8⁺T cells are a major component of insulitis (row 4 of [Figure 2.E](#) and [Figure 2.F](#)) and are capable of directly and indirectly killing β -cells. A comprehensive description of the activation profiles of CD8⁺T cells could provide insight into their roles in T1D pathogenesis. To obtain extremely high-quality marker quantification, we trained a neural network on manually labelled images of single T cells ([Figure 3.A](#), Supplemental Figure 3.A). Using our neural network, we quantified the expression of T cell markers on islet CD8⁺T cells ([Figure 3.B](#)).

PD-1, TOX, CD45RO, CD69, and CD44—markers of antigen experience—were the markers most frequently expressed by islet-infiltrating CD8⁺T cells ([Figure 3.B](#), Supplemental Figure 3.B). CD8⁺T cells expressing CD45RA (which are either naïve or terminally differentiated effector memory cells (TEMRA)) were detectable in islets, as previously reported¹⁸ ([Figure 3.B](#)). In addition, we observed a rare population of CD45RO⁺ CD8⁺T cells co-expressing Lag-3, Granzyme-B, and ICOS ([Figure 3.B](#) bottom clade). Lastly, a rare population of CD57⁺ CD8⁺T cells was present but these cells rarely co-expressed LAG-3, Granzyme-B, or ICOS ([Figure 3.B](#) top clade). These populations resemble the two exhausted T cell populations identified in the peripheral blood of T1D patients that were associated with responsiveness to alefacept⁴⁰. Therefore, the activation profiles of islet-infiltrating T cells are heterogeneous.

We reasoned that the islet microenvironment may dictate the activation state of CD8⁺T cells by specifically recruiting T cells of a particular state or inducing changes after they enter the islet. If so, islets would contain specific combinations of CD8⁺T cell states. To interrogate this, we performed UMAP only on Inflamed islets, using the frequencies of CD8⁺T cells expressing each functional marker. We identified four inflamed sub-clusters, I-IV, ([Figure 3.C](#) top). Here, the term “sub-cluster” is used to highlight that these groups were all contained within the previously defined “Inflamed” cluster and the roman numerals do not imply a temporal ordering. Inflamed-I contained only CD8⁺T cells that did not express any of the functional markers analyzed ([Figure 3.C](#) bottom, top row). Inflamed-II was characterized by a high frequency of CD45RA⁺CD8⁺T cells ([Figure 3.C](#) bottom, second row from top and [Figure 3.D](#) top row). Inflamed-III was characterized by a low frequency of CD45RA⁺ cells and high frequency of CD45RO⁺ and PD-1⁺ cells ([Figure 3.C](#) bottom, third row from top and [Figure 3.D](#) middle row). Inflamed-IV was characterized by an enrichment of CD8⁺T cells expressing CD57, LAG-3, ICOS, Granzyme-B, PD-1 or CD45RO ([Figure 3.C](#) bottom, bottom row and [Figure 3.D](#) bottom row). In summary, the phenotypes of CD8⁺T cells are coordinated across islets.

Regulation of insulitis sub-states by the islet microenvironment

To identify cellular or molecular factors that regulate the state of CD8⁺T cells in islets, we first inspected the distribution of inflamed sub-clusters in each patient. Each donor possessed islets that belonged to multiple inflamed islet sub-clusters ([Figure 3.E](#)). Therefore donor-level factors such as genetics, the location within the pancreas (i.e. head, body, or tail), or time since T1D onset, are not associated. Instead, these insulitis sub-states are conserved among T1D patients.

Next, we asked if each T cell marker is enriched in CD8⁺T cells in islets compared to CD8⁺T cells in the peri-islet and exocrine space. We computed the frequencies of each CD8⁺T cell state inside islets of each inflamed sub-cluster and in separate swaths 0-25 μ m, 25-50 μ m and 50-100 μ m away from the islets (Supplemental Figure 3.C). We found that for islets of Inflamed-II, -III, and -IV, functional markers characterizing their CD8⁺T cells were expressed more frequently inside than in the surrounding tissue areas. Although the different functional markers were all enriched on islet-infiltrating T cells compared to T cells outside islets, the degree of this enrichment varied across the markers measured (Supplemental Figure 3.C). The markers of Inflamed-IV, LAG-3, ICOS, and Granzyme-B, were highly enriched inside islets (~20% of islet-infiltrating CD8⁺T cells vs <5% of extra-islet CD8⁺T cells). CD45RA and CD69 in Inflamed-II were slightly less enriched in islets

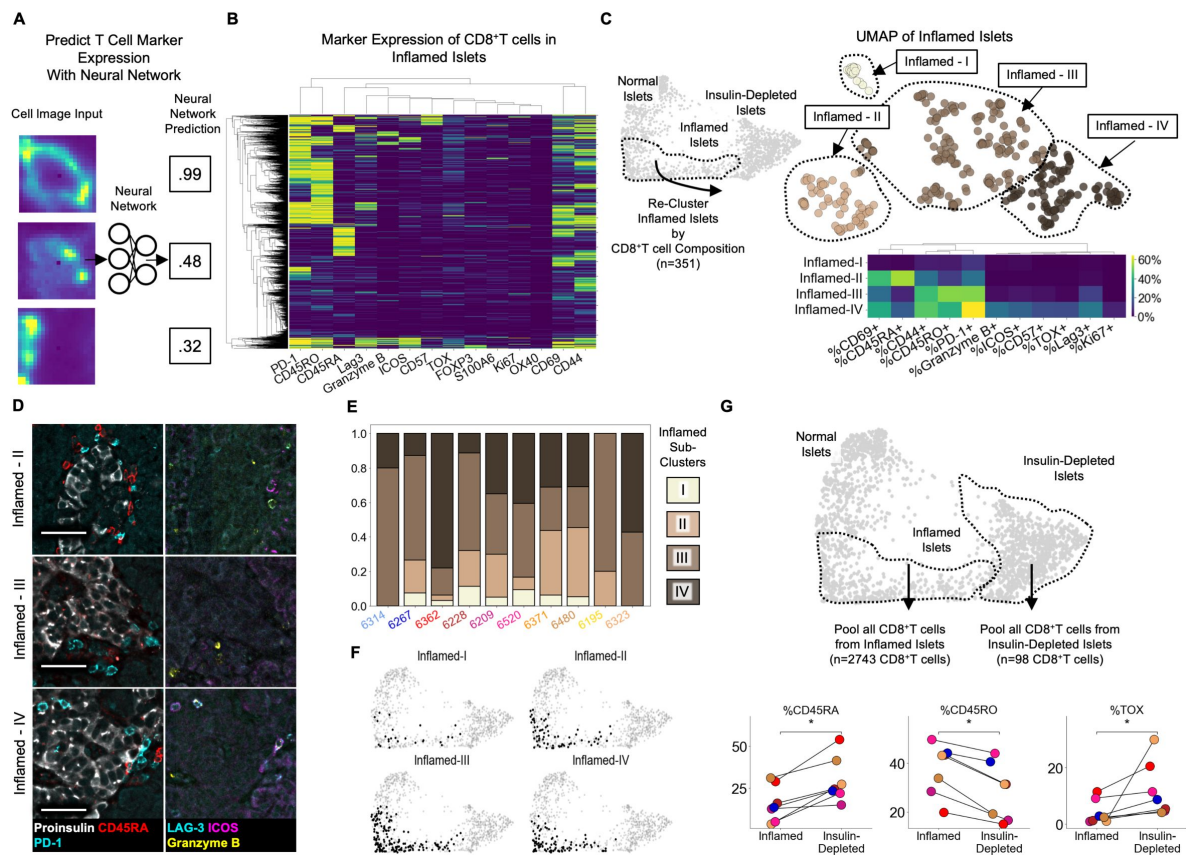


Figure 3

Insulitis has sub-states, characterized by CD8⁺T cell functionality

Figure 3.A Schematic of marker-quantification with a ResNet50 neural network. Raw images are input, and the neural network outputs a number between 0 and 1 indicative of the level of confidence that the cell expresses that marker with 1 indicating the highest confidence.

Figure 3.B Heatmap of all 2,855 Inflamed Islet CD8⁺T cells, hierarchically clustered according to marker expression predicted by the neural network.

Figure 3.C Top) UMAP of Inflamed Islets based on frequencies of markers on CD8⁺T cells in islets. Bottom) Mean frequencies of each marker on CD8⁺T cells in islets of each inflamed sub-cluster.

Figure 3.D Representative images of islets from each subcluster with associated immune markers. Scale bars indicate 50 μ m.

Figure 3.E Frequencies of islets from each subcluster per donor in AA⁺ and T1D samples. Color indicates subcluster as in panel C.

Figure 3.F Distribution of the islets of Inflamed-I through -IV on the PAGA force-directed layout shown in Figure 2.B.

Figure 3.G Differences in marker expression frequencies between CD8⁺T cells in islets from the Inflamed group and from the Insulin-Depleted + Immune group. T cells from all islets of the specified groups were pooled within each donor to compute the frequencies of marker expression. Significance was determined using the Wilcoxon signed-rank test (* p<0.05, ** p<0.01, *** p<0.001) and was not corrected for multiple hypothesis testing.

(~28% of islet-infiltrating and ~20% of extra-islet CD8⁺T cells). CD45RO and PD-1 in Inflamed-III islets were the most abundant but had the least enrichment in islets (~45% of islet-infiltrating CD8⁺T cells vs ~35% of extra-islet CD8⁺T cells) (Supplemental Figure 3.C). This demonstrated that the specific differences in the compositions of CD8⁺T cell states in different islets were attributable to the islet microenvironment and not the surrounding extra-islet spaces.

Although macrophage/DCs are abundant in islets from the Inflamed group (**Figure 2.E**, **Figure 2.F**) and can interact with T cells by presenting antigen or secreting cytokines, neither the expression of markers of macrophage/DC activity nor macrophage/DC abundance was significantly associated with any of the inflamed sub-clusters (Supplemental Figure 3.D). Similarly, no other cell-type nor the vascular expression of IDO was linked to CD8⁺T cell programs in islets (Supplemental Figure 3.D). Accordingly, the four inflamed sub-clusters had identical distributions throughout the original PAGA force-directed layout (**Figure 3.F**). Therefore, the activation states of islet-infiltrating T cells are independent of the abundance of any of the other cell types we could identify.

Lastly, we compared CD8⁺T cells in islets from the Insulin-Depleted + Immune group to CD8⁺T cells in islets from the Inflamed group. Insulin-Depleted + Immune islets contained a higher frequency of CD45RA⁺ CD8⁺T cells and a lower frequency of CD45RO⁺ CD8⁺T cells than Inflamed islets (**Figure 3.G**). TOX was expressed by a higher frequency of CD8⁺T cells in Insulin-Depleted + Immune islets than CD8⁺T cells in Inflamed islets (**Figure 3.G**). Importantly, CD45RA⁺ and TOX were never co-expressed on the same CD8⁺T cell (**Figure 3.B**). These data indicate that the activation state or persistence of these two populations in islets depends on insulin expression.

Vasculature, nerves, and Granzyme-B⁺/CD3-cells outside islets are associated with lobular patterning

In the pancreas, islets are grouped within lobules. In some donors, the destruction of islets in T1D exhibits a strong lobular patterning: that is, insulin-positive, immune-infiltrated, and insulin-negative islets are each primarily found in different lobules⁴¹. However, other donors are on the other end of this spectrum and have lobules comprised of a random mixture of islets from different stages of pathogenesis.

It is unknown why some cases exhibit lobular patterning. One explanation could be an islet-intrinsic mechanism where the expression of programs sensitizing beta cells to immune-killing (i.e. stress) are correlated across islets in the same lobule. Alternatively, it could be mediated by cells outside islets if they facilitate extravasation into the lobule or trafficking from one afflicted islet to the next.

To systematically investigate lobular patterning in T1D, we used a neural network to segment lobules and assign each single cell and islet to its lobule. We first quantified the degree of lobular patterning within each donor using the intra-class correlation coefficient (ICC). The ICC ranges from 0 to 1 where cases with values closer to 1 have islets whose states are more synchronized within lobules (**Figure 4.A**). Islets of non-T1D cases and 6314, 6195, and 6323 did not have appreciable variability in their pseudotimes, but in the remaining cases, the ICCs ranged from 0.17 to 0.74 (**Figure 4.B**). This highlights that the magnitude of lobular patterning ranges widely across T1D cases with insulinitis.

We employed hierarchical linear modeling (HLM), a statistical framework designed to identify relationships between levels of multi-level data. HLMs are standard in fields where multi-level data are common such as Education, in which students are grouped into classrooms, which are grouped into schools (Gelman et al. 2014) and have been applied in biomedical settings^{43,44}. We were interested in cell-types if their abundance in a lobule correlated with the lobule's average islet pseudotime. Importantly, we omitted the islet region itself from the calculation of a cell type's

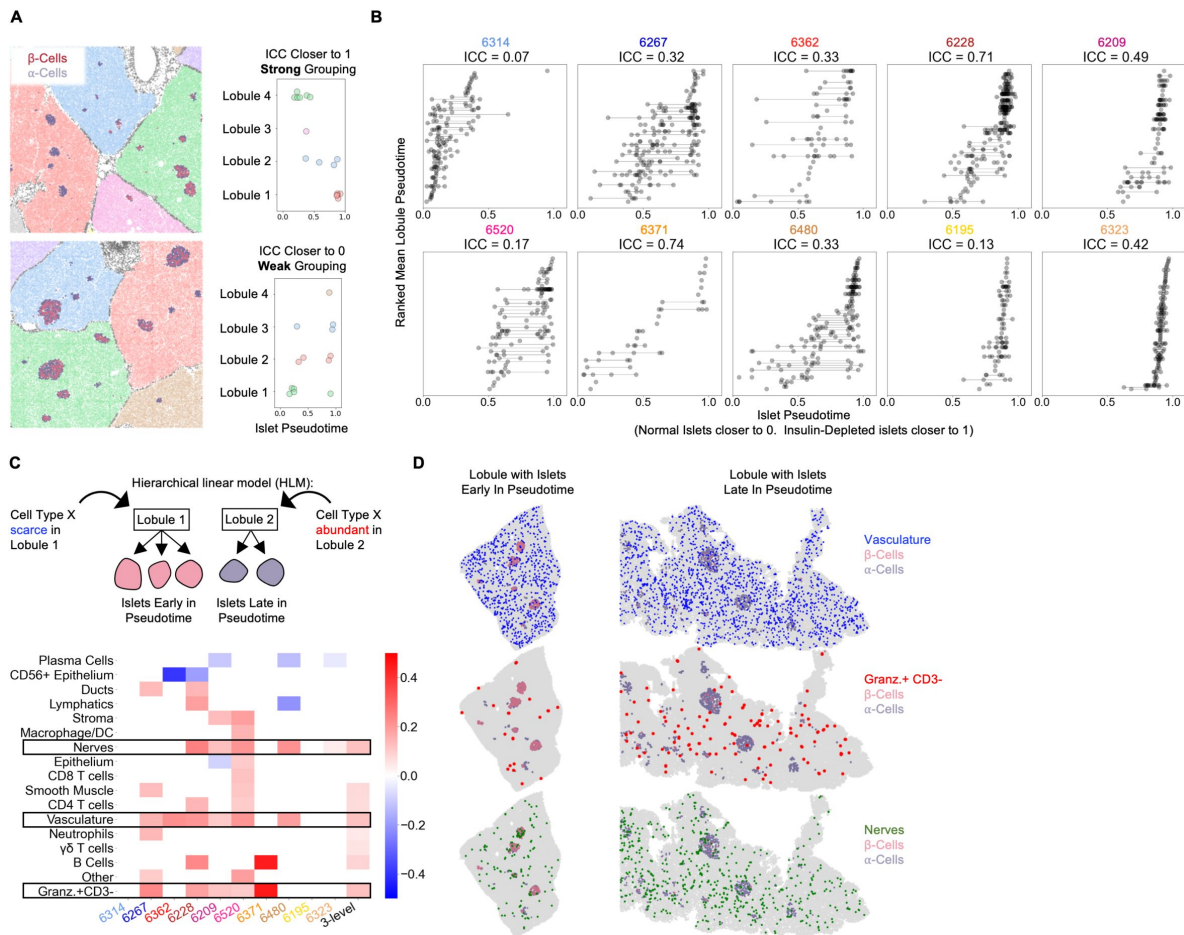


Figure 4

Vasculature, nerves, and Granzyme-B⁺/CD3-cells in the extra-islet pancreas are associated with the lobular patterning of islet pathogenesis

Figure 4.A A schematic of the method for quantifying lobular patterning of insulinitis. Lobules were segmented and colored accordingly. The islets are colored according to their composition of b-cells and a-cells. Top: A region from case 6228 with a strong lobular grouping effect and an ICC closer to 1. Bottom: A region from case 6267 with a weak lobular grouping effect and an ICC closer to 0.

Figure 4.B Lobular patterning of insulinitis within each donor. Each point represents an islet. The x-axis represents the islet pseudotime. The y-axis is ordinal, representing the rank of each lobule according to the mean pseudotime of its islets. Violin plots per lobule are overlaid. Abbreviations: ICC: Intraclass correlation coefficient.

Figure 4.C Cell types associated with lobular patterning. Top: Schematic of the hierarchical linear model. Cells in islets were omitted when computing the lobular abundance of each cell-type. Bottom: Coefficients of two-level models trained on each donor separately (columns labeled by donor) and a three-level model (right column). Color corresponds to the coefficient and features with $p > 0.05$ are white. Significance was determined using Satterthwaite's method in the lmerTest R package. No adjustment for multiple hypothesis testing was applied.

Figure 4.D Visualization of vasculature (top), Granzyme-B/CD3-cells (middle), and nerves (bottom) in two lobules. The left lobule represents lobules with islets earlier in pseudotime and a lower abundance of the given cell type in the lobule. The right lobule represents lobules with islets late in pseudotime and a greater abundance of the given cell type in the lobule.

lobular abundance because we were interested in identifying features in the extra-islet space that were associated with islet destruction. For each cell type, we estimated the effect of its total abundance in a lobule (the number of cells divided by the number of acinar cells to normalize for lobule area) on the pseudotimes of islets in that lobule. We performed this analysis in two-level HLMs for each donor and a three-level HLM considering all donors together (**Figure 4.C** [↗](#)).

We identified three cell types — vasculature, nerves, and Granzyme-B⁺/CD3-cells — that were significantly associated with lobules across multiple T1D tissue donors. All three were more abundant in lobules with islets late in pseudotime (**Figure 4.C** [↗](#) boxed rows, **Figure 4.D** [↗](#)). Samples from cases 6323 and 6195 which had very few insulin-containing islets had increased abundances of vasculature, Granzyme-B/CD3-cells, and nerves in their extra-islet spaces compared to non-T1D controls (Supplemental Figure 1C), indicating these changes persist through the point when the entire tissue is afflicted. In addition, vasculature, Granzyme-B⁺/CD3-cells, and nerves were increased in Inflamed islets compared to Normal islets indicating that they may serve a role in islets in addition to their role in the extra-islet compartment (Supplemental Figure 4A). Note that Supplemental Figure 4A differs from Supplemental Figure 1C because islets are broken up according to pseudotime, not donor, and not all islets in T1D donors are undergoing insulinitis.

Counterintuitively, although CD8⁺T cells and macrophage/DCs were higher in the extra-islet compartments of T1D cases vs non-T1D cases (Supplemental Figure 1C), they were not associated with lobular patterning (they were not more abundant in the extra-islet space of lobules with more advanced insulinitis) (**Figure 4.C** [↗](#)). These data raise the possibility that vasculature, Granzyme-B/CD3-cells, and nerves outside islets help predispose lobules to insulinitis or are affected by extensive insulinitis.

Immature tertiary lymphoid structures are enriched in subjects with T1D

We hypothesized that T cells' interactions with certain cell types in specific areas of the pancreas may be important for their functionality. Therefore, we identified Cellular Neighborhoods (CNs) ^{30,45}, tissue regions that are homogeneous and have defined cell-type compositions. To identify CNs, single cells were clustered according to the cell-type composition of their twenty nearest spatial neighbors and automatically annotated with the names of enriched cell types (**Figure 5.A** [↗](#), See Methods). This resulted in 75 CNs. Throughout the manuscript, CNs are referred to with the nomenclature (Cell Type A|Cell Type B|...) to indicate all the cell types that are enriched in them (See Methods).

Next, we identified CNs that were more abundant in T1D than non-T1D tissues (**Figure 5.B** [↗](#)). The top three CNs (fold change of abundance in T1D relative to abundance in non-T1D) were (CD8⁺T cells|B Cells), (Macrophage|Stromal Cells|B Cells), and (Vasculature|B Cells) (**Figure 5.B** [↗](#), **Figure 5.C** [↗](#)). We asked whether these three CNs were commonly adjacent to each other as this could indicate that they act as components of a larger structure ⁴⁵. Measuring the frequency with which the three B cell CNs were adjacent to each other throughout the tissues demonstrated that the (CD8⁺T cells|B Cells) CN is predominantly found adjacent to both the other CNs but that (Macrophage|Stroma|B Cells) and (Vasculature|B Cells) are less commonly adjacent to each other (**Figure 5.D** [↗](#)).

We next asked whether these CN assemblies corresponded to either peri-vascular cuffs ^{46,47} or tertiary lymphoid structures (TLSs) ^{17,46,48}, as these are two B Cell-rich structures commonly present in autoimmune conditions. Although the (CD8⁺T cells|B Cells) CN was adjacent to vessels (**Figure 5.D** [↗](#), **Figure 5.E** [↗](#)), it was not in the perivascular space, as is the case with perivascular cuffs (**Figure 5.E** [↗](#)). In our samples, the (CD8⁺T cells|B Cells) CN did not have segregated T cell and B cell zones as seen in mature TLSs, consistent with ¹⁷.

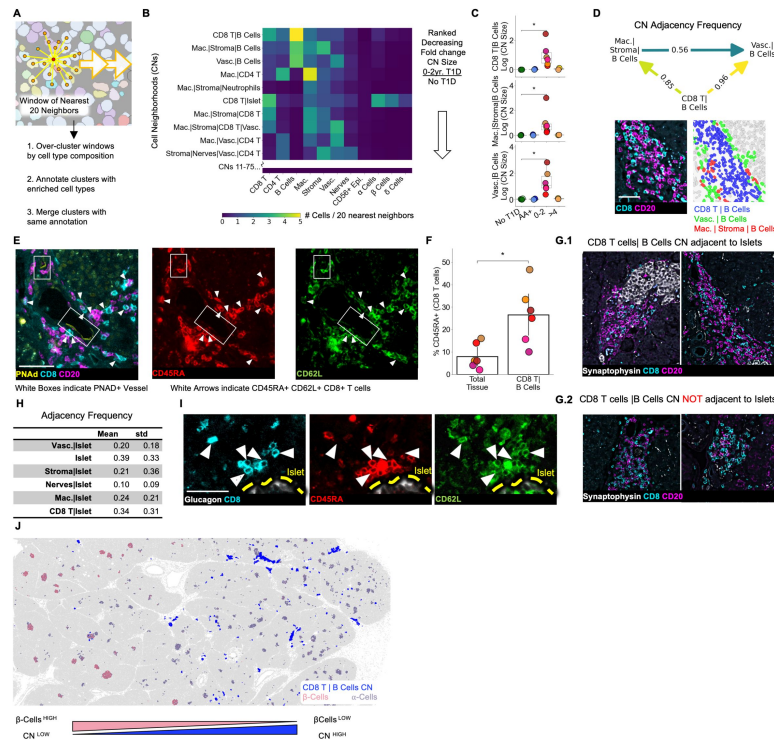


Figure 5

Immature tertiary lymphoid structures far from islets are potential staging areas for islet-destined CD8⁺T cells

Figure 5.A Schematic of algorithm for identifying CNs. Red point indicates index cell for the CN. Orange points indicate the nearest neighbors of the index cell. Windows are collected for each cell in the dataset (indicated by orange arrows).

Figure 5.B Cell-type compositions of the top CNs organized in decreasing order of the fold increase in abundance in T1D vs. non-T1D samples. Each column in the heatmap indicates the mean density of that cell type in the 20 nearest spatial neighbors of cells assigned to the CN designated for that row. CN abundance was calculated as the number of cells assigned to the given CN divided by the number of acinar cells. Abbreviations: Vasc.: vasculature; Mac.: macrophage/DCs; Lym.: lymphatics; Neu.: neutrophils; CD8 T: CD8⁺T cells; CD4 T: CD4⁺T cells. Endocrine cell types were merged during CN annotation and are labeled "Islet".

Figure 5.C Mean abundances of the CD8⁺T cell and B cell CNs per donor. Abundance was calculated as the number of cells assigned to the given CN divided by the number of acinar cells. Significance was determined using the Mann-Whitney U test (* p<0.05, ** p<0.01, *** p<0.001). No adjustment for multiple hypothesis testing was applied.

Figure 5.D Top: Adjacency frequencies of (CD8⁺T cells | B Cell CN) with (Macrophage | Stroma | B Cells) and (Vasculature | B Cells) CNs. The adjacency frequency was calculated as the number of instances of the source CN adjacent to the destination CN divided by the total number of instances of the source CN. Bottom Left: Raw image of a representative assembly of the three CNs (CD8⁺T cells | B Cell CN), (Macrophage | Stroma | B Cells), and (Vasculature | B Cells) displaying CD8 and CD20 staining. Bottom Right: The same assembly as to the left colored by CN. Scale bar indicates 50μm.

Figure 5.E Representative images of co-localization of PNAd⁺ endothelium and CD45RA⁺ CD62L⁺ CD8⁺T cells located in the (CD8⁺T cells | B Cells) CN. Scale bar indicates 50μm.

Figure 5.F Frequency of CD45RA expression on CD8⁺T cells located in (CD8⁺T cells | B cell) CN relative to CD8⁺T cells throughout the tissue. Significance was determined with a Wilcoxon signed-rank test (* p<0.05, ** p<0.01, *** p<0.001).

Figure 5.G Representative images of (CD8⁺T cells | B Cells) instances adjacent to islets (G.1) and not adjacent to islets (G.2). Scale bars indicate 200μm.

Figure 5.H Quantification of the adjacency frequencies between the (CD8⁺T cells | B Cells) CN and CNs rich in endocrine cells (i.e. "Islet CNs"). Mean, std: mean and standard deviation adjacency frequency across T1D donors. Abbreviations: Vasc.: vasculature; Mac.: macrophage.

Figure 5.I Representative images of islet-adjacent CD45RA⁺ CD62L⁺ CD8⁺T cells. Scale bar indicates 50 μm.

Figure 5.J Image showing the spatial distribution of the (CD8⁺T cells | B cells) CN instances relative to islets and the enrichment of (CD8⁺T cells | B cells) CN instances in areas of the pancreas with islets lacking β-Cells.

In summary, the (CD8⁺T cells|B Cells) CN is more abundant in T1D tissues from patients with diabetes durations of 0-2 years compared to non-T1D tissues and T1D tissue from patients who had T1D for more than 4 years.

Immature tertiary lymphoid structures are in the extra-islet pancreas, and are enriched in CD45RA⁺ /CD8⁺ T cells

We next asked whether the (CD8⁺T cells|B Cells) CN had high endothelial venules (HEV), specialized blood vessels that are commonly found in TLSs that enable naïve lymphocytes to extravasate into peripheral tissues. We observed expression of peripheral lymph node addressin (PNAd), an HEV marker, in the vessels associated with the (CD8⁺T cells|B Cells) CN (**Figure 5.E** [↗](#) left image) but not in other vessels (data not shown). Although we could not assess the presence of other TLS traits such as follicular dendritic cells, fibroblastic reticular cells, or follicular helper T cells, the aggregation of B cells and presence of HEVs, but the lack of compartmentalized B and T cell zones indicate that instances of the (CD8⁺T cells|B Cells) CN are immature TLSs.

Next, we asked if immature TLSs could support the entry of naïve CD8⁺T cells into the pancreas. We observed CD8⁺T cells co-expressing CD45RA and CD62L (the ligand for PNAd) near PNAd⁺ vasculature (**Figure 5.E** [↗](#), middle and right image respectively). Thus, CD45RA⁺/CD8⁺T cells in the pancreas can adhere to HEVs. Furthermore, CD45RA⁺ was enriched three-fold on CD8⁺T cells in the (CD8⁺T cells|B Cells) CN relative to CD8⁺T cells in the tissue as a whole (**Figure 5.F** [↗](#)), providing additional evidence that CD45RA⁺ T cells may enter the pancreas through HEVs.

We found immature TLS both adjacent (**Figure 5.G** [↗](#).1) or not adjacent (**Figure 5.G** [↗](#).2) to islets. Quantifying the frequency of this adjacency revealed that fewer than half of the immature TLS were adjacent to islets (**Figure 5.H** [↗](#)). We did not identify any differences in the cellular composition of the immature TLS that were or were not adjacent to islets (Supplemental Figure 5A). We reasoned that even if immature TLSs were far from islets, extravasating cells may migrate to islets. Accordingly, islet-adjacent CD45RA⁺ CD8⁺T cells (that were not in islet-adjacent TLSs) co-expressed CD62L, suggesting that they originated from the (CD8⁺T cells|B Cell) CN (**Figure 5.I** [↗](#)). Consistent with this, in one notable tissue donor, regions of the pancreas with Insulin-Depleted islets were enriched in the (CD8⁺T cells|B Cell) CN relative to regions of the pancreas with β -cell containing islets (**Figure 5.J** [↗](#)). This spatial correlation between the (CD8⁺T cells|B Cells) CN and the destruction of islets implicates immature TLS with islet pathology even if they are not adjacent to islets (**Figure 5.J** [↗](#)).

Discussion

We have performed CODEX imaging and comprehensive computational analysis of whole cadaveric pancreata from T1D subjects. Our data support several conclusions.

First, our results are consistent with the model previously proposed by Damond et al, who proposed a single trajectory for insulinitis, characterized by an enrichment in HLA-ABC expression, CD8⁺T cells, and macrophage/DCs¹⁸ [↗](#).

Second, we are the first to report that IDO⁺ vasculature is present in inflamed islets but not in normal islets or islets that have lost insulin-expression (**Figure 2.G** [↗](#), **Figure 2.H** [↗](#)). Furthermore, islets with IDO⁺ vasculature contained higher frequencies of CD8⁺T cells and higher expression of HLA-ABC, but not higher frequencies of macrophage/DCs compared to inflamed islets that did not contain IDO⁺ vasculature, suggesting that IDO is induced by a cytokine produced by infiltrating CD8⁺T cells such as IFN- γ (**Figure 2.I** [↗](#)). Given IDO's established tolerogenic role, these data

suggest that the loss of IDO on vasculature could be a prerequisite for β -cell death. Leveraging this checkpoint to protect transplanted β -cells from rejection has shown promise⁴⁹ and could be combined with similar approaches using programmed death-ligand 1^{50,51}.

We did not observe IDO expression on β -cells, in contrast to Anquetil et al. that report this in healthy patients⁵². Our observations are consistent with data demonstrating that IDO needs to be induced. First, it has been shown with western blot and RT-PCR that human islets require cytokine stimulation to express IDO⁵³. Second, the human protein atlas has tested multiple IDO antibodies and demonstrated that IDO is negative in human islets via immunohistochemistry: (<https://www.proteinatlas.org>). In addition, the human protein atlas's single-cell RNAseq atlas only report IDO transcripts in immune cells in healthy pancreas.

Third, we performed the first high-dimensional spatial phenotyping of CD8⁺T cells in T1D islets. We found that most T cells were antigen experienced. A small population expressed CD45RA and CD69, which could be naïve, TEMRA, or Tscm cells (**Figure 3.B**). Another population expressed Lag-3, Granzyme-B, and ICOS. It is notable that only a small population of islets had Granzyme-B-expressing T cells. This could indicate that alternative mechanisms are contributing to the elimination of β -cells.

Fourth, the insulinitis trajectory is comprised of four sub-clusters, each characterized by the activation profile of the islet-infiltrating CD8⁺T cells (**Figure 3.C**). Multiple of these inflamed sub-clusters were present in all T1D donors. One potential explanation for this observation is that sub-clusters are capable of inter-converting (**Figure 3.E**). The factors that regulate the conversion of a given islet between sub-clusters could correspond to immunoregulatory checkpoints that are critical to the progression of T1D. Unfortunately, our search for such features failed to generate any candidates (Supplemental Figure 3.D). By phenotyping T cells at different distances from the islet edge, we were able to determine that the T cell activation profiles characterizing each sub-cluster were only present in the islet, not in the surrounding tissue (Supplemental Figure 3.C). Unfortunately, from our data, we cannot speculate whether the insulinitis sub-clusters arose due to differential stimulation of T cells that had already entered islets, differential recruitment of pre-activated T cells, or both.

Fifth, pancreatic lobules affected by insulinitis are characterized by distinct tissue markers. We discovered that lobules enriched in β -cell-depleted islets were also enriched in nerves, vasculature, and Granzyme-B⁺/CD3-cells, suggesting these factors may make lobules permissive to disease (**Figure 4.C**). The role of islet enervation in T1D has been studied but such work has focused on nerves in the islet rather than on nerves in the exocrine tissue⁵⁴. The Granzyme-B⁺/CD3-cells could be natural killer cells; if so, they are most likely of the CD56dim subset as CD56 was not detected on these cells. It is noteworthy that the cell types linked with direct islet invasion were distinct from those linked to lobule patterning even though both sets of cell types were found across islet and extra-islet regions. Therefore, for insulinitis to consume every islet, crosstalk may be required between the cell types in the islet and extra-islet compartments. Conversely, inhibiting this interaction might contain pathology to isolated lesions.

Finally, we identify immature TLS away from islets where CD45RA⁺ CD8⁺T cells aggregate. We also observed an inflamed islet-subcluster characterized by an abundance of CD45RA⁺/CD8⁺ T cells. It will be important to determine whether the CD45RA⁺ T cells localized around islets may have originated from immature TLS. In mice, blocking immune egress from lymph nodes reduced the size of TLSs and halted diabetes⁵⁵. Thus, therapeutic targeting of immune cell trafficking to TLS could help mitigate autoimmunity in human T1D.

Together, these data illuminate relationships between insulinitis, the local islet microenvironment and inflammation at distal sites.

A major limitation for the study is the cohort size. Cases with documented insulinitis are very rare, significantly limiting the feasibility of curating large cohorts³⁵. Due to this limitation, factors such as the donors' histories of drug use, durations of stay in the intensive care unit, and BMIs could not be balanced or statistically adjusted for but should be considered as they may affect exocrine inflammation but not the prevalence of insulinitis^{29,56}. In addition, only one of the AA+ cases, Case 6267, had detectable insulinitis (**Figure 2.C**). The other case, 6314 tested positive for only one autoantibody, GADA+, and therefore had a significantly lower probability of progressing to overt T1D⁵⁷. Age and diabetes duration are also important criteria to consider when interpreting key results (Supplemental Figure 5B-C).

Another limitation is our limited perspective on myeloid cell populations. Although antibodies in our panel detect numerous myeloid markers, we failed to identify any heterogeneity in myeloid populations during insulinitis. This was likely due in part to the difficulty of segmenting myeloid cells and quantifying marker expression due to their morphology. Spatial transcriptomics could be used in future studies to better define the myeloid populations and inform additions to future CODEX panels.

Lastly, our samples are 2-dimensional sections which could affect some of the adjacency relations.

In conclusion, using a data-driven approach, we mapped conserved sub-states of insulinitis and integrated the spatial pathology of islet and extra-islet regions into a single model of T1D pathogenesis. The tools and computational pipelines developed here will enable further investigation of immune pathology at the tissue scale that may lead to development of therapies for T1D.

Methods

Human tissues

Cadaveric pancreatic FFPE tissue sections were obtained through the nPOD program, sponsored by the Juvenile Diabetes Research Fund. Case numbers cited herein are assigned by nPOD and comparable across nPOD-supported projects. 17 cases in the nPOD biorepository had been previously documented to contain insulinitis. For each of these 17 cases, we examined the triple stained immunohistochemistry images (CD3, Insulin, and Glucagon) using nPOD's online pathology database to select blocks in which insulinitis was present. To ensure that the tissue regions still contained insulinitis (and had not been sectioned extensively after their images were uploaded to the nPOD pathology database), we re-sectioned and visualized CD3, Insulin, and Glucagon. The use of cadaveric human tissue samples is approved by Stanford University's Institutional Review Board.

CODEX data collection

CODEX Antibody Generation and Validation. Oligonucleotides were conjugated to purified, carrier-free, commercially available antibodies as previously described^{30,58}. For validation experiments, human tonsils and non-diabetic pancreata were co-embedded in FFPE blocks so both tissues could be stained and imaged simultaneously. Each antibody in the CODEX panel was validated by co-staining with previously established antibodies targeting positive and negative control cell types. Once validated, the concentration and imaging exposure time of each antibody were optimized. The tissue staining patterning was compared to the online database, The Human Protein Atlas, and the published literature. The specificity, sensitivity, and reproducibility of CODEX staining has been previously validated^{30,31,58,60}.

CODEX Staining. Staining and imaging was conducted as previously described^{30,58–60}. Briefly, FFPE tissues were deparaffinized and rehydrated. Heat-induced epitope retrieval (HIER) antigen retrieval was conducted in Tris/EDTA buffer at pH9 (Dako) at 97°C for 10 minutes. Tissues were blocked for 1 hour with rat and mouse Ig, salmon-sperm DNA, and a mixture of the non-fluorescent DNA oligo sequences used as CODEX barcodes. Tissues were stained with the antibody cocktail in a sealed humidity chamber overnight at 4°C with shaking. The next day, tissues were washed, fixed with 1.6% paraformaldehyde, 100% methanol, and BS3 (Thermo Fisher Scientific), and mounted to a custom-made acrylic plate attached to the microscope.

CODEX Imaging. Imaging was conducted using the Keyence BZ-X710 fluorescence microscope with a CFI Plan Apo λ 20 $\times$ /0.75 objective (Nikon). “High resolution” mode was selected in Keyence Navigator software, resulting in a final resolution of 37744 $\mu\text{m}/\text{pixel}$. The exposure times are listed in Supplemental Table 3. Regions for imaging were selected by rendering HLA-ABC, Proinsulin, and CD8 and selecting large regions (averaging 55mm²). The full antibody panel and cycle-ordering is detailed in (Supplemental Tables 3 and 4). Biotinylated hyaluronan-binding protein was rendered by adding streptavidin-PE at 1:500 concentration to the 96 well plate containing fluorescent oligos in the last cycle and running the CODEX program normally. DRAQ5 was added to the last cycle because we found it stained nuclei more evenly than HOECHST which slightly improved segmentation. Each tissue took between 3 and 7 days to image depending on the tissue area.

Characterization of Tertiary Lymphoid Structures. A serial section from case 6228 was imaged in a separate CODEX experiment using an antibody panel tailored for characterizing tertiary lymphoid structures, as described in Supplemental Table 3. These data were acquired and analyzed identically to the main dataset.

Image Pre-processing. Drift compensation, deconvolution, z-plane selection was performed using the CODEX Toolkit uploader (github.com/nolanlab/CODEX). Cell segmentation using the DRAQ5 nuclear channel and lateral bleed compensation was performed with CellSeg³³.

Cell Type Clustering and Annotation

Marker expression was z-normalized within each donor and subsequently clustered in two steps. First, cells were projected into 2 dimensions using the markers indicated in Supplemental Table 2 and Parametric Uniform Manifold Approximation and Projection (pUMAP)⁶² was applied on a downsampled dataset. The fit model was used to transform the remaining cells. Cell types were gated using Leiden clustering and manual merging. The cluster containing immune cells was sub-clustered using the markers detailed in Supplemental Table 2. Acinar cells contaminating the Immune cluster were gated out and merged with the Acinar cluster from the previous step. The Endocrine class was sub-clustered into α -, β -, and δ -Cells using Glucagon, Proinsulin, and Somatostatin respectively. Clusters were annotated according the heatmap marker expression, and overlaying annotations onto raw images (Supplemental Figure 1.A) using custom scripts in Fiji.

Islet Segmentation and Pseudotime

Preprocessing. Windows consisting of the twenty nearest spatial neighbors surrounding each single cell were clustered according to their cell-type composition using Mini Batch K Means with $k=200$. For this analysis, α -, β -, and δ -Cells were combined into one ‘Endocrine’ cell type. One cluster was highly enriched in Endocrine cells and accurately defined the islet area. Individual islets were identified using the connected components algorithm and filtering out islets that had fewer than ten total cells.

PAGA Analysis. For each islet, the number of each cell type inside the islet and between the islet edge and 20 μ m beyond were extracted. To adjust for variation due to the islet size, the cell type counts were divided by the number of endocrine cells inside the islet. Data were then log-transformed. The PAGA embedding was computed using the default parameters except for the following: The neighborhood search was performed using cosine distance and 15 nearest neighbors; Leiden clustering used a resolution of 1. For computing the pseudotime values (used in the colormap in Figure 2B, the x-axis in Figure 2F, and Figure 4), the path through the inflamed islet was isolated by temporarily omitting 25 islets positioned in the middle of the map between Normal and Insulin-Depleted islets. Only 9 of these were from T1D or AA+ donors.

Quantification and Validation of Functional Marker Gating

Annotation of Ground-Truth Dataset. 4000 CD8⁺T cells were labelled for fifteen markers by an immunologist familiar with the staining patterns of each marker using VGG Image annotator⁶³.

Automated Thresholding. For each functional marker of interest, the lateral-bleed-compensated mean fluorescence³³ of cell types known to not express the marker in question were used to calculate a background distribution. Marker-positive cells were defined as those whose expression was greater than the 99th percentile of the background distribution.

Gating with Neural Network: 22 μ m x 22 μ m cropped images of each single cell were used as training data. The marker that the image corresponded to was not included as an input in the neural network and one classifier was trained for all markers. Cells were split into training, validation, and test splits (60/15/25 respectively). ResNet50 architecture and initial weights were imported from the Keras library pre trained on ImageNet. Image augmentation consisted of random flips, rotations, zooms, contrast, and translation (+/- ten pixels only). All weights were unfrozen, and the model was trained for 100 epochs (see accompanying source code for training details).

Sub-clustering of Inflamed Islets with Cell-Type specific Functional Markers

For each Inflamed Islet (n=351), the frequency of each marker expressed by CD8⁺T cells was computed. Single cells inside the islet and within 20 μ m from the islet's edge were combined before the frequency was measured. The subsequent matrix underwent z-normalization followed by UMAP gating using Bokeh. Insulin-Depleted + Immune Islets were defined as islets without β -cells with greater than two CD8⁺T cells and greater than seven macrophage/DCs. These thresholds correspond to the 95th percentiles of CD8⁺T cells and macrophage/DCs in Normal islets.

Identification of Cellular Neighborhoods

Previously, CNs³⁰ were identified by, for each single cell, defining its 'window' as the 20 spatial nearest neighbors. Cells were clustered according to the number of each cell type in their windows using Mini Batch K-Means. The output clusters corresponded to CNs. To ensure our method was sensitive to rare neighborhoods, we adapted this algorithm by intentionally over-clustering, using k=200 in the K-Means step rather than using a k ranging from 10-20 as used elsewhere^{31,45,64}. Next, to determine which cell types were characteristic of each cluster, we identified, for each cluster, the set of cell-types that were present in more than 80% of the windows allocated to that cluster. We named the clusters according to this set of cell-types and merged all clusters with the same name, resulting in seventy-five CNs. Acinar cells and epithelial cells were used in the kNN graph and in the clustering but were not considered when merging clusters. Note that this method does not differentiate neighborhoods that have the same combination of cell types but different stoichiometries.

Lobule Segmentation

A training dataset was generated by manually tracing the edges of lobules in ImageJ using the ROI function. The ROI were then floodfilled in Python and used as masks for training. For each tile, the blank cycle was selected to distinguish tissue from background coverslip. A U-Net model was trained for 10 epochs (see attached source code for training details). After stitching together all masks, the resulting images required slight refinement where lobules were not completely separated, and this was done manually in ImageJ. The connected components in the stitched image defined the lobule instances. Cells were assigned to a lobule by indexing the lobule mask with their X and Y coordinates. Cells in the inter-lobular space were assigned to one “edge” lobule. This resulted in 464 lobules.

Formulation of Hierarchical Linear Models

For each lobule, the number of each cell type in the extra-islet space was divided by the number of acinar cells in the extra-islet space. For all HLMs, the *lme4* package for R was used⁶⁵ and statistical significance was computed using the *lmerTest* package for R⁶⁶. Lobular cell-type abundance was z-normalized within each donor and the pseudotime was z-normalized across the entire dataset prior to fitting.

The ICC was computed using the model: $pseudotime_{islet} \sim 1 | lobuleID$ with the *performance* package in R. A value of 0 indicates that the variation in pseudotimes of islets within the same lobule is equal to the variation across all islets in the donor and a value of 1 indicates that the variation in pseudotimes of islets within the same lobule is much smaller than that of all islets in the donor.

For each cell type, a two-level, random intercept HLM within each donor was constructed with the following formulation (in R formula syntax): $pseudotime_{islet} \sim celltype_{lobule} + (1 | lobuleID)$ and a three-level random intercept, random slope HLM including islets from all donors was formulated: $pseudotime_{islet} \sim celltype_{lobule} + (1 + celltype_{lobule} | donorID) + (1 | lobuleID)$. Here, $pseudotime_{islet}$ equals the pseudotime of each islet, $celltype_{lobule}$ equals the number of the given cell type in a particular lobule divided by the number of acinar cells in that lobule, z-normalized within each donor, and $lobuleID$ and $donorID$ are categorical variables specifying the lobule and donor that the given islet belongs to.

Neighborhood Adjacency

The adjacency between neighborhoods was computed as in⁴⁵. The only modification was that neighborhood instances were identified using connected components of the k-NN graph with k=5 rather than from the thresholded images.

Data Availability

Processed single cell dataframes are accessible at: <https://www.dropbox.com/sh/8sqef3iwjb6f4wp/AAAQzFgDOgMBiWh9Jqcb81U8a?dl=0>.

The raw and stitched, processed data is hosted by the BioImage Archive (<https://www.ebi.ac.uk/bioimage-archive/>) with the accession number S-BIAD859.

Code Availability

All primary code is attached with the supplementary information and will be posted on the Nolan Lab's github upon acceptance. The code is completely open source. The code is not intended as a software tool and so no small example data set is applicable.

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Paul Bollyky (pbollyky@stanford.edu).

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Additional information

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G.L.B curated data, wrote software, and analyzed data. D.P. and C.M.S contributed significantly to validating the antibody panel and assisted with analysis. S.D., S.S.B, A.Y., H.A.M., G.K.K., N.N., S.R., and J.M. assisted with analysis. G.L.B., J.A.B., G.P.N., and P.L.B., conceptualized the study and wrote the manuscript. All authors have read and approved the final version of the manuscript.

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Additional files

Supplemental figures [↗](#)

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Reviewer #1 (Public review):

Summary:

Barlow and coauthors utilized the high-parameter imaging platform of CODEX to characterize the cellular composition of immune cells in situ from tissues obtained from organ donors with type 1 diabetes, subjects presented with autoantibodies who are at elevated risk, or non-diabetic organ donor controls. The panels used in this important study were based up prior publications using this technology, as well a priori and domain specific knowledge of the field by the investigators. Thus, there was some bias in the markers selected for analysis. The authors acknowledge that these types of experiments may be complemented moving forward with the inclusion of unbiased tissue analysis platforms that are emerging that can conduct a more comprehensive analysis of pathological signatures employing emerging technologies for both high-parameter protein imaging and spatial transcriptomics.

Strengths:

In terms of major findings, the authors provide important confirmatory observations regarding a number of autoimmune-associated signatures reported previously. The high parameter staining now increases the resolution for linking these features with specific cellular subsets using machine learning algorithms. These signatures include a robust signature indicative of IFN-driven responses that would be expected to induce a cytotoxic T cell mediated immune response within the pancreas. Notable findings include the upregulation of indolamine 2,3-dioxygenase-1 in the islet microvasculature. Furthermore, the authors provide key insights as to the cell:cell interactions within organ donors, again supporting a previously reported interaction between presumably autoreactive T and B cells.

Weaknesses:

These studies also highlight a number of molecular pathways that will require additional validation studies to more completely understand whether they are potentially causal for pathology, or rather, epiphenomenon associated with increased innate inflammation within the pancreas of T1D subjects. Given the limitations noted above, the study does present a rich and integrated dataset for analysis of enriched immune markers that can be segmented and annotated within distinct cellular networks. This enabled the authors to analyze distinct cellular subsets and phenotypes in situ, including within islets that peri-islet infiltration and/or intra-islet insulinitis.

Despite the many technical challenges and unique organ donor cohort utilized, the data are still limited in terms of subject numbers - a challenge in a disease characterized by extensive heterogeneity in terms of age of onset and clinical and histopathological presentation. Therefore, these studies cannot adequately account for all of the potential covariates that may drive variability and alterations in the histopathologies observed (such as age of onset, background genetics, and organ donor conditions). In this study, the manuscript and figures could be improved in terms of clarifying how variable the observed signatures were across each individual donor, with the clear notion that non-diabetic donors will present with some similar challenges and variability.

<https://doi.org/10.7554/eLife.100535.2.sa3>

Reviewer #2 (Public review):

Summary:

The authors aimed to characterize the cellular phenotype and spatial relationship of cell types infiltrating the islets of Langerhans in human T1D using CODEX, a multiplexed examination of cellular markers

Strengths:

Major strengths of this study are the use of pancreas tissue from well-characterized tissue donors, the use of CODEX, a state-of-the-art detection technique of extensive characterization and spatial characterization of cell types and cellular interactions. The authors have achieved their aims with the identification of the heterogeneity of the CD8⁺ T cell populations in insulinitis, the identification of a vasculature phenotype and other markers that may mark insulinitis-prone islets, and characterization of tertiary lymphoid structures in the acinar tissue of the pancreas. These findings are very likely to have a positive impact on our understanding (conceptual advance) of the cellular factors involved in T1D pathogenesis which the field requires to make progress in therapeutics.

Weaknesses:

A major limitation of the study is the cohort size, which the authors directly state. However, this study provides avenues of inquiry for researchers to gain further understanding of the pathological process in human T1D.

Comments on revisions:

The authors have responded well to the 3 critiques. They have addressed my specific comments in their revised text.
I have no further comments.

<https://doi.org/10.7554/eLife.100535.2.sa2>

Reviewer #3 (Public review):

Summary:

The authors applied an innovative approach (CO-Detection by indEXing - CODEX) together with sophisticated computational analyses to image pancreas tissues from rare organ donors with type 1 diabetes. They aimed to assess key features of inflammation in both islet and extra-islet tissue areas; they report that the extra-islet space of lobules with extensive islet infiltration differs from the extra-islet space of less infiltrated areas within the same tissue section. The study also identifies four sub-states of inflamed islets characterized by the activation profiles of CD8+T cells enriched in islets relative to the surrounding tissue. Lymphoid structures are identified in the pancreas tissue away from islets, and these were enriched in CD45RA+ T cells - a population also enriched in one of the inflamed islet sub-states. Together, these data help define the coordination between islets and the extra-islet pancreas in the pathogenesis of human T1D.

Strengths:

The analysis of tissue from well-characterized organ donors, provided by the Network for the Pancreatic Organ Donor with Diabetes, adds strength to the validity of the findings.

By using their innovative imaging/computation approaches, key known features of islet autoimmunity were confirmed, providing validation of the methodology.

The detection of IDO+ vasculature in inflamed islets - but not in normal islets or islets that have lost insulin-expression links this expression to the islet inflammation, and it is a novel observation. IDO expression in the vasculature may be induced by inflammation and may be lost as disease progresses, and it may provide a potential therapeutic avenue.

The high-dimensional spatial phenotyping of CD8+T cells in T1D islets confirmed that most T cells were antigen experienced. Some additional subsets were noted: a small population of T cells expressing CD45RA and CD69, possibly naive or TEMRA cells, and cells expressing Lag-3, Granzyme-B, and ICOS.

While much attention has been devoted to the study of the insulinitis lesion in T1D, our current knowledge is quite limited; the description of four sub-clusters characterized by the activation profile of the islet-infiltrating CD8+T cells is novel. Their presence in all T1D donors, indicates that the disease process is asynchronous and is not at the same stage across all islets. Although this concept is not novel, this appears to be the most advanced characterization of insulinitis stages.

When examining together both the exocrine and islet areas, which is rarely done, authors report that pancreatic lobules affected by insulinitis are characterized by distinct tissue markers. Their data support the concept that disease progression may require crosstalk

between cells in the islet and extra-islet compartments. Lobules enriched in β -cell-depleted islets were also enriched in nerves, vasculature, and Granzyme-B+/CD3- cells, which may be natural killer cells.

Lastly, authors report that immature tertiary lymphoid structures (TLS) exist both near and away from islets, where CD45RA+ CD8+ T cells aggregate, and also observed an inflamed islet-subcluster characterized by an abundance of CD45RA+/CD8+ T cells. These TLS may represent a point of entry for T cells and this study further supports their role in islet autoimmunity.

Weaknesses:

As the author themselves acknowledge, the major limitation is that the number of donors examined is limited as those satisfying study criteria are rare. Thus, it is not possible to examine disease heterogeneity, and the impact of age at diagnosis. Of 8 T1D donors examined, 4 would be considered newly diagnosed (less than 3 months from onset) and 4 had longer disease durations (2, 2, 5 and 6 years). It was unclear if disease duration impacted the results in this small cohort. In the introduction, the authors discuss that most of the pancreata from nPOD donors with T1D lack insulinitis. This is correct, yet it is a function of time from diagnosis. Donors with shorter duration will be more likely to have insulinitis. A related point is that the proportion of islets with insulinitis is low even near diagnosis. Finally, only one donor was examined that while not diagnosed with T1D, was likely in the preclinical disease stage and had autoantibodies and insulinitis. This is a critically important disease stage where the methodology developed by the investigators could be applied in future efforts.

While this was not the focus of this investigation, it appears that the approach was very much immune-focused and there could be value in examining islet cells in greater depth using the methodology the authors developed.

Additional comments

Overall, the authors were able to study pancreas tissues from T1D donors and perform sophisticated imaging and computational analysis that reproduce and importantly extend our understanding of inflammation in T1D. Despite the limitations associated with the small sample size, the results appear robust, and the claims are well supported.

The study expands the conceptual framework of inflammation and islet autoimmunity, especially by the definition of different clusters (stages) of insulinitis and by the characterization of immune cells in and outside the islets.

Comments on revisions:

I have not felt the need to update the initial review.

However, I note that the paragraph describing the nPOD repository (lines 154-158) can be misinterpreted that insulinitis is infrequent in T1D (17 of 200 donors had it) without the clarification that insulinitis is present around the time of diagnosis in most patients and it subsides over time. Thus, authors are urged to clarify that the presence of insulinitis and its severity are impacted by the disease stage and disease duration.

The last sentence of this paragraph, lines 164-165, although linked to the previous sentence about the cause of death in the donors, may be misconstrued in the context of this paragraph, and it is unclear what data support this statement. Please delete this sentence.

<https://doi.org/10.7554/eLife.100535.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

Summary:

Barlow and coauthors utilized the high-parameter imaging platform of CODEX to characterize the cellular composition of immune cells in situ from tissues obtained from organ donors with type 1 diabetes, subjects presented with autoantibodies who are at elevated risk, or non-diabetic organ donor controls. The panels used in this important study were based on prior publications using this technology, as well as a priori and domain-specific knowledge of the field by the investigators. Thus, there was some bias in the markers selected for analysis. The authors acknowledge that these types of experiments may be complemented moving forward with the inclusion of unbiased tissue analysis platforms that are emerging that can conduct a more comprehensive analysis of pathological signatures employing emerging technologies for both high-parameter protein imaging and spatial transcriptomics.

Strengths:

In terms of major findings, the authors provide important confirmatory observations regarding a number of autoimmune-associated signatures reported previously. The high parameter staining now increases the resolution for linking these features with specific cellular subsets using machine learning algorithms. These signatures include a robust signature indicative of IFN-driven responses that would be expected to induce a cytotoxic T-cell-mediated immune response within the pancreas. Notable findings include the upregulation of indolamine 2,3-dioxygenase-1 in the islet microvasculature. Furthermore, the authors provide key insights as to the cell:cell interactions within organ donors, again supporting a previously reported interaction between presumably autoreactive T and B cells.

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Despite the many technical challenges and unique organ donor cohort utilized, the data are still limited in terms of subject numbers - a challenge in a disease characterized by extensive heterogeneity in terms of age of onset and clinical and histopathological presentation. Therefore, these studies cannot adequately account for all of the potential covariates that may drive variability and alterations in the histopathologies observed (such as age of onset, background genetics, and organ donor conditions). In this study, the manuscript and figures could be improved in terms of clarifying how variable the observed signatures were across each individual donor, with the clear notion that non-diabetic donors will present with some similar challenges and variability.

Thank you to all reviewers and editors for their thoughtful and constructive engagement with our manuscript. We agree that patient heterogeneity and the sample size limited the impact of this study. In the future, more cases with insulinitis will become available and spatial technologies will become more scalable.

Given these constraints, we have made a significant effort to illustrate the individual heterogeneity of the disease by using the same color for each nPOD case ID throughout the manuscript and showing individual donors whenever feasible (e.g. Figures 1D-E, 2C, 2I, 3E, 3G, 4B-C, 5C, and 5F). For figures related to insulinitis, we do not typically include non-T1D controls since they did not have any insulinitis (Figure 2C). We also explicitly discuss the differences in the two autoantibody-positive, non-T1D cases: one closely resembled the T1D cases with respect to multiple features and the other more closely resembled the non-T1D, autoantibody-negative controls.

Reviewer #2 (Public review):

Summary:

The authors aimed to characterize the cellular phenotype and spatial relationship of cell types infiltrating the islets of Langerhans in human T1D using CODEX, a multiplexed examination of cellular markers

Strengths:

Major strengths of this study are the use of pancreas tissue from well-characterized tissue donors, and the use of CODEX, a state-of-the-art detection technique of extensive characterization and spatial characterization of cell types and cellular interactions. The authors have achieved their aims with the identification of the heterogeneity of the CD8+ T cell populations in insulinitis, the identification of a vasculature phenotype and other markers that may mark insulinitis-prone islets, and the characterization of tertiary lymphoid structures in the acinar tissue of the pancreas. These findings are very likely to have a positive impact on our understanding (conceptual advance) of the cellular factors involved in T1D pathogenesis which the field requires to make progress in therapeutics.

Weaknesses:

A major limitation of the study is the cohort size, which the authors directly state. However, this study provides avenues of inquiry for researchers to gain further understanding of the pathological process in human T1D.

Thank you for your analysis. We point the reader to our above description of our efforts to faithfully report the patient variability despite the small sample size.

Reviewer #3 (Public review):

Summary:

The authors applied an innovative approach (CO-Detection by indEXing - CODEX) together with sophisticated computational analyses to image pancreas tissues from rare organ donors with type 1 diabetes. They aimed to assess key features of inflammation in both islet and extra-islet tissue areas; they reported that the extra-islet space of lobules with extensive islet infiltration differs from the extra-islet space of less infiltrated areas within the same tissue section. The study also identifies four sub-states of inflamed islets characterized by the activation profiles of CD8+ T cells enriched in islets relative to the surrounding tissue. Lymphoid structures are identified in the pancreas tissue away from islets, and these were enriched in CD45RA+ T cells - a population also enriched in one of

the inflamed islet sub-states. Together, these data help define the coordination between islets and the extra-islet pancreas in the pathogenesis of human T1D.

Strengths:

The analysis of tissue from well-characterized organ donors, provided by the Network for the Pancreatic Organ Donor with Diabetes, adds strength to the validity of the findings.

By using their innovative imaging/computation approaches, key known features of islet autoimmunity were confirmed, providing validation of the methodology.

The detection of IDO+ vasculature in inflamed islets - but not in normal islets or islets that have lost insulin-expression links this expression to the islet inflammation, and it is a novel observation. IDO expression in the vasculature may be induced by inflammation and may be lost as disease progresses, and it may provide a potential therapeutic avenue.

The high-dimensional spatial phenotyping of CD8+T cells in T1D islets confirmed that most T cells were antigen-experienced. Some additional subsets were noted: a small population of T cells expressing CD45RA and CD69, possibly naive or TEMRA cells, and cells expressing Lag-3, Granzyme-B, and ICOS.

While much attention has been devoted to the study of the insulinitis lesion in T1D, our current knowledge is quite limited; the description of four sub-clusters characterized by the activation profile of the islet-infiltrating CD8+T cells is novel. Their presence in all T1D donors indicates that the disease process is asynchronous and is not at the same stage across all islets. Although this concept is not novel, this appears to be the most advanced characterization of insulinitis stages.

When examining together both the exocrine and islet areas, which is rarely done, authors report that pancreatic lobules affected by insulinitis are characterized by distinct tissue markers. Their data support the concept that disease progression may require crosstalk between cells in the islet and extra-islet compartments. Lobules enriched in β -cell-depleted islets were also enriched in nerves, vasculature, and Granzyme-B+/CD3-cells, which may be natural killer cells.

Lastly, authors report that immature tertiary lymphoid structures (TLS) exist both near and away from islets, where CD45RA+ CD8+T cells aggregate, and also observed an inflamed islet-subcluster characterized by an abundance of CD45RA+/CD8+ T cells. These TLS may represent a point of entry for T cells and this study further supports their role in islet autoimmunity.

Weaknesses:

As the authors themselves acknowledge, the major limitation is that the number of donors examined is limited as those satisfying study criteria are rare. Thus, it is not possible to examine disease heterogeneity and the impact of age at diagnosis. Of 8 T1D donors examined, 4 would be considered newly diagnosed (less than 3 months from onset) and 4 had longer disease durations (2, 2, 5, and 6 years). It was unclear if disease duration impacted the results in this small cohort. In the introduction, the authors discuss that most of the pancreata from nPOD donors with T1D lack insulinitis. This is correct, yet it is a function of time from diagnosis. Donors with shorter duration will be more likely to have insulinitis. A related point is that the proportion of islets with insulinitis is low even near diagnosis. Finally, only one donor was examined that while not diagnosed with T1D, was likely in the preclinical disease stage and had autoantibodies and insulinitis. This is a critically important disease stage where the methodology developed by the investigators could be applied in future efforts.

While this was not the focus of this investigation, it appears that the approach was very much immune-focused and there could be value in examining islet cells in greater depth using the methodology the authors developed.

Additional comments:

Overall, the authors were able to study pancreas tissues from T1D donors and perform sophisticated imaging and computational analysis that reproduce and importantly extend our understanding of inflammation in T1D. Despite the limitations associated with the small sample size, the results appear robust, and the claims well-supported.

The study expands the conceptual framework of inflammation and islet autoimmunity, especially by the definition of different clusters (stages) of insulitis and by the characterization of immune cells in and outside the islets.

Thank you for your feedback. We agree that it would be very informative to expand on our analysis of autoantibody-positive cases and look at additional non-immune features.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

(1) Do any of the observed cellular or structural features correlate with age of onset or disease duration? While numbers of subjects are low, considering these as continuous variables may clarify some of the findings.

Thank you for the suggestion. In Supplemental Figure 5B-C, we plotted the key immune signatures from the manuscript against the diabetes duration and age of onset.

(2) The IDO is an interesting observation and has prior support in the literature. The authors speculate this may be induced as a feature of IFN γ expressed by lymphocytes in the local microenvironment. Can any of these concepts be further validated by staining for transcription factors or surrogate downstream markers associated with Th1 skewing (e.g., Tbet, CXCR3, etc)?

The only other interferon-stimulated gene in our panel is HLA-ABC. We updated Supplemental Figure 2F to include HLA-ABC expression in IDO- and IDO+ islets (within the “Inflamed” group). Consistent with the hypothesis that IDO is stimulated by interferon, HLA-ABC is also significantly higher in IDO+ islets than IDO- islets. PDL1, another interferon-stimulated gene, was included in the panel but we did not detect any signal. This antibody was very weak during testing in the tonsil, so we couldn’t confidently claim that PDL1 was not expressed.

(3) The authors discuss the potential that CD45RA may be expressed in Temra populations. This could use additional clarification and a distinction from Tscm if possible.

Unfortunately, we did not have the appropriate markers to distinguish naïve, TEMRA, or Tscm cells from each other. We updated the text in the discussion to include this consideration (Line 432).

(4) Supplemental Figure 5 is not informative in the current display.

Thank you, we replotted these data.

(5) Supplemental Table 1 could be expanded with additional metadata of interest, including the genetic features of the donors (e.g, class II diplotype and GRS2 values) that are published and available in the nPOD program.

Some genetic data are only available to nPOD investigators. We think it is more appropriate to request the data directly from them.

Reviewer #2 (Recommendations for the authors):

(1) I had only a few specific comments. I think the statement in Lines 317 and 318 is too strong. It implies that each lobe is always homogeneous for having all islets with insulitis or not having insulitis. Some lobes are certainly enriched for islets with insulitis but insulin+ islets without insulitis in some lobes in some T1D donors are seen. Please soften that statement.

We apologize for our lack of clarity. We have edited the text (line 305-309) to better articulate that organ donors fall on a spectrum. Thank you for raising this point as we think the motivation for our analysis is much clearer after these revisions.

(2) Please cite and discuss In't Veld Diabetes 20210 PMID: 20413508. While the main point of the paper is that there is beta cell replication after prolonged life support, another observation is that there is a correlation between prolonged life support and CD45+ cells in the pancreas parenchyma. This might indicate that not all immune cells in the parenchyma are T1D associated in donors with T1D.

Thank you, we have added this citation to our discussion of the importance of duration of stay in the ICU (Line 471).

(3) Can you rule out that CD46RA+/CD69+ CD8+ T cells in the islets are not TSCM?

(See above)

Reviewer #3 (Recommendations for the authors):

Similar studies in experimental models may afford increased opportunity to evaluate the significance of these findings and model their potential relevance for disease staging and therapeutic targeting.

We agree that the lack of experimental data limits the ability to interpret and validate the significance of our findings. We hope that our study motivates and helps inform such experiments.

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