Immune modulation and monitoring of cell therapy in inflammatory disorders

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Pancreatic pathology in pre-diabetic patients

Heterogeneity and lobularity of pancreatic pathology in type 1 diabetes during the prediabetic phase: a case study

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Abstract

Type 1 diabetes (T1D) is an autoimmune disease in which insulin-producing beta cells are destroyed in the islets of Langerhans. One of its main pathological manifestations is the hyper-expression of MHC class I by beta cells, which was first described over 3 decades ago, yet its cause still remains unknown. It might not only be a sign of beta-cell dysfunction but could also render them susceptible to autoimmune destruction for example by islet-infiltrating CD8 T cells. In this report we studied the pancreas of a 22 year old non-diabetic male at high risk of developing T1D in which auto-antibodies against GAD and IA-2 were detected. Pancreas sections were analyzed for signs of inflammation. Multiple insulin containing islets were identified, which hyper-expressed MHC-I. However, islet density and MHC I expression exhibited a highly lobular and heterogeneous pattern even within the same section. In addition, many islets with high expression of MHC-I presented higher levels of CD8 T cell infiltration than normal islets. These results demonstrate the heterogeneity of abnormalities occurring early during the pre-diabetic, autoantibody positive phase and should contribute to the understanding of human T1D pathology.

Abbreviations

Ab+, Autoantibody positive; MHC-I, Major Histocompatibility Complex I; BMI, Body Mass Index; ICIs, Insulin Containing Islets; IDIs, Insulin Deficient Islets; T1D, type 1 diabetes; T2D, type 2 diabetes; nPOD, Network for Pancreatic Organ donors with Diabetes; NOD, Non-Obese Diabetic Mouse.

Introduction

Pathological changes take place before the complete destruction of insulin-producing beta cells in the pancreatic islets of pre-diabetic individuals, and might offer us insight into the earlier events underlying diabetes development. These coincide with the appearance of autoantibodies, which constitute nowadays the most common tool to predict future diabetes development (82). Usually, antibodies against insulin (IA) appear first, followed by glutamate decarboxylase (GAD), insulinoma-associated protein 2 (IA-2) and zinc transporter 8 (ZnT8) (83). Around the time of diagnosis, beta cell function is relatively rapidly lost, but in most cases a significant residual number of functional beta cells can still be present over many years (26, 84-86). It is known that during the early pre-diabetic state, beta cells can show an abnormal phenotype with one pathognomonic sign being the increase in MHC-I expression in both insulin-deficient and insulin-containing islets (26, 87, 88). This phenomenon was described 30 years ago by Bottazo et al. and by Foulis and colleagues (88, 89). The trigger or cause for this elevated expression is still not understood.

As the disease progresses, a lymphocytic infiltration can be observed in some islets. This phenomenon, described more than 100 years ago by Schmidt (90), was named insulitis by von Meyenburg in 1940 (91) and studied by LeCompte and Gepts in 1958 (92) and in 1965 (93). It is somewhat better characterized today and we know that the most frequent cell types are CD8 lymphocytes followed by macrophages, B cells and CD4 T cells (94). However, only a few studies have been carried out in non-diabetic, autoantibody positive donors with the majority of them showing no leukocytic infiltration or beta cell damage (95-97). The Network for Pancreatic Organ Donors with Diabetes (nPOD) has now opened up the unique possibility of investigating and characterizing the histopathological presentation during all the stages of the disease, from the pre-diabetic to the chronic state. In the present study we investigated the pancreas of a double autoantibody positive donor at high risk of developing T1D. We show that high MHC-I expression and CD8 T cell infiltration are remarkably heterogeneously distributed and differentially affect islets situated in different regions of the pancreas creating a multifocal pattern. The cause(s) for

this lobularity remain unclear, among them the potential for viral infections, the inflammatory milieu in the pancreas as well as a potentially yet unknown intrinsic etiology.

Material and methods

Subject

Human pancreata were collected from a cadaveric organ donor through the Network for Pancreatic Organ donors with Diabetes (nPOD). Six µm sections from frozen pancreas samples from 3 different blocks from the head (02, 04 and 06), body (02, 06 and 08) and tail (02, 04 and 06) region were obtained. All experimental procedures were approved by the La Jolla Institute for Allergy and Immunology Institutional Review Board-approved protocol number DI3-054-1112.

Immunofluorescence for insulin, HLA-ABC and CD8

Sections were subject to a standard immunofluorescence staining protocol. Briefly, sections were fixed with 0.4% paraformaldehyde and blocked with goat serum. Staining for insulin and HLA-ABC (MHC-I) was performed at room temperature for 1h using the following antibodies: Polyclonal guinea pig anti-Insulin (1/140; Dako, Carpinteria, CA) and mouse monoclonal (clone W6/32) IgG2a against a monomorphic epitope on the 45 kD polypeptide products of the HLA-A, B and C loci (1/100; Dako, Carpinteria, CA). Detection was done at room temperature for 45 minutes using polyclonal goat anti-guinea pig IgG, highly cross-adsorbed, Alexa Fluor 488 (1/1000 Invitrogen, Grand Island, NY) and polyclonal goat anti-mouse IgG2a, isotype-specific Alexa Fluor 594 (1/1000 Invitrogen, Grand Island, NY). Finally, sections were incubated for 1h at room temperature with mouse monoclonal (clone RFT8) IgG1 conjugated to Alexa Fluor 647 (1:50, Birmingham, AL). After washing, sections were mounted with ProLong Gold antifade reagent (Molecular Probes, Grand Island, NY). Control pancreatic tissue sections with only secondary antibodies were used to determine background staining.

Image acquisition and analysis

Images were acquired using a Zeiss Axio Scan Z.1 slide scanner (10x objective) and Zen software, Blue edition (Zeiss, Oberkochen, Germany). Whole tissue section images were divided into multiple smaller areas using a grid in order to facilitate analysis. Islets were then counted manually based on insulin staining and islet density calculated per section based on the number of islets and the total area of the tissue determined by using a custom macro developed for ImageJ (National Institutes of Health). MHC-I expression was qualitatively evaluated and every islet classified based on the intensity of MHC-I staining. Infiltrating CD8 T cells were manually counted in each islet and matched to the MHC-I pattern. The percentage of elevated and hyper-expressing islets combined (abnormal islets) was calculated and represented as a heat map by using Excel conditional format tool (Microsoft, Redmond, WA, USA). The lowest values were assigned green color tones and the highest values red color tones. The same process was applied to CD8 T cell values per abnormal islet section.

Statistical analysis

Group differences were analyzed by using Kruskal-Wallis non-parametric test followed by Dunn test for multiple comparisons. Correlation analysis was done by using Spearman correlation with two-tailed significance test. All analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego California USA). Data in bar graphs are presented as mean ± SD. Findings were assumed statistically significant at $p \leq 0.05$.

Results

Demographic characteristics of case 6197

The donor described in this manuscript was male, 22 years old, with BMI of 28.2, African American, positive for IA-2 and GAD autoantibodies. High HLA resolution analysis showed the following genotype: A*02:02, 24:02; DRB1*03:02, 07:01; DQA1*02:01, 04:01; DQB1*02:02, 04:02. The nPOD pathology core indicated the presence of multiple insulin and glucagon positive islets at first screening. Some insulitis was also reported (rare) as well as islet hyperemia. Finally, a mild, multifocal chronic pancreatitis was found. The donor was found to be positive for cytomegalovirus (CMV) and Epstein-Barr virus (EBV) IgG in serum.

Systematic histological analysis of pancreatic sections

Frozen pancreatic sections from 3 different blocks from the head, body and tail of the pancreas were analyzed (head blocks #02, 04 and 06; body blocks #02, 06 and 08 and tail blocks #02, 04 and 06). Each section was divided into several quadrants by using a grid. In each of these areas, islets were manually counted (Fig. 1). The number of total islets was very variable depending on the section and region. A minimum of 183 islets were counted in the head block #04 while 656 islets were detected in the tail block #04 (Fig. 2A). Islet density was then calculated based on the total tissue area and ranged from 2.04 islets/mm^2 up to 4.80 islets/mm^2 and was found to be higher in the pancreatic head and tail (3.61 vs 3.33 islets/mm^2) while it was lower in the body (2.41 islets/mm^2) (Fig. 2A). Islets were then classified based on the level of MHC-I expression as normal (no detectable expression in endocrine cells), elevated (high expression of MHC-I by endocrine cells not affecting the whole islet area) and hyper-expressing islets (high expression of MHC-I by endocrine cells affecting all the islet area) (Fig. 2B).

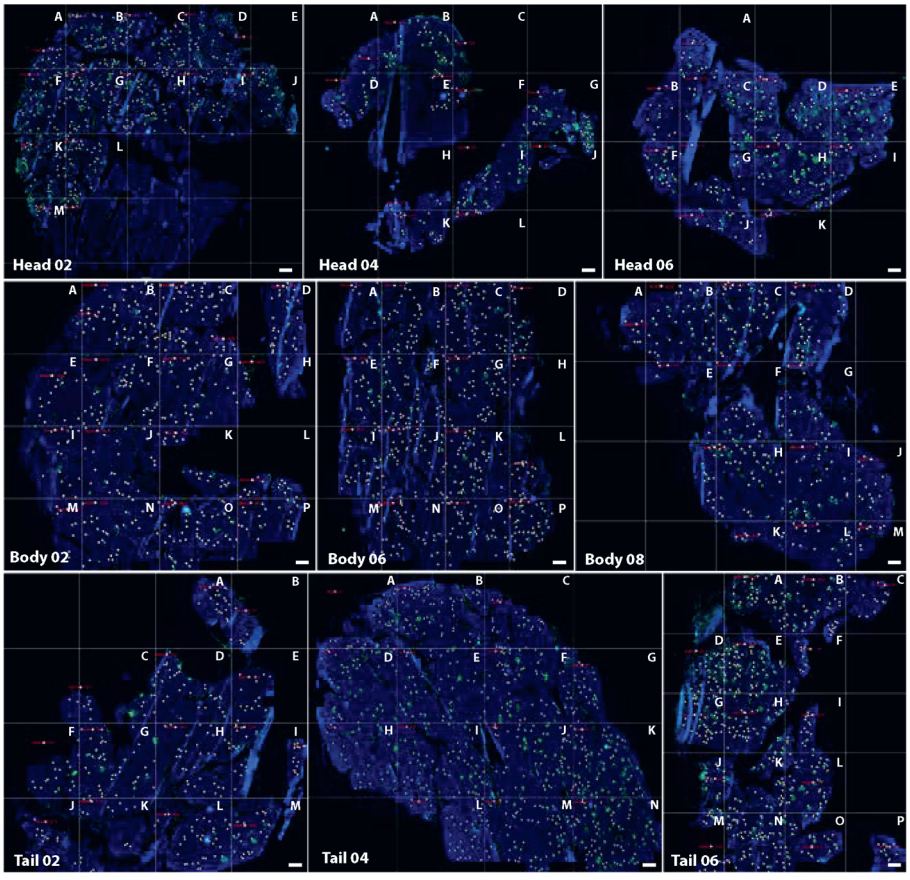

Figure 1. Whole frozen pancreatic sections were stained for insulin (green), MHC-I (red), CD8 (white) and DAPI (blue) following a standard immunofluorescence protocol. Sections were scanned and divided into smaller areas by using a grid in order to facilitate analysis. A letter was assigned to each quadrant (A-P). Whole tissue images are shown for each of the sections. Only insulin and DAPI staining are depicted for clarity purposes. White dots indicate islets. Scale bar, 1000 μm in all images.

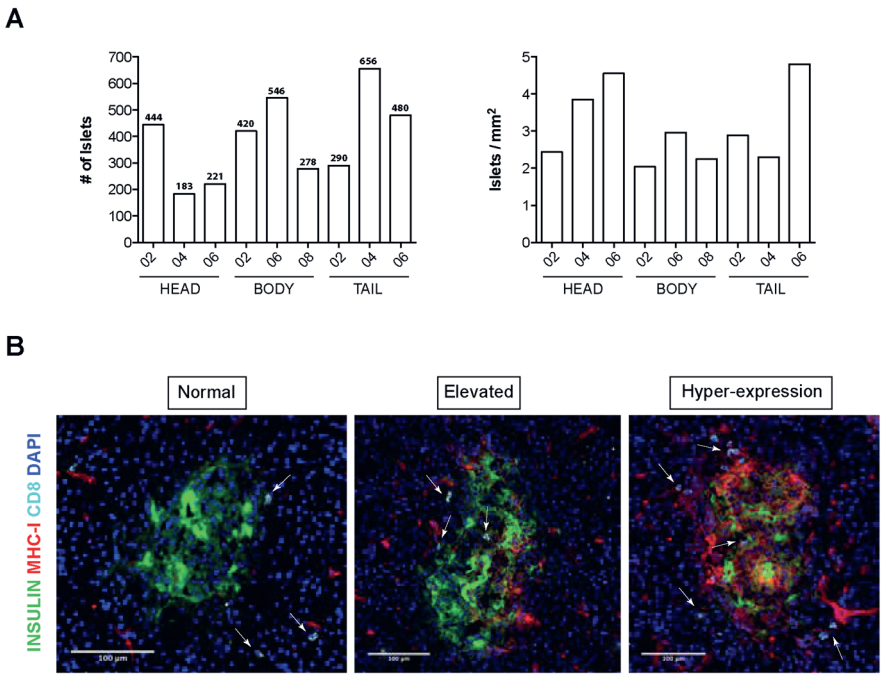

Figure 2. A) The absolute number of islets for each section and block is shown as bar graphs. The specific number of islets is indicated on top of each bar (left panel). Islet density (Islets/mm^2) was calculated based on the number of islets and total tissue area and it is shown as bar graphs (right panel). B) Islets were classified based on the level of MHC-I expression as normal (left panel, no detectable expression in endocrine cells), elevated (center panel, high expression of MHC-I by endocrine cells not affecting the whole islet area) and hyper-expressing islets (right panel, high expression of MHC-I by endocrine cells affecting all the islet area). Arrows indicate the presence of CD8 T cells. Scale bar, 100 µm in all images.

MHC-I hyper-expression is heterogeneously distributed across multiple islets and regions in the pancreas

In order to study islet MHC-I expression and localization in the pancreas, staining for insulin and MHC-I was performed across multiple sections from the head, body and tail. The number of islets and the expression of MHC-I was analyzed manually for each section and represented as bar graphs and heat maps (Figs. 3 and 5) (see Materials and Methods for details). In head block #02, 444 islets were counted. Of these, 5.63 (0-32%, A to M) and 10.81 % (0-50%, A to M) of the islets showed elevated Class I expression and hyper-expression respectively (Fig. 3A). In head block #04 and #06, 183 and 221 islets were counted. While elevated islets constituted 13.11 (0-33.3%, A to L) and 5.43 % (0-21.7%, A

to K) respectively, hyper-expression was detected in 8.74 (0-36.3%, A to L) and 3.62 % (0-47.6%, A to K) in these two blocks (Fig. 3B and C). Overall, elevated and hyper-expressing islets (abnormal phenotype) constituted 13.43 % of the islets in the head region.

Next, we performed the same analysis for body and tail regions. A total of 420, 546 and 278 islets were counted in body blocks #02, 06 and 08 respectively. Of this, 9.52 (0-20.6%, A to P), 7.88 (0-17%, A to P), and 12.59 % (0-34.8%, A to M) of the islets presented elevated MHC-I expression while a lower number of islets was found to hyper-express it (5.24 (0-11.3%, A to P), 3.11 (0-40%, A to P), and 6.83 % (0-33.3%, A to M) respectively) (Fig. 3D, E and F). In the tail, 290, 656 and 480 islets were counted in tail block #02, 04 and 06, with 4.14 (0-12.5%, A to M), 9.15 (0-33.3%, A to N) and 7.29 % (0-37.5%, A to P) of them presenting increased MHC-I expression. Hyper-expressing islets were found to be lower in this region with 0.69 (0-9.5%, A to M), 8.38 (0-32.1%, A to N) and 3.75 % (0-34.8%, A to P) of the islets for blocks #02, 04 and 06 respectively (Fig. 3G, H and I). No significant differences were found between different blocks except for tail block #02 which presented a lower percentage of abnormal islets than other blocks (Fig. 4A, B and C). Summarized information can be found in Table 1. Finally, values from all the regions were put together in a heat map in which the areas with higher percentage of abnormal islets are shown in red color tones and were present in head blocks #02 and 04, body block #08 and tail block #04. Conversely, those areas containing mainly normal islets are depicted as green (Fig. 7A) and were scattered across all the blocks.

Affected islets (elevated and hyper-expressing MHC-I) constituted 13.4 % of the islets in the body, which was almost the same percentage as found in the head (14.4 %) while the tail presented a slight reduction (11.9 %) mainly due to the smaller amount of hyper-expressing islets found in Tail block #02 (data not shown). When values were grouped according to the main anatomical regions (head, body or tail), islet hyper-expression was found to be higher in the head while elevated MHC-I was predominant in the body (Fig. 4D, E and F). However, no significant differences were detected between head, body and

tail regions regarding the percentage of elevated, hyper-expressing or normal islets (Fig. 4D, E and F).

Table 1. Summarized information for the % of elevated, hyper-expressing and abnormal islets for each block and region is shown.

	MHC-I (% islets)		
Elevated	**HEAD**	**BODY**	**TAIL**
Block A	5.63	9.52	4.14
Block B	13.11	7.88	9.15
Block C	5.43	12.59	7.29
Hyper-expression	**HEAD**	**BODY**	**TAIL**
Block A	10.81	5.24	0.69
Block B	8.74	3.11	8.38
Block C	3.62	6.83	3.75
Abnormal	13.40	14.40	11.90

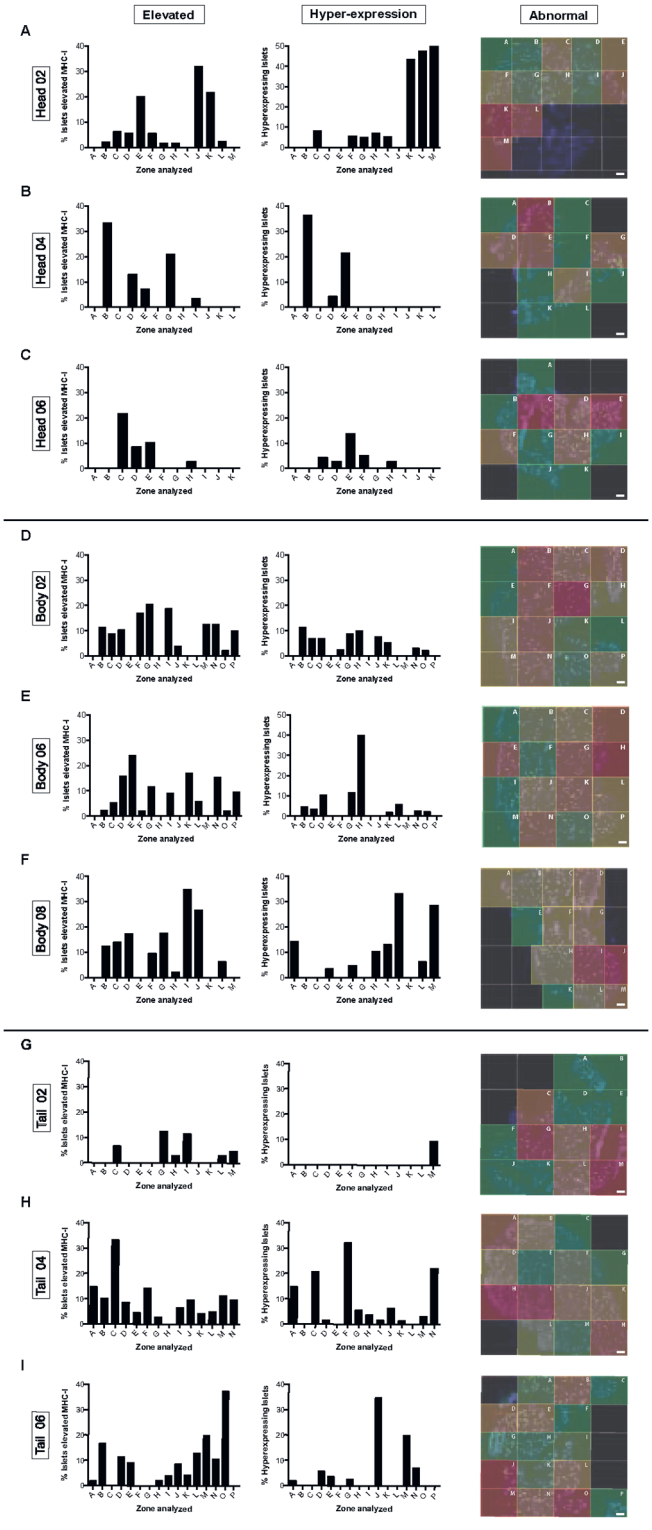
Elevated
Hyper-expression
Abnormal
A
Head 02
B
Head 04
C
Head 06
D
Body 02
E
Body 06
F
Body 08
G
Tail 02
H
Tail 04
I
Tail 06
% Islets elevated MHC-I
% Hyperexpressing islets
Zone analyzed

Figure 3. The percentage of elevated (left panel) and hyper-expressing (center panel) islets from each quadrant of the pancreatic sections depicted in figure 1 was calculated and it is shown as bar graphs. Heat maps showing the areas with higher (red) or lower (green) percentage of abnormal islets within the section are shown on the right. A) Head Block # 02; B) Head Block #04; C) Head Block #06; D) Body Block # 02; E) Block #06; F) Body Block #08; G) Tail Block # 02; H) Tail Block #04; I) Tail #06. Scale bar, 1000 µm in all images.

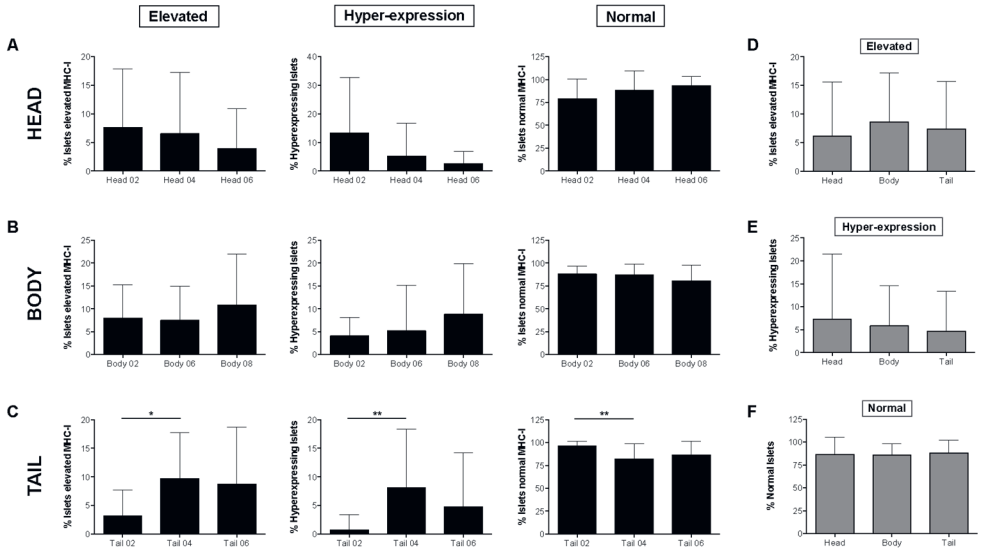

Figure 4. The mean percentage of elevated (left panel), hyper-expressing (center panel) and normal (right panel) islets is shown as bar graphs for each block in A) Head; B) Body and C) Tail. In D, E and F, overall head, body and tail values are shown for elevated, hyper-expressing and normal islets. *significant difference between groups ($P \leq 0.05$). **significant difference between groups ($P \leq 0.01$).

CD8 T cells preferentially infiltrate islets with elevated levels of MHC-I expression

To evaluate the possible link between high MHC-I expression and infiltration, CD8 T cells were quantified on a per islet basis and correlated with their MHC-I pattern. High infiltration was only noticed in some islets. Similar values of CD8 T cells per elevated or hyper-expressing islet were found in head block #02 (3.40 and 3.71 CD8 T cells per islet respectively). In head block #04 a mean of 1.79 and 3.25 cells were found to infiltrate elevated and hyper-expressing islets. Furthermore, almost identical numbers of CD8 T cells were found in head block #06 for abnormal islets (2.75 and 2.63 CD8 T cells per elevated and hyper-expressing islet respectively) (Fig. 5A, B and C). Accordingly, similar values were found for elevated islets in body block #02 and 06 (2.33 vs 2.16) while block #04 had lower level of infiltration (1.20 CD8 T cells/elevated islet section). In contrast hyper-expressing islets were infiltrated by a mean of 4.73, 2.76 and 0.79 CD8 T cells/islet section in blocks #02, 04 and 06, being this region the most variable (Fig. 5D, E and F). Finally, no major differences were found in the tail between regions. Infiltration ranged from 1.67 to 2.67 CD8 T cells/elevated islet and from 2.28 to 3.33 CD8 T cells/hyper-expressing islet in the tail blocks analyzed (Fig. 5G, H and I). Summarized information for all the blocks can be found in Table 2.

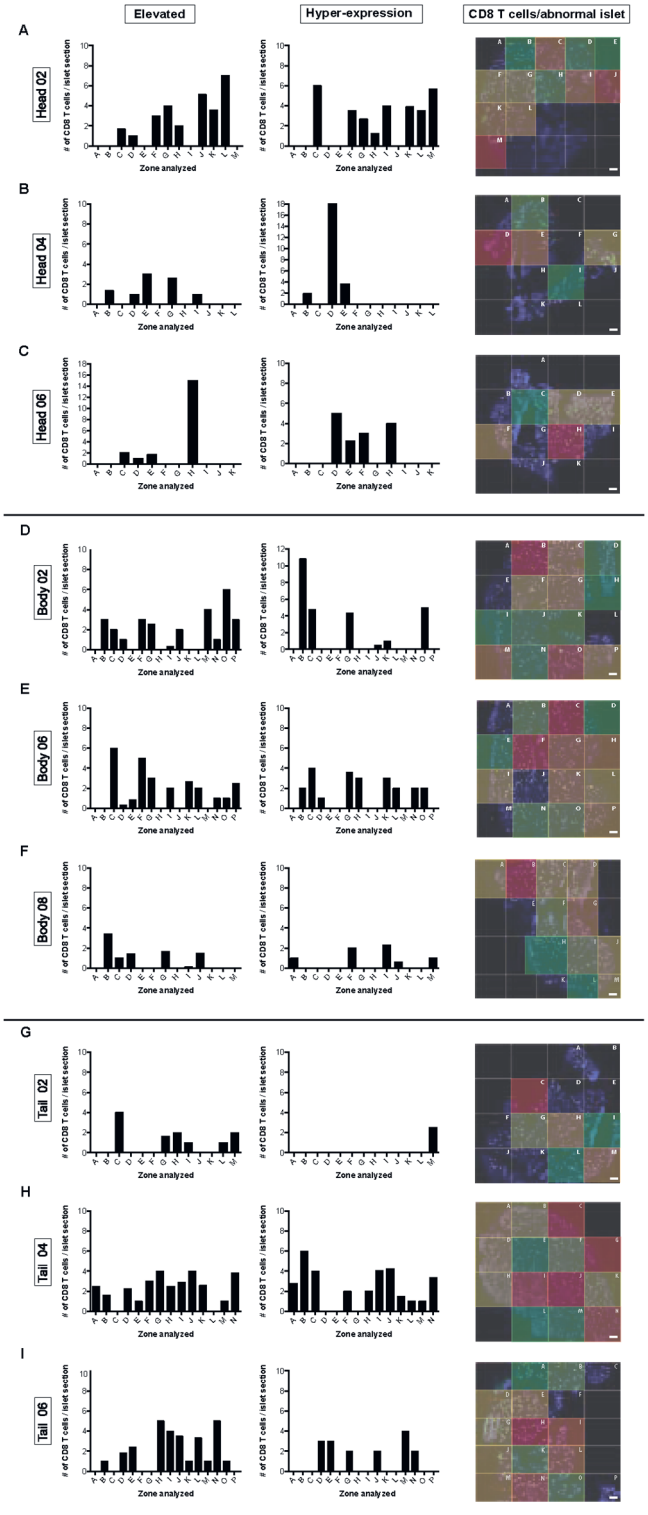
Elevated
Hyper-expression
CD8 T cells/abnormal islet
A
Head 02
B
Head 04
C
Head 06
D
Body 02
E
Body 06
F
Body 08
G
Tail 02
H
Tail 04
I
Tail 06
# of CD8 T cells / islet section
Zone analyzed

Figure 5. The mean number of CD8 T cells per elevated (left panel) and hyper-expressing (center panel) islet from each quadrant of the sections depicted in figure 1 was determined and it is shown as bar graphs. Heat maps showing the areas with higher (red) or lower (green) number of CD8 T cells per abnormal islet (elevated and hyper-expressing) within the section are shown on the right. A) Head Block # 02; B) Head Block #04; C) Head Block #06; D) Body Block # 02; E) Block #06; F) Body Block #08; G) Tail Block # 02; H) Tail Block #04; I) Tail #06. Scale bar, 1000 µm in all images.

Table 2. Summarized information for the number of CD8 T cells per elevated, hyper-expressing and abnormal islet for each block and region is shown.

	CD8 T cells (# per islet section)		
Elevated	**HEAD**	**BODY**	**TAIL**
Block A	3.40	2.33	1.67
Block B	1.79	1.20	2.67
Block C	2.75	2.16	2.20
Hyper-expression	**HEAD**	**BODY**	**TAIL**
Block A	3.71	4.73	2.50
Block B	3.25	2.76	3.33
Block C	2.63	0.79	2.28
Abnormal	2.89	2.19	2.33

Across the 3 blocks examined in each of the 3 regions, normal islets presented similar numbers of infiltrating CD8 T cells ranging from 0.33 to 0.66 CD8 T cells/islet section for all the blocks except for tail block #04 which presented a slightly higher value (1.24 CD8 T cells/islet section) (data not shown).

Overall, elevated and hyper-expressing islets presented a significantly higher number of infiltrating CD8 T cells than normal islets in all the blocks. In addition, these differences were significant in head, body and tail regions. No differences were found between elevated and hyper-expressing islets in any analyzed area (Fig. 6). Lastly, values from all the regions were placed together in a heat map in which the areas with higher number of

infiltrating CD8 T cells per islet section are shown in red color tones and were present in head block #02 and tail block #04 and 06. The quadrants containing less CD8 T cells are depicted in green (Fig. 7B) and were scattered across all the blocks being less predominant in tail block #06.

Correlation analysis between the percentage of abnormal islets and the number of CD8 T cells per islet section in each quadrant showed strong correlation (r=0.6) with a moderate linear fit (R^2=0.44) (Fig. 7C).

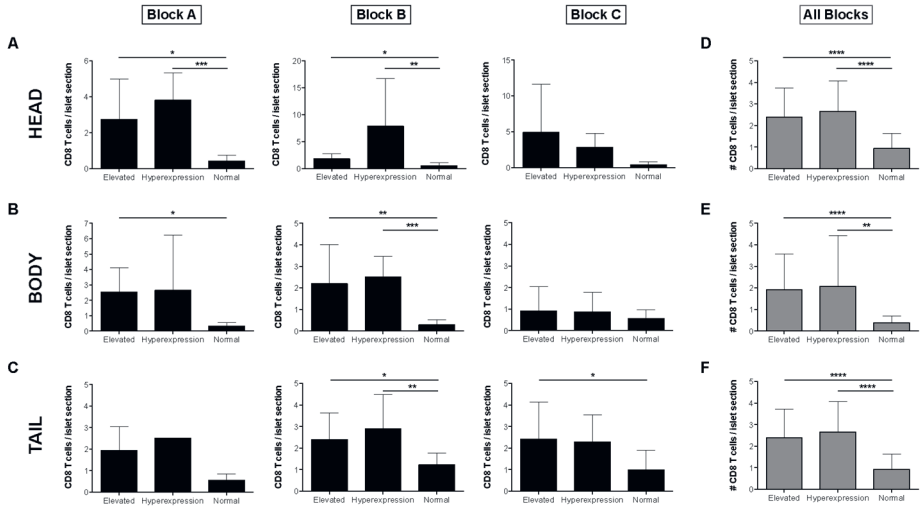

Figure 6. The mean number of CD8 T cells per elevated, hyper-expressing and normal islet is shown as bar graphs for the 3 blocks examined (named A, B and C here) from A) head; B) Body and C) Tail. The overall mean number of CD8 T cells per elevated, hyper-expressing and normal islet is shown for all the blocks combined in D (Head), E (Body) and F (Tail). *significant difference between groups (P ≤ 0.05). **significant difference between groups (P ≤ 0.01). ***significant difference between groups (P ≤ 0.001). ****significant difference between groups (P ≤ 0.0001).

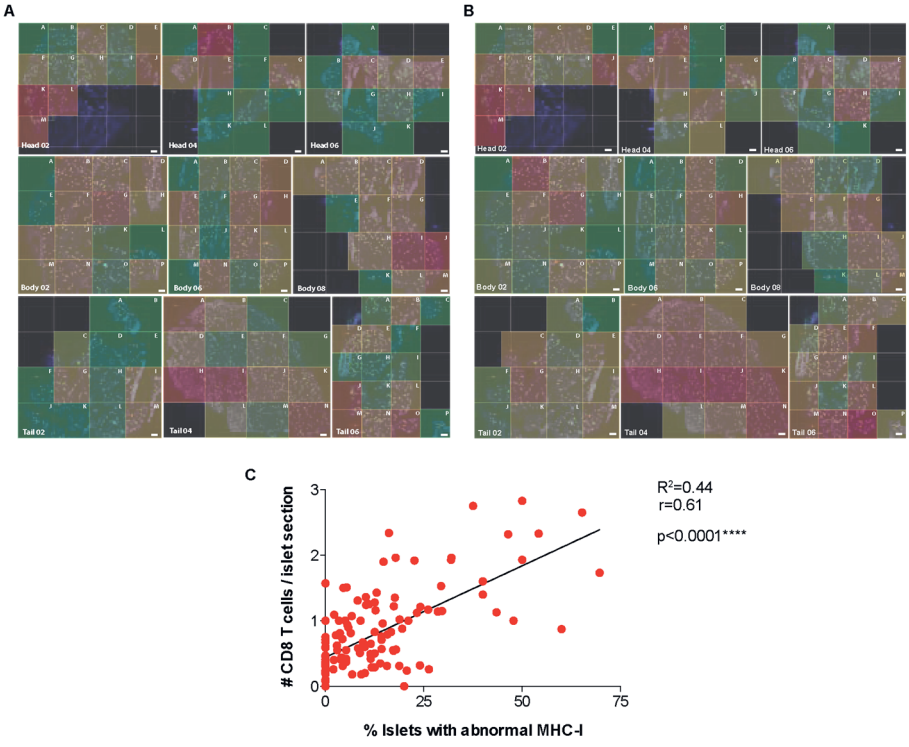

Figure 7. A) The overall percentage of islets with abnormal MHC-I expression (elevated and hyper-expressing islets) is represented as a heat map. Areas with higher (red) or lower (green) presence of affected islets are shown for all the sections together. B) The overall mean number of CD8 T cells per islet section (elevated, hyper-expressing and normal) is represented as a heat map. Areas that contained islets with higher (red) or lower (green) presence of CD8 T cells are shown for all the sections together. C) Correlation analysis of the percentage of islets with abnormal MHC-I and the number of CD8 T cells per islet section (r=0.61; R^2=0.44). Scale bar, 1000 µm in all images. ****significant difference (P ≤ 0.0001).

Discussion

In the present study, we have performed a systematic analysis of two pathognomonic signs of early diabetes, MHC-I expression and CD8 T cell infiltration into islets. Pancreatic samples from a double Ab+ individual with no clinical signs of diabetes showed high MHC-I expression affecting approximately 14 % of the islets in head and body and 12 % in the tail. Areas of islets with normal MHC-I expression were frequently continuous to areas with hyper-expressing islets. In some of these areas almost 50 % of the islets hyper-expressed Class I. In addition, we observed an intermediate level of expression that was apparent in some islets. In these so-called "elevated islets", not all the endocrine cells within the islet expressed MHC-I. Their distribution was also scattered across the pancreatic sections. Hence, islets with an abnormal phenotype (elevated and hyper-expressing MHC-I) presented a patchy and sometimes lobular distribution. CD8 T cell infiltration, although mild, was also detected in the affected islets and was on average higher in islets with clear MHC-I hyper-expression compared to islets with just elevated and normal MHC class I. In 1985, a case report by Botazzo and colleagues described the pathological findings in the pancreas of a 12 year old girl with newly-diagnosed T1D, who died within 24 hours of diagnosis (89). In this important study, "a marked increase" in Class I (HLA-A, B and C) expression was also observed in some islets. In addition, analysis of islet infiltration showed the presence of a predominant cytotoxic T cell population (89). In 1987 Foulis et al. confirmed the presence of an abnormally high expression of MHC-I molecules on endocrine cells from type 1 diabetic donors (88). Even at that time, the possible underestimation of pathological alterations based on the sole analysis of one tissue section from one particular block was a concern (88). In the present study we have performed a comprehensive and systematic analysis in multiple blocks from the pancreatic head, body and tail to overcome this possible limitation. As shown here, the lobularity and heterogeneity of the human disease increases the complexity of possible pathological interpretations and therefore conclusions obtained from the study of one tissue section should, in general, be interpreted with caution.

Insulitis is present in both mice and humans, but major differences have been reported. In mouse models, infiltration starts in the peri-islet area and it is comparatively massive while in humans, as described here, it is usually mild. Additionally, we and others have shown that only a small percentage of islets is inflamed during the pre-diabetic phase (26, 95). Conversely, in the NOD mice model, at 18 weeks-old, almost all of the islets are heavily infiltrated (98, 99). Furthermore, in both Non-Obese Diabetic (NOD) mice and humans, islet beta cells up-regulate MHC-I during the inflammatory response and this process is important for the disease progression. As reported by Hamilton-Williams et al, MHC-I influences beta cell destruction by infiltrating cytotoxic T cells (100). NOD mice lacking MHC-I expression on beta cells presented the same level of insulitis as those with normal expression, but hyperglycaemia was delayed or abolished. Our results additionally show that there is a strong correlation between islet MHC-I expression and infiltrating CD8 T cells in humans. Areas containing mostly normal islets presented a lower but still evident number of CD8 T cells per islet section than those with higher percentage of abnormal islets. This could indicate that CD8 T cells infiltrate the islets even before the up-regulation of MHC-I occurs. The differences between mice and human pathology are therefore intriguing. They could reside on the aetiology or the primary cause of the disease, but could also be just reflective of an overall slower disease course in humans (years versus months in mice).

The link between MHC-I hyper-expression and virus infections has often been debated over the past 30 years. Our results here cannot confirm the presence of a viral infection but the lobular pattern is indicative of the usual patchy appearance of viral antigen found during infection in a solid organ (101-103). Infection of some islets might trigger an antiviral response and therefore an increase in MHC-I (104). For example, growing evidence suggests that enteroviruses (EV) or neutralizing antibodies against them can be detected in pancreata and in the serum from T1D donors (103, 105-109). EV can effectively infect beta cells, which express the coxsackievirus and andenovirus receptor (CAR) (110, 111). Infection of beta cells could trigger the release of interferons and islet antigens (112) that might be recognized by auto-reactive T cells therefore initiating the

autoimmune destruction. However, no viral proteins or genome were detected in the pancreas of this particular autoantibody positive donor by immunohistochemistry and PCR in the blocks analyzed (Richardson S. and Oikarinen M., personal communication). We cannot completely rule out the presence of a viral infection since not all the regions of the pancreas were analyzed. In addition, it has been shown that EVs can persist due to deletions in their genome that make the virus an extremely slow replicator and that displace the wild type forms during a chronic infection (113). Additional and new techniques will be needed to detect these terminally deleted viruses in pancreata from pre- or diabetic individuals and their possible role in the pathogenesis of T1D. Slow-replicating, endogenous and/or latent viruses like herpesviruses could also be good candidates and may have a role in the induction of autoimmune diabetes (114-116). The MHC-I pattern shown here could match a possible lobular spreading of these viruses.

In conclusion, in this systematic study, MHC-I expression and CD8 T cell infiltration have been analyzed on a per islet basis in whole tissue sections from multiple regions of the pancreas. Our data demonstrate that during the pre-diabetic state, islets undergo important pathophysiological changes that occur in a patchy, almost 'vitiligo-like' fashion (117) and highlights the importance of understanding the precise cause for these changes. More insight into autoantibody positive cases could reveal early pathological events of T1D and thus could also inform future preventive strategies.

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