Immunohistochemical identification of interleukin-1β immunoreactive cells and their distribution in pancreatic biopsies from volunteers with new-onset type 1 diabetes: comparison with donors without diabetes and with longer duration of disease

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ABSTRACT

Aims/hypothesis Although interleukin-1β (IL-1β) is considered a key mediator of beta cell destruction, its cellular expression in islets during early type 1 diabetes remains unclear. We compared its expression in rare pancreatic biopsies from new-onset living volunteers with non-diabetic autoantibody-positive and -negative cases and long-standing disease.

Methods Pancreatic biopsy sections from 6 new-onset volunteers (group 1) and cadaveric sections from 13 non-diabetic autoantibody–negative (group 2), 4 non-diabetic autoantibody-positive (group 3) and 9 diabetic donors (0.25-12 years of disease; group 4) were triple-immunostained for IL-1β, insulin and glucagon. Intra- and peri-islet IL-1β-positive cells in insulin-positive and -negative islets and in random exocrine fields were enumerated.

Results In all cases, the overall mean number of IL-1β-positive cells in peri- and intra-islet regions per islet was <1.25 and <0.5, respectively and <31% of the islets were IL-1β-positive. In diabetic cases (groups 1 and 4), higher mean number of IL-1β-positive cells occurred in insulin-positive than –negative islets. In group 2, 70-90% of islets in 3/13 cases had weak to moderate IL-1β staining in alpha cells but was virtually absent or substantially reduced in the remaining groups. The mean number of exocrine IL-1β-positive cells in group 1 was lower than in the remaining groups.

Conclusions/interpretation During early type 1 diabetes, the low number of islet-associated IL-1β-positive cells may be insufficient to elicit beta cell destruction. Its variable expression in alpha cells in groups 2-4 suggests their cellular heterogeneity and probable physiological role. The significance of a higher but variable number of exocrine IL-1β-positive cells in non-diabetic and long-term type 1 diabetic cases remains unclear.

Key words Cytokines • Immunohistochemistry • Interleukin-1β • Islet cells • New-onset type 1 diabetes

Research in context (120 words)

What is already known?

- Long-term exposure of human islets in culture to interleukin-1β (IL-1β) with interferon-γ (IFN-γ) and/or tumour necrosis factor-α (TNF-α) leads to beta cell toxicity
- Exposure to lower concentrations of IL-1β and shorter incubation period results in glucose-stimulated insulin release.

What is the key question?

- Is IL-1β expressed within and around the islets during early type 1 diabetes?

What are the new findings?

- A low number of IL-1β-positive cells are present in and around the islets of new-onset cases.
- There is an increase in IL-1β-positive cells in exocrine regions of some non-diabetic and longer-term diabetic cases, probably contributed by pre-existing complications and hospitalisation stay.
- Selective macrophages, T cells and alpha cells express IL-1β.

Impact on clinical practice in the foreseeable future?

- Successful prevention of type 1 diabetes clinically may require blockade of as yet unidentified multiple beta cell destructive pathways.

Abbreviations

DAB	Diaminobenzidine
DKA	Diabetic ketoacidosis
DiViD	Diabetes Virus Detection Study
ER	Endoplasmic reticulum
ESM	Electronic supplementary material
IFN-γ	Interferon-γ
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-17	Interleukin-17
LPS	Lipopolysaccharide
MAP kinase	Mitogen activated protein kinase
NFκB	Nuclear factor kappa beta
nPOD	Network for Pancreatic Organ Donors with Diabetes
PBS	Phosphate-buffered saline
STAT 1	Signal transducer and activator of transcription 1
TNF-α	Tumour necrosis factor-α
T1D	Type 1 diabetes
T2D	Type 2 diabetes

Introduction

Interleukin-1β (IL-1β), an important pro-inflammatory cytokine, is expressed by a variety of cell-types and displays pleiotropic actions, including key roles in innate and adaptive immunity, immune cell communication, inflammation and in neuronal, metabolic and endocrine processes [1]. Several earlier studies employing cultured islets demonstrated its toxicity towards beta cells and led to the premise that it is of vital importance in the pathogenesis of human type 1 diabetes [2–7]. Prolonged exposure of human islets to IL-1β, in the presence of either interferon-γ (IFN-γ) or tumour necrosis factor-α (TNF-α), impairs glucose-stimulated insulin secretion and promotes beta cell death [5–7]. Binding of IL-1β to its cognate receptor on beta cells in vitro results in activation of prominent downstream intra-cellular gene networks and signalling events [8]. These include activation of mitogen activated protein kinase (MAPK), signal transducer and activator of transcription 1(STAT-1) and nuclear factor kappa beta (NFκB), resulting in endoplasmic reticulum (ER) and mitochondrial stress and local production of various oxidant species, such as nitric oxide and superoxide anions [4, 7, 9, 10].

Is IL-1β toxic to the beta cell during early type 1 diabetes? If so, are the deleterious processes mediated, in part, by IL-1β, within or close to the islet? In vivo effects of IL-1β on beta cell destruction are complicated by studies which point to bi-modal opposing effects of the cytokine on beta cells; at lower concentrations and shorter exposure period it promotes insulin release, anti-apoptotic effects and beta cell proliferation, while at higher concentrations and longer exposure period, it inhibits glucose-stimulated insulin release, and promotes beta cell death [7, 10–13].

Crucial evidence indicating a direct pathogenic role of IL-1β originating within the islet during development of type 1 diabetes has been hindered by limited studies clarifying the level of cytokine expression in human pancreas with early disease. Most studies have analysed isolated islets from normal and type 2 diabetic pancreas from deceased subjects at the mRNA level, which may not correlate with the translated protein [9, 14]. Since mRNA and Western blotting analysis involve initial disruption of islet integrity, vital information regarding the spatial juxtaposition of cytokine-expressing cells in relation to the islets and islet cells is unavailable. Based on findings from pancreatic sections from patients with T1D, we speculated that islet-infiltrating leukocyte subsets in the peri- and intra-islet regions may release a variety of beta cell toxic factors, including IL-1β and various oxidants involved in nitrosative stress [15, 16].

The deleterious effects of IL-1β on human beta cells in vitro and in animal studies have prompted recent multicentre clinical trials in volunteers with recent-onset type 1 diabetes, using strategies involving systemic blockade of IL-1β or its receptor [10, 17]. The inefficacy of such interventions calls for a re-examination of the pathogenic role of IL-1β in type 1 diabetes and we ask whether the cytokine is indeed expressed locally at a level toxic to beta cells [17]. Studies on its cellular expression within the islets during the early stages of disease have been severely limited by the scarcity of suitable pancreatic samples. In a previous report from Japan, six pancreatic biopsy samples from adult living donors showed the presence of IL-β and TNF-α predominantly in selective macrophages and dendritic cells, but a clear cellular enumeration was not fully established [18]. The expression patterns reported in this previous study may not be applicable to other new-onset cases in view of the known heterogeneous immunopathogenesis of type 1 diabetes, disparate rates of beta cell destruction and differing ethnic and environmental determinants. Thus, there is an urgent need to examine additional high stringency pancreatic samples from new-onset cases with different demographics and geographic locations.

Here, we applied combined immunohistochemical techniques to investigate the expression of IL-1β in sections of surgically-retrieved pancreatic biopsies from six living volunteers with recent-onset type 1 diabetes of Norwegian descent, from the Diabetes Virus Detection (DiViD) Study [19]. We also quantified and determined the spatial distribution of IL-1β-positive cells within and close to beta cell-positive and negative islets and exocrine areas, and compared our findings with cadaveric pancreatic samples from patients with longer duration of disease and autoantibody-negative and -positive non-diabetic cases supplied by the Network of Pancreatic Organ Donors with Diabetes (nPOD) [20].

Methods

Experimental protocol and microscopy We immunostained and analysed paraffin-embedded pancreatic sections (5 μm) from formalin-fixed biopsy specimens retrieved laparoscopically from 6 newly-diagnosed cases with type 1 diabetes, from the DiViD Study and sections of formalin-fixed cadaveric pancreas from 26 nPOD cases [19, 20]. Approval was granted by the Norwegian Government's Regional Ethics Committee and for the Auckland study by the New Zealand Ministry of Health and Disability Ethics Committee (approval number NTX/11/EXP/092/AMO2). The main study groups are summarised in Table 1 and patient demographics in ESM Table 1.

Relevant immunohistochemical steps, reagents and incubation times are summarised in ESM Table 2. Immediately prior to immunohistochemistry, sections were de-paraffinised, rehydrated and subjected to antigen retrieval with citrate buffer containing 0.05% (v/v) Tween-20 (Sigma-Aldrich), washed twice with distilled water and exposed to 3% (v/v) H_2O_2 for 15 min. Following a further wash in distilled water, sections were equilibrated with immunohistochemical wash buffer (0.1% (v/v) Tween-20 in phosphate-buffered saline (PBS), pH 7.4 for 5 min and blocked with 5% (v/v) normal goat serum (constituted in wash buffer) for 1 h at 37^0C. Sections were incubated with mouse anti-IL-1β (IgG concentration: 1 μg/ml) and then washed and incubated with anti-mouse IgG horseradish peroxidase polymer. After further washing, sections were exposed to SignalStain diaminobenzidine (DAB) substrate (Cell Signaling, catalogue number 8059) for 4-5 minutes. A mixture of guinea pig anti-insulin serum and rabbit anti-glucagon serum in 5% v/v normal goat serum in PBS was then applied. Highly cross-adsorbed species-specific goat anti-guinea pig IgG-Alexa 568 + goat anti-rabbit IgG-Alexa 488 in 5% (v/v) normal goat serum in PBS was applied and incubated as in the previous step and sections washed and prepared for microscopy.

The immunohistochemical specificity of anti-IL-1β has been reported by Cell Signaling (catalogue number 12242). Thus, by immunohistochemistry, it successfully detects IL-1β immunoreactive cells in human colonic sections from patients with chronic colitis. Further, by Western blotting, the same antibody detects mature recombinant human and mouse IL-1β and the human precursor (molecular weight of 34 kD) in extracts of Raw 264.7 and human monocyte-derived THP-1 cells, following exposure to Brefeldin and lipopolysaccharide (LPS), respectively.

Substitution of anti-IL-1β with diluent acted as negative controls. As further controls, paraffin sections of THP-1 cells, harvested after incubation with LPS (100 ng/ml for 3 h) and without (from Cell Signaling), were evaluated in this laboratory for IL-1β immunohistochemical specificity.

Immunostaining protocol for IL-1β was tested with formalin-fixed tonsil sections, supplied by Department of Surgery, University of Auckland.

Sections from selected pancreas and tonsil were immunostained for IL-1β in two separate sections. They were then exposed to rabbit anti-CD68 or rabbit anti-CD3, followed by donkey anti-rabbit IgG-biotin and then streptavidin-Alexa 568. Pancreatic sections were immunostained subsequently for insulin as above, followed by donkey anti-guinea pig IgG-Alexa 488.

Immunostained sections were examined with a Nikon Eclipse E600 epifluorescence-bright field microscope and digital images recorded. Group assignment was blinded to the investigator, except groups 1 and 4 where many islets were insulin-deficient. From each pancreatic section, islets with approximately ≥ 20 endocrine cells were imaged for the presence of IL-1β, insulin, glucagon, CD68 and CD3 cells. Each of the specific image sets from multiple acquisitions were merged with Adobe Photoshop CS4, following conversion of IL-1β immunostained cells to a greyscale fluorescence mode. 15 random exocrine fields in each pancreatic section were imaged at 20x objective for the presence of IL-1β-positive cells, enumerated and the mean number per field per case determined.

For each section, the number of IL-1β-positive cells in the peri- and intra-islet regions was enumerated and the mean number of cells per insulin-positive and –negative islet determined. Islets which showed immunostaining for IL-1β in alpha cells were scored microscopically as negative, weakly or moderately stained.

Data analysis For each case, the percentages of insulin-positive islets were represented as bar graphs. The mean ± SD number of IL-1β-positive cells per islet, in peri- and intra-islet locations and in insulin-positive and -negative islets was determined, tabulated and represented as bar graphs. The percentage of islets with IL-1β-positive cells for each case was determined. The number of IL-1β-positive cells in each exocrine field and the mean value per case were depicted graphically. Statistical analysis was performed using a generalized linear mixed model due to Poisson distribution of data to determine significance. Fixed effects were insulin-positivity and -negativity of the islet, location of IL-1β (peri-; intra-islet) and the case type (4 study groups) with case chosen as a random effect. Pairwise comparisons were also performed between fixed effects. The same statistical techniques were applied to exocrine IL-1β-positive cell counts.

Results

Immunohistochemical staining pattern for IL-1β Application of immunohistochemical procedure for IL-1β to THP-1 cells showed positive cytokine staining following prior exposure to LPS but not without (ESM Fig. 1a, b). In tonsil sections, weak to strong expression of the cytokine was observed in various regions, being co-localized in CD68 cells and absent in T cells (ESM Fig. 1c-f). Pancreatic sections from case 6070 showed a few cells positive for IL-1β in the peri-islet and exocrine regions but not upon substitution of the primary antibody with diluent (ESM Fig. 1g-j)

The distribution of IL-1β-positive cells within or close to islets and in the exocrine regions of DiViD cases 1 and 2 are shown in Fig. 1 and for cases 3-6 in ESM Fig. 2 and 3. In case 1, almost all islets were devoid of beta cells, with only a small minority of islets harbouring one or two IL-1β-positive cells either in the peri- or intra-islet areas (Fig. 1a, b, d, e). IL-1β-positive cells were also rare in the exocrine region (Fig. 1c, f). This cellular distribution pattern was also similar in DiViD cases 2-6, where a larger number of insulin-positive islets were observed. The distribution pattern was independent of the presence of remaining beta cells within islets or the presence of autoantibodies and their antigenic specificities (Fig. 1g-l, ESM Fig. 2 and 3; ESM Table 1).

The distribution of IL-1β immunoreactive cells in peri- and intra-islet regions and in exocrine areas in 3 non-diabetic cases is illustrated in Fig. 2a-i. In all 3 cases, only rare IL-1β-positive cells located in the peri- and intra-islet regions were present (Fig. 2a, b, d, e, g, h), with a few scattered cells in the exocrine region, usually in the pancreatic septa (Fig. 2c, f, i).

In nPOD diabetic cases 6209 and 6039, only occasional IL-1β-positive cells were present in the peri- and intra-islet regions and exocrine areas (Fig. 3a-l). However, the number of islet-associated IL-1β-positive cells was slightly higher than in non-diabetic (group 2) and new-onset DiViD cases. In diabetic case 6070 (type 1 diabetes: 7 years), IL-1β-positive cells in the peri-islet and intra-islet areas were also rare, irrespective of the presence of residual insulin cells (ESM Fig. 4a, b, d, e, g, h). However, there was a marked increase in the number of IL-1β-positive cells in the exocrine region, usually in inter-acinar stroma and adjacent to pancreatic septa and pancreatic ducts, consistent with pancreatitis and diabetic ketoacidosis (DKA) of the donor (ESM Fig. 4c, f, j, k; ESM Table 1).

In some nPOD cases, IL-1β immunolabelling of variable intensity was present in selective alpha cells and examples are shown as paired images (Fig. 4a-t). In the non-diabetic autoantibody-negative group, although moderate and weak immunolabelling in alpha cells were prominent in specific cases (6160: Fig. 4a-f; 6178: Fig. 4i, j) it was absent or weak in other cases (6055: Fig. 4

g, h). Expression was slightly more attenuated but variable in non-diabetic autoantibody-positive group (6267: Fig.4 k-n) and most long-term diabetic cases (6088 and 6211: Fig. 4o-t).

Dual staining of selected pancreatic sections showed the expression of IL-1β in macrophages and occasionally T cells (ESM Fig. 5a-l).

Cellular analysis Various morphometric analysis of islets for each case are shown, including the number of islets examined per case (ESM Tables 3-6). The percentage of insulin-positive islets in each case is shown in Fig. 5a-d. In the non-diabetic groups, all islets had insulin-positive cells (Fig. 5b, c) but was highly variable in new-onset and long-standing cases (islets from DiViD case 1 and 3 nPOD cases 6262, 6220, 6039 from group 4 were insulin-negative; Fig. 5a, d). The number of peri- and intra-islet IL-β-positive cells was extremely low (usually less than 2 cells per islet region); the percentages of islets with IL-1β-positive cells in peri- and intra-islet regions are also included. In group 1, 2/6 cases showed a higher percentage of islets with IL-1β-positive cells in peri-islet than in intra-islet regions (Fig. 5a) while in groups 2 , 3 and 4, the percentages in the peri-islet region were higher in 4/13, 3/4 and 6/9 cases, respectively (Fig. 5b, c, d) In group 1, the 5 cases with insulin-positive islets showed a higher mean number of IL-1β-positive cells per islet in insulin–positive than in insulin-negative islets (Fig. 6a). This trend was also observed in 2/6 cases from group 4 (Fig. 6d). Case 6070 (type 1 diabetes: 7 years) showed a higher number of IL-1β-positive cells (approximately 2.25 cells per insulin-positive islet), probably attributable to the donor's clinical history of repeated hospitalization, pancreatitis, DKA, metabolic de-compensation and higher pancreatic infiltrates. In the same case, the overall mean number of IL-1β-positive cells was also higher in peri-islet than in intra-islet regions.

In group 1, the overall mean number of IL-1β-positive cells (peri-and intra-islet) was higher in 5/6 cases with insulin-positive islets (0.205 ± 0.449) than in insulin-negative islets (0.09 ± 0.364; ESM Table 3). In long-term diabetic cases, the values in 5/9 cases with residual insulin-positivity were 0.565 ± 1.03 and 0.257 ± 0.581, respectively (first 5 cases in Table 6). These differences were not statistically significant.

The percentage of islets showing IL-1β staining in alpha cells in various cases are shown (Fig. 7a-d and ESM Tables 3-6). In group 1, it was almost absent. In group 2, 3/13 cases showed weak to moderate staining in alpha cells in >70% of islets while in groups 3 and 4, the percentages were markedly reduced (4.8-31%, 0-52.4%, respectively).

IL-1β-positive cells (peri- and intra-islet non-alpha cells) were significantly higher in group 4 than in group 3 ($p = 0.01$). In insulin-positive islets, the cell counts were significantly higher in group 3 than group 1 ($p = 0.02$) and group 4 ($p = 0.001$). Insulin-negative islets from group 4 had a higher IL-1β-positive cell count than group 1 ($p = 0.041$). However, the statistical difference may be due to the unusually higher cell counts in some long-term diabetic cases (group 4).

The number of IL-1β-positive cells in 15 random exocrine fields and the mean number per case are shown (Fig. 8a-d). The mean values per exocrine field were significantly lower in the new-onset group than in the remaining 3 groups ($p = 0.003$). Although the overall exocrine cell counts in group 2 were lower than in group 4 ($p = 0.031$), inclusion of the unusually higher values for case 6070 (group 4) may have contributed to statistical significance.

Discussion

A role for IL-1β in beta cell integrity or its demise is plausible due to the presence of its receptor at a high density on the beta cell surface [21]. Previous in vitro studies demonstrating deleterious effects on beta cells when exposed to higher levels of IL-1β, in combination with TNF-α and IFN-γ, led to the rationale that IL-1β blockade may arrest further destruction of beta cells in human type 1 diabetes [6, 10, 12]. However, IL-1β at lower levels may have a trophic effect on beta cells and thus enhance functional integrity and exert insulinotrophic actions [13]. The true role of IL-1β is further complicated by the presence of circulating soluble IL-1β receptors which may neutralize peripheral and tissue IL-1β bioactivity [7].

To clarify the proposed pathogenic role of IL-1β in early type 1 diabetes in vivo, we first determined if the cytokine is expressed within the islet or in close proximity, in rare well-preserved pancreatic biopsies from six living volunteers with new-onset type 1 diabetes. Secondly, we determined if this expression was dependent on the presence of residual beta cells within islets.

We demonstrate that, overall, the expression of IL-1β-positive cells is low within the peri- and intra-islet regions of new-onset cases. The few sparsely-distributed IL-1β-positive cells in peri- and intra-islet non-alpha cells and in exocrine regions were independent of the presence of beta cells within the islets. In the islets of new-onset and long-term diabetic cases with residual beta cells, a slightly higher overall mean number of IL-1β-positive cells were noted in insulin-positive than in insulin-negative islets. Further analysis involving larger cohorts harbouring residual beta cells will be necessary to verify these differences. The markedly reduced number of IL-1β-positive cells in the islets of most cases from the 4 study groups suggests low but ongoing constitutive expression of the cytokine, independent of disease status. Thus, minimally-expressed IL-1β within and around the islet may be trophic to beta and other islet cells.

Our findings are in close agreement with a recent careful study which quantified the level of various cytokine and chemokine genes, including IL-1β, in laser-captured insulitic islets and in isolated islets from the same DiViD cases as employed here [22]. This previous study demonstrated extremely low levels of IL-1β mRNA in laser-captured islet sections as well as in isolated islets; IL-1β protein levels in isolated islets were also low. In laser-captured islet sections from deceased nPOD donors with type 1 diabetes, IL-1β mRNA was also undetectable (personal communication, Dr Ivan Gerling, University of Tennessee, Memphis, USA). Previous studies showed only low basal levels of IL-1β mRNA in normal human islets cultured for 48 hours to 7

days, with increasing concentrations of glucose [9]. Interpretation of the latter data requires some caution, since mRNA and protein levels may change during islet isolation and culture.

Our demonstration of weak to moderate immunolabelling of IL-1β in selective alpha cells in 3/13 non-diabetic autoantibody-negative cases and its virtual absence in the remaining 10 cases, including new-onset cases is novel but intriguing. The variable immunostaining also observed in non-diabetic autoantibody-positive and longer duration diabetic cases may imply intrinsic heterogeneity of alpha cells within and between cases. It is unclear if IL-1β from selective alpha cells functions as an autocrine or paracrine factor. Additional sources of IL-1β are macrophages and some T cells shown here and dendritic cells shown in the Japanese study and resident macrophages in human islets exposed to IFN-γ + LPS + TNF [18, 23]. The origin of IL-β in some T cells, in a mouse model of autoimmune encephalomyelitis, also supports our observations [24]. The extremely rare expression of IL-1β in beta cells from some non-diabetic and long-term diabetic cases observed in this study is in contrast to a higher degree of expression reported in Japanese new-onset type 1 diabetic volunteers and in humans with type 2 diabetes and may reflect differences in subject characteristics [14, 18]. The overall number of IL-1β-positive cells in the Japanese study was also low. A direct comparison between this previous study and our findings is not possible since the absolute number of IL-1β-positive cells per insulin-positive and negative islet was not reported. In addition, differences in environmental exposure of donors in the two studies, time of diagnosis, ethnicity and genetic background may not justify true comparisons.

A recent study has compared mRNA expression patterns for IL-1β, TNF-α, IL-6, IL-17 and IFN-γ in four animal models of type 1 diabetes with cadaveric sections from humans with and without disease [25]. In this study, pancreas from human diabetic cases of unknown duration, showed the expression of IL-β and TNF-α in immune cells infiltrating islets by in situ hybridization, which may not always correlate with immunohistochemistry.

During the preparation of this manuscript, an immunohistochemical analysis of pancreatic sections from nPOD donors with type 1 and type 2 diabetes and non-diabetic cases with or without autoantibodies showed that all islet alpha cells were the major pancreatic source of IL-1β [26]. Our observations showing its variable expression in selective alpha cells suggest heterogeneity of this cell-type. The published report showing uniform staining of IL-1β in alpha cells employed anti-IL-1β from Abcam, whereas we obtained it from Cell Signaling. The latter antibody was validated for immunohistochemical monospecificity in our laboratory and by the suppliers by Western blotting. The low level of IL-1β mRNA and protein reported previously in isolated human

islets and in the same biopsy samples from the DiViD Study are in agreement with our findings [9, 22]. Differences in properties of the antibody from the two sources may explain some of the differing results.

The role of systemic sources of IL-1β, as effectors of beta cell destruction remains unclear. The few reported studies show an increase in circulating IL-1β at the protein and mRNA levels in the peri-diagnostic period [27, 28]. The net bioactivity of systemic IL-1β may be confounded by oscillating levels and variable turnover of soluble receptors and receptor antagonists.

The significance of the unexpected lower IL-1β-positive cell density in the exocrine region of new-onset cases than in autoantibody-positive non-diabetic and long-term diabetic groups remains unclear. The expression pattern of cytokines in cadaveric pancreas may be profoundly influenced by the metabolic state of the donor preceding brain death and during cold ischaemia. These confounding variables were likely to be absent in biopsy samples from new-onset volunteers, who remained in an optimised metabolic state and were pancreatitis-free. It is possible that acute or chronic pancreatitis documented in some nPOD donors (6250, 6267, 6070) may have contributed to the increase in exocrine IL-1β-positive cell density. An increase in CD8 T cells in the exocrine region has been reported in long-term type 1 diabetic donors from nPOD [29]. We have shown an increase in the number of leukocytic infiltrates in the same region of several longer-term type 1 diabetic cases [15]. This pathology may be consistent with the emerging view of plasticity of exocrine macrophages, potentially driving them to an inflammatory phenotype, including cytokine production or even towards an anti-inflammatory protective mode [30].

This study has carefully examined the cellular expression of a key cytokine implicated in beta cell destruction during type 1 diabetes. We analysed sections from one of the first carefully-retrieved pancreatic biopsies from young adults at onset and compared our findings with non-diabetic autoantibody-positive and –negative cases and long-standing diabetic cases. An overall decrease in the mean number of IL-1β-positive cells in the new-onset group compared with non-diabetic autoantibody-positive group, may suggest a pathological change, but requires further verification due to differences in tissue retrieval. Given the small sample size of this study, limited by the rarity of biopsies from living donors and the known marked intra- and inter-patient heterogeneity of pathogenic processes within the islets during type 1 diabetes, generalization of our findings to all new-onset cases is premature. Nonetheless, it offers some key perspectives on the biology and dynamics of this important cytokine within the islet in new-onset disease and during longer duration. We are cognisant that the DiViD cases were in better metabolic homeostasis during and

immediately prior to biopsy whereas in cadaveric donors with long-term diabetes, variable degrees of metabolic de-compensation, hyperglycaemia-driven oxidative stress and cold ischaemia, may have influenced the expression pattern of this labile cytokine. We also recognize the limitations of cross-sectional studies and that there may be regional differences in the frequency of IL-1β-positive cells and its juxtaposition to the islets. Future non-invasive deep tissue molecular imaging techniques may shed valuable information regarding the real-time IL-1β dynamics within the early diabetic pancreas. Nonetheless, the present study provides new impetus, to delineate the precise role of a key cytokine with reported opposing concentration-dependent biological effects on beta cells. Our immunohistochemical snapshot argues against locally-produced IL-1β as a major mediator of beta cell destruction during early type 1 diabetes, but implicates its purported role in beta cell survival and function.

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Contribution statement SR conceived and designed the experimental studies, carried out a considerable portion of them, acquired and analysed the data, wrote and revised the manuscript critically for publication, and led and directed the study. FW, LK and K D-J made significant co-contribution to the conception of the study and its design and data interpretation. They also assisted in critically reading and offering valuable advice in revising the manuscript for intellectual content. CM, RH, JC and HW assisted in designing the studies, performed part of the experimental studies, acquired multiple microscopic images from several samples, carried out further data analysis, read and revised the relevant parts of the manuscript for scientific content. In addition, following discussions with SR, CM prepared all figures and carried out careful statistical analysis. All

authors have given their final approval of the version to be published. SR is the guarantor of the work.

All original data are available from the corresponding author (SR) on request.

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Fig. 1 Immunohistochemistry: IL-1β-positive cells (brown or white) in pancreatic sections from DiViD cases 1 (**a-f**) and 2 (**g-l**), co-stained for insulin (red) and glucagon (green). Case numbers and duration of diabetes in weeks (w) are specified in (**a, g**). (**a, d, g, j**) Arrows indicate a few IL-1β-positive cells in peri- and intra-islet regions; islet boundary is stippled. (**b, e, h, k**) Same field as (**a, d, g, j**) showing IL-1β-positive cells (white arrows), merged with islet cells positive (**h**) and negative (**b, e, k**) for insulin. (**c, f, i, l**) Exocrine region where arrowheads indicate a few IL-β-positive cells. Scale bar in (**i**), 50 μm, applies to all micrographs IL-1β; interleukin-1β; Ins, insulin; Glu, glucagon; T1D, type 1 diabetes

Fig. 2 Immunohistochemistry: IL-1β-positive cells (brown or white) in pancreatic sections from selected non-diabetic autoantibody-negative nPOD cases, co-stained for insulin (red) and glucagon (green). Case numbers are specified in (**a, d, g**), where arrows and arrowheads indicate a few IL-1β-positive cells in peri-islet and exocrine cells, respectively; islet boundary is stippled. (**b, e, h**) Same field as (**a, d, g**) showing IL-1β-positive cells (white arrows and arrowheads), merged with insulin and glucagon cells. (**c, f, i**) Exocrine region where arrowheads indicate a few IL-β-positive cells. Scale bar in (**i**), 50 μm, applies to all micrographs IL-1β; interleukin-1β; Ins, insulin; Glu, glucagon; ND, non-diabetic

Fig. 3 Immunohistochemistry: IL-1β-positive cells (brown or white) in pancreatic sections from 2 nPOD cases with longer duration of T1D, co-stained for insulin (red) and glucagon (green). (**a-f**) nPOD case 6209 and (**g-l**) nPOD 6039. (**a, d, g, j**) Arrows and arrowheads indicate a few IL-1β-positive cells in peri- and intra-islet and exocrine cells, respectively; islet boundary is stippled. **(b, e, h, k)** Same field as (**a, d, g, j**) showing IL-1β-positive cells in white (arrows), merged with islets positive (**b**) and negative for insulin. (**c, f, i, l**) Arrowheads indicate a few IL-1β-positive cells in exocrine region. (**a, g**) Duration of diabetes is indicated in years (y). Scale bar in (**l**), 50 μm, applies to all micrographs IL-1β, interleukin-1β; Ins, insulin; Glu, glucagon; T1D, type 1 diabetes

Fig. 4 Immunohistochemistry: Columns 1 and 3: Merged images of islet sections stained for insulin (red) and glucagon (green); columns 2 and 4: corresponding islet sections as in columns 1 and 3 triple-stained for IL-1β (brown). Examples are from non-diabetic autoantibody-negative nPOD cases 6160, 6055 and 6178, non-diabetic autoantibody-positive nPOD case 6267 and diabetic nPOD cases 6088 (T1D 5y) and 6211 (T1D 4y) and show negative, weak and moderate

immunolabelling for IL-1β in alpha cells. In (**d, h, l, n, p, r, t**) islet boundary is stippled. Black and white arrows point to weak and moderate staining for IL-1β in alpha cells while black and white arrowheads indicate negative staining in alpha cells. (**r, t**) Red arrows indicate IL-1β-positive cells in non-alpha cells. (**q, r**) Alpha cells in an islet negative for IL-1β. Scale bar in (**t**), 50 μm, applies to all micrographs. AAb+, autoantibody-positive; AAb-, autoantibody-negative; ND, non-diabetic; T1D, type 1 diabetes; y, years

Fig. 5 Insulin-positive islets (%) shown as black bars in each pancreatic section from cases belonging to groups 1 (**a**), 2 (**b**), 3 (**c**) and 4 (**d**).The total number of islets examined in each case are indicated in the second column of ESM Tables 3-6. White and checkered bars indicate percentage of islets with peri-islet and intra-islet (non-alpha cells) IL-1β-positive cells, respectively. GAD, anti-glutamic acid decarboxylase; IA-2, anti-insulinoma associated antigen; IAA, insulin autoantibodies; ZnT8, anti-zinc transporter 8

Fig. 6 Mean number of IL-1β-positive cells in insulin-positive (black bars) and insulin-negative islets (light grey bars) in each pancreatic section from cases belonging to groups 1 (**a**), 2 (**b**), 3 (**c**) and 4 (**d**). Also shown are mean number of IL-1β-positive cells in peri-islet (white bars) and intra-islet non-alpha cells (checkered bars). GAD, anti-glutamic acid decarboxylase; IA-2, anti-insulinoma associated antigen; IAA, insulin autoantibodies; ZnT8, anti-zinc transporter 8

Fig. 7 Percentage of islets with weak (black bars) and moderate (striped bars) staining for IL-1β in alpha cells in each pancreatic section from cases belonging to groups 1 (**a**), 2 (**b**), 3 (**c**) and 4 (**d**). GAD, anti-glutamic acid decarboxylase; IA-2, anti-insulinoma associated antigen; IAA, insulin autoantibodies; ZnT8, anti-zinc transporter 8

Fig. 8 Number of IL-1β-positive cells in 15 random exocrine fields in each pancreatic section from cases belonging to groups 1 (**a**), 2 (**b**), 3 (**c**) and 4 (**d**). Horizontal bars indicate the mean values per case. GAD, anti-glutamic acid decarboxylase; IA-2, anti-insulinoma associated antigen; IAA, insulin autoantibodies; ZnT8, anti-zinc transporter 8

Table 1 Summary of main study groups, including sex and age distribution

Study groups	Number of females and males	Mean age (years)	Median age (years)	Range (years)
Group 1: New-onset cases	4F, 2M	28.83	28	24-35
Group 2: Non-diabetic AAb-negative cases	6F, 7M	24.74	24.5	5-44
Group 3: Non-diabetic AAb-positive cases	2F, 2M	26.41	25.5	17.65-37
Group 4: Long-term diabetic cases	5F, 4M	26.54	26.4	5-44

Abbreviations: AAb, autoantibody; F, female; M, male