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This is the peer reviewed version of the following article:

Original:

Nigi, L., Maccora, C., Dotta, F., Sebastiani, G. (2020). From immunohistological to anatomical alterations of human pancreas in type 1 diabetes: New concepts on the stage. DIABETES/METABOLISM RESEARCH AND REVIEWS, 36(4), 1-18 [10.1002/dmrr.3264].

Availability:

This version is available <http://hdl.handle.net/11365/1089477> since 2020-01-16T14:40:07Z

Published:

DOI:10.1002/dmrr.3264

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Review

**From Immunohistological to anatomical alterations of human pancreas in Type 1 Diabetes:
new concepts on the stage**

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Keywords: Type 1 Diabetes,, autoimmunity, insulinitis, pancreas

Word count: 10721

Figures: 3

Tables: 1

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Abstract

The histological analysis of human pancreatic samples in type 1 diabetes (T1D) has been proven essential to move forward in the evaluation of *in situ* events characterizing T1D. Increasing availability of pancreatic tissues collected from diabetic multiorgan donors by centralized biorepositories which shared tissues among researchers in the field, allowed a deeper understanding of T1D pathophysiology, using novel immune-histological and high-throughput methods. In this review, we provide a comprehensive update of the main recent advancements in the characterization of cellular and molecular events involving endocrine and exocrine pancreas as well as the immune system in the onset and progression of T1D. Additionally, we underline novel elements which provide evidence that T1D pathological changes affect not only islet β -cells but the entire pancreas.

1. Introduction

The study of human pancreas using histological techniques is of pivotal importance, since it provides valuable contributions to the description of the scenario occurring in the pancreas of patients with Type 1 Diabetes (T1D). The generation and expansion of pancreatic tissue biorepositories, resulted in crucial advancements in the understanding of the disease processes and progression, with major contributions coming from tissues sharing, collaborative studies and consortia establishment. The analysis of pancreatic tissues collected through highly standardized operating procedures aimed at preserving morphological quality and molecular scenario, coupled with the use of cutting edge technologies, allowed the identification of novel disease mechanisms which led to several paradigm shifts in T1D (1,2).

Noteworthy, even though analysis of pancreatic histological specimens represents the gold standard for the study of T1D pathogenesis at the level of the target organ, recent advances in non-invasive imaging techniques (e.g. molecular studies applied to Magnetic Resonance Imaging, Positron Emission Tomography and optical imaging) suggest that diagnostic imaging may represent a future valuable approach in the analysis of some specific aspects of the disease (e.g. determination of β -cell number, β -cell mass and function, immune cells activity) (3,4).

In this review, we report the main latest research advancements in T1D pathophysiology, obtained through the analysis of pancreatic specimens.

2. Insights into Type 1 Diabetes pathogenesis: major contribution from tissue biobanks of pancreatic organ donors

T1D is a chronic autoimmune disease, characterized by the progressive destruction and dysfunction of pancreatic insulin-producing β -cells in genetically susceptible individuals, leading to hyperglycemia, to the need of life-long exogenous insulin replacement therapy and to the risk of chronic complications development (5,6). T1D clinical onset occurs after a highly variable time window, ranging from few weeks to many years (5), during which the immune-mediated attack

against β -cells starts and perpetuates. In this pre-clinical/prodromic phase, a progressive decline of functional β -cell mass (secondary to the immune-mediated inflammatory destructive process) occurs, alongside with the appearance, in various titers and combinations, of disease-specific though non-pathogenic autoantibodies against β -cell autoantigens (7). In infants and in young children a more aggressive disease process seems to develop, resulting into overt disease as low as few months after the appearance of the first diabetes-associated autoantibody, whereas in older subjects the clinically silent phase may continue for more than 20 years (8–10). However, it should be noted that, although the majority of children positive for two or more islet autoantibodies will develop T1D (11), with some specific autoantibody combinations conferring higher risk to progress to disease onset (12), not all islet autoantibody positive subjects will advance to overt T1D.

The immunohistological analysis of T1D donors pancreas is of pivotal importance in order to characterize molecular events occurring at the different stages of the disease. A strong contribution to the detailed characterization of T1D pancreas histopathology has been obtained through the analysis of tissues collected within historical T1D pancreatic tissue biorepositories and, more recently, by the establishment of specialized networks for T1D pancreatic organ donors collection, aimed at distributing tissue samples to the researchers upon specific requests and according to their experimental requirements.

The world's biggest historical T1D pancreatic tissues biobank is represented by the retrospective UK collection of T1D pancreas obtained at autopsy, known as Exeter Archival Diabetes Biobank (EADB, UK) (13). EADB includes 169 cases, including 80 with a disease duration less than 1 year and with a mean age at onset of 12 years. Although many significant results have been obtained through the analysis of EADB collected tissues, pancreatic autoptic samples show several limitations; indeed, they are often subjected to variable degrees of autolysis and prolonged cold ischemia time, thus resulting into a reduction of morphological quality and stability of certain proteins/epitopes and of other classes of molecules (5,14,15). Additionally, EADB collection is exclusively composed of pancreatic tissue samples processed using formalin-fixation and paraffin

embedding method, thus excluding the application of other techniques requiring snap frozen fixation procedure (13,16).

In order to overcome these difficulties, pancreas with transplantation-grade quality obtained from brain-dead multiorgan donors have started to be used for research purposes, alongside with additional collected tissues, such as pancreatic lymph nodes, spleen and duodenum (17,18).

The first established network is represented by the “Network for Pancreatic Organ Donors with Diabetes” (nPOD, USA), founded in 2007 thanks to the support of the Juvenile Diabetes Research Foundation (JDRF, www.jdrf.npod.org) (18). nPOD mission is to collect tissues from T1D and from islet autoantibody positive and islet autoantibody negative non-diabetic organ donors, as well as from organ donors affected by type 2 diabetes (T2D), gestational diabetes, pancreatitis or by other pancreas-affecting diseases, in order to establish a biobank of tissues available to investigators worldwide (17,18). An added value of this resource is that nPOD network team established multiple processing methods which follow rigorous standard operating procedures (SOPs) for the collection and handling of tissue samples, in order to render them ready to be analyzed using different methodologies (17,19).

Similarly, the more recent “European Network for Pancreatic Organ Donors with Diabetes” (EU-nPOD) project has been launched in the context of the INNODIA consortium (www.innodia.eu) (20), by adopting nPOD tissue processing workflows and making the collected tissues available to the consortium partners according to their needs and upon a specific project approval.

An additional biorepository network inspired to nPOD has been established in 2016 in US. Indeed, Human Pancreas Analysis Program (HPAP) (<https://hpap.pmacs.upenn.edu>, USA) collects pancreas from T1D, T2D, islet autoantibody positive and islet autoantibody negative non-diabetic multiorgan donors with the aim to analyze islets function/physiology and pancreatic endocrine/exocrine tissue histology adopting the latest technologies, in order to perform a deep phenotyping of the human pancreas and its interactions with the immune system. Moreover, HPAP research results related to

each analyzed tissue are available for the scientific community through a fully accessible user-friendly web-database.

Increasing evidence showed that post-mortem samples and multiorgan donors pancreatic tissue biorepositories significantly improved T1D research through the increased availability of high quality processed tissues from diabetic donors. However, at present, an intrinsic limitation is that the access to such tissues is often possible only several years after T1D onset, with only a minor fraction of collected tissues available from newly diagnosed diabetic donors or from multiple islet autoantibody positive non-diabetic donors. Such obvious intrinsic characteristic, limits the possibility to analyze pancreatic tissues in the early stages of the disease. This issue could be partially overcome by biopsying pancreas of patients with new-onset T1D. In 2014, the Diabetes Virus Detection study (DiViD, Norway) successfully collected bioptic samples by adopting laparoscopic pancreatic tail resection from 6 new-onset T1D patients (less than 6 weeks from diagnosis). DiViD researchers processed pancreas bioptic tissue for multiple uses (formalin-fixed paraffin-embedded, OCT frozen, snap frozen, collagenase islet isolation, electron-microscopy grade, laser capture microdissection etc.), then shared tissues with many researchers worldwide in order to deeply characterize them (21). Unfortunately, the difficulty to perform pancreas biopsies, alongside with ethical considerations regarding the safety of the procedure, led to the closure of the DiViD study. However, despite the low number of patients recruited (n=6), analyses of T1D DiViD cases, as the result of multiple collaborative studies (21–29), led to an improved characterization of human pancreas during the early stages of T1D.

3. Insulinitis, β -cell loss and β -cell persistence in T1D

3.1 Insulinitis

Insulinitis is the main histopathological feature of T1D. It represents the typical sign and the hallmark of autoimmune attack against β -cells, and it is characterized by adaptive and innate immune cells surrounding and/or infiltrating pancreatic islets. Insulinitis is a heterogeneous phenomenon; indeed, it is detected only in a subsets of T1D donors and, frequently, in a lobular manner (30,31) (**Table 1**).

In 2011, a metaanalysis of studies (from 1902 to 2010) which characterized insulinitis in pancreas obtained from T1D donors, observed that insulinitis was reported in 73% of young donors (<14 years) with a short disease duration (<1 month from diagnosis) and in 29% of T1D donors between 15-40 years with a similar disease duration. In young T1D patients (<14 years), the proportion of those showing signs of insulinitis, dropped from 73% of subjects with a short disease duration (<1 month from diagnosis) to 4% of those with longer disease duration (>1 year) (32). A more recent insulinitis frequency analysis of pancreas collected within nPOD, aimed at reproducing data reported in the previous metaanalysis, partially confirmed such inverse correlation with age and disease duration. In this study Campbell-Thompson ML and colleagues determined insulinitis frequency by adopting criteria used in the 2011 metaanalysis and analyzed 159 pancreas (80 from T1D, 18 from islet-autoantibody positive and 61 from autoantibody negative non-diabetic organ donors). They found a significant inverse correlation between insulinitis and diabetes duration but no significant correlation with age at onset. Of note, only 23% of T1D donors (age range 3- 26.3 years) showed evidence of insulinitis. In these donors, insulinitis was found in 33% of insulin-positive islets while only 2% of insulin-negative islets showed signs of lymphocytic infiltration (31).

In DiViD study, Krogvold L and colleagues found evidence of insulinitis in all pancreatic specimens derived from n=6 T1D donors recruited within 6 weeks from diagnosis; however, they observed a wide variability in terms of number of islets which satisfied insulinitis criteria (5-58% of total), thus underlining the elevated heterogeneity of insulinitis detected in pancreas of T1D donors and

highlighting the “patchy” islet inflammation scenario occurring in autoimmune diabetes. Several additional observations were also reported: (i) a total of 82% of inflamed islets were insulin-positive; (ii) lymphocytic infiltrates affected preferentially the islet periphery rather than the islet parenchyma; (iii) numerous CD3⁺ cells were found scattered within the exocrine tissue [most of the CD3⁺ cells (absolute numbers) resulted located within the exocrine tissue]. Collectively, these studies demonstrated that insulinitis is more frequently observed in recent-onset donors vs. those with long-standing T1D, while no significant difference was observed between young and adult donors; additionally, insulinitis is significantly associated with insulin-positivity being detected more frequently in insulin-containing islets vs. insulin-deficient islets (**Figure 1**) (23).

Although several studies analyzed in details pancreatic lymphocytic infiltration in T1D and evaluated insulinitis presence based on CD3⁺ cells, in 2013 specific consensus guidelines have been published in order to better define the presence of insulinitis in T1D organ donors (33). The main statement provided focused on the positivity for CD45 (thus including also CD20⁺ B cells) and a threshold number of CD45⁺ cells/islet (≥ 15 based on lymphocytes counting in a cohort of control donors pancreas). Indeed, at present, insulinitis positivity is ascertained when pancreatic lymphocytic infiltration consists of at least 15 CD45⁺ cells/islet in a minimum of 3 islets.

Insulinitis frequency and characterization studies revealed the extreme heterogeneity of this phenomenon (23,31,34). Additionally, detailed characterization of immune cells composing insulitic infiltrates has been reported to be heterogeneous as well. However, although insulinitis composition greatly varies among T1D donors and among different islets of the same donor, CD8⁺ cytotoxic T cells represent the predominant immune cells population followed by macrophages, CD4⁺ T cells and B-cells. Immunohistochemical analysis of T1D pancreatic samples from EADB collection represents a milestone study for the characterization of insulinitis composition. Islet inflammatory infiltrates were described to be mostly composed of CD8⁺ cytotoxic T cells during all T1D stages. Macrophages (CD68⁺) were lower vs. CD8⁺ T cells, while CD4⁺ T cells were present but less abundant compared to CD8⁺ T cells and macrophages. A limited number of B cells

(CD20⁺) were observed in the early stages of insulinitis but their frequency increased alongside with the progression of β -cell death (24). Presence of Natural Killer (NK) cells has been reported (35) and, finally, FOXP3⁺ regulatory T-cells were detected only in a single donor, whereas widely identified in a murine model of T1D (Non-Obese Diabetic –NOD- mouse) (36). The authors observed that CD8⁺ T cells increased in number as β -cells started to be destroyed and then declined dramatically once β -cells were no longer present within the islets (**Figure 2**), while macrophages have been described as the most prominent immune cell type infiltrating β -cell-depleted islets. Interestingly, B cells could be detected only in the presence of CD8⁺ T cells (with the exception of a single islet only), demonstrating a potential link between B cells and the presence of CD8⁺ cytotoxic T cells. Overall, the observed scenario suggests a progressive sequence of recruited immune cells to islets during T1D, with both CD8⁺ cytotoxic T cells and macrophages contributing to β -cell death, especially during the early stages of insulinitis. Of note, CD20⁺ B cells, scarcely represented during the early stages and recruited in greater numbers during the late stages of the infiltrating process, may be addressed as developers/facilitators of insulinitis and (indirectly) of β -cell death. More interestingly, later studies performed in Exeter, nPOD and DiViD cohorts (24,37) observed that CD20⁺ B cells primarily contribute to the identification of two insulitic profiles, defined as CD20^{lo} (pancreatic islet infiltrates with a low frequency of CD20⁺ cells) and CD20^{hi} (pancreatic islet infiltrates with a high frequency of CD20⁺ cells). Such insulitic profiles distinguished patients diagnosed before the age of 7 years, characterized by higher proportion of CD20⁺ B cells and low residual β -cell content, from those diagnosed after the age of 13 years who showed a lower proportion of CD20⁺ cells and higher β -cell content. Importantly, the proportion of insulin-deficient islets increased with lower age at onset, thus being also positively correlated with B cells frequency. These data suggest that the two patterns of insulinitis may reflect different degrees of severity, since patients with higher proportion of CD20⁺ B cells lose β -cells at a more rapid rate (24). Therefore, higher proportions of B cells in insulitic lesions potentially represent a marker of an early aggressiveness of the autoimmune process or of a rapid rate of β -cell loss (38).

The identification and quantification of immune cells infiltrating the pancreas during T1D progression has also been recently reported by Damond N. and colleagues (39). Indeed, they adopted a novel technology for the characterization of tissue histology in a high-throughput manner. They combined laser ablation and mass cytometry (40) to simultaneously evaluate up to 35 typical markers of endocrine and exocrine pancreas and of immune cells, in pancreatic sections stained with metal-tagged antibodies. Firstly, they confirmed the increased number of immune cells in recent-onset T1D donors vs. non-diabetic and vs. donors with long-standing T1D. In recent-onset donors, T cells and macrophages represented the most prevalent immune cells population while B cells were scarcely represented. In donors with long-standing T1D, neutrophils represented the highest fraction of immune cells, followed by macrophages and T cells. Importantly, immune cells density was generally higher in exocrine tissue respect to the islets, except for B cells whose frequency was higher in islets than in exocrine tissue during all disease stages, thus underlining the importance of B cells in insulitic process and islet inflammation.

Similar observations were reported in a parallel study adopting the same technology and analyzing pancreas deriving from nPOD and HPAP cohorts (41).

3.2 β -cell loss

Histological evidence generated throughout the different cohorts analyzed (EADB, nPOD, DiViD and others), significantly addressed β -cell loss as the most compelling sign which characterizes pancreas histopathology in T1D.

β -cell loss has been addressed as the consequence of the insulitic process and inflammation. Indeed, although highly variable, β -cell loss has been reported in all T1D tissue collections, pediatric T1D donors usually are characterized by a marked disease severity vs adults donors, who retain a significant proportion of β -cells (40-60%) at disease diagnosis. Although β -cells destruction is a progressive phenomenon showing a decline of β -cell mass with increasing disease duration, it does

not necessarily lead to a complete loss of functional β -cell mass; indeed, several studies clearly reported the persistence of residual β -cells even in donors with long-standing T1D (31,42–49).

A recent study performed on pancreas derived from 47 nPOD T1D donors with a disease duration ranging from 0 to 41 years, found that 64% of them showed persistent insulin-containing islets, even though the total β -cell mass/area was reduced by 88-95% (49).

Additionally, as previously observed (42,48,49), “patchy” and regional β -cell preservation areas were detected in distinct pancreatic lobes/lobules of some T1D donors. Of note, β -cell mass reduction was more pronounced in T1D donors with younger age at onset, thus underlining the diversity of disease progression between young and adults. The progressive loss of β -cells is paralleled by an increase of α -cells number and by the appearance of pseudoatrophic islets (**Figure 1 and Figure 2**).

Such results have been recently confirmed and better defined by Damond N. and colleagues using the novel high-throughput histological analysis method described above (39). Using this approach nPOD pancreatic tissue sections from 4 control donors, 4 recent-onset T1D and 4 donors with long-standing T1D were analyzed. By taking into consideration data generated through the evaluation of multiple markers in 1581 islets, a time-resolved map of islets heterogeneity and of interactions between endocrine tissue and immune cells was developed, finally modeling the evolution of T1D at single-islet level based on cross-sectional data. The analysis of islets composition revealed a marked inter- and intra-donor heterogeneity: β -cell content was reduced by 62% in recent-onset T1D donors and, as expected, donors with long-standing T1D were mostly devoid of β -cells. As previously observed, the increase in α -cells proportion was clearly confirmed. Additionally, the generation of islet evolution profiles over time (pseudotime) based on multiple endocrine markers, allowed the classification of islets based on 3 different T1D stages (defined as ‘pseudostages’). Of note, the authors observed that the progression to β -cell death is preceded by a significant downregulation of insulin (INS), Proinsulin (PINS), Islet Amyloid polypeptide (IAPP) and Protein Tyrosine Phosphatase Receptor Type N (PTPRN) expression which occurred between pseudostage-

1 and -2, while pan-endocrine markers and β -cell specific transcription factors remained unchanged. These data suggest that an initial β -cell phenotypic change, resembling an early de-differentiation event, may occur at the beginning of the autoimmune process during pseudostage-1, then leading to the progression to pseudostage-2 and finally to pseudostage-3 and to β -cell death. Importantly, while the abundance of β -cell markers decreased with time, eventually leading to β -cell death, a striking overlap was observed between islet profiles/pseudostages of recent-onset donors and those with long-standing T1D, thus demonstrating that islet profiles are not necessarily associated with disease duration (39).

3.3 β -cell persistence

Although β -cell loss in T1D is a well-established phenomenon, several studies have demonstrated the presence of residual β -cells in donors with long-standing T1D (**Figure 3**). In 2010, the analysis of post-mortem pancreas from 9 T1D Medalist cohort donors (with >50 years of disease duration), highlighted the presence of insulin-positive cells in all cases examined. Such finding was associated to the evidence of detectable random serum C-peptide levels in the majority of T1D patients belonging to this cohort.

More recently, an expanded study of Joslin Medalist cohort analyzed 68 post-mortem pancreas from long-standing T1D donors, in order to further evaluate the presence of residual β -cells and their function. All cases analyzed (68/68) showed scattered singlet/doublet insulin-positive cells, while in 59/68 donors scattered β -cells were also identified in some islets. Of note, 14/68 donors showed the presence of several β -cells in some islets of few lobes. The authors classified T1D Medalist donors into 3 different categories based on residual β -cells presence, and associated them to increasing C-peptide levels measured close to tissue procurement. Although some donors with elevated C-peptide levels showed most numerous insulin-positive islets, it was reported that this was not always the case, with some subjects showing solely scattered singlet/doublet cells and high values of serum C-peptide or viceversa. These results suggest that even though the presence of insulin-

positive islets in several donors with long-standing T1D positively correlated with pre-mortem C-peptide values (indicating a fully functional phenotype), the scenario is heterogeneous and the function of residual β -cells (both scattered and within islets) should be further studied (50).

Residual β -cell persistence in long-duration T1D (**Figure 3**) led researchers to investigate the mechanisms which may contribute to β -cell survival and/or regeneration after many years from disease onset. A long debated issue regards the contribution of replication to β -cell survival and persistence during T1D progression. It is now well established that the rate of β -cell proliferation is high during fetal development, then rapidly declines during the postnatal period and remains low throughout the adult life (51). However, some evidence suggests that the rate of β -cell replication may increase temporarily in consequence of insulinitis (52). Willcox A. et al. analyzed autptic pancreatic tissues obtained from pediatric patients with recent-onset T1D (<18 months, n=10), compared to non-diabetic donors (n=14) derived from EADB cohort, in order to establish whether islet cell proliferation was increased during the disease process. Paraffin-embedded pancreatic sections were stained using specific antibodies recognizing the proliferation marker Ki-67 and the minichromosome maintenance protein-2 (MCM-2). The authors observed that in recent-onset T1D donors the fraction of islets showing Ki-67 positive cells was markedly increased vs. age-matched non-diabetic donors. Interestingly, increased Ki-67 positivity occurred both in α -cells and in β -cells and was positively correlated with the presence of insulinitis in T1D donors. A significant increased proliferation of islet cells in T1D donors was also observed through MCM-2 staining. Noteworthy, T1D donors with longer disease duration or T2D donors or donors with pancreatitis did not show evidence of increased islet cell proliferation vs. non-diabetic controls. These results suggest that islet cell proliferation can occur during the autoimmune process in the early stages of T1D natural history. We can speculate that infiltrating immune cells might mediate an enhanced proliferative response of islet cells through signals which promote cell mitosis (52). In contrast to these results, in a study aimed at analyzing the proliferation rate of islet cells in nPOD donors cohort, Lam and colleagues did not observe any difference in terms of Ki67⁺ β -cells between T1D and control

donors (both young and adults), thus highlighting that β -cell proliferation is unaltered in T1D donors vs. age-matched controls and that additional mechanisms may mediate β -cell persistence in T1D (49). The observed discrepancy in proliferation rate between these two studies may be due to different donors conditions (autopsy vs. brain dead), differences of time in ICU or pancreas harvesting experimental conditions (e.g. type of fixative, fixation time etc) (53).

Additional explanations for β -cell persistence in T1D may be: (i) high regeneration potential (sustained by different mechanisms such as transdifferentiation, neogenesis from a not yet identified progenitor cells or resurrection of β -cells from a de-differentiated phenotype); (ii) protection from autoimmune destruction through elusive mechanisms adopted by specific β -cell subsets.

Further studies are needed in order to clarify the mechanisms contributing to β -cells persistence during T1D progression.

4. β -cell and α -cell responses to islet inflammation in T1D

4.1. β -cells

Advanced histological analysis of high quality pancreatic tissues, coupled to innovative methods and technologies, allowed the identification of novel factors playing a role in the progression of T1D:

- (i) β -cells actively participate in their own demise and death;
- (ii) During inflammation and T1D progression some β -cells acquire a “protected phenotype”;
- (iii) responses to inflammatory milieu in T1D pathogenesis do not involve only β -cells but include the entire islet, thus extending the focus on other islet cell types.

One of the typical pathognomonic signs of islet/ β -cell responses in T1D is represented by HLA class I hyperexpression. Although islet HLA class I hyperexpression in T1D was reported several decades ago, and then consistently debated for a long time, it is a phenomenon undoubtedly

observed in multiple studies (29,54–56). In 2016, a seminal paper by Richardson and colleagues (26) confirmed that islets of T1D donors express high levels of HLA class I. They adopted multiple in-situ approaches to analyze HLA class I expression in pancreas derived from three different organ biorepositories (nPOD, DiViD and EADB), and concluded that HLA class I hyperexpression is a “defining feature of T1D” (26). Noteworthy, thanks to the entire bulk of studies performed and the availability of pancreas collected within repository biobanks, we know that HLA class I hyperexpression shows several peculiar characteristics:

- (i) hyperexpression occurs only in insulin-containing islets both in β -cells and in α -cells;
- (ii) it is a marked phenomenon in recent-onset T1D donors and in donors with long-standing T1D up to 11 years of disease duration and declining thereafter;
- (iii) it is not correlated to evidence of insulinitis (26); however, at least in donors with double positivity for autoantibodies, HLA class I expression was positively correlated with the presence of cytotoxic CD8⁺ T cells, in line to what previously observed.
- (iv) it has a lobular expression pattern (potentially associated to the expression of insulin), with some lobules containing islets which express high levels of HLA class I while others don't (56);
- (v) HLA class I hyperexpression is not confined to classical isoforms (HLA-ABC), but includes other isoforms, such as HLA-F which is hyperexpressed both at mRNA and at level.

Additionally, it has been demonstrated that HLA class I hyperexpression can be potentially induced by a variety of inflammatory mediators or stressors (57,58). As a matter of fact, in the same study (25) a significant correlation between HLA class I and STAT1 hyperexpression in β -cells of DiViD T1D donors was observed; STAT1 is an intracellular protein which mediates antiviral responses triggered by interferons. Therefore, such correlation suggests that viral infections may represent a potential triggering factor leading to HLA class I hyperexpression. Although HLA class I hyperexpression should be considered an epiphenomenon which follows to other events during the progression of T1D, one can speculate that β -cells which express high levels of HLA class I could be significantly exposed to the immune system due to a sort of increased “visibility”.

In addition, HLA class I hyperexpression in β -cells seems to be associated also to a recently discovered mechanism occurring in T1D: Thompson et al. identified a subpopulation of “senescent” β -cells actively promoting the immune-mediated destruction process (59). Interestingly, it was observed that during the natural history of T1D, both in humans and in NOD mice, a subset of β -cells acquires a senescence-associated secretory phenotype pattern (SASP), thus defining them as SASP β -cells. The exposure of β -cells to a variety of inflammatory cytokines derived from infiltrating immune cells, triggers strong stress responses which, in turn, induce a senescent fate characterized by the activation of a DNA damage response (DDR). Of note, persistent DDR leads to chromatin molecular changes and to the acquisition of a specific secretome pattern phenotype composed of cytokines, chemokines, growth factors, proteases and extracellular matrix components, commonly known as senescence-associated secretory phenotype. The presence of senescent cells within pancreatic islets can disrupt tissue architecture and lead to β -cell dysfunction, thus representing a potential actor in T1D pathogenesis. Using different approaches, including immunohistochemistry, it was demonstrated that β -cells display the hallmarks of DNA-damage induced senescence. Elimination of SASP β -cells stopped the immune-mediated β -cells destruction, thus generating protection. These data support the contribution of SASP β -cells to disease progression and suggest that clearing such cells may represent a novel therapeutic approach for T1D (59). In pancreatic sections derived from nPOD donors, SASP β -cells have been identified through the immunohistochemical co-detection of CDKN1A, SERPINE1 and IL-6, whose presence gradually increased from non-diabetic controls to islet autoantibody positive and T1D donors. Collectively, these data show that the appearance of SASP β -cells is triggered by inflammatory mediators (60), which are particularly active on a specific subtype of β -cells, thus enhancing islet inflammation and resembling a β -cell fragility model and an inflammatory loop. As a matter of fact, SASP β -cells have been demonstrated to express and secrete pro-inflammatory chemokines as well. In particular, the pro-inflammatory chemokine CXCL10 seems to be directly induced by acquisition of a senescent phenotype and is involved in the recruitment of autoreactive T-lymphocytes in

pancreatic islets, thus contributing to insulinitis and β -cell destruction. In accordance to these results, previous studies showed that CXCL10 is expressed in islet endocrine cells of T1D donors and plays a critical role in disease pathophysiology (61–63). Roep et al. observed that the elevated levels of this chemokine in islets of recent-onset T1D donors, corresponded with the juxtaposed infiltration of lymphocytes bearing its cognate receptor CXCR3; of note, both CXCL10⁺ endocrine cells and CXCR3⁺ lymphocytes were undetectable in pancreas of non-diabetic control donors (64,65). In line with these data, it has been reported that the chemokine CCL2 is also expressed and secreted by β -cells, thus contributing to the recruitment of specific CCR2⁺ immune cells to the site of inflammation (66).

An accurate evaluation of inflammatory effects on β -cell response is of fundamental importance in order to identify therapeutic strategies. Indeed, several studies have elucidated the role of cytokines (e.g. TNF- α , IL-1 β and IFN- γ) in the pathogenesis of T1D, particularly on β -cell apoptosis or dysfunction (67). As a matter of fact, concordant results showed that inflammatory cytokines induce an initial dysfunction followed by a pro-apoptotic signal through the activation of several intracellular mediators (68,69). Additionally, as previously reported, cytokines have been shown to induce the expression and secretion of chemokines (CXCL10, CCL2, CXCL1-2) (70,71) on β -cells, thus enhancing insulinitis and inflammation.

Recently, it has been demonstrated that inflammatory mediators are able to activate checkpoint inhibitory signals between β -cells and immune cells, thus suggesting a tentative preservation of tolerance toward pancreatic β -cell antigens as a mechanism of intrinsic β -cell defense and escape. The Programmed Death receptor-1 (PD-1) and its ligand Programmed Death-Ligand 1 (PD-L1) have been recently suggested to play a crucial role in this phenomenon. Colli M et al. evaluated the expression of PD-L1 in pancreatic sections from T1D donors (from DiViD study and from EADB UK cohort) and from non-diabetic controls (EADB UK cohort) (72). Interestingly, in T1D donors, PD-L1 expression was exclusively observed within pancreatic islets, preferentially in insulin-positive cells (occasionally in α -cells), and was negatively correlated with the degree of islet CD8⁺

T cell infiltration. Of note, PD-L1 was not expressed in insulin-deficient islets, gradually decreased with disease duration and was absent or barely detectable in islets from non-diabetic donors, suggesting that islet inflammation is a requisite for PD-L1 expression. As a matter of fact, in-vitro, PD-L1 expression was induced by exposure of human islets or EndoC- β H1 cells to interferon- α or interferon- γ . These results suggest that β -cell specific PD-L1 expression in patients with T1D may occur as a response to the inflammatory process and contribute to the dialogue between β -cells and immune cells during inflammatory insults (72). Such results have been confirmed in nPOD cohort, comprising T1D, non-diabetic, islet autoantibody-positive, -negative and T2D donors. This analysis confirmed the PD-L1 expression pattern in T1D cases and further demonstrated that PD-L1 was absent in islets from non-diabetic and T2D donors, while it was specifically expressed in β -cells and gradually increased in non-diabetic autoantibody positive and T1D donors (73). As suggested by several authors, we can speculate that the expression of PD-L1 on β -cells during disease progression can be associated to the acquisition of a de-differentiated phenotype (39,74–76). Indeed, increasing evidence showed that under certain conditions (e.g. prolonged subtle inflammatory stress), β -cells lose the expression of their specific markers while acquiring those associated to a de-differentiated phenotype, thus leading to the loss of specialized functions (e.g. glucose-induced insulin secretion) and to the acquisition of a protective phenotype (76,77). Although such concept is taken into consideration by T1D research community, it requires further evidence on human samples and additional in-vitro studies.

4.2. α -cells

An important role for glucagon in T1D pathophysiology has been suggested. Indeed, increased plasma glucagon in response to mixed-meal test was observed in children and adolescents with recent-onset T1D (78). In such context, α -cell dysfunction seems to be increasingly important, resulting in the exacerbation of hyperglycemia due to paradoxical hyperglucagonemia and to potentially severe hypoglycemic episodes, as a consequence of a defective counterregulatory

system. Of note, during autoimmune diabetes progression, α -cells have been described as hypertrophic and hyperplastic, both in humans and in multiple rodent models. An explanation for α -cell hypertrophy/hyperplasia could reside in the loss of insulin-mediated inhibition of α -cells activity, leading to their aberrant expansion and to hyperglucagonemia (79). In order to gain insight into α -cell function in T1D, Brissova M. et al.. (80) analyzed pancreas and isolated islets collected from the same T1D donors. They observed an impaired glucagon secretion that correlated with specific alterations of α -cell gene expression profiles. More in detail, although T1D islets showed 2-fold more α -cells than non-diabetic controls, glucagon secretion was decreased. In addition, α -cell response was reduced when normalized to islet glucagon content and failed the appropriate increase at low glucose concentrations following 30-min high glucose inhibition. These functional changes were associated with a reduced expression of multiple α -cell transcription factors (e.g. ARX, MAFB, RFX6) and of their downstream targets, suggesting both direct and indirect impact on glucagon secretory pathways. Immunofluorescence was employed to analyze the expression and localization of α -cell transcription factors (MAFB and ARX) in formalin-fixed paraffin-embedded pancreatic sections obtained from nPOD T1D donors with different disease duration (3, 6 and 31 years). Interestingly, it was found that the fraction of α -cells positive for such transcription factors decreased progressively with disease duration. Furthermore, a progressive increase of α -cells expressing the β -cell specific transcription factor NKX6.1 was observed. Finally, the authors demonstrated that α -cell gene expression alterations were partially restored when T1D islets were transplanted into a normoglycemic non autoimmune environment (NSG mice). These results suggest that: (i) in T1D, an intrinsic α -cell defect may explain the dysregulated glucagon secretion previously observed in T1D patients; (ii) such defects are correlated with the progressive loss of α -cell specific transcription factors; (iii) NKX6.1 expression in α -cells raises the possibility of a partial switch toward a β -cell phenotype, even though additional stimuli may be needed in order to obtain a complete transdifferentiation α -to- β ; (iv) the observed molecular changes are reversible upon removal of stress stimuli, thus suggesting the possibility of a therapeutic intervention (80).

Importantly, it has been shown that α -cells may contribute to the islet inflammatory environment. Anquetil F and colleagues analyzed pancreatic tissue samples from nPOD donors (9 non-diabetic islet autoantibody negative, 5 non-diabetic islet autoantibody positive, 6 T1D and 6 T2D donors) in order to evaluate intra-islet expression of pro-inflammatory cytokine IL-1 β and to determine its cellular origin. Although IL-1 β expression (both at mRNA and at protein level) was independent of disease status, results revealed that the main source of IL-1 β was retained by α -cells (40-68% of total α -cells) respect to β -cells (1-21% of total β -cells) (81). Potentially, IL-1 β produced by α -cells could exert different paracrine effects on β -cells expressing IL-1 receptor, thus representing a potential crosstalk mediator between α - and β -cells (81).

Collectively, these studies suggest that α -cells are of importance in T1D progression, thus requiring further studies in order to explore the causes and the origin of α -cell dysfunction in T1D.

5. Adaptive Immune cells: deciphering *in-situ* autoreactive CD8⁺ T cell specificities

Autoreactive cytotoxic CD8⁺ T-lymphocytes appear to be the main mediators of β -cell destruction, showing an exclusive specificity toward islet autoantigens (55). In 2012 Coppieters KT and colleagues provided the first histological *in-situ* evidence of autoreactive CD8⁺ T cells against β -cell autoantigens, thus underlining their contribution to T1D pathogenesis. Of relevance, the use of *in-situ* immuno-histochemical tetramers (TMRs) staining which adopts specific targeting peptides loaded on MHC class I and linked to a detectable marker, allowed the identification of CD8⁺ T cells bearing a T cell Receptor (TCR) specificity against typical β -cell autoantigens. Using this approach, the authors demonstrated that specific CD8⁺ T cells directed against β -cell autoantigens (GAD-65, insulin, PPI, IA-2, IGRP) are within or in close proximity to pancreatic islets of T1D adult donors. The identification of CD8⁺ autoreactive T cells in insulitic infiltrates was associated to by insulin containing islets and HLA class I hyperexpression, the latter reported as a typical pathognomonic sign of recent onset T1D (see above). Additionally, it was found that CD8⁺ T cell diversity in targeting specific autoantigens, increased concomitantly with T1D progression, being higher in

donors with longer disease duration; indeed, insulinitic lesions of donors with long-standing T1D vs. those with recent-onset diabetes were characterized by the presence of CD8⁺ T cells autoreactivity to multiple β -cell autoantigens, thus suggesting the complexity of immune response throughout the natural history and progression of T1D (55). More recently, additional evidence highlighted and confirmed the in-situ identification of specific autoreactive CD8⁺ T cells, both in collagenase-isolated human islets (82) and in pancreatic tissue sections derived from T1D donors from nPOD tissue biobank (83,84). Interestingly, in the latter, Culina S et al. showed that the frequency of a autoreactive CD8⁺ T cells directed against a specific peptide of ZnT8 (Zinc Transporter 8) protein (186-194aa) was increased in pancreas but not in peripheral blood of T1D patients vs. islet autoantibody positive and islet autoantibody negative- non-diabetic controls. Of note, such pancreas-infiltrating specific autoreactive CD8⁺ T cells also showed an antigen-experienced phenotype, being positive for CD45RO and demonstrating the potential effector function of these cells in the pancreas. Of note, in peripheral blood, non-diabetic subjects showed similar frequencies of ZnT8-specific autoreactive CD8⁺ T cells vs. T1D patients, thus moving the focus at the level of the target organ and introducing for the first time the concept of “benign autoimmunity” in non-diabetic subjects. In regard to this, it has been hypothesized that in non-diabetic individuals, these potentially pathogenic self-reactive circulating CD8⁺ T cells are controlled by multiple mechanisms of peripheral tolerance (83), including the suppression by regulatory T (T_{reg})-cells, which play a key role in maintaining self-tolerance (85). Interestingly, although patients with T1D display similar numbers of peripheral T_{reg}-cells compared to non-diabetic subjects, T_{reg}-cells isolated from T1D patients show a decreased suppressive activity in vitro, suggesting an impaired function of this type of immune cells in T1D individuals (86,87); alternatively, effector T cells may be resistant to such immune regulation (85). However, the increased frequency of autoreactive CD8⁺ T cells in the target-organ, not paralleled by peripheral blood differences between T1D patients and non-diabetic subjects, suggests the involvement of additional factors, which may contribute to the diabetogenic effects of CD8⁺ T cells in the pancreas, ranging from a different T cell regulation and tolerance

induction (as described above) to enhanced β -cell fragility/vulnerability or defects which increase their visibility to the immune system (84).

Noteworthy, the plethora of newly discovered β -cell autoantigens targeted by the immune system in T1D is currently expanding and latest findings indicate the presence of other relevant neoantigens and corresponding autoreactive specific $CD8^+$ T cells, which dictate direct β -cell damage. In a collaborative study using a peptidomic approach, Gonzalez-Duque S and colleagues uncovered a set of β -cell neopeptides (secretogranin V, secretogranin V splice form 009, proconvertase-2, urocortin-3, ISL-1 and islet amyloid polypeptide transpeptidation) whose HLA class I mediated presentation by β -cells is increased under inflammatory circumstances. Correspondingly, using tetramers immunostaining, they identified specific $CD8^+$ T cells directed against IAPP_{15-17/5-10}, ISL1₂₇₆₋₂₈₄ and UCN3₁₋₉ and whose presence in pancreatic tissue sections was more abundant in T1D and in non-diabetic islet autoantibody positive donors respect to non-diabetic controls. Additionally, fluorescent confocal microscopy on pancreas sections from a T1D donor, showed that 61.7% of $CD8^+$ cells were also $CD45RO^+$ and that 5.4% of them were positive for pooled tetramers directed against IAPP_{15-17/5-10}/ISL1₂₇₆₋₂₈₄/UCN3₁₋₉, thus demonstrating also the antigen-experienced phenotype of these cells (84).

6. Innate Immune cells: novel players in T1D pathogenesis

Although the presence and involvement of $CD8^+$ autoreactive T cells has been confirmed and associated to T1D pathogenesis, the immunological scenario might be more complex, involving additional immune cells. Therefore, the need to explore other potential factors participating to T1D pathogenesis, prompted research efforts to focus also on innate immunity and inflammation. In this regard, recent evidence highlighted a potential role for neutrophils and mast cells in T1D pathogenesis.

Neutrophils have been recently associated to the development and progression of T1D and their presence has been clearly highlighted in pancreas of T1D donors deriving from multiple organ

biobank collections (28,88,89). A strong trigger to the initial evaluation of neutrophils in T1D, derives from the fact that such cells are more active immunological mediators than previously thought. Indeed, their role has been found critical in multiple human autoimmune diseases (e.g. lupus erithematosus, vasculitis, rheumatoid arthritis etc) and causally associated to the onset of such diseases in several animal models (90,91). In animal models of T1D, neutrophils recruitment in pancreas during autoimmune diabetes progression has been shown to be essential in disease onset and severity (88). In human T1D, even though neutrophils dynamics and causal relationship are still missing, we certainly know that they can infiltrate the pancreas and are strongly increased in T1D donors vs. islet autoantibody positive and negative non-diabetic controls, while such increase has not been reported in T2D pancreas (89). In such context, neutrophils were mostly found in exocrine portion of the pancreas being tightly associated with acinar tissue (28,89); however, some of them were also found in close proximity or within insulitic lesions, thus ascribing neutrophils as potentially involved in insulitic immune network. Although the exact mechanism of neutrophils involvement in human T1D initiation or progression has not yet been clarified, it is believed that they are not simply bystander elements. Indeed, it is well known that neutrophils are key cells in recognition and elimination of pathogens and they can be activated into a mechanism defined as NETosis, namely by extracellularly extruding macromolecules complexes composed of decondensed DNA, associated with specific proteins (citrullinated proteins, elastin, Mieloperoxidase), commonly known as Neutrophils Extracellular Traps (NETs) (92). Such mechanism is also adopted by neutrophils to sense self-components, thus being involved in tissues repair and clearing (93). Although beneficial, in certain conditions this process may trigger or amplify autoimmune reactions. Vecchio F and colleagues recently demonstrated that neutrophils are increased in pancreatic tissues deriving from T1D donors vs. islet autoantibody positive and negative non-diabetic control donors and that a proportion of them generates NETs, thus co-expressing citrullinated histone H₃, mieloperoxidase and decondensed DNA (28). Moreover, the increase of neutrophils in pancreas of T1D organ donors, concomitantly correlates with the

reduction of peripheral blood circulating neutrophils, thus leading to the hypothesis of a pancreas-centered massive migration (tissue sequestration) of these cells under specific pathological conditions. Such reduction has been recently highlighted also in another longitudinal T1D study cohort (TEDDY) which observed a significant reduction of peripheral blood circulating neutrophils in at-risk children positive for multiple islet autoantibodies, therefore indirectly corroborating the hypothesis of neutrophils massive migration to the pancreas (94). Although the evidence for a role of neutrophils are increasingly important, additional studies are needed to decipher a mechanistic link between neutrophils migration to the pancreas and triggering or amplification of autoimmunity in T1D.

Innate immune cells infiltration in T1D includes also mast cells which seem to be involved in the pathogenesis of disease (95,96). Although mast cells are mostly considered the first defense line, mainly present in peripheral tissues near body surfaces and linked to allergic reactions, they have been strikingly associated to autoimmune diseases as well. Mast cells are able to directly interact with different immune cells (e.g. T cells, B cells) by inducing their differentiation and/or activation. Moreover, increasing evidence showed that mast cells are involved in the pathogenesis of several diseases like rheumatoid arthritis (RA) or multiple sclerosis (MS) (97); such evidence led some authors to re-define the role of mast cells within autoimmune reactions, thus attributing them equal importance in immune response exacerbation respect to T cells (98). Recently, a study investigated the presence of mast cells in pancreas obtained from adult donors with T1D (mostly long-standing donors - range disease duration: 2-10y -), T2D and age-matched non-diabetic donors. In this study, Martino L and colleagues demonstrated that mast cells preferentially infiltrate pancreas of T1D donors, being more numerous in T1D vs. T2D and non-diabetic donors. The presence of these cells was demonstrated in exocrine tissue as well as in peri- and intra-islet area. More interestingly, T1D donors showed a higher number of islets having at least one mast cell in peri- or intra-islet area respect to T2D and non-diabetic donors, thus showing that these cells represent an active component of immune cells insulinitis infiltration (99). By visualizing them using electron

microscopy, pancreas-infiltrating mast cells appeared to be also partially degranulated (a specific feature of active mast cells during inflammation resolution), then potentially contributing to islet/beta-cell damage through degradative enzyme production (tryptase, histamine) or through complement production and activation. As a matter of fact, a specific subset of mast cells expressing C4 mRNA complement family member, has been described to infiltrate gut mucosa during autoimmune Crohn's disease and showed to actively participate in immune-mediated tissue damage (100). Interestingly, a 2013 report analyzing nPOD pancreas tissue sections highlighted the presence of complement C4d deposition in pancreas of T1D donors which was higher vs. T2D, islet autoantibody positive and negative non-diabetic organ donors (101) and in line with the increased mast cell infiltration in T1D pancreas sections. Such evidence suggest a potential function of mast cells in the production of complement members and their role as active immune cells in T1D.

A relevant role of NK cells in T1D is currently emerging as well, contributing to define a scenario in which β -cells, innate and adaptive immune cells are engaged each other (102). The presence of NK cells has been shown in pancreas of mouse model of autoimmune diabetes, as well as in human pancreas from T1D donors (35). NK cells represent the interface between innate and adaptive immunity and their presence has been associated to viral infections, being involved in the defense against viruses. In this regard, the hypothesis that viruses could play a key role in the pathogenesis of T1D has a long history and enteroviruses, especially Coxsackievirus B-group (CVB), seem to trigger pancreatic islet autoimmunity through several mechanisms, including direct destruction of pancreatic β -cells, bystander activation of autoreactive T-cells, molecular mimicry and/or viral persistence (not mutually exclusive) (103). Both *in vitro* and *in vivo* studies have shown the capability of enteroviruses to infect human pancreatic β -cells, with consequent effects ranging from functional damage to cell death (104). As a matter of fact, in 2007, Dotta F. et al. isolated for the first time a strain of CVB4 from a pancreatic sample of a recent-onset T1D organ donor. CVB4 positivity was associated with NK cells islets infiltration (35). Moreover, capsid protein VP1 was detected in islets (but not in exocrine tissue) of pancreatic autopsy specimens from patients with

T1D, as well as in β -cells (but not in α -cells) of islets from pancreatic biopsies of recent-onset T1D patients recruited in the DiViD study (29). We can hypothesize a scenario in which enteroviruses (in particular CVBs) could infect pancreatic β -cells by binding to specific receptors (such as human Cocksackie- and adenovirus receptor, hCAR) (105). After internalization into β -cells, viruses could be recognized by innate immune pattern recognition receptors (PRRs), triggering inflammatory signaling cascades and leading to the production of pro-inflammatory chemokines and cytokines, with subsequent expression of interferon-stimulated genes (ISGs), whose products could limit infection. Although local inflammation and activation of antiviral defenses, should eradicate the viral infection, in some genetically susceptible individuals these cellular attempts to neutralize the invading virus might be characterized by exaggerated inflammatory response, defective triggering of intracellular anti-inflammatory or defective anti-apoptotic responses, then inducing progressive inflammation and β -cell loss (106).

7. Exocrine pancreas in T1D: not just a matter of islet cells

7.1. Exocrine-Endocrine pancreas interconnection

T1D is commonly considered a disease affecting the endocrine pancreas. However, increasing evidence suggest a critical involvement of the exocrine pancreas as well. Indeed, even though pancreatic endocrine and exocrine compartments are considered two separate entities (exocrine~98%, endocrine ~2% of the pancreatic tissue), they are not independent of each other. Indeed, they are anatomically and functionally related and the endocrine compartment seems to play a significant role in pancreatic digestive activities.

First of all, pancreatic islet vessels are connected with those vascularizing the exocrine tissue through a network of capillaries. In addition, the interplay between the endocrine and the exocrine pancreas may be mediated by humoral factors and neurotransmitters also (107,108). In 1981, a paper by Henderson et al. described the effects of islet hormones on the exocrine pancreas homeostasis and function (109). However, to date, little is known about the potential role of the exocrine pancreas in the pathogenesis of T1D, nor if this involvement may be a consequence or a cause of the chronic inflammation and of autoimmune reaction associated with β -cell destruction (107,110). Consequently, studies clarifying the hypothetical active involvement of the pancreatic exocrine tissue in T1D development represent a novel and very interesting area of research.

7.2 Exocrine pancreas inflammation

Some evidence from literature suggest that pancreatic exocrine tissue can be affected by the same inflammatory process that classically characterizes islets in T1D. Nakanishi K et al. described an infiltration of LCA (leucocyte common antigen)-positive cells surrounding pancreatic acinar cells in autoptic samples derived from T1D patients (6 out of 12 patients studied). The same cells were not detectable in 5 non-diabetic subjects (with or without other pancreatic diseases). More recently, pancreatic exocrine pancreas was observed to be infiltrated by lymphocytes in 22/47 T1D patients

of a Japanese cohort (111). In addition, few CD3⁺ T cells were found scattered in the exocrine pancreas of 42 autoptic long-standing T1D patients. In this study the authors identified macrophages within the exocrine tissue and adjacent to scattered β -cells in the exocrine compartment (112).

Summers KL et al. described dendritic cells (DC) scattered in the exocrine tissue surrounding islets (but not within islets) in autoptic pancreatic samples from T1D patients. In contrast, DCs were not detectable in non-diabetic subjects. Authors argued that the presence of these cells in patients with T1D could suggest their contribution (either direct or indirect) to β -cell destruction, once migrated into the pancreas (113). The presence of DCs (identified as CD11c⁺ cells), together with T cells, were also investigated by Rodriguez-Calvo T. et al. in pancreatic sections of T1D and non-diabetic [both islet autoantibody positive (AAb⁺) and islet autoantibody negative (AAb⁻)] organ donors from the nPOD cohort (110). Indeed, they found that CD8⁺ T cells (the predominant cell type identified in human pancreas in T1D), CD4⁺ T cells and CD11⁺ cells were described to infiltrate the exocrine pancreas at a higher density in T1D (recent-onset as well as long-standing) compared to AAb⁻ non-diabetic donors. Moreover, AAb⁺ non-diabetic donors showed a significantly higher density of CD11c⁺ cells compared to AAb⁻ non-diabetic donors. However, in T1D donors, both pancreatic DCs and CD4⁺ T cells were detected at a high frequency in the exocrine pancreas; of note, a strong correlation between them was found, thus supporting the critical role of DCs as antigen-presenting cells to naïve CD4⁺ T cells. Interestingly, T1D donors showed a significant high number of CD8⁺ T cells in the exocrine tissue also, independently of age, BMI, ICU time, disease duration and presence of prominent insulinitis or insulin-containing islets. These important observations regarding the distribution and localization of immune cell in T1D pancreas, were more recently confirmed by Damond N et al. and Wang YJ et al. (39,41) by adopting wide spectrum mass-cytometry approach, as described above.

Detection of CD8⁺ T cells even in the absence of insulin-containing islets, suggests that their presence within the pancreas is not only antigen-driven; in addition, their location in high numbers

within the exocrine tissue supports the hypothesis that this pancreatic component may be involved in T1D pathogenesis. In support to this, finally, preliminary data showed an increase of MHC class I expression in the pancreatic exocrine tissue of T1D donors, which may be related to the CD8⁺ T cells infiltration in the same compartment (110).

Interestingly, Wiberg A et al. investigated the presence of CD45⁺ cells in pancreatic samples obtained from non-diabetic donors (29 AAb⁺ and 31 age-matched AAb⁻ donors). CD45⁺ cells were identified in all donors, mostly scattered within the exocrine and the connective tissues; however, AAb⁺ donors with high GADA (glutamic acid decarboxylase antibodies) titers, showed significantly higher CD45⁺ cell numbers in the exocrine tissue compared to AAb⁻ subjects. Several donors showing high numbers and/or high density of CD45⁺ cells in the exocrine tissue (6 AAb⁺ and 6 AAb⁻) were selected to further evaluate the presence of neutrophils, CD8⁺, CD4⁺ and regulatory T cells. CD8⁺ T cells represented the most abundant cell-type within the exocrine tissue of all donors, followed by neutrophils and CD4⁺ T cells, while regulatory T cells were present in a very few number. Of note, no differences were identified regarding analyzed immune cell type between AAb⁺ and AAb⁻ donors.

In 1986, Foulis AK et al. identified polymorphonuclear cells in the interstitial tissue and surrounding ducts of 6 T1D donors (5 with recent-onset disease) from a cohort of 96 autoptic T1D cases (13). More recently, Valle A et al. showed the presence of neutrophils [identified by myeloperoxidase (MPO) positivity] in pancreatic exocrine tissue of three T1D donors (but not in T2D and non-diabetic donors). Neutrophils were mainly identified in small blood vessels but also interspersed among acinar cells (89). These findings were confirmed by another study reporting a massive neutrophils infiltration within pancreatic exocrine tissue of two new-onset T1D donors and, more recently, by Vecchio F et al. as described above (28).

Collectively, these results indicate that immune cells can be extensively found within the exocrine pancreas of T1D donors, independently of the presence of insulin-containing islets, suggesting that β -cells are not necessarily required for the homing of cells of the immune system in the exocrine

pancreas (110). However, several authors argued that immune cells would be recruited to pancreatic islets through the secretion of chemokines by β -cells and the subsequent generation of a chemoattractant gradient (114,115) which disappears once β -cells have been destroyed (62).

Overall, a high number of T1D patients show clinically important inflammatory processes within the exocrine pancreas. As a matter of fact, Gepts W et al. identified focal to diffuse lesions of acute pancreatitis associated with mild atrophy of exocrine tissue in patients with “acute juvenile diabetes” (with short disease duration) compared to “chronic juvenile diabetic” patients, characterized by intra- and peri-lobular sclerosis (116). Interestingly, Gaglia JL et al. described a non-invasive imaging method to detect pancreatic inflammation, based on magnetic resonance imaging (MRI) of the magnetic nanoparticle ferumoxytol (which reflects nanoparticle uptake by macrophages within pancreatic inflammatory lesions), evaluated in mouse models of autoimmune diabetes. In a recent study, it has been showed a higher accumulation of nanoparticles in recent-onset T1D patients compared to non-diabetic healthy individuals, characterized by a little or no nanoparticle accumulation. Moreover, there was a high heterogeneity of intra- and inter-pancreatic signal in T1D patients, whereas pancreas appeared essentially homogenous in non-diabetic subjects (117). We can speculate that pancreatic exocrine inflammation may be associated to the loss of pancreas parenchyma and function in T1D patients.

7.3- Exocrine pancreas functional alterations

In addition to the inflammatory environment, functional and histological alterations of the exocrine pancreas have been described in T1D. Indeed, in 1940, Pollard HM et al. described a reduction of amylase and lipase levels after pancreozymin-secretin stimulation (107). The most common alterations observed by performing direct pancreatic function tests were represented by a reduction in amylase and bicarbonate output and a decreased of maximum bicarbonate concentration in T1D patients. Moreover, a mild to moderate decrease of lipase output has been described in few studies, as well as a major prevalence of decreased FE1 levels in T1D patients compared to non-diabetic

subjects (118). However, it has not been clarified yet whether these modifications are caused by the same pathogenic factors participating in β -cell destruction or represent a consequence of β -cell loss. Interestingly, autoantibodies against exocrine antigens (e.g. carbonic anhydrase and lactoferrin) have been detected in several studies (119,120).

In a recently published paper, Lundberg M et al. found a reduced density of parasympathetic axons (assessed by immunofluorescence and morphometry) in the exocrine pancreas of 6 recent-onset T1D patients undergoing pancreatic biopsy and 2 recent-onset T1D organ donors compared to 7 long-standing T1D and 7 non-diabetic donors. In contrast, there was no difference in islet-associated parasympathetic axons per islet (axons within islets or surrounding them) between groups, suggesting that islet parasympathetic innervation is not affected in T1D. Moreover, the non-reduction of axons in long-standing T1D donors could reflect a re-innervation, or, alternatively, be caused by a reduced pancreatic volume with an unaltered amount of axons (121).

8. Pancreas anatomical alterations in T1D

In 1909, Cecil RL described a reduction in size (atrophy) and weight of the pancreas in diabetic patients compared to non-diabetic controls (122). Given the small contribution of the endocrine portion in determining pancreas size, the main responsible for this phenomenon has been suggested to reside within the exocrine pancreas. In consideration of the paracrine trophic effect of the islet hormones on acinar cells (109,123), the destruction of β -cells which occurs in T1D could induce the loss of the insulinotropic impact on the exocrine tissue (107,124). Particularly, severe atrophy of acinar cells has been observed next-to insulin-deficient islets, a phenomenon that has not been observed in the acini adjacent to insulin-containing islets (125). Indeed, in the presence of high numbers of insulin-deficient islets, the exocrine pancreas is exposed to an altered balance of islet hormones, thus favoring atrophy. Moreover, diabetic microangiopathy has been suggested to be involved in pancreatic exocrine tissue atrophy, reflecting the vascular connections between the islets and the exocrine tissue. Finally, several authors hypothesized that pancreas volume is already

reduced years before the clinical onset of T1D, suggesting an immune-mediated insult to the exocrine pancreas as well as to the endocrine counterpart (110). Exocrine pancreas fibrosis is also frequently observed in T1D patients; it can be of various degrees and localized only in the proximity of the ducts, or extended to the interlobular pancreas as well.

8.2 Pancreas Volume reduction in T1D

Over the years, several studies evaluated the size and weight of pancreas in patients with T1D. In 1985, Fonseca V et al. measured the area of pancreatic head and body in 22 T1D patients and in 19 non-diabetic healthy subjects, using ultrasound techniques. In T1D patients, pancreas size resulted to be reduced in comparison to non-diabetic individuals, without any correlation with body weight or diabetes duration (126). Later, Williams AJK et al. performed a study including 20 adult recent-onset T1D patients and 24 non-diabetic healthy controls (all males) matched for age, to analyze pancreatic volume between these two groups. Using a non-invasive MRI, authors determined pancreatic volume and after an adjustment for body weight, they observed a significant reduction (26%) in T1D patients compared to healthy controls, without any correlation with diabetes duration, number or level of islet autoantibodies and markers of β -cell function (e.g. C-peptide); moreover any exocrine insufficiency was observed (124). The same authors, using MRI, observed a reduction (-48%) in the pancreatic volume of 12 long-standing T1D patients (evaluated 10 years after diagnosis) compared with 12 non-diabetic subjects (all males, age matched) (127). Moreover, Gaglia JL et al., using MRI, determined the mean pancreatic volume index (PVI) by dividing pancreatic volume by body-surface area in a cohort of 10 new-onset T1D patients and 10 non-diabetic healthy controls (age-matched). A 31% reduction in the mean pancreatic volume index was found in T1D patients compared to controls, without any correlation between PVI and diabetes duration, age or islet autoantibodies titer (128). Recently, Regnell SE et al. observed a reduction of pancreatic volume (-27%) evaluated by MRI in 22 children with T1D and without exocrine

pancreatic disorders compared to 29 non-diabetic children not correlating with diabetes duration (129).

All the studies described above suggest that pancreatic atrophy could be a process starting before the clinical onset of diabetes; moreover, the exogenous insulin administration may decelerate pancreatic atrophy, as hypothesized by the authors of the last study who found a positive correlation between the reduction of pancreatic volume and insulin units/kg body weight/day in T1D patients (129). Finally, a recent meta-analysis by Garcia T.S. et al., including data from 8 selected studies evaluating pancreatic size (2 in terms of diameter, 2 area and 4 volume) determined by ultrasound, computed tomography (CT) or MRI in adult T1D patients compared to healthy controls, showed that pancreatic size was reduced in T1D patients, in particular in those with a long disease duration (130). These results are in agreement with those reported by Virostko J et al. in a study evaluating pancreatic volume (by MRI or CT) in 25 T1D patients and 25 age, sex and weight matched non-diabetic subjects. Authors described a reduction (-47%) of pancreatic volume (normalized by body weight, body mass index and body surface area) in T1D patients compared to non-diabetic individuals. This reduction of pancreatic volume was particularly evident in long-standing T1D patients; moreover, a progressive reduction over time was observed in 4 T1D patients subjected to multiple imaging scans (separated temporally by 1 to 7 years). In contrast, 5 non-diabetic individuals did not show the same progressive reduction in pancreas volume over the years (131). Finally, in a recent study evaluating pancreas volume (PV) by MRI, Campbell-Thompson ML et al. described for the first time a significantly lower PV adjusted for BMI (PV-to-BMI ratio) in non-diabetic T1D first-degree relatives (FDRs) with or without one/multiple autoantibodies positivity compared with unrelated control subjects. Moreover, patients with recent-onset T1D showed lower PV-to-BMI ratio compared to FDRs (132).

8.3 Pancreas weight reduction in T1D

Concerning the studies evaluating pancreatic weight, in 1965 Gepts W et al. observed a weight reduction in autopsic pancreas obtained from long-standing T1D patients (despite, however, of some variability) compared to recent-onset T1D and non-diabetic subjects (116). Moreover, pancreatic weight did not correlate with the age at disease onset, duration of diabetes and severity of pancreatic fibrosis (116). This data support the notion that pancreatic weight reduction in T1D patients could be attributable to a prolonged total absence of insulin or due to the chronic inflammation associated with insulinitis. However, Campbell-Thompson ML et al. disputed these concepts with 2 studies describing a loss of weight in T1D donors, already present at the onset of disease. In the first study a reduced mean weight (adjusted for age and BMI) of pancreas was described not only in T1D but also in single AAb⁺ compared to AAb⁻ non-diabetic donors, suggesting that pancreatic atrophy could be an early important feature in the natural history of T1D. Subsequently, Campbell-Thompson ML et al. compared pancreatic weight of 90 T1D organ donors (15 AAb⁻, 36 single AAb⁺ and 36 multiple AAb⁺) with 86 non-diabetic and 40 T2D donors. In order to normalize confounding inter-subject factors (such as age, sex, BMI and diabetes duration) the pancreas-to-body weight ratio (relative pancreas weight) was calculated after determination of the entire-pancreas and head, body and tail weight. Authors found that relative pancreatic weight was significantly reduced in T1D compared to non-diabetic donors, while the relative weight of pancreatic head, body and tail was lower in T1D compared not only to non-diabetic but also to T2D donors. In addition, in T1D donors, relative pancreatic weight did not correlated with diabetes duration and neither with C-peptide levels (when detectable) (133).

Taken together, these studies suggest that pancreatic weight and volume are reduced in patients with T1D and in subjects at high risk to develop the disease. Since islets constitute only a small portion of the pancreatic mass, these reductions seem to be largely attributable to a significant exocrine tissue loss. In support of this concept, a decline in exocrine function (evaluated by chymotrypsin and elastase stool measurement) has been observed (47,125). Finally, an exocrine pancreatopathy (defined as a moderate-to-severe subclinical fibrosis and modest exocrine

dysfunction, occurring in the absence of clinical or histopathological evidence of chronic pancreatitis) has been described to be associated with pancreatic size in T1D. Several authors, indeed, described the presence of pancreatic fibrosis in T1D patients (especially in those with long disease duration) (111,116,122,134) which appears quite intra-lobular and inter-acinar and sometimes diffuse. In 2 studies, in particular, pancreatic fibrosis was compared between T1D and non-diabetic individuals and scored from mild to severe. T1D subjects were more frequently affected by a moderate to severe fibrosis compared to non-diabetic subjects.

9. Conclusions

Increased tissues availability from organ donors affected by T1D together with an improved quality of pancreatic tissue sections from novel biobanks worldwide coupled to the use of recent and innovative technologies, significantly expanded our understanding of the disease, challenging some previously accepted dogmas. Taken together, the data reported in the studies mentioned above, suggest that we can consider T1D as a disease affecting the whole pancreas and not only of the endocrine component, with the participation of both adaptive and innate immunity. Such observations should be taken into consideration as a starting point to elaborate novel therapeutic approaches for autoimmune diabetes prevention and treatment.

Authors' Contribution Statement: LN wrote the manuscript and drafted figures and tables. CM contributed to write the manuscript and to literature database search. GS contributed to write the manuscript, edited and revised the final version. FD edited and revised the final version of the manuscript.

Conflict of Interest Statement: The authors declare no conflict of interest related to this manuscript.

Acknowledgements. This project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement No 115797 (INNODIA). This Joint Undertaking receives support from the Union's Horizon 2020 research and innovation program and "EFPIA", "JDRF" and "The Leona M. and Harry B. Helmsley Charitable Trust"

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Table 1. Main characteristics of Insulinitis. Insulinitis is defined as lymphocytic infiltration of the islets which consists of at least $n=15$ $CD45^+$ cells/islet in a minimum of $n=3$ islets. The immunohistochemical positivity for leucocyte common antigen $CD45$ has been preferred over $CD3$ positivity, because of the presence of $CD20^+$ B in insulitic lesion other than T cells.

Main characteristics of Insulinitis
<ul style="list-style-type: none"> • More frequently observed in recent-onset vs. long-standing T1D donors • More frequently detected in insulin containing islets vs. insulin-deficient islets • Elevated heterogeneity among T1D donors and among different islets of the same donor • Predominant lymphocytic infiltration ($CD8^+$ T cytotoxic T cells > macrophages > $CD4^+$ T cells > $CD20^+$ B-lymphocytes) • Presence of evolutionary phases (<i>Figure 2</i>) • Presence of pseudoatrophic islets (insulin deficient islets having a wide glucagon-positive area) • Predominance of “peri-islets” insulinitis (within islet periphery) vs. “intra-islets” insulinitis (within islet parenchima)

Figure Legends

Figure 1. Insulin-Containing and Insulin-Deficient islets. Representative images reporting confocal microscopy immunofluorescence analysis of insulin (green), glucagon (red) and nuclei (grey) in a FFPE pancreatic section of a new-onset T1D donor. *Left panel:* pancreatic islet containing β -cells (green) and α -cells (red), identified as a typical insulin-containing islet or ICI. Immune cells infiltrate is clearly detectable (due to density, shape and size of the nuclei) on the right peripheral side of the islet. Scale bar size: 70 μ m. *Right-panel:* pancreatic islet showing no residual β -cells, identified as an insulin-deficient islet or IDI. Insulin-deficient islets are devoid of β -cells, mainly composed of α -cells and usually termed as pseudoatrophic islets due to their size (larger than usual) and/or to their altered cellular composition. Scale bar size: 50 μ m

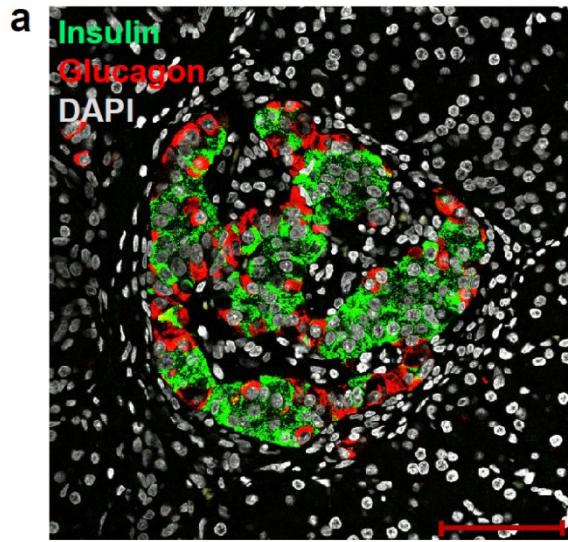
Figure 2. Progressive sequence of recruited immune cells to pancreatic islets relative to β -cell dysfunction/destruction during T1D. The scheme reports immune cells dynamics during insulinitis and T1D progression according to the latest findings (43), which described insulinitis as a progressive phenomenon characterized by several pseudo-stages (Pseudostage 2: early insulinitis; Pseudostage-3 in T1D recent onset: late stage insulinitis; pseudostage-3 in T1D long-duration: very late stage insulinitis. Dysfunctional (dedifferentiated) β -cells (narrow red) have been described during the initial phases of insulinitis while transitioning to destruction (43), being replaced by α -cells (dark blue), with a consequent reduction of fully functional β -cells (red). Insulinitis is present in 30% of β -cell rich islets while decreasing in β -depleted islets. Islet inflammatory infiltrates have been described to be composed abundantly by cytotoxic CD8⁺ T cells (violet) which increase alongside β -cell destruction then decline dramatically once β -cells are no longer present within islets. Macrophages are present, in fewer numbers, while increasing during progression of insulinitis. CD4⁺ T cells are also present but less abundantly compared to CD8⁺ T cells and macrophages. B cells have been observed in small numbers throughout the stages of insulinitis (32). Neutrophils are present both in exocrine and in insulinitic area and increased during T1D progression (43).

Figure 3. Insulinitis heterogeneity and β -cell persistence in a donor with longstanding T1D. Presence of residual β -cells is typical in donors with long-standing T1D. The image reports an immunohistochemical staining for Insulin (red) and CD45 (brown) performed in a FFPE section of

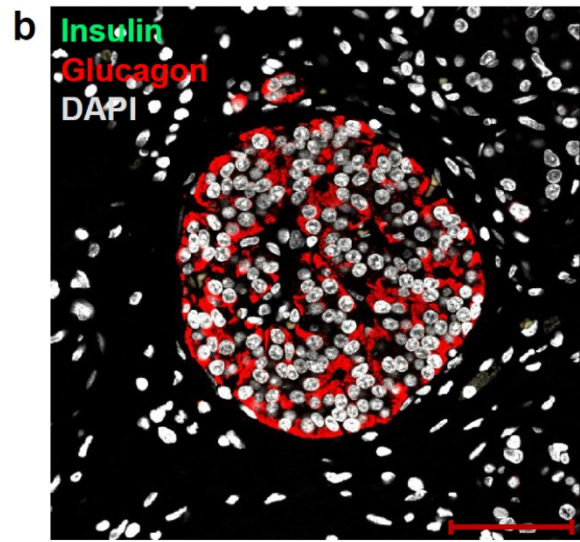
the head of the pancreas collected from a donor with long-standing T1D (21 years of disease duration, 39y, Female, GADA+ IA-2A- ZNT8-) belonging to INNODIA-EUnPOD collection.

(a) Low magnification (4X) of INS-CD45 immunostaining of FFPE pancreas section showing the presence of residual β -cells; heterogeneity of residual β -cells distribution is clearly evident. Scale Bar 750 μ m. **(b)** Magnification (20X) of an islet with many residual β -cells presenting n=5 CD45⁺ infiltrating immune cells, thus not satisfying criteria to be defined an insulitic islet. Scale Bar 150 μ m. **(c)** Islet with a patchy distribution of residual β -cells presenting >15 CD45⁺ infiltrating immune cells (insulitic islet). Scale Bar 150 μ m. **(d)** Magnification of a region of the pancreas lobule showing n=3 insulin-positive scattered cells; the presence of scattered insulin-positive cells is the hallmark of donors with long-standing T1D (49). Scale Bar 150 μ m. Blue arrows indicate some CD45⁺ cells in **(b)** and **(c)**. Black arrows indicate insulin-positive scattered β -cells in **(d)**.

FIGURE 1



Insulin-containing islet



Insulin-deficient islet

FIGURE 2

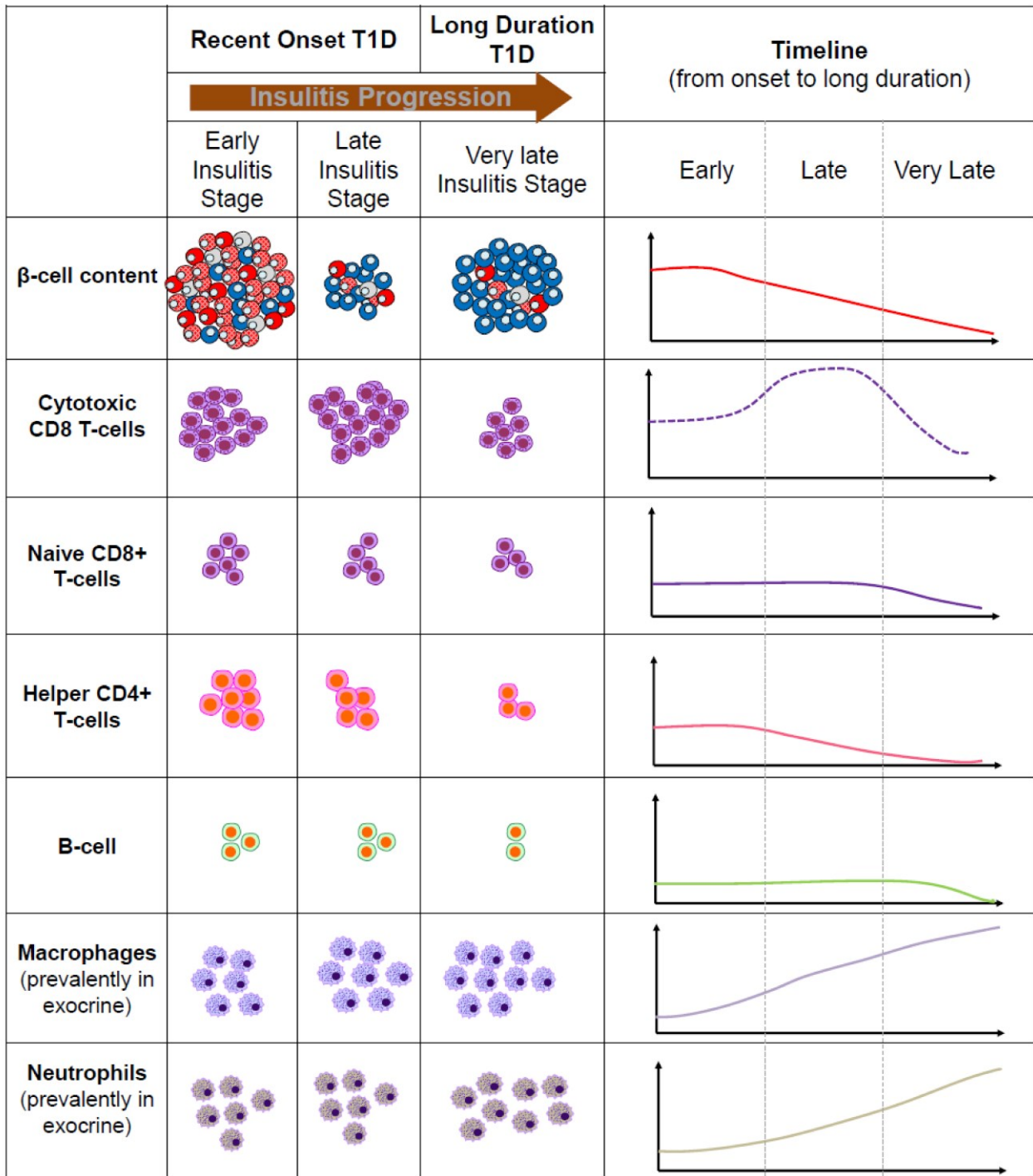


FIGURE 3

