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Deciphering the Pathogenesis of Human Type 1 Diabetes (T1D) by Interrogating T Cells from the "Scene of the Crime"

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Abstract
Purpose of Review Autoimmune-mediated destruction of insulin-producing β-cells within the pancreas results in type 1 diabetes (T1D), which is not yet preventable or curable. Previously, our understanding of the β-cell specific T cell repertoire was based on studies of autoreactive T cell responses in the peripheral blood of patients at risk for, or with, T1D; more recently, investigations have included immunohistochemical analysis of some T cell specificities in the pancreas from organ donors with T1D. Now, we are able to examine live, islet-infiltrating T cells from donors with T1D.
Recent Findings Analysis of the T cell repertoire isolated directly from the pancreatic islets of donors with T1D revealed pro-inflammatory T cells with targets of known autoantigens, including proinsulin and glutamic acid decarboxylase, as well as modified autoantigens.
Summary We have assayed the islet-infiltrating T cell repertoire for autoreactivity and function directly from the inflamed islets of T1D organ donors. Design of durable treatments for prevention of or therapy for T1D requires understanding this repertoire.

Keywords Human · T cell · Autoreactivity · Islets of Langerhans

This article is part of the Topical Collection on *Pathogenesis of Type 1 Diabetes*

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Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by the activation of lymphocytes that infiltrate and destroy insulin-producing beta (β)-cells within the pancreatic islets [1]. Loss of β cell number and function results in insulin deficiency, and requires life-long insulin administration. Because people with T1D require frequent blood glucose monitoring along with intensive insulin therapy and the disease incidence is dramatically increasing, especially in young children [2], there is an urgent need for effective therapies. With this goal in mind, many well-designed clinical trials have been completed using immune therapies to prevent or stop β-cell destruction, before or soon after disease onset [3]. To date, no therapies have proven to be clinically beneficial. The need exists to understand the immune cells infiltrating islets of human patients with T1D to define specific targets for therapies and biomarkers of disease activity.

The human pancreas, and the islets which are naturally embedded throughout the organ, needs to be accessed to effectively study and characterize islet-infiltrating lymphocytes and fully understand the mechanisms of insulitis/β-cell death. However, the pancreas is a retroperitoneal organ, predominantly involved in the exocrine production of enzymes for the digestion of proteins, carbohydrates, and fat, making it extremely difficult to access or biopsy. A recent trial involving pancreatic biopsies of patients with new-onset T1D was halted because of surgical complications [4]. Attempts to visualize or

image islets using non-invasive methods are being developed, but have not fully resolved nor defined insulitis, which is the lymphocytic infiltration of the islets in T1D [5–7]. By necessity, the study of autoreactive T cells in human T1D has come from the analysis of autoantigen-specific T cells from peripheral blood [8–16]. Almost all of the studies evaluating cellular infiltrates in the islets are gleaned from histology sections from cadavers.

Compared to humans, animal models of autoimmune diabetes provide straightforward access to the target organs. The non-obese diabetic (NOD) mouse model of spontaneous autoimmune diabetes shares many similarities with humans including MHC genes conferring disease risk, the development of insulin autoantibodies and insulitis [17]. Experiments evaluating NOD mouse islet-infiltrating T cells indicate the vast majority are specific for β-cell antigens and transfer diabetes to immune deficient (lacking T cells and B cells) NOD mice [18, 19]. By mutating or knocking out β-cell antigens, such as insulin or chromogranin A, $CD4^+$ T cell responses to these proteins have been shown to be necessary for the development of autoimmune diabetes [20, 21]. Additionally, islet-derived $CD8^+$ T cells have been shown to be a major driver of β-cell destruction by directly targeting and killing the β-cells [22, 23]. Furthermore, only β-cell antigen-specific $CD8^+$ T cells infiltrate NOD mouse islets. In another animal model of T1D, the biobreeding (BB) rat, a variety of lymphoid cells infiltrate the islets following a viral infection or innate immune system activation [24].

Studying immune cells from the inflamed islets in animal models has led to many therapies capable of preventing and even reversing diabetes. Importantly, using antigens and peptides that stimulate islet-infiltrating T cells, known as antigen-specific immunotherapy, can induce long-lasting diabetes remission. Unfortunately, the therapeutic results in animal models have not translated to humans [25]. Studying the functional biology of human islet-infiltrating T cells "at the scene of the crime" will provide powerful new insights into the autoimmune basis of human disease with the potential to improve prevention efforts.

Pancreatic Histology and Autoreactive T cells in T1D

The unusual infiltration of cells in the islets of a young child who died from ketoacidosis was noted over a hundred years ago (the history of insulitis is comprehensively reviewed in [26]). In this review, In't Veld makes the salient point that as of 2011, our knowledge of the histology and composition of insulitis comes from approximately only 150 donors, and that insulitic lesions are rare and heterogeneous, even in recent onset donors. Here, we will highlight the donor tissue collections that have aided in implicating islet-infiltrating immune cells, including $CD4^+$ and $CD8^+$ T cells, as the mediators of pathogenesis in T1D.

The Willy Gepts collection consists of 22 pancreas tissue samples from new or recent onset donors with T1D in Brussels, Belgium in the 1960s (11 with insulitis) [27]. This collection remains an important resource for studies into the disease processes leading to T1D [27–30]. Stained pancreatic sections from this collection are available in a digitized format as part of the Diabetes BioBank Brussels (http://www.diabetesbiobank.org/).

The Alan Foulis collection is a collection of autopsy pancreas samples recovered from nearly 200 individuals with new or recent onset T1D, from across the UK, who died shortly after receiving a diagnosis of T1D. This collection is now housed at the University of Exeter Medical School. Examples of infiltrated islets from cases can be seen here (http://foulis.vub.ac.be/index.php). From this collection, immune dysregulation was seen within the islets from new-onset donors with T1D by noting the upregulation of human leukocyte antigen (HLA) class II, and especially HLA class I, within inflamed islets and the pro-inflammatory phenotype of immune cells infiltrating the islets [31–36]. This collection is still in use today.

The Network of Pancreatic Organ Donors with Diabetes (nPOD) (http://www.jdrfnpod.org/) was established by the Juvenile Diabetes Research Foundation (JDRF) in 2007 in order to collect tissue from donors with T1D and to distribute these tissues to investigators while fostering collaboration and interaction to understand the etiology of human T1D. To date, there are over 160 cases of donors with T1D across the disease spectrum: islet autoantibody positive (30 donors) in the absence of diabetes, recent onset and established disease (130 donors. For a complete list of donors see, http://www.jdrfnpod.org/for-investigators/donor-groups/. In histopathological analyses of the pancreata from donors with T1D, detection of insulitis was rare (in 3–18% of islets) [37, 38]. Insulitis can be found in both islets containing insulin and islets without insulin and restricted to a single pancreatic lobe or located in several lobes of different areas of the pancreas [39, 40]. The current histopathological definition of insulitis is "the presence of ≥ 15 $CD45^+$ leukocytes/islet (alternatively ≥ 6 $CD3^+$ lymphocytes) in three islets with the presence of pseudoatrophic (insulin-negative) islets" [38], though continuing discussions and efforts examine this issue [41•].

Pancreas Tissue From Living Patients Two studies (the Osaka Study and the DiViD Study) have sought to examine pancreas tissue from living donors; the most recent of these was terminated due to adverse events suffered by the donors [4]. While these studies were not without controversy [42], they generated important information on the nature of the pancreas, islets, and infiltrates in patients with recent onset T1D, which is quite rare.

Osaka University Collection While acknowledging that difference in HLA genotypes, age, or other pathogenic factors may play a role in the etiology of T1D, these studies from Japan are included here as these collections are rare. In addition to observation of rare insulitis and frequent lymphocytic infiltrate in the exocrine tissue in the pancreas from donors with T1D [43, 44], researchers from Osaka University Medical School recovered by pancreas tissue by biopsy from patients with newly diagnosed T1D and found detectable insulitis in approximately 50% of donors [45, 46]. The insulitis was composed of $CD4^+$ and $CD8^+$ T cells, B cells, and macrophages with noted HLA class I molecule hyperexpression in islets, intercellular adhesion molecule-1 (ICAM-1) expression on endothelial cells [47], and markers of an activated and pro-inflammatory environment were detected on infiltrating immune cells. Specifically, the costimulatory molecules CD80 and CD86 were expressed on $CD3^+$ islet-infiltrating T cells [45] and Fas ligand was expressed by islet and endothelial cells within the islets [48]. Specific T cell receptor (TCR) clonotypes were over-represented in infiltrated islets with interferon-γ (IFN-γ) mRNA present [49]. CXC chemokine ligand 10 (CXCL10) was expressed on $insulin^+$ cells within islets and CXCR3 was expressed on $CD3^+$ T cells [50]. In addition, tumor necrosis factor alpha (TNF-α) expressing, islet-infiltrating macrophages, and dendritic cells were seen [51].

In the DiViD study [4, 52•], insulitis was detected in all six recent onset donors with 5–58% of the insulin-expressing islets having insulitis of ≥ 15 T cells/islet [53•] with different patterns of B cell composition of the insulitis [54•]. The $CD8^+$ T cells were tissue resident memory T cells (T_{RM}) ($CD8^+CD69^+CD103^+$), but without expression of mRNA species associated with acute cytotoxicity or inflammation [55•]. In contrast, interferon-stimulated genes and CXCL10 were shown to be upregulated in the islet core as compared to peri-islet tissue [56•].

HLA Associations in T1D Genetic association studies have revealed that the HLA class II region has the strongest impact on risk of T1D [57•]. HLA-DQ2 (DQA*05:01, DQB*02:01) and DQ8 (DQ*A03:01, DQB*03:02) confer the greatest risk of developing T1D of any HLA alleles [58]. Individuals who are heterozygous for HLA-DQ2 and HLA-DQ8 are at greater risk of developing T1D than those with either HLA-DQ2 or HLA-DQ8 alone [59]. Antigen-presenting cells from HLA-DQ2, DQ8 heterozygous individuals express an HLA-DQ8 *trans*dimer composed of the DQ2α chain paired with the HLA-DQ8β chain (DQA*05:01; DQB*03:02) and a DQ2 *trans*dimer where the DQ8β pairs with DQ2α (DQA1*03:01; DQB1*02:01) [60]. These *trans*dimers may promote β-cell autoimmunity by presenting unique diabetogenic epitopes, or the high density of T1D-promoting HLA molecules (DQ2, DQ8, DQ2*trans* and DQ8*trans*) may promote autoimmune $CD4^+$ T cell responses against β-cell antigens [60, 61].

It is now clear that T cell responses to (pro)insulin are essential for the development of T1D in the NOD mouse [21, 62, 63]. Evidence continues to accumulate to support the role of (pro)insulin as an autoantigen in human T1D [64]. Genetic association studies have implicated proinsulin because a T1D susceptibility locus maps to a polymorphism of variable number of tandem repeats upstream of the insulin gene [65, 66]. This polymorphism is believed to modulate proinsulin expression in the thymus affecting central tolerance to this molecule [65–67]. Many studies have attempted to detect proinsulin specific $CD4^+$ T cell responses in the peripheral blood mononuclear cells (PBMC) of patients with T1D and healthy control subjects [16]. Using sensitive methods capable of detecting very rare T cells, some investigators could detect weak responses to proinsulin peptides [68]. However, these $CD4^+$ T cells could not be analyzed in detail. Furthermore, T cells isolated from the pancreatic lymph nodes (PLN) of deceased tissue donors with T1D were reported to be insulin specific and HLA-DR4 restricted [69].

Autoreactive $CD8^+$ T Cells Detected In Situ in Islets The antigen specificity of human islet-infiltrating T cells was first addressed by Coppetiers et al. [39] by using HLA-A2 tetramers loaded with known β-cell epitopes to stain pancreas sections from organ donors with T1D. This seminal work showed for the first time that $CD8^+$ T cells infiltrated human islets in T1D and that these T cells were specific for epitopes of human glutamic acid decarboxylase 65 (GAD65), islet antigen 2 (IA-2, previously known as ICA-512), insulin and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP). However, this study did not address the specificity of all islet-infiltrating $CD8^+$ T cells or the T cell receptor (TCR) genes used by these cells.

Cloning Islet-Infiltrating T Cells Identification of clinically relevant T cells and their antigens/epitopes has progressed slowly because β-cell antigen specific T cells are present at low frequencies in peripheral blood, pushing even the most sensitive assays to their limits [13, 16]. Moreover, it requires knowledge of the antigen or access to quantities of human islets. Evidence for a T1D-associated response in PBMC were invariably weak [14, 70, 71]. Isolating $CD4^+$ T cell clones, based on their responses to β-cell antigens [11], allowed for some epitopes to be defined in detail [8, 12, 15]. It has become clear that a detailed understanding of the T cell responses against β-cells within the pancreatic islets would be essential to gain insights into the immunopathogenesis of human T1D. In doing so, one must consider the sample purity and T cell

source when interpreting data derived from such studies since islets from control individuals [72•] and acinar tissue [73••] in pancreata can contain immune cells.

The first report describing the isolation and analysis of viable human islet-infiltrating $CD4^+$ T cells was published in 2015 [74••]. Islets isolated from a 19-year-old tissue donor who had T1D for 3 years were cultured with IL-2 and IL-15 for 10 days. Under these conditions, T cells emerged from some of the islets. These T cells were cloned by fluorescent-activated cell sorting (FACS) sorting. Cloned cells were screened against a panel of overlapping peptides that mimicked the entire sequence of proinsulin. In addition, 26 peptides from GAD65, IA-2, IGRP, zinc transporter 8 (ZnT8), and heat-shock protein 6 (HSP-6) that were previously reported to be $CD4^+$ T cell epitopes in earlier studies were tested, but none of them stimulated any of the T cell clones. Remarkably, all of the $CD4^+$ T cell clones for which an epitope could be identified were restricted by HLA-DQ8, or HLA-DQ8 *trans*dimers—HLA molecules strongly implicated in the pathogenesis of human T1D (Table 1, Fig. 1 and [74••]).'

Table 1 Islet donor characteristics and specific autoreactivity of islet-derived T cells

Organ donor ID	Age (years)	Sex	T1D duration (years)	Donor HLA	T cell	Autoantigen	HLA restriction	Reference
Donor A	19	M	3	A1, A2	8 $CD4^+$ T cell clones	$Proinsulin_{42–50}$	DQ8	[74••]
				B8, B51	1 $CD4^+$ T cell clone	$Proinsulin_{41–51}$	DQ8	[74••]
				DR3, DR4	1 $CD4^+$ T cell clone	$Proinsulin_{41–49}$	DQ8	[74••]
				DQ2, DQ8	1 $CD4^+$ T cell clone	$Proinsulin_{50–59}$	DQ8	[74••]
					1 $CD4^+$ T cell clone	$Proinsulin_{50–58}$	DQ8	[74••]
					2 $CD4^+$ T cell clones	$Proinsulin_{52–62}$	DQ8*trans*[a]	[74••]
					1 $CD4^+$ T cell clone	HIP C-peptide: IAPP2[b]	DQ8	[75••]
					1 $CD4^+$ T cell clones	HIP C-peptide: IAPP2[b]	DQ8	[75••]
nPOD6342	14	F	2	A2, A68	1 $CD4^+$ T-cell clone	$GAD_{274–286}$	DR4[c]	[76••]
				DR1, DR4	1 $CD4^+$ Transductant (GSE.20D11)[d]	Insulin $B_{9–23}$	DQ8	[77••]
				DQ5, DQ8				
nPOD6367	24	M	2	A2, A29	1 $CD4^+$ T cell line	HIP C-peptide:A-chain[b]	ND	[76••]
				DR4, DR7				
				DQ2, DQ8				
nPOD6268	12	F	3	A2, A68	3 $CD8^+$ T cell lines	Pooled insulin $B_{10–18}$, $IA\text{-}2_{797–805}$, $IGRP_{265–273}$ tetramers	A*02:01	[76••]
				DR17, DR13				
				DQ2, DQ6				
nPOD69	6	F	3	A2, A26	1 $CD4^+$ T cell line	$Proinsulin_{76–90}$[e]	DR4[c]	[76••]
				DR4, DR7	1 $CD4^+$ T cell line	Chromogranin A[f]	ND	[76••]
				DQ2, DQ8	2 $CD4^+$ T cell lines	Proinsulin[f]	ND	[76••]
nPOD6323	22	F	6	A1, A25	1 $CD4^+$ T cell line	Chromogranin A[f]	ND	[76••]
				DR4, DR17	1 $CD4^+$ T cell line	HIP C-peptide:IAPP1[b]	ND	[76••]
				DQ2, DQ8	1 $CD4^+$ T cell line	HIP C-peptide: IAPP2[b]	ND	[76••]
					1 $CD4^+$ T cell line	$GRP78_{292–305(Arg\text{-}Cit\ 297)}$	ND	[76••]
					1 $CD4^+$ Transductant (GSE.8E3)[d]	$Proinsulin_{49–65}$	DQ8*trans*[a]	[77••]
					1 $CD4^+$ Transductant (GSE.6H9)[d]	Insulin $B_{9–23}$	DQ8	[77••]
T1D.6	20	M	7	A2, –	1 $CD4^+$ T cell line	$GAD_{555–567}$	DR4[c]	[76••]
				DR17, DR4	1 $CD4^+$ T cell line	HIP C-peptide:NP-Y[b]	DQ8	[75••, 76••]
				DQ2, DQ8				
T1D.7	27	M	17	A1, A3	1 $CD4^+$ T cell clone	$IAPP_{65–84(Arg\text{-}Cit\ 73,\ 81)}$	ND	[76••]
				DR17, DR4	1 $CD4^+$ T cell clone	$GAD_{115–127}$	ND	[76••]
				DQ2, DQ8	1 $CD4^+$ T cell clone	$IA\text{-}2_{545–562(Gln\text{-}Glu\ 548,\ 551,\ 556)}$	ND	[76••]

The age, gender, HLA, and duration of T1D of the islet donors are shown along with the original citations. In this on-going project, these are the autoreactivities of islet-infiltrating T cell lines or clones identified, to date, and the donors from which the islet-infiltrating T cells were derived. (Adapted from: Babon JA, et al. Nat Med. 2016;22:1482–1487) [76••]

ND not determined

[a] HLA-DQ8*trans*: DQA1*05:01/DQB1*03:02

[b] *HIP* hybrid insulin peptide: fusion of a human insulin C-peptide fragment (N-terminus ELGGG) with a fragment of another peptide (*A-chain* insulin A-chain fragment, *IAPP1 and 2* two islet amyloid polypeptide fragments, *NP-Y* neuropeptide Y fragment)

[c] HLA-DR4 were all HLA-DRB1*04:01

[d] Clonal $CD4^+$ T-cell receptor transductant

[e] $Proinsulin_{76\text{-}90}$ (SLQPLALEGSLQKRG) is designated $Proinsulin_{52\text{-}66}$ by numbering starting with the B chain

[f] Epitopes not identified

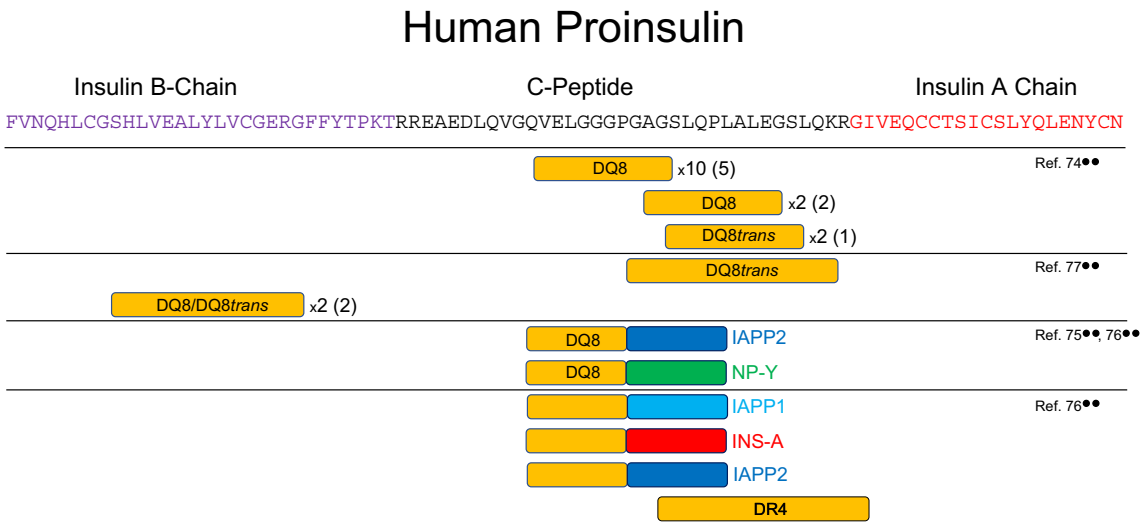

Fig. 1 Proinsulin-derived epitopes recognized by human islet-infiltrating CD4$^+$ T cells. The *boxes* indicate regions of human proinsulin for which CD4$^+$ T cell epitopes have been mapped examining human islet-infiltrating T-cells from multiple donors with T1D. *Two-colored boxes* indicate hybrid insulin peptides (HIPs) and are placed to align with the proinsulin part of the epitope, with the other half of the HIP is as labeled: islet amyloid polyprotein (IAPP), neuropeptide Y (NP-Y), insulin A-chain (INS-A). *Horizontal lines* indicate the epitopes described in each study (references shown on the right). For epitopes that an HLA restriction have been determined, the restricting HLA allele is shown within the box. In some cases, several clones have been isolated that recognize the same, or very similar epitopes indicated by the *numbers* (i.e., x2). The number of unique TCRαβ sequences expressed by these clones is shown in parenthesis

Using a similar strategy [76••], the isolated islets from nine donors with T1D (2–20 years duration of T1D, received 2–5 days following brain death) were handpicked for increased purity and divided into two aliquots that were treated in two parallel methods. The first aliquot of 100 isolated handpicked islets were dispersed with enzyme, stained for viability and immune cell surface markers, and then immediately detected and sorted by FACS. By doing so, an "ex vivo" or "ex islet" profile of islet-infiltrating T cells could be seen along with single T cell sorting for expansion. From these donors, there were 202 ± 404 CD4$^+$ T cells and 119 ± 189 CD8$^+$ T cells (per 100 islets) for a CD4$^+$:CD8$^+$ ratio of 1.7:1. From the isolated, handpicked islets of seven control donors and from two donors with type 2 diabetes, a few CD8$^+$ T cells were seen from only one of the control donors. The second aliquot of 100 handpicked islets was plated on a gel matrix with T cell receptor stimulation and cytokines for growth. After 10 days in culture, cellular outgrowths were seen only in the islets from donors with T1D, with an average of 26% of the plated islets. These outgrowths were collected, characterized for CD4$^+$ and CD8$^+$ T cells, and expanded.

The autoreactivity from 50 lines (grown from individual islets from donors) or from sorted clones from donor islets was tested with panels of known islet-protein associated peptide targets and to modified peptides using either HLA-matched Epstein Barr virus (EBV)-transformed B cells or autologous splenic EBV-transformed B cells. To date, we have identified the reactivity of 18 of the T cell lines or clones (Table 1, Fig. 1 and [76••]).

Ex vivo Sequencing of TCR From Islet - Infiltrating T Cells An alternate, but complementary approach to study islet-infiltrating T cells was carried out by single cell sorting islet-infiltrating CD4$^+$ and CD8$^+$ T cells after short-term culture, followed by TCR sequencing of individual cells [77••]. Subsequently, the TCR α/β chains were transduced in a TCR null cell line, termed TCR transductants, and tested for antigen specificity to overlapping preproinsulin peptides and other well-characterized islet antigens. Isolated islets from three recent onset T1D organ donors were studied in this manner, all of which were also evaluated by Babon and colleagues by functional T cell analysis (Table 1). It was possible to isolate hundreds to thousands of T cells from 500 islet equivalents. Analysis of α/β TCR sequences revealed diversity within CD4$^+$ T cells with about 15–20% of sequences detected more than two times from two separate donors [77••]. CD8$^+$ TCR sequences revealed more clonality with 1/3 to 1/2 of all sequences in the same donor repeated > 2 times [77••]. Interestingly, the majority of repeatedly detected TCR sequences were found from separate islet preps in the same donor, indicating that clonally expanded T cells have the ability to migrate to different islets in the pancreas. None of the TCR sequences, CD4$^+$ or CD8$^+$, were shared between patients. This could be due to the fact that only three patients with slightly different HLA genes were studied and larger numbers may reveal more clustering of TCR usage.

Others have studied TCRβ chain usage among islet-infiltrating T cells from histologic sections [78] and isolated islets [79] or from the PLN [69] from donors with T1D,

finding some skewing of certain TCRVβ families with higher frequencies than in the spleen or peripheral blood. Some skewing of TCRVα chains has been seen [49]. The largest effort to profile TCR sequences from donor tissues from individuals with T1D comes from Brusko and colleagues within the nPOD consortium [80••]. Tissue donors with T1D ($n = 18$) and non-diabetic controls ($n = 9$) had PLN, nonpancreatic lymph nodes, spleen, and peripheral blood FACS sorted into T cell subsets and TCRVβ chains sequenced. Within a single individual, there was evidence of TCR clonal expansion that could be traced from PLN to spleen to peripheral blood, especially within $CD8^+$ T cells; however, there was limited TCR clonal sharing across T1D donors. However, the TCRVβ CDR3 region of a known GAD-restricted $CD4^+$ T cell clone [81] was identified within the PLN of seven donors. From these studies, it appears that larger numbers of HLA-matched patients need to be studied with a focus on targeted searched for antigen-specific T cells.

Autoantigen Specificity of Islet-Infiltrating T Cells

Proinsulin Epitopes Proinsulin, the precursor of insulin, has been a strong candidate antigen in the pathogenesis of human T1D for many years [64, 82, 83]. Several lines of evidence suggest that proinsulin is recognized by the adaptive immune response that drives β-cell destruction. For example, autoantibodies to (pro)insulin precede the onset of T1D [84] and genetic polymorphisms in the insulin promoter modulate risk of T1D [65]. Now that several human islet-infiltrating $CD4^+$ T cell clones specific for proinsulin epitopes have been described, the evidence against proinsulin is very strong. All but one epitope recognized by human islet-infiltrating $CD4^+$ T cells derived from the C-peptide region of proinsulin, which is not present in administered insulin (Table 1 and Fig. 1**)**. Two $CD4^+$ TCR transductants responding to insulin B chain amino acids 9–23 (B:9–23) presented by either DQ8 or DQ8*trans* were identified from two separate tissue donors with T1D [77••]. This epitope is known to play a critical role in NOD mouse diabetes development [21] and has been well-characterized from the peripheral blood of patients with T1D [85–87]. Another identical antigen-specific $CD4^+$ T cell response has been reported to amino acids 19–35 within C-peptide presented by DQ8*trans* from two separate patients identified in different laboratories [74••, 77••] (Table 1 and Fig. 1). This indicates the distinct possibility of common epitopes stimulating islet-derived $CD4^+$ T cells, even after the clinical onset of T1D.

Additional Known Islet Epitopes A large bank of T cells directly sorted from or directly grown from individual islets of nine donors with T1D includes a total of 236 lines or clones: 111 $CD4^+$ T cell lines or clones, 23 $CD8^+$ T cell lines or clones, and 102 lines grown from individual islets that were mixtures of both $CD4^+$ T cells and $CD8^+$ T cells. Initial analysis found a broad repertoire of T cell autoreactivity to a number of known target epitopes and to a number of modified epitopes [76••]. To date, we have identified the reactivity of 15 $CD4^+$ T cell lines or clones and three $CD8^+$ T cells lines (Table 1). Proinsulin was the target of four of the islet-infiltrating lines: a $CD4^+$ T cell line reactive with an HLA DRB1*04:01 restricted proinsulin$_{76–90}$ epitope and two $CD4^+$ T cell lines reactive with as-yet-unidentified proinsulin epitope(s). Other known $CD4^+$ targets included three epitopes of GAD65 and a $CD4^+$ T cell line reactive with an unidentified epitope of chromogranin A. Three $CD8^+$ T cell lines reacted with pools of HLA-A2 multimers loaded with previously identified [88] peptides from insulin, IA-2 and IGRP. It should be noted that all donors (Table 1) were recovered after diagnosis of T1D and have been on an insulin regimen since diagnosis. In addition, we must consider epitope spreading as a mechanism of multiple targets of autoimmunity after diagnosis [89]. The remaining islet-infiltrating $CD4^+$ T cell and $CD8^+$ T cell lines and clones from this bank are under current investigation.

Post-Translationally Modified Epitopes Epitopes generated by post-translational modification have been implicated in the pathogenesis of many autoimmune diseases [90], including T1D [91, 92]. Epitopes formed by post-translational disulfide bond rearrangement in insulin [12], glutamine deamidation of several islet-associated proteins [93•], and the conversion of arginine to citrulline have all be reported for GAD65 [91]. From the large bank of islet-infiltrating T cells [76••], islet-derived $CD4^+$ T cell lines and clones were reactive to an epitope of glucose-regulated protein 78 (GRP78) with an arginine to citrulline modification (GRP78$_{292–305(\text{Arg-Cit } 297)}$), an epitope of islet amyloid polypeptide (IAPP) with two arginine to citrulline modifications (IAPP$_{65–84\ (\text{Arg-Cit } 73,81)}$), and an epitope of IA-2 with three glutamine to glutamic acid deamidations (IA-2$_{545\text{-}562(\text{Gln-Glu } 548,\ 551,\ 556)}$).

Hybrid Insulin Peptide (HIP) Epitopes A new type of post-translation modification, the formation of hybrid peptides by transpeptidation, was recently reported to generate neo-epitopes recognized by NOD mouse and human $CD4^+$ T cells [75••]. Human islet-infiltrating $CD4^+$ T cells, isolated from the residual pancreatic islets of deceased organ donors who suffered from T1D were found to recognize hybrid insulin peptides (HIPs). Two HIPs were shown to be the targets of a human islet-derived $CD4^+$ T cell clone and a human islet-derived $CD4^+$ T cell line: a C-peptide:IAPP2 and a C-peptide:neuropeptide-Y HIP, respectively [75••]. Synthetic peptides of these sequences were very also potent stimulators of these T cells, with responses being detected at low nanomolar concentrations. Interestingly, some of these clones

are restricted by HLA-DQ8, which is strongly associated with risk of T1D in humans [57•, 58].

The presence of human HIP specific $CD4^+$ T cells in the pancreatic islets of organ donors who suffered from T1D was confirmed recently. Babon et al. [76••] reported that T cell responses to HIPs formed by the fusion of C-peptide and peptides from IAPP1, IAPP2, or insulin A-chain could all be detected in $CD4^+$ T cell lines derived the islets of organ donors who suffered from T1D.

Function of Islet-Infiltrating T Cells In addition to the autoantigenic reactivity of islet-infiltrating T-cells, identification of their effector functions is critical for understanding and intervening with their function in potential therapies. In examining the autoreactive $CD4^+$ T cells from the large bank of islet-infiltrating T cells from nine donors with T1D, we found that, upon stimulation with specific peptide-pulsed HLA-matched or autologous EBV-transformed B cells, all autoreactive $CD4^+$ T cell lines or clones secreted interleukin (IL)-2, IFN-γ and/or TNF-α and none of lines or clones secreted any detectable IL-4, IL-5, IL-10, or IL-17a [76••]; this was done with low passage number lines and clones. This will be an important line of investigation to continue with the inclusion of a variety of methods to fully understand the function of the islet-infiltrating T cells.

Pathogenicity There is a strong "circumstantial" case to be made that human islet-infiltrating T cells cause T1D. Recurrence of autoimmunity has been seen following islet transplantation [94–96], indicating that autoimmunity must be controlled in those with long-term T1D for whom islet regeneration or replacement may be a therapeutic option. The best possible evidence linking human T cells to the development of T1D is to analyze them directly from infiltrated islets. This has the advantage that no bias, due to selection based on antigen specificity, is introduced: T cells are selected solely by their location within the affected tissue of individuals with the disease.

Future Directions This is an on-going analysis of large banks of islet-infiltrating T cells from a number of donors across three laboratories that will include analyses such as epitope discovery, functional analyses, and transcriptome analyses for both $CD4^+$ and $CD8^+$ islet-derived T cell clones, lines, and transductants. These analyses will most likely expand to other laboratories as additional techniques and expertise is required to obtain a comprehensive analysis of the islet-infiltrating T cell repertoire. For example, these studies can be paired with in situ staining of pancreata from the same donors for global phenotype and specific autoreactivity with HLA multimers of islet-infiltrating T cells and transcriptome analyses of islets recovered by laser microcapture.

We anticipate the recovery of more donors with T1D. In order to begin to define common antigens that may be targeted early in the disease process, through the efforts of nPOD, the recovery of donors with circulating T1D-associated autoantibody, but without a diagnosis of T1D will be pursued. Nonetheless, the isolation of islet-infiltrating T cells from these samples may be challenging [97–100].

A major goal of any immunotherapy is to monitor patients' responses to that therapy, which can only be done by sampling peripheral blood. However, to perform the correct comparison of the T cell repertoire infiltrating a donor's islets to the repertoire found in that donor's peripheral blood is a challenge. For these tissue donors, peripheral blood is either unavailable or in quantities insufficient for current analyses. To overcome this, we must first understand the islet-infiltrating T cell repertoire and then examine the peripheral blood of HLA-matched individuals at risk for T1D and at different stages of T1D. Ultimately, we will apply this knowledge to develop biomarkers of disease activity and improve antigen-specific therapy.

Conclusions

Through the collaborative efforts of many individuals, consortia, institutions, and families of donors, we are now able, for the first time, to directly assay the repertoire and function of islet-infiltrating immune cells. Here, across three laboratories, we have isolated both $CD4^+$ and $CD8^+$ T cells directly from the islets of donors with T1D and have seen remarkable similarity in $CD4^+$ autoreactivity to known islet-associated proteins (peptides from proinsulin, GAD65, and, chromogranin A), with post-translationally modified peptides, with arginine-citrulline modifications or deamidations, peptides from islet-associated proteins (GRP78, IAPP, IA-2), or to a number of hybrid insulin peptides. Both $CD4^+$ and $CD8^+$ T cell clonality has been observed, but with noted diversity of the TCR from islet-infiltrating T cells. To date, the islet-infiltrating $CD4^+$ T cells have exhibited a pro-inflammatory phenotype. This is an active, on-going investigation that will yield critical information on the repertoire and function of islet-infiltrating T cells and inform the design of therapies for T1D.

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Compliance with Ethical Standards

Conflicts of Interest Sally C. Kent, Jenny Aurielle B. Babon, and Stuart I. Mannering declare that they have no conflict of interest.

Aaron W. Michels reports conflicts outside the submitted work, including a patent Compounds That Modulate Autoimmunity and Methods of Using the Same licensed to ImmunoMolecular Therapeutics, a patent Methods of Preventing and Treating Autoimmunity licensed to ImmunoMolecular Therapeutics, and a patent Insulin Mimotopes and Methods of Using the Same pending. He is the scientific founder and owns shares in ImmunoMolecular Therapeutics, LLC.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of each institutional review board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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- •• Of major importance

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