AUTOIMMUNE DIABETES: MOLECULAR MECHANISMS AND NEOANTIGENS

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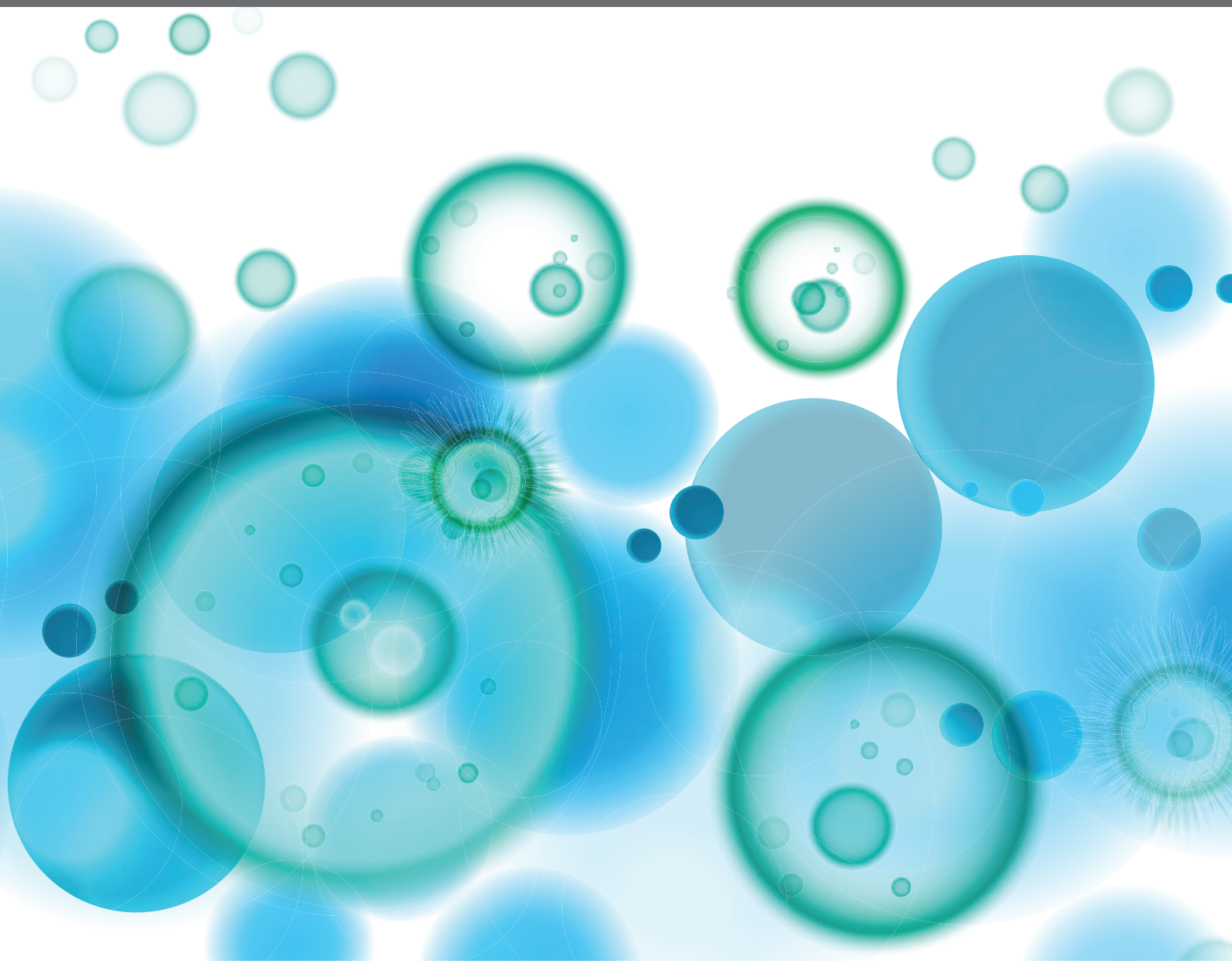

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AUTOIMMUNE DIABETES: MOLECULAR MECHANISMS AND NEOANTIGENS

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EDITORIAL
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Editorial: Autoimmune Diabetes: Molecular Mechanisms and Neoantigens

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Keywords: type 1 diabetes, neoantigens, beta cell (β cell), autoimmunity, immunotherapy, T cells, stress

Editorial on the Research Topic

Autoimmune Diabetes: Molecular Mechanisms and Neoantigens

Hundred years after the discovery of insulin by Banting, Best, and McLeod that fostered hope across type 1 diabetic population, there is still no cure for type 1 diabetes (T1D). Perhaps worse, the molecular mechanisms involved in the destruction of insulin producing cells in the pancreas remain poorly understood hampering the development of novel therapeutic strategies. While the scenario is clear and characterized by the selective and progressive destruction of the insulin-producing pancreatic beta cells by the invading immune cells, our view on the participation of the different actors has evolved over the years. From the view that T1D is a solely T cell mediated disorder, in which an impaired thymic education or the participation of low affinity T-cells were believed to be responsible for the immune attack, increasing evidence has positioned the beta cell as main player in their own destruction with physiological/metabolic stress on the beta cell as a major contributor to beta cell dysfunction The increased splicing events in beta cells when maintained under pathophysiological conditions and the T-cell autoreactivity detected against defective ribosomal products, deamidated/citrullinated autoantigens or fusion epitopes illustrate that transcriptional, translational, post-transcriptional and post-translational processes can be affected during insulitis and support the participation of neoantigens in T1D pathology and position the beta cell as the main actor in their destruction process. In addition, the roles of multiple islet-infiltrating immune cell subpopulations in the pathophysiology of beta cell dysregulation and death are now coming into focus. While the development of therapies targeting beta cell dysfunction/destruction and the autoimmune attack for T1D remains challenging, this increased understanding of these processes in individuals at-risk for or with T1D is beneficial.

In this Research Topic, Rodriguez-Calvo et al. propose an overview of the different causes and molecular mechanisms involved in the generation of these neoepitopes and speculate on how those non-native proteins could drive autoimmunity and precipitate beta cell destruction. The evaluation of their role as biomarkers for disease progression and target for immune intervention is also discussed. Among the different processes that participate to the generation of neoantigens, many studies have investigated the impact of citrullinated proteins to the development of autoimmunity. Yang et al. detail the role of calcium-dependent peptidylarginine deiminase (PAD) enzymes in the generation of citrullinated proteins and their impact on the antigenicity of beta cell antigens, echoing studies on other autoimmune diseases (e.g. rheumatoid arthritis (RA), systemic lupus

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erythematosus (SLE), multiple sclerosis (MS), psoriasis, Sjögren's syndrome (SS), anti-phospholipid syndrome (ALS) and inflammatory bowel disease (IBD) in which citrullination of self-protein has been implicated). Building on convincing data from NOD mice, they show the potential of the chemical inhibition of PAD enzyme in preventing and delaying T1D incidence, the authors elaborate on the potential of PAD inhibitor as therapeutic strategy for new onset T1D patients. Furthermore, while You et al. are questioning the role of the microbiome composition in triggering autoimmunity exploring, in NOD mice models, the participation of gut leakage and neutrophil extracellular traps (NETs) in T1D progression, they identified PAD4 as important player during NETosis, providing additional proofs for therapeutic approach targeting PAD enzymes.

Also in this Research Topic, Mannering et al. reviewed the historical discovery of T1D associated hybrid insulin peptides (HIPs) and discussed the mechanisms leading to the formation of non-contiguous peptides formed by cis/trans peptidation or reverse proteolysis during protein processing and degradation in crinosomes. Despite the large number of possible combinations and the low concentration of HIPs in beta cells that render their detection challenging by peptidomics analyses, Wiles et al. identified a novel non-contiguous C-peptide epitope ($insC_{4\text{-}26}$ – $insC_{1\text{-}3}$) in human islets and measure a CD4 peptide response in T1D patients by tetramer staining illustrating the important role of HIP peptides in T cell immunity to beta cells.

The concept of peptide fusion significantly expands the peptidome diversity that may participate to the break in tolerance observed in T1D. Revisiting the molecular mimicry hypothesis, Mishto et al. assess whether cis peptides derived viruses could trigger T-cell mediated autoimmunity. Using in silico prediction they compared zwitter cis spliced peptides with sequences from medullary thymic epithelial cells (mTEC)and beta cells and identify potential diabetogenic viral-human hybrid antigens.

In this issue, Arif et al. propose an elegant combinatorial approach (phenotypic, transcriptomic and clonotypic analysis) to evaluate the peripheral T cell responses to native versus PTM dependent auto antigenic peptides in T1D and understand the break in peripheral tolerance to beta cell antigens. Despite an evident T cell response mounted against beta cells, Rodriguez-Calvo et al. questioned the participation of non-islet reactive T cells to T1D and discuss a role as "partners in crime" in the constitution of the inflamed islets' microenvironment.

An important mechanism by which cytokines contribute to beta cell death and amplification of the inflammation is *via* induction of ER stress and subsequent unfolded protein response pathway. Like the endoplasmic reticulum, mitochondria are complex and dynamic organelles that play a key role in beta cell function by coupling glucose metabolism to insulin secretion and regulating apoptotic cell death. As described by Vig et al. the interaction between the ER and mitochondria during the adaptive mechanism to environmental stress indicate that both organelles orchestrate the communication between the beta cells and the immune system compartment. Prolonged exposure to metabolic or inflammatory stress promotes additional coping mechanisms including initiation of recycling programs and selective secretion of proteins and small RNA in microvesicles. In this Research Topic, Grieco et al. discuss the biogenesis and mechanisms of action of these extracellular vesicles and examine their role as mediator between the beta cells and the immune cells in particular as primary event in activating APCs. In addition, Leenders et al. describe another aspect of the stress response demonstrating a role of oxidative stress in the change of beta cell identity. Using primary human islets, they show that hydrogen peroxide triggers a beta cell dysfunction associated with a loss of beta cell specific markers.

Altogether the different studies presented in this issue, provide an update on the latest developments in fundamental and clinical T1D research. Yet, as conclusion, Thomas et al. identify the remaining challenges, focusing on antigen specific immunotherapy, emphasizing the hurdles encountered in the bench to bed side transition but also the novel opportunities from T1D consortia that facilitate the identification of subjects who could benefit from such immunotherapies.

AUTHOR CONTRIBUTIONS

SCK and AZ act as guest editor of this issue and wrote the editorial. Both authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Potential Mimicry of Viral and Pancreatic β Cell Antigens Through Non-Spliced and *cis*-Spliced *Zwitter* Epitope Candidates in Type 1 Diabetes

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Increasing evidence suggests that post-translational peptide splicing can play a role in the immune response under pathological conditions. This seems to be particularly relevant in Type 1 Diabetes (T1D) since post-translationally spliced epitopes derived from T1D-associated antigens have been identified among those peptides bound to Human Leucocyte Antigen (HLA) class I and II complexes. Their immunogenicity has been confirmed through $CD4^+$ and $CD8^+$ T cell-mediated responses in T1D patients. Spliced peptides theoretically have a large sequence variability. This might increase the frequency of viral-human *zwitter* peptides, *i.e.* peptides that share a complete sequence homology irrespective of whether they originate from human or viral antigens, thereby impinging upon the discrimination between self and non-self antigens by T cells. This might increase the risk of autoimmune responses triggered by viral infections. Since enteroviruses and other viral infections have historically been associated with T1D, we investigated whether *cis*-spliced peptides derived from selected viruses might be able to trigger $CD8^+$ T cell-mediated autoimmunity. We computed *in silico* viral-human non-spliced and *cis*-spliced *zwitter* epitope candidates, and prioritized peptide candidates based on: (i) their binding affinity to HLA class I complexes, (ii) human pancreatic β cell and medullary thymic epithelial cell (mTEC) antigens' mRNA expression, (iii) antigen association with T1D, and (iv) potential hotspot regions in those antigens. Neglecting potential T cell receptor (TCR) degeneracy, no viral-human *zwitter* non-spliced peptide was found to be an optimal candidate to trigger a virus-induced $CD8^+$ T cell response against human pancreatic β cells. Conversely, we identified some *zwitter* peptide candidates, which may be produced by proteasome-catalyzed peptide splicing, and might increase the likelihood of pancreatic

β cells recognition by virus-specific $CD8^+$ T cell clones, therefore promoting β cell destruction in the context of viral infections.

Keywords: T1D, antigen presentation, tolerance, mimicry, spliced peptides, virus

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease with a pivotal T cell activity. Indeed, $CD4^+$ and $CD8^+$ T cell-mediated responses in T1D patients play a prominent role in pancreatic β cell death, and the consequent insulin-dependent disease. $CD4^+$ and $CD8^+$ T cells recognize fragments (peptidic epitopes) of antigens, which are presented to T cell receptors (TCRs) αβ through Human Leucocyte Antigen class I and class II (HLA-I and -II) complexes. Since autoreactive T cells selectively recognize antigenic epitopes specific for pancreatic β cells, part of the research in T1D sails in uncharted waters to discover neoantigens, epitopes and their presentation mechanisms, which can explain why $CD4^+$ and $CD8^+$ T cells build an autoreactive immune response in T1D. In the last decade, huge progress in mass spectrometry and bioinformatics has allowed the identification of unconventional antigenic peptides, *i.e.* peptides that could not be directly identified in the human proteome. Cryptic peptides derived from putative non-coding regions, usage of alternative open reading frames, as well as post-translational modifications emerged as a sizeable portion of the peptides that are presented by HLA-I and -II complexes to T cells (1–5). Among them, post-translationally spliced epitopes derived from T1D-associated antigens represent an attractive source of neoantigens. These peptides are produced by fusion of two non-contiguous peptide fragments of either an antigen – i.e. *cis*-spliced peptides – or two distinct antigens, *i.e. trans*-spliced peptides (6) (**Figures 1A, B**). Hybrid insulin peptides (HIPs) identified by Delong and colleagues (7), belong to the latter category. Indeed, they are formed by the fusion of a splice-reactant of insulin, and another derived from other T1D-associated antigens. HIPs are presented by major histocompatibility complexes class II molecules (MHC-II) in nonobese diabetic (NOD) mice and by HLA-II (HLA-DP, HLA-DM, HLA-DOA, HLA-DOB, HLA-DQ, and HLA-DR) in humans (7–9). A handful of known HIPs can trigger a $CD4^+$ T cell response both in NOD mice and in T1D patients (7–11). The enzymes (or biochemical reactions) catalyzing their production are not fully understood, although pioneer studies suggest that HIPs or HIPs' precursors might be produced in β cell's insulin crinosomes or professional antigen presenting cell (APC)'s lysosomes (9, 12, 13). Their identification, however, is still controversial, and the employment of different mass spectrometry data analysis strategies has led to contradictory results in HIPs' identification (12, 14).

Many more antigenic *cis*-spliced peptides have been identified in the context of HLA-I antigen processing and presentation (APP) pathway since their first appearance in the literature in 2004 (6, 15, 16). They can target *in vivo* $CD8^+$ T cell responses against bacterial antigens, otherwise neglected in a mouse model of *Listeria monocytogenes* infection (17). *Cis*-spliced epitopes derived from either melanoma-associated or T1D-associated antigens are recognized by $CD8^+$ T cells in peripheral blood of melanoma patients and T1D patients, respectively (18–20).

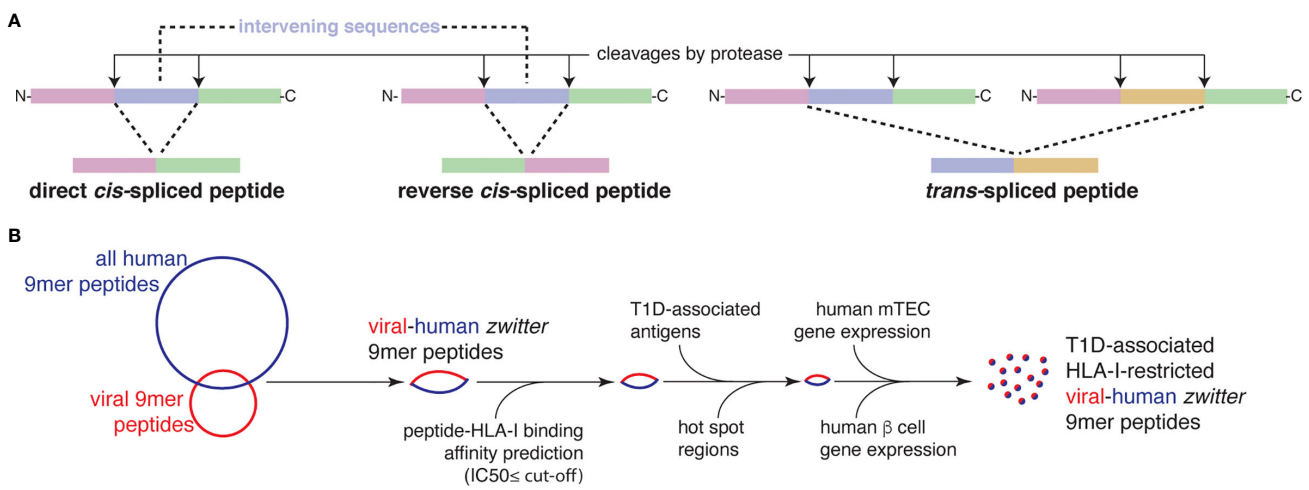

FIGURE 1 | Post-translationally spliced peptides and *in silico* pipeline for the identification of T1D-associated *zwitter* epitope candidates. **(A)** Post-translationally spliced peptides can be formed by: i *cis*-peptide splicing, when the two splice-reactants, *i.e.* the non-contiguous peptide fragments ligated by a protease, derive from the same polypeptide molecule; the ligation can occur in normal order, *i.e.* following the orientation from N- to C-terminus of the parental protein (direct *cis*-spliced peptides), or in the reverse order (reverse *cis*-spliced peptides); ii. *trans*-peptide splicing, when the two splice-reactants originate from two distinct protein molecules or two distinct proteins (6). **(B)** *In silico* pipeline to identify a pool of *zwitter* non-spliced or *cis*-spliced epitope candidates associated with T1D.

Cis-spliced as well as non-spliced peptides bound to HLA-I complexes are mainly produced by proteasomes (16, 18, 21). During *in vitro* digestions of synthetic polypeptides, proteasomes seem to produce almost as many *cis*-spliced as non-spliced peptides in terms of number of different peptide products (22). However, on average, *cis*-spliced peptides are generated in lower amounts than non-spliced peptides (23, 24), thereby suggesting that peptide splicing is catalyzed by proteasomes less efficiently than peptide hydrolysis. The identification of *cis*-spliced peptides among peptides bound to HLA-I complexes - *i.e*. HLA-I immunopeptidomes – is at least as controversial as that of HIPs; depending on the method used for *cis*-spliced peptide identification, their frequency in HLA-I immunopeptidomes was estimated to range from 1% to 34% (25).

The large variety of *cis*-spliced peptide sequences might have been problematic for models of self/non-self discrimination and tolerance of $CD8^+$ T cells (26). Indeed, the vast human and virus *cis*-spliced peptide database might significantly increase the theoretical number of viral-human *zwitter* peptides. *Zwitter* is the German word for hybrid. It is etymologically derived from *zwi*-, "duplex". For example, in chemistry, a zwitterion is an ion which possesses both positively- and negatively-charged groups. We use the term '*zwitter* peptides' to describe peptides with a sequence that may be generated from viral antigens as well as from human antigens (6, 27). A large number of viral-human *zwitter cis*-spliced peptides might impinge upon $CD8^+$ T cell repertoires and the recognition of viral antigens through central and peripheral tolerance. Nevertheless, according to a preliminary *in silico* study, this does not seem to be a frequent occurrence (27). This is in part due to the fact that only a tiny fraction of all theoretical *cis*-spliced peptides is *de facto* produced by proteasomes, as suggested by *in vitro* digestions of synthetic polypeptides with proteasomes measured by mass spectrometry (22).

Nonetheless, even a few viral-human *zwitter* (*cis*-spliced) epitopes may act as the targets of a $CD8^+$ T cell autoreactive response, triggered by a viral infection. A growing number of immunological studies suggest that thymic clonal deletion prunes but does not completely eliminate autoreactive $CD8^+$ T cells (28–30). Therefore, naïve $CD8^+$ T cell clones specific to viral-human *zwitter* epitopes may circulate in the body and be controlled by peripheral tolerance. Central and peripheral tolerance, however, does not completely eliminate the risk that some of these naïve $CD8^+$ T cell clones may recognize the cognate epitopes during a viral infection, be primed, proliferate, and then carry out an autoreactive response, for instance during a second or multiple viral infections. This might be the case of T1D, wherein $CD8^+$ T cells could recognize viral-human *zwitter* epitopes which are also presented by pancreatic β cells. T1D has been historically associated with viral infections and especially with enteroviruses (EVs), such as Coxsackievirus B1 and B4 (CVB1 and CVB4; **Table S1**) (31). However, to date, the detection of circulating or infiltrating EV-reactive T cells has been challenging. $CD8^+$ T cells predominate in the islets, many of which express high levels of HLA-I molecules at disease onset, likely contributing to exacerbated antigen presentation. EVs are known to infect pancreatic β cells and induce an inflammatory response in the islets (32). Sequence similarity has been previously described through the study of canonical non-spliced epitope candidates, between the 2C non-structural protein (P2C) of CVB4 and the glutamate decarboxylase 2 (GAD2; a.k.a. GAD65) antigen, which is predominantly expressed in pancreatic β cells, although no evidence of cross-recognition by $CD8^+$ T cell clones was demonstrated in that report (33). Other members of the Picornaviridae family, such as Parechoviruses (HPeV2), have also been associated to T1D (34). Similarly to EVs, a possible link was described between Rotaviruses – such as Rotavirus C (RVC) - and T1D, because of a potential molecular mimicry between the VP7 protein of a human rotavirus strain and I-A2 (35) and GAD65 (36), although in the latter case the response was limited to $CD4^+$ T cells.

Viral and β cell epitopes might be generated and presented during viral infections, where β cell destruction could be triggered. Even if the infection is cleared and regulatory mechanisms are in place, additional infections could trigger further waves of β cell destruction. This might explain why T1D has been defined as a relapsing-remitting disease, where β cells may be killed only when a certain trigger (i.e. a viral infection) is present (37). Moreover, persistent infections are likely to be problematic, as they could perpetuate inflammation, immune activation and β cell destruction. Many herpesviruses produce lifelong infections and remain in their host in a latent state. Reactivations occur upon immune dysfunction, but might also be concomitant with newer infections. Cytomegalovirus (HCMV) is one of the most prevalent viruses of this family, infecting over half of adults in the United States by the age of 40 (https://www.cdc.gov/cmv/overview.html). Several case reports have associated HCMV infection with T1D. Pak and colleagues (38) showed a strong correlation between HCMV genome and islet autoantibodies, while HCMV-positive cells were found in the islets of subjects with fulminant T1D (39). HCMV-specific $CD8^+$ T cells have been found in the pancreas of T1D subjects at the onset of the disease (40). Conversely, several reports have found neither an association with the disease (41) nor even a delay in progression of T1D (42). It is therefore possible that more virus-specific cells are present in the islets during disease progression than previously expected. Other highly prevalent herpesviruses like Epstein-Barr virus (EBV), human herpesvirus 6A (HHV-6A) and 6B (HHV-6B) have also been associated with T1D. High circulating antibody titers against EBV have been detected in patients with T1D when compared to non-diabetic controls (43). Interestingly, HHV-6B glycoprotein B (gB) was more expressed in the islets and exocrine pancreas of donors with T1D, as compared to non-diabetic subjects (44). However, due to the persistent and latent nature of these viruses, a direct involvement in the pathogenesis of T1D is likely to be hard to prove.

If viral-human *zwitter* epitopes associated with multiple viruses existed, immune responses could be constantly triggered, even if they were of low magnitude, with potential implication for the etiopathogenesis of T1D. Therefore, we investigated *in silico* the theoretical existence of T1D-associated

viral-human *zwitter* peptides, and their potential in triggering $CD8^+$ T cell-mediated autoimmunity in T1D.

MATERIALS AND METHODS

Peptide-HLA-I Binding Affinity Prediction and Immune Epitope Database (IEDB)

The study focused on non-spliced and *cis*-spliced 9 amino acid long (9mer) peptides and HLA-A*01:01, -A*02:01, -A*03:01, -A*11:01, -A23:01, -A*24:02, -B*07:02, -B*08:01, -B*15:01, -B*35:01, -B*39:06, -B*40:01, -B*44:02, -B44:03 complexes. This pool of HLA-I alleles covers over 90% of the Caucasian population. For each HLA-I allele, we computed a cut-off comparable to the threshold of a predicted $IC_{50} \leq 500$ nM of peptide-HLA-A*02:01 complex as follows: we downloaded all 9mer peptides detected through peptide elution from HLA-I complexes, and reported in the IEDB database (45). We restricted the analysis to the HLA-I alleles specified above. For each peptide-HLA-I complex, we predicted the inhibitory constant (IC_{50}) of these 9mer peptide sequences by using NetMHCpan-BA4.0 algorithm (46). IC_{50} estimates the binding affinity of HLA-I-peptide complexes. The lower the IC_{50}, the higher the binding affinity between peptide and HLA-I complex. To have a similar IC_{50} cut-off among HLA-I alleles, we determined the quantile of the HLA-A*02:01 for IC_{50} = 500 nM, which resulted in 91.4%-ile of peptides present in the HLA-A*02:01-specific HLA-I immunopeptidome database of the IEDB. We then applied this quantile to the predicted IC_{50} distributions of all other peptide-HLA-I complexes (**Figure S1**), thereby identifying the IC_{50} cut-offs of each HLA-I allele, which corresponded to the peptide-HLA-A*02:01 IC_{50} = 500 nM. Values are displayed in **Table S2**.

For the identification of peptides already determined in HLA-I immunopeptidomics or analyzed (with positive outcome) for T cell recognition, we consulted the IEDB. We downloaded and selected all HLA-I-restricted peptides for which a positive T cell assay was reported (45). The latter included experiments, for example, performed through tetramer staining, IFN-γ assays with co-culture of APCs pulsed with synthetic peptide candidates and either peripheral blood mononuclear cells (PBMCs) or $CD8^+$ T cell clones as well as Cr^{51} cytotoxicity. For the computation of antigenic hotspot regions see below.

Estimation of Viral-Human *Zwitter* Peptides

Viral proteomes were obtained *via* ViralZone, and the Human proteome referred to Swiss-Prot Version 2016 excluding protein isoforms (47, 48). Only viruses with human tropism and association to T1D were included in any downstream analysis here presented (n = 8; **Table S1**). The Human proteome database contained 20,191 protein entries with a total of 11,323,862 amino acid residues.

We focused our study on 9mer peptides since they represent the majority of non-spliced and *cis*-spliced peptides in HLA-I immunopeptidomes (21, 49, 50).

We defined as viral-human *zwitter* 9mer peptide any 9mer peptide that had a sequence that could be obtained by either peptide hydrolysis or *cis*-peptide splicing, both from self-proteins and from viral proteins.

We first computed all possible non-spliced 9mer peptides from viral and human proteomes, and all normal and reverse *cis*-spliced 9mer peptides - with an intervening sequence length ≤ 25 amino acids - that could be derived from the viral and human proteomes. *Cis*-spliced peptides were computed *in silico* as previously described (49). We used an intervening sequence length restriction of 25 amino acid residues to be consistent with our previous study on HLA-I immunopeptidomes and *in cellulo* study on a tumor-associated spliced epitope (49, 51).

After, we compared all viral peptides with human peptides by aligning their sequences.

Two 9mer peptides were considered as identical, *i.e.* as viral-human *zwitter* peptides, if all of their 9 amino acid residues were exactly matching. The relative frequency of viral-human *zwitter* peptides (*Fv*) was calculated as:

$$F_v = 100\frac{z_v}{p_v},$$

where z_v is the number of all unique viral-human *zwitter* peptides of a given virus *v*; and p_v is the number of all possible unique 9mer peptides derived from virus *v*. The number of viral-human *zwitter* peptides, z, was computed for the comparison of non-spliced peptides only, of *cis*-spliced peptides only, of non-spliced viral peptides compared to *cis*-spliced human peptides, and of *cis*-spliced viral peptides compared to non-spliced human peptides. Additionally, the relative frequency of all (non-spliced and *cis*-spliced) viral-human *zwitter* peptides was computed.

Estimation of Viral-Human *Zwitter* Epitope Candidates Considering Antigenic Hotspots and the Potential Antigen Repertoire of Human mTECs and Pancreatic β Cells

To determine the potential hotspot regions among antigens that might be the origin of *zwitter* epitope candidates, we collected all peptide sequences present in IEDB's human HLA-I immunopeptidome database and mapped them to the reference proteome database. For each amino acid in the reference proteome database, we counted how many unique peptides of IEDB's human HLA-I immunopeptidome database contained that residue. For any given *zwitter* 9mer peptide we computed the average count over the 9 residues on its sequence, which was our hotspot score. Finally, we applied a cut-off score of 1 to define hotspot regions. Therefore, a hotspot score of 1 was computed if each residue of a given 9mer peptide was identified at least once in IEDB's human HLA-I immunopeptidome database.

To determine the potential antigen repertoire of human medullary thymic epithelial cells (mTECs) and pancreatic β cells, we extracted gene expression values from the RNA sequencing dataset of human mTECs and pancreatic β cells, published by Gonzalez-Duque and colleagues (19), for each antigen in our study. We filtered all antigens based on their

expression values, such that the expression was smaller than 0.1 RPKM in mTECs and larger than 5 RPKM in pancreatic islets.

Predicted Protein Structures

For visualization purpose, the structure of HCMV DNA primase (*UL70*) and human IA-2 (a.k.a. PTPRN) antigens was determined using iTasser (52) with default settings without inclusion or exclusion of structural templates.

Database Source and Data Availability

The human mTEC's and pancreatic β cell RNA sequencing data were provided by Gonzalez-Duque et al. (19), as well as the T1D-associated antigen list (**Table S3**).

The list of *zwitter* non-spliced and *cis*-spliced 9mer peptides and related information (**Tables S4, S5**) are accessible in the repository Mendeley dataset: http://dx.doi.org/10.17632/z9g9knjxgw.1

RESULTS

Estimation of Viral-Human *Zwitter* Epitope Candidates Potentially Associated to T1D

For a systematic estimation of the potential number of viral-human *zwitter* epitope candidates that could (i) be presented by HLA-I complexes, (ii) be involved in an autoimmune $CD8^+$ T cell response in T1D patients, and (iii) be at least in part triggered by viral infection, we started from the foundations: we computed the number of 9mer peptides that might originate from human proteome, as well as those that might originate from T1D-associated viruses (**Table S3**). We focused on 9mer peptides because this is the predominant length in HLA-I immunopeptidomes. We neglected TCR degeneracy (see Discussion), and therefore we focused only on peptides that might be derived from either human proteome and virus proteome with the exact same sequence of amino acids, here named viral-human *zwitter* peptides. With these restrictions, 332 non-spliced peptides were obtained that might be viral-human *zwitter* non-spliced 9mer peptides (**Figure 2A** and **Table S4**). Only HHV-6A and -6B, EBV and HCMV potentially carried these peptides. Among them, 45 were predicted to efficiently bind at least one of the selected HLA-I variants, which represents a large section of the Caucasian population (**Figure 2B**). Twelve viral-human *zwitter* non-spliced 9mer epitope candidates have already been eluted from HLA-I complexes and identified by mass spectrometry, and for one peptide a positive T cell assay has been described, according to the IEDB (**Figures 2C, D**). Six viral-human *zwitter* non-spliced 9mer peptides could be derived from a list of T1D-associated antigens proposed by Gonzalez-Dunque et al. (19), (**Figure 2E**). None of these latter peptides were predicted to efficiently bind the selected HLA-I alleles, although two of them were identified in HLA-I immunopeptidomes, according to the IEDB (**Figures 2F, G**). No viral-human *zwitter* non-spliced 9mer epitope candidates derived from T1D-associated antigens has been tested through T cell assays with a positive outcome (**Figure 2H**).

As expected, the scenario changed when we included *cis*-spliced peptides (**Table S5**). Indeed, the number of *zwitter* peptides that may be produced by *cis*-peptide splicing of either both viral and human antigens or only one or the other – herein defined as *zwitter cis*-spliced peptides - increased in each of the categories analyzed. Almost two million viral-human *zwitter cis*-spliced 9mer peptides were computed, which could be derived from the investigated viruses (**Figure 2A**), and more than

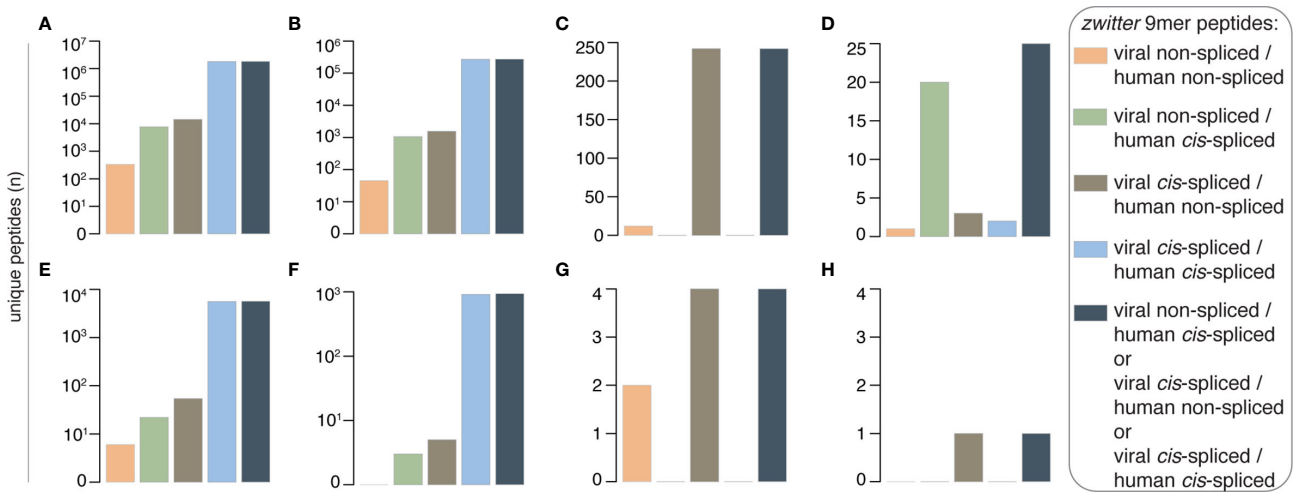

FIGURE 2 | Theoretical viral-human *zwitter* 9mer peptide frequency and potential association with T1D. **(A–H)** Number of theoretical viral-human 9mer (non-spliced or *cis*-spliced) **(A)** *zwitter* peptides, **(B)** *zwitter* epitope candidates predicted to efficiently bind selected HLA-I complexes, **(C)** *zwitter* epitope candidates described in published HLA-I immunopeptidomes, **(D)** *zwitter* epitope candidates that showed a positive T cell response in published studies, **(E)** *zwitter* epitope candidates derived from T1D-associated antigens, **(F)** *zwitter* epitope candidates predicted to efficiently bind selected HLA-I complexes and derived from T1D-associated antigens, **(G)** *zwitter* epitope candidates described in published HLA-I immunopeptidomes and derived from T1D-associated antigens, **(H)** *zwitter* epitope candidates that may be derived from T1D-associated antigens and showed a positive T cell response in published studies. For the identification of epitope candidates already identified in HLA-I immunopeptidomics or analyzed (with positive outcome) for T cell recognition, we consulted the IEDB database.

270,000 of them were predicted to efficiently bind the selected HLA-I variants (**Figure 2B**). 242 viral-human *zwitter cis*-spliced 9mer epitope candidates have already been eluted from HLA-I complexes, and identified by mass spectrometry, according to the IEDB (**Figure 2C**). However, they all belonged to the *zwitter* viral *cis*-spliced/human non-spliced peptide category; hence, they were all identified as human non-spliced peptides in human HLA-I immunopeptidomes (**Table S5**). For 25 viral-human *zwitter cis*-spliced 9mer epitope candidates, we identified studies showing a positive T cell assay (**Figure 2D**). Among them, 20 were viral non-spliced human *cis*-spliced epitope candidates, and the response has been detected against the viral non-spliced epitopes. The remaining five were viral *cis*-spliced epitopes and either human non-spliced or human *cis*-spliced epitope candidates in our database (**Table S5**). For them, the positive T cell response reported by other groups was either against the human non-spliced peptide, or a viral non-spliced peptide derived from a different viral strain than what was included in our database (**Table S1**).

Over 5,000 viral-human *zwitter cis*-spliced 9mer peptides could be derived from the Gonzalez-Dunque's et al. T1D-associated antigen list (**Figure 2E**). Almost a thousand of these latter peptides were also predicted to efficiently bind the selected HLA-I alleles (**Figure 2F**), and four of them were identified as non-spliced peptides in human HLA-I immunopeptidomes, according to the IEDB (**Figure 2G**). One viral-human *zwitter cis*-spliced 9mer peptide derived from T1D-associated antigens has been tested through T cell assays with a barely positive outcome (**Figure 2H**). It was the antigenic peptide [LLPPLLEHL], which may be generated through peptide hydrolysis from the human insulinoma-associated antigen 2 (IA-2; a.k.a. PTPRN) as well as, according to our computation, from the DNA primase (*UL70*) antigen of HCMV through *cis*-peptide splicing (**Figure 3**). This antigenic peptide is presented by HLA-A*02:01 complex (predicted IC_{50} = 45 nM; measured IC_{50} = 444 nM) (53, 54). In a standard IFN-γ ELISpot assay with PBMCs, only 1 out of 11 T1D HLA-A*02:01 patients showed a $CD8^+$ T cell response above the cut-off, whereas no response was detected in healthy donors against this epitope (53).

Prioritization of Viral-Human *Zwitter* Peptide Candidates Potentially Associated to T1D

Not all non-spliced and *cis*-spliced epitope candidates that might be generated are in fact produced and presented by HLA-I complexes. APP pathway has many steps that filter peptides based on their sequence and amount (5). The number of peptide molecules in HLA-I immunopeptidomes seems to depend on the amount of the antigen, its degradation rate, and the location of the peptide within antigens. The latter is well represented by "hotspot" regions in antigens, which are overrepresented by antigenic non-spliced and *cis*-spliced peptides in HLA-I immunopeptidomes (49, 55). Furthermore, RPKMs of RNA sequencing measurements, which may be an indirect indicator of protein translation, showed a certain degree of correlation with HLA-I immunopeptidomes (55). According to the current models of thymic negative selection, we could speculate that autoreactive $CD8^+$ T cell clones may be more likely to recognize self-antigenic peptides that are not (or barely) presented by mTECs or thymic dendritic cells (DCs), which would reduce the risk of being eliminated during thymic negative selection. If the same antigenic peptides were highly expressed by β cells, the risk of autoreactive $CD8^+$ T cell response against these cells would be higher, although several peripheral tolerance pathways are in place to repress undesired autoimmune reactions.

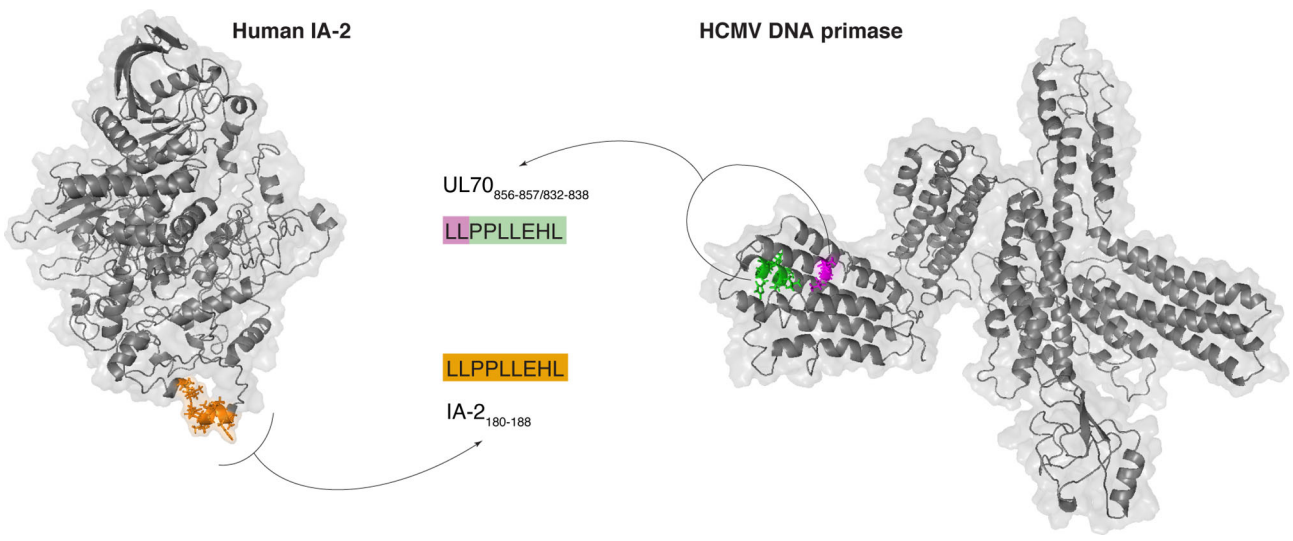

FIGURE 3 | Example of potentially immunogenic *zwitter* viral *cis*-spliced/human non-spliced peptide. Predicted crystal structure of the human IA-2 (a.k.a. PTPRN) and HCMV DNA primase (*UL70*) and theoretical localization of the viral-human *zwitter* peptide candidate LLPPLLEHL. This peptide may be generated through peptide hydrolysis from human IA-2 and through peptide splicing from HCMV DNA primase. The *zwitter* non-spliced peptide candidate $IA\text{-}2_{180\text{-}188}$ [LLPPLLEHL] is depicted in orange. For the *zwitter cis*-spliced peptide candidate $UL70_{856\text{-}857/832\text{-}838}$ [LL][PPLLEHL], the two splice-reactants are depicted, too.

With this in mind, we prioritized viral-human *zwitter* 9mer peptide candidates (predicted to bind the selected HLA-I variants) based on: (i) RNA sequencing data of human mTECs and primary pancreatic islets (for antigen selection); (ii) localization of epitope candidates within hotspot regions of antigens; (iii) antigen association with T1D. The former and the latter databases were derived from Gonzalez-Duque and colleagues (19). The localization within antigenic hotspot regions was computed based on published HLA-I immunopeptidome databases (see Material and Methods section). The distribution of RPKM of genes coding for antigens from which viral-human *zwitter* 9mer epitope candidates may be derived - as measured in mTECs and primary pancreatic islets - is reported in **Figure 4** and **Figures S2–S14**.

When we considered a cut-off for gene expression with a RPKM larger than 5 in islets and smaller than 0.1 in mTECs, which mimicked what was proposed by Gonzalez-Duque and colleagues (19), we obtained no viral-human *zwitter* non-spliced 9mer epitope candidates predicted to efficiently bind the selected HLA-I variants (**Figure 5A**). When we considered only epitope candidates predicted to bind the HLA-I variants and located in hotspot regions, sixteen viral-human *zwitter* non-spliced 9mer peptide candidates could be identified (**Figure 5B**). Again, the outcome is very different if *cis*-spliced peptides are included. Over 900 epitope candidates that were predicted to efficiently bind the selected HLA-I alleles, and theoretically derived from antigens preferentially expressed in pancreatic islets over mTECs – i.e. with RNA sequencing RPKM larger than 5 in islets and smaller than 0.1 in mTECs – may be viral-human *zwitter cis*-spliced 9mer epitope candidates (**Figure 5A**). Over 60,000 viral-human *zwitter* 9mer epitope candidates predicted to efficiently bind the selected HLA-I alleles and located in hotspot regions may have at least one of the two paired peptides produced by peptide splicing (**Figure 5B**). Among them, over a hundred were predicted to efficiently bind the selected HLA-I alleles, derived from antigens preferentially expressed in pancreatic islets over mTECs and located in hotspot regions. None of the viral-human *zwitter* non-spliced 9mer epitope candidates had these characteristics (**Figure 5C**). When we focused our *in silico* investigation on T1D-associated antigens (**Table S3**), although no non-spliced peptides were predicted to efficiently bind the

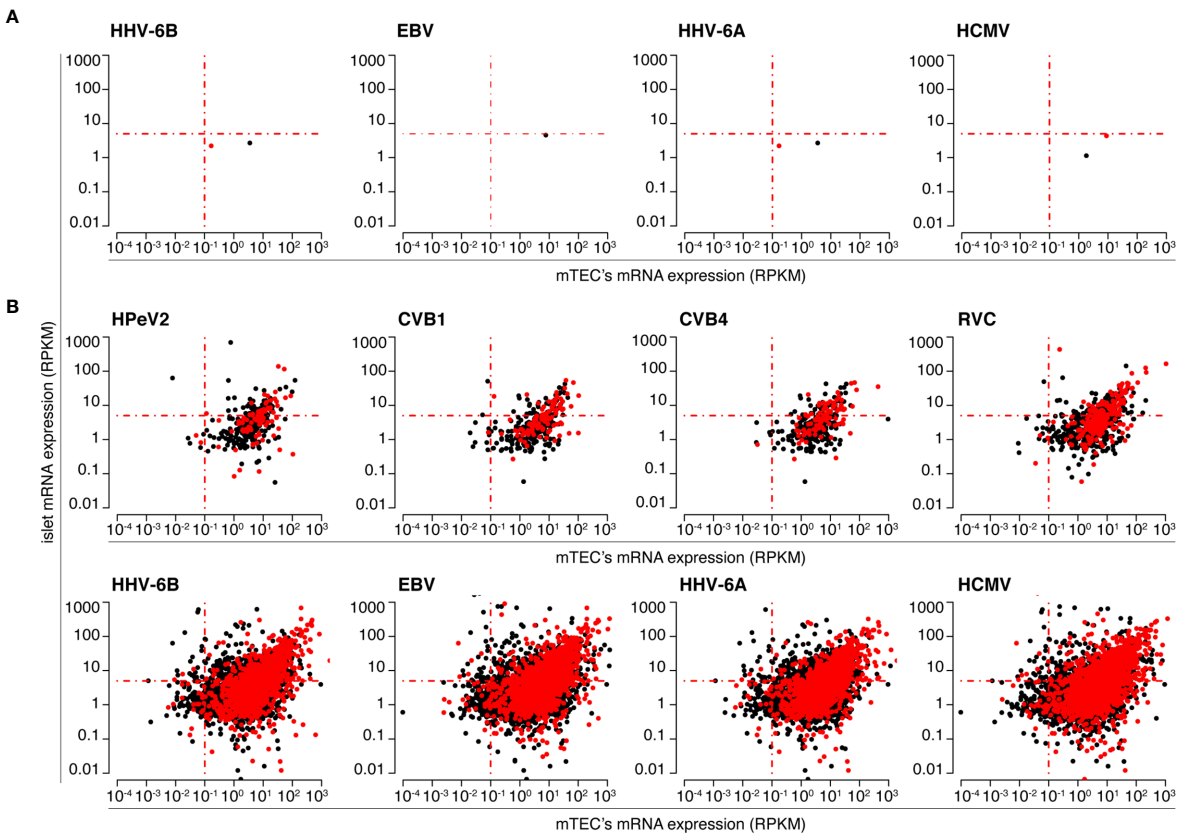

FIGURE 4 | Human pancreatic islets and mTECs' mRNA expression of antigens potentially carrying HLA-A*02:01-restricted viral-human non-spliced and *cis*-spliced *zwitter* peptide candidates. The scatter plots depict the distribution of RPKM of mRNA of human antigens, as measured by Gonzalez-Duque and colleagues (19) in human pancreatic islets and mTECs, that theoretically can carry viral-human *zwitter* **(A)** non-spliced and **(B)** *cis*-spliced epitope candidates. Scatter plots are divided based on the corresponding theoretical virus origin. In **(A)** only four out of eight viruses are shown because for four viruses no viral-human non-spliced peptide candidates with the required characteristics were identified. Black dots represent antigens carrying epitope candidates predicted to bind the HLA-A*02:01 allele. Red dots represent antigens carrying epitope candidates predicted to bind the HLA-A*02:01 allele and located in hotspots, according to the IEDB database.

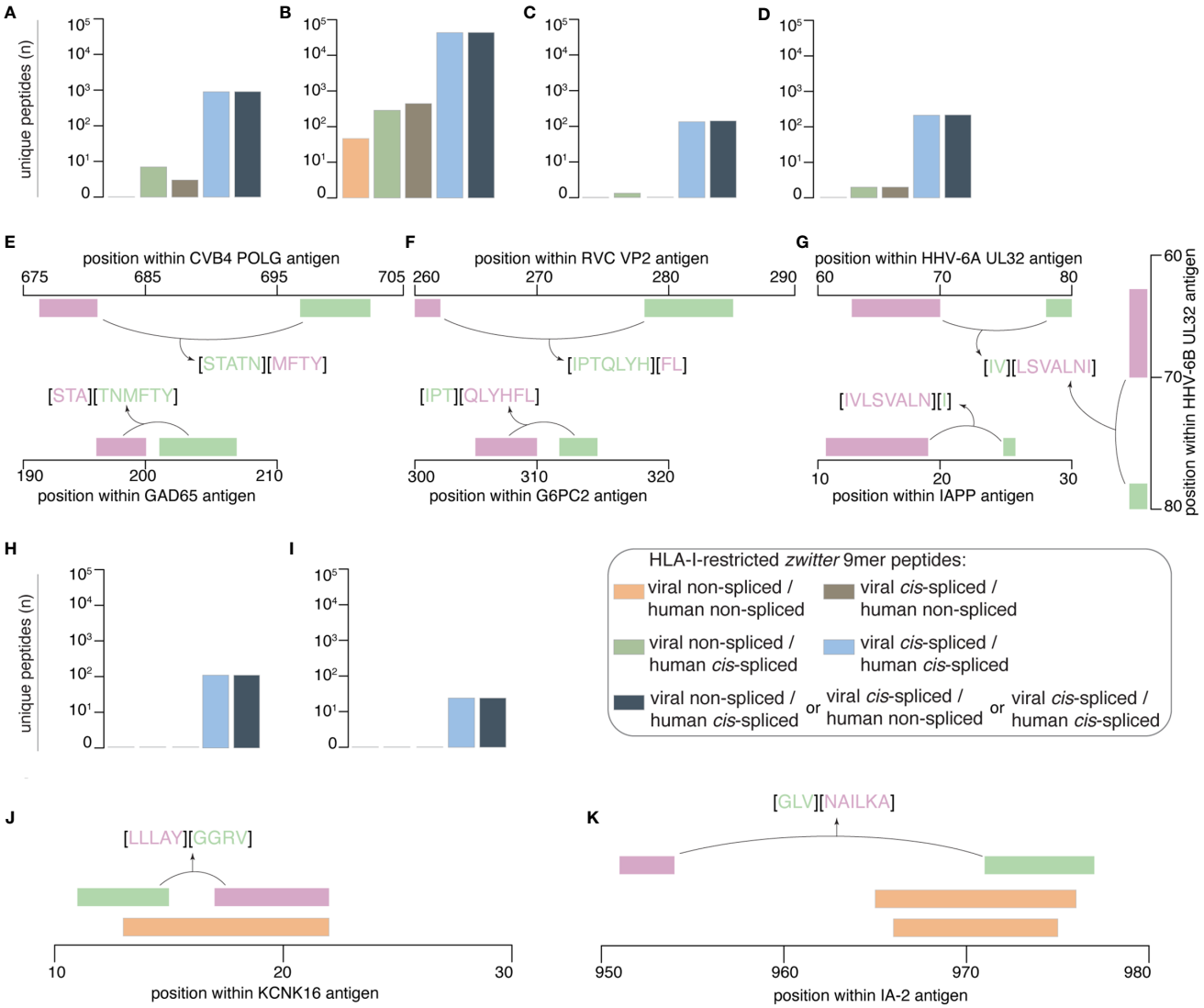

FIGURE 5 | Prioritization of viral-human *zwitter* 9mer peptide candidates and examples. **(A–D)** Number of theoretical viral-human *zwitter* non-spliced or *cis*-spliced 9mer epitope candidates predicted to efficiently bind selected HLA-I variants and either **(A)** derived from antigens preferentially expressed in pancreatic islets over mTECs, or **(B)** located in hotspot regions, or **(C)** located in hotspot regions of antigens preferentially expressed in pancreatic islets over mTECs, or **(D)** derived from T1D-associated antigens preferentially expressed in pancreatic islets over mTECs. **(E–G)** Examples of *zwitter* viral-human *cis*-spliced epitope candidates derived from T1D-associated antigens preferentially expressed in pancreatic islets over mTECs: **(E)** $GAD65_{198\text{-}200/202\text{-}207}$ [STA][TNMFTY] and CVB4-derived $POLG_{698\text{-}702/677\text{-}680}$ [STATN][MFTY], **(F)** $G6PC2_{312\text{-}314/305\text{-}310}$ [IPT][QLYHFL] and RVC-derived $VP2_{278\text{-}284/260\text{-}261}$ [IPTQLYH][FL], as well as **(G)** $IAPP_{11\text{-}18/25}$ [IVLSVALN][I], HHV-6A-derived $UL32_{79\text{-}80/64\text{-}70}$ [IV][LSVALNI] and HHV-6B-derived $UL32_{79\text{-}80/64\text{-}70}$ [IV][LSVALNI] *cis*-spliced peptides. **(H, I)** Number of theoretical viral-human *zwitter* non-spliced or *cis*-spliced 9mer epitope candidates derived from T1D-associated antigens, predicted to efficiently bind the selected HLA-I variants and either **(H)** located in hotspot regions, or **(I)** located in hotspot regions of antigens preferentially expressed in pancreatic islets over mTECs. **(J, K)** Examples of *zwitter* viral-human *cis*-spliced peptide candidates potentially associated to T1D and located in hotspots: **(J)** $KCNK16_{17\text{-}21/11\text{-}14}$ [LLLAY][GGRV] and **(K)** $IA\text{-}2_{951\text{-}953/971\text{-}976}$ [GLV][NAILKA] *cis*-spliced epitope candidates are located in an area where non-spliced antigenic peptides (orange bars) have been identified by mass spectrometry in HLA-I immunopeptidomes by others. In **(E–G, J, K)** bars' color code corresponds to that used in **Figure 1A**.

selected HLA-I alleles, and may be derived from antigens within the genes' RPKM cut-offs, over 200 viral-human *zwitter* 9mer epitope candidates with such characteristics may have at least one of the two paired peptides produced by peptide splicing (**Figure 5D**).

From the latter, we mention the example of the *zwitter cis*-spliced epitope candidate, which may be derived from the human GAD65 antigen as [STA][TNMFTY] and from the CVB4 Genome Polyprotein POLG as [STATN][MFTY] (**Figure 5E**). This *zwitter cis*-spliced 9mer epitope candidate was predicted to bind HLA-A*01:01, -A*11:01 and -B*35:01 with IC_{50} < 100 nM (**Table S5**). In this pool of epitope candidates, we also have a peptide that may be derived from RVC, and specifically from the Inner Capsid Protein VP2 as $VP2_{278\text{-}284/260\text{-}261}$ [IPTQLYH][FL].

The human counterpart would be the peptide [IPT][QLYHFL] derived from Glucose-6-phosphatase 2 ($G6PC2_{312\text{-}314/305\text{-}310}$), which was predicted to bind HLA-B*07:02 and -B*35:01 with IC_{50} < 200 nM (**Table S5**, **Figure 5F**). This pool of *zwitter cis*-spliced 9mer epitope candidates also contained many peptides that may be derived from HHV-6A and -6B. In some cases, the viral *zwitter cis*-spliced peptide may be derived from both HHV-6A and -6B, as the peptide [IV][LSVALNI], *i.e.* from HHV-6A Packaging protein UL32 and HHV-6B UL32 homolog (**Figure 5G**). The human counterpart would be the *cis*-spliced peptide [IVLSVALN][I] derived from Islet amyloid polypeptide (IAPP), which is predicted to efficiently bind the HLA-A*02:01 complex (**Table S5**).

When we considered peptides derived from hotspot regions of T1D-associated antigens and predicted to efficiently bind the selected HLA-I alleles, no non-spliced and over 100 *cis*-spliced epitope candidates were identified (**Figure 5H**). Among them, twenty-five may be derived from antigens whose genes were highly expressed in pancreatic islets and marginally expressed in mTECs (**Figure 5I**). The viral epitope candidates may be derived from either EBV or HCMV antigens (**Table S5**). The two T1D-associated antigens are IA-2 and Potassium channel subfamily K member 16 (KCNK16). For the latter, we mention the example of the human $KCNK16_{17\text{-}21/11\text{-}14}$ *cis*-spliced peptide candidate [LLLAY][GGRV], which may also be annotated as EBV Glycoprotein 42 $BZLF2_{21\text{-}25/34\text{-}37}$. If these two *cis*-spliced epitope candidates were actually generated by proteasomes, it would be through the ligation of the same splice-reactants [LLLAY] and [GGRV]. The $KCNK16_{17\text{-}21/11\text{-}14}$ *cis*-spliced peptide was predicted to efficiently bind HLA-A*02:01 complex (predicted IC_{50} = 93 nM; **Table S5**). It partially overlapped with the HLA-A*03:01-restricted non-spliced peptide $KCNK16_{13\text{-}21}$ [RVLPLLLAY] (**Figure 5J**), which was identified in HLA-I immunopeptidomes of human ECN90 pancreatic β cell line upon IFN-γ stimulation (19).

The other half of the viral-human *zwitter cis*-spliced epitope candidates that are included in this final list may be derived from IA-2 protein, which was largely expressed in human pancreatic islets whereas it was barely expressed in human mTECs (**Figure 4**). Among them, we briefly describe the *zwitter* $IA\text{-}2_{951\text{-}953/971\text{-}976}$ *cis*-spliced peptide candidate [GLV][NAILKA], which may also be annotated as EBV Major DNA-binding protein $DBP_{853\text{-}855/835\text{-}840}$. Also, in this case, the peptide splicing reaction would be between the same two splice-reactants [GLV] and [NAILKA]. The *zwitter cis*-spliced peptide $IA\text{-}2_{951\text{-}953/971\text{-}976}$ was predicted to efficiently bind HLA-A*02:01 complex (**Table S5**). The C-terminal splice-reactant of this *cis*-spliced peptide may be derived from an IA-2 area where the non-spliced epitope candidates $IA\text{-}2_{966\text{-}974}$ [VAEEVNAIL] and $IA\text{-}2_{965\text{-}975}$ [AVAEEVNAILK] were also found (**Figure 5K**). The latter non-spliced epitope candidates have been predicted to bind other HLA-I alleles, and were identified in HLA-I immunopeptidomes by mass spectrometry (19, 56).

IA-2 and KCNK16 antigens are not overrepresented in the IEDB's HLA-I immunopeptidome database (data not shown), therefore their predominance in this latter group of optimal viral-human *zwitter cis*-spliced 9mer epitope candidates may be due to a partial sequence homology between their sequence and the viral antigen sequences. This is true for IA-2 antigen, which has one of the largest pools of theoretical viral-human *zwitter cis*-spliced 9mer peptide candidates among the T1D-associated antigens (**Table S5**).

DISCUSSION

Our study is the first attempt to evaluate the potential role of antigenic *cis*-spliced peptides in a $CD8^+$ T cell-mediated autoimmune response triggered by viral infections. Due to the theoretically extremely large pool of *cis*-spliced peptide sequences, and the limited knowledge of proteasome-catalyzed peptide hydrolysis and peptide splicing dynamics (6, 22, 23, 57–59), any *in silico* analysis of *zwitter cis*-spliced epitope candidates faces further hurdles and a higher degree of complexity when compared to analysis of canonical peptides. Therefore, in this study we neglected multiple layers of complexity of the $CD8^+$ T cell response and HLA-I-restricted APP pathway, and focused only on *zwitter* 9mer peptides that share a complete homology between viral- and human-derived peptide candidates. This analysis provided a first estimation of the scale of the pool of viral-human *zwitter cis*-spliced epitope candidates potentially associated with T1D.

Where we disregarded APP pathway and antigen expression, this theoretical pool varied from a few hundred non-spliced peptides to millions of *cis*-spliced peptides. When we considered that, in the context of the $CD8^+$ T cell cytotoxicity against pancreatic β cells, immunogenic epitopes are supposed to efficiently bind HLA-I clefts and to be derived from antigens (preferentially from antigen hotspots) that are expressed in pancreatic β cells and, ideally, barely expressed in mTECs, this initial figure seems to decrease. No viral-human *zwitter* 9mer non-spliced epitope candidates, and a hundred *cis*-spliced epitope candidates were left. On the one hand, this figure can further shrink if we considered that not all possible non-spliced and *cis*-spliced epitope candidates are actually produced by proteasomes. Based on *in vitro* digestion experiments with synthetic polypeptides and purified proteasomes, measured by mass spectrometry, we estimated that around one fourth of non-spliced and less than 0.4% of *cis*-spliced peptides that might have been produced were in fact produced by proteasomes in those conditions, and many of them in such a small amount that make them most likely immunologically irrelevant (22, 27). On the other hand, $CD8^+$ TCRαβ are prone to a certain degree of degeneracy of their specificity. This might lead to cross-recognition of multiple antigenic peptides, thereby increasing the immunological overlap between self and non-self antigens. The immunological relevance of $CD8^+$ TCRαβ cross-reactivity is still a matter of debate (60–63), although seminal studies on potential cross-reactivity of T1D-relevant $CD8^+$ T cell clones for T1D-associated human antigens and pathogens have already been published (28, 64, 65). In future analyses of *cis*-spliced epitope candidates, including TCR degeneracy would represent a computational challenge, although it might significantly

increase the number of viral-human *zwitter* epitope candidates potentially associated to T1D. With today's limited knowledge of TCR degeneracy, we can also speculate that its introduction in the analysis would also increase the number of false viral-human *zwitter* epitope candidates. Additionally, future experimental analyses should consider the islet microenvironment, where proinflammatory molecules could promote immune activation and antigen presentation to APCs, further refining the pool of epitope candidates that could effectively be presented.

Regarding the *zwitter* epitope candidates derived from T1D-associated viruses and pancreatic β cell antigens described in this study, we found interesting examples potentially derived from CVB4, HHV-6A and -6B as well as RVC. Of course, the *in silico* identification of *zwitter* epitope candidates derived from T1D-associated viruses and pancreatic β cell antigens, even if confirmed *in vitro* and *in cellulo*, would not represent the only key to understanding T1D pathogenesis. It is likely that the strong genetic component of this disease plays a crucial role. We think, however, that in some T1D patients, an autoimmune response could be triggered by viral infection, which in turn might target viral-human *zwitter cis*-spliced epitopes. Although the exact mechanisms by which this occurs are currently unknown, it is possible that type I interferons, secreted in response to viral infections, play an important role. Exposure of human β cells to IFN-α leads to changes in chromatin accessibility, mRNA and protein expression, and the subsequent activation of pathways involved in protein modification, degradation and ER stress (66). IFN-α is capable of shaping the islet microenvironment by inducing the upregulation of several RNA-binding proteins with direct effects in immune cells and the potential to induce extensive changes in alternative splicing, activation and differentiation (66). In addition, the hyper-expression of HLA-I and other anti-viral response markers is associated with islet immune infiltration, which suggests that inflammatory and anti-viral responses play a crucial role in creating an islet microenvironment that potentially attracts APCs and favors antigen presentation (67). The mechanisms leading to epitope formation and presentation, as well as its possible outcomes in terms of T cell activation and cytotoxicity remain elusive, and thus will need to be elucidated in future.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JL and MM designed the study. JL and AM carried out the data analysis and MM the data mining. MM and TR-C critically revised the immunological implication of the analysis. JL, TR-C, and MM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.656451/full#supplementary-material

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REVIEW
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Endoplasmic Reticulum-Mitochondria Crosstalk and Beta-Cell Destruction in Type 1 Diabetes

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Beta-cell destruction in type 1 diabetes (T1D) results from the combined effect of inflammation and recurrent autoimmunity. In response to inflammatory signals, beta-cells engage adaptive mechanisms where the endoplasmic reticulum (ER) and mitochondria act in concert to restore cellular homeostasis. In the recent years it has become clear that this adaptive phase may trigger the development of autoimmunity by the generation of autoantigens recognized by autoreactive CD8 T cells. The participation of the ER stress and the unfolded protein response to the increased visibility of beta-cells to the immune system has been largely described. However, the role of the other cellular organelles, and in particular the mitochondria that are central mediator for beta-cell survival and function, remains poorly investigated. In this review we will dissect the crosstalk between the ER and mitochondria in the context of T1D, highlighting the key role played by this interaction in beta-cell dysfunctions and immune activation, especially through regulation of calcium homeostasis, oxidative stress and generation of mitochondrial-derived factors.

Keywords: endoplasmic reticulum, ER stress, mitochondria, Type 1 diabetes (T1D), beta-cell, inflammation, cytokines

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease that targets pancreatic beta-cells, leading to their progressive loss (1). For years, impaired thymic education or low affinity T cells were thought to be responsible of the immune attack directed against native self-proteins. However, accumulating evidence suggest that local inflammation or other forms of stress, like viral infection, toxic chemical exposure or dysglycemia, combined with genetic predisposition may lead to the generation and accumulation of aberrant or modified proteins to which central tolerance is lacking, thereby triggering autoimmunity (2–4).

The endoplasmic reticulum (ER), is the hub for protein synthesis, folding, modification and transport as well as for phospholipid and cholesterol biosynthesis (5). Alterations in ER homeostasis due to inflammatory stress, accumulation of misfolded proteins, and/or alterations in the cellular Ca^{2+} or redox balance triggers an unfolded protein response (UPR) through activation of ER transmembrane proteins [*e.g.* double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1α (IRE1α) and activating transcription factor 6 (ATF6)]. These

central mediators of the UPR sense the accumulation of misfolded proteins in the ER lumen and activate mechanisms to inhibit protein synthesis, restore expression of chaperones, like the 78-kDa glucose regulated protein [also known as binding immunoglobulin protein] (GRP78/BiP), and initiate ER associated degradation pathway to eliminate newly synthesized proteins through proteasome-mediated degradation (6, 7). Persistent stimulation of the UPR in response to ER stress induces apoptosis *via* activation of C/EBP homologous protein (CHOP), c-jun N-terminal kinase (JNK), death protein 5 (DP5) and other pro-apoptotic signals (8, 9). Several studies, have demonstrated that this adaptive phase disturbs (post)-transcriptional, (post)-translational and degradation processes, increasing the complexity of the beta-cell proteome and peptidome, promoting the generation of neoantigens (10, 11).

Like the ER, mitochondria are complex and dynamic cellular organelles that play a key role in beta-cell functions, notably by coupling glucose metabolism to insulin secretion, but also in regulating apoptotic cell death *via* the production of reactive oxygen species (ROS) and release of cytochrome C (12, 13). In most eukaryotic cells, including beta-cells, mitochondria form dynamic networks that are continually reshaped by fission and fusion processes, under the control of specific mitochondrial membrane anchor proteins. Induction of the mitochondria UPR (UPRmt) plays an essential role in the maintenance of the mitochondrial integrity, dynamics and function in response to various stressors (14, 15). Currently, little is known regarding the impact of pro-inflammatory stimuli on mitochondrial dynamics/bioenergetics and UPRmt in human beta-cells. Yet, the interaction between the ER and mitochondria during the adaptive mechanism to environmental stress indicates that both organelles orchestrate the communication between the beta-cells and the immune system. Therefore, further exploring the regulatory mechanisms involved in mitochondria-ER interaction and in particular those controlling Ca^{2+} homeostasis and mitochondrial homeostasis, is required for a better understanding of the pathophysiology of beta-cell failure and its immune-related consequences in T1D.

ER-MITOCHONDRIA CROSSTALK IN BETA-CELL (DYS)FUNCTIONS

The ER and mitochondria are organelles that physically interact in a highly dynamic and regulated manner, forming specific microdomains, termed mitochondria and ER contact sites (MERCs) or mitochondria-associated membranes (MAMs) when studied at the molecular level (16). It is well established that MAMs play a central role in cellular Ca^{2+} homeostasis (17–19) and, more recently, they have also been shown to regulate mitochondrial dynamics and bioenergetics (20), ROS production (21), mitochondrial-mediated apoptosis (22), and inflammation (22, 23). MAMs are composed of membrane fractions from both the ER and the outer mitochondrial membrane (OMM) containing a large range of cell-specific molecular components involved in the tethering complex (16). Alterations in the MAMs composition and abnormal ER-mitochondria interaction have been reported to be associated with different pathological conditions, especially in type 2 diabetes (T2D) where organelle miscommunication has been suggested to underlie beta-cell inflammation, cell death and impaired metabolic function (24).

ER-Mitochondria Tethering, Ca^{2+} Homeostasis and Beta-Cell Dysfunction

The regulation of Ca^{2+} homeostasis is essential for proper beta-cell functions, because of its role in driving insulin granule biogenesis, trafficking and exocytosis but also by triggering multiple intracellular signaling pathways essential for the maintenance of beta-cell identity and survival (25). Cytosolic Ca^{2+} concentration is tightly controlled and results from a balance between its cellular influx and efflux, and its intracellular uptake and release by various organelles, such as ER, Golgi and the mitochondria, through specific exchangers, pumps, and channels (**Figure 1**). It is still unclear whether the mitochondria can play a significant role in directly buffering cytosolic Ca^{2+} in a quantitative manner under physiological conditions (26). However, acute and/or long-lasting modulation of inter-organelle communication, particularly under pathological conditions, may impact Ca^{2+} homeostasis in beta-cells. As such, channeling of the cation in between subcellular compartments, notably from the ER to the mitochondria, represents another way by which large quantities of Ca^{2+} can be conveyed and exert key regulatory roles on the organelle functions. Under homeostatic conditions, a transient increase in beta-cell mitochondrial matrix Ca^{2+} levels promotes ATP production by oxidative phosphorylation (OXPHOS). This occurs principally through direct activation of several tricarboxylic acid (TCA) cycle dehydrogenases and contributes to K_{ATP} channel-mediated opening of L-type voltage-gated Ca^{2+} channels (L-VGCCs), increased cytosolic Ca^{2+} and sustained glucose-stimulated insulin secretion (GSIS) (27). However, any perturbations of this highly regulated spatio-temporal process would result in an altered mitochondrial homeostasis that may ultimately lead to bioenergetic dysfunction, enhanced oxidative stress and cell death. The ER-mitochondrial connectivity was shown to involve a set of interacting proteins located in the MAMs (**Figure 1,** insert), allowing ER and mitochondria to share their content, especially Ca^{2+}, through the 75-kDa glucose-regulated protein (GRP75)-mediated coupling of the ER inositol trisphosphate receptor (IP3R) with the mitochondrial voltage-dependent anion-selective channel 1 (VDAC1) (28). The association formed by the ER vesicle-associated membrane protein-associated protein B (VABP) with the OMM protein tyrosine phosphatase-interacting protein-51 (PTPIP51) could also contribute in the organelle interaction (29). Moreover, proteins involved in mitochondrial dynamics were also shown to be involved in this tethering, such as mitofusin 2 (MFN2) and mitochondrial fission 1 protein (FIS1). Indeed, FIS1 can directly interact with the ER B-cell receptor-associated protein 31 (Bap31), promoting Ca^{2+} transfer from ER to the mitochondria and subsequent induction of apoptosis (30).

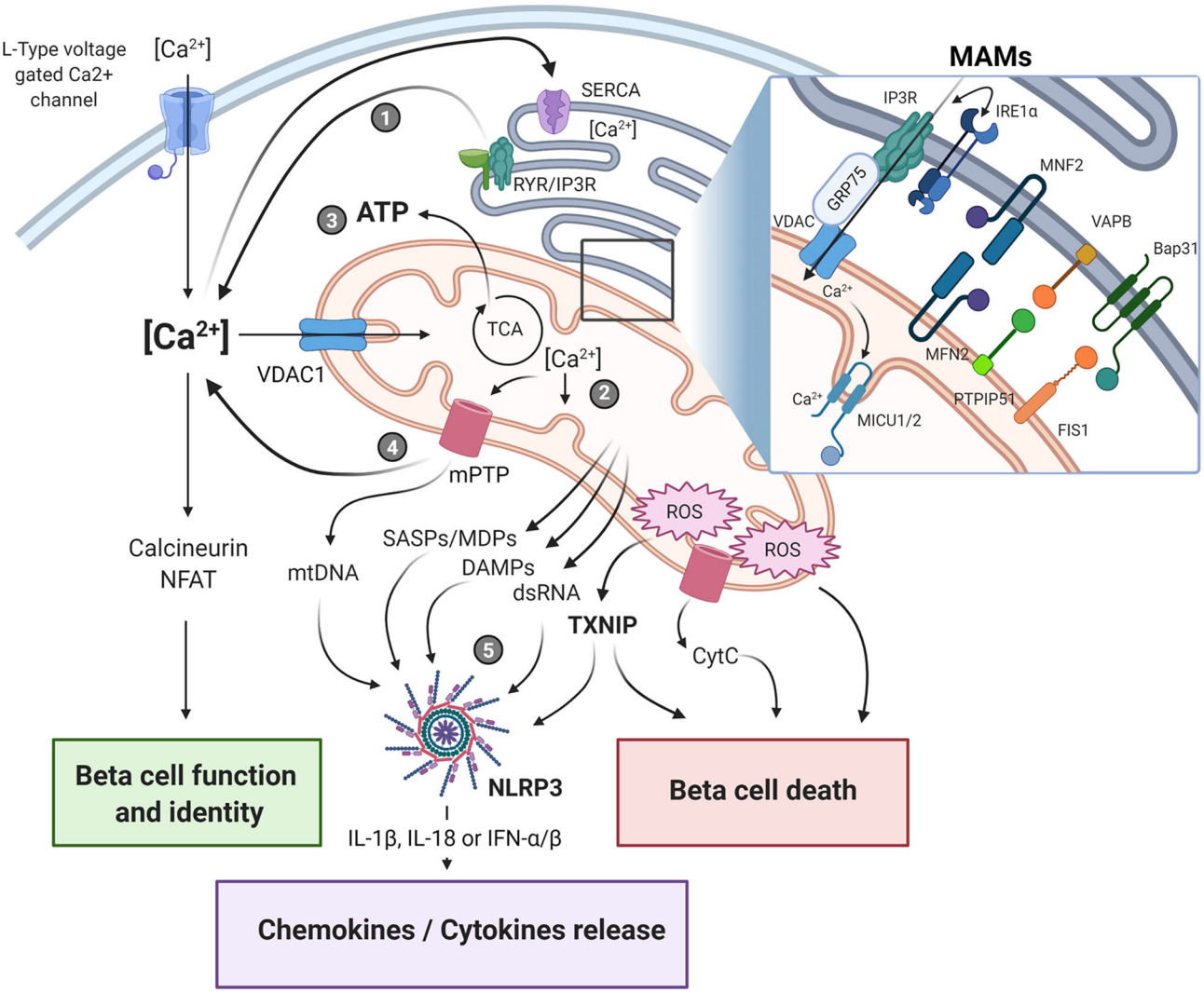

FIGURE 1 | Endoplasmic reticulum-mitochondria crosstalk in beta-cell dysfunction and immune activation in type 1 diabetes. The crosstalk between the endoplasmic reticulum (ER) and the mitochondrial network is playing a central role in cellular Ca^{2+} homeostasis and beta-cell function. The physical ER-mitochondria interaction involves a set of proteins located in the mitochondrial-associated membranes (MAMs, insert) which allows the exchange of organelle respective content. Modulation of MAM assembly has been shown to regulate mitochondrial dynamics and bioenergetics, ROS production, and release of various mitochondrial-derived factors. Alterations of these highly regulated processes in response to ER/mitochondria stress, can contribute to beta-cell dysfunction and immunogenicity by triggering apoptotic beta-cell death and NLRP3 activation, ultimately promoting pro-inflammatory cytokine/chemokine production, immune cells recruitment within the islet microenvironment, and beta-cell destruction. Upon ER stress induction, the depletion of ER Ca^{2+} store (1) induces mitochondrial Ca^{2+} accumulation (2). This increase in mitochondrial matrix Ca^{2+} concentration triggers oxidative phosphorylation and ATP production (3) in order to restore cellular and ER homeostasis. If this adaptive response fails to restore homeostasis, prolonged Ca^{2+} accumulation in the mitochondria would promote the opening of the mPTP, leading to increased mitochondrial membrane permeability and leakage of mitochondrial-derived factors, such as DAMPS, mtDNA, dsRNA (4) that ultimately activate downstream sensors/inflammasome (5). SASPs, senescence-associated secretory phenotype; DAMPs, damage-associated molecular patterns; MDPs, mitochondrial-derived peptides; ROS, reactive oxygen species; mtDNA, mitochondrial DNA; mPTP, mitochondrial permeability transition pore; CytC, cytochrome C. The figure was created with BioRender.com.

Interestingly, it has recently been reported that besides its canonical function in UPR, IRE1α also acts as a scaffold within the ER-mitochondria junction by directly interacting with IP3R, the main ER Ca^{2+} channel, ultimately affecting ER-mitochondria Ca^{2+} signaling (31). Indeed, the interaction between the respective C-terminal cytosolic domains of monomeric IRE1α and IP3R proteins within the MAMs allows for a VDAC1-mediated transfer of Ca^{2+} from the ER lumen to the mitochondrial matrix, leading to increased Ca^{2+} concentrations that trigger activation of TCA cycle and ATP production (31). Interestingly, it has been shown in pancreatic tissue sections that the number of IP3R-VDAC1 complexes assessed by *in situ* proximity ligation assay was significantly lower in beta-cells from individuals with T2D as compared to non-diabetic controls, suggesting that alterations in the ER-mitochondria interaction may contribute to beta-cell dysfunction under

diabetic conditions (32). Furthermore, induction of ER stress by treatment with the long-chain fatty acid palmitate significantly reduced ER-mitochondria interactions and altered GSIS in the murine Min6-B1 beta-cell line (32). More recently, the same team has also reported that both acute or prolonged exposure to glucose may promote ER-mitochondria interactions while having opposite impact on mitochondrial Ca^{2+} homeostasis and beta-cell insulin secretion (33). Indeed, it was shown that an acute glucose challenge enhanced MAMs assembly, as evidenced by increased VDAC1-IP3R2 proximity, which was associated with higher ATP-stimulated ER-mitochondrial Ca^{2+} transfer, intramitochondrial Ca^{2+} accumulation and GSIS in rat INS-1E beta-cell line. This ER-mitochondria interaction was abolished when MAM tethering protein GRP75 was knocked down by siRNA, demonstrating that organelle coupling is crucial for beta-cell Ca^{2+} homeostasis and insulin exocytosis under homeostatic conditions (33). Counterintuitively, extending the duration of glucose exposure to mimic diabetogenic conditions also resulted in increased MAM formation in both INS-1E cells and human pancreatic islets. However, despite higher organelle interactions, chronic glucose exposure triggered ER stress and disruption of ER-mitochondrial Ca^{2+} exchange, characterized by a progressive depletion of the ER Ca^{2+} store and increased Ca^{2+} levels in fragmented mitochondria, ultimately leading to altered mitochondrial respiration and impaired GSIS (33). Remarkably, this feature also resembles the effect of pro-inflammatory cytokines in the context of T1D, with Ca^{2+} depletion in the ER being associated with enhanced protein misfolding, UPR activation, and beta-cell death (34). Of note, treatment with the ER stress inducer tunicamycin also increased VDAC1-IP3R2 interactions in INS-1E cells, suggesting that increased organelle tethering could constitute an adaptive mechanism for restoring ER-mitochondria Ca^{2+} homeostasis (33). Whether beta-cell ER stress and IRE1α-mediated UPR associated with islet inflammation in T1D would be the cause or the consequence of impaired MAM assembly that leads to altered mitochondrial Ca^{2+} homeostasis and mitochondria-mediated immune activation, remains however to be investigated.

ER-Mitochondria Crosstalk, Oxidative Stress and Beta-Cell Death

MAMs have also been identified as a critical hub in the regulation of beta-cell death, because ER stress and alterations in mitochondrial Ca^{2+} homeostasis are linked to increased local ROS production and induction of intrinsic apoptotic pathways (35, 36). IRE1α was shown to be involved in the regulation of the OMM B cell lymphoma-2 (Bcl-2) protein family. Once activated, IRE1α can bind the TNF receptor associated factor 2 (TRAF2) adaptor and activate the JNK signaling pathway, triggering apoptosis *via* upregulation of CHOP and downregulation of the anti-apoptotic protein Bcl-2 (10). Additionally, CHOP promotes Bcl-2 phosphorylation which impairs its inhibitory interaction with the pro-apoptotic proteins, Bcl-2 associated X protein (Bax) and Bcl-2 homologous antagonist killer protein (Bak). These proteins induce IP3R-mediated cytosolic release of Ca^{2+} from the ER which is next channeled into the mitochondria through VDAC1 (37, 38). This causes an increase in mitochondrial matrix Ca^{2+} concentration that triggers the opening of the mitochondrial permeability transition pore (mPTP), a non-specific channel located in the inner mitochondrial membrane (IMM), ultimately leading to cytochrome C-mediated activation of apoptotic pathway and cell death (39, 40). In addition to IRE1α, the other ER stress-driven UPR arms, involving PERK and ATF6, can also participate in MAM assembly and ER-mitochondria crosstalk. Indeed, ATF6 has been shown to promote mitochondrial biogenesis through interaction with peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) (41, 42), an effect that would presumably promote mitochondrial network remodeling and impact the ER-mitochondria interaction. PERK has also been reported to be located in the MAMs fraction and involved in mediating ER-mitochondria interaction during ROS-induced oxidative stress (43). Moreover, ATF4, located downstream of PERK, has been shown to regulate mitochondrial dynamics by controlling the expression of the ubiquitin ligase Parkin, that is responsible for the removal of the damaged mitochondria by mitophagy during ER stress (44). Interestingly, Parkin also mediates ER-mitochondria crosstalk during ER stress by increasing MAM microdomains assembly for maintaining intra-organelle Ca^{2+} transfer (45). In addition, ATF4 can participate to the regulation of the oxidative stress response and apoptosis in beta-cells through interaction with the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) (46–48). In fact, under inflammatory conditions, the increase in oxidative stress may lead to oxidation of cysteine residues on Kelch-like ECH associated protein 1 (KEAP1), leading to conformational changes and disruption of its interaction with NRF2. Next, NRF2 can translocate to the nucleus where it exerts a dual function. On one hand, it can form a heterodimer with ATF4 and regulate antioxidant genes (heme oxygenase-1, NAD(P)H: quinone oxidoreductase, glutathione S-transferase) and prevent cell death by activating anti-apoptotic genes (B-cell lymphoma-extra large (Bcl-xL), Bcl-2). On the other hand, it can also compete with ATF4 and inhibit its binding to the Amino Acid Responsive Element (AARE) on the CHOP promoter, ultimately limiting cytochrome C release and apoptosis (49).

Mitochondria form a very dynamic and plastic network that is continuously re-organized owing to tightly regulated processes involving fission, fusion and mitophagy-mediated clearance of dysfunctional organelles. On one hand, mitochondrial network remodeling, especially in beta-cell, is important for organelle bioenergetics and functions, including OXPHOS-driven ATP synthesis, Ca^{2+} signaling and cell survival. On the other hand, mitochondrial dynamics and their interaction with other subcellular structures are also regulated through intrinsic organelle metabolic activity and ROS production, with disrupted OXPHOS and increased oxidative stress promoting mitochondrial fission and mitophagy (50). In beta-cells, changes in the expression of mitochondrial fission/fusion proteins in response to both inflammatory and metabolic stresses have been shown to affect mitochondrial dynamic and ultrastructure, leading to impaired organelle energetics and altered beta-cell GSIS and survival (51). For instance, prolonged exposure to high

glucose and/or lipotoxic environment increases the expression of the fission protein dynamin-related protein 1 (DRP1), reduces GSIS, and increases apoptotic cell death in INS-1E cells (52, 53). Similarly, DRP1 expression was found to be increased in beta-cells from dysfunctional pancreatic islets in a model of hyperglycemic T2D mice (54). In line with this, overexpression of DRP1 or FIS1 in various *in vitro* models of murine beta-cells was generally associated with increased mitochondrial fission, impaired mitochondrial bioenergetics and increased apoptosis (**Table S1**). More recently, the expression of the orphan nuclear receptor Nor1/Nr4a3, which was previously identified as a negative regulator of beta-cell mass (55), was found to be increased by proinflammatory cytokines and involved in beta-cell mitochondrial dysfunction (56). Indeed, following mitochondrial translocation, Nor1 promotes the disruption of mitochondrial network, leading to reduction in glucose oxidation and ATP production that ultimately contribute to apoptotic beta-cell death (56). By contrast, a balanced expression of the fission protein FIS1 was shown to be required for maintenance of mitochondrial network remodeling, beta-cell survival and efficient GSIS in INS-1 cells (57). Interestingly, dampening high glucose/lipid-induced beta-cell stress restores mitochondrial dynamics by increasing fusion and reducing fission, leading to decreased apoptosis in mouse islets (58).

FROM MITOCHONDRIA DYSFUNCTION TO BETA-CELL IMMUNOGENICITY?

The prokaryotic origin of mitochondria positions the organelle as a potential source of intracellular mediators that can trigger innate immunity through release of various damage-associated molecular patterns (DAMPS) and/or pathogen-associated molecular patterns (PAMPS). Among these mitochondrial-derived molecules, specific phospholipids from the OMM (59), N-formyl methionine containing peptides which are legacy from prokaryotic translation initiation phase (60), and also mitochondrial DNA (mtDNA) containing hypomethylated CpG motifs that closely resemble bacterial CpG DNA can all trigger downstream immune activation, either directly or *via* pattern recognition receptor (PRRs)-mediated activation of the inflammasome.

In response to environmental stresses that lead to altered mitochondrial homeostasis, the organelle integrity is maintained, like in the ER, by chaperones (HSP60, HSP70) and proteases (ClpXP, ATP-dependent AAA protease LON) that control protein synthesis, folding and degradation and activate antioxidant mechanisms for ROS detoxification (61). Interestingly, the humoral and T cell reactivity against both HSP60 and HSP70 reported in newly diagnosed T1D patients may illustrate the important role played by mitochondrial dysfunction and stress in triggering autoimmunity (62, 63). During ER and/or mitochondrial stress, disruption in the Ca^{2+} homeostasis opens the mPTP which may lead to cytoplasmic release of mtDNA or mitochondrial double-stranded RNA (dsRNA) from mitochondria and induction of a programmed cell death. In line with this observation, it has been shown that activation of Bax/Bak during apoptosis led to the formation of a macropores on the OMM which allows the IMM to swell out into the cytosol and release mitochondrial factors, including mtDNA and dsRNA, without caspases activation (64). Classically, the presence of cytosolic mtDNA is sensed by cyclic GMP-AMP synthase (cGAS), resulting in stimulator of interferon genes (STING) activation and downstream phosphorylation of tyrosine kinase non receptor 1 (TNK1) and interferon regulatory factor 3 (IRF3), ultimately leading to increased transcription of type 1 IFN genes (65). Alternative pathways can trigger TLR9 activation within endosomes, leading to NFκB-mediated inflammasome (NLRP1 and NLRP3) activation, increased caspase 1 activity and subsequent IL-1β and IL-18 processing (66). Although little is known on the beta-cell inflammasome, studies conducted in both INS-1E cells and human islets have shown that prototypical inflammatory cytokines of the insulitis microenvironment may differentially regulate NLRP1 and NLRP3 *via* ATF4 and NFκB pathways (67). Similarly, cytosolic accumulation of dsRNA, generated after bidirectional transcription of circular mtDNA, can be detected by several cellular sensors, including protein kinase R (PKR), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), to inhibit protein synthesis *via* phosphorylation of the eIF2α, recruitment of TANK-binding kinase-1, inducible IκB kinase (IKKϵ), and activation of IFN regulatory factor 3 and 7 and NFκB, to promote type I IFN secretion (68). Altogether, these findings highlight a potential mechanism by which ER stress-induced release of mitochondrial genomic material might lead to the activation of inflammatory pathways in beta-cells, contributing to their eventual demise.

The production of IL-1β, IL-18 or IFN-α/β by islet resident innate immune cells and/or endocrine cells (69–73), among which beta-cell is probably not the major source, facilitates the recruitment of immune cells [*e.g.* monocytes/macrophages, dendritic cells (DC)] to the inflammatory microenvironment within pancreas where they might contribute to the priming and activation of adaptive immune cells (74). Type I IFNs signature has been highly associated with disease development and progression from prediabetic stage (75). Moreover, among the 50 loci linked to T1D, several are expressed in beta-cells and involved in innate immunity [*e.g.* Protein Tyrosine Phosphatase, Non-Receptor Type 2 (PTPN2), Tyrosine Kinase 2 (TYK2), Interferon Induced Helicase C Domain 1 (IFIH1) and BTB Domain and CNC Homolog 2 (BACH2)]. In addition, a recent meta-analysis comparing RNAseq data from tissues of patients with T1D (beta-cells), systemic lupus erythematosus (SLE, kidney cells), multiple sclerosis (MS, optic chiasm) and rheumatoid arthritis (RA, joint tissue), identified a type I IFN signature as a common feature, further highlighting a more global role of type I IFN signaling in the development of autoimmune disease (76).

Although it is tempting to speculate that mitochondria permeability and subsequent genetic material leakage may participate to immune activation by beta-cells, strong

supportive data are currently lacking to clearly implicate these processes in T1D development. However, a recent study has reported that inhibition of VDAC1 oligomerization during mitochondrial stress reduced accumulation of cytosolic mtDNA, expression of type I IFN genes, and circulating auto antibody levels in a mouse model of SLE, highlighting the possible role of cytosolic mtDNA in triggering autoimmunity (77). Furthermore, a relation between mitochondria content and beta-cell autoimmune destruction in T1D has also been suggested by the identification of a SNP located within the mitochondrial gene for NADH dehydrogenase 2 (*mt-Nd2*) that was associated with T1D (78). In this study, the presence of the *mt-Nd2a* (resistant) allele prevented both T1D after adoptive transfer of diabetogenic CD4+ T cell clones in NOD mice and beta-cell destruction by CD8 T cells *in vitro* (78). In addition, depletion of mtDNA from βlox5 cells lowered cytokine-mediated destruction and prevented CD8 T cell-mediated cytotoxic killing (79), positioning mtDNA a potentially important trigger of beta-cell destruction.

Alterations in intracellular Ca^{2+} homeostasis by ER/mitochondria stress may also have broad consequences on the cell visibility to the immune system by generating neoantigens, through regulation of mRNA splicing and protein synthesis or proteasomal degradation (80–82). Several studies have indeed highlighted the role of Ca^{2+} as activator for post translational enzymes transglutaminase 2 (TG2) and protein arginine deiminases (PADs) involved in deamidation and citrullination, respectively. These enzymes are mainly involved in coeliac disease and RA but have also been extensively studied in the context of T1D as important component of beta-cell-directed autoimmunity (3, 83, 84). In these processes, ROS participates in the regulation of post-translation modification (PTM) enzymes, inhibiting TG2 ubiquitination in endothelial cells or controlling activity of PAD4 (85, 86). Deamidation of glutamine and asparagine residues in the insulin B chain modifies the structural properties of insulin-derived epitopes and generates perfect anchors for peptide presentation in HLA-DQ2/-DQ8 predisposing haplotype (87). Similarly, citrullination has been shown to turn the GRP78/BiP ER chaperone into an important autoantigen in T1D by an ER stress-independent mechanism involving alteration of Ca^{2+} homeostasis and activation of PADs (84). In addition, changes in cytosolic Ca^{2+} level activate nuclear factor of activated T cells (NFAT) *via* the calcineurin/calmodulin pathway which plays a critical role in beta-cell proliferation and maintenance of beta-cell mass and function. As such, immunosuppressive drugs used in islet transplantation, such as cyclosporin A or FK506, inhibit calcineurin pathway, impair beta-cell function and trigger the development of post-transplantation diabetes mellitus (New Onset Diabetes After Transplantation [NODAT]) (88). Of note, new calcineurin inhibitors, such as voclosporin, has been recently reported to have less deleterious effects on islet functions (89). Increased cytosolic Ca^{2+} also promotes phospholipase C activation and production of IP3, which stimulates Ca^{2+} mobilization and subsequent activation of protein kinase C (PKC) and downstream MAP kinases (JNK and ERK) that ultimately leads to activation of NFκB-dependent transcriptional program and increased expression of the chemokines IL8, CCL2, CXCL10 and CXCL12 (25). Although Ca^{2+} chelation had serious deleterious effect on beta-cell function and GSIS *in vitro*, it prevented DC migration to insulinoma (90) and PAD enzymes activation (4), highlighting its role as a cellular mediator in the communication with the immune compartment. This also suggests that restoration of intracellular Ca^{2+} homeostasis may inhibit immune cell trafficking to the pancreatic islets and reduce beta-cell immunogenicity. Interestingly, chemical inhibition of the NFκB pathway reduced IL-8 production by stressed human beta-cells and prevented neutrophil migration, an effect mediated by the proton exchanger GRP68 and the transcription factor RFX6 which are also involved in the regulation of Ca^{2+} homeostasis (91).

Another consequence of ER stress and mitochondria dysfunction is the induction of cell senescence (92), secondary to increased ROS production and impaired redox status (21, 93, 94). Cellular senescence is a complex cell fate response that is characterized by the release of senescence-associated secretory phenotypes (SASPs) in response to multiple types of endogenous and exogenous stressors. The SASP components are of diverse nature, including cytokines and chemokines but also a large range of soluble and insoluble factors, and can contribute to immune activation, by promoting infiltration of immune cells. Although further studies are definitely required, it has been recently reported that islets from T1D mice as well as beta-cells from T1D donors display increased markers of senescence during disease progression (95), suggesting that beta-cell senescence may be an adaptive response to prolonged cellular stress that can contribute to autoimmunity through SASPs (96). Interestingly, studies aiming at eliminating senescent beta-cells by using specific senolytic drugs show remarkable results in limiting T1D progression in mice models (95, 97). These effects were associated with reduction in insulitis and improvements of both glucose metabolism and beta-cell function (95, 97). In response to metabolic activation and stress-associated UPRmt, mitochondria can also secrete mitochondria-derived peptides (MDPs), which are small bioactive peptides encoded by mtDNA that are mainly acting as retrograde signals to regulate mitochondrial energetics (98, 99). Although supporting data are currently sparse, especially in beta-cells, one can speculate that alterations in MDPs by inflammatory microenvironment may also contribute to modulate the cell communication with the immune compartment through various signaling pathways.

CONCLUSION

Dysfunctional mitochondria has been particularly studied in the context of metabolic disorders and T2D, where it has been associated for decades to insulin resistance and beta-cell failure. Although the beta-cell failure in pancreatic islets differs in many aspect between T1D and T2D [for review (100)], the presence of an islet-specific inflammatory microenvironment characterized by elevated local concentrations of type 1 cytokines (67, 101), together with enhanced recruitment and/or activation of tissue-resident innate and adaptive immune cells

(*e.g.* macrophages, B cells and T cells) (102) and accumulation of amyloid deposit (103, 104) represent common features to both pathologies. Consequently, the possibility of repurposing T2D drugs for T1D, to improve blood glucose management for relapsing metabolic and oxidative stress on beta-cells and limiting further immune destruction is worth considering. Ongoing studies using metformin, GLP-1 analogues, SGLT-2 inhibitors, or the L-VGCC inhibitor verapamil, which are either modulating mitochondrial bioenergetics/ROS/mPTP [metformin; (105–108)], ER stress [GLP-1 analogues; (109, 110)], glucose toxicity [SGLT-2 inhibitors; (111, 112)] or cellular Ca^{2+} homeostasis [verapamil; (113, 114)] are currently explored in the framework of clinical trials. These conceptually attractive novel approaches would also require further supportive mechanistic *in vitro* molecular studies in human primary beta-cells/islets and/or relevant beta-cell line models.

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All the authors contributed equally to the review. SV and JL wrote the first draft of the manuscript. BG and AZ edited and wrote sections of the manuscript. AZ designed the figure. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.669492/full#supplementary-material

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Neoepitopes in Type 1 Diabetes: Etiological Insights, Biomarkers and Therapeutic Targets

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The mechanisms underlying type 1 diabetes (T1D) pathogenesis remain largely unknown. While autoantibodies to pancreatic beta-cell antigens are often the first biological response and thereby a useful biomarker for identifying individuals in early stages of T1D, their role in T1D pathogenesis is not well understood. Recognition of these antigenic targets by autoreactive T-cells plays a pathological role in T1D development. Recently, several beta-cell neoantigens have been described, indicating that both neoantigens and known T1D antigens escape central or peripheral tolerance. Several questions regarding the mechanisms by which tolerance is broken in T1D remain unanswered. Further delineating the timing and nature of antigenic responses could allow their use as biomarkers to improve staging, as targets for therapeutic intervention, and lead to a better understanding of the mechanisms leading to loss of tolerance. Multiple factors that contribute to cellular stress may result in the generation of beta-cell derived neoepitopes and contribute to autoimmunity. Understanding the cellular mechanisms that induce beta-cells to produce neoantigens has direct implications on development of therapies to intercept T1D disease progression. In this perspective, we will discuss evidence for the role of neoantigens in the pathogenesis of T1D, including antigenic responses and cellular mechanisms. We will additionally discuss the pathways leading to neoepitope formation and the cross talk between the immune system and the beta-cells in this regard. Ultimately, delineating the timing of neoepitope generation in T1D pathogenesis will determine their role as biomarkers as well as therapeutic targets.

Keywords: type 1 diabetes, neoepitopes, autoantigens, pathogenesis, beta-cell, biomarker

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THE EVOLVING UNDERSTANDING OF THE PATHOGENESIS OF HUMAN T1D

The immune system plays a pivotal role in type 1 diabetes (T1D), as demonstrated by numerous studies and experiments *in vitro*, *ex vivo*, in animal models, and in humans (1, 2). However, our understanding of the disease continues to evolve, with a greater recent appreciation for the role of pancreatic beta-cell factors and beta-cells themselves in disease etiology (3, 4). Our knowledge of the

immunopathology of T1D is incomplete, partially due to the difficult access to human pancreas samples. This limitation has been partially overcome in recent years thanks to the emergence of several large tissue biobanks like the Network for Pancreatic Organ Donors with Diabetes (nPOD) (5, 6) and the Exeter Archival Diabetes Biobank (EADB) (7), which now permit the investigation of immune cell populations in the human pancreas. The presence of immune cells in the islets, known as insulitis, is a hallmark of T1D (8–11). The importance of CD8+ T-cells in T1D is evident by their abundance in islets that have remaining beta-cells, as well as in those with only a few beta-cells left. CD8+ T-cells are also found in the exocrine portion of the pancreas in individuals with T1D, even when beta-cells are lost (12). While CD4+ T-cells are also present in the islets, they are not considered a major component of the immune infiltrate in established diabetes; this is not unexpected given the more prominent role of CD4+ T-cells in disease initiation (13) rather than in disease progression/amplification. In addition, their potential role in sustaining the effector functions of CD8+ T-cells should not be neglected (14). Both CD4+ and CD8+ T-cell populations decline with beta-cell loss, suggesting that their presence is driven by a beta-cell antigen (11, 12, 15). However, the exact role infiltrating T-cells play in the pathogenesis of the disease remains to be determined both in terms of specificity and function.

Antigen-specific CD8+ T-cells recognizing diverse islet antigens have been detected in the pancreas of individuals with T1D (16). T-cells with single specificity were detected early in the disease process, whereas in long-standing donors, islets usually contained multiple islet-reactive specificities indicating epitope spreading (16). Interestingly, in recent onset cases, different islets harbored different reactivities, which could reflect different stages of the autoimmune process. More recently, a high proportion of preproinsulin (PPI) specific cells have been detected in the islets of donors with T1D (17, 18), confirming previous data obtained from blood samples and highlighting the role of PPI as one of the most prominent antigens in disease pathogenesis (15, 19–22). Attempts to detect neoantigens *in situ* have not been reported so far but are on the horizon. Characterizing the frequency and localization of neoantigens at different disease stages therefore remains an important goal.

THE EXPANDING CATALOGUE OF EPITOPES WITH PUTATIVE ROLES IN T1D

The exact mechanisms and timing of the antigenic events, the initial loss of tolerance, as well as the role of autoimmunity to both native and modified epitopes in the pathogenesis of T1D remains unknown. Recently, a comprehensive overview of the known T1D epitopes and neoepitopes was published (22). Sixteen CD8+ T-cell conventional epitopes have been identified and five of these are contained within the major known antigens insulin, glutamate decarboxylase (GAD), insulinoma-associated antigen 2 (I-A2), zinc-transporter 8 (ZnT8) and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP). CD8+ T-cells reactive against epitopes from islet amyloid polypeptide (IAPP), insulin gene enhancer protein (ISL1), urocortin-3 (UCN3) and SLC30A8 (also known as ZnT8) have been also identified in the pancreas (23, 24). Their frequencies were higher in T1D donors compared to non-diabetic donors and their phenotype was predominantly antigen-experienced. In addition, CD8+ T-cell reactivity against other granule proteins such as prohormone convertase 2 (PCSK2), secretogranin 3 (SCG3) and 5 (SCG5) have been recently reported, highlighting the immunogenic potential of beta-cell granule proteins (25). The specificity of islet infiltrating T-cells directly sorted or grown from individual islets isolated from donors with T1D has been also investigated (26). Several CD4+ T-cell clones were obtained, which reacted against proinsulin, GAD65 and chromogranin A (ChgA). CD8+ T-cell clones recognized epitopes from insulin, IA-2 and IGRP. The differential antigenic drivers, including number of targets, diversity of epitopes, environmental triggers, host genetics and even clinical age, may contribute to these varying antigen recognition and disease pathways (27).

GENERATION OF NEOEPITOPES THROUGH POST-TRANSLATIONAL MODIFICATIONS

In the case of T1D, autoantibodies to native proteins are highly prognostic of future disease but there is little evidence of their pathogenic role (28–30). While loss of tolerance to insulin has long been thought to be involved early in disease pathogenesis, responses to modified proteins may add to disease heterogeneity, in terms of variations in risk and rate of progression to clinical T1D (31). However, clinical evidence has shown that not all cases of T1D start with reactivity to insulin, and reactivities to different non-conventional antigens may be due to different clinical and pathogenic features (32).

PTMs are part of normal physiological processes. However, they can also be formed as a result of an inflammatory assault restricted to inflamed tissues, and as such, be associated with autoimmunity. As neoepitopes are exclusively expressed in the peripheral target tissue, and not present in their modified form in the thymus, their escape from thymic deletion through negative selection in medullary thymic epithelial (mTEC) cells accounts for the lack of central tolerance to PTMs (33, 34). In the context of T1D, several neoantigens generated through PTMs have been described (**Figure 1**). In some cases, autoantibodies against modified proteins can be both pathogenic and predictors of disease onset (35). Antibodies directed against citrullinated proteins can predate clinical onset of rheumatoid arthritis (RA) by up to two decades (36). The most extensively studied enzymatically mediated PTMs are citrullination, the conversion of arginine into citrulline residues, and deamidation, the conversion of glutamine into glutamic acid residues. These PTMs are mediated by peptidylarginine deiminase (PAD) and tissue transglutaminase (TGM) enzymes, respectively. Citrullinated proteins are preferentially bound by RA-

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PTM	Mediator	Antigen	How is recognized	Identification	Species	Reference
Hybrid peptides	unknown	Insulin + secretory granule peptides	Mouse beta cell extracts, CD4 T cells, islet infiltrating CD4 T cells	LC-MS/MS, tetramer assays, IFN-γ response of CD4 T cell clone	Mouse/Human	Delong 2016
			Islet infiltrating CD4 T cells	IFN-γ response of CD4 T cell lines	Human	Babon 2016
			CD4 T cells	MHC tetramers	Mouse	Baker 2018
			Islets	LC-MS/MS	Mouse/ Human	Wiles 2019
			CD4 T cells	HLA-binding, HLA tetramers	Human	Baker 2019; Arribas-Layton 2020
			CD4 T cells	MHC-peptidomics, ELISPOT (IL-2/IFN-γ)	Mouse	Wan 2020
			CD4 T cells	ELISPOT (IL-10/IFN-γ)	Human	Mitchell 2021
	Cat-L	secretory granule peptides + WE14 (or IAPP)	CD4 T cells	In vitro transpeptidation, MHC-binding, IL-2 response of BDC T cell clones	Mouse	Reed 2021
Oxidation	ROS	Insulin	CD4 T cells	Antigen-specific T cell analysis	Human	Mannering 2005
			AutoAbs	ELISA	Human	Strollo 2015, 2017
Crosslinking	TGM enzymes	ChgA (WE14)	CD4 T cells	IFN-γ response of CD4+ T cell clone	Mouse	Delong 2012
		ChgA (WE14)	CD4 T cells	ELISPOT (IFN-γ)	Human	Gottlieb 2014
Deamidation	TGM enzymes, ER stress, cytokines, spontaneous	Proinsulin	CD4 T cells	Binding affinity, proliferation, ELISPOT (IFN-γ/IL10)	Human	Van Lummel 2014
		GAD65	CD4 T cells	HLA-binding, HLA tetramers	Human	McGinty 2014
		IA-2	CD4 T cells	HLA-binding, HLA tetramers	Human	Marré 2018
		Ins1 C-peptide	CD4 T cells	HLA-peptidomics, IL-2/IFN-γ response	Mouse	Wan 2020
Citrullination	PAD enzymes, ER stress, cytokines	GAD65	CD4 T cells	HLA binding, HLA-tetramers	Human	McGinty 2014
		GRP78	AutoAbs, CD4 T cells	ELISA, IFN-γ response of splenocytes	Mouse	Rondas 2015
		GRP78	Islet infiltrating CD4 T cells	IFN-γ response of CD4 T cell line	Human	Babon 2016
		IAPP	Islet infiltrating CD4 T cells	IFN-γ response of CD4 T cell clone	Human	Babon 2016
		GRP78	Islets, AutoAbs, CD4 T cells	LC-MS/MS, ELISA, HLA-binding, HLA tetramers	Human	Buitinga 2018
Spliced peptides	trans-peptidation	IAPP + IAPP	HLA-A2 binding, CD8 T cells	HLA-peptidome, HLA multimers	Human	Gonzalez-Duque 2018
		IA-2 + IA-2	HLA-A3 binding, CD8 T cell	HLA-peptidome, HLA multimers	Human	Azoury 2020
Alternative splicing	cytokines	IGRP	AutoAbs, CD8 T cells	Radiobinding assays, HLA multimers	Human	De Jong 2013
		SCG5	CD8 T cells	HLA-peptidome, HLA multimers	Human	Gonzales-Duque 2018; Azoury 2020
DRiP	ER stress	insulin	CD8 T cells	HLA-binding, HLA tetramers	Human	Kracht 2017

B

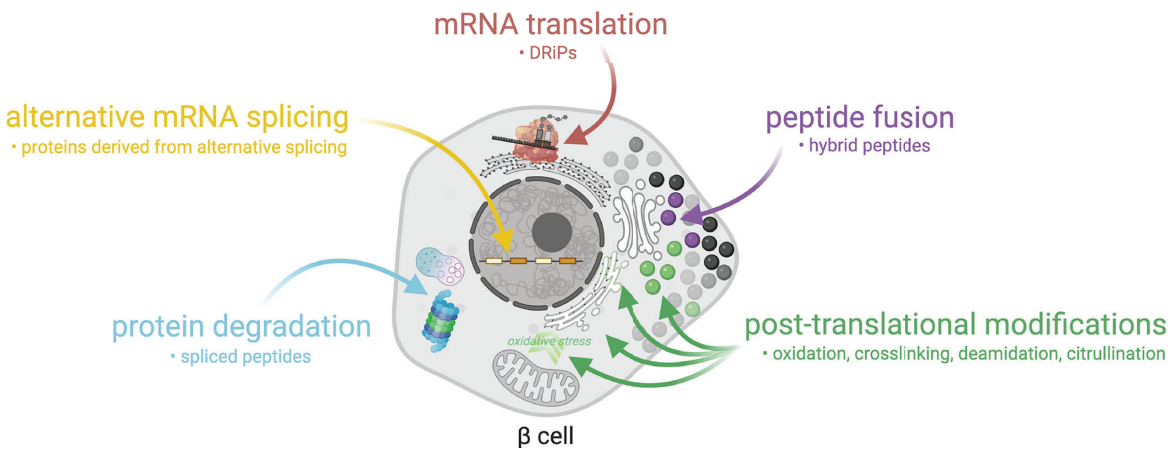

FIGURE 1 | A catalogue of neoepitopes and their production sites/mechanisms in beta-cells. **(A)** Referenced list of known neoepitopes in type 1 diabetes where the type of PTM, the potential mediator, the antigen and how it is recognized and identified, the species in which it was identified and the reference to the original publication are shown. Cat-L, cathepsin L; ROS, reactive oxygen species; AutoAbs, autoantibodies; TGM, tissue transglutaminase; ChgA, chromogranin A; ER, endoplasmic reticulum; PAD, peptidylarginine deiminase; GAD: glutamate decarboxylase; IA-2: insulinoma-associated antigen 2; ZnT8, zinc-transporter 8; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; GRP78, glucose-regulated protein 78; IAPP, islet amyloid polypeptide; SCG5, secretogranin 5. **(B)** Schematic representation of a beta-cell showing the current view on sub-cellular origin of specific classes of neoepitopes, as well as the types of products produced.

susceptible HLA class II and presented to T-cells, implicating them in disease pathogenesis (37). With similar HLA-susceptible haplotypes shared between RA and T1D, citrullination of multiple proteins has also been implicated in T1D pathogenesis (38, 39). Autoantibodies and circulating and islet infiltrating CD4+ T-cells have been found to react against citrullinated glucose-regulated protein 78 (GRP78) epitopes in T1D individuals (38, 40), following up on earlier observations in NOD mice (41). In addition, circulating CD4+ T-cells reactive against citrullinated GAD65 (39) and islet CD4+ T-cells reactive against citrullinated IAPP have been detected in people with T1D (40). Deamidated peptides, in which glutamine was converted into glutamic acid, were described in both murine T1D (42) and human T1D (39, 43), with several autoantigens identified (39, 42–44).

Oxidative post-translational modifications and cross-linking of proteins are important mechanisms that may contribute to autoreactivity in T1D (**Figure 1**). TGM-mediated crosslinking of the naturally processed ChgA cleavage product WE14 increased its immunogenicity, eliciting strong CD4 T-cell responses both in NOD mice (45) and T1D patients (46). Oxidation of the insulin A-chain, resulting in a disulfide bond formation between two adjacent cysteines, was shown to be responsible for recognition of the peptide by a T-cell clone isolated from the circulation of a T1D patient (47). More recently, autoantibodies against oxidized insulin were detected in prediabetic children (48) and in recently diagnosed T1D subjects (49).

Hybrid insulin peptides (HIPs) are also potential neoantigens, formed through a covalent cross-linking reaction between the C-terminal carboxylic acid group of proinsulin fragments and the N-terminal amine group of other secretory granule peptides (26). HIPs have been identified by LC-MS/MS in human (50) and mouse islets (51), as well as in the mouse MHC-peptidome (42). An increasing number of studies have shown autoreactive responses against such HIPs, both in NOD mice and in human T1D (26, 52). CD4+ T-cells isolated from pancreatic islets recognized different proinsulin C-peptide fragments fused to IAPP1, IAPP2, neuropeptide-Y or insulin A chain (40, 51). Increased reactivity to several HIPs was shown in peripheral blood of T1D patients (53, 54), Furthermore, in genetically at risk individuals, HIPs were detectable, and were shown to have a predominantly pro-inflammatory profile in those that progressed to developing disease (55), making them interesting candidates for novel biomarkers. Finally, a recent report suggests that transpeptidation of beta-cell antigens, mediated by cathepsin L, generates chimeric epitopes through fusion of secretory granule proteins with WE14, for diabetogenic CD4 T-cells (56). In regard to HLA class I epitopes, spliced peptides, generated in the proteasome through a process referred to as transpeptidation by which two different regions of a protein or of two different proteins are fused (57, 58), have been identified in a human beta-cell line by HLA-peptidomics (21, 25) and were recognized by circulating and pancreas-infiltrating CD8+ T-cells from T1D donors. Furthermore, defective ribosomal products (DRIPs) (24) are also regarded as potential neoepitopes against which CD8+ T-cells were shown to be reactive in human subjects.

BETA-CELL STRESS AS A CONTRIBUTOR TO NEOEPITOPE GENERATION

There are many factors that could theoretically lead to the production of and reactivity to autoantigens in a subset of beta-cells (**Figure 1**). Beta-cells could produce a modified protein *via* genetic or epigenetic up-regulation of transcription, or increased translation. Alternatively, errors in mRNA transcription, splicing, or translation processes could generate neoantigens from modified proteins (24, 59), and they could build up as a result of impaired quality control of the modified protein in the endoplasmic reticulum (ER), post-ER compartments, or during proteosomal degradation (**Figure 1**). Autoantigens may also result from increased expression or activity of PTM modifying enzymes (44). In the case of insulin, the T1D at-risk allele at rs3842753 (60–62) was reported to increase insulin production in a small number of human pancreata (60, 63, 64), a result supported by recent meta-analysis of single-cell RNA sequencing data (65). One might speculate that increased insulin production could both increase neoepitope production and beta-cell vulnerability to ER stress (66). This may be a contributing factor to the increased risk of T1D development observed with childhood obesity (67).

Increasing evidence points to the beta-cell itself as an active player in mediating such processes, thereby participating in its own destruction (3). Beta-cells have a highly developed ER, making it possible for individual cells to react rapidly to changes in metabolic demand and produce enormous amounts of insulin in a short time. On the other hand, this highly specialized secretory task makes the most active beta-cells highly vulnerable to ER stress, which is present to some degree even in normal basal conditions (66). When the demand for protein synthesis and folding overwhelms the capacity of the ER, the cell-autonomous Unfolded Protein Response (UPR) is initiated, aiming to restore ER homeostasis (68). This UPR response is mediated by three UPR 'sensors', which are inactive in physiological conditions through their association with the abundant ER chaperone GRP78. With an increased load of unfolded proteins in the ER, GRP78 is released from these UPR sensors, thereby initiating the UPR. When an excessive level of stress is maintained, the UPR fails, and beta-cell apoptosis is triggered (69, 70). ER stress has also been implicated in the generation of neoantigens (71), with increasing recognition of autoreactive T-cell clones specific for deamidated peptides in multiple experimental systems (44, 72). Of importance, such increased immunogenicity was observed when beta-cells were stressed with thapsigargin, but not with tunicamycin, an ER stressor acting through the blocking of glycoprotein synthesis. A similar increase in activity of TGM2 upon inflammatory stress with cytokines has been observed in rodent MIN6 cells and was associated with an increased number of deamidated peptides. Moreover, an increase in non-enzymatically mediated deamidations was observed in this model upon cytokine exposure (73). Finally, insulin DRIP polypeptides increase by Ca-2+ -mediated ER stress, shown by exposure of INS-DRiP-GFP transfected cells to thapsigargin (24).

As to the role of alternative splicing, it has been shown that the beta-cell alternative spliced repertoire is largely affected by the pro-inflammatory cytokines, interleukin-1β (IL1β) and interferon-γ (IFNγ), changing the expression of more than 30 RNA binding proteins, thereby affecting the splicing of more than 3000 genes involved in beta-cell function and survival (3, 59). In addition, PTMs, alternative splicing and first exon usage are induced by interferon-1α (IFNα) (74). The fact that such transcriptional regulation could lead to neoantigen formation was shown for IGRP, with generation of autoantibodies and CD8 T-cells against a pancreas specific IGRP alternative spliced form (75). Recent evidence further showed that the 'alternative splicing signature' is also seen in the immunopeptidome of HLA-A2 and HLA-A3 restricted epitopes, leading to the generation of islet-reactive CD8+ T-cells both in T1D patients and healthy subjects (21, 25). All these studies point to a role for ER stress in increasing the prevalence of a variety PTMs where pro-inflammatory cytokines are likely to expand the repertoire of proteins and transcripts generated by beta-cells.

ENVIRONMENTAL FACTORS IN THE GENERATION OF NEOAUTOANTIGENS

Viruses and other environmental triggers have been implicated in T1D and may not only contribute to beta-cell stress and production of neoepitopes, but may be responsible for antigen-specific targeting. One such potential pathway is molecular mimicry, where viruses, microbiota or other environmental targets express epitopes similar to those expressed on beta-cells. Cross-reactive T-cells against these epitopes have the potential to eliminate both the environmental stimulus (i.e. viruses) and pancreatic beta-cells (76–78). Cross-reactivity between epitopes present in Coxsackievirus B (CVB) and GAD65 have been reported (79, 80). Furthermore, a dominant epitope present in IA-2 elicited T-cell responses in relatives and shared sequence similarity with a protein of human rotavirus (81). This IA-2 epitope also had some identity and similarity to sequences in Dengue, cytomegalovirus, measles, hepatitis C, and canine distemper viruses, and the bacterium *Haemophilus influenzae*. Interestingly, two other IA-2 epitopes were similar to amino acid sequences in milk, wheat, and bean proteins (81). More recently, ZnT8-reactive CD8+ T-cell clonotypes were found to cross-recognize a *Bacteroides stercoris* mimotope (23). Based on this evidence, environmental factors have the potential to elicit autoreactive immune responses. In this scenario, environmental cues could be a requirement or a contributing factor for neoepitope formation in the pancreas.

DOES NEOEPITOPE GENERATION LEAD TO AUTOIMMUNITY AND BETA-CELL KILLING IN TYPE 1 DIABETES?

The identification and timing of T-cells reactive against antigens in the pancreas remains a challenging task. Further studies are needed, as illustrated by the apparent lack of correlation between the presence of antigen-specific cells in the periphery and in the pancreas (23). Given that most cells present in the islets have unknown reactivities, it becomes clear that we might be only looking at the tip of the autoreactive iceberg. Models indicate that only 1-2% of antigen-specific cells are enough to achieve effective killing (82). On the other hand, if this is the case, why does it take so long for T-cells to kill their target? It could be that T-cell access to islets is asynchronous due to the expression of different antigens in different islets, at different times. Or perhaps T-cells are not attracted to a given islet unless there is inflammation or a triggering event locally, one that might be able to generate neoantigens. Furthermore, there is little clinical evidence to suggest whether these processes are taking place before disease onset, at a later stage of the disease, or both. A recent longitudinal study on individuals at-risk indicate the presence of neoepitopes in early stages of T1D. Individuals progressing to T1D showed a predominant pro-inflammatory T-cell reactivity against few of the HIPs analyzed (55).

It is clear that individuals carrying certain HLAs are prone to autoreactivity. In this context, it is tempting to speculate that the fragility of beta-cells and their susceptibility to environmental insults and conditions of incremental stress are likely to further unbalance an already compromised genetic system. Considering that neoepitopes are likely to arise before or during disease development, we envision a scenario in which beta-cells themselves actively contribute to neoepitope formation. The upregulation of HLA-I in the islets prior to clinical diagnosis is a good indicator of the potential capacity of beta-cells to present self-epitopes to the immune system, a phenomenon that is not well understood, but that many are actively investigating.

In this regard, and closing the speculative circle, we hypothesize that an environmental insult (i.e. viral infection/s) could induce an anti-viral response and the local production of cytokines (i.e. type I interferons). Anti-viral response molecules can induce the upregulation of HLA-I. At the same time, viruses could induce a translational arrest in beta-cells, hampering insulin production. Also, other forms of environmental stress, such as chemicals, dietary components may cause an initial trigger leading to ER or oxidative stress.

These environmental insults may lead to the formation of a first wave of non-conventional proteins. Impaired clearance of such stressed or dying beta-cells expressing modified proteins will cause activation of APCs and presentation of neoepitopes to CD4+ T-cells, which in turn can trigger several immune responses, including B cell activation with autoantibody production, and the activation of antigen-specific effector T cells that can directly kill beta-cells presenting modified islet peptides. This first cascade of immune activation may in this way cause further beta-cell stress or death, generating an autoreactive loop, with further modification of beta-cell proteins and disease exacerbation. Moreover, epitope spreading to native epitopes may further amplify the immune response (Yang et al, submitted). This proposed model could provide evidence for a role of neoepitopes both in initiation and exacerbation of disease (**Figure 2A**). It may indeed be that different ways of stress are needed to activate specific types of PTMs, at different times and with unknown duration, during the disease course.

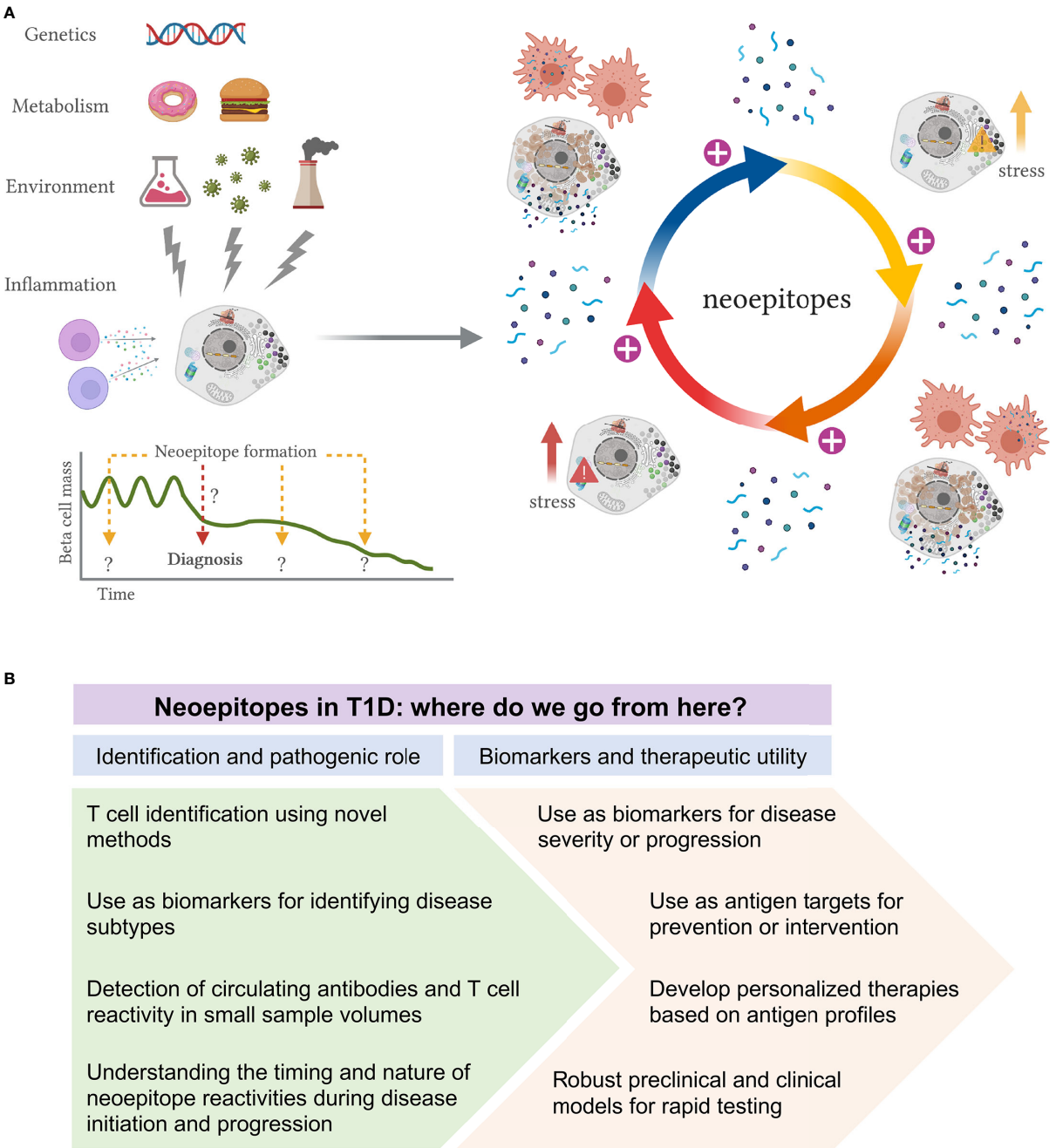

FIGURE 2 | Proposed model of neoepitope generation and roadmap to their identification and utility in T1D. **(A)** Schematic representation of how neoepitope generation could lead to autoimmunity and beta-cell killing in T1D. Genetic predisposition, metabolism, environmental insults and immune inflammation are some of the factors that may lead to the formation of a first wave of non-conventional proteins. Impaired clearance of such stressed or dying beta-cells expressing modified proteins will cause activation of APCs and presentation of neoepitopes to T-cells, which can trigger several immune responses. This first cascade of immune activation may cause further beta-cell stress or death, generating an autoreactive loop, with further modification of beta-cell proteins and disease exacerbation. This proposed model could provide evidence for a role of neoepitopes both in initiation and exacerbation of disease. It may indeed be that different ways of stress are needed to activate specific types of PTMs, at different times and with unknown duration, during the disease course. **(B)** Proposed roadmap to identification and utility of neoepitopes in T1D. Multiple neoepitopes have been identified in T1D and yet their precise role in the disease remains ambiguous. We envision two major areas of research: 1) to improve the identification of neoepitopes and to better delineate their role in disease pathogenesis; 2) to evaluate their use as biomarkers and their therapeutic utility.

WHERE DO WE GO FROM HERE?

To get a full picture of the neoepitope landscape, more studies should be carried out evaluating such responses in the early stages of T1D and through their progression to T1D to further delineate and describe the importance and timing of neoepitope formation in T1D. Elucidating their role in pathogenesis may also enable their use to improve the current staging paradigm, which is largely based on the presence of autoantibodies to classical islet antigens. Finally, while antigen-specific approaches have shown limited efficacy to date in T1D prevention and treatment, it is feasible that targeting neoepitopes in addition to or instead of classic antigens may provide a better therapeutic benefit.

Understanding how neoantigens are involved in the break of tolerance will lead us towards tolerogenic therapies for T1D. Multiple antigens have been used in T1D trials (83, 84). While no trial achieved its primary outcome, two trials have shown partial successes. First, in a trial where oral insulin was administered to individuals with stage 1 T1D, a pre-defined subgroup had a significantly delayed time to diabetes development (85). Furthermore, in a trial with intranodal injection of GAD65, a predefined subgroup showed preservation of beta-cell function (https://www.diamyd.com/docs/pressClips.aspx?ClipID=3768129). Other groups are exploring combination of antigens and/or antigens plus immunomodulators. Given the limited success of single native antigen in inducing tolerance, the presence of epitope spreading in T1D and the evidence for neoantigen generation and autoreactivity, it is quite feasible that targeting multiple (neo) antigens could aid in promoting antigenic tolerance.

Based on current evidence, we wish to outline two major areas of research to better delineate their exact role, which is a prerequisite for their therapeutic utility (**Figure 2B**). First, to identify novel epitopes that are most relevant to T1D progression, it will be necessary to develop and use novel detection and analysis methods with improved sensitivity and capacity to identify the nature of the peptides that are presented (86). In turn, being able to assay for T-cell reactivity, even in the periphery, remains a major challenge. The identification of novel epitopes could open opportunities for the characterization of disease subtypes and broaden our understanding of the disease pathogenesis. More efforts on the assessment of circulating antibodies and T-cell reactivity in longitudinal samples are needed to fully understand the timing and nature of neoepitope autoreactivity. Although sample volume continues to be a limitation, especially in studies of pediatric population, we anticipate that new technologies will be able to significantly improve our capacity to detect autoreactive cells in these challenging samples.

It is conceivable that neoepitopes may be used alone or in conjugation with other antigens as biomarkers for disease severity and/or progression, with evident potential to become immune-modifying therapies to induce tolerance in T1D. We could imagine a scenario in which each diabetic patient has its own antigenic profile, which could be used towards personalized medicine. However, more robust preclinical models or *in vitro* systems to test antigenic candidates should be developed prior to entering clinical studies. In all, neoepitopes possess a yet untapped mechanism to provide better biomarkers for staging progression as well as therapeutic targets, and could be the key to understanding the loss of tolerance in T1D.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

TR-C, JDJ, LO and JLD conceived the concept and co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Identifying New Hybrid Insulin Peptides (HIPs) in Type 1 Diabetes

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In 2016 Delong et al. discovered a new type of neoepitope formed by the fusion of two unrelated peptide fragments. Remarkably these neoepitopes, called hybrid insulin peptides, or HIPs, are recognized by pathogenic $CD4^+$ T cells in the NOD mouse and human pancreatic islet-infiltrating T cells in people with type 1 diabetes. Current data implicates $CD4^+$ T-cell responses to HIPs in the immune pathogenesis of human T1D. Because of their role in the immune pathogenesis of human T1D it is important to identify new HIPs that are recognized by $CD4^+$ T cells in people at risk of, or with, T1D. A detailed knowledge of T1D-associated HIPs will allow HIPs to be used in assays to monitor changes in T cell mediated beta-cell autoimmunity. They will also provide new targets for antigen-specific therapies for T1D. However, because HIPs are formed by the fusion of two unrelated peptides there are an enormous number of potential HIPs which makes it technically challenging to identify them. Here we review the discovery of HIPs, how they form and discuss approaches to identifying new HIPs relevant to the immune pathogenesis of human type 1 diabetes.

Keywords: hybrid insulin peptides (HIPs), $CD4^+$ T cell, autoimmunity, type 1 diabetes, epitope

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease caused the T-cell mediated destruction of the pancreatic beta cells (1). How and why the immune system turns against otherwise healthy tissue, to cause an autoimmune disease, is currently not known. However, for T1D it is clear that T-cells infiltrate the islet of Langerhans within the pancreas and destroy the insulin producing beta cells (2).

Genetic studies have revealed that risk of developing T1D is associated with class II HLA (3). The strongest genetic risk for developing T1D is associated with the HLA class II haplotypes HLA-DR3-DQ2 (Odds ratio (OR)>3.6) and HLA-DR4-DQ8 (OR>11.37) (3). Within these haplotypes the HLA-DQ alleles confer the majority of the risk (4). Intriguingly, people who are HLA-DR3-DQ2; DR4-DQ8 heterozygous are at greatest risk (OR>16.6) of developing type 1 diabetes (3). HLA-DQ2/

8 heterozygous antigen presenting cells express four types of HLA-DQαβ heterodimers. The 'regular' HLA-DQ2*cis* and –DQ8*cis* (α and β chains encoded on the same chromosome) and the '*trans*' dimers (α and β chains encoded on different chromosomes) (5). Collectively, this work suggests that $CD4^+$ T-cell responses against beta cell derived antigens play a central role in the pathogenesis of T1D, the identity of the antigens targeted by pathogenic $CD4^+$ T cells has not been fully resolved.

Insulin and its precursor, proinsulin, have long been considered to be central to the autoimmune pathogenesis of T1D (1, 6). Proinsulin is the major protein product of beta cells accounting for ~10% of the cell's protein (7). Mature insulin forms when the central 'C-peptide' of proinsulin is excised in the beta-cell granules leaving mature insulin and 'free' C-peptide (8). A genetic susceptibility locus maps to a variable number of tandem repeats (VNTR) upstream of the insulin gene and regulates its expression in the thymus, which in turn affects central tolerance to (pro)insulin (9). This locus is strongly associated with risk of developing type 1 diabetes (OR>2.5), second only to the HLA (10). In 2015, Pathiraja et al. (11) were the first to show that proinsulin specific $CD4^+$ T cells infiltrate human islets in type 1 diabetes. Human islet-infiltrating $CD4^+$ T cells recognize several epitopes derived from the C-peptide presented by HLA-DQ8, or DQ8*trans* (11).

THE DISCOVERY OF HYBRID INSULIN PEPTIDES (HIPS)

An important twist in proinsulin's role in T1D came in 2016 when Delong et al. (12) described hybrid insulin peptides (HIPs). A hybrid insulin peptide, or HIP, is a $CD4^+$ T-cell epitope that is formed by the posttranslational fusion of two peptide fragments [reviewed by (13)]. To date, as the name suggests, at least one of the peptide fragments derives from insulin or proinsulin. The peptide fragments that form a HIP can be derived from the same parental protein molecule(s), or from two distinct proteins. Delong and Haskins' goal was to identify the epitopes recognized by a family of T-cell clones, known as BDC, that had been isolated from NOD mice (14). Importantly, many of the BDC lines were known to cause diabetes (15). Because of their clear pathogenic role, the BDC clones have been well studied, particularly BDC-2.5 (16, 17). However, the naturally arising agonist epitope for BDC-2.5, and many other BDC clones, remained elusive. Several mimotopes peptides were identified that stimulated BDC-2.5 (18–20), but a broadly accepted, naturally arising, target antigen was not found. Despite this, it became clear that an epitope from the beta-cell secretory granule protein, chromogranin-A (ChgA) was, at least, a partial target (21, 22). More specifically, it was clear that the WE14 fragment of chromogranin-A could stimulate the BDC-2.5 cells albeit weakly. Synthetic peptide analogues of the WE14 fragment were made with amino acid substitutions at each position and tested for their capacity to stimulate BDC-2.5 cells. This analysis showed that substitutions at the carboxyl terminus of the putative epitope impacted upon BDC-2.5 stimulation (22). Furthermore, a membrane extract from a beta-cell tumor was a more potent stimulator of BDC-2.5 than synthetic WE14 peptide (22) - clearly something was missing.

Delong, Haskins and co-workers resolved the enigma of the natural agonist for BDC-2.5 (and several other BDC lines) when they made the intuitive leap that $CD4^+$ T-cell epitopes could form by fusion of two peptide fragments. They showed, by mass spectrometry and functional T-cell assays, that the missing half of the epitope was a fragment of the C-peptide from proinsulin (LQTLAL). The optimal agonist for BDC-2.5 (and BDC-9.46 and BDC-10.1) was a peptide formed by the fusion of a fragment of C-peptide with a fragment derived from WE14 (LQTLAL-WSRMD). A similar HIP (LQTLAL-NAARD) formed by the same C-peptide fragment (LQTLAL) fused to a fragment of IAPP2 (NAARD) was also identified. These new types of peptides were called 'hybrid insulin peptides' or HIP. Titration experiments, using synthetic peptides, revealed that a Cpeptide-ChgA HIP was an extremely potent (at nM concentrations) stimulator of BDC-2.5 $CD4^+$ T cells, making it a very plausible candidate for the natural agonist for BDC-2.5. Solving the mystery of the ligand for BDC-2.5 was interesting, but it was not clear if HIP specific $CD4^+$ T cells arose in people with T1D and if they played any role the pathogenesis of T1D. We addressed this question by testing a panel of 16 putative human HIPs, designed based on the HIPs identified in the NOD mouse, against of human islet-infiltrating $CD4^+$ T-cell clones. From these experiments we identified two clones that responded to a Cpeptide-IAPP2 HIP (12). Independently, Sally Kent's group screened the same panel of 16 HIPs and identified an islet-infiltrating $CD4^+$ T-cell line that responded to a Cpept-NYP HIP (12). Subsequently, further evidence for a pathogenic role of $CD4^+$ T-cell responses to HIPs has accumulated. For example, further screening of this panel of 16 HIPs against human islet-infiltrating T-cells lines revealed responses to Cpept-IAPP1, Cpept-InsA and Cpept-IAPP2 HIPs (23) (See **Table 1**). Analysis of HIP specific responses in the PBMC of people with recent onset T1D revealed responses to several HIPs that were less frequently detected in control subjects who did not have T1D (25).

HOW DO HIPS FORM?

Protease-mediated peptide splicing, or transpeptidation, has been described in bacteria (26), plants (27), *in vitro* (28) and in humans. In humans, proteasome-mediated protein splicing generates epitopes recognized by tumor specific $CD8^+$ T cells (29, 30). In all cases energy released during proteolysis is 'recycled' to drive the formation of a peptide bond (30). Under most circumstances this 'reverse proteolysis' is very inefficient, but high protein concentrations in a confined environment favor protease mediated peptide fusion (31). Beta-cell granules are extremely densely packed with insulin, C-peptide and several other proteins including chromogranins and amyloid proteins (32). Furthermore, the beta-cell granules are the site of very active proteolysis. Many proteins found in the beta cell granules,

TABLE 1 | Summary of currently known HIPs.

Proteins	HIP amino acid sequence	HLA	Origin of T cells	Ref
Cpept-IAPP2	GQVELGGG-NAVEVLK	DQ8	Islet-infiltrating T cells	(12)
Cpept-Neuropept Y	GQVELGGG-SSPETLI	ND	Islet-infiltrating T cells	(12)
Cpept-InsA	GQVELGGG-GIVEQCC	ND	Islet-infiltrating T cells	(23)
Cpept-IAPP1	GQVELGGG-TPIESHQ	ND	Islet-infiltrating T cells	(23)
Cpept-IAPP2	GQVELGGG-NAVEVLK	ND	Islet-infiltrating T cells	(23)
InsB-SG1	VCGERGFF-EELVARSE	DR4	PBMC	(24)
InsB-SG1	HLVEALYL-EELVARSE	DR4	PBMC	(24)
InsA-InsB	ICSLYQLE-FVNQHLCG	DR4	PBMC	(24)
Cpept/InsA-SG1	SLQKRGIV-EELVARSE	DR4	PBMC	(24)
InsA-SGV	CSLYQLEN-SVPHFSDE	DR4	PBMC	(24)
Cpept-GRP78	QPLALEGS-ALSSQHQA	DR4	PBMC	(24)

including proinsulin (PI), chromogranins (Chg) and islet amyloid polypeptide (IAPP), are cleaved by the granule proteases to generate an array of bio-active peptides (32). However, Wan et al. (33) reported that analysis of mass spectrometry data from beta-cell crinosomes revealed the presence of HIPs suggesting that HIPs form in the crinosomes. More recently, Reed et al. (34) reported that when the appropriate peptides are digested by the lysosomal protease, cathepsin L, HIPs that activate the lines BDC-2.5 and BDC-6.9 are generated. This led to the suggestion that HIPs form when senescent insulin granules fuse with lysosomes to form crinophagic granules. The high concentration of insulin granule proteins and cathepsin-L favors the formation of HIPs. Crinophagic granules can be taken up by antigen presenting cells allowing the HIPs to be presented to $CD4^+$ T cells (35). Nonetheless, the precise location(s) of HIP formation remain unclear. C-peptide can be cleaved at sites other than the dibasic residues at the B-C and C-A chain junctions (36). This indicates that other proteases may mediate the formation of HIPs. It also remains possible that transpeptidation and HIP formation occurs in both granules and crinosomes.

IDENTIFYING NEW HIPS

Identification of 'regular' T-cell epitopes has been scientific challenge, but over the past decades significant progress has been made (37). Progress has been facilitated by advances in peptide synthesis techniques, T-cell cloning (38), TCR expression, recombinant DNA techniques and mass spectrometry. Mapping of 'regular' T-cell epitopes usually reduces the target epitope from a cell, or microbe, to a protein and from there an ~8-14 amino acid peptide. However, in the case of HIPs the process is more complex, because, theoretically at least, any protein fragment can fuse with any other protein fragment. HIPs can form from fragments of the same proteins, or from two different proteins, and the fragments can fuse in either order. These possible combinations give rise to an enormous array of HIPs that are, theoretically at least, possible. For example, from a protein of 100 amino acids we calculate that there are 9,756 possible HIPs (**Figure 1**). This only included possible HIPs formed by fragmentation and fusion of a single 100 amino acid protein. Once other candidate proteins are considered as potential HIP fragment donors the number of possible HIPs becomes extremely large. Below we discuss the different approaches to identifying and validating novel HIPs at scale.

Mass spectrometry has been invaluable for the identification of HIPs. The advantage of mass spectrometry is that a large number of sequences can be identified rapidly. Candidate T cell epitopes have been identified by elution of peptides from HLA/MHC on the surface of antigen-presenting cells. However, this approach is not feasible with HIPs which are present at very low concentrations in beta cells. Furthermore, since HIPs aren't directly encoded new algorithms have been developed to facilitate the identification of HIPs, or $CD8^+$ T-cell epitopes formed by proteosome mediated peptide splicing (39, 40). Nonetheless, a rigorous mass spectrometry work flow for the identification of HIPs from beta-cell extracts has been developed (41).

One of the disadvantages of mass spectrometry is large numbers of cells are usually required to isolate sufficient material for analysis. This makes the analysis of primary beta cells difficult. Delong et al. solved this problem by using beta cell tumors from RIP-TAg mice. More recently they have adapted their work-flow to allow for smaller numbers of cells (41) and developed internal controls to allow quantifiable confidence when matching synthetic and biologically derived peptides (42). Mass spectrometry analysis provides good evidence for the presence of a peptide, but it does not give any insights into the capacity of T cells to respond to a particular peptide for their relationship between a $CD4^+$ T-cell response to an epitope and the disease pathogenesis. For this reason, validation of candidate HIPs with functional T-cell assays, as described above, is essential.

Arribas-Layton et al. (24) used a tetramer-based approach to identify new HIPs. They started with a database of 7,654 candidate HIPs formed by fusion of fragments of proinsulin with a variety of beta cell granule proteins. This list of peptides was stratified by predicted binding to HLA-DR4 (DRB1*04:01). The 50 peptides with the strongest predicted binding affinity to HLA-DR4 were then screened in competitive HLA binding assays and 30 confirmed HLA-DR4 binding peptides were chosen. These 30 HIPs were used to make HLA-DR4/HIP

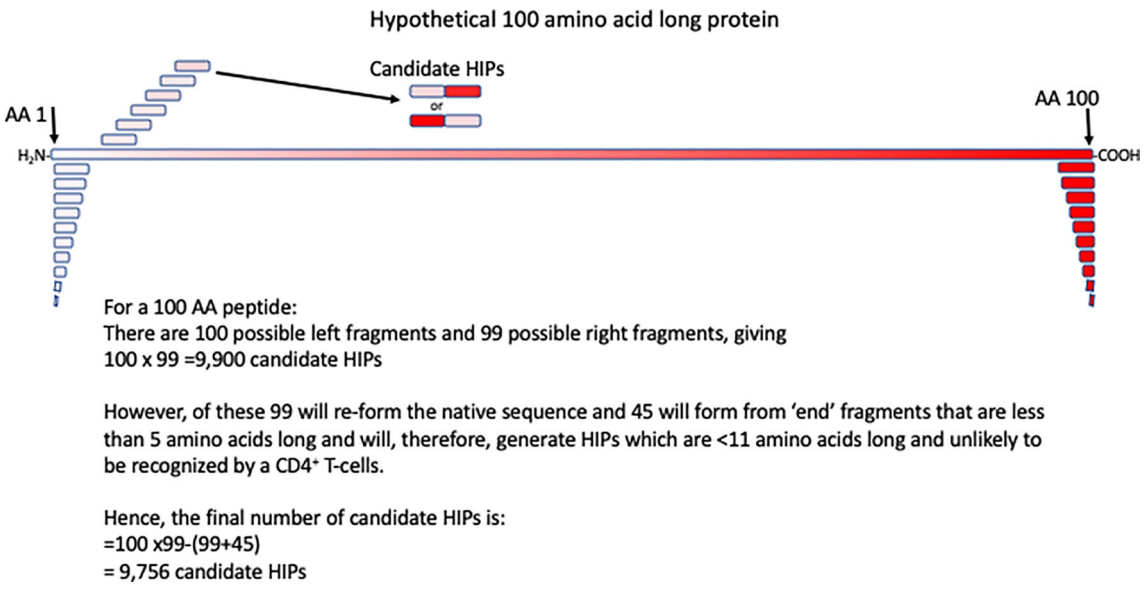

FIGURE 1 | Calculating the number of HIPs that could be generated from a 100 amino acid (AA) long protein. A hypothetical 100 amino acid long peptide is indicated by the red box. The first few of all possible 10 amino acid fragments, derived from this protein are shown in red. Potential HIPs, in both orientations of the composite fragments are shown. Assumptions made in calculating the number of potential HIPs that may form from a 100 amino acid protein are shown.

tetramers. Finally, PBMC from individuals with T1D were stimulated with pools of HIP peptides and cytokines for two weeks, then the expanded cells were stained with DR4/HIP tetramers. This work led to the identification of six new, HLA-DR4 restricted HIPs (**Table 1**).

DISCUSSION

The work summarized above highlights the power of combining functional T-cell responses with proteomic analysis. Observations using T-cell responses support the proteomics data and, in turn, the proteomics supports the immunology. This work brings us to the current question: is it possible to develop a high throughput and efficient protocol for identifying HIPs that are relevant to the immune pathogenesis of human T1D? If so, what would such a protocol look like? The primary challenge, as outlined above, is the enormous number of potential HIPs to be screened. Proteomic approaches use fractionation of tissue lysates and screening with disease-relevant T cells. While this approach worked for Delong et al. (12), who had the BDC lines and RIP-Tag beta cells, it will be challenging to apply this to human studies because sufficiently large numbers of beta cells are not readily available and $CD4^+$ T cells of clear clinical relevance are not available. $CD4^+$ T cells that infiltrate human islets (11, 43) are available, but screening large numbers of candidate HIPs against large numbers of islet-infiltrating T cells remains a challenging task.

The development of improved synthetic peptide and DNA technologies increases the feasibility of screening approaches. Large libraries of candidate HIPs can be generated *in silico* and as peptides or synthetic DNA. For example, Arribas-Layton et al. refined their pool of over 7,000 candidates HIPs by predicting and then confirming binding to HLA-DR4. This gave them a manageable number of HIPs to screen with PBMC from people with T1D using DR4/HIP tetramers. The limitation of this approach is that it focuses on a single HLA restriction and relies upon the accuracy of the HLA-binding predictions.

An ideal HIP-identification protocol would have the following features: (i) it would allow the screening of very large numbers of candidate HIPs, reducing the incentive to use HLA binding predictions to enrich for candidates with higher predicted binding capacities. (ii) it would screen full haplotypes of T1D-associated HLA alleles at one time, avoiding the need to screening for a particular restriction. (iii) it would identify epitopes based on their capacity to stimulate disease relevant T cells, such as $CD4^+$ T cells isolated from the islets of organ donors who had T1D. The frequency of beta cell antigen specific T cells in peripheral blood is too low to allow these cells to be used in screening assays. Expansion of cells of possible relevance biases the screen towards the specificities already thought to be important. To our knowledge, an assay that meets all these criteria does not currently exist. Recent developments may make such an assay feasible. For example, the T-Scan protocol (44), which can screen large numbers of candidate $CD8^+$ T-cell epitopes, has many of the desirable feature. Our challenge now is to develop a comparable assay that can be used to identify human $CD4^+$ T-cell epitopes, including HIPs.

The discovery of HIP peptides has revealed a new array of targets for the autoimmune responses underlying type 1 diabetes. Discoveries made using the NOD mouse have rapidly been supported by experiments using human materials. It is now

clear that HIPs do form in beta cells and can be the targets of autoimmune responses. However, it seems very likely that we have only identified a fraction of the HIPs that may play a role in T1D. Emerging approaches to identify and validate new HIPs. Once defined these epitopes will be of great interest, and utility, in T cell assays to monitor changes in beta cell autoimmunity (45) and antigen-specific therapies for T1D.

AUTHOR CONTRIBUTIONS

SM and PB conceived the scope and focus of the review. SM wrote the first draft. AR, RW and PB contributed to the editing and preparation of the final manuscript. All authors contributed to the article and approved the submitted version.

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Characterization of Human CD4 T Cells Specific for a C-Peptide/ C-Peptide Hybrid Insulin Peptide

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Hybrid Insulin Peptides (HIPs), which consist of insulin fragments fused to other peptides from β-cell secretory granule proteins, are CD4 T cell autoantigens in type 1 diabetes (T1D). We have studied HIPs and HIP-reactive CD4 T cells extensively in the context of the non-obese diabetic (NOD) mouse model of autoimmune diabetes and have shown that CD4 T cells specific for HIPs are major contributors to disease pathogenesis. Additionally, in the human context, HIP-reactive CD4 T cells can be found in the islets and peripheral blood of T1D patients. Here, we performed an in-depth characterization of the CD4 T cell response to a C-peptide/C-peptide HIP (HIP11) in human T1D. We identified the TCR expressed by the previously-reported HIP11-reactive CD4 T cell clone E2, which was isolated from the peripheral blood of a T1D patient, and determined that it recognizes HIP11 in the context of HLA-DQ2. We also identified a HIP11-specific TCR directly in the islets of a T1D donor and demonstrated that this TCR recognizes a different minimal epitope of HIP11 presented by HLA-DQ8. We generated and tested an HLA-DQ2 tetramer loaded with HIP11 that will enable direct *ex vivo* interrogation of CD4 T cell responses to HIP11 in human patients and control subjects. Using mass spectrometric analysis, we confirmed that HIP11 is present in human islets. This work represents an important step in characterizing the role of CD4 T cell responses to HIPs in human T1D.

Keywords: CD4, hybrid insulin peptides, autoimmunity, mass spectrometry, type 1 diabetes, neoepitope, tetramer

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease driven by T cells specific for pancreatic β-cell autoantigens. Identifying β-cell antigens targeted by autoreactive T cells is an important step in understanding the etiopathogenesis of T1D. Post-translational modification of proteins can generate neo-epitopes in the periphery that are not represented in the thymus, providing a mechanism by which autoreactive T cells can evade central tolerance mechanisms and mediate β-cell destruction in the pancreatic islets. Hybrid Insulin Peptide (HIP) formation is a recently discovered modification that may

play a key role in the generation of primary targets of autoimmunity in T1D (1). HIPs are formed by covalent fusion of the N-terminus of β-cell secretory granule peptides (a right peptide) to the C-terminus of truncated insulin peptides (a left peptide) through a peptide bond. The resulting HIPs contain amino acid sequences not encoded by the genome, making them plausible targets for autoreactive T cells in T1D. We have identified HIPs in murine and human islets by mass spectrometry (1, 2), and a separate group recently demonstrated that HIPs can be found in the major histocompatibility complex (MHC) class II peptidome of mice (3).

In contrast to conventional post-translational modifications, such as phosphorylation or deamidation, which alter the side chain of a single amino acid residue, HIP formation leads to the replacement of an entire sequence of amino acids within insulin. This could have marked consequences for how a HIP, when compared to the unmodified precursor, binds to a given human leukocyte antigen (HLA) molecule and interacts with a specific T cell receptor (TCR). This, in turn, could result in recognition of a HIP in the periphery by T cells that did not recognize the corresponding unmodified peptides in the thymus during negative selection.

The role of HIPs as autoantigens in the non-obese diabetic (NOD) mouse model of T1D is clearly established. We have demonstrated that HIPs are the peptide ligands of diabetogenic CD4 T cell clones isolated from NOD mice (1). HIP-specific CD4 T cells display an inflammatory phenotype and can be detected in the pancreatic lymph nodes (pLN), islets, and spleen of prediabetic and diabetic NOD mice using MHC class II tetramers (4). The frequency of HIP-specific CD4 T cells in the blood of NOD mice increases with age, as do the percentages of HIP-specific CD4 T cells that display an activated phenotype, suggesting that these cells may serve as biomarkers of disease progression (4).

Evidence supporting an important role for HIPs as autoantigens in human T1D is also mounting. Multiple CD4 T cell clones/lines specific for different HIPs have been isolated from the residual islets of human organ donors with T1D (1, 5–7). As recently reported, we screened peripheral blood mononuclear cells (PBMCs) from T1D patients or controls for reactivity to a panel of HIPs by IFN-γ enzyme-linked immunosorbent spot (ELISpot) analysis (6). We observed statistically significant responses from patient PBMC to four of the HIPs tested. One of those HIPs (HIP11) is a hybrid peptide formed by the fusion of a C-peptide fragment with the N-terminus of C-peptide. Robust responses to HIP11 were detected in samples from more than 20% of the 35 patients tested (6). Here, using the CD4 T cell clone E2 isolated from the PBMCs of a new onset T1D patient (6), we describe the further characterization of the CD4 T cell response to HIP11, advancing our understanding of HIPs as autoantigens in T1D and providing a foundation for future efforts to investigate responses to HIPs during the development of T1D.

MATERIALS AND METHODS

Peptides

The list of peptides used in this study were obtained commercially at >95% chromatographic purity and are listed in **Supplementary Table 1**.

HIP Nomenclature

To avoid ambiguity and confusion, we developed a systematic nomenclature to describe the large diversity of potential HIP sequences. HIPs are identified using standard abbreviations for the proteins from which the left and right peptides originated (*e.g.*, Ins, IAPP, CgA), separated by a dash. Next to each abbreviation, a subscript is used to describe the residues from the parent protein that span the sequence. For easy reference, insulin peptides are designated as "insA", "insB", or "insC", indicating that the peptide originated from the A-chain, B-chain, or C-peptide region of insulin, respectively, and numbering is based on the position of the residues within the specific peptide. For peptides from all other proteins, residues are numbered based on their position in the pre-proprotein, with position 1 being the N-terminal residue of the signal peptide. For example, the HIP sequences DLQVGQVELGGGPGA GSLQPLAL-EAE and SLQPLAL–EAEDLQV (hyphen indicates the HIP junction) referenced in this study are designated $insC_{4\text{-}26}$ – $insC_{1\text{-}3}$ and $insC_{20\text{-}26}$ – $insC_{1\text{-}7}$, respectively.

The defining feature of any individual HIP is its junction region. Trimming of a HIP – as may occur within an antigen-presenting cell, the proteasome, or with a protease used during sample preparation prior to LC-MS/MS analysis – could lead to the generation of peptides of different lengths that all represent the same HIP (*i.e.*, they contain the same junction). Common names can be used to refer to a specific HIP junction. For example, the HIP generated when an insulin C-peptide fragment ending with residue 26 is fused to the N-terminus of C-peptide ($insC_{x\text{-}26}$ – $insC_{1\text{-}x}$) has been designated HIP11 (1). Both $insC_{4\text{-}26}$ – $insC_{1\text{-}3}$ and $insC_{20\text{-}26}$ – $insC_{1\text{-}7}$ are considered HIP11 peptides.

Sequencing and Generation of E2a and E2b Transductants

TCRα and TCRβ chain genes were amplified by 5'RACE-PCR, followed by sequencing on an MiSEQ sequencer (Illumina) as previously described (6). V- and J-gene usages as well as CDR3 sequences were then analyzed by IMGT/V-QUEST (http://www.imgt.org). The determined TCRα and TCRβ chain genes were expressed on a 5KC hybridoma cell line transduced with the NFAT-ZsGreen-1 reporter construct using a retroviral expression system as previously described (8). Briefly, a murine stem cell virus-based retroviral vector encoding TCRα and TCRβ chain genes connected by a porcine-2A peptide was generated, and the reporter 5KC cell line was spinfected with a virus supernatant, followed by MACS-based enrichment of transduced cells using magnetic beads conjugated with anti-CD3 antibody. The E2a and E2b transductants (2×10^4 cells/well) were cultured with or without peptides along with K562 cells expressing DQ2 (DQA1*05:01-DQB1*02:01) or DR3 (DRA1*01:01-DRB1*03:01) at 5×10^4 cells/well in a round-bottom 96 well plate overnight, followed by evaluating ZsGreen-1 expression by the TCR transductants on a Cytoflex flow-cytometer (Beckman Coulter).

GSE.8E3 Transductant Activation Assay

GSE.8E3 transductant cells (2×10^4 cells/well), produced by the same method used to generate the E2a and E2b transductants,

were cultured with various concentrations of peptides in the presence of K562 cells expressing DQ8 (DQA1*03:01-DQB1*03:02) or DQ8*trans* (DQA1*05:01-DQB1*03:02) at 5 x 10^4 cells/well in a round-bottom 96 well plate overnight. ZsGreen-1 expression by the TCR transductants was analyzed on a Cytoflex flow-cytometer (Beckman Coulter).

T Cell Clone Assays

Early-passage CD4 T cell clones (E2, a HIP11-reactive clone and B11b, a HIP4-reactive clone) were thawed and restimulated with HIP11 or HIP4, respectively, in the presence of irradiated autologous EBV-transformed B-cell line as antigen-presenting cells (APC). Twenty-four hours later, IL-2 and IL-4 were added to the T cell cultures for T cell expansion. T cells were maintained in culture for 14-28 days before functional assays were performed. For T cell assays, CD4 T cell clones (0.5 – 5 x 10^5) were incubated either with the irradiated autologous EBV-transformed B-cell line (0.5 – 5 x 10^5 cells) or with irradiated PBMC from a partially HLA matched donor, in the presence or absence of antigen at indicated concentrations. For IFN-γ and TNF-α ELISA, supernatants were collected 24-48 hours after culture and analyzed using a kit from eBiosciences according to manufacturer's protocol. To determine the DR- or DQ-HLA restriction of HIP responses, the antigen assay was performed in the presence and absence of anti-DR (L243) or anti-DQ (SPV-L3) antibodies. The EBV-transformed cell lines were first pulsed with peptide (50 μg/ml) and washed twice with AIM V medium. Antibodies were added at a final concentration of 1 μg/ml.

Flow Cytometry

For the CD25 upregulation assay, 1 x 10^5 CD4 T cells were cultured with an autologous EBV-transformed B cell line (1 x 10^5 cells) in the presence or absence of peptide. After 24 hours, cells were harvested, washed, and stained for CD4, CD25 and viability (efluor780, Invitrogen) before analysis. Antibodies used for staining of T cells were anti-CD4 BV711 (SK3; BD Biosciences) and anti-CD25 BV421 (M-A251; BD Biosciences). Gating strategies are indicated in each figure; the lymphocyte gate was based on FSC/SSC properties and the singlets gate was based on the FSC-A/FSC-H. Samples were run on a BD Fortessa X-20 flow cytometer (5 lasers), a Cytek Aurora (5 lasers) or a CytoFlex (Beckton Dickinson) flow cytometer (3 lasers), and 5,000 – 50,000 cells were acquired for analysis. Data were analyzed using FlowJo v10 software (Tree Star, USA).

Preparation of Islet Proteins

Islets from a non-diabetic human donor (64 year-old male) were obtained from the University of Alberta Diabetes Institute Islet Core (Edmonton, Alberta, Canada). Upon arrival, islets were washed with phosphate-buffered saline (PBS) and then pelleted. The supernatant was removed and tubes were placed in liquid nitrogen to rapidly freeze the islet pellets. Islets were stored at -80°C until use. Islets were processed as described previously (2). Briefly, islets were thawed and lysed in 50% trifluoroethanol with heat and sonication, and cellular debris was pelleted by high-speed centrifugation. The supernatant, which contained extracted islet proteins, was fractionated by size exclusion chromatography (SEC). SEC fractions were then digested overnight at 37°C with the endoproteinase AspN (cleaves at the N-terminal side of aspartic acid residues) in buffer supplemented with zinc sulfate. Digested samples were dried in a vacuum concentrator and then reconstituted in loading buffer (2.7% acetonitrile/0.1% formic acid/water), sonicated, and spun at 17,000 x g for 2 minutes to remove any insoluble material. The supernatant was then analyzed by liquid chromatography tandem-mass spectrometry (LC-MS/MS).

LC-MS/MS Analysis of Human Islet Proteins

Samples were analyzed by LC-MS/MS using an Agilent 1200 series UHPLC system with a nanoflow adapter and an Agilent 6550 Q-TOF equipped with a nano-ESI source. Samples were separated online by reversed-phase liquid chromatography using a trap forward-elute configuration (trap column: Thermo Acclaim Pepmap 100, 75 μm x 2 cm, 3 μm particles, 100Å pores; analytical column: Thermo Acclaim Pepmap RSLC C18 analytical column, 75 μm inner diameter, 2 μm particles, 100Å pores). A water/acetonitrile gradient was used (buffer A: 0.1% formic acid in water; buffer B: 0.1% formic acid and 90% acetonitrile in water). Mass spectrometric data was collected in positive ion mode with an MS scan range of 290-1700 m/z, an MS acquisition rate of 5 spectra/sec, an MS/MS scan range of 50-1700 m/z, and a minimum MS/MS scan rate of 3 spectra/sec. Abundance dependent accumulation was enabled with a target of 80,000 counts/spectrum. Using auto-MS/MS mode, the ten most abundant precursors per cycle were selected for fragmentation (absolute threshold: 3000 counts; relative threshold: 0.01%). Singly-charged precursors were excluded.

Analysis of Mass Spectrometric Data

Data were searched using the Spectrum Mill MS Proteomics Workbench (Agilent, Rev B.06.00.201) and the SwissProt human proteome database. For data extraction, spectrum merging was enabled based on precursor selection purity, spectral similarity, retention time (2-minute window), and m/z. Search settings were as follows: instrument = Agilent ESI Q-TOF; precursor mass tolerance = +/- 10 ppm; product ion mass tolerance = +/- 20 ppm; digest = no enzyme. Matches were considered valid if the following thresholds were satisfied: score > 10, percentage scored peak intensity (SPI) > 70%, and rank 1 minus rank 2 (R1-R2) score > 2.5. Spectra that could not be confidently matched to unmodified peptides in the SwissProt database were subjected to a second round of searches using the settings listed above, except oxidized methionine and deamidated asparagine/glutamine were considered as variable modifications and digest was set to AspN (maximum of two missed cleavages). Spectra that remained unmatched according to the validation filters listed above were searched against a custom database containing hypothetical human HIPs (2) using the same settings as in the first search.

Validation of HIP11 Identification Using Synthetic Peptide and the P-VIS Approach

The HIP11 peptide-spectrum match (PSM) was first evaluated by applying our previously-published criteria (2). A synthetic version of the HIP11 peptide (GenScript, >95% pure) was then

used to validate the match by applying the novel **P**SM **V**alidation with **I**nternal **S**tandards (P-VIS) approach recently developed by our group (9). In bottom-up proteomics, proteins from a biological spectrum are digested using a protease such as trypsin. The resultant peptides are analyzed by LC-MS/MS to generate fragmentation spectra. These spectra are then used to search a protein database, generating a large number of PSMs. Traditionally, to confirm that the correct sequence interpretation has been assigned to a particular fragmentation spectrum, the fragmentation spectrum of a synthetic version of the candidate sequence is compared to the fragmentation spectrum of the biological peptide in question using a metric such as the Pearson correlation coefficient. Since two spectra for the same peptide will not be entirely identical, the investigator decides subjectively if the similarity is high enough to indicate that the synthetic peptide and the biological peptide are the same. In the P-VIS approach (9), a set of internal standard peptides is spiked into both the biological sample and the synthetic peptide sample prior to LC-MS/MS analysis. For each internal standard peptide, the fragmentation spectra from the two samples are compared and the Pearson correlation coefficient is calculated. This information is used to determine a prediction interval for the Pearson correlation coefficient of a correct match. A similar process is used to determine if the chromatographic retention time of the biological peptide and synthetic peptide are close enough to indicate that the peptides are the same. PROCAL peptides (JPT Peptide Technologies) were used as internal standard peptides (10). LC-MS/MS analysis was performed as described above, except precursor selection was limited to those masses predicted for the peptides of interest. PSM_validator v1.4 (https://github.com/Delong-Lab/PSM_validator/releases) was used for data analysis with the following settings applied: pre_mz_tol = 20, pro_mz_tol = 20, abund_thresh = 500, PCC_abund_thresh = 10, min_score = 30, min_weighted_score = 25, min_pairs_PCC = 8, min _PCC = 0.7, RTtol = 0.6, min_RT = 10, max_RT = 90, manual_RTdev_thresh = 0, min_intstd = 10, percentile_thresh = 5, ion_type = "b/y".

RESULTS

Response to HIP11 by the E2 CD4 T Cell Clone Is DQ2-Restricted

The HIP11-reactive CD4 T cell clone E2 was isolated from the peripheral blood of a patient with T1D (patient 3196) and in a previous study, we determined that the E2 response to HIP11 was restricted to HLA-DQ (6). We further confirmed these findings using thymidine incorporation as a read-out for T cell proliferation (**Figure 1A**). Since patient 3196 carries one copy of the DQ2 haplotype (DQA1*05:01-DQB1*02:01) and one copy of the DQA1*03:02-DQB1*03:03 haplotype, our goal was to determine the presenting HLA element. Using donor PBMCs partially matched for each haplotype as APCs, we tested the ability of the two haplotypes to stimulate the E2 CD4 T cell clone to HIP11. The E2 T cell clone proliferated when stimulated with the HIP11 peptide $insC_{20-26}$ – $insC_{1-7}$ (SLQPLAL–EAEDLQV) presented by PBMCs homozygous for DQ2, but not when presented by PBMCs homozygous for DQA1*03:02-DQB1*03:03 (**Figure 1B**). As an additional control, we determined that mis-matched PBMCs homozygous for DQ8 (DQA2*0301-DQB2*0302) did not stimulate the E2 T cell clone either at the 1 μM or the 10 μM concentration. To provide additional evidence of the presenting element, we used an artificial antigen-presenting line (K562) devoid of any HLA molecules and transduced with DQ2 or HLA-DRB1*03:01 (DR3) as a negative control (8). Each K562 line (expressing either DQ2 or DR3) was pulsed with the HIP11 peptide and cultured with the E2 T cell clone (**Figure 1C**). After 18h, upregulation of CD25 was assessed by flow cytometry as a measure of T cell activation. The E2 CD4 T cell clone upregulated CD25 in response to HIP11 only when the K562 line was transduced with DQ2, but not DR3, confirming our previous observation that the E2 T cell clone is stimulated by HIP11 in the context of DQ2.

Identification of TCR Responsible for Recognition of HIP11 by the E2 Clone

TCR sequencing of the E2 CD4 T cell clone revealed that two different TCRα chains – along with a single TCRβ chain – were expressed at the RNA level (6). It has been shown that dual TCRα-expressing T cells can contribute to autoimmunity through different processes, including escape of central tolerance (11) and failure to generate thymically-derived Tregs reactive to self-antigens (12). To determine which TCRα was responsible for the recognition of HIP11, each TCRα chain was expressed individually in combination with the cognate TCRβ chain in a TCR-deficient hybridoma (5KC) engineered with a ZsGreen-1 fluorescent protein gene, under the NFAT promotor (8). Under these conditions, the TCR transductants express ZsGreen-1 when the TCR is engaged with the peptide-MHC complex, enabling monitoring of T cell activation by flow cytometry. The resulting transductants, E2a and E2b (expressing either TRAV6 or TRAV8-2/8-4 respectively), were then challenged with HIP11-pusled artificial APCs (K562 line expressing DQ2). The E2b – but not the E2a – transductant recognized HIP11 in the context of DQ2 (**Figure 1D**), demonstrating that the combination of the TRAV8-2/8-4 TCRα chain with the TRBV5-4 TCRβ chain is responsible for recognizing HIP11.

Characterization of a HIP11-Specific TCR From the Islets of a T1D Donor

We previously isolated T cell clones and lines from the residual islets of human T1D donors recognizing four different HIPs containing a sequence from the C-peptide linked to insulin A-chain, Islet Amyloid Polypeptide, or Neuropeptide Y (1, 5). Although the E2 clone, which was isolated from the peripheral blood of a T1D patient, is specific for HIP11, isolating a HIP11-reactive T cell clone from the islets – the actual site of autoimmune destruction – would further establish the relevance of HIP11 as an autoantigen in human T1D. Michels et al. previously generated transductants expressing TCRs identified by direct TCR sequencing of islet-infiltrating T cells

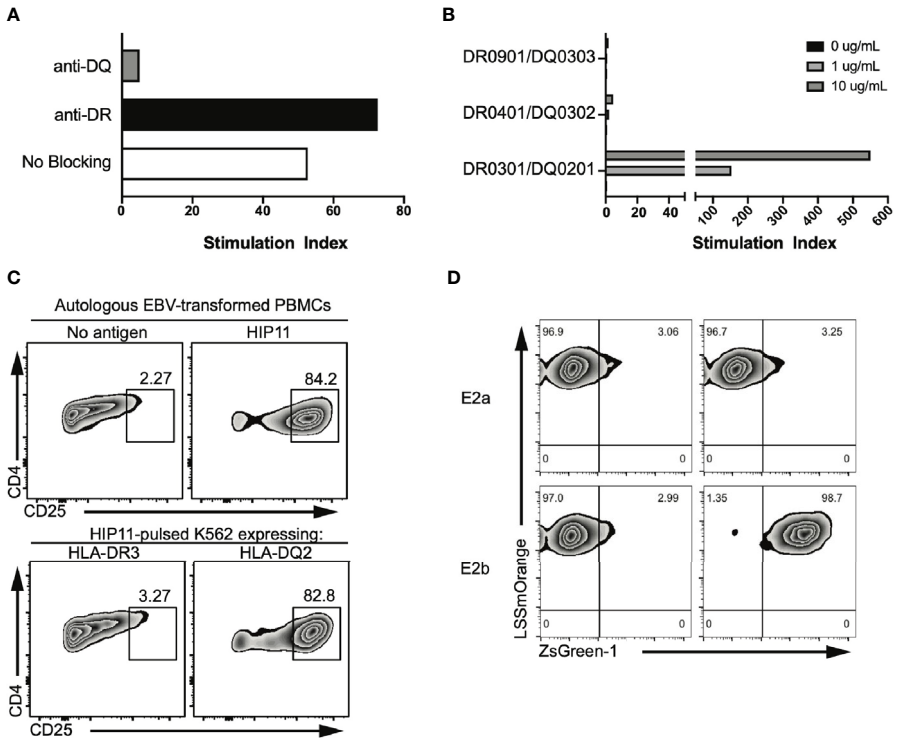

FIGURE 1 | The E2 TCR consisting of the TRAV8-2/8-4 TCRα chain and the TRBV5-4 TCRβ chain recognizes HIP11 in the context of DQ2. **(A)** Peptide-specific proliferation of the E2 T cell clone was assessed using cells from an autologous EBV transformed B cell line as antigen presenting cells and pulsing with 10 μg/mL of the cognate HIP11 peptide in the presence or absence of anti-DR (black bar) or anti-DQ (grey bar) blocking antibody. **(B)** HLA restriction was further defined using cells from partially HLA-matched third-party donors as antigen presenting cells. Irradiated PBMC from each third party donor were pulsed with the cognate HIP11 peptide at concentrations of 0, 1, and 10 μg/mL. Substantial proliferation was observed only in response to the DQA1*05:01-DQB1*02:01 (HLA-DQ2) positive antigen presenting cells, indicating a DQA1*05:01-DQB1*02:01-restricted response. All data are represented as stimulation index (SI) values, calculated by normalizing the proliferation of the T cell clone based on [3H] thymidine incorporation of un-stimulated wells. **(C)** To confirm DQ2 restriction, the E2 T cell clone was cultured in the presence of either an autologous B cell line or K562 lines expressing DR3 or DQ2. After 18h, cells were harvested and stained for CD4 and CD25, and CD25 expression was monitored by flow cytometry. Data is representative of 3 independent experiments. **(D)** Two TCR alpha sequences and one beta sequence were identified from the E2 clone. Each TCR alpha/beta combination, E2a and E2b, was expressed in 5KC T-hybridoma cells with an NFAT-reporter. ZsGreen-1 is expressed upon T cell activation. The E2a and E2b TCR transductants were cultured with the HIP-11 peptide in the presence of K562 cells expressing DQ2 followed by evaluation of ZsGreen-1 expression by flow cytometry.

in organ donors with T1D (13). Several of the CD4 T cell transductants were shown to be reactive to proinsulin peptides. The CD4 T cell transductant GSE.8E3 responded to the peptide $insC_{17\text{-}33}$ (GAGSLQPLALEGSLQKR), a region of the C-peptide prone to HIP formation (2). We previously tested reactivity of human PBMCs to eight HIPs containing that region of the C-peptide (6) and found that reactivity to two of them (HIP11 and HIP16) was increased (compared to no antigen) in T1D patients but not in control subjects (6). We hypothesized that post-translational modification of the C-peptide fragment through hybrid peptide formation would generate a more potent antigen for GSE.8E3. Eight HIPs containing the insulin C-peptide fragment $insC_{20\text{-}26}$ as the left peptide were tested for antigenicity. The GSE.8E3 transductant strongly reacted to the HIP11 peptide $insC_{20\text{-}26}$ – $insC_{1\text{-}7}$ (SLQPLAL–EAEDLQV) in the context of both DQ8 and DQ8*trans* (**Figure 2**). When DQ8 was the presenting element, GSE.8E3 did not respond to the native insulin peptide $insC_{16\text{-}31}$ (PGAGSLQPLALEGSLQ) (**Figure 3A**) except at very high concentrations (**Figure 2**). GSE.8E3 did respond to $insC_{16\text{-}31}$ when presented by DQ8-trans (**Figure 3B**). The E2 clone did not respond to native C-peptide when presented by DQ2 (**Figure 3C**).

E2 and GSE.8E3 Recognize Different Minimal Epitopes of HIP11

Although the E2 and GSE.8E3 TCRs both recognized HIP11, it was possible that they were specific for different HIP11 epitopes, particularly since recognition by these TCRs was restricted to different HLA molecules. Identifying the precise epitope recognized by each TCR could facilitate future efforts to characterize the immune response to HIP11. For example, it could enable the generation of HLA class II tetramers that allow for detection of different subsets of HIP11-reactive T cells in human subjects. To determine the minimal epitope recognized by the E2 T cell clone and the islet-derived GSE.8E3 transductant, we tested reactivity to HIP11 peptides truncated

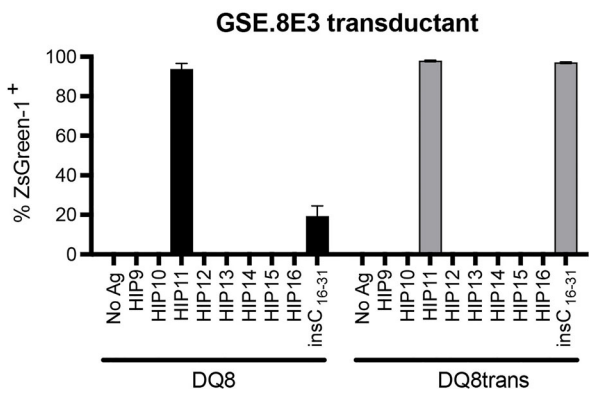

FIGURE 2 | The GSE.8E3 transductant recognizes HIP11 in the context of DQ8 and DQ8-trans. The GSE.8E3 TCR clonotype identified from a CD4 T cell in the islets of a T1D organ donor was expressed in 5KC T-hybridoma cells with an NFAT-reporter. The GSE.8E3 TCR transductant was cultured with or without various HIPs (10 μM) or a native proinsulin peptide (100 μM) in the presence of K562 cells expressing DQ8 (black bars) or DQ8-trans (gray bars). ZsGreen-1 expression was evaluated by flow cytometry. Each result is the mean for three independent experiments. Error bars indicate the standard error of the mean.

either on the N- or the C-terminus. Our data show that the response by the E2 T cell clone was completely abrogated when the glutamine on the N-terminus was missing (**Figure 4A**) and when the aspartic acid (D) on the C-terminal region was truncated (**Figure 4B**), indicating that the minimal HIP11 epitope seen by the E2 T cell clone is QPLALEAED. For the GSE.8E3 transductant, our data show that the minimal epitope presented by DQ8 is LQPLALEAE (**Figures 4A, B**). Results were similar when testing the GSE.8E3 response using DQ8*trans* as the presenting element (**Supplementary Figure 1**). Residues flanking these minimal epitopes also seem to contribute to overall activity, possibly as TCR contact residues outside of the peptide binding groove. Based on these data and published data regarding preferred binding residues for DQ2 and DQ8 (14, 15), we propose that HIP11 is presented in two different binding registers depending on whether DQ2 or DQ8 is the presenting element, as shown in **Figure 4C**.

Design and Validation of an HLA-DQ2/HIP11 Tetramer

The study of antigen-specific T cells is a high priority in T1D research as CD4 T cells reactive to islet antigens could potentially serve as biomarkers of disease progression in at-risk subjects. While ELISpot analysis can detect cytokine-secreting cells independent of MHC restriction, HLA class II tetramers allow for the detection, enumeration, and phenotyping of antigen-specific T cells directly *ex vivo* (16). Based on the discovery that the response of the HIP11-reactive CD4 T cell clone E2 to HIP11 was DQ2-restricted, we designed an HLA class II tetramer loaded with the HIP11 peptide and tested its ability to stain the E2 T cell clone. As a negative control we used the T cell clone B11b, which is reactive to HIP4, a C-peptide/A-chain hybrid peptide (**Supplementary Figure 2**). While the HIP11-reactive T cell clone stained with the DQ2/HIP11 tetramer, the HIP4-reactive T cell clone B11b did not (**Figure 5A**). Neither of the CD4 T cell clones stained with a negative control DQ2 tetramer loaded with the CLIP peptide (**Figure 5A**). To determine whether antigen stimulation of the T cell clone could improve tetramer binding, the E2 and B11b T cell clones were stimulated with HIP11 or HIP4 respectively in the presence of autologous EBV-transformed PBMCs. T cells were stained with either the HIP11 or the CLIP tetramers 8, 12 and 23 days after stimulation and tetramer staining was monitored by flow cytometry (**Figure 5A**). Our data indicate that the strongest staining is obtained within a week after stimulation. Finally, PBMCs from two DQ2+ T1D patients (with T1D onset less that 2 years) were cultured with medium only or in the presence of HIP4 or HIP11. After 10 days of culture, cells were harvested and stained with the HIP11 tetramer. A substantial population of CD4 T cells present in one of the patients stained with the HIP11/DQ2 tetramer when PBMCs were stimulated with HIP11, but not when stimulated with an irrelevant HIP (**Figure 5B**).

HIP11 Is Present in Human Islets

A critical step in confirming that HIP11 is a natural antigen in human T1D was to identify HIP11 in human islets by mass spectrometry (LC-MS/MS). Proteins were isolated from non-diabetic human donor islets and digested with the protease AspN to generate peptides suitable for mass spectrometric analysis. Resultant

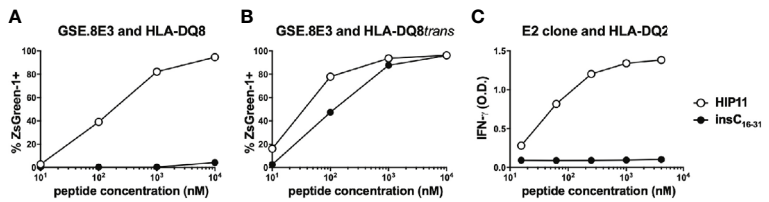

FIGURE 3 | Response of HIP11-reactive T cells to insulin C-peptide. **(A, B)** The GSE.8E3 TCR transductant was cultured with various concentrations of a HIP11 peptide or an insulin C-peptide sequence in the presence of K562 cells expressing DQ8 **(A)** or DQ8*trans* **(B)**. ZsGreen-1 positivity was assessed by flow cytometry as a measure of activation. Data are representative of three independent experiments. **(C)** The E2 T cell clone was cultured with various concentrations of a HIP11 peptide or an insulin C-peptide sequence in the presence of an autologous EBV-transformed B cell line. After 48 hours, supernatants from T cell cultures were harvested and IFN-γ concentrations were measured by ELISA. Results are representative of two independent experiments.

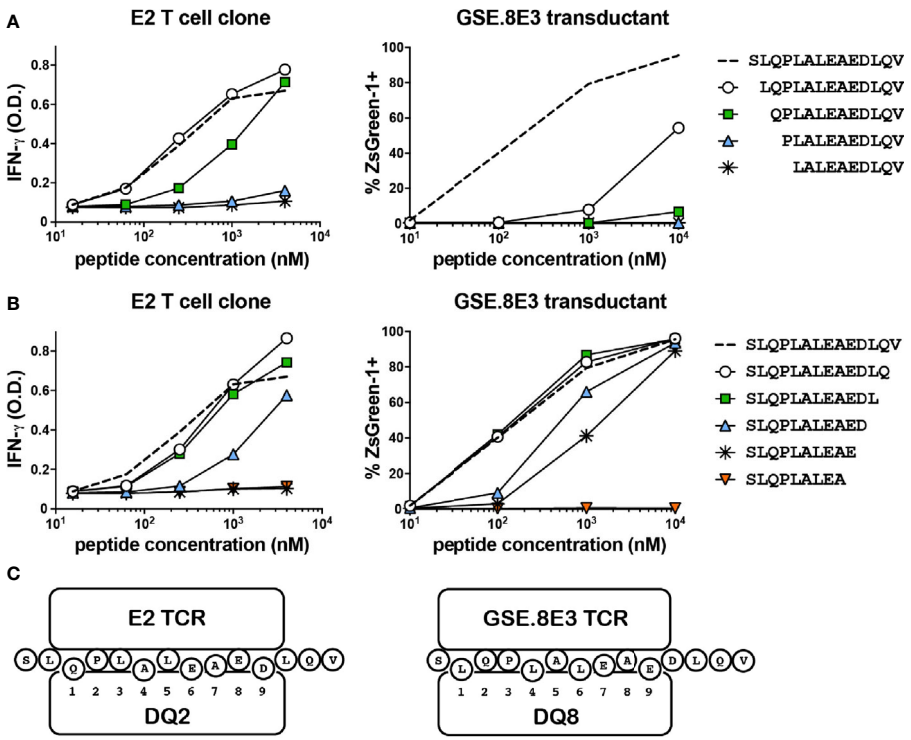

FIGURE 4 | The E2 CD4 T cell clone and the GSE.8E3 TCR transductant recognize different minimal epitopes. N-terminally **(A)** and C-terminally **(B)** truncated HIP11 peptides were tested with E2 and GSE.8E3 for antigenicity. Results are representative of three independent experiments. **(C)** Proposed binding registers for HIP11 in the context of DQ2 or DQ8 when recognized by E2 or GSE.8E3, respectively.

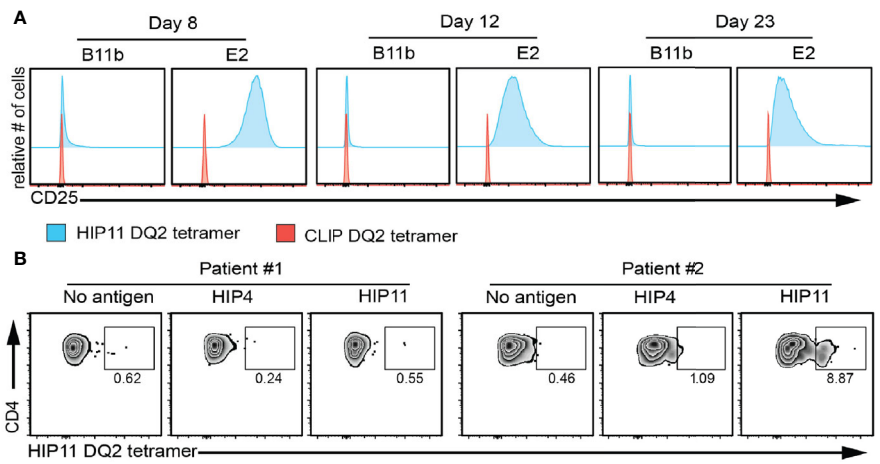

FIGURE 5 | HIP11/DQ2 tetramer stains the E2 T cell clone and PBMCs from a T1D patient. **(A)** The E2 or B11b T cell clones were stimulated with HIP11 or HIP4 respectively and an irradiated autologous EBV-transformed B cell line. T cells were then maintained in culture and harvested at 8, 12 and 23 days post-stimulation and stained with the DQ2/HIP11 tetramer (blue histograms) or DQ2/CLIP tetramer (red histograms), CD4, and a fixable viability dye. Tetramer staining was assessed by flow cytometry by gating on live, $CD4^+$ cells. Data are representative of two independent experiments. **(B)** PBMCs from two DQ2+ patients were isolated, stained with CFSE and cultured with medium only, HIP4, or HIP11. After 10 days, cells were harvested and stained with the DQ2/HIP11 tetramer, anti-CD4 and anti-CD25 antibodies, and a fixable viability dye before flow cytometry analysis. Gates were set on live, $CD4^+$ $CD25^+$ $CFSE^{low}$ events and the percentage of tetramer positive events is reported.

peptides were then analyzed by LC-MS/MS, and data were searched against a custom database to find peptides describing the HIP11 hybrid junction. Our analysis identified the peptide ins$C_{4\text{-}26}$ – ins$C_{1\text{-}3}$ (DLQVGQVELGGGPGAGSLQPLAL-EAE) with high confidence (**Figure 6A** and **Supplementary Table 2**). This peptide is the predicted AspN cleavage product of HIP11. We previously established a set of rigorous criteria to confidently identify HIPs by mass spectrometry (2), and the putative HIP11 match satisfied all of these criteria (**Supplementary Table 3**). To further confirm the validity of the mass spectrometry-based HIP11 identification, we applied a novel approach – **P**eptide-spectrum match **V**alidation with **I**nternal **S**tandards (P-VIS) – recently developed by our group (9). Using this approach, the fragmentation spectrum of the peptide found in human islets was found to be highly similar to the spectrum of synthetic ins$C_{4\text{-}26}$ – ins$C_{1\text{-}3}$ peptide (**Figure 6A**), with a Pearson correlation coefficient of 0.953 (**Figure 6B**). Based on the data for internal standard peptides, this degree of correlation indicated that the peptide found in human islets was indeed the HIP11 peptide ins$C_{4\text{-}26}$ – ins$C_{1\text{-}3}$ (**Figure 6C**). This finding was confirmed by P-VIS chromatographic retention time analysis (**Supplementary Figure 3**).

DISCUSSION

The discovery of HIPs in human islets (2) and the detection and isolation of HIP-reactive CD4 T cells from the peripheral blood and islets of individuals with T1D (1, 5–7) indicate a role for HIPs as autoantigens in T1D. Here, we used LC-MS/MS analysis

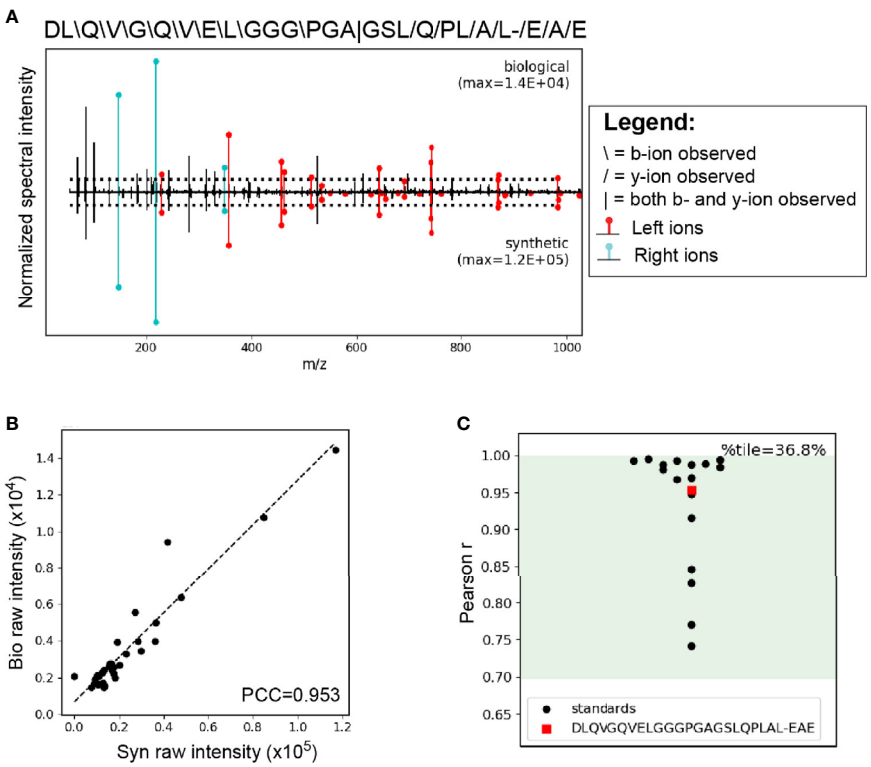

FIGURE 6 | LC-MS/MS analysis using the P-VIS approach confirms that HIP11 is present in human islets. Proteins were extracted from human islets, fractionated by size exclusion chromatography (SEC), and digested with the protease AspN, which cleaves at the N-terminus of aspartic acid (D) residues. Samples were analyzed by LC-MS/MS and data were searched using Agilent Spectrum Mill software. The predicted HIP11 AspN cleavage product DLQVGQVELGGGPGAGSLQPLAL-EAE was identified with high confidence and validated using the P-VIS approach. **(A)** Mirror plot generated by PSM_validator displaying the spectrum from the biological sample (positive y-axis) and the spectrum from the validation sample (negative y-axis). Peak intensities are normalized to the most intense peak in each spectrum. The intensity of the tallest peak in each spectrum is indicated. In the sequence map, "\", "/", and "|" indicate that the spectrum from the biological sample contained a b-ion, y-ion, or both, respectively, corresponding to fragmentation at a given bond. Horizontal dotted lines indicate the user-defined threshold for consideration of peaks in the Pearson correlation coefficient (PCC) calculation. Ions corresponding to fragmentation of a bond to the left (L ions) or right (R ions) of the hybrid junction are labeled in red and cyan, respectively. **(B)** Peaks that were present above the intensity threshold in at least one of the spectra were used to calculate the Pearson correlation coefficient (PCC) between the two spectra. For each peak, the intensity in the biological peptide spectrum (bio raw intensity) and the intensity in the spectrum for the synthetic peptide (syn raw intensity) were paired, and the pairs for all peaks were plotted. Calculation of the PCC for these values indicated that the spectra were highly correlated (PCC = 0.953). **(C)** Comparison of the PCC for the HIP11 match to the distribution of PCCs for internal standard peptides (ISPs). Data for the ISPs passed the D'Agostino-Pearson omnibus normality test ($p = 0.229$; $p > 0.05$ indicates that the null hypothesis cannot be rejected and the data can be treated as normal). Green shading indicates the 95% prediction interval based on one-tailed analysis of Fisher's z-transformed PCC values. The PCC comparing the biological spectrum and the validation spectrum is shown (red square), and the percentile (%tile) is reported.

to report for the first time that HIP11, a C-peptide/C-peptide HIP, is present in human islets. We determined the TCR Vα and Vβ usage and the HLA class II restriction of the HIP11-reactive E2 clone previously isolated from the peripheral blood of a T1D donor. We also demonstrated that HIP11 is a potent epitope for a proinsulin-specific T cell receptor (GSE.8E3) identified by analysis of islet-infiltrating T cells from a T1D organ donor (13). Notably, we found that GSE.8E3 and the E2 clone each recognize a different minimal epitope of HIP11 and recognition by these clones was restricted to HLA-DQ8/HLA-DQ8*trans* or HLA-DQ2, respectively. HLA-DQ8 and HLA-DQ8*trans* consist of the same β chain paired with different α chains. A previous study (17) demonstrated that CD4 T cell clones (isolated from T1D donors and specific for immunodominant islet epitopes) could recognize their cognate peptide epitopes presented by either DQ8 or DQ8*trans*, with presentation by DQ8*trans* eliciting a stronger response. The DQ8*trans* α chain lacks the Arg52 residue present in the DQ8 α chain. The authors demonstrated that the absence of Arg52α led to differences in the orientation of TCR contact residues in the bound peptide, resulting in a higher affinity between the TCR and the peptide-DQ8*trans* complex (17). The GSE.8E3 transfectoma was more sensitive to N-terminal and C-terminal truncations of the HIP11 peptide SLQPLAL-EAEDLQV when DQ8 was the presenting element, also suggesting possible differences in the orientation of crucial TCR contacts when either DQ8 or *DQ8trans* was the presenting element. However, structural characterization, such as by X-ray crystallography, would be needed for definitive characterization of the interactions between the TCR, the peptide, and the different HLA molecules.

As more than 73% of all patients with T1D carry either HLA-DQ8 (DQA2*0301-DQB2*0302), HLA-DQ2 (DQA1*0501-DQB1*0201), or both (18), presentation by two different HLA molecules strongly associated with disease susceptibility further supports the case for HIP11 as a relevant autoantigen in T1D. Of note, individuals that carry both DQ2 and DQ8 alleles have a higher risk of developing T1D (19) and investigation of the relevance of HIP11 in this population is currently underway in our lab. Like I-A^{g7} in the NOD mouse model of autoimmune diabetes, position 57 of the β-chain of HLA-DQ8 and HLA-DQ2 has a non-aspartic acid (D) amino acid polymorphism that favors the presentation of an acidic amino acid in p9 of the binding groove (20). While there are clear amino acid preferences for binding to either molecule in pockets 1, 4, 6, 7 and 9 (14, 15, 21), the possibility of forming HLA trans dimers offers additional possibilities for peptide binding while maintaining the β57 non-D amino acid (22).

Formation of HIPs in pancreatic β-cells is likely the result of the cellular environment where unique peptides, such as the insulin C-peptide, are present at high local concentrations inside secretory granules. This could lead to the formation of specific HIPs that do not form in the thymus. Thus, central tolerance to these HIPs may not be established, allowing for the development of autoreactivity to HIPs in the periphery. Cross-reactivity of HIP-reactive T cells to unmodified peptides such as C-peptide may then lead to a break in tolerance to these unmodified insulin peptides, potentially resulting in a more robust autoimmune response. We observed, for example, that the GSE.8E3 transductant, which responded to HIP11 in the context of either HLA-DQ8 or HLA-DQ8*trans*, also responded to native C-peptide but only when presented by DQ8*trans* and not by DQ8. One could speculate that the ability of HLA-DQ8*trans* to present both a HIP and an unmodified insulin peptide to an autoreactive T cell clone in a stimulatory fashion suggests a possible mechanism in which carrying both the HLA-DQ2 and HLA-DQ8 alleles increases the risk of developing T1D by facilitating T cell cross reactivity to HIPs and unmodified peptides. It should be noted that HIP11 could be found in the islets of a non-T1D organ donor, suggesting that HIP formation is not limited to humans with autoimmunity. Similarly, insulin has long been recognized as an autoantigen in T1D, even though it is expressed in all individuals. Elucidating how various factors such as HIP formation and HLA genotype act in concert to promote disease development could be key to future progress in T1D research.

The former gold standard for validating mass spectrometry-based peptide identifications was to compare the fragmentation spectrum and chromatographic retention time for a synthetic version of a putative peptide sequence to those observed for the biological peptide. Our P-VIS approach (9) adds rigor and objectivity to this process by using comparisons for internal standard peptides to evaluate if the observed degree of similarity between the biological and synthetic peptides is high enough to indicate a correct match. Although we confidently identified HIP11 in human islets, the observed fragment provides limited information about the origin and mechanism of formation of the biological peptide since human islet proteins were digested with the AspN protease prior to LC-MS/MS analysis. Could HIP11 be the product of intramolecular transpeptidation and therefore the generation of a cis-hybrid? In this scenario, a protease would remove the five C-terminal residues from a C-peptide molecule and fuse the N-terminus of the same molecule to the newly-formed C-terminus, thereby generating a cyclic peptide. Cleavage of this cyclic peptide by a protease or the proteasome complex could then generate a linear HIP11 peptide suitable for presentation by MHC molecules. Alternatively, HIP11 could be formed by intermolecular transpeptidation (formation of a trans-hybrid), in which one C-peptide molecule is cleaved, generating the $insC_{1\text{-}26}$ fragment, and the N-terminus of a separate C-peptide molecule is fused to the C-terminus of the $insC_{1\text{-}26}$ fragment. Various other details of how HIP11 is formed are also unknown including for example, the specific enzyme responsible for formation of HIP11 in human islets.

In combination with our previous work, our results provide a thorough analysis of a disease-relevant HIP in human T1D, including identification of the HIP in islets, investigation of CD4 T cell responses, and molecular characterization of epitope recognition. The validation of an HLA-DQ2/HIP11 tetramer opens the path for further characterization of the role of HIP11-reactive T cells in T1D. To our knowledge, this is one of the first DQ2 tetramers loaded with a T1D disease-relevant antigen. This work provides a precedent for in-depth characterization of

responses to HIPs as important components of the autoimmune response in T1D.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. The data presented in the study are included in the article figures, tables, and supplementary materials. The T cell receptor alpha and beta chain sequences and corresponding epitope data were submitted to the immune epitope database (https://www.iedb.org/home_v3.php) and can be accessed as submission ID 1000868. Further inquiries about the data can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Colorado Multiple Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RB, TW, MN, and EJ designed experimental work. AH, RB, TW, MD, LL, RP, and MN performed experimental work. RB, TW, KH, and TD wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Citrullination and PAD Enzyme Biology in Type 1 Diabetes – Regulators of Inflammation, Autoimmunity, and Pathology

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The generation of post-translational modifications (PTMs) in human proteins is a physiological process leading to structural and immunologic variety in proteins, with potentially altered biological functions. PTMs often arise through normal responses to cellular stress, including general oxidative changes in the tissue microenvironment and intracellular stress to the endoplasmic reticulum or immune-mediated inflammatory stresses. Many studies have now illustrated the presence of 'neoepitopes' consisting of PTM self-proteins that induce robust autoimmune responses. These pathways of inflammatory neoepitope generation are commonly observed in many autoimmune diseases including systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, and type 1 diabetes (T1D), among others. This review will focus on one specific PTM to self-proteins known as citrullination. Citrullination is mediated by calcium-dependent peptidylarginine deiminase (PAD) enzymes, which catalyze deimination, the conversion of arginine into the non-classical amino acid citrulline. PADs and citrullinated peptides have been associated with different autoimmune diseases, notably with a prominent role in the diagnosis and pathology of rheumatoid arthritis. More recently, an important role for PADs and citrullinated self-proteins has emerged in T1D. In this review we will provide a comprehensive overview on the pathogenic role for PADs and citrullination in inflammation and autoimmunity, with specific focus on evidence for their role in T1D. The general role of PADs in epigenetic and transcriptional processes, as well as their crucial role in histone citrullination, neutrophil biology and neutrophil extracellular trap (NET) formation will be discussed. The latter is important in view of increasing evidence for a role of neutrophils and NETosis in the pathogenesis of T1D. Further, we will discuss the underlying processes leading to citrullination, the genetic susceptibility factors for increased recognition of citrullinated epitopes by T1D HLA-susceptibility types and

provide an overview of reported autoreactive responses against citrullinated epitopes, both of T cells and autoantibodies in T1D patients. Finally, we will discuss recent observations obtained in NOD mice, pointing to prevention of diabetes development through PAD inhibition, and the potential role of PAD inhibitors as novel therapeutic strategy in autoimmunity and in T1D in particular.

Keywords: type 1 diabetes, neoepitopes, post-translational modification, citrullination, peptidylarginine deiminase

ORIGIN AND IMPLICATIONS OF POST-TRANSLATIONAL MODIFICATIONS IN AUTOIMMUNITY

At a simplistic level, the success of immunity relies on distinguishing 'self' from 'non-self', originating primarily by purging the autoreactive repertoire both in central lymphoid organs, the thymus and bone marrow, as well as by peripheral tolerance mechanisms. Classical mechanisms of immune tolerance rely on the processing and presentation of self-peptides by antigen presenting cells (APCs), resulting in deletion or anergy of the autoimmune repertoire. However, it is clear that many self-proteins, or post-translational modifications (PTMs) to self-proteins, are not expressed in primary lymphoid organs. For example, PTMs specific to peripheral tissues may not be expressed in the thymus and thus these PTMs never tolerize or delete the emerging thymocytes. While we know that proteins are fundamentally assembled from 20 amino acid structures, the addition of PTMs pushes that group above 140 structurally unique amino acids (1). These observations have altered the concepts and the breadth of immune tolerance to self-proteins, particularly in the perspective of autoimmune syndromes. Many factors influence the rate at which PTMs arise in self-proteins, including the amino acid sequence, flanking amino acid motifs, and variables such as the tissue microenvironments. PTMs also alter specific peptides that arise by antigen processing and, subsequently, the specificity of ongoing B- and T-lymphocyte immunity (2–4).

Some PTMs arise by enzymatic processes, as with citrullination reviewed herein, or with N-linked glycosylation or phosphorylation, critical to biological functions of many host proteins. In contrast, some modifications arise spontaneously, under physiologic pH and temperature, such as the formation of isoaspartyl modifications [reviewed by (5)]. Also, some modifications can be processed either enzymatically or spontaneously, such as deamidation (6). As illustrated herein, PTMs may trigger aberrant autoimmunity as well as alter the biological functions of self-proteins in selected tissues, including transcriptional and translational events. The text that follows will review the conditions that elicit citrulline PTMs, including their role in biological and immunological processes, with specific focus on their implications in type 1 diabetes (T1D).

Citrullination, also known as deimination, is a PTM in which an arginine residue is converted into a citrulline (**Figure 1**). This modification, which was first described in 1939 (7), leads to a loss of one positive charge and a reduction in mass of 0.984 Da per modified arginine. This type of modification can alter intra- and inter-molecular interactions of the protein (8), having an impact on its structure, function and its interaction with other proteins (9). Citrullination is catalyzed by peptidylarginine deiminase (PAD) enzymes in a Ca^{2+}-dependent manner (10), which is thought to be an irreversible process (11).

A role for citrullinated self-proteins has been associated with several autoimmune diseases, such as T1D, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), psoriasis, Sjögren's syndrome (SS), antiphospholipid syndrome (ALS) and inflammatory bowel disease (IBD) (**Table 1**). Among these different autoimmune diseases, citrullination as an autoimmune biomarker in RA has been most extensively described. RA is a chronic autoimmune disease characterized by inflammation of the synovial joints. Proteomic analysis of the cellular and soluble components of RA synovium identified the full RA citrullinome, with more than 100 citrullinated proteins, amongst which vimentin, enolase, fibrinogen and fibronectin (46). Some of these were shown to induce autoantibodies and/or autoreactive T-cell responses in RA (11, 22, 29, 30). The presence of anti-citrullinated protein antibodies (ACPAs) in the serum of RA patients is one of the most specific diagnostic marker for the disease (47). ACPAs can be detected years before clinical symptoms appear (48). Apart from being a prognostic biomarker for disease development, some ACPAs have been described as useful in predicting the severity of joint destruction during the first five years after RA onset (49). ACPAs can also be detected in a small percentage of patients with SS (3 to 9.9%), and the presence of such

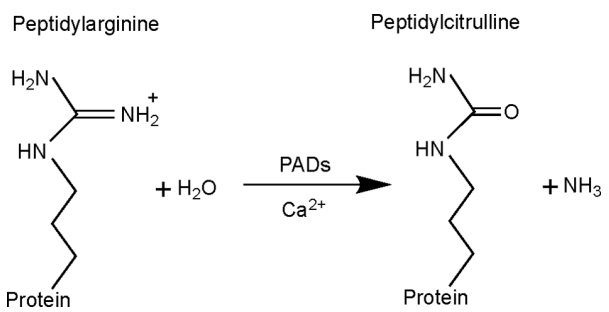

FIGURE 1 | Citrullination reaction catalyzed by peptidylarginine deiminase (PAD) enzymes. With the conversion of arginine into citrulline, the primary ketamine group of arginine (=NH) is replaced by a ketone group (=O), with production of ammonia as a side-product. This results in a mass difference of 0.984 Da and loss of one positive charge. Citrullination is catalyzed by PAD enzymes, requiring $Ca2^+$ as cofactor.

TABLE 1 | Citrullination in autoimmune diseases.

Diseases	Target proteins	References
Type 1 diabetes (T1D)	GAD65	(12–14)
	IA-2	(13)
	GRP78	(15–17)
	IGRP	(13)
	IAPP	(17, 18)
Rheumatoid arthritis (RA)	fibrinogen	(19–21)
	vimentin	(22–24)
	histone H1, H2B, H3 and H4	(25–27)
	type 2 collagen	(28)
	α-enolase	(29)
	GRP78	(30)
Systemic lupus erythematosus (SLE)	LL37	(31)
	histone H1 and H3	(26, 32)
Multiple sclerosis (MS)	MBP	(33–36)
	GFAP	(36)
	neurogranin	(37)
	histone H3	(38)
Psoriasis	keratins	(39)
	filaggrin	(40)
Sjögren's syndrome (SS)	histone H1	(41)
	α-enolase	(42)
Antiphospholipid syndrome (ALS)	vimentin	(43)
Inflammatory bowel disease (IBD)	vimentin	(44, 45)

GAD65, glutamic acid decarboxylase 65; IA-2, islet antigen-2; GRP78, glucose-regulated protein 78; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; IAPP, islet amyloid polypeptide; MBP, myelin basic protein; GFAP, glial fibrillary acidic protein.

autoantibodies indicates a risk of developing RA (50). As listed in **Table 1**, emerging citrullinated target proteins are proposed as autoimmune disease specific biomarkers. Interestingly, the serological level of citrullinated vimentin in patients with ulcerative colitis (UC) was decreased compared to patients with another important IBD, Crohn's disease (CD), and non-IBD control subjects. Therefore, citrullinated vimentin has also been suggested as differentiating marker between CD and UC to improve the diagnostic accuracy in IBD (44).

PEPTIDYLARGININE DEIMINASES: TYPES AND MECHANISM OF ACTION

Peptidylarginine deiminases are a group of 5 enzymes encoded by genes localized on chromosome 1p36.1 in human and chromosome 4E1 in mice, located in a cluster of 350 kb and 240 kb, respectively (51). They were first described in 1977 as the enzymes mediating the conversion of arginine into the non-classical amino acid citrulline in proteins, from studies performed in mammalian hair follicles (52). The enzyme responsible for this reaction was for the first time partially purified by Fujisaki and Sugawara in 1981 and named peptidylarginine deiminase (PAD) (53). Since then, five different PAD isozymes have been described in mammals, designated as PAD1 to PAD4 and PAD6, which display 50-70% sequence identity (54). PADs replace the primary ketamine group of arginine (=NH) by a ketone group (=O) and yield ammonia as a side-product (**Figure 1**), leading to the loss of one positive charge of the target protein. This can lead to changes in the function and fate of the citrullinated target protein.

Although the PAD enzymes are widely expressed throughout the body, and have been implicated in a variety of physiological processes, each isozyme has specific tissue distribution, functions, and substrates under physiological conditions (8) (**Figure 2** and **Table 2**). As such, PAD1 is predominantly expressed in skin epidermis, uterus and hair follicles, targeting keratin and filaggrin (86). PAD2 is the ubiquitous member of the family, being expressed in multiple tissues such as brain, skeletal muscle, spleen, uterus, secretory glands and leukocytes (60, 65, 71). Importantly, PAD2 and PAD4 are the only PAD isozymes expressed in immune cells. Amongst other targets, PAD2 citrullinates myelin basic protein (MBP) in brain, vimentin in skeletal muscle and macrophages, actin in neutrophils and histones in various cell types (71). PAD3 is found in hair follicles and epidermis, and citrullinates filaggrin, trichohyalin, apoptosis-inducing factor (AIF) and vimentin (86). PAD4 is expressed in leukocytes, mainly granulocytes (like neutrophils and eosinophils), and monocytes and macrophages (63, 73). It is the only PAD isotype with a nuclear localization signal sequence, which is located at its N-terminus. PAD4 targets several nuclear proteins, such as histones, nucleophosmin and nuclear lamin C (71). With its high expression in neutrophils, PAD4 plays an important role in the generation of neutrophil extracellular traps (NETs) and thereby in the first line of defense against bacterial pathogenic invaders (see in more detail below). PAD6 is mainly expressed in eggs, embryo and ovary. It is the only PAD isozyme for which until today no protein substrates have been identified (86) and, *in vitro*, no catalytic activity can be measured (89). Also, no known association with autoimmunity or other diseases has been reported for PAD6.

Although all 5 PAD enzymes target arginine residues in proteins, they do have different substrate specificities. The basis

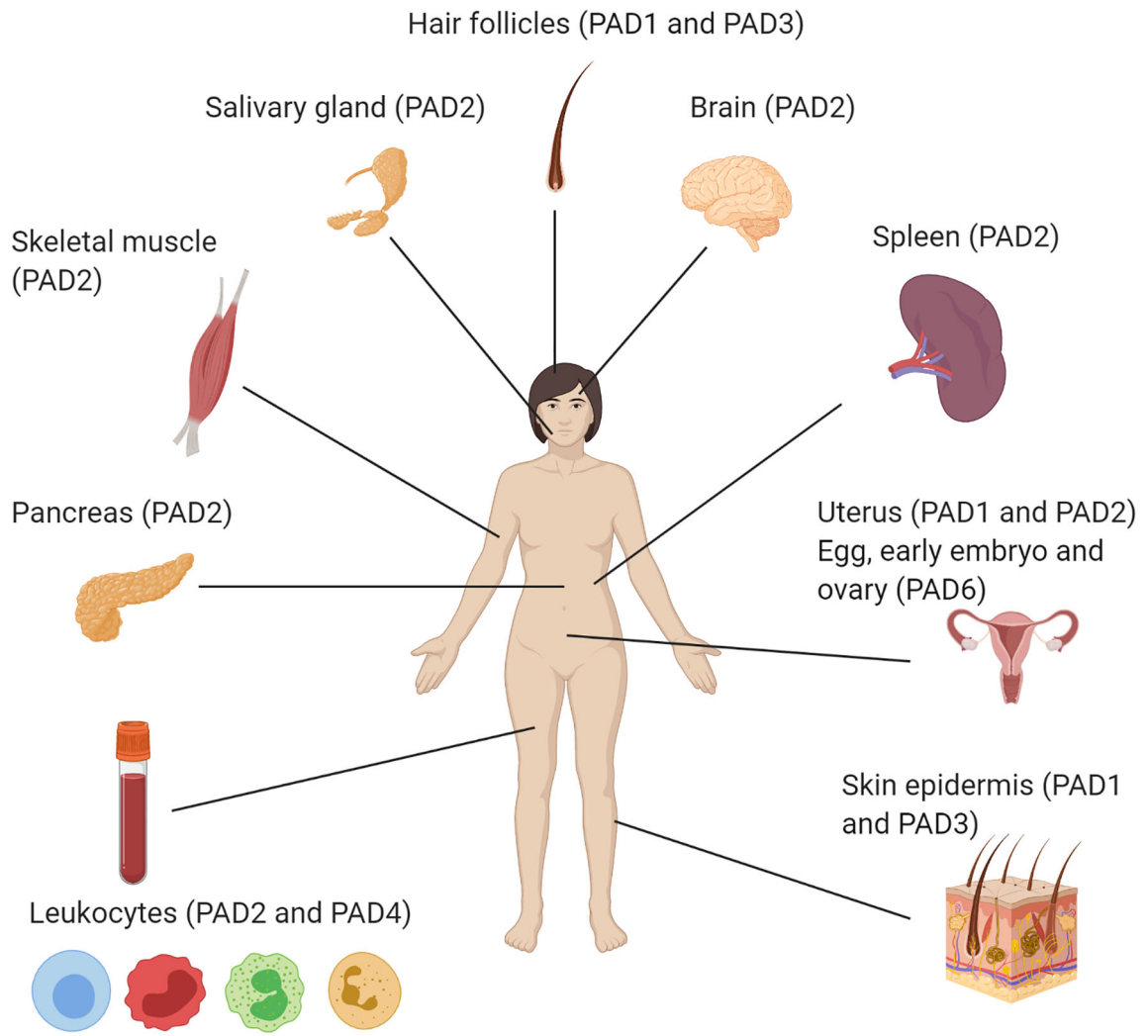

FIGURE 2 | Illustration of the organ-specific protein expression of peptidylarginine deiminase isozymes in humans.

TABLE 2 | PAD isozymes tissue distribution, target substrates, physiological functions and disease association.

Isozyme	Tissue distribution (Protein level)	Target substrates	Physiological functions	Disease association
PAD1	Skin epidermis, uterus (55) and hair follicles (56)	Keratin and filaggrin (57, 58)	Skin keratinization (59)	Psoriasis (39)
PAD2	Brain, skeletal muscle, spleen, spinal cord, uterus, secretory glands and pancreas (55, 60–62), leukocytes [macrophages (63), neutrophils (64) and T cells (65)]	Myelin basic protein (66), vimentin (63), actin (67), histones (68), fibrinogen and α- enolase (69)	Disassembly of vimentin filaments (70), CNS plasticity (9), epigenetic and transcriptional regulation (71), immune response (65, 72)	Rheumatoid arthritis, multiple sclerosis (73), Alzheimer disease (74) and prion diseases (75)
PAD3	Skin epidermis and hair follicles (55, 56)	Filaggrin, trichohyalin (56, 57), apoptosis-inducing factor (76)	Regulation of epidermal functions (57)	Unknown
PAD4	Leukocytes [mainly granulocytes, such as neutrophils and eosinophils (77, 78), monocytes, macrophages (63) and T cells (65)] and neurons (79)	Histones, nucleophosmin (80), nuclear lamin C (81), antithrombin (82), ING4 (83), NF-Kb (84), fibrinogen and α- enolase (69)	Epigenetic and transcriptional regulation (71), NET formation (85), immune response (65, 85)	Rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis (86) and cancers (87)
PAD6	Egg, early embryo and ovary (88)	No substrates identified; no activity *in vitro* (89)	Oocyte cytoskeletal formation and female fertility (90)	Unknown

ING4, inhibitor of growth 4; CNS, central nervous system; NET, neutrophil extracellular trap.

for this difference is not fully understood, but cannot solely be explained by their difference in tissue distribution or subcellular location (54). Additional influencing factors include the enzyme kinetics, the conformation of the secondary structure of the target protein and the flanking amino acid composition surrounding the arginine residues, as revealed from *in vitro* studies with recombinant PAD enzymes. In regard to the latter, it was shown for instance that a glutamic acid accompanying the arginine residue decreases the chance of being citrullinated, whereas a flanking aspartic acid residue increases the citrullination level (91). In this regard, PAD4 was found to have a higher substrate specificity as compared to PAD2 (92, 93).

The most important regulator of PAD activity is calcium, with micro to millimolar calcium concentrations needed for their full activation (53, 54, 94). However, under physiological conditions, with intracellular cytosolic calcium concentrations usually around 100-fold lower, a basal PAD activity can also be measured, in line with the occurrence of low levels of citrullination and their role during normal physiological processes. This raises questions as to how exactly calcium is mediating the activity of PADs. One thought is that calcium may alter the confirmation of PADs. Under low calcium conditions, PADs may be present in a conformation that selects only high efficiency substrates, thus allowing citrullinations to happen, but limiting aberrant citrullination events (11). In the presence of high calcium, all calcium binding sites of the PAD enzyme are occupied, promoting extensive rearrangement and dimerization of the enzyme, leading to its full activation, as shown for PAD4 (95, 96). Alternatively or additionally, other factors are known to regulate PAD enzymes (9), which could act as cofactors modulating their calcium sensitivity and specificity (11). Considering the importance of calcium in PAD activation, pathways that alter intra- or extracellular calcium levels, such as endoplasmic reticulum (ER) and inflammatory stress, are important in many of the autoimmune diseases in which citrullination is implicated (see in more detail below) and underscore the important link between citrullination and stress pathways. Indeed, changes in calcium fluxes may lead to activation of PAD activity in stressed or dying cells. Moreover, the externalization and activation of intracellular PAD enzymes into the extracellular space may occur in the surroundings of dying cells. This may explain the citrullination of extracellular proteins, as observed for instance during NET formation (NETosis) (see in detail below) or the citrullination of beta-cell proteins as observed in T1D (15). Similar observations in RA show that PADs are highly activated by Ca^{2+} ion deposition in the inflamed joints, particularly during apoptotic cell death (97, 98), leading to elevated levels of citrullinated proteins and peptides.

PROTEIN CITRULLINATION DETECTION

The first antibody-based methodology for citrullinated protein detection was described in 1992 by Senshu et al. (99). The antibody does not recognize citrulline residues directly but instead binds a chemically modified form, diacetyl monoxime and antipyrine derivatized-citrulline, now available in commercialized kit form. The approach utilizes protein samples separated by SDS-PAGE, transferred to a microporous membrane. The membrane bound citrulline-containing proteins are modified in the presence of 2,3-butanedione monoxime and antipyrine in acid condition. In a similar concept known as the "Senshu" method, Moelants et al. developed a sandwich ELISA format to detect citrullinated proteins utilizing antibody recognizing 2,3-butanedione-modified citrulline (100). Another citrulline-specific labeling chemical probe, rhodamine-tagged phenylglyoxal derivative (Rh-PG), was developed for detection of citrullinated proteins in both purified protein sample and complex mixtures including serum (101, 102).

There are now commercially available anti-peptidyl citrulline antibodies for detection of citrullinated proteins including mouse monoclonal IgM antibody, clone F95, from Millipore (MABN328), and rabbit polyclonal antibody from Upstate (07-377) or Abcam (ab10092 and ab6464). Clone F95 antibody is extensively used in tissue staining, immunoblot and ELISA (25, 103, 104). The specificity and sensitivity of the above commercial anti-peptidyl citrulline antibodies for differentiating arginine citrullination and lysine carbamylation were recently discussed (105). Of note, carbamylation is a non-enzymatic PTM converting lysine to homocitrulline, which shares similar structural features with citrulline. For specific citrullinated target proteins, a variety of commercially available or in-house produced antibodies are available, such as those specific to citrullinated-histones, hypoxia-inducible factor 1-α, vimentin, fibrinogen, MBP and GRP78.

Upon citrullination, 0.984 Da mass increase from the parent peptide can be identified by mass spectrometry. As this is exactly the same increase in mass as for deamidated peptides, caution is needed when analyzing liquid chromatography-tandem mass spectrometry (LC-MS/MS) data, not to misinterpret a citrullinated peptide from a deamidated peptide on a closeby N- or Q residue. On top of that, C^{13} isotopes, with an increase in mass of 1 Da compared to C^{13} parent ions, can also be wrongfully identified as deamidated or citrullinated residues, even with the use of very accurate mass spectrometry instrumentation and strict settings. A recent study by Callebaut et al. (6) outlines critical parameters to detect deamidated residues by LC-MS/MS, through minimizing artificial *in vitro* occurring deamidations and manual inspection of spectra. This same method can also be applied for critical evaluation of citrullinated peptides from LC-MS/MS analysis. Another issue to be considered is that the loss of one positive charge of the arginine residue due to citrullination will affect protease cleavage efficiency for proteases which cleave after arginine, such as trypsin. This will result in mis-cleavages by trypsin, in case the arginine is citrullinated (106). Of particular interest is the dual search delta score method developed by Qian laboratory which integrates several critical parameters for identifying citrullinated and deamidated peptides in an automated way, thereby decreasing false discovery rates (FDR) (107). A different method used for facilitating LC-MS/MS-based

detection of citrullinated proteins is chemical derivatization of citrulline residues by 2,3-butanedione alone or combined with antipyrine, resulting in a mass increase of 50 or 238 Da, respectively (108, 109). Chemical derivatization of citrulline residues can also be used for enrichment of citrullinated proteins, through the use of biotin-conjugated phenylglyoxal (BPG), prior to LC-MS/MS analysis (101, 102). This method, however, requires large amounts of starting material. In addition, a BPG-based ELISA platform recently developed will validate the mass spectrometry proteomic data for citrullination detection (110).

INFLAMMATORY PATHWAYS INCREASE CITRULLINATION AND OTHER PTMS

Simply put, autoimmunity is initiated when cellular and soluble components of the immune system interact to trigger the recognition and robust response to self-proteins leading to tissue pathology. Additionally, a large number of heritable genetic risk traits have been defined by genome wide association studies (GWAS) in many autoimmune syndromes. However, autoimmune syndromes and T1D, in particular, are not entirely explained by a defined collection of heritable genetic traits. Indeed, poorly defined environmental influences and epigenetic factors, which may or may not be inherited, also influence the early onset and progression of T1D (111). Of importance, the amplification of PTMs in T1D autoimmunity is clearly linked to oxidative tissue environments. Reactive oxygen species (ROS), including superoxide anion ($O_2^{\cdot-}$), hydrogen radicals (OH.), and hydrogen peroxide (H_2O_2) are a product of a dynamic balance of endogenous anti-oxidant cellular compounds that control their tissue concentrations and biological effects. These anti-oxidants include superoxide dismutase (SOD), glutathione peroxidase, catalase, peroxiredoxins, as well as other small molecule anti-oxidants, including vitamins E and C. There are potentially a number of sources of specific ROS in tissue autoimmunity, including the infiltration of activated phagocytic cells (neutrophils, macrophages and dendritic cells) which have been demonstrated to be important in the progression and tissue pathology of T1D as well as many other autoimmune syndromes.

Oxidative stress can amplify the modification of certain proteins, or protein motifs, or, alternatively, alter metabolic pathways. As described in detail below, there are secondary effects of ROS on apoptosis, NETosis, and cellular metabolic pathways, affecting the progression of autoimmune responses and tissue pathology. ROS affected proteins may be changed in solubility, reduce the ability of proteins to be cleared, or increase immunogenicity. As detailed below, oxidation and subsequent citrullination can also provoke changes at both the level of DNA transcription and translation.

Citrullination of histone H3 by PAD4 in granulocytes, leading to formation of NETosis, is a process which is promoted by intracellular ROS (85, 112). Also, exogenous H_2O_2 was shown to induce citrullination and NETosis in mouse neutrophils (85). Depending on the stimulus used, the level and time course of ROS production was shown to be important for subsequent H3 citrullination and NET formation (113). However, seemingly contradictory to this notion, *in vitro* studies showed a direct inhibitory effect of PAD2 and PAD4 activity in the presence of H_2O_2 concentrations above 40 μM. In addition, PMA-stimulated leukocytes could effectively citrullinate recombinant fibrinogen, although this process was markedly enhanced when ROS formation was inhibited by the NADPH oxidase inhibitor diphenyleneiodonium (DPI) (114). These findings suggest that although ROS is important intracellularly for mediating citrullination of histones, supraphysiological levels of ROS may inhibit citrullination extracellularly. Discrepancies in different studies on the exact role of ROS in PAD activation and citrullination may need further examination to resolve the impact of intra- vs. extracellular ROS concentrations.

Another important factor mediating PAD activity is the local redox balance, with a reducing environment needed for efficient PAD activation. As such, *in vitro* studies, making use of the non-physiological reducing agent dithiothreitol (DTT), have shown that PADs can be activated under reducing conditions. Also more physiological reducing agents, like thioredoxin (115) and reduced glutathione (GSH) (116) can activate PAD enzymes. Kinetic characterization of PAD4 using thioredoxin as reducing agent produced results equivalent to those obtained with DTT (115).

Clearly, ER stress is a key factor in the amplification of PTMs in T1D. The insulin producing beta-cell, i.e. the immune targeted cell in T1D, is particularly sensitive to ER stress, because of its highly developed ER needed to cope with the high demands for protein translation and folding (117–119), in response to acute changes in blood glucose levels. As such, beta-cells can increase the translation of preproinsulin up to 25-fold (120), reaching almost 1 million molecules of preproinsulin per minute (121), when blood glucose levels are high. In order to restore the equilibrium between the cellular demand for protein synthesis and the ER folding capacity, cells under ER stress activate the unfolded protein response (UPR) (122). The UPR is activated by three transmembrane protein sensors, activating transcription factor 6 (ATF6), protein kinase RNA-like ER kinase (PERK) and inositol-requiring kinase 1 (IRE1). Under physiological conditions, these three sensors are kept in an inactive state through binding to the chaperone glucose-regulated protein 78 (GRP78; also known as binding immunoglobulin protein (BiP) or heat shock 70kDa protein 5 (HSPA5)) (123). When misfolded proteins accumulate in the ER lumen, GRP78 releases from these sensors inducing their activation and downstream signaling (123) leading to ER stress attenuation. When this UPR fails to restore ER homeostasis, usually in conditions of intense or chronic stress, this adaptive UPR will change to a terminal UPR (124), activating pro-apoptotic signaling pathways that lead to cell death (122).

Next to ER stress induced by high demands of protein translation, typical for secretory cells such as beta-cells, environmental factors associated with T1D can trigger

additional ER stress in beta-cells (125), including coxsackie viral infection, dysglycemia, inflammation, ROS and exposure to chemicals such as streptozotocin and alloxan (125, 126). Apart from inducing beta-cell dysfunction and death, ER stress can also induce PTMs in beta-cells, a process that has been described for thapsigargin induced ER stress, showing increased activation of tissue transglutaminase (TGM2) and PAD, both Ca^{2+} dependent enzymes (127, 128). Also, inflammatory cytokines, which act at least in part through activation of ER stress pathways, were shown to induce citrullination of beta-cell proteins (15, 16, 118, 129) (**Figure 3**). Interestingly, thapsigargin induced ER stress of human beta-cells leads to increased immunogenicity, as measured by IFNγ response of T-cell clones specific for deamidated peptides (127, 128).

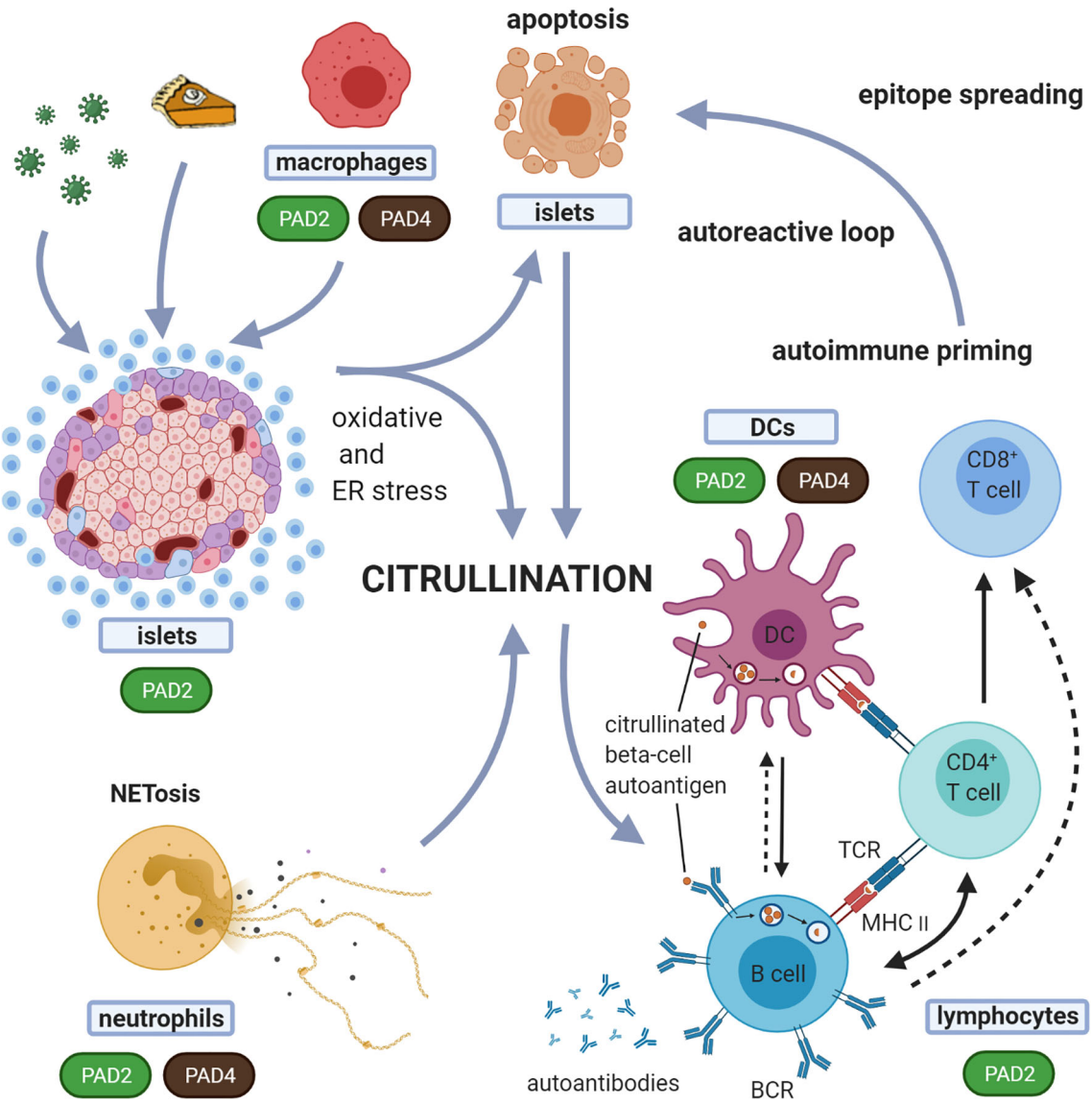

FIGURE 3 | Suggested model for the role of citrullination in the induction of autoreactive responses in T1D. In this proposed model, different forms of cell death, i.e. beta-cell apoptosis and neutrophil death (NETosis), are both implicated in the generation of citrullinated proteins. Any form of environmental stress, such as viruses, inflammatory cytokines or high metabolic demand, can induce oxidative and ER stress in the beta-cells. Oxidative stress induces NETosis. Both beta-cell apoptosis and NETosis have been shown to be involved in T1D initiation and propagation. With expression and activation of PADs during stress conditions, this model suggests that both beta-cells and neutrophils, can induce citrullination. These citrullinated peptides/proteins may be processed by the immune system in different immunogenic ways, forming a source of citrullinated autoantigens. Processing of these modified proteins/peptides by antigen presenting cells, and subsequent presentation to T cells, can in turn trigger several immune responses, including activation of B cells (producing islet autoantibodies) and islet antigen-specific (effector/memory) T cells that can directly kill beta-cells presenting citrullinated islet peptides. The expression of PAD enzymes in beta-cells and neutrophils, and their activation during processes of cellular stress, underscores the relevance of this model. Dashed arrows indicate the potential interactions between B cells and CD8+ T cells and between B cells and DCs. BCR, B cell receptor; TCR, T-cell receptor.

CITRULLINATED PROTEINS AND THEIR ROLE IN FUNCTIONAL PATHWAYS

Epigenetic Role and Biological Functions of Histone Citrullination

Histones are highly basic proteins, due to the abundance of lysine and arginine residues, assembling with DNA to form nucleosomes. Changes of the positive net charge of histone due to PTMs will affect its electrostatic interaction with chromatin and chromatin accessibility such as phosphorylation, acetylation and citrullination. Of note, citrullinated histones account for about 10% of all histones in granulocytes (80). Extensive studies demonstrate that PAD catalyzed histone citrullination regulates chromatin structure (condensation versus decondensation), transcriptional regulation, and a variety of biological pathways (**Table 3**).

Histone H1 function relies on basic amino acid residues to interact with chromatin and is responsible for the formation of higher-order chromatin structure. Linker histone H1 citrullinated at residue 54 (H1Cit54; mediated by PAD4) results in activation of several pluripotency genes, such as klf2, Tcl1, Tcfap2c and Kit, due to chromatin decondensation (130, 131). Linker H1Cit54 has also been found in breast cancer cells (138) and in activated neutrophils (41). Relevant to autoimmune syndromes, autoantibodies against citrullinated linker H1 were found in about 6% of sera from patients with SLE and SS (41).

The protein core of nucleosomes is composed by core histones, H2A, H2B, H3 and H4. H2A function is finely regulated by several PTMs such as acetylation, phosphorylation and methylation (139, 140). For example, protein arginine methyltransferase 5 (PRMT5) methylates H2A at residue arginine 3 (H2AR3) which serves as an epigenetic activator to promote prostate cancer growth (140). Of note, PAD4-catalyzed citrullinated histone H2ACit3, is identified in activated neutrophils (77). Compared to other histone proteins, citrullination on histone H3 has been extensively investigated. Different PAD isozymes catalyze H3 at different arginine residues and regulate various biological functions. Both PAD1 and PAD2 citrullinate histone H3 at 2, 8 and 17 residues, H3Cit2,8,17. Citrullination of histone tails at H3R2,8,17 and H4R3 was significantly reduced in 2- and 4-cell embryos after PAD1-morpholino knockout or treatment with a PAD1 specific inhibitor. Deficiency of PAD1 resulted in mouse embryo cells arrested at the 4-cell stage (133). In human mammary epithelium cells (MCF7 cells), PAD2-catalyzed H3R2,8,17 regulates gene expression of pleotropin (PTN) and melanoma associated antigen A12 (MAGEA12) (141). In addition, PAD4-mediated H3Cit8 diminishes the binding of heterochromatin protein 1α (HP1α) to methylated histone H3K9 and leads to the suppression of gene expression of human endogenous retroviruses (HERVs) and cytokines in MCF7 cells such as TNFα, IL-1A, IL-8, IL-16 and IL-23 (136). PAD2-catalyzed H3Cit26 results in chromatin decondensation and transcriptional activation of estrogen receptor α-regulated genes in breast cancer cells (68). Therefore, H3Cit26 is believed to be a potential prognostic biomarker for estrogen receptor positive (ER+) breast cancer (137). Similar to PAD1-catalyzed H3Cit2,8,17, PAD4-catalyzed H4Cit3 also facilitates early embryo genome transactivation (133). Moreover, PAD4-catalyzed H4Cit3 regulates the p53 pathway in apoptosis and in carcinogenesis (81).

Citrullination and Its Role in NETosis

NETosis is a cellular clearance mechanism distinct from apoptosis, which occurs when neutrophils encounter microorganisms and produce highly modified chromatin webs (142), immobilizing and killing the pathogens. The extruded DNA webs carry a number of bound bactericidal proteins (lactoferrin, elastase, proteinase 3, myeloperoxidase, cathepsin G, etc.) as well as histones and granule proteins. Thus, NETs serve as the first line defense mechanism of innate immunity to protect the host from bacteria, fungi, viruses and protozoa. Emerging evidence reveals that PTMs of histones in neutrophils regulate NETosis, and is associated with the development of autoimmune diseases such as RA, MS and SLE (**Table 3**). In leukocytes, both PAD2 and PAD4 are expressed (60), with PAD2 being mainly expressed in macrophages and PAD4 in

TABLE 3 | Biological functions of histone citrullination.

Histone	PAD isozyme	Physiological or pathological roles	References
Linker H1Cit54	PAD4	impairs binding to nucleosomal DNA chromatin decondensation in pluripotent stem cells	(130, 131)
H2ACit3	PAD4	occurs in calcium ionophore A23187-stimulated neutrophils	(77, 132)
H3Cit2,8,17	PAD1	facilitates early embryo genome transactivation	(133)
H3Cit2,8,17	PAD2	regulates lactation associated genes during diestrus in mammary epithelial cells	(69)
H3Cit2,8,17	PAD4	neutrophil extracellular trap (NET) formation and NETosis	(134, 135)
H3Cit8	PAD4	transcriptional repression of cytokines genes and human endogenous retroviruses (HERVs) *via* heterochromatin protein 1α (HP1α)	(136)
H3Cit26	PAD2	chromatin decondensation and transcriptional activation of estrogen receptor (ER) α-regulated gene potential prognostic biomarker for ER positive (ER+) breast cancer	(68) (137)
H4Cit3	PAD4	facilitates early embryo genome transactivation regulates p53 pathway in apoptosis and in carcinogenesis neutrophil extracellular trap (NET) formation and NETosis	(133) (81) (12–14, 134)

H, histone; Cit, citrullination; PAD, peptidylarginine deiminase.

monocytes, macrophages, eosinophils and neutrophils (63, 78). In neutrophils, citrullination is crucial for NET formation and release (85, 143). Activation of PAD4 leads to hypercitrullination of histones and consequently, decondensation and release of DNA structures coated with neutrophil granule proteins, the NETs (134, 143).

NETosis has been indicated as the major autoantigen source in SLE. NETs induce moderate levels of autoantibodies against H3Cit2, 17 and H3Cit26 in MRL/lpr mice, a spontaneous murine model of lupus (32). Moreover, autoantibodies against H1R53 were detected in patients with SLE (41). Recently, abundant citrullinated LL37 was identified in SLE target tissue (skin and kidney) and autoreactive T cells against both native and citrullinated LL37 were detected in patients with SLE, but not in RA (31). As mentioned earlier, NETosis is enhanced in RA circulating and synovial neutrophils and correlates with ACPA titers (144). H1Cit53 and H3Cit8,17,26 were found in RA and SLE neutrophil NETs (26). One study showed the presence of anti-citrullinated H2B antibodies in the anti-CCP2 positive sera from patients with RA (25). In addition, both H3Cit2,8,17 and citrullinated H4 from NETs were found as the targets of autoantibodies in patients with RA (27, 145).

The involvement of neutrophils and NETosis in T1D has been pointed out by several studies. In the non-obese diabetic (NOD) mouse, a spontaneous mouse model of autoimmune mediated beta-cell destruction and diabetes development, neutrophils and formation of NETs in the pancreas have been shown to be present during early stages of disease development and are required for diabetes development (146). In human T1D, one recent study illustrated a reduction in serum components of NETs [neutrophil elastase (NE) and proteinase 3 (PR3)], consistent with a reduced overall neutrophil count in early onset T1D (147). Conflicting studies report increases in these same NET components in T1D and a positive correlation of the circulating levels of these components with titers of autoantibodies against IA-2 and GAD65 (148). Neutrophil count is decreased in newly diagnosed T1D adult and pediatric patients (147, 149) as well as in pre-symptomatic autoantibody individuals (150). This reduction correlates with a decline in beta-cell function (151). Additionally, neutrophils infiltrate the pancreas before disease onset and during disease progression (151) and a significant fraction of these pancreas-infiltrating neutrophils forms NETs (54% and 50% in autoantibody-positive and T1D donors, respectively) (151). Relevant to citrullination, protein expression of PAD4 was elevated in neutrophils from patients with T1D and T2D (152). Given all these data indicating that neutrophils and NETosis are involved in diabetes development, the role of PADs and citrullination has also been implicated in T1D through their high expression in neutrophils and essential role in NET formation (**Figure 3**).

Of note, besides neutrophils, macrophages can release extracellular traps, a process called macrophage extracellular trap formation (METosis). The contribution of METosis in T1D has not been investigated yet, but METosis and PAD4 have been shown to contribute to self-antigen citrullination and ACPA production in autoimmune arthritis (153).

Citrullination in Transcriptional Regulation in Immune Cells

Besides NETosis, citrullination has other functions in the immune system. In neutrophils, citrullination of NF-kB p65 enhances its nuclear translocation and transcriptional activity, increasing Toll-like receptor (TLR)-induced expression of IL-1β and TNFα (84). Citrullination of the transcription factors GATA3 and RORγt by PAD2 determines the fate of differentiating Th cells. As such, citrullination of GATA3 on R330 weakens its DNA binding ability, thereby decreasing transcription of Th2 cytokines, attenuating differentiation of Th2 cells. On the other hand, citrullination of RORγt on R56,59,77, 90 strengthens its DNA binding ability, increasing the transcription of IL-17A/F thereby enhancing the differentiation of Th17 cells (72). Also, citrullination of RNA polymerase II by PAD2 favors an efficient transcription of genes related to cellular proliferation (154). Citrullination can also reduce the potency of chemokines, such as CXCL8, CXCL10, CXCL11 and CXCL12, when compared to their native form (155–157), thereby dampening inflammatory reactions. Additionally, PADs can citrullinate the cytokine TNFα (158), reducing its capacity to stimulate the production of inflammatory chemokines, and TNFα can induce the translocation of PAD4 from the cytosol to the nucleus (38).

Citrullinated Proteins and Their Role in Increasing Antigenicity in T1D

A primary function of the immune system is to differentiate between self and non-self-proteins. This is achieved by mechanisms that deplete the immune system of lymphocytes that react too strongly to self-antigens being present in the thymus and bone marrow, resulting in tolerance towards self-proteins. For achieving T-cell tolerance, the medullary thymic epithelial cells (mTECs) play an important role in this so-called negative selection (159). This mechanism works effectively for many self-antigens that are expressed in the thymus, through mTEC specific transcriptional regulator AIRE (autoimmune regulator), which drives expression of tissue-restricted genes such as islet specific genes, in the thymus (160). However, whether post-translationally modified self-proteins (**Tables 1** and **4**) are also expressed in the thymus has not been extensively investigated. If not, this could create a novel autoantigenic proteome for which immune tolerance has not been established in the thymus. This concept, previously described as 'autoantigenesis' is a term described to proteins that 'evolve' and acquire PTMs in a disease related target tissue, during the course of disease development, leading to B and/or autoreactive T-cell responses (4). Evidence for such antigenic modifications was shown in a mouse model of tissue-restricted self-antigen collagen type II, where the PTM reactive T cells escaped thymic selection (162). In regard to citrullination, it has been shown that PAD2 and PAD4 are expressed in murine mTECs, as measured by qPCR, thereby demonstrating that the prerequisites for negative selection of citrulline-specific T cells in the thymus are met in C57Bl6 mice (163). However, whether these enzymes are active and able to convert arginine into

TABLE 4 | Citrullination in T1D.

Target proteins	Affected immune responses	References
GAD65	target of autoreactive T cells (HLA-A2 and HLA-DRB1*04:01)	(12, 13, 17)
IAPP	target of autoreactive cells (HLA-DR and HLA-DQ8)	(17, 127, 161)
IA-2	potential target of autoreactive T cells (HLA-A2)	(13)
GRP78	target of autoreactive T cells (HLA-DRB1*04:01) recognized by autoantibody	(15–17)
IGRP	potential target of autoreactive T cells (HLA-A2)	(13)

GAD65, glutamic acid decarboxylase 65; IAPP, islet amyloid polypeptide; IA-2, islet antigen-2; GRP78, glucose-regulated protein 78; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein.

citrulline in proteins locally in the thymus, and whether this PAD expression and citrullination capacity is defective in autoimmune strains, such as the NOD mouse, needs further investigation.

In T1D, progressive loss of B and T-cell tolerance to beta-cell specific antigens leads to the destruction of insulin producing beta-cells. A growing number of studies suggest that immune recognition of non-conventional generated peptides/proteins (164), amongst which citrullinated proteins (12, 15, 16, 165) are an important component of that loss of tolerance (**Table 4**). As such, citrullinated proteins, generated through different stress pathways in beta-cells or neutrophils, as outlined above, could be a source of citrullinated antigens. The expression of PAD enzymes both in beta-cells and neutrophils, their activation during processes of cellular stress, and the role for both beta-cell apoptosis and NETosis in initiation and propagation of T1D, fit with such view (as schematically shown in **Figure 3**). The citrullinated peptides/proteins may be processed by the immune system in different immunogenic ways, forming a source of citrullinated autoantigens. Presentation of citrullinated proteins/peptides by antigen presenting cells, and subsequent presentation to T cells, can trigger several immune responses, including activation of B cells (producing islet autoantibodies) and islet antigen-specific (effector/memory) T cells that can directly kill beta-cells presenting citrullinated islet peptides.

Already in 1993 it was shown that the insulin B chain is prone to citrullination in the bacterial model *Porphyromonas gingivalis* (166), however, only during the last decade several publications described the link between citrullinated beta-cell proteins and autoreactive responses in T1D. In comparison with native peptides, citrullinated peptides present higher binding affinity to HLA-A2 (13) and HLA-DRB1*04:01 (12, 13, 167), diabetes-associated HLA class I and class II molecules, respectively. Of interest, there is a significant overlap in genetic susceptibility between T1D and RA, with HLA-DRB1*04:01 haplotype being a high-risk haplotype in both diseases. The antigenicity of citrullinated beta-cell antigens that bind to HLA-A2 has been demonstrated by *in vitro* activation of $CD8^+$ T-cell clones, expanded from peripheral blood of HLA-$A2^+$ T1D subjects, when cultured with citrullinated peptides derived from IA-2, GAD65 and IGRP (13). One citrullinated peptide of GAD65 with higher binding affinity to HLA-DRB1*04:01 was shown to be recognized by $CD4^+$ T cells in the peripheral blood of T1D patients making use of *ex vivo* tetramer assays. These $CD4^+$ T cells exhibited an antigen-experienced phenotype and were less- or non-responsive to the native form of the epitope (12). Shortly thereafter, Rondas et al. revealed that the ER chaperone GRP78, a major ER chaperone and a key mediator of the UPR pathway, can be citrullinated in INS-1E beta-cells under inflammatory stress, as shown by 2D-Western blotting using a citrulline specific antibody (16). This confirmed earlier proteomic studies showing an increase in PTM for GRP78 in INS-1E after IFNγ plus IL1β exposure (129). Citrullinated GRP78 was shown to be immunogenic in diabetes-prone NOD mice with appearance of both autoreactive T cells and autoantibodies (16). Furthermore, it was shown that NOD mice have significantly higher levels of *Padi2* mRNA and PAD activity in islets, already at the age of 3 weeks, as compared to normoglycemic C57Bl6 mice (16, 165). Absence of IL1β and IFNγ expression at this young age, indicated no or minor immune infiltration in the islets, suggesting expression of PAD in the endocrine cells. Further proof for this was provided in a later study, in which $CD45^+$ islet infiltrating immune cells from 10-week-old NOD mice were separated from beta and alpha cells by FACS sorting. Expression of *Padi2* mRNA was evident in all 3 fractions, with comparable levels in beta-cells and $CD45^+$ immune cells (Sodré et al., 2021). Further *in vitro* studies showed that GRP78 was not only citrullinated upon inflammatory stress, but was also translocated from the ER to the plasma membrane and secreted, at which level the modification of GRP78 was even much more pronounced (16, 118). This opens the view that GRP78 may become citrullinated once exposed on the plasma membrane or even after its secretion into the extracellular space. First evidence for a role of citrullinated GRP78 in human T1D, came from the discovery of a $CD4^+$ T-cell clone, isolated from an islet outgrowth of a deceased T1D patient, which recognized citrullinated GRP78 epitope 292-305 (citrullinated at position 297) (17). The same study also showed autoreactive $CD4^+$ T cells against citrullinated IAPP. Direct evidence for citrullination of human GRP78 at arginine residue 510 in cytokine-exposed islets was shown by targeted LC-MS/MS. Using *ex vivo* tetramer and ELISA assays, the same study showed that a subpopulation of T1D patients presents higher frequencies of $CD4^+$ T cells against a citrullinated GRP78 epitope and elevated titers of autoantibodies against citrullinated GRP78 compared to

healthy subjects (15). Moreover, T-cell responses and autoantibodies against citrullinated GRP78 were more frequent in long-standing T1D than in patients with new-onset T1D. Although the number of patients included in this study is too low to make hard conclusions, these last findings may indicate that immune responses against citrullinated GRP78 contribute to aggravation and/or acceleration of the disease rather than to the initiation of the disease development. Longitudinal studies on at-risk subjects are awaited to further evaluate the exact role of citrullinated antigens in disease staging.

PTMS CAN TRIGGER MORE EXTENSIVE AUTOIMMUNITY, SUCH AS EPITOPE SPREADING

Several investigators have identified significant differences between the T- and B-cell responses that develop against PTM self-proteins, or cryptic epitopes (5, 13, 128, 168–170). In general, T-cell responses to PTM determinants tend to be specific for the modified peptide only and more rarely cross-react with the unmodified form of the peptide. This concept was originally demonstrated in mice immunized with the isoaspartyl PTM form (isoAsp) of snRNP D lupus autoantigen protein, showing T cells only proliferate in response to the isoAsp PTM, but are unresponsive to the native (Asp) peptide (168). Alternatively, B-cell and autoantibody responses are often more promiscuous in their binding to both the PTM modified and native self-protein. This phenomenon may be due to the features of antibodies to bind flanking amino acid sequences in both modified and native protein forms. As one example, human SLE and lupus-prone MRL/lpr mice exhibit autoantibodies that bind both isoAsp and Asp forms of histone H2B (171). Moreover, it was demonstrated that autoimmune responses originating from the PTM self-protein diversify in an intra- and extra-molecular manner to other self-protein determinants. Therefore, breaking immune tolerance to a PTM self-protein promotes 'epitope spreading', a mechanism where autoimmunity diversifies to epitopes beyond the initial site(s) initiating the response (170). Similarly, there is both intra- and inter-molecular B- and T-cell epitope spreading in T1D, SLE, and MS (168, 172). Classical studies have described epitope spreading of autoantibody responses with the progression of autoimmune disease (173). As illustrated earlier, apoptotic and necrotic cells are rich sources of PTM-altered self-proteins in various microenvironments, notably conditions of oxidative stress (174) or altered pH (175).

In both of the collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) mouse models, citrullination of joint and brain proteins creates neoantigens that become additional targets in epitope spreading of autoimmune responses (172). Citrullination of aggrecan, vimentin, fibrinogen, and type II collagen, known target proteins in RA, initiates epitope spreading by promoting T-cell responses to both citrullinated peptides and the corresponding control peptides (176). In addition, citrullination-induced conformational changes of HSP90 protein unmasks cryptic epitopes to bypass B-cell tolerance in RA (177). Epitope spreading may also apply for citrullinated epitopes in triggering T1D autoimmunity (**Figure 3**). This is shown for example for citrullinated GRP78, where circulating $CD4^+$ T cells and autoantibodies against both naïve and citrullinated GRP78 peptides are detectable in patients with T1D, suggesting that epitope spreading due to citrullination also occurs in T1D (15).

PTMS IN ANTIGEN PROCESSING AND PRESENTATION

It is clear that the specificity of ongoing immunity relies on the efficient and accurate antigen processing pathways in epitope generation (178). The presence (or absence) of a PTM of an amino acid residue has been shown to alter the recognition and cleavage by proteases. Additionally, the presence of PTM within an intracellularly processed peptide alters the binding to specific MHC motifs. For example, it has been demonstrated that the absence of N-glycosylation of the neuronal glutamate receptor subunit 3 in Rasmussen's encephalitis, a severe form of pediatric epilepsy, exposes a granzyme B cleavage site. This is just one example of how PTMs may create a novel autoantigen (neoepitope) (179). As another example, most proteases and peptidases *do not* recognize the peptide linkage connecting isoAsp residues to its neighboring amino acid (180). Finally, Moss and coworkers demonstrated that the deamidation of asparagine residues in tetanus toxin C fragment inhibits the processing by asparagine endopeptidase and results in decreased antigen processing (181). The role of PTMs in antigen processing was examined years ago in studies of model proteins in immunity (168, 182–184). An isoaspartylated form of cytochrome c protein is cleaved differently by cathepsin D compared to the (normal) aspartyl form of the same protein (2). Granzyme B cleavage of autoantigens may also generate new epitopes based on the presence or absence of PTMs in self-protein (185). Simply put, the presence of PTMs that affect proteolytic enzyme recognition generates a completely new repertoire of peptides during antigen presentation.

Specific subsets of APCs and even the compartments within the cells in which the antigen is processed may shape the type of PTM acquired and whether modified peptide is presented on MHC. Ireland and colleagues (186) demonstrated that B-cell autophagy was required for the generation and presentation of a citrullinated peptide (but not required for the non-modified peptide form). Mamula and others have demonstrated the unique APC functions of B cells in presenting antigen to T cells. In particular, it is clear that B cells both present antigens directly, as well as transfer antigens to other APC, such as macrophages and dendritic cells. Thus, different APCs may dictate the self PTM epitopes generated and eventually presented by the immune system (187–189).

It is not fully understood as to how a PTM will affect HLA binding. The 'fit' of the PTM peptide versus native peptide for an

HLA molecule can vary based on elements including charge and structural changes imposed by PTMs. The HLA binding of various PTM T1D autoantigens has been studied and reviewed by James et al. (12, 13). For deamidated and citrullinated residues specifically, the introduction of a negative charge enhances the binding affinity at specific positions in HLA-DR or HLA-DQ molecules. As such, HLA-DRB1*04:01 and DRB1*15:01 prefer citrulline at key positions of their binding motifs, whereas deamidation results in preferential loading onto HLA-DQ molecules (13). This was also shown for citrullinated peptides of vimentin, a RA autoantigen, which has greater affinity for HLA-DRB1*0401 than the unmodified peptide (190).

Among other autoimmune syndromes, specific PTMs of MBP result in either low, intermediate or a similar affinity for MHC compared with the corresponding wild-type peptide (191). Notably, acetylated MBP peptide (Ac 1-11) is required to incite pathogenic T cells in murine MS, though the unmodified peptide binds MHC with virtually identical kinetics. Similarly, isoaspartic acid residues in cytochrome c or snRNP D peptides (SLE autoantigens) bind MHC class II in a manner identical to the unmodified peptides (168). However, immune tolerance is maintained to the native peptides. The overall lesson is that PTM self-peptides alter processing and binding by MHC in distinct manner from unmodified (native) peptide.

TARGETING CITRULLINATION: TYPES OF PAD INHIBITORS AND THEIR POTENTIAL AS THERAPEUTIC AGENT IN PRECLINICAL MODELS OF AUTOIMMUNITY

Given the involvement of citrullination and PAD enzymes in several autoimmune diseases, it has been of great interest to generate compounds targeting PAD activity, i.e. PAD inhibitors. The development of such compounds was made possible with the discovery of the crystal structure of PAD enzymes (95, 192). Since then several pan-PAD inhibitors have been developed, with increasing potency, specificity and metabolic stability, of which the 4 most extensively studied irreversible inhibitors are shown in **Figure 4**. More recent efforts also include the development of specific PAD inhibitors, targeting for instance PAD2 or PAD4, as well as non-covalent reversible inhibitors. For a detailed overview of all developed PAD inhibitors we refer to a recent review specifically focusing on these developments (197).

Considering that PAD dysregulation is associated with several autoimmune diseases (86), numerous studies have evaluated the efficacy, safety and mechanism of action, mainly making use of irreversible pan-PAD inhibitors in animal models of autoimmunity, with promising results. As such, amelioration or even reversal of disease in case of intervention therapies, and delayed initiation or complete protection in case of preventive therapies, was shown in mouse models of RA, MS, SLE, UC, inflammatory bowel disease, and recently also in T1D [reviewed by (198, 199)]. In general, mechanistic insights from these studies have taught us that targeting PAD making use of pan-PAD inhibitors effectively decreases protein citrullination levels in the inflamed target tissues, as measured mainly by LC-MS/MS, PAD activity assays or Western blotting using anti-citrulline Ab (200–203). Such decreased protein citrullination can evidently have an effect on the autoreactive responses against citrullinated autoantigens, both in terms of autoantibody and T-cell responses, as shown in some published studies (200, 201). Next to this, it has become clear that other mechanisms are involved in the observed protection, pointing towards more general effects on innate and adaptive immunity. Not surprisingly, based on the high levels of PAD4 in neutrophils and their important role in histone 3 citrullination and induction of NETosis, as outlined above, *in vivo* PAD inhibition was shown to reduce NET formation and associated NET-induced tissue damage (204, 205). Other ameliorating disease effects were associated with a decrease in circulating pro-inflammatory cytokine levels, such as IL-6, TNFα and IL-1β (206, 207), an increase in Treg populations (200) or a shift in T-lymphocyte populations from Th1/Th17 towards Th2 (201, 208). The latter is thought to be mediated through direct inhibition of citrullination of the transcription factors RORγT and GATA3 (see also above) (72).

Of interest in the field of T1D, a recent study from the Overbergh laboratory showed a complete protection against diabetes development in the NOD mouse, by daily subcutaneous injections of BB-Cl-amidine (1μg/g body weight) (200). BB-Cl-amidine is a pan-PAD inhibitor that, similar to its mother compound (Cl-amidine), irreversibly inactivates PAD enzymes through covalent modification of an important cysteine for the activity of the enzymes (209). Remarkably, diabetes protection was observed when starting treatment at 8 weeks of age, a time point at which insulitis is already ongoing, but hyperglycemia has not developed (200). This observation tempts us to conclude that citrullination may play a role in amplification of the disease rather than being an initial trigger in breaking immune tolerance. BB-Cl-amidine treatment was associated with decreased citrullination levels in the pancreas as demonstrated by LC-MS/MS and western blotting with anti-citrulline Ab, as well as decreased levels of circulating autoantibodies against citrullinated GRP78, a known citrullinated autoantigen in T1D (as outlined above, **Table 4**) (15, 16). These findings confirm the effective direct action of BB-Cl-amidine on inhibition of PAD activity. Furthermore, bone-marrow derived neutrophils isolated from BB-Cl-amidine treated mice showed less potential for spontaneous NET formation when compared to the control group. Disease protection was associated with preservation of pancreatic insulin levels, although only a marginal reduction in insulitis was observed, suggesting a less aggressive form of insulitis, in line with reduced $CD4^+$ effector memory T cells and reduced IFNγ-producing T cells in the pancreas infiltrates. In the periphery, a shift from Th1 towards Th2 cytokine levels and increased frequency of regulatory T cells was observed.

Taken together, the promising results obtained with pan-PAD inhibition in the NOD mouse, as well as in other autoimmune mouse models, indicate that disease protection is

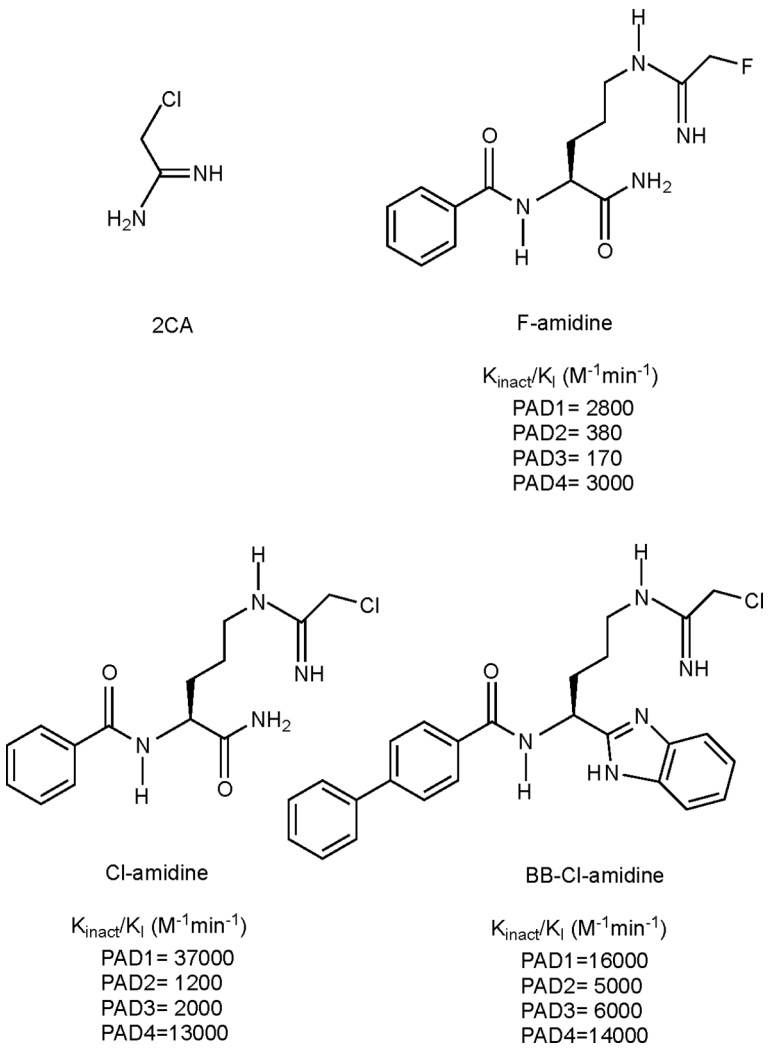

FIGURE 4 | Structures and k_{inact}/K_I values for some irreversible pan-PAD inhibitors. 2CA (193), F-amidine, Cl-amidine (194) and BB-Cl-amidine (195). k_{inact}/K_I values have been described as the best measure of potencies for irreversible inhibitors (196). k_{inact}: rate of enzyme inactivation; K_I: inhibition constant.

mediated by effective inhibition of citrullination in the target inflamed tissue, leading to decreased autoreactive responses. This further underscores the observation that citrullination is not a specific disease-related event, but rather an inflammation-dependent process occurring preferentially in autoimmune target tissues (210). Next to this, the extensive data in different preclinical autoimmune models indicate that pan-PAD inhibitors also have more general effects on immune cells, thereby dampening inflammation and reshaping the immune response towards tolerance. Whereas this may be an additional benefit for the use of PAD inhibitors, caution is also warranted. Since pan-PAD inhibitors act both intra- and extracellularly, and citrullination is important in many physiological processes, such as epigenetic and transcriptional regulation, as outlined above, the concern about possible adverse side effects cannot be neglected. Therefore, more studies are needed to evaluate more specifically potential adverse effects before translating to the human situation. Of interest in this regard is the development of isozyme-specific PAD inhibitors, which are hoped to still be protective in disease development, but allow physiological citrullination to occur. Great progress has been made in this path, with development of a high number of PAD specific inhibitors. For instance, a PAD1-selective inhibitor (211), D-Cl-amidine, and a specific PAD2 inhibitor (194), AFM30a, both based on the structure of Cl-amidine. PAD4-specific inhibitors include TDFA (212) and GSK199, with GSK199 being a reversible PAD4 inhibitor that targets the apo state of the enzyme, showing potent inhibition of PAD4 at low concentration of calcium (0.2mM) (143). GSK199 has been demonstrated to be effective in blocking murine arthritis (213), however, more studies are needed to evaluate the efficacy and safety of such specific inhibitors.

CONCLUDING REMARKS

In summary, we have attempted to illustrate and summarize various biochemical, immunologic, and transcriptional aspects of citrulline modifications. How citrullination alters these unique biologic processes in spontaneous autoimmune syndromes are beyond the prediction of simple genetics or other stochastic factors. Citrullination, as well as many other PTMs and cellular pathways, are affected by inflammatory cytokines and reactive oxygen species that inhabit the tissue microenvironments in T1D and other autoimmune diseases. More indirect pathways are also affected by citrulline modified proteins, including downstream transcriptional events. The emerging technologies of detecting citrulline modifications as well as other PTMs in proteomics and tissue analyses will undoubtable change the landscape of autoimmunity in the coming months and years. These analyses, notably the presence of serum anti-citrulline autoantibodies, have already contributed to the clinical diagnoses and assessment of the progression of disease, and tissue pathology. With the identification of specific biomarkers and an understanding of their origins, the field will now have potential therapeutic pathways as targets to modify these autoimmune diseases, including the reducing tissue inflammation and use of inhibitors of citrullination prior to destruction of the pancreatic islets.

AUTHOR CONTRIBUTIONS

M-LY, FS, MM, and LO conceived the concept and co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Extracellular Vesicles in Immune System Regulation and Type 1 Diabetes: Cell-to-Cell Communication Mediators, Disease Biomarkers, and Promising Therapeutic Tools

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Extracellular vesicles (EVs) are generated by cells of origin through complex molecular mechanisms and released into extracellular environment. Hence, the presence of EVs has been described in multiple biological fluids and in most cases their molecular cargo, which includes non-coding RNAs (ncRNA), messenger RNAs (mRNA), and proteins, has been reported to modulate distinct biological processes. EVs release and their molecular cargo have been demonstrated to be altered in multiple diseases, including autoimmune diseases. Notably, numerous evidence showed a relevant crosstalk between immune system and interacting cells through specific EVs release. The crosstalk between insulin-producing pancreatic β cells and immune system through EVs bidirectional trafficking has yet started to be deciphered, thus uncovering an intricate communication network underlying type 1 diabetes (T1D) pathogenesis. EVs can also be found in blood plasma or serum. Indeed, the assessment of circulating EVs cargo has been shown as a promising advance in the detection of reliable biomarkers of disease progression. Of note, multiple studies showed several specific cargo alterations of EVs collected from plasma/serum of subjects affected by autoimmune diseases, including T1D subjects. In this review, we discuss the recent literature reporting evidence of EVs role in autoimmune diseases, specifically focusing on the bidirectional crosstalk between pancreatic β cells and immune system in T1D and highlight the relevant promising role of circulating EVs as disease biomarkers.

Keywords: extracellular vesicles, exosomes, type 1 diabetes, autoimmunity, immune regulation, pancreatic islets

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INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by chronic hyperglycaemia, caused by β cells destruction due to specific autoreactive T cells and other immune cells. Despite this simplified definition, the striking heterogeneity of T1D pathogenetic mechanisms has been investigated and reported in multiple studies (1, 2). Such heterogeneity is clinically characterized by different age at onset, disease progression timing and severity (3). The elucidation of additional pathogenetic mechanisms may help to further understand the pathological bases of T1D heterogeneity both at molecular and clinical level.

Extracellular vesicles (EVs) have been recently reported to be involved in multiple diseases and shown to represent pivotal mediators of several pathogenetic mechanisms. Different types of EVs can be identified in biofluids based on their size, content, and surface markers. They are mainly classified in exosomes and ectosomes, both potentially involved in cell-to-cell communication. As a matter of fact, recent evidence demonstrated that under certain conditions, immune cells can communicate with each other and with other cell types through specific mechanisms driven by EVs release and uptake. Specifically, pioneering studies showed that during T1D progression, pancreatic islet infiltrating lymphocytes secrete a distinct subset of EVs capable of transferring a specific cluster of microRNAs (miRNAs) to β cells, thus causing apoptotic pathways activation. On the other hand, β cell EVs release may influence immune cells function through direct lymphocytes activation, or distinct EVs containing autoantigens release and subsequent uptake by local antigen-presenting cells (APCs). Taken together, such studies indicate that an active communication channel between β cell and immune system, based on EVs secretion, may be involved in the pathogenesis and progression of T1D. Of note, it has been also demonstrated that EVs released by pancreatic β cells can contain autoantigens which are transported to neighboring immune cells, thus potentially contributing to the typical autoimmune response (4–6).

Considering EVs ability to mediate signals exchange among different cell types of multiple tissues/organs, it is conceivable that EVs cargo could be an interesting tool to evaluate pathogenetic mechanisms involved in T1D and other autoimmune diseases as well as for the identification of potential biomarkers for the prediction and the progression of the disease. The communication features of EVs could also be used to develop novel therapeutic tools thus opening the way to innovative avenues for the cure or prevention of T1D. As a matter of fact, several evidence of EVs therapeutic ability to induce immunomodulatory effects or immunotolerance have been reported in different diseases.

EXTRACELLULAR VESICLES (EVs) BIOGENESIS, CLASSIFICATION, AND COMPOSITION

Extracellular vesicles (EVs) are a highly heterogeneous population of phospholipid bilayer membrane-enclosed vesicles, currently classified based on their biogenesis, release mechanism, size, content, and function (7–10). Although the classification of EVs is continuously evolving, they are usually classified into two major categories: ectosomes and exosomes (11, 12).

Ectosomes are directly shed from the plasma membrane and include microvesicles and large vesicles ranging from ~50 nm to 1 μm in diameter. On the contrary, exosomes have endosomal origin and are characterized by a ~40 to 160 nm diameter (~100 nm on average) (13, 14). Their biogenesis and secretion include specific intracellular processes determining selective molecules cargo loading that potentially defines their composition and, consequently, their function (15–17). Exosomes are generated through a process of double invagination of the plasma membrane. The first step leading to the invagination process results into *de novo* formation of an early-sorting endosome (ESE) characterized by cell-surface proteins and soluble proteins (18, 19). Moreover, since ESE continuously exchanges cargo constituents with endoplasmic reticulum (ER), trans-Golgi network (TGN), and mitochondria, it is specifically enriched for proteins and molecules typical of these organelles. Subsequently, the early-sorting endosome can mature into a late-sorting endosome through a second invagination of the plasma membrane leading to the generation of the Multi Vesicular Bodies (MVBs) composed by multiple intraluminal vesicles (ILVs). Based on the invagination volume, ILVs show different sizes and content (20, 21).

MVBs play an essential role both as intermediate progenitor extracellular vesicles and as part of the degradative lysosomal pathway. In details, MVBs can: (*i*) fuse with lysosomes resulting in the digestion of their ILVs or (*ii*) fuse with the plasma membrane thus leading to the release of ILVs as exosomes (**Figure 1**) (22, 23).

The endosomal sorting complex required for transport (ESCRT) plays an essential role in the generation of MVBs. ESCRT mechanism consists of 4 protein subcomplexes (ESCRT-I to IV) acting in a progressive fashion to mediate exosome generation (37–43). The presence of relevant ESCRT proteins in purified exosomes [e.g., tumor susceptibility gene 101 protein (TSG101) and apoptosis-linked gene 2-interacting protein X (ALIX)], represents the evidence of their MVBs origin (18, 44–46). Several studies suggested that ESCRT-independent mechanisms are also involved in MVBs and ILVs biogenesis. These mechanisms involve lipids, tetraspanins and/or heat shock proteins (HSPs) (47–51), thus opening to the presence of multiple parallel mechanisms of exosomes biogenesis.

As a result of their endosomal origin, exosomes carry proteins associated with MVBs formation (Alix and TSG101, as previously cited) and/or in membrane transport and fusion (Rab proteins and annexins) as well as proteins mostly associated with lipid microdomains and exposed to the external exosomes surface, such as integrins and tetraspanins (e.g., CD63, CD9, and CD81) (24–28) (**Figure 1**). However, the identification of novel exosomal molecules is rapidly evolving. Indeed, recent studies demonstrated that Annexin A1 is a specific marker of microvesicles shed from the plasma

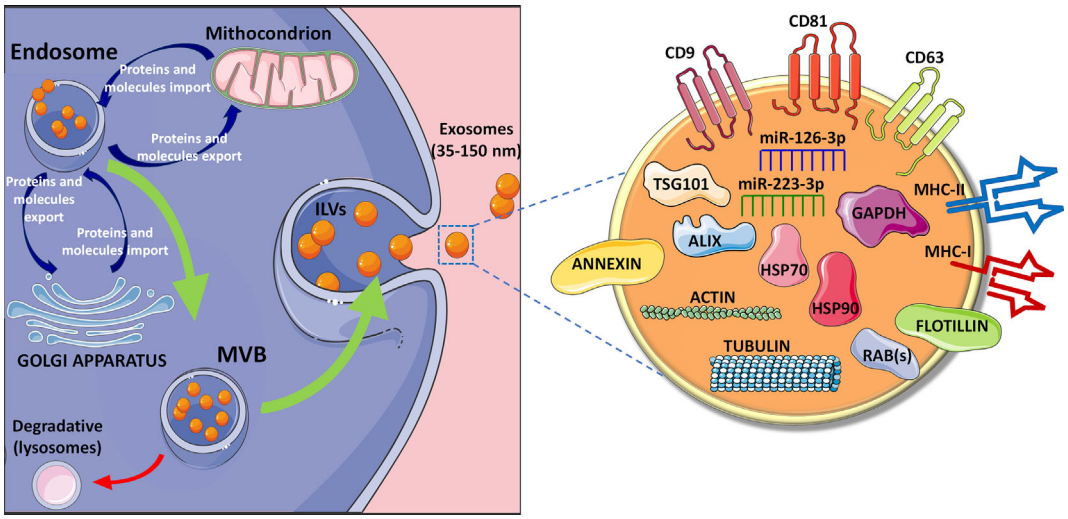

FIGURE 1 | Exosomes biogenesis and composition. Exosomes biogenesis is based on a process of double invagination of the plasma membrane. The first step leading to the invagination process results into *de novo* formation of an early-sorting endosome characterized by cell-surface proteins and soluble proteins. Following the continuous exchange (import and export) of specific proteins and molecules from endosomes and mitochondria as well as with Trans Golgi network, the early-sorting endosome can mature into a late-sorting endosome through a second invagination of the plasma membrane leading to the generation of the Multi Vesicular Bodies (MVBs) composed by multiple intraluminal vesicles (ILVs). Then, MVBs can: (*i*) fuse with lysosomes resulting in the digestion of their ILVs or (*ii*) fuse with the plasma membrane thus leading to the release of ILVs as exosomes (22, 23). Exosomes expose several membrane proteins, such as tetraspanins (CD9, CD81, CD63) (24–28) and MHC molecules, both class I and class II, as well as Annexin and Flotillin. Moreover, exosomes content is highly variable depending on the cell of origin, but usually they contain cytoskeleton proteins (e.g., actin and tubulin), heat shock proteins such as HSP70 and HSP90, proteins associated to metabolism (GAPDH) (29–31), as well as specific proteins (e.g., ALIX, TSG101 and RAB proteins). Exosomes also contains different RNA species such as microRNAs, among which the most reported are miR-126-3p and miR-223-3p (32–36).

membrane, while the tetraspanin CD63 is a typical marker of those vesicles released through ESCRT-independent mechanisms, thus rendering these markers not specific for a pure exosome population (52–55). Other proteins identified in almost all exosomes, independently of their origin, are Heat Shock Proteins (HSPs) (e.g., HSP90), cytoskeleton proteins (actin and tubulins), metabolic proteins (GADPH), and major histocompatibility complex (MHC) class I and II molecules (29–31).

Importantly, exosomes also contain different species of RNAs that can be transferred to recipient cells (56–62). Among these, microRNAs (miRNAs), small non-coding RNAs approximately 19 to 24 nts in length, have generated a particular interest in the scientific community for their regulatory function. Two putative pathways of miRNAs sorting into exosomes have been suggested: (i) neural sphingomyelinase 2 (nSMase2)-dependent pathway; (ii) sumoylated heterogeneous nuclear ribonucleoprotein (hnRNP)-based sorting. Although additional studies are needed, it was demonstrated that overexpression of nSMase2 causes an increase in the amount of exosomal miRNAs, while its inhibition leads to a reduced number (63, 64). Regarding hnRNP-based sorting, it has been reported that sumoylated hnRNPA2B1 recognizes the GGAG motif in the 3' portion of miRNAs sequences triggering a specific sorting into EVs (65). Such evidence underlines that the 3'-end of the miRNA sequence contains an important sorting signal motives, which strongly contributes to the miRNAs sorting into EVs (66). However, the presence of specific miRNAs sequence motives is not the only leading cue determining its loading into EVs; indeed, loading mechanisms can also be modulated by miRNA-induced silencing complex (miRISC). Of note, the specific knockout of ribonucleoprotein complex Ago2 (main component of RISC complex) induced the reduction of some EVs enriched miRNAs (miR-142-3p, miR-150, and miR-451) from HEK293T cells (67), thus indicating that the presence of a fully functional miRISC protein complex is fundamental for miRNAs sorting through EVs.

Different miRNAs, such as miR-126-3p and miR-223-3p, have been reported to be enriched and secreted through EVs (**Figure 1**), even though additional confirmatory studies are needed to definitely characterize specific EVs miRNAs content (32–36).

Interestingly, in order to regularly update information about EVs miRNAs, several databases across different biofluids and isolation methods have been generated, such as the extracellular vesicles miRNA database (http://bioinfo.life.hust.edu.cn/EVmiRNA), Vesiclepedia (http://microvesicles.org/), ExoCarta (http://exocarta.org/), and miRDB (http://mirdb.org/) (26, 27, 68, 69).

EVs ROLE IN IMMUNE SYSTEM HOMEOSTASIS AND IMMUNE CELLS CROSSTALK

In order to support cellular homeostasis and provide host defense, immune system has developed direct and indirect

communication mechanisms, such as the release of soluble factors or the transfer of information through EVs (70). The latter has been extensively investigated in macrophages, dendritic cells (DCs), B cells and T cells. Indeed, EVs secreted by all these cells are highly interconnected and act in several molecular mechanisms controlling immune system function, mainly through the modulation of initiation, expansion, and maintenance of innate and adaptive immune responses (9, 71, 72).

Several studies have shown that macrophages infected with different intracellular pathogens, such as *Mycobacterium tuberculosis*, Bacillus-Calmette-Guèrin (BCG), *Salmonella*, *Toxoplasma*, or *Leishmania*, can release EVs carrying pathogens-associated molecular patterns (PAMPs). The exposure of naïve macrophages to such EVs leads to an increased production of cytokines *via* Toll-like receptors (TLR) activation, then promoting the exacerbation of the immune response (73, 74). As a matter of fact, in other two studies, it was demonstrated that EVs released by monocytes exposed to interferon alpha (IFN-α), lipopolysaccharide (LPS), or a combination of both, were able to enhance the production of proinflammatory cytokines by not-treated monocytes as well as by epithelial cells, mainly through the activation of TLR4/nuclear factor kappa-light-chain-enhancer of activated B (NF-kB) cells pathway (75, 76).

Bacteria and parasites can also release specific EVs [e.g., outer-membrane vesicles (OMV)] carrying specific molecules having immunomodulatory properties, such as LPS and lipoproteins (77, 78). Furthermore, bacterial-derived EVs contribute to the exacerbation of bacterial infections by carrying and releasing elements inducing resistance to antimicrobials and complement system factors, as well as by transferring virulent factors. In fact, these EVs seem to be a vehicle for the diffusion of PAMPs which consequently lead to different events: (*i*) triggering of pattern recognition receptors (PRRs) signalling and inflammasome activation; (*ii*) triggering of stimulator of interferon genes (STING) pathways and (*iii*) activation of host innate immune cells, finally leading to the amplification of tissue damage and inflammation (79–82).

Furthermore, macrophage- and monocyte-derived EVs are also involved in inflammatory processes. Indeed, it was reported upon LPS stimulation, they can transfer chemokines, such as tumor necrosis factor α (TNF-α), C-C motif chemokine ligand 3 (CCL3), C-C Motif Chemokine Ligand 5 (CCL5), granulocyte colony-stimulating factor (G-CSF), interleukin-1 receptor α (IL-1Rα), and C-X-C motif chemokine ligand 2 (CXCL2) that induce the synthesis of additional pro- or anti-inflammatory mediators and act as homing signals for other immune cells (83). These data suggest that EVs secreted by activated macrophages strongly work as signal for immune response by host cells (83).

Interestingly, in inflammatory processes, macrophages-derived EVs are also able to induce the activation of memory $CD4^+$ and $CD8^+$ T cells. In fact, intranasal administration of exosomes isolated from BCG-infected macrophages of *M. bovis* induces the proliferation of memory $CD4^+$ and $CD8^+$ T cells (84).

In addition to monocytes/macrophages, also dendritic cells (DCs) have been reported to secrete EVs with essential roles for immune regulation processes. DCs, which are the main antigen-presenting cells (APC), canonically activate T lymphocytes through MHC-peptide complex (85). However, APCs can also indirectly activate lymphocyte, through the release of specific EVs (86, 87) carrying the entire MHC-peptide complex (88). However, it was also demonstrated that T cell stimulation by DCs-EVs is less effective than that by APCs (89, 90). It was hypothesized that this weaker stimulatory ability could be ascribed to a low capacity of EVs to T cell receptor (TCR) cross-linking, possibly due to their small size or to a different membrane composition (91, 92).

Given their function in several immune processes, the potential role of DCs-EVs in autoimmunity was taken into consideration for the presentation of intracellular antigens to immune cells. Importantly, several studies have shown that antigens presented by APCs can undergo intracellular transfer, where they are internalized by EVs and delivered to autoreactive T cells (93–95).

As lymphocytes can be targeted and activated by EVs released by other immune cells, they also release specific EVs involved in the crosstalk mechanisms regulating immune response in different contexts. The first evidence of lymphocyte-released EVs was reported in 1996 by Raposo et al., who demonstrated that human B cell–derived EVs are efficiently able to present MHC class II (MHC-II) peptide complexes (pMHC-II) to CD4 T cells *in vitro* (88). Later, it was demonstrated that the release of EVs by primary B cells, requires pMHC-II ligation with cognate TCR on $CD4^+$ T cells (96), CD40/IL-4 signalling, and relies on pathways mediated by NF-kB for T cell–induced B cell proliferation (97). Moreover, Rialland and colleagues showed that IgG-mediated B Cell Receptor (BCR) cross-linking in DOHH2 immortalized B cell line, stimulates EVs release, although to a minor extent with respect to TCR–pMHC-II interaction (98), which is the major determinant for EVs release by B cells. As an example, one of the potential targets of B cell–derived EVs are follicular DCs (FDCs) which are activated upon B cell-EVs exposure. As a matter of fact, B cell EVs are specifically uptaken by FDCs which, in turn, present MHC-II peptides to $CD4^+$ T cells, leading to T cell differentiation, expansion or silencing depending on the pathological context (99).

An essential role in immune regulation is also played by T cells-derived EVs containing TCR/CD3 complexes (100) and mainly associated to antiviral immune response. It was recently observed that upon activation by DCs, T cells can release EVs containing genomic and mitochondrial DNA, which are then transferred to DCs, leading to the enhancement of antiviral responses *via* the cGAS (cyclic GMP-AMP Synthase)/STING cytosolic DNA-sensing pathway and subsequently inducing interferon regulatory factor 3 (IRF3)-dependent interferon-regulated genes expression (101). These results suggest a potential feedback mechanism by which T cells enhance the activity of APCs, priming them to a more efficient response to the infections (71). We can speculate that this immunomodulatory

mechanism could also occur toward Enteroviruses infection, which is one of the most investigated and described environmental risk factor in the pathogenesis of T1D.

Recent studies demonstrated that EVs subpopulations play different roles in immune cells, thus generating multiple immune responses. For instance, Whalund et al. observed that when DCs are loaded with the whole ovoalbumin (OVA) antigen, exosomes from OVA-pulsed DCs are more efficient in inducing antigen-specific $CD8^+$ T-cells activation, and in eliciting antigen-specific IgG production respect to other microvesicles types. In this case, this was probably due to the greater ability of exosomes to carry high levels of the antigen, while in other microvesicles it was barely detectable (102).

Furthermore, similarly to healthy cells, also apoptotic cells have been reported to release extracellular vesicles (termed apoptotic extracellular vesicles, Apo-EVs) (103). The release of these EVs seems to occur during apoptosis and differs from apoptotic bodies formation. Apo-EVs have similar characteristics to those EVs released by healthy cells, in terms of cargo delivery and immune regulation; indeed, this peculiar EVs population mainly acts on inflammation, cancer and autoimmunity (103–105). Of note, Apo-EVs formation has also been proposed as a mechanism to facilitate the transport of autoantigens to APC, thus putatively playing a key role in the initiation of autoimmune reaction *in situ*. As an example, Dieudè and colleagues recently discovered a novel class of vesicles, namely apoptotic exosome-like vesicles, much more active than most known apoptotic bodies and released by endothelial cells following serum-free starvation induced-apoptosis. As a matter of fact, the injection of these class of vesicles into C57Bl/6 mice was able to trigger the production of anti-LG3 autoantibodies (subunit of perlecan), thus enhancing the severity of graft rejection. Importantly, apoptotic exosome-like vesicles are particularly enriched of 20s proteasome subunit, and since LG3 behaves as a proteasome substrate, authors further demonstrated that proteasome activity within apoptotic exosome-like vesicles directly regulates anti-LG3 autoantibody formation and allograft inflammation (106).

Overall, these data suggest that immune cells-derived EVs can exert similar functions as their parental cells, including immune stimulation or inhibition. Indeed, the crosstalk between immune cell populations through EVs physiologically contribute to the maintenance of immune system homeostasis, whose alterations can lead to several disorders, including autoimmune diseases.

EVs AND AUTOIMMUNE DISEASES

Accumulating evidence reveal that EVs from both immune and non-immune cells could play a key role into the regulation of immunity (107). As reported above, EVs are involved in the establishment, maintenance and modulation of autoimmune processes through different mechanisms (108).

EVs can be secreted by APC and may represent a source of autoantigens leading to the activation of autoreactive T lymphocytes (109–111). In fact, several *in vitro* studies showed the ability of APC EVs to directly or indirectly stimulate T cells in order to establish a "dialogue" required for their activation (89, 108, 112). Alternatively, EVs can interact with APC through multiple independent molecular mechanisms involving TCR complexes-MHC interaction (92), co-stimulatory molecules binding (108), or by direct EVs cargo internalization and processing (7, 92). Due to this variety of mechanisms, EVs can contribute to the pathogenetic molecular mechanisms of different immune-mediated diseases including multiple sclerosis (MS), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).

Since EVs are enriched in the cerebrospinal fluid (CSF), they could also play a relevant role in nervous system autoimmune diseases such multiple sclerosis (MS) (93). MS, which represents the most common autoimmune demyelinating disease of the CNS (113), characterized by lesions of the blood-brain barrier (BBB) thus contributing to the trans-endothelial migration of autoreactive T cells into the CNS, inducing chronic neuro-inflammation and demyelination (93, 114, 115). In this context, endothelial cells and platelet-released EVs resulted increased in the peripheral blood of MS patients during relapses phases of the disease, consequently causing enhanced endothelial permeability and increased migration of immune cells (116, 117). In addition, it has been demonstrated that EVs released by infiltrating T cells, containing C-C chemokine ligand 5 (CCL5) and arachidonic acid, are able to enhance immune cells recruitment thus contributing to further BBB disruption (117). Finally, microglia-derived EVs, containing MHC class II molecules, also contribute to restimulate infiltrated lymphocytes thus leading to the spreading of neuronal antigens outside the CNS (115, 118).

Another autoimmune disorder in which EVs have been reported to play a pivotal role is rheumatoid arthritis (RA), a chronic inflammatory autoimmune disease characterized by joint inflammation and destruction associated with systemic symptoms (113). Of interest, synovial fluid-derived EVs carry fibrinogen components and vimentin, thus stimulating autoantibodies production and contributing to the generation of immune complexes (110, 119). Therefore, pathogenetic molecular mechanisms of RA are strongly related to EVs-mediated cell-to-cell communication among immune cells, synoviocytes, endothelial cells, and chondrocytes, through different mechanisms (93, 110, 113, 119). On the other side, EVs derived from synoviocytes released in inflammatory conditions may stimulate adjacent cells to secrete mediators of inflammation, leading to cartilage damage. Furthermore, EVs also contribute to RA pathogenesis by promoting the survival of autoreactive T lymphocytes. In fact, inflamed tissue-resident fibroblasts secrete TNF-α^+ exosomes which are uptaken by autoreactive T lymphocytes, thus becoming resistant to the activation induced cell death (AICD) (120). Moreover, the enzymatic cargo of EVs can also contribute to RA pathogenesis, leading to the degradation of the cartilage matrix caused by the secretion of proteinase and glycosidase enzymes by synovial fibroblasts (115, 121, 122). On the other side, in the RA synovial fluid an increase of leukocyte derived EVs has also been

described (123). In particular, EVs derived from T lymphocytes and macrophages are able to induce the secretion of metalloproteinases, proinflammatory mediators, and proangiogenic chemokines by synovial fibroblasts (121). Finally, it can be also hypothesized a role for platelet-derived EVs in RA angiogenesis occurring in synovia, promoting development and growth of synovial membrane and subsequent cartilage and bone destruction as well as articular remodeling (108, 110). Indeed, synovial fluid of RA patients has been reported to contain proinflammatory immune complexes generated by the association of platelet-derived microvesicles and citrullinated peptides autoantibodies (124). Furthermore, Boilard et al. (125) also demonstrated that platelets-released microvesicles play an active role in RA inflammatory processes being detected exclusively in joints of RA patients but not in those from control individuals. Of note, depletion of platelets in K/BxN mice (rheumatoid arthritis animal model), was able to reduce arthritis severity, mainly through the reduction of IL-1 (125).

The same research group also demonstrated that activated platelets are able to release respiratory-competent mitochondria encapsulated in microparticles or as free organelles. Since mitochondria are a substrate for secreted phospholipase A2 IIA (sPLA2-IIA), the hydrolysis of the mitochondrial membrane by sPLA2-IIA yields inflammatory mediators (i.e., lysophospholipids, fatty acids, and mtDNA) that promote leukocyte activation and RA typical inflammation (126).

A specific role of EVs has also been reported in systemic lupus erythematosus (SLE). SLE is a chronic systemic autoimmune disease characterized by the presence of autoantibodies (anti-double stranded DNA and antinuclear antibodies), resulting in the deposition of immune complexes in several tissues, thus consequently leading to organs damage (93, 119). Of note, the role of EVs in SLE has recently been investigated (108). In fact, it has been shown that EVs released from apoptotic cells carry antigenic nuclear determinants, thus being targeted by anti-DNA and anti-nucleosome antibodies from SLE affected mice and from plasma of patients with SLE (127). Moreover, in SLE patients, circulating proinflammatory EVs, are able to induce TNF-α and IFN-α secretion by peripheral blood mononuclear cells (PBMC) through a TLR-mediated mechanism (115, 128, 129). In addition, SLE-specific EVs cargo, composed of metalloproteinases, tissue factor and CD40, are able to induce VEGF production and chemokines secretion thus exacerbating pathological angiogenesis typical of SLE (130, 131). As an example, it has been reported that in advanced SLE vascular lesions, atherosclerotic plaques can release CD40 ligand$^+$ (CD40L)-microvesicles which can further stimulate endothelial cell proliferation and angiogenesis, thus contributing to the transition from stable to unstable plaques and to the worsening of SLE vascular lesions (130).

EVs AND TYPE 1 DIABETES

As already mentioned, EVs are essential components of cell-to-cell communication, thus being involved in the crosstalk between different cell types. Among these, β cells have been reported to actively communicate *via* EVs with other cells, including immune cells. Of note, several recently published studies specifically revealed a bidirectional crosstalk between β cells and immune cells *via* EVs, both in physiological and pathological conditions (**Table 1**).

EVs From Pancreatic β Cells to Immune Cells

The first evidence of EVs secretion by pancreatic β cells was reported in 2011 (132). In this study, Sheng H. and colleagues showed that the murine β cell line mouse insulinoma-6 (MIN6) was able to secrete EVs characterized by the typical pattern of exosomal markers, alongside with pancreatic islet β cell autoantigens including GAD65. Of note, the exposure of splenocytes obtained from Non Obese Diabetic mice (NOD 6-8 weeks) to EVs isolated from MIN6 cells, induced the secretion of multiple inflammatory molecules including IL-6, IFN-γ, TNF-α, MCP-1, and IL-10. Of interest, cytokines release was completely abolished when splenocytes were isolated from TLR-innate signalling pathway MyD88-KO NOD mice; similarly, lymphocytes proliferation assay showed a significant reduction of the proliferation rate when MyD88-KO NOD mice splenocytes were exposed to MIN6 EVs respect to wild type splenocytes, thus suggesting that the inflammatory stimuli enclosed within MIN6 EVs may activate TLR signals *via* MyD88-dependent pathways, then favoring lymphocytes proliferation. Splenocytes exposure to MIN6 EVs also generated an increase of MHC class II, CD80 and CD86 costimulatory molecules on APC cells, paralleled by an increase of IFN-γ-secreting T cells. Importantly, authors also demonstrated that the intravenous injection of MIN6 EVs in Non Obese Resistant (NOR) mice induced a significant increase in insulitis. Indeed, the severity of insulitis or lymphocyte infiltration was three-fold higher in the NOR mouse group treated with MIN6 EVs for 7 days in comparison to untreated group, demonstrating a tight crosstalk between MIN6 and immune cells activation in autoimmune diabetes through EVs.

Among EVs cargo molecules released by pancreatic islet β cells, insulin is of particular interest. In 2015, Vomund and colleagues demonstrated that NOD mouse β cells can transfer vesicles containing insulin or its catabolites to tissue-resident APCs. Importantly, this result was confirmed also in human pancreatic islets. In this case, the EVs transfer, which required a close contact interaction between β cells and APCs, was increased upon glucose stimulation and requires mobilization of intracellular Ca^{2+}. Additionally, the authors observed that $CD4^+$ T cells of diabetic NOD mice exposed to various peptides of the insulin B chain, are able to recognize the APCs vesicles-transferred antigens, thus reporting a potential mechanism through which β cells become recognizable by autoreactive T cells in T1D upon autoantigens transfer through EVs (**Figure 2**) (4).

Recent studies further demonstrated the presence of islet autoantigens in β cell-derived EVs and their specific release upon proinflammatory conditions. Cianciaruso and colleagues

TABLE 1 | EVs are involved in the crosstalk between β-cells and immune cells.

Vesicle type	EVs isolation methods	Cells of origin	Recipient Cells	EVs Function/Cargo	References
Small microparticles	UC	MIN6	Splenocytes	Trigger secretion of inflammatory molecules	132
EVs	DUC	NOD mouse β cells	Tissue-resident APCs	Insulin	4
Exosomes	DUC	MIN6, INS-1 β cell lines and human and rat islets	APCs	GAD65, proinsulin and IA2	5
EVs	UC	Human islets	N/A	GAD65, ZnT8, Glut2	133
EVs	DUC	Human islets	PBMCs	Trigger proinflammatory immune responses	134
Exosomes	ExoQuick-TC	Human islets	N/A	mRNAs, lncRNAs, miRNAs, piRNAs, snoRNAs and tRNAs	135
Exosomes	UC	MIN6, INS-1 and human islets	MIN6 β cells	miRNAs released following cytokines exposure	136
Exosomes	DUC	NOD mouse β cells	Splenocytes	Trigger secretion of TNF-α *via* miR-29b	137
EVs	DUC+UF+SEC	MIN6	APCs derived from bone marrow	Increased cargo of insulin, proinsulin and β cell autoantigens	138
EVs	UC	Human islets	Human islet endothelial cells	Insulin, C-peptide, GLP1R, VEGFa, eNOS, and miR-27b, miR-126, miR-130, and miR-296	139
Exosomes	UC	T-lymphocytes	Rodent and human pancreatic β cells	Trigger chemokine expression and apoptosis of β cells *via* miR-142-3p/-5p and miR-155	6

Table reporting the studies demonstrating a cross-talk among β cells and other cell types, with a potential role in T1D. In details, column 1 lists described EV types; in second column EV isolation method is reported; third and fourth columns respectively illustrate origin and recipient cell types; in fifth column main cargo molecules and/or EVs played role are described; in the last column belonging reference is reported.
UC, ultracentrifugation; DUC, differential ultracentrifugation; UF, ultrafiltration; SEC, size exclusion chromatography.

showed the presence of islet autoantigens (GAD65, proinsulin, and IA2) in EVs isolated from MIN6 and INS-1 β cell lines supernatant as well as from those isolated from human and rat islets culture medium (5). The induction of ER stress by proinflammatory cytokines in rat islets increased EVs secretion. Of note, mouse islet EVs can be uptaken by APCs leading to their activation. Interestingly, APCs activation was increased when exposed to EVs derived from proinflammatory cytokines treated islets vs. non treated control samples, thus demonstrating that inflammatory stress induced EVs cargo molecules modulation as well.

The presence of GAD65 autoantigens in EVs released by human pancreatic islets was also confirmed by Hasilo and colleagues who were also able to detect the autoantigen ZnT8, as well as the β cell glucose transporter 2 (Glut2) (133).

Human pancreatic islet EVs can be actively internalized by PBMCs, particularly by $CD14^+$ cells and by $CD19^+$ B-cells. Of note, IL-6, TNF-α and IL-1β cytokines expression were significantly increased in $CD14^+$ cells upon EVs internalization. Such inflammatory pathways triggering also involved the upregulation of IFN-γ, IL-4, and IL-17 expression in $CD4^+$ T cells and a significant increase of $CD19^+$ cell proliferation upon human islets EVs PBMCs exposure. Interestingly, both Th1 cytokines expression and $CD19^+$ cells proliferation were further increased in T1D derived PBMCs exposed to human islets EVs. Additionally, human islets EVs are able to specifically activate memory T cells derived from T1D patients but not those from non-diabetic control subjects, as documented by a significant increase of CD69 MFI in both memory T-cells (CD4+CD45RO+CD45RA−CCR7−CD62L−) and B-cells (CD19+IgD−CD27+) observed in T1D PBMCs (134). Since memory B and T-cells activation upon EVs exposure is selective to T1D patients and not observed in non-diabetic controls, these results strongly suggest a pivotal role of EVs in antigen-specific responses.

These data demonstrate the role of EVs in the transfer of autoantigens, or other inflammation-relevant molecules, from islets to immune cells thus representing a novel mechanism of autoimmune reaction triggering in T1D pathogenesis.

As described previously, miRNAs and other RNA molecules are critical components of EVs cargo and could play a key role in the dialogue between pancreatic islets and immune cells (143). Notably, given their intrinsic regulatory function, the evaluation of miRNAs and other small non-coding RNAs enclosed in islet-derived EVs is of particular relevance. In 2019, the group of Evans-Molina characterized the RNA content of exosomes secreted by *in-vitro* cultured human pancreatic islets (135). In this study, Krishnan and colleagues performed both total RNA and small RNA sequencing analysis of human pancreatic islets released exosomes pool, showing a prevalence of mRNA transcript, long non-coding RNAs and miRNAs. Of note, they highlighted the presence of additional small RNA species such as tRNA fragments and piRNAs, showing the high complexity of human islets exosomal RNAome. A critical question related to exosomal RNAome is about its potential alteration following stress stimuli typical of T1D, such as proinflammatory cytokines (IL-1β+ IFNγ). Of interest, they found a total of 133 mRNAs,

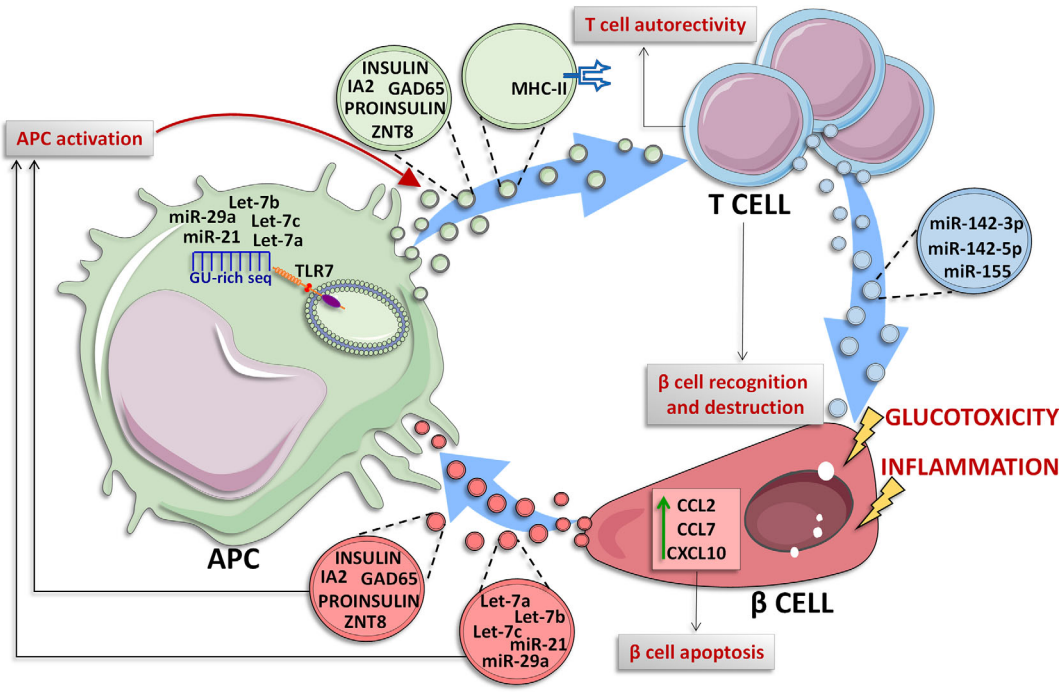

FIGURE 2 | Crosstalk between β cells and immune cells through EVs. In T1D context, β cells and immune cells tightly communicate each other. β cells are subjected to glucotoxic and/or inflammatory stress and can release EVs containing specific miRNAs characterized by a GU-rich sequence (let-7a/b/c, miR-21 and miR-29a) which are transferred to resident Antigen Presenting Cells (APCs), where these miRNAs can bind to endosomal Toll Like Receptor 7 (TLR7) leading to the activation of inflammatory signals (140–142). Moreover, under inflammatory stress, β cells secrete and transfer EVs to APCs. Such EVs can contain specific autoantigens such as insulin, IA2, ZNT8, GAD65 and proinsulin (5) thus leading to their transfer to APC which can present these antigens for adaptive immunity activation. Activated APC can also lead to $CD4^+$ T cells activation through two different mechanisms: (*i*) release of EVs containing insulin, IA2, ZNT8, GAD65 and proinsulin autoantigens (4), or (*ii*) release of EVs exposing MHC-II on their surface through which APCs present autoantigens to $CD4^+$ T cells (86, 87), leading to autoreactive T cell activation and subsequent β cell destruction. In T1D context, pancreatic islet-infiltrating T cells secrete a specific subpopulation of EVs carrying miR-142-3p/5p and miR-155 which can be transferred to β cells; these miRNAs cause the upregulation of inflammatory molecules such as CCL2, CCL7 and CXCL10 leading to β cell apoptosis (6).

31 lncRNAs, 19 miRNAs, 25 piRNAs, 8 snoRNAs, and 20 tRNAs as differentially expressed in EVs secreted from cytokines-treated human pancreatic islets respect to not-treated control samples. Among differentially expressed miRNAs in EVs released from human pancreatic islets in response to cytokines, Krishnan and colleagues identified miR-155-5p as the most upregulated miRNA, while miR-4485 was the most downregulated. Although such data cannot confirm the direct origin of EVs small RNAs content alterations from β-cells, they certainly indicate a global contribution of islet EVs to potential pathogenetic mechanisms and cell-cell communication pathways in T1D. As a matter of fact, other evidence previously showed that exosomal small RNAs cargo is profoundly shaped by proinflammatory stressors in β cells. Importantly, exosomal miRNAs do not simply represent cellular miRNAs expression changes. Guay C. and colleagues demonstrated that MIN6 exosomal miRNAs are significantly different upon proinflammatory stress respect to untreated control and that specific miRNAs are preferentially released in exosomes only after cytokines exposure while others are selectively retained by the cells (136). Of interest, exosomes released from cytokines-treated MIN6 β cells can be horizontally transferred to not stressed MIN6 cells, inducing the same type of apoptotic response and demonstrating also the transfer of exosomes among β cells (136). A point of interest resides in the evaluation of the contribution of small RNAs transfer mediated by exosomes from β cell to other cells. Salama and colleagues demonstrated that the exposure of splenocytes isolated from diabetes-prone NOD mice to β cell-released exosomes is able to increase the synthesis and secretion of TNF-α (137); such effect was dependent on the presence of miR-29b within β cell EVs. The inhibition of exosomal miR-29b reversed the TNF-α increase in splenocytes, thus demonstrating the role of exosomes-transferred miRNAs in the activation of immune cells toward a proinflammatory phenotype.

In the light of the high heterogenous pattern of vesicles secretion by cells of origin, an interesting aspect is the identification of molecules cargo associated to different types of vesicles secreted from the same cell. A recent study analyzed the entire spectrum of vesicle populations (apoptotic bodies, microvesicles and exosomes) secreted by MIN6 cells following the exposure to various stress conditions (proinflammatory stress, UV exposure, hypoxia) (138). Interestingly, the authors reported that proinflammatory stress is able to enhance all EVs

population secretion, therefore, leading to an increased vesicles number. Most importantly, EVs released upon inflammatory stress exposure showed changes of cargo molecules respect to the physiologic ones. Of note, apoptotic bodies and exosomes convey not only a higher cargo of insulin, proinsulin, and β cell autoantigens upon stresses but also an increased amount of TLR binding miRNAs (e.g., GU-rich miRNAs let-7a/b/c, miR-21, and miR-29a). TLR binding miRNAs have a specific sequence which can be recognized as a DAMP by endosomal localized TLRs, thus triggering the downstream associated immune signalling. Specifically, Toll-like Receptor 7 (TLR-7) can recognize these EVs-miRNAs thus playing an essential role in the activation of autoimmune processes (140–142). As a matter of fact, EVs secreted from MIN6 cells exposed to inflammatory stress are able to activate primary APCs derived from bone marrow of NOD/ShiLtJ mice (138). Such mechanism is also partially driven by increased cytokines and chemokines cargo levels found in EVs upon MIN6 proinflammatory treatment respect to control. Among them, monocyte chemoattract protein-1 (MCP-1) is one of the most hyperexpressed (138).

Interestingly, it should be noted that human pancreatic islets communicate *via* EVs also with other non-immune cell types. Indeed, Figliolini and colleagues showed that pancreatic islets secreted EVs can be uptaken by local endothelial cells mediating the transfer of several mRNAs (e.g., VEGF and NOS) and miRNAs (e.g., miR-126a, miR-27a), thereby modulating endothelial cells proliferation and function (139). Since human islet endothelial cells represent a pivotal interface directly interacting with immune-cells (144) and playing a critical role in the early phase of T1D by increasing the expression of surface leucocyte-homing receptors, we can speculate that the alteration of pancreatic islet EVs content in response to proinflammatory stressors may indirectly induce endothelial cell dysfunction and, in turn, putative deregulation of their interaction with immune cells. Overall, these studies demonstrated that the specific cargo of different populations of EVs secreted by β cells can activate several immune cell types, thus rendering insulin-producing cells visible and exposed to immune-mediated destruction and leading to the exacerbation of inflammation and autoimmune diabetes.

EVs From Immune Cells to Pancreatic β Cells

Immune cells are able to secrete unique EVs populations which can be transferred to other cell types, including insulin-producing β cells. A seminal study by Guay and colleagues, reported that three miRNAs, namely miR-142-3p, miR-142-5p, and miR-155, are particularly enriched in T lymphocytes of 8 weeks NOD mice with respect to mouse pancreatic islets (145). Importantly, authors also demonstrated that NOD mice $CD4^+$/$CD25^-$ T lymphocytes specifically release a population of exosomes enriched of miR-142-3p, miR-142-5p, and miR-155 (6), and that this release is independent from cytokines secreted by infiltrating immune cells, thus hypothesizing that T cells could be able to transfer a specific population of EVs to β cells. In order to test this hypothesis, dispersed murine islets were treated with exosomes isolated from T cells of NOD mice (exoNOD), and MIN6 cells were subjected to EVs isolated from Jurkat human T cell line (exoT). In both cases they observed an increased expression of miR-142-3p/-5p, and miR-155, thus confirming that these miRNAs are actively transferred from T lymphocytes to β cells in autoimmune diabetes. Importantly, a higher apoptotic rate was observed in both dispersed mouse and human islet cells treated with exosomes secreted by activated primary human T cells (exo-hT), thus indicating a detrimental role for this set of miRNA in β cells.

The pro-apoptotic effect of exosomal miR-142-3p/5p and miR-155 is potentially due to their ability to induce the upregulation of several cytokines (CCL2, CCL7, and CXCL10) in murine and human pancreatic islets β cells. As a matter of fact, the *in vitro* inhibition of these three miRNAs was able to reduce apoptosis rate induced by exoT in primary rat islet cells. The same protective effect was also observed in 4-week-old NOD mice upon inhibition of miR-142-3p/5p and miR-155-5p through adenoviral associated miRNA sponges treatment. Of note, the injection of miRNA sponges reduced autoimmune diabetes development rate (10% in AAV-treated mice vs. 60% of not treated mice) *via* a significant decrease of inflammation and insulitis primarily driven by a specific reduction of CXCL10 protein levels in pancreatic islet β cells.

EVs as a Potential Source of Novel Biomarkers in T1D

EVs' secretion and their presence in many biological fluids prompted the scientific community to investigate their role as novel biomarkers (146). Indeed, circulating EVs number and content could be altered in several disease conditions, thus representing not only a vehicle of communication and interaction between different cell types but also a possible source of biomarkers (147).

It is well established that the underlying autoimmune process of T1D occurs long before the clinical onset. Efficient biomarkers for the early identification and stratification of high-risk T1D subjects are still lacking (148). Of note, numerous studies explored the potential usefulness of whole plasma/serum miRNAs analysis to detect novel biomarkers of T1D (149–152). However, the detection of miRNAs associated to different plasma/serum components could be the key to uncover novel and more specific biomarkers. In such context, EVs may represent an optimal source of novel and specific biomarkers. As a matter of fact, different studies demonstrated that EVs' size, number, and enclosed cargo are altered in several diseases, including T1D (153).

In an effort to characterize circulating EVs of T1D patients, Garcia-Contreras and colleagues analyzed EVs isolated using ultracentrifugation approach from plasma of long-standing T1D patients (~25 years disease duration) and age- and gender-matched non diabetic subjects. They observed reduced EVs

concentration and increased average size in plasma of T1D respect to CTR subjects (148). Moreover, the same authors found an altered expression of several miRNAs in circulating EVs isolated from T1D subjects. In details, miR-16-5p, miR-302d-3p, miR-378e, miR-570-3p, miR-574-5p, and miR-579 were downregulated, while miR-25-3p was upregulated in EVs from plasma of T1D subjects respect to controls.

In a more recent work, Tesovnik et al. investigated the potential alterations of plasma EVs miRNAs cargo in children with newly diagnosed T1D (<6 months) (nT1D) and in T1D young adults subjects (max 10 years of disease duration, 10y-T1D) in comparison to healthy subjects. Following EVs small RNAs sequencing, the authors found a higher expression of miR-122-5p and miR-192-5p in nT1D cohort compared to controls, as well as the differential expression of several miRNAs in nT1D versus 10y-T1D, namely the downregulation of miR-193b-5p, miR-195-3p, miR-122-5p, and miR-445-5p and the upregulation of miR-21-5p. Furthermore, miR-195-3p and miR-455-5p were both downregulated while miR-185-5p expression was upregulated in 10y-T1D vs control subjects (154).

The importance to distinguish miRNAs enriched in EVs fraction respect to whole biofluid (serum or plasma) was mainly highlighted in a work published by Lakhter and colleagues (155). In details, they reported a significant upregulation of miR-21-5p in EVs secreted by MIN6 murine β cell line, EndoC-βH1 and human pancreatic islets treated with a proinflammatory cytokines mix. Of note, miR-21-5p was increased in whole serum as well as in serum-EVs from NOD diabetic mice respect to control mice. In T1D subjects, the expression of miR-21-5p was discordant between whole serum and serum-isolated EVs; indeed, miR-21-5p resulted significantly reduced in whole serum T1D respect to controls while it was upregulated in serum-isolated EVs, thus demonstrating that analyzing miRNAs expression by distinguishing different fractions (EVs, ribonucleoproteins, lipoproteins, etc.) could be of critical importance in order to identify potential circulating biomarkers of several diseases, including T1D (155).

Another important work published in 2019 by Mirza and colleagues, showed that several miRNAs can be differentially expressed in the EVs derived from breast milk of T1D mothers respect to non diabetic ones. Particularly, they detected a significant hyperexpression of four miRNAs, namely miR-4497, miR-3178, miR-1246, and miR-133a-3p. Importantly, *in vitro* overexpression of miR-4497 and miR-3178 in macrophages resulted in their activation, thus consequently stimulating the secretion of cytokines (TNF-α and IL-1β) (156). Importantly, since it has been previously demonstrated that newborn food intake could affect the potential future development of T1D (157, 158), it could be useful to further investigate the potential role of these breast milk EVs miRNAs in children breastfed by T1D mothers.

Overall, these data demonstrate that the altered expression of miRNAs in EVs of T1D subjects, both recent onset and long standing, could represent valid biomarkers for the prediction and the diagnosis of T1D. However, further studies are needed to better characterize EVs obtained from T1D patients in order to use them as biomarkers.

EVs AS INNOVATIVE THERAPEUTIC TOOLS FOR IMMUNE SYSTEM MODULATION AND T1D

EVs have recently emerged as novel and attractive therapeutic tools in immune therapy, regenerative medicine and drug delivery (159). A growing number of preclinical and clinical studies investigated EVs therapeutic applications in a wide range of diseases including T1D (**Table 2**) (146).

The most investigated source of EVs as therapeutic tools aimed at immune modulation are mesenchymal stem cells (MSCs) (172). As a matter of fact, MSCs are extensively studied for their regenerative and anti-inflammatory roles (173). The therapeutic properties of MSCs have been largely attributed to their differentiation and self-renewal ability, upon specific environmental stimuli, as well as to their immunomodulatory actions, both toward innate and adaptive immunity (172, 174, 175). However, increasing evidence showed that EVs are responsible of most of the beneficial effects of MSCs-based therapy, as demonstrated by the appearance of regenerative effects following administration of MSCs supernatant in damaged tissues (174–176). EVs efficiently mimic the biological activity of parental MSCs and exhibit the same surface receptors, signaling and cell adhesion molecules or associated antigens of parental cells thus representing an interesting cell-free alternative to MSC-based therapy (172, 174). The use of MSC-EVs therapy, although investigated in a small number of clinical trials, showed several advantages over MSC-based therapy, such as higher safety profile, lower immunogenicity, better biodistribution, no need for engraftment, ability to evade from clearance by reticulo-endothelial system, reduced systemic and off-targets side effects, and the capacity to cross biological barriers, while avoiding risks of oncogenesis, embolism, and immune rejection (172, 173, 176–178). Indeed, several promising results in preclinical studies have kicked off numerous clinical trials on the therapeutic use of EVs in several diseases from autoimmune and inflammatory diseases to cancer.

MSC-EVs are reported to induce an imbalance in the secretory pattern of cytokines from immune cells, in favor of anti-inflammatory ones, while inhibiting the secretion of proinflammatory cytokines (172, 174). Of note, MSC-EVs effects on dendritic cells (DCs) seems to be largely mediated by miRNAs (i.e. miR-21-5p, miR-142-3p, miR-223-3p, and miR-126-3p), resulting in the inhibition of maturation, and consequently to an impairment of antigen uptake and antigen presentation capacity (179). Moreover, MSC-EVs stimulate macrophages M2 polarization meanwhile reducing recruitment, activation, and M1 polarization of macrophages and monocytes (180, 181). Furthermore, MSC-EVs inhibit the activation and proliferation of T and B lymphocytes (182), increase the ratio of Treg cells on effector T cells and enhance the function of Treg (171), inhibit maturation of B cells and the consequent immunoglobulin secretion. In addition, MSC-EVs is also able to re-induce self-tolerance through the transfer of anti-inflammatory and tolerogenic molecules into autoreactive T cells, promoting generation of Tregs and apoptosis of

TABLE 2 | EVs and therapeutic applications in T1D or in T1D complications.

Cells of Origin	EVs cargo	Target organ	Therapeutic function	References
Human urine-derived stem cells	Growth factor, transforming growth factor-β1, angiogenin and bone morphogenetic protein-7	Kidney	Reduction of the urine volume and urinary microalbumin excretion; prevention of podocyte and tubular epithelial cell apoptosis in diabetic rats.	160
Bone-marrow stromal cells (BMSCs)	miR-145	Brain	Improved functional outcome and promoted neurorestorative effects	161
Human umbilical cord blood-derived EPCs	N/A	Skin	Enhancement of Angiogenesis Through Erk1/2 Signaling	162
Human umbilical cord blood-derived EPCs	N/A	Skin	Enhancement of the proliferation, migration and tube formation of vascular endothelial cells	163
Bone-marrow mesenchymal stem cells	N/A	Brain	Improvement of cognitive impairments by repairing damage neurons and astrocytes	164
HSP20 overexpressing cardiomyocytes	HSP-20	Heart	Improvement of cardiac function and angiogenesis	165
Mesenchymal stem cells	let-7c	Kidney	Attenuation of renal fibrosis	166
Human circulate fibrocyte	HSP90-Alpha; miR-126; miR-130a; miR-132; miR-124a; miR-125b: miR-21	Skin	Activation of diabetic dermal fibroblast; induction of migration and proliferation of diabetic keratinocyte; accelerate wound closure	167
Mouse serum	miR-106b-5p and miR-222-3p	Endocrine pancreas	Improvement of hyperglycaemia *via* pancreatic beta cell proliferation	168
Human bone marrow mesenchymal stem cells	N/A	Endocrine pancreas	Inhibition of immune rejection	169
Endothelial progenitor cells	miR-126 and miR-196	Endocrine pancreas	Enhancement of neo-angiogenesis of human pancreatic islets	170
Adipose tissue-derived mesenchymal stem cells	N/A	Spleen	Increase of regulatory T-cell population and their products without a change in the proliferation index of lymphocyte	171

Table reporting the potential therapeutic applications of EVs. In particular, we included: (i) Origin cells or biofluid from which vesicles are isolated; (ii) the main cargo of these vesicles; (iii) organ targeted by the specific EVs population; (iv) potential therapeutic function and/or mechanisms acted by released EVs.

activated T cells (182). Taken together, these findings suggest that MSC-EVs directly influence the immune state of adaptive immune cells and promote an anti-inflammatory phenotype of antigen presenting cells (APCs) also inducing immunotolerance of T and B lymphocytes.

Although the use of MSC-EVs in autoimmune diseases therapy is still at early stages, several clinical trials are evaluating their efficacy and safety. As a matter of fact, a first study published by Rahman and colleagues in 2014 demonstrated the immunostimulatory effect of EVs released by mesenchymal stem cells derived from *in vitro* culture of 8-week-old NOD mice pancreatic islets. Indeed, authors demonstrated that vesicles released by these MSCs and injected into 1 week old NOD mice are able to enhance inflammatory reactions, resulting in the increase of cytokines release (IL-6, IFN-γ, TNF-α, and MCP-1), stimulation of surface TLRs and other innate receptors as well as increased B cells proliferation rate. Therefore, authors hypothesized that MSC-EVs could potentially contain and transport immunostimulatory autoantigens, leading to enhanced immune response (183).

However, more recent evidence suggest therapeutic efficacy of EVs in T1D, through immunomodulatory effects (171), protection of pancreatic islets from autoimmune targeting, and consequent slowdown of disease progression (160, 161, 163–167). Furthermore, adipose tissue MSCs-derived EVs were proved to reduce hyperglycaemia and islet degeneration in mice with autoimmune diabetes (168); in addition, the levels of IL-17 and TNF-α were significantly decreased while IL-4, IL-10, and TGF-β levels, as well as Treg number are significantly increased in spleen of EV-treated mice (171).

Of note, EVs could represent a promising tool to overcome possible failure of islet transplantation due to the immune rejection and loss of function of transplanted islets (169). Indeed, MSC-EVs administration alongside with islet transplantation, is able to improve islet functions simultaneously inducing transplant tolerance by enhancing Treg function (184). In accordance with these observations, MSC-EVs treatment resulted in increased graft survival and normalized blood glucose levels in T1D mice (178).

More recently, EVs have also emerged as vectors of bioactive molecules, given their intrinsic stability, biocompatibility, and target homing ability. Several studies investigated whether EVs could deliver anti-inflammatory compounds in central nervous system (185, 186). Indeed, purified EVs can be loaded with therapeutically active cargo molecules *ex vivo*, e.g., through electroporation of small molecules into EVs or, alternatively, the molecules of interest can be incorporated in EVs *in vivo*, during vesicles biogenesis (187).

Despite these encouraging evidence, clinical translation of EVs has been hindered by challenges in isolation, purification, and high-yield production. Dose-finding is also critical for clinical application, since EVs effects seemed to be dose-dependent (188). Moreover, despite quality control and manufacturing practice improvement, EVs heterogeneity is still difficult to eliminate. With the aim to bypass these limitations, researchers have focused on synthetic EVs production, such as liposomes—the most biocompatible and least toxic artificial vesicles created from cholesterol and phospholipids and cholesterol—and biomimetic nanoparticles—which are hybrid nanostructures coated with exosomes-like membranes with desired targeting or functional characteristics (189). Exosome-mimetic nanosystems showed important advantages in production efficiency and functionality (190). Another critical aspect in the clinical use of EVs relates to the integrity of the cargo, which could be reduced/damaged by common loading methods, thus making it crucial to develop novel loading efficiency evaluation systems (191, 192).

TABLE 3 | Outstanding questions on T1D and EVs: how to push the field forward?

Key Question	Comments	References
Which is the best methodology to isolate a pure fraction of EVs from cell culture supernatant and plasma?	Absolute purification and isolation of EVs from their origin biological fluids, is an unrealistic goal at present. Each method and approach has advantages, as well as disadvantages and each of this influences the amount, type and purity of recovered EVs. It is important to choose based on the downstream applications and scientific questions of planned experiments.	193, 194
Are there markers characterizing exosomes released by human β-cells? If so, can we specifically isolate them from cell culture supernatant or plasma samples?	It has been reported that exosomes derived from β-cells are enriched of a cargo composed by different specific β-cells markers (insulin and proinsulin); nevertheless, no surface exosomal marker specific for β-cells has been identified yet. However, as future goal, a potential approach could be that of performing a proteomic profile on exosomes isolated from culture medium of pure human β-cell in order to identify a specific β-cell membrane marker on exosomes surface.	4, 5
Which set(s) of stress stimuli drives differential EVs release and content secreted from primary human β-cells?	It has been reported that proinflammatory cytokines mix induces an increase of miR-21-5p in exosomes derived from Endocβ-H1 and human islets, without significant differences in term of quantity and size of exosomes, suggesting a selective enrichment of EVs miR-21-5p in response to inflammatory stress. Moreover, Krishnan et al. demonstrated that exosomes released from human pancreatic islets treated with IL-1β and IFNγ showed differential expression of several RNAs species, with respect to untreated human islets. However a wide range of stress test on primary islets are still missing and require further studies.	135, 155
Can we use engineered EVs to target β-cells or specific immune cell subsets in order to protect β-cells or to restore immune tolerance in T1D?	EVs have also emerged as vectors of bioactive molecules, given their intrinsic stability, biocompatibility and target homing ability. Cantaluppi et al. demonstrated that EVs released by endothelial progenitor cells carried the proangiogenic miR-126 and miR-296 that enhance neoangiogenesis of human pancreatic islets thus improving their function. A potential mechanism to improve β-cell function may reside in the engineering of endothelial progenitor-like EVs overexpressing miR-126 and miR-296 in order to ameliorate islet function.	170

CONCLUDING REMARKS

EVs are involved in multiple mechanisms of immune system function and regulation through the transfer of bioactive cargo molecules which include cytokines, chemokines, lipids, metabolites, and small RNAs. The alterations of EVs mediated cell-cell communication mechanisms have been shown to be critical in multiple autoimmune diseases including T1D. Importantly, EVs have been shown to be involved in the bidirectional crosstalk between pancreatic β cells and immune cells, thus representing an interesting mechanism to be further studied during the pathogenesis of T1D.

EVs have been also explored as a novel potential source of disease biomarkers. Indeed, multiple small RNAs have been found altered exclusively in circulating exosomal component; such results indicate the needs to differently fractionate plasma/serum components [EVs, ribonucleoproteins (e.g., AGOs family), lipoprotein particles], in order to discover novel disease biomarkers, whose alteration can be hindered by a dilution effect when analyzing samples as a whole. It should be underlined the urgent need for standardization of isolation and purification of circulating EVs, quality control checks, and storage protocols, as well as the development of sensitive and scalable downstream methodologies to analyze EVs content, including small RNAs, proteins, and metabolites. Moreover, additional studies about the role and functionality of EVs cargo, and in particular on the ability of non-coding RNAs to further regulate cellular processes into recipient cells, are needed in order to shed light onto their mechanisms and functions.

Multiple critical questions surrounding EVs and T1D research (see **Table 3**) are still requiring adequate studies in order to move forward to large-scale clinical application of EVs, as well as to define rigorous criteria for quality control and standardized strategies for quantification and characterization of EVs. Nonetheless, EVs represent a relatively novel and still largely unexplored research field encompassing biomarkers, pathogenetic and therapeutic area in T1D research, thus strongly requiring focused attention by the research community.

AUTHOR CONTRIBUTIONS

GEG, DF, CF, LN, CM, GL, and NB: writing and drafting the manuscript. GS and FD: manuscript writing, drafting and revising. All authors contributed to the article and approved the submitted version.

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Means, Motive, and Opportunity: Do Non-Islet-Reactive Infiltrating T Cells Contribute to Autoimmunity in Type 1 Diabetes?

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In human type 1 diabetes and animal models of the disease, a diverse assortment of immune cells infiltrates the pancreatic islets. $CD8^+$ T cells are well represented within infiltrates and HLA multimer staining of pancreas sections provides clear evidence that islet epitope reactive T cells are present within autoimmune lesions. These *bona fide* effectors have been a key research focus because these cells represent an intellectually attractive culprit for β cell destruction. However, T cell receptors are highly diverse in human insulitis. This suggests correspondingly broad antigen specificity, which includes a majority of T cells for which there is no evidence of islet-specific reactivity. The presence of "non-cognate" T cells in insulitis raises suspicion that their role could be beyond that of an innocent bystander. In this perspective, we consider the potential pathogenic contribution of non-islet-reactive T cells. Our intellectual framework will be that of a criminal investigation. Having arraigned islet-specific $CD8^+$ T cells for the murder of pancreatic β cells, we then turn our attention to the non-target immune cells present in human insulitis and consider the possible regulatory, benign, or effector roles that they may play in disease. Considering available evidence, we overview the case that can be made that non-islet-reactive infiltrating T cells should be suspected as co-conspirators or accessories to the crime and suggest some possible routes forward for reaching a better understanding of their role in disease.

Keywords: type 1 diabetes, insulitis, autoreactive T cells, non-islet reactive T cells, β cell destruction

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INTRODUCTION

In criminal investigations, establishing means, motive, and opportunity are necessary elements to prove a suspect's guilt. In this construct, means refers to available tools or methods to commit the crime; motive refers to a plausible reason for committing the crime, and opportunity is the occasion that presents itself to allow the crime to occur (1). In this perspective, we explore the role and function of non-islet-reactive immune cells in human insulitis. Type 1 diabetes (T1D) involves the criminal activity of murder of the insulin-producing pancreatic islet β cells. In this context, "means" is established by showing that a suspect possesses an adequately potent murder weapon (effector function); "motive" is established by showing that the suspect was driven by some plausible impulse to commit the crime (typically, an islet-reactive antigen receptor); and "opportunity" is demonstrated by placing the suspect at the scene of the crime and eliminating any "alibi". There is increasing evidence suggesting that β-cell metabolic dysfunction may directly contribute to β-cell stress and demise (2), and, potentially, to the autoimmune attack ("assisted suicide") (3, 4). Furthermore, some evidence suggests that neonatal remodeling events (waves of apoptosis) may lead to "incompetent repairmen", leaving vulnerabilities that set the stage for the crime (revealed when the perpetrators "case the joint") (5). However, the focus of this mini-review is to investigate the role of T cells at the scene of the crime. We will present ample evidence of opportunity: cells with no discernable evidence of islet antigen reactivity that may be non-self-reactive, are present in human insulitic lesions. Establishing means and motive is a more nuanced venture, as their possible roles could be either pathogenic or protective, passive, or co-conspirators to the crime. Nevertheless, we will conclude that credible evidence exists for a role of non-islet-reactive T cells in disease.

INVESTIGATING THE SCENE OF THE CRIME: EVIDENCE OF CRIMINAL ACTION WITHIN ISLETS IN T1D

The Victims: Pancreatic Islet β Cells

In T1D, a scenario in which the scene of the crime is the pancreatic islet and the victim is the β cell can be contemplated. Arriving on this scene, our investigator is confronted by a chilling scene: a small crowd of infiltrating cells has gathered (first termed insulitis in 1902 by Schmidt (6), observing the pancreas of a 10-year-old child with diabetes). These infiltrates are diverse in location and composition. Insulitis is present in a small percentage of islets (even at diagnosis), making it easy to miss when examining small numbers of islets. The nature of β-cell destruction seems sporadic, almost random. Analysis of pancreas biopsies from adult living individuals taken only weeks from diagnosis (7) showed that insulitis [defined as ≥15 $CD45^+$ cells/islet (8)] affected only 11% of the islets at diagnosis. At onset, insulitis is present in 25.8% of the insulin containing islets (ICIs) versus 2.9% of the insulin deficient islets (IDIs) (8). Thus, these snapshots of evidence of insulitis are concentrated in islets with insulin expression, indicating focused aggression against functional β cells.

Factors in the islet microenvironment that could serve to provoke immune attack remain under study. As mentioned above, remodeling events (waves of apoptosis that prime the immune system: "remodeling gone wrong") that occur early in life could set the stage for subsequent unlawful entry (5). Once the stage has been set, islet α and β cells in T1D could express the CXCL10 chemokine. CXCR3 expression in T cells has been identified in inflamed pancreata and pancreatic draining lymph nodes from donors with T1D (9, 10) along with islet β-cell expression of monocyte chemoattractant protein-1 [MCP-1 (11)]. Further potential contributors to a chemoattractive/pro-inflammatory environment are the expression of IFN-α (12), and later in disease progression, IL-1β (13), the latter potentially secreted by virally infected β cells or by α cells (14). In addition, the scene in the islets appears altered with increased HLA Class I (HLA-I) expression in both α and β cells (15) and changes in the extracellular matrix (16) (among others) that can be interpreted as signs of a confrontation.

Gathering Evidence at the Scene of the Crime

A survey of human insulitis reveals a diverse array of suspects, composed of $CD8^+$ and $CD4^+$ T cells, B cells, macrophages, and other immune cells (17–22) [along with data sets from consortia such as the Network of Pancreatic Organ Donors with Diabetes (nPOD; https://www.jdrfnpod.org/), the Exeter Archival Diabetes Biobank (EADB; https://foulis.vub.ac.be/) and the Human Islet Research Network (HIRN; https://hirnetwork.org/)]. Islet-reactive T cells emerge as prime suspects of β cell killing. These have been unambiguously identified in the pancreas using HLA-I multimers (MMrs) (23–27), and implicated through *in vitro* assays using HLA class II restricted peptides with many specificities defined in peripheral blood which have been confirmed among islet infiltrating T cells (28–30). Insulitic $CD8^+$ T cells are present in higher numbers than other immune cell types. They increase in number as insulin decreases and decline once insulin positive cells can no longer be detected (21). This suggests a major role, but did these prime suspects act alone or with assistance from co-conspirators? This surge in T cell numbers represents an 'opportunity' for non-islet reactive T cells to also enter the islets (in response to the chemoattractive and pro-inflammatory factors), interact with antigen presenting cells (APCs) and reinforce islet-reactive T cell responses.

Other immune cell populations follow a similar trend, albeit in lower numbers. These include $CD4^+$ T cells with only a few detected in human islets. Their numbers are slightly increased in the pancreas at the autoantibody positive stage, along with an increase in $CD11c^+$ cells (31). This could indicate that $CD4^+$

cells and APCs play a role in disease initiation, which would be consistent with Non Obese Diabetic (NOD) mouse studies (32) and a recent single cell analysis that demonstrated a progressive increase of intra-islet leukocyte infiltration and significant heterogeneity in cell type and function (33). $CD20^+$ B cells, macrophages and other immune cells are also present in insulitis or in peri-insulitis. Their presence and numbers are associated with length of disease and age at disease diagnosis (18–20, 34–37). Only a few studies have attempted to identify $CD4^+$ regulatory T cells (Tregs) in the human pancreas with some success (38), but other studies did not confirm the presence of conventional $CD4^+$ Tregs in human insulitis (18, 20). It remains to be demonstrated (with better tools) whether regulatory cells are overwhelmed by the developing effector response, incapable of providing adequate T cell regulation, or if they are absent altogether. Such dereliction of duty by immune regulation/Tregs would be a notable contributing factor to the crime that took place.

Arraigning the Key Perpetrators/Prime Suspects: Islet Reactive T Cells in Human T1D

Although the number of antigens reported to be recognized by insulitic $CD4^+$ and $CD8^+$ T cells from donors with T1D is limited, $CD8^+$ epitopes from native and neoepitopes from islet-derived proteins have been identified (25, 26, 39) along with broad $CD4^+$ T cell reactivity (28–30); see (40) for a review of islet proteins with native and neoantigen epitope targets. Two reports documented higher numbers of pancreas-infiltrating cells reactive to zinc transporter 8 and epitopes derived from previously unidentified targets in donors with T1D than healthy controls (25, 26). Bender et al. demonstrated that preproinsulin reactive $CD8^+$ T cells were enriched in the islets of donors with T1D. These cells preferably infiltrated ICI, with a mean frequency of 40% (of total $CD8^+$ T) cells compared to 22% in IDIs. Preproinsulin-reactive $CD8^+$ T cells were also present in the exocrine pancreas of non-diabetic donors, and their numbers increased in donors with islet autoantibodies and T1D (23). What is the role of T cells found in the pancreatic exocrine tissue in T1D? To date, this is unknow, but the identification of the reactivity and extended phenotype of these T cells, their possible reactivity to islet targets, or exocrine or duct components, will require investigation to understand their role in T1D. Rodriguez-Calvo et al. showed that 10% of $CD8^+$ T cells react against preproinsulin in the pancreas of living individuals with T1D whose specimens were obtained only weeks from diagnosis (27). Although some of these $CD8^+$ T cells express the memory marker CD45RO in human pancreas specimens (23, 26), the question of whether this memory phenotype is a universal feature and its precise nature remains unsettled. Islet-reactive $CD8^+$ T cells have been shown to produce inflammatory and cytotoxic mediators (25, 26), indicating sufficient means to commit the crime. Thus, islet-reactive T cells have the means, motive, and opportunity to commit the crime of β cell damage.

INVESTIGATING ALLEGED CO-CONSPIRATORS: EVIDENCE OF CRIMINAL ACTION BY NON-ISLET-REACTIVE T CELLS IN T1D

Islet reactive T cells were arraigned as our prime suspects but did these suspects act alone or with assistance from accomplices? Here, we consider potential roles for non-islet-reactive T cells, including active involvement, indirect contributions that either promoted or potentiated the crime, and modestly heroic attempts to regulate the aggressiveness of the islet-reactive T cell attack on β cells. To build our case, we will draw on T cell data from animal models and from human T1D.

Non-Islet-Reactive T Cells in Animal Models

Although animal models of T1D do not completely reflect the human disease, they are a crucial tool to perform real-time exploration of mechanisms of immune regulation in the pancreas and associated lymphoid organs. In the lymphocytic choriomeningitis virus-rat insulin promotor-glycoprotein (LCMV-RIP-GP) mouse model of T1D, as little as 1% of the islet T cell infiltrate consists of $CD8^+$ T cells specific for the driver antigen in this monospecific model (41). In this transgenic (i.e. β cells transgenically expressing experimental driver antigens), non-islet-reactive $CD8^+$ T cells with known specificity towards irrelevant antigens were transferred and activated *in vivo* during disease onset (42). Titration of the numbers of non-islet-reactive $CD8^+$ T cells revealed that these cells halted T1D onset in a dose-dependent manner. High numbers of non-islet-reactive infiltrating $CD8^+$ T cells left β cells largely unharmed despite islet-specific $CD8^+$ T cells being also present at the site. Another study showed that inflammation induced by diabetogenic CD4+ T cells alone was not sufficient to mediate non-islet reactive CD4+ T cell islet entry and/or retention (43). In addition, islet antigen expression was a key factor in determining the ability of a given T cell population to accumulate in the islets (43). Some evidence suggests that non-islet-reactive T cells may have different entry requirements than islet-reactive T cells. For example, in one study, the entrance of non-islet-reactive T cells required a chemokine response and VCAM-1 expression by the islets (44).

In multiple sclerosis (MS) and the experimental autoimmune encephalomyelitis (EAE) mouse model of the disease, the Davis lab reported an opposing immune response against the autoimmune $CD4^+$ T cells driving the disease (45). Clonal expansion of endogenous $CD8^+$ T cells not reactive to the myelin driver antigen was found, and the target peptides were identified for two clones. Co-immunization of mice with the disease-driving antigen myelin oligodendrocyte glycoprotein and the newly discovered peptides protected mice from EAE through expansion of regulatory CD8+ T cells expressing CD122 and Ly49. These markers have been found to be expressed on regulatory $CD8^+$ T cells in other studies (46).

It is evident from these model studies and others on islet-infiltrating T cells (and from those infiltrating most other inflammatory sites) that T-cell recruitment to islets (or other

sites of inflammation) is antigen independent (e.g., viral reactive cells at autoimmune sites), with an antigen-dependent component (43), with the likelihood that both mechanisms rely on diverse interstitial chemokines and upregulation of integrins on the vascular endothelium (47, 48).

Non-Islet-Reactive T Cells in Insulitis in Humans

Establishing an opportunity that would allow the crime to occur boils down to showing that the suspect was present at the scene of the crime. Non-islet-reactive T cells would be drawn to a pro-inflammatory and chemoattractive environment in islets: this establishes 'opportunity' for non-islet-reactive T cells to be present *in situ*. Although informative, the strategies [MMr staining (23–27), growth from islets (28, 30), sorted T cells for T cell receptor (TCR) transductant generation (29)] to identify the targets of autoreactive T cells in human insulitis have not yet defined the relative representation of islet specificities among the total number of islet-infiltrating $CD4^+$ and/or $CD8^+$ T cells. Moreover, these strategies are inherently biased by their focus on epitopes known to be targeted by circulating T cells, which could be different in the pancreas (26). The antigen specificities for a large fraction (96%) of TCR transductants generated from single T cells seen in (29) or T cell lines and clones derived from insulitis remain unassigned (23, 25–28). This raises two possibilities: 1) a significant proportion of islet infiltrating T cells recognize self-antigens that are not yet appreciated as being disease relevant or 2) a significant proportion of T cells found in the islets are not islet-reactive. Collectively, the relative representation of islet-infiltrating T cells recognizing islet *vs.* non-islet antigens and the hierarchy of the islet antigens recognized remain open questions.

Interacting With the Crime in Progress: Indirect Roles for Non-Islet-Reactive T Cells in T1D

Non-islet-reactive T cells are present at the scene of the crime in animal models of T1D, in human T1D, and in other human autoimmune diseases, but what are their possible roles in the disease process? We consider three: 1) active regulatory cell function or secretion of regulatory cytokines/factors, 2) potentiation of the pro-inflammatory environment, and 3) bystander consumption of cytokines/growth factors/nutrients *in situ* (potentially dampening autoreactive T cell function) (**Figure 1**).

Regulation of the Microenvironment

As well-meaning citizens, active Tregs or T cells with regulatory cytokine secretory, or 'exhausted' properties may suppress autoreactive T cells *in situ*. In NOD mice, the suppressive mechanism of infiltrating Tregs seems to be of a less specific nature. Islet-reactive $CD8^+$ T cells in the pancreas of protected mice showed greatly reduced proliferation rates and lower amounts of islet-reactive cells present, with some signs of exhaustion (42). In EAE, on the other hand, a perforin-dependent mechanism of suppression was found (45). Tregs strongly impact T1D pathogenesis in mouse models, with a gradual decrease in the Treg:T conventional (Tconv) cell ratio in inflamed islets (49). Tang et al. suggested that the decline in pancreatic Tregs reflected apoptosis; a reduced influx of Treg *vs.* Tconv cells may also be possible. In human insulitis, the data point to an absence of classical $CD4^+FOXP3^+$ Tregs. Current data from animal models supports multiple regulatory mechanisms, but the common thread is that Treg activity is inadequate to prevent the crime.

Another mode of microenvironment regulation in diverse mouse model systems is the local secretion of IL-4. Expression of IL-4 in β cells under the control of the human insulin promoter abrogated development of T1D (50, 51), but conversely, increased antigen presentation within islets (52). The use of an IL-4-Ig fusion protein suppressed proinflammatory cytokine production, but augmented islet infiltration (53). Interestingly, expression of CD1d, the ligand for NKT cells, under the control of the insulin promoter in β cells in NOD mice was associated with an IL-4-secreting phenotype of NKT cells and abrogation of T1D development (54). Collectively, these data in murine models suggest that skewing of the microenvironment with regulatory cytokines can have a beneficial effect for the development of T1D, but requires its own regulation.

Potentiation of the Proinflammatory Microenvironment

Non-islet-reactive T cells may be propagators of violence ("egging on the perpetrators") by secretion of pro-inflammatory cytokines (e.g. IFN-γ) or IL-2, which can potentiate and support the replication and function of $CD8^+$ autoreactive T cells. IL-6 has an unclear role in the development of T1D as the expression of IL-6 from both α and β cells was found to be reduced in islets of donors with T1D as compared to those of control donors (55). In mouse models, overexpression of IL-6 has been found to be insufficient to induce T1D, but rather increased islet infiltration by predominantly B cells, but also $CD4^+$ T cells and macrophages (56) whereas IL-6 inhibition has been found to reduce incidence (57). Proinflammatory cytokines, possibly secreted by bystander T cells, are known to contribute to β cell stress (58–60) and to directly damage β cells (61–63).

A potential source of bystander T cell activation in the pancreas is viral infection. Bystander activated $CD8^+$ T cells have been investigated in NOD mice infected with Coxsackievirus B3 and diabetes acceleration was observed. However, this required a pre-existing level of insulitis at the time of infection (62, 63). Of note, viral infections have also been found to be protective in T1D models (64), suggesting that the specific influence from non-islet reactive T cells may be largely dependent on situation and timing. In different models, bystander activated $CD8^+$ T cells have been implicated as either benefiting or harming the microenvironment (65), but the strongest evidence seems to suggest a harmful role.

Depletion of Nutrients and Growth Factors

Non-islet-reactive T cells could contribute to the crime scene by "taking up space". The tumor microenvironment is highly metabolically active to support tumor growth. This leads to a rapid depletion of nutrients in tumors with high glycolytic rates,

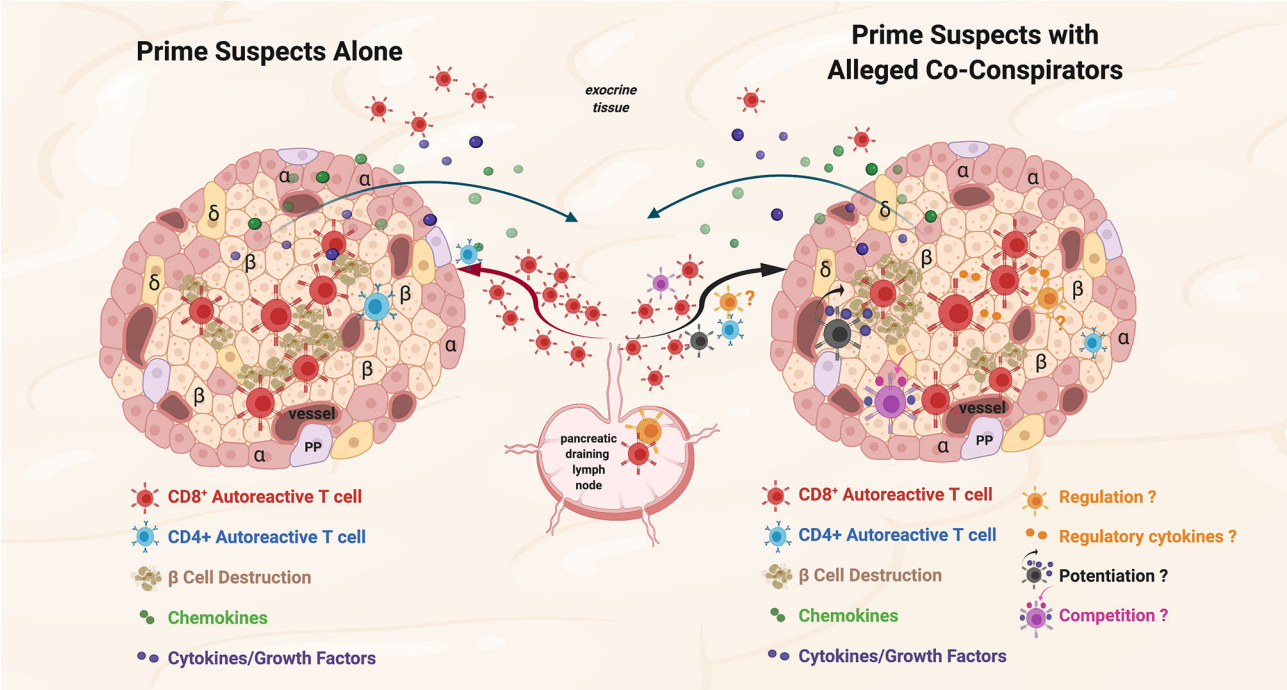

FIGURE 1 | Prime suspects and alleged co-conspirators at the scene of the crime: function of islet-reactive T cells and potential function(s) of non-islet-reactive T cells in insulitis. Proposed schema for islet and non-islet cell reactive T cell functions in insulitis. Representative alpha (α), beta, (β) and delta (δ); cells, and blood vessels are labeled and shown in the represented islet on the left and on the right. The islets are depicted as sources of chemoattractants/cytokines gradients (depicted in green and purple symbols, respectively) through venules with the resultant egress of lymphocytes from the pancreatic draining lymph nodes (pLN) and migration to the islets. Autoreactive $CD4^+$ T cells are depicted in blue. On the left, the prime suspects, β–cell cytolytic function of islet-reactive $CD8^+$ T cells (depicted in brick red) with β cell destruction depicted in brown. For both representative islets, auto-reactive $CD8^+$ T cells are shown in the exocrine tissue. On the right, three potential functions of the alleged co-conspirators, non-islet-reactive T cells, on β–cell destruction by the prime suspects, are depicted. A pro-inflammation 'potentiating' T cell (depicted in black) with cytokine/growth factor secretion (depicted in green and purple) and increased β–cell destruction are depicted in brown. The potential suppressive function of a Treg on β–cell destruction and the potential regulatory effects of cytokines are depicted in orange. The competitive function(s) of a resource/nutrient/growth factor consuming T cells are depicted. The three proposed functions of non-islet-reactive T cells in insulitis are not necessarily mutually exclusive.

and the microenvironment becomes metabolically restrictive for infiltrating T cells leading to resistance to T cell cytotoxicity (64). Could similar mechanisms be at play in an islet infiltrate consisting of a large amount of non-islet-reactive cells? Competition over nutrients can occur between immune cells. One example is the interaction between T cells and APCs, where APCs can become nutrient-starved by clusters of neighboring activating $CD8^+$ T cells (65). Similar clustering was observed in mouse models of suppression (42). Depletion of arginine and tryptophan in the microenvironment may also shift the inflammatory response. Arginine is utilized for nitric oxide (NO) production in inflammatory macrophages, and is important for T cell responses (66). Tryptophan is depleted by the enzyme indoleamine 2,3-dioxygenase (IDO), and the expression of IDO, for example, in tolerogenic APCs leads to inhibition of T cells through both lack of tryptophan and the presence of the resulting metabolite kynurenine (67). T cells increase their metabolic demand upon stimulation and activation. Consequently, depletion of nutrients is a potential regulatory factor worthy of further exploration in the context of insulitis. Competition for nutrients and growth factors by non-islet reactive T cells in insulitis could contribute to the indolent nature of T1D development in humans.

EVIDENCE OF CRIMINAL ACTION BY NON-TARGET REACTIVE T CELLS IN OTHER AUTOIMMUNE DISEASES AND IN VIRAL INFECTIONS: IS THERE A COMMON MODE OF ACTION?

In rheumatoid arthritis (RA), epidemiologic and clinical observations suggest that Th1 responses are primary drivers of disease (68). This is illustrated by the amelioration of RA in pregnant women, who present improved symptoms and diminished inflammation driven by a shift from Th1 to Th2 T cell responses (69). However, given that relapses occur six months after delivery in most cases, the beneficial Th2 response is not maintained long term. These observations have

implications for disease pathogenesis, as they indicate that T cell responses (antigen-specific and bystander) have plasticity and can be effectively modulated. If modulation could be maintained, T cells might no longer find a motive to go to the target tissues or might have more limited means to cause injury. In RA, a disease setting in which sampling disease-proximal joint tissues is feasible, MMr staining of T cells derived from the synovial fluid revealed a notable population of influenza-reactive $CD4^+$ T cells (70). This indicates that non-tissue specific T cells are drawn to sites of inflammation. These T cells displayed a memory phenotype, but were CD25-negative, suggesting that they had not been recently activated.

MS has been linked to EBV infection and, although up to 95% of the general population is seropositive, disease risk is 15-fold greater in seropositive compared to seronegative individuals (71). Patients show increased T cell responses against EBV nuclear antigen (EBNA1), which cross-recognize myelin antigens, whereas influenza and CMV-specific responses are not altered (72). Analogously, *in situ* MMr staining showed that 5% of $CD8^+$ T cells in the pancreas of 4 recent onset T1D subjects were reactive against CMV (27). These cells were located predominantly in the exocrine tissue, whereas their presence around or inside the islets was rare. Likewise, a recent study identified T cells that could be activated either by influenza or GAD65 (73), but these cross-reactive cells were from peripheral blood and not documented within the islets.

The role of virus-reactive T cells in the pathogenesis of human autoimmune diseases remains unclear. Given the potential cyclical activation of T cells due to new infections or viral reactivation, virus-reactive T cells might be important contributors to tissue inflammation. T1D has been historically associated with enterovirus infections (74). Investigating the location and phenotype of enterovirus-reactive cells has potentially important implications for disease pathogenesis and prevention. Although molecular mimicry between microbial and β-cell epitopes has been described, the arrival of virus-reactive T cells could be driven by viral antigens and/or chemokines/cytokines, with the aim of eliminating infected β cells. Their ongoing activity could contribute to islet inflammation by activating anti-viral responses (interferon-mediated), favoring antigen presentation (increasing HLA-I expression), and inducing β cell destruction. With ~96% of the reactivities of islet infiltrating T cells undefined, the question remains open as to the identity and *in situ* function of this large portion of T cells.

CONCLUSIONS AND FUTURE PERSPECTIVES

Collectively, there is some consensus that in T1D (and other autoimmune diseases), T cells that lack any documented self-reactivity are present within inflamed tissues. Some of these might recognize unknown self-antigens, but others have been shown to recognize epitopes from common viruses. It cannot be completely ruled out that non-islet reactive infiltrating T cells could actively suppress autoreactive T cells or restrain their activity by depleting nutrients and growth factors/regulatory cytokines or competing for 'space'. While we cannot exclude that multiple mechanisms may be at play, the most likely conclusion is that non-islet reactive T cells potentiate and support the replication and function of destructive autoreactive T cells by secreting pro-inflammatory cytokines and growth factors. It should be noted that nutrient consumption and cytokine secretion within the islets could also have a direct negative effect on β cells, leading to a direct, but non-antigen specific contribution to autoimmunity in T1D. Although the current evidence is inadequate to convict, we assert that further investigative efforts to scrutinize the motive and opportunity of non-specific islet infiltrating T cells is warranted. Analogous to conducting interviews, constructing a psychological profile, and petitioning the court for a search warrant, investigators should assess the function of non-islet-reactive T cells, their activation state, their ability to secrete regulatory, pro-inflammatory or growth factors, their interactions with other immune cell types, their cell surface phenotype, TCR repertoire, and their function in animal models. These studies should be paired with companion analysis of the 'scene of the crime' in T1D to confidently determine the complicity and culpability of these T cells so that they can be brought to justice.

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Mapping T Cell Responses to Native and Neo-Islet Antigen Epitopes in at Risk and Type 1 Diabetes Subjects

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Aims: Recent studies highlight the potentially important role of neoepitopes in breaking immune tolerance in type 1 diabetes. T cell reactivity to these neoepitopes has been reported, but how this response compares quantitatively and phenotypically with previous reports on native epitopes is not known. Thus, an understanding of the relationship between native and neoepitopes and their role as tolerance breakers or disease drivers in type 1 diabetes is required. We set out to compare T cell reactivity and phenotype against a panel of neo- and native islet autoantigenic epitopes to examine how this relates to stages of type 1 diabetes development.

Methods: Fifty-four subjects comprising patients with T1D, and autoantibody-positive unaffected family members were tested against a panel of neo- and native epitopes by ELISPOT (IFN-γ, IL-10, and IL-17). A further subset of two patients was analyzed by Single Cell Immune Profiling (RNAseq and TCR α/β) after stimulation with pools of native and neoepitope peptides.

Results: T cell responses to native and neoepitopes were present in patients with type 1 diabetes and at-risk subjects, and overall, there were no significant differences in the frequency, magnitude, or phenotype between the two sets of peptide stimuli. Single cell RNAseq on responder T cells revealed a similar profile in T1D patients stimulated with either neo- or native epitopes. A pro-inflammatory gene expression profile (TNF-α, IFN-γ) was dominant in both native and neoepitope stimulated T cells. TCRs with identical clonotypes were found in T cell responding to both native and neoepitopes.

Conclusion/Interpretation: These data suggest that in peripheral blood, T cell responses to both native and neoepitopes are similar in terms of frequency and phenotype in patients with type 1 diabetes and high-risk unaffected family members. Furthermore, using a combination of transcriptomic and clonotypic analyses, albeit using a limited panel of peptides, we show that neoepitopes are comparable to native epitopes currently in use for immune-monitoring studies.

Keywords: neoepitopes, T cells, cytokines, T cell receptor, genes, type 1 diabetes, autoantibody, transcriptome

INTRODUCTION

Type 1 diabetes arises as a result of immune-mediated loss of β cell mass and function. A lack of self-tolerance against islet autoantigens facilitates this destruction; one of the hallmarks of type 1 diabetes pathogenesis is the autoantigen-specific clonal expansion of T cells (1). Upon stimulation with peptides of islet autoantigens, these T cells respond and secrete cytokines such as interferon-γ, IL-10, and IL-17 which can be measured in subjects with type 1 diabetes and used to illustrate the heterogeneity of the disease (2–4) Furthermore, T cell responses elicited against peptides of proinsulin distinguish age-related heterogeneity in type 1 diabetes pathogenesis (4). Previous studies have suggested that T cell epitopes such as proinsulin peptide C13–32 elicit pro-inflammatory responses in approximately 70% of patients (4), and responses to this epitope can also be detected in autoantibody-positive unaffected family members (3).

Recent studies have highlighted the role of the β cell in orchestrating its own sustained attrition and demise by enabling a repertoire of non-germline encoded "neoepitopes" previously not encountered by the immune system (5).

Neoepitopes in T1D can be generated through several mechanisms including post-translational modification of peptides, for example *via* enzymatic deamidation or citrullination which can augment binding to HLA molecules and boost T cell responses (6); alternative RNA-splicing results in proteins being encoded from an alternative open reading frame (7) and peptide fusion of islet-derived peptides (8). Peptide fusion is a post-translational modification in which peptide fragments of proteins such as proinsulin are modified through fusion with peptides from other secretory granule proteins resulting in novel peptidic species termed hybrid insulin peptides (HIPs).

The fact that neoepitope specific T cells have been isolated from the islets of subjects with type 1 diabetes after death (8, 9) has provided a strong implication that these antigenic drivers and T cells directed against them could be relevant to disease pathology.

Similarly, T cell reactivity to neoepitopes such as modified glucose-regulated protein 78 (GRP78), HIPS and post-translationally modified GAD peptides has been demonstrated in the peripheral blood of subjects with type 1 diabetes and is observed in a greater frequency in these individuals compared to healthy donors (10–12).

In some cases, reactivity to neoepitopes has been shown to be more enhanced than that directed to the native epitopes (10, 12); for example, CD4 T cells directed against citrullinated GRP78 elicited T cell responses at a greater frequency in patients compared to the native epitope. Thus, growing evidence suggests that neoepitopes are relevant in type 1 diabetes pathology, but whether these are germane to the pathological response, are early drivers of disease or arise secondarily, and whether they serve as biomarkers remain issues to be addressed.

It is not clear how T cell responses to native and neoepitopes interlink and indeed which comes first; hence, an understanding of the relationship between native and neoepitopes and their role as tolerance breakers or disease drivers in type 1 diabetes is required. In order to gain this knowledge, both sets of epitopes need to be tested in parallel on the same patient cohorts.

In this study, we set out to evaluate T cell response to neoepitopes and established native epitopes in patients with type 1 diabetes and high-risk autoantibody-positive non-diabetic subjects to determine whether an epitope hierarchy exists. We then proceed to analyze T cells responding to native and neoepitopes at the single cell level and compare TCR usage to help address the phenotypic, transcriptomic, and clonal nature of the responses.

METHODS

Subjects

Blood was obtained from 54 subjects, 41 of whom had type 1 diabetes, and 13 subjects comprised of autoantibody-positive unaffected family members (UFMs); both groups consisted of children (≤16 years of age) and adults (≥16 years of age). In the type 1 diabetes group there were 14 participants in the group comprising of children (disease duration <1.5 months; median age 11 years; nine males) and 27 in the adult group (disease duration <8 months; median age 24 years; 14 males). In the UFM, there were five children (median age 11; 5 males) and eight adults (median age 37; six males). Forty-six subjects were recruited through the INNODIA natural history study (13). HLA and autoantibody data on all the subjects are shown in **Table 1**.

In addition, to examine the immune signature at the single cell level using transcriptomics, we obtained 130–160 ml of blood from two of the subjects with type 1 diabetes described above (two females, ages 30 and 34 years; duration of diabetes: 6 and 12 months) and identified as ND01 and ND02.

Ethical approval for this study was granted by the Ethics committee and institutional review board, and informed consent/assent was obtained from all subjects enrolled or their guardians.

Detection of β-Cell-Specific CD4+ T Cells by Cytokine ELISPOT

Peptides representing native proinsulin epitopes (PIPs) or neoepitopes: hybrid insulin peptides (HIPs) (PIPs: C13–32; C19-A3; C22-A5, and HIPs: C peptide-IAPP1; C peptide-IAPP2; C peptide-NPY) were synthesized and purified by high-performance liquid chromatography (Thermo Hybaid, Germany).

Interferon-γ (IFN-γ), interleukin-17A (IL-17A), and IL-10 production by CD4+ T cells was detected by enzyme-linked immunospot (ELISPOT) assay, performed as described previously (2, 4), in triplicate for each peptide. Briefly, fresh PBMCs supplemented with peptide were dispensed into 48-well plates at a density of 3×10^6 in 0.5 ml RPMI-1640 supplemented with antibiotics (TC medium; Life Technologies Ltd.) and 10% human AB serum (Sigma, Dorset, United Kingdom) and incubated at 37°C to a final concentration of 10 μg/ml. Control

TABLE 1 | HLA and autoantibody status of subjects with type 1 diabetes and unaffected family members (UFM).

Subject	HLA-DR/DQ				AUTOANTIBODIES			
	DR4+	DQ8+	DR3+	DQ2+	IAA	GADA	IA2A	ZnT8A
ND01	N	N	Y	Y	NEG	POS	NEG	POS
ND02	Y	Y	Y	Y	POS	NEG	NEG	NEG
ND03	Y	Y	N	N	N/A	N/A	N/A	N/A
ND04	N	N	Y	Y	N/A	N/A	N/A	N/A
ND05	N	N	Y	Y	POS	POS	POS	POS
ND06	N	N	Y	Y	POS	POS	POS	POS
ND07	N	N	Y	Y	POS	POS	POS	NEG
ND08	N	N	N	N	POS	POS	POS	POS
ND09	N	N	N	N	POS	NEG	NEG	NEG
ND10	N	N	N	N	NEG	NEG	NEG	NEG
ND11	N	N	Y	Y	POS	POS	POS	POS
ND12	N	N	N	N	POS	NEG	POS	POS
ND13	N	N	N	N	POS	POS	POS	NEG
ND14	N	N	N	N	POS	NEG	NEG	POS
ND15	N	N	Y	Y	NEG	POS	POS	POS
ND16	N	N	N	N	POS	POS	POS	POS
ND17	Y	N	N	N	NEG	POS	POS	POS
ND18	Y	Y	N	N	POS	POS	POS	POS
ND19	Y	Y	N	N	POS	NEG	POS	POS
ND20	Y	Y	Y	Y	POS	POS	POS	POS
ND21	Y	Y	Y	Y	POS	POS	POS	POS
ND22	Y	Y	Y	Y	NEG	POS	NEG	POS
ND23	Y	Y	N	N	POS	POS	NEG	POS
ND24	Y	Y	Y	Y	NEG	NEG	NEG	NEG
ND25	Y	Y	Y	Y	NEG	POS	NEG	NEG
ND26	Y	Y	Y	Y	POS	NEG	NEG	NEG
ND27	Y	Y	Y	Y	POS	POS	NEG	NEG
ND28	Y	N	N	N	POS	POS	NEG	POS
ND29	Y	Y	Y	Y	NEG	POS	NEG	POS
ND30	Y	N	N	N	POS	NEG	NEG	NEG
ND31	Y	Y	N	N	POS	NEG	POS	NEG
ND32	Y	Y	N	N	POS	POS	POS	NEG
ND33	Y	Y	Y	Y	POS	POS	POS	POS
ND34	Y	Y	Y	Y	NEG	POS	POS	POS
ND35	Y	Y	Y	Y	POS	POS	POS	POS
ND36	N	N	Y	Y	NEG	POS	POS	NEG
ND37	Y	Y	N	N	NEG	POS	NEG	NEG
ND38	Y	Y	N	N	N/A	POS	POS	POS
ND39	Y	Y	N	N	N/A	POS	POS	NEG
ND40	Y	Y	N	N	N/A	POS	POS	N/A
ND41	Y	Y	Y	Y	N/A	GAD	NEG	N/A
UFM01	N	N	N	N	NEG	POS	NEG	NEG
UFM02	Y	Y	N	N	POS	NEG	NEG	NEG
UFM03	N	N	Y	Y	NEG	NEG	POS	NEG
UFM04	N	N	Y	Y	NEG	POS	POS	POS
UFM05	N	N	Y	Y	NEG	POS	NEG	NEG
UFM06	Y	Y	N	N	POS	NEG	NEG	NEG
UFM07	N	N	Y	Y	NEG	POS	NEG	NEG
UFM08	Y	Y	N	N	NEG	POS	NEG	NEG
UFM09	Y	Y	N	N	POS	NEG	NEG	NEG
UFM10	N	N	Y	Y	POS	NEG	NEG	NEG
UFM11	N	N	Y	Y	POS	POS	POS	POS
UFM12	N/A	N/A	N/A	N/A	NEG	POS	NEG	NEG
UFM13	N	N	Y	Y	NEG	POS	POS	POS

wells contained TC medium with an equivalent concentration of peptide diluent alone (DMSO). Pediacel, a penta-vaccine, was obtained from Sanofi Pasteur Ltd. (Guildford, U.K.) and used at 1 µl/ml to examine anamnestic responses induced by vaccination or infection as previously described (4).

Pre-warmed TC medium/10% AB serum was added 24 h later, and 48 h after stimulation, cells were washed and resuspended in TC medium containing 10% human AB serum and brought to a concentration of 10^6/300 µl; 100 µl was dispensed in triplicate into wells of 96-well ELISA plates

(Nunc Maxisorp; Merck Ltd., Poole, United Kingdom) pre-blocked with 1% BSA in PBS and precoated with monoclonal anti-IFN-γ, anti-IL-10, or anti-IL17 capture Ab (U-Cytech, Utrecht, The Netherlands). After capture at 37°C for 20–22 h, plates were washed in PBS/Tween 20, and spots developed according to the manufacturer's instructions. Plates were dried, and spots of 80–120 µm were counted in a BioReader 3000 (BioSys, Karben, Germany), and data were expressed as stimulation index (SI) values (mean spot number of test peptide/mean spot number of diluent). The SI takes account of background, spontaneous responsiveness in the negative control, and ROC plots can be used to assign cut-off values as described previously; SI values ≥3 was taken to indicate a positive response for IFN-γ and IL-10 and ≥2 for IL-17A.

We have previously demonstrated that CD4+ T cells elicit the responses to the native peptides used in this assay by showing that depleting CD4+ cells abolished the cytokine responses (14).

Isolation and Single Cell Transcriptional Profiling of β-Cell-Specific CD4+ T Cells

Peptides were pooled into two groups, those consisting of native epitopes in one and neoepitopes in the second group. The pools comprised of the three proinsulin peptides and included peptides of GAD (115–127 and 265–284) in the native group (pool 1) and deamidated and citrullinated versions of these in the neoepitopes pool [GAD 115–127 (120E) and GAD 265–284 (272cit.)] together with C-peptide IAPP1 and C-peptide-NPY peptides (pool 2); these were synthesized and purified by high-performance liquid chromatography (Thermo Hybaid, Germany). Each of the two experiments was conducted using blood obtained from two individuals with type 1 diabetes; PBMCs at 2×10^6/ml were added to 20 wells of a 48-well plate; pool 1 peptides were added at a final concentration of 10 µg/ml to each well; this was repeated for an additional 20 wells where pool 2 was added at a final concentration of 10 µg/ml. Pediacel was used as positive control, and 1 µl/ml was added to a well containing cells at 2×10^6/ml, and medium alone was used as a negative control. Anti-CD40 (Biolegend, clone G28.5, 303611) was added at a final concentration of 2 µg/ml to each well, and the cells were incubated for 18–20 h.

The cells were then harvested and washed with phosphate buffered saline (PBS). An antibody staining master mix consisting of anti-CD19 (M5E2, 561391), anti-CD3 (SK7, 641415), anti-CD4 (SK3, 345768), anti-CD45-RO (UCHL1, 337168), anti-CD95 (DX2, 561978), anti-CD154 (TRAP1, 555700), anti-CD69 (FN50, 555530) (BD Biosciences, Oxford, UK), and anti-CD27 [(O323, 302830) Biolegend, London UK] was prepared and spilt into five tubes. Anti-human Hashtag 1 (TotalSeqC 0251), anti-human Hashtag 2 (TotalSeqC 0252), anti-human Hashtag 3 (TotalSeqC 0253), anti-human Hashtag 4 (TotalSeqC 0254), and anti-human Hashtag 5 (TotalSeqC 0255) (Biolegend, London, UK) were added at a concentration of 0.1 µg/µl to each of the five tubes. The hashtags were used to barcode the samples as follows: patient 1 pool 1 (Hashtag 1); patient 1 pool 2 (Hashtag 2) patient 2 pool 1 (Hashtag 3); patient 2 pool 2 (Hashtag 4), and Pediacel (patients 1 and 2; Hashtag 5). The hashtag barcoded cells were incubated for 20 min in the dark and washed three times in FACS buffer (PBS with 3% FCS and 2% human AB) (both from Sigma, UK). CD4+ T cells responding to stimulation were identified by co-expression of CD69, and CD154 were sorted into 35 µl PBS using a BD FACSAria III cytometer as previously described (15).

Sorted cells were characterized using the 10× Chromium Controller Single-cell Immune Profiling system which simultaneously defines gene expression and T cell receptor profiles from single cells in the same sample; this was conducted at the Genomics facility at Guy's and St Thomas' Biomedical Research Centre. Briefly, single-cell suspensions were used to generate full-length cDNA in gel beads (containing Unique Molecular identifiers—UMI) in an emulsion master mix from 5′ V1 chemistry. The cDNA was amplified and subsequently size selected at the clean-up stage: smaller fragments were separated and stored for feature-barcoding/cell-hashing library preparation and the large fragments for gene expression (GEX) and T cell VDJ according to the manufacturer's instructions.

The GEX libraries were enzymatically fragmented and indexed while the T cell library was first enriched [using Chromium Single Cell V(D) J Enrichment Kit, Human T Cell, PN-1000005] and then fragmented and indexed for further sequencing. All the three final indexed libraries (feature barcoded + GEX + VDJ) were diluted to 4 nM concentrations and then pooled together at a ratio of 1:4:1 (feature barcoded library: GEX library: VDJ library) before being sequenced at 10 pM on HisSeq Rapid flow cell using an Illumina's HiSeq2500 instrument.

Cell ranger software (version 4) was used for calculating 5′ genes, and VDJ sequence construction and downstream secondary analysis were performed using Loupe Cell Browser (version 3.1.1.) and Seurat_3.2.2 in R (version 4.0.3).

Cell Ranger output was filtered to remove doublets, genes expressed in less than four cells, cells expressing less than 700 genes, and cells with more than 10% counts from mitochondrial gene transcription. We also eliminated all MAIT cells (identified by their TCR alpha TRAV1-2) and cells with double TCRB as potential doublets and cells identified as contaminating non-T cells in the first round of clustering. We then mapped all the remaining cells to a PBMC reference (16) (**Supplementary Figure 1**) with symphony and harmony and excluded all cells which, according to the outcome of knnPredict() function, were not CD4 T cells. We also assigned T cell subtypes whenever possible. Barcodes of cells used for the downstream analysis are included in **Supplementary File 1**.

For clustering purposes, each of 10× runs was split by individual and reintegrated with Seurat to remove individual effects.

Dimension reduction (PCA, UMAP) and clustering were performed on this cleaned dataset with exclusion of TCR, mitochondrial genes, and ribosomal protein genes. UMAP was performed on first 30 PCA axes and with 50 neighbors. Clusters were determined by SNN algorithm, with resolution of 0.2. Gene expression between clusters was compared with FindConservedMarkers() function in Seurat by MAST, and top

genes (all with adjusted p values in each individual <0.0005 and fold change >2) are shown in **Figure 4C**. Comparison of gene expression between native and neoepitope pools was also performed in Seurat with MAST, with each individual included as a latent variable (results included in **Supplementary Table 1**).

Original Cell Ranger-reported clonotypes require all TCRB and TCRA chains of two cells to be identical to pertain to the same clone; we assumed that two cells are from the same TCR clone if they shared both TCR chains and there was no conflict in their B chains (so if they shared identical TCRA and TCRB, or two TCRA when at least one of the cells did not have TCRB reported). TCR sequences were compared using three-point matches (V-CDR3-J) and TCR chains within our experiments and against external databases when the data was available.

Statistical Analysis

The proportion of individuals responding to individual peptides was compared using Fisher's exact test and one-way ANOVA. Responses to native and neoepitopes were compared using paired t-tests. Differential gene expression between clusters and in response to peptide pools was tested with MAST method as implemented in Seurat, with individual as a latent variable. Frequency of clonotypes was determined by dividing the number of clonotypes by the total number of cells.

Data Availability

A 10× data was submitted to Array Express (E-MTAB-10323).

RESULTS

Comparison of T Cell Responses to Neoepitopes and Native Epitopes in Type 1 Diabetes and Preclinical Subjects by Cytokine ELISPOT

We set out to compare the magnitude, prevalence, and phenotype of the T cells responding to native and neoepitopes derived from islet autoantigens in individuals with or at risk of developing clinical type 1 diabetes using a sensitive cytokine ELISPOT. We selected native peptides of proinsulin (PIPs), representing naturally processed and presented epitopes known to elicit CD4 responses in PBMC from individuals with type 1 diabetes (2–4) and hybrid insulin peptides (HIPs), known to activate CD4 T cells isolated from pancreatic islets from deceased organ donors with type 1 diabetes (8, 9).

Firstly, we compared responses to native and neoepitopes in all individuals (pre-clinical and clinical type 1 diabetes). As depicted in **Figure 1A** and **Supplementary Figures 2A–C**, all peptides stimulated responses for all three cytokines in at least a proportion of individuals tested. All the peptides show a similar overall level of stimulation. Responses to Pediacel were detected in all of the patients for all three cytokines (**Supplementary Figures 3** and **4**).

As it is possible that the hierarchy of epitope immunodominance may be influenced by the stage of disease, we separated the subjects into patients with type 1 diabetes and

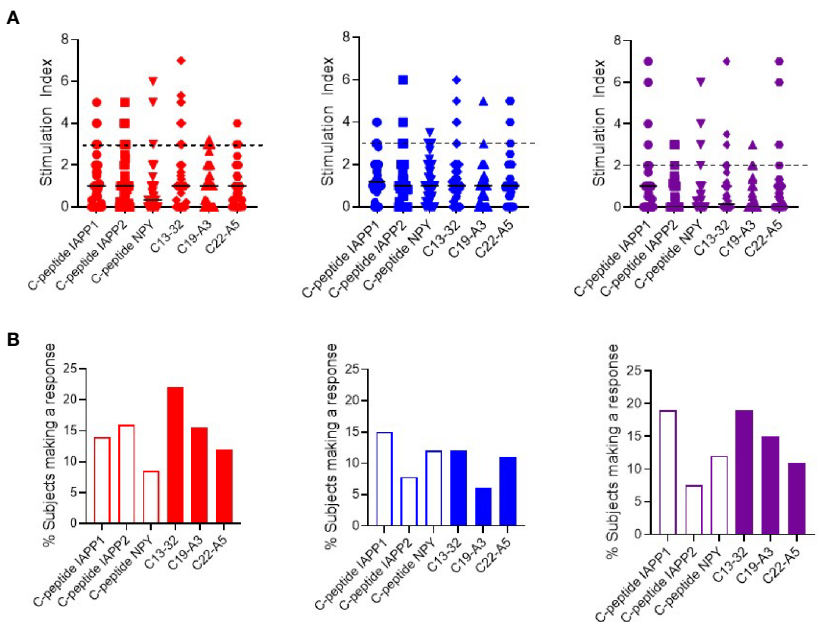

FIGURE 1 | Cytokine T cell responses were measured by ELISPOT, and data are expressed as SI [stimulation index (mean spot number of test peptide/mean spot number of diluent)]. **(A)** Magnitude of IFN-γ (red) IL-10 (blue) and IL-17 (purple) responses in preclinical and subjects with type 1 diabetes against neo- (C-peptide-IAPP1, C-peptide-IAPP2 and C-peptide-NPY) and native epitopes (C13–32, C19-A3, C22-A5). The dashed line represents the cut-off for positivity for the stimulation index (three for interferon-γ and IL-10 and two for IL-17). **(B)** Prevalence of IFN-γ (red) IL-10 (blue) and IL-17 (purple) responses measured by ELISPOT in preclinical and subjects with type 1 diabetes against individual peptides of neo- (open bars) and native (filled bars) epitopes.

those with preclinical disease. In subjects with type 1 diabetes, C-peptide-NPY elicits the highest interferon-γ response, C-peptide-IAPP2 the highest IL-10 and IL-17 responses were observed at a similar magnitude for C-peptide-IAPP1, C13-32, and C22-A5 (**Supplementary Figure 5A**). In preclinical subjects, C13–32 elicited the highest interferon-γ and IL-10 responses and C22-A5 the highest IL-17 response (**Supplementary Figure 6A**). However again, none of these differences reached statistical significance.

We have previously applied a ROC plot approach to establish criteria for defining a positive or negative response to individual peptide stimulation in cytokine ELISPOT assays (2, 4). We applied these criteria to the current data to determine the prevalence of positive responses in all the individuals tested and show that although there is a significant response to each peptide, there is no clear peptide immunodominance (**Figure 1B**). For interferon-γ responses, neoepitopes elicit a response in 8–14% of subjects and native epitopes in 12–22%. For IL-10, neoepitopes elicit a response in 8–15% of subjects and native epitopes in 6–12%, and finally IL-17 responses are present against 8–19% of neoepitopes and 11–19% of native epitopes.

Examining positive responses based on clinical group, in the subjects with type 1 diabetes, interferon-γ responses were observed in 6–16% against neoepitopes and 10–5% of native epitopes. IL-10 responses in 8–11% against neoepitopes and 0–12% of native epitopes and IL-17 against 10–26% of neoepitopes and 9–20% of native epitopes (**Supplementary Figure 5B**). In preclinical subjects, interferon-γ responses were observed against 8–23% of neoepitopes and 8–17% of native epitopes; IL-10 against 8–23% of neoepitopes and 8–31% of native epitopes, and no IL-17 responses were observed against neoepitopes and 8–15% subjects responded to native epitopes (**Supplementary Figure 6B**).

As responses to individual peptides were low, we pooled responses into two groups representing neo- or native epitopes and compared the magnitude of the response (**Figure 2A**) and the proportion of subjects mounting a positive response (**Figure 2B**). Although the overall magnitude of the response is similar, in terms of prevalence of positive responses, we observed a higher proportion of interferon-γ and IL-17 responses in response to native epitopes compared to neoepitopes (37 *vs.* 22% and 31 *vs.* 23% respectively). IL-10 responses were similar to both native and neoepitopes.

When segregating pooled responses by clinical group, the magnitude of response to neo- and native epitopes was similar in both groups (**Supplementary Figures 7A** and **8A**). Examining the proportion of individuals with a positive response, we observed that subjects with type 1 diabetes trended towards higher interferon-γ responses to native *vs.* neoepitopes (45 *vs* 36% respectively). In contrast, in preclinical subjects, IL-10 and IL-17 responses were more prevalent to native epitopes (85 *vs* 62% (IL-10) and 31 *vs* 8% (IL-17) while interferon-γ responses were similar to both neo- and native epitopes (54%) (**Supplementary Figures 7B** and **8B**).

Next, we compared the relative production of each cytokine stimulated by individual peptides to investigate if any single or group of epitopes led to a distinct polarization of the immune response.

When examining the magnitude of response, each peptide was capable of stimulating a range of cytokines in different individuals with no peptide clearly eliciting a stronger response to a particular cytokine (**Figure 3A**). However, when examining the proportion of individuals who mount a positive response, we note that C-peptide-IAPP2 trends towards increased stimulation of a

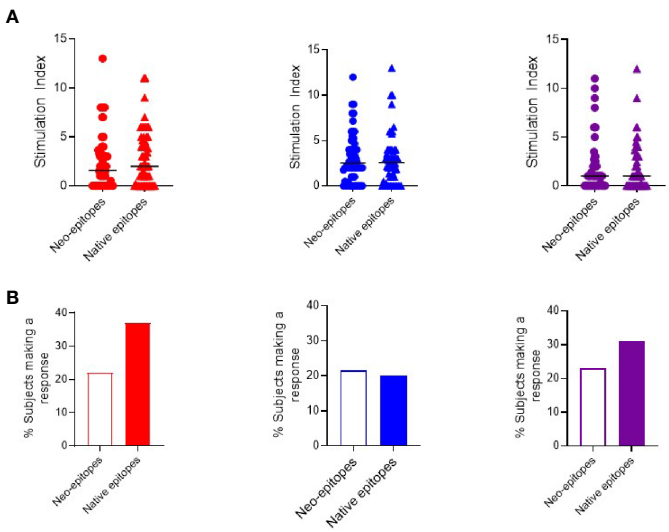

FIGURE 2 | Cytokine T cell responses were measured by ELISPOT, and data are expressed as SI [stimulation index (mean spot number of test peptide/mean spot number of diluent)]. **(A)** Magnitude of responses as depicted by SI for IFN-γ (red) IL-10 (blue) and IL-17 (purple) responses against pooled neo- and native epitopes in preclinical and subjects with type 1 diabetes. **(B)** Prevalence of IFN-γ (red) IL-10 (blue) and IL-17 (purple) responses in preclinical and subjects with type 1 diabetes against pooled peptides of neo- (open bars) and native (filled bars) epitopes.

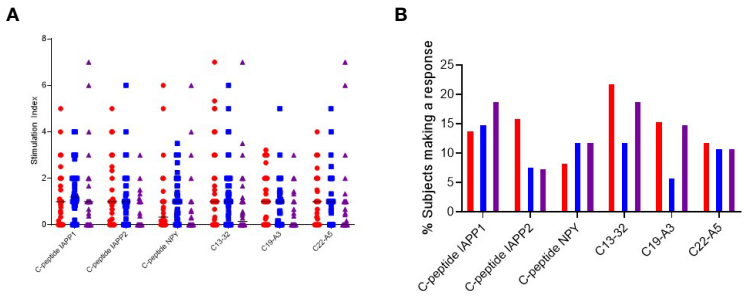

FIGURE 3 | Cytokine T cell responses were measured by ELISPOT, and data are expressed as SI [stimulation index (mean spot number of test peptide/mean spot number of diluent)]. **(A)** Magnitude of IFN-γ (red) IL-10 (blue) and IL-17 (purple) responses depicted as stimulation indices for individual peptides of neoepitopes (three on the left) and native (three on the right) in preclinical and subjects with type 1 diabetes. **(B)** Frequency of IFN-γ (red) IL-10 (blue) and IL-17 (purple) responses to individual peptides of neoepitopes (three on the left) and native (three on the right) in preclinical and subjects with type 1 diabetes.

significant IFN-γ response, C13-32 and C19-A3 trend towards increased IFN-γ and IL-17 compared to IL-10, whereas the remainder showed no particular polarization (**Figure 3B**).

All the peptides except C19-A3 elicited multiple cytokines: interferon-γ and IL10 were detected in 3.8% of patients. C-pep-IAPP1 was the only peptide to elicit IL-10 and IL-17 responses, and these were detected in 3.8% of patients; interferon-γ and IL17 responses were only detected in one patient (1.9%), and in one patient, responses to all three cytokines were observed.

Finally, we examined responses to native and neoepitopes specifically in children as we have previously reported that native proinsulin epitopes are preferentially targeted by interferon-γ producing T cells in this group (4). We show that 11/14 (79%) of children made a response to the native epitopes compared to 8/14 (57%) to neoepitopes ($p = 0.04$). Furthermore, despite the small numbers in the current study, we confirm that as reported previously, the native epitope C13–32 elicits interferon-γ response in more than 40% of children with type 1 diabetes (**Supplementary Figure 9A**). Indeed, two thirds of all interferon-γ responses in all the subjects are detected in children. An interferon-γ response is also seen against the native epitope, C19-A3; in contrast, the neoepitopes tend to elicit IL-17 responses albeit at a lower frequency. IL-10 responses were seen in preclinical subjects particularly to C-pep-IAAP1; however, the number of subjects here are small (**Supplementary Figure 9B**).

Although the native peptides were initially identified as being HLA-DR4 restricted, we now know that these promiscuous responses are observed in non-HLA DR4 individuals, and for both native and neoepitopes we observed responses in non-HLA DR4/DQ8 individuals (**Supplementary Table 3**).

Single Cell Transcriptional Profiling of CD4 T Cells Responding to Neo- and Native Epitopes

To further investigate the phenotype of neo- and native epitope-specific T cells, we conducted an unbiased transcriptional analysis including TCR clonotyping. Responding T cells were isolated based on CD154 and CD69 co-expression following brief *ex-vivo* culture with a pool of peptides representing native or neoepitopes and profiled using scRNA-seq *via* the 10× Genomics pipeline. As a positive control we used T cells activated by Pediacel from the same patients.

Data from donors were filtered and integrated as described in *Methods*, and cells were projected in two dimensions (UMAP). Data for subjects ND01 and ND02 are shown in **Figure 4A**. We performed cell clustering based on gene expression and independently inferred cell subtype by mapping to a reference PBMC dataset (16). The main clusters corresponded to memory CD4 and naïve T cells (**Figure 4B**). In concordance with this, cluster markers are genes preferentially expressed in memory (S100A10, S100A4, LGALS3) or naïve (FABP, SELL) cells (**Figure 4C**; there is also a very small cluster—cluster 3 which comprises of memory-like cells with expression of PLZF.

Cells from both individuals are similarly split between clusters, although ND01 has higher fraction of Tregs and ND02 more naïve cells (**Supplementary Figures 10A, B**).

We then compared cells stimulated with peptide pools in each subject (**Figure 4D**) and show that for each subject, the composition of native and neoepitope stimulated samples does not differ in reference-based assignment or clusters.

Importantly, T cells responded to native or neoepitopes co-cluster (**Figure 4E**) for each individual, implying no general distinction between the responding cells.

We explored this further by comparing gene expression profiles of cells stimulated with either neo- or native epitopes for both patients and found no significant differences in gene expression (**Supplementary Table 1**).

We then examined expression of selected pro-inflammatory genes including *IL-17A, IL-22, TNF-α, INF-γ,* and *IL-32* and anti-inflammatory genes *IL-4* and *IL-10* in cells stimulated with either neo- or native epitopes (**Figure 5A**). *IL-17A* expression was generally low, although expression was slightly higher in patient ND01; *IL-22* expression was greater than *IL-17A*; similarly, expression of this was higher in patient ND01; there were no differences in cells stimulated with either neo- or native epitopes. *TNF-α* and *INF-γ* expression was higher in patient

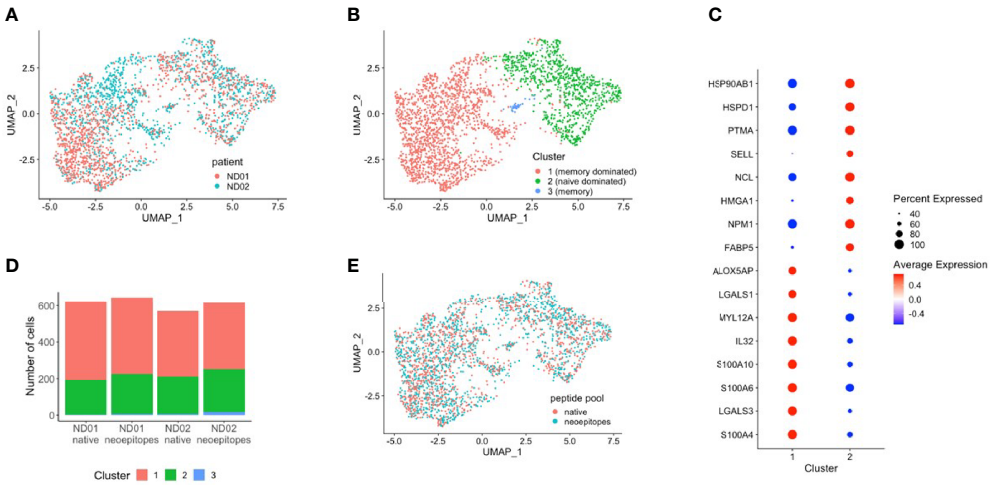

FIGURE 4 | PBMCs from subjects ND01 and ND02 were stimulated with native (proinsulin peptides: C13–32, C19-A3, C22-A5; GAD peptides: 115–125, 265–284) and neoepitopes [C-pep-IAPP1, C-pep-NPY, GAD 115–127 (120E), GAD 265–284 (272 cit)] and each patient/pool was labeled with a different hashtag as described in *Methods*. Antigen-responsive cells were identified by expression of CD154/CD69, and immune profiling was subsequently conducted on single cells. Cells from subjects **(A)** ND01 and ND02 separate into **(B)** three clusters (0–3) based on gene expression similarity. **(C)** Clusters differ by expression of memory and naïve cell specific genes. **(D)** Cells reacting to native and neoepitopes are similarly split between clusters and **(E)** have similar patterns of expression.

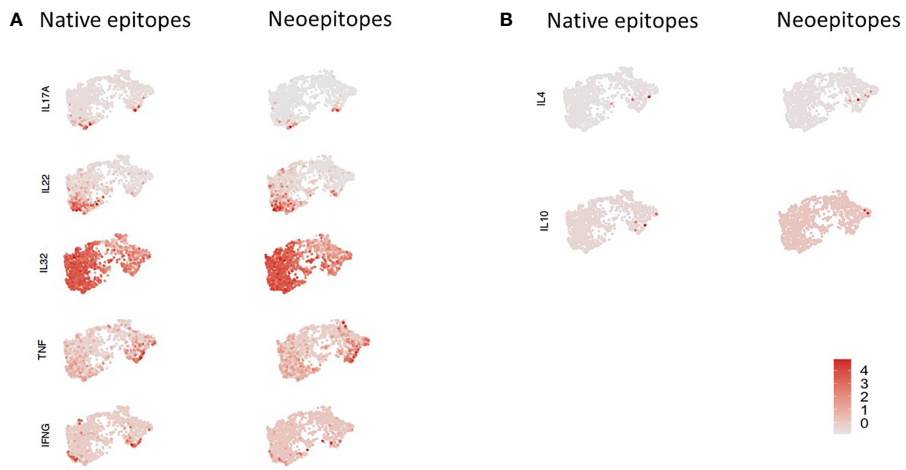

FIGURE 5 | PBMCs from subjects ND01 and ND02 were stimulated with native [proinsulin peptides: C13–32, C19-A3, C22-A5; GAD peptides: 115–125, 265–284) and neoepitopes (C-pep-IAPP1, C-pep- NPY, GAD 115-127 (120E), GAD 265-284 (272 cit)]. Antigen-responsive cells were identified by expression of CD154/CD69, and immune profiling was conducted on single cells. **(A)** Cells were stimulated with native (right) and neoepitopes (left), and gene expression (depicted in pink) of **(A)** *IL-17A, IL-22, IL-32, TNF-α*, and *IFN-γ* and **(B)** *IL-4* and *IL-10* was examined. Level of expression corresponds to greater intensity of color.

ND02, and expression in cells stimulated with either neo- or native epitopes was similar.

Interestingly, *IL-32* gene expression was by far the most extensive and present in both patients in response to both neo- and native epitope stimulated cells.

In contrast, expression of the anti-inflammatory cytokines (**Figure 5B**), *IL-4* and *IL-10* was undetectable in patient ND01 and very low in ND02 for both native and neoepitopes.

TCR Use of Cells Responding to Neo- and Native Epitopes

The Single Cell Immune Profiling system allows the synchronized identification of gene expression and T cell receptor profiles from single cells in the same sample. We used this approach to assess the number and profile of TCR clonotypes and determine whether any are shared between T cells stimulated with either neo- or native epitopes.

For patient set ND01/ND02 we identified 76 clonotypes shared between T cells responding to native and neoepitopes in subject ND01 and seven such clonotypes for ND02 (**Table 2**). Each of these TCR receptors was present in 2–30 cells, and again there was no preference towards either native or neoepitopes. Of the 76 clonotypes in ND01, four of the TCRB sequences (highlighted in red) corresponded to sequences in the JDRF npod database (http://clonesearch.jdrfnpod.org) where they were present in the pancreatic lymph nodes and spleen of both patients with type 1 diabetes and healthy controls (17) (**Table 2**). Representative data are shown in **Figure 6**.

For both sets of patients, stimulating T cell with native or neoepitopes did not lead to a preferential expansion of specific clonotypes shared between peptide pools.

Finally, we also investigated TCR clones that were expanded by one peptide pool only (**Supplementary Table 2**). Subject ND01 had identical number of clonotypes, with similar number of cells expanded to either native or neoepitope pools. In contrast, ND02 had more expanded clones in the native epitope pool compared to the neoepitope pool (eight *versus* one). Eight of the TCRB chains were also present in the JDRF npod database, where they were present in the pancreatic lymph nodes and spleen of both patients with type 1 diabetes and healthy controls at frequencies ranging from 0.0008 to 0.002% which is comparable to the 0.004–0.005% for both native and neoepitopes in the present study. Taken together, these data show the need for testing more patients to determine whether there is a quantitative difference in expanded clonotypes between native and neoepitopes.

DISCUSSION

In agreement with observations made by others we can detect T cell responsive to HIPs in PBMCs and that these cells respond with a range of cytokine production. We wanted to determine if they were present at a higher frequency or with a different phenotype to PIPS and demonstrated that this is not the case. Our findings contrast with those recently published by Mitchell et al. (18); however, we did not test all the HIPs that they used. It is possible that some HIPs elicit stronger responses than those we tested, but equally some PIPs may elicit stronger responses than the ones they tested—there was only one PIP in their panel and that differed in amino acid sequence to the three used in our panel. Moreover, in that study there was no difference between the negative control (no antigen) and B9-23 in either autoantibody-positive or autoantibody-negative individuals for either IFN-γ or IL-10; for C10-24, they report very low reactivity too indicating that overall, they saw very low reactivity to native peptides in their study.

In the present study, through a detailed phenotypic analysis, we show that HIPs are not better than conventional islet epitopes in eliciting IFN-γ, IL-10, or IL-17 specific T cell responses in both patients with type 1 diabetes and high-risk unaffected family members. On the contrary, pro-inflammatory responses tend to be more frequent to islet epitopes when responses are measured collectively. Furthermore, in terms of epitope hierarchy, the native epitope, C13-32, elicits the most frequent IFN-γ and IL-10 responses in patients with type 1 diabetes and preclinical subjects respectively consistent with our previous studies (3, 4). This was confirmed in a subgroup analysis on children with type 1 diabetes where interferon-γ responses to all the PIPs including C13–32 were higher than those observed against HIPS despite comparable HLA-DR4 and HLA-DQ8 prevalence.

The three HIPs used here were selected based on two previous studies which described IFN-γ producing CD4 T cell clones specific for these HIPS isolated from islets of type 1 diabetes patients (8, 9).

During the course of this study, Baker et al., published data measuring IFN-γ T cell responses by ELISPOT against a series of HIPs including the three used in the present study and showed minimal reactivity: C-peptide-IAAP1 elicited IFN-γ responses in 0/35 patients and both C-peptide-IAAP2 and C-peptide NPY in 1/35 patients each (3%) (11). In contrast, we detected reactivity in 16% of subjects with type 1 diabetes for C-peptide-IAAP1; reactivities to C-peptide-IAAP2 and C-peptide NPY were also higher than those reported by Baker et al. (14% and 6% respectively). We also show frequent IL-17 T cell responses to C-peptide-IAAP1 which suggests that this epitope may be relevant in type 1 diabetes as it elicits multiple cytokine reactivity.

Baker et al. also tested responses against the native Ins75–90 peptide and report reactivity in ~10% of patients; this peptide corresponds to Ins75–92 (C19-A3) which we have used not only in the present study but extensively in the past and reported reactivity in more than 50% of subjects with type 1 diabetes (4). Due to limitations in the volume of blood available, it was beyond the scope of this study to test a wider selection of peptides presented by overlapping HLAs, but the frequencies of DR4 and DQ8 were compatible (63% and 56% respectively). Furthermore, using a single peptide concentration for stimulation does not allow us to differentiate between higher and lower affinities of the native and neoepitopes for MHC.

These variations in frequency of responses can be attributed to differences in assay design; Baker et al. stimulated their cells for 96 h prior to transfer to anti-IFN-γ antibody coated plates whereas we stimulate for 48 h, and they also report a much higher background response which could be due to differences in the serum/media used, during cell transfer to the anti-IFN-γ coated plate and the longer incubation time. The fact that cytokine responses to Pediacel were detected in all the subjects does validate the robustness of the ELISPOT assay used in the current study.

The lack of phenotypic differences in T cells responding to either native or neoepitopes is further substantiated by the Single Cell Immune-Profiling data which detects subtle differences in gene expression. Intriguingly, when focusing on pro-inflammatory cytokines, we noted that IL-32 expression was very high in patients responding to both native and neoepitopes. This ubiquitous expression could be because of culture conditions, but it is unlikely as its expression is dependent on stimulation (19). A potential role for IL-32 in type 1 diabetes

TABLE 2 | T cell clonotypes shared amongst native and neoepitopes.

Patient Set & Clonotype	Subject	TRA	TRB	Cells' native epitopes	Cells Neo-epitopes
ND01/ND02 1	ND01	CALISGGGADGLTF	CSVGSQPQHF	21	11
ND01/ND02 4	ND01	CAAINAGNNRKLIW	CAWNKKKGLTYNEQFF	5	5
ND01/ND02 5	ND01	CATDAMNSNYQLIW	CASTTGPQNEKLFF	2	7
ND01/ND02 9	ND01	CAVGTGTASKLTF	CASSTTSLSSYEQYF	4	2
ND01/ND02 12	ND01	CAVSTGGGNKLTF	CSARLGAQNTGELFF	4	3
ND01/ND02 13	ND01	CAVPRSGNTGKLIF	CASSLPGVTNYGYTF	3	4
ND01/ND02 20	ND01	CAANMYALNF	CASSPGQGANTGELFF	4	2
ND01/ND02 21	ND01	CAGIQGAQKLVF	CASSFIAGGPSDTQYF	1	1
ND01/ND02 27	ND01	CILRDSGATNKLIF	CAAGQPNTGELFF	3	3
ND01/ND02 273	ND01	CAVPDQTGANNLFF	CASSTHRVNAEAFF	1	1
ND01/ND02 276	ND01	CALMNQGGKLIF	CSVEDPDSRTDTQYF	1	1
ND01/ND02 19	ND01	CAVISNTGKLIF	CASSRGQGAEKLFF	3	2
ND01/ND02 23	ND01	CATDPWSGANSKLTF	CASGFTGGYNEQFF	3	1
ND01/ND02 230	ND01	CASSGGSYIPTF	CASSLGRGASTEAFF	1	1
ND01/ND02 234	ND01	CVARRGGGGNKLTF	CASSPGTGQETQYF	1	1
ND01/ND02 2351/3227	ND01	CAVRSNQAGTALIF	CASSAAGASSYEQYF	1	1
		CAVGGNTNAGKSTF			
		CAVRSNQAGTALIF	CASSAAGASSYEQYF		
ND01/ND02 28	ND01	CLVGDISTSGTYKYIF	CASRHARQPQHF	3	2
ND01/ND02 287	ND01	CAGSGTMNYGGSQGNLIF	CASSGQGGGNTEAFF	1	1
ND01/ND02 30	ND01	CVVTGYNNNDMRF	CAIREGYGYTF	2	3
ND01/ND02 37	ND01	CAGPDMDSNYQLIW	CASRYRGGSGRELFF	2	3
ND01/ND02 38	ND01	CAFIWGSSNTGKLIF	CASRRGQANYGYTF	2	1
ND01/ND02 48	ND01	CILARSGAGSYQLTF	CASSYFGRGTDTQYF	3	2
ND01/ND02 18	ND01	CVVNTVTGGGNKLTF	CASSLRGPYGYTF	2	2
ND01/ND02 22	ND01	CAVRIQGAQKLVF	CASSYSEVYNEQFF	2	2
ND01/ND02 26	ND01	CALSASKIIF	CASSLSRESNQPQHF	1	1
ND01/ND02 263	ND01	CALSDRPGSARQLTF	CSASPTPQVGGTEAFF	1	1
ND01/ND02 264	ND01	CALPLSKTGANNLFF	CASSSTGGYEQYF	1	1
ND01/ND02 221	ND01	CALRSGANSKLTF	CASSLSLAPSDEQFF	1	1
ND01/ND02 2297/6	ND01	CAVSDTGGFKTIF	CATSETGKYQETQYF	4	5
		CAVGPAGTGGFKTIF	CATSETGKYQETQYF		
		CAVSDTGGFKTIF			
ND01/ND02 29	ND01	CAVNPGNTPLVF	CSARDDRTAAEAFF	3	1
ND01/ND02 31/42	ND01	CAVADSNYQLIW	CASSSLTGGLYNEQFF	4	5
		CAVSYTGGGNKLTF			
		CAVSYTGGGNKLTF	CASSSLTGGLYNEQFF		
ND01/ND02 32	ND01	CAVRDGGTGGFKTIF	CASSLGAQYTGELFF	1	1
ND01/ND02 33	ND01	CAAPLKTSYDKVIF	CASSLDRGVQPQHF	2	2
ND01/ND02 335	ND01	CAVEDGGNDYKLSF	CASSAGTGRHTDTQYF	1	1
		CAVVPPFTGGGNKLTF			
ND01/ND02 349	ND01	CALSEARDAGNMLTF	CSARGQGVATSNQPQHF	1	1
ND01/ND02 319	ND01	CLVGDIRGGGYQKVTF	CSVEVDRAGTEAFF	1	1
ND01/ND02 43	ND01	CAASGWGSARQLTF	CASSYRAPGDNSPLHF	2	2
ND01/ND02 47	ND01	CALKAAGNKLTF	CASAGEHTGELFF	3	1
ND01/ND02 49	ND01	CAPRGNDYKLSF	CSAIDNTDTQYF	2	2
ND01/ND02 53	ND01	CAVDAAGNKLTF	CASSADILLREQYF	3	1
ND01/ND02 54	ND01	CAAGGATNKLIF	CASSPRALENTEAFF	1	1
ND01/ND02 57	ND01	CAVSNQAGTALIF	CASSLRGTGGYTF	1	1
ND01/ND02 58	ND01	CALSENTNAGKSTF	CSAQGGGDTEAFF	1	2
ND01/ND02 62	ND01	CALSRTGYSTLTF	CASVVLGNTEAFF	3	1
ND01/ND02 69	ND01	CAVFTGGFKTIF	CATSVRGDYNEQFF	2	2
ND01/ND02 11	ND01	CAVYHAGNMLTFT	CASSTGTGGYEQYF	1	2
ND01/ND02 113	ND01	CAGAGGSYIPTF	CASSPGGPVGNTIYF	1	1
ND01/ND02 116	ND01	CAMRKGDYKLSF	CASSAGRGSDYGYTF	1	1
		CAVLGGGGFKTIF			
ND01/ND02 117	ND01	CAASAIKYGGSQGNLIF	CASSQDTASGAYEQYF	1	1
ND01/ND02 140	ND01	CAASAALQTGANNLFF	CASSLGQGAEAFF	2	1
ND01/ND02 142	ND01	CALTSRGGFGNVLHC	CSAREGAGANVLTF	2	1
ND01/ND02 151	ND01	CAVKEVDSSYKLIF	CASSTGTGAEMNTEAFF	1	1
ND01/ND02 153	ND01	CARGNNDYKLSF	CASSATLQGGGYTF	1	1

(Continued)

TABLE 2 | Continued

Patient Set & Clonotype	Subject	TRA	TRB	Cells' native epitopes	Cells Neo-epitopes
ND01/ND02 1584/52	ND01	CILRDAFGNEKLTF CILRDAFGNEKLTF CAGHNNAGNMLTF	CSARRDLGNQPQHF CSARRDLGNQPQHF	2	1
ND01/ND02 247	ND01	CAAMNTGGFKTIF	CASSELSSGRNNEQFF	1	1
ND01/ND02 248	ND01	CAVGAGYGGATNKLIF	CASSRGVTEAFF	1	1
ND01/ND02 257	ND01	CALMNTGFQKLVF CSLRYSGAGSYQLTF	CASSFGLRQGGRVGEEYF	1	1
ND01/ND02 1878/44	ND01	CASQRGSQGNLIF CALNTGTASKLTF CALNTGTASKLTF	CASSSGLAGGLEQYF CASSSGLAGGLEQYF	1	1
ND01/ND02 190	ND01	CALRDTGGFKTIF	CASSAGTGGLFGELFF	1	1
ND01/ND02 199	ND01	CAVASNTGKLIF	CSVEDSGNTIYF	1	1
ND01/ND02 205	ND01	CALSSQGTYKYIF CAGVFMRF	CASSETGRGIEQYF	1	1
ND01/ND02 207	ND01	CATHASGGSYIPTF	CASKNQGTYGYTF	1	1
ND01/ND02 209	ND01	CAVGIAGNTPLVF	CASSPSWDFHGYTF	1	1
ND01/ND02 64	ND01	CAVPNQAGTALIF	CASSQRGTYEQYF	2	1
ND01/ND02 65	ND01	CATDTSYTGANSKLTF	CASSRILTSGNRGVTQYF	2	1
ND01/ND02 67	ND01	CAVGVSGNTPLVF	CASSVLSGGETQYF	1	2
ND01/ND02 75	ND01	CVVSDRDTGFQKLVF	CASSVAGSVSDTQYF	2	1
ND01/ND02 77	ND01	CALISGGGADGLTF		1	1
ND01/ND02 78	ND01	CVVGDFGNEKLTF	CASSLVQAYYSGNTIYF	2	1
ND01/ND02 79	ND01	CALSGGGSARQLTF	CASSSSLRRFNYGYTF	2	1
ND01/ND02 82	ND01	CAFRGTGNQFYF	CASSITGTTYEQYF	1	2
ND01/ND02 83	ND01	CAGRSGGYQKVTF	CSVERRGGDTQYF	1	2
ND01/ND02 91	ND01	CAVIPMNSGYSTLTF	CASSPRPQGQSYNEQFF	2	1
ND01/ND02 92	ND01	CAVGPWGNEKLTF	CASSLDSKPEAFF	2	1
ND01/ND02 94	ND01	CAASLGAGSYQLTF	CSARGAGGLTYEQYF	1	2
ND01/ND02 98	ND01	CAASIRSGTYKYIF	CASSLRLTGGNNEQFF	2	1
ND01/ND02 99	ND01	CAANDQTGANNLFF	CASRKAGGPYEQYF	1	2
ND01/ND02	ND02	CASSPQGGSEKLVF	CASSFFRGRNTEAFF	1	2
ND01/ND02 8	ND02	CAVYSGNTPLVF	CASKAQGPGNTIYF	3	7
ND01/ND02 81	ND02	CAVGQQGGSEKLVF	CASRSPRDRNTEAFF	2	2
ND01/ND02 194	ND02	CAASRDTGFQKLVF	CASSRTGRTDQPQHF	1	1
ND01/ND02 196	ND02	CAMREGPNDYKLSF	CSARDFLRLAGGEETQYF	1	1
ND01/ND02 200	ND02	CAVGTQGGSEKLVF	CASRSNRDRNTEAFF	1	1
ND01/ND02 362	ND02	CIVGYSTLTF	CASSLARLVAGGDNEQFF	1	1

Sequences highlighted in red correspond to those listed in the JDRF npod TCR database.

has been suggested in two recent studies describing gene upregulation in pancreatic β cells (20) and the detection of *IL32* transcripts preceding the onset of autoantibodies in type 1 diabetes (21). IL-32 has been implicated in inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) (22) where it is thought to orchestrate a panoply of other cytokines; indeed, in RA, IL-32 participates in the interplay with IL-17 in disease pathogenesis by amplifying inflammation in the synovium (23). Based on these data and previous studies on the role of IL-17 in T1D (2, 24), the elevated expression of IL-32 described in the present study warrants further investigation, and future studies will address whether IL-32 is produced by T cell stimulated with islet autoantigenic peptides. Overall, the lack of differences in gene expression and the similarity in composition of cells within clusters further substantiate that there is no difference in T cells responding to native or neoepitopes.

Finally, we were able to assess the number and profile of clonotypes present in immune response upon stimulation. Further evidence of the lack of distinction between T cells responding to native compared to neoepitopes comes from the T cell clonotype data which shows no preferential expansion of clonotypes. For this part of the study the T cells were stimulated with native peptide pools consisting of proinsulin and GAD or neoepitope peptide pools consisting of HIPs and modified GAD epitopes. There is very little sequence homology between the proinsulin and hybrid insulin peptides in the native and neoepitopes pools, whereas there is only one amino acid difference between the two GAD epitopes (deamidation and citrullination) in the two pools thus implying that the shared clonotypes must be targeting one of the GAD peptides. This is supported by the fact that clonotype 1 in patient ND03 has the TCRB chain sequence: CASSPATGGSSYNEQFF which has been previously identified in T cells stimulated with GAD (25).

Interestingly, four of the shared clonotypes in the current study have TCRB sequences found in the nPOD TCR database where they are present in the pancreatic lymph nodes and spleens of patients with type 1 diabetes. The shared TCR clonotypes between cells stimulated by the native and neoepitope pools suggest a lineage relationship between cells recognizing the two types of epitopes, although the specificity of

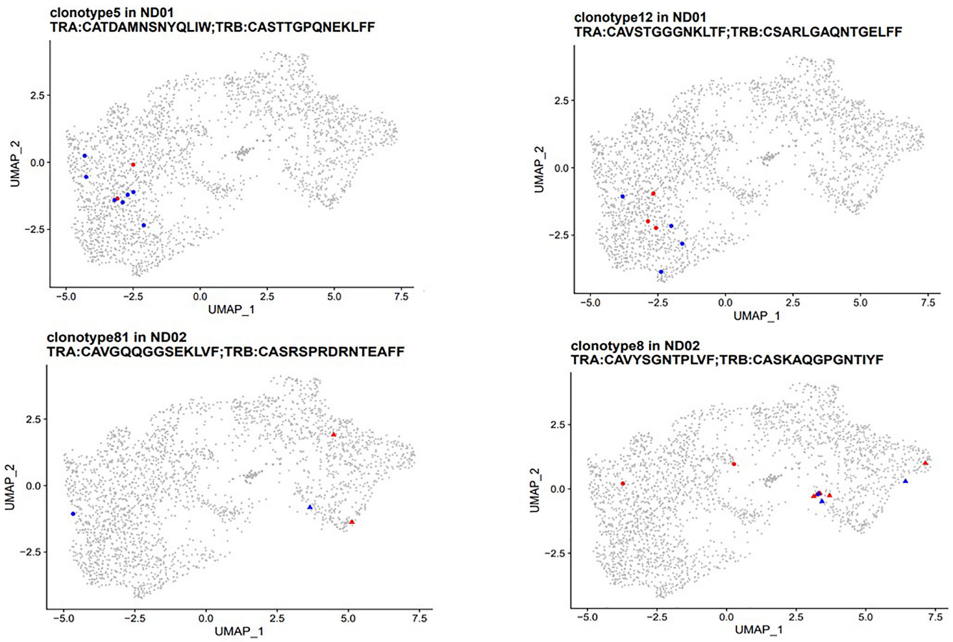

FIGURE 6 | PBMCs from subjects with type 1 diabetes were stimulated with native [proinsulin peptides: C13–32, C19-A3, C22-A5; GAD peptides: 115–25, 265–284) and neoepitopes (C-pep-IAPP1, C-pep-NPY, GAD 115–127 (120E), GAD 265–284 (272 cit)]. Antigen-responsive cells were identified by and sorted on expression of CD154/CD69. Immune profiling was conducted on single cells from which cDNA was synthesized, followed by library construction and sequencing as described in *Methods*. TCR clonotypes (both α and β chains) were examined in patients ND01 (top panel) and ND02 (bottom panel). T cells responding to native epitopes are shown in red and those responding to neoepitopes are shown in blue. Clusters are marked by point shapes (circle, triangle, and square).

these clonotypes has not been demonstrated. TCRs unique to either native or neoepitopes were also detected, but the specificity is not known. Future work will address the exact specificity of the epitope by transducing the TCR sequences into immortalized TCR−/− cell lines such as Jurkat cells and screening the relevant peptides.

We were only able to test two patients in the single Cell Immune profiling system, and our peptide panel for both native and neoepitopes had to be limited as we were constrained in the volume of blood available especially from juvenile subjects—this is a potential weakness in the study; however, the Immune Profiling data can be regarded as exploratory and can be used to guide future studies.

In summary, neoepitopes in T1D have a significant role in type 1 diabetes pathology; however, as the list of potential targets grows there is an even more pressing need to appraise and validate these epitopes and determine whether any have biomarker potential. By using a combination of phenotypic, transcriptomic, and clonotypic analyses we show that as it stands, neoepitopes are comparable to the native epitopes currently in use for immuno-monitoring studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National Research Ethics Committee, Bromley NRES Committee, reference number 08/H0805/14. Written informed consent to participate in this study was provided by the participant or the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SA designed and performed experiments, analyzed data, and wrote the manuscript. IP-A designed experiments and analyzed the data, YK, EW, NY, CD-V, YS, EP, and LK performed experiments and analyzed data. AL analyzed data and reviewed the manuscript. SA and MP designed research studies. SA and MP conceived ideas and reviewed the manuscript. SA and MP oversaw research. TT reviewed and edited the manuscript. MP is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

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Overcoming Obstacles in the Development of Antigen-Specific Immunotherapies for Type 1 Diabetes

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Antigen-specific immunotherapy (ASI) holds great promise for type 1 diabetes (T1D). Preclinical success for this approach has been demonstrated *in vivo*, however, clinical translation is still pending. Reasons explaining the slow progress to approve ASI are complex and span all stages of research and development, in both academic and industry environments. The basic four hurdles comprise a lack of translatability of pre-clinical research to human trials; an absence of robust prognostic and predictive biomarkers for therapeutic outcome; a need for a clear regulatory path addressing ASI modalities; and the limited acceptance to develop therapies intervening at the pre-symptomatic stages of disease. The core theme to address these challenges is collaboration—early, transparent, and engaged interactions between academic labs, pharmaceutical research and clinical development teams, advocacy groups, and regulatory agencies to drive a fundamental shift in how we think and treat T1D.

Keywords: T1D, autoimmunity, immunotherapy, tolerance, precision medicine

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INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by T-cell-dependent immune destruction of insulin-producing beta-cells, leading to dysregulated glucose homeostasis. T1D is triggered by complex genetic and environmental factors, progressing from asymptomatic to autoantibody-positive to overt dysglycemia. Since the 1920s, people diagnosed with T1D have had few options beyond exogenous insulin therapy. While the ever-evolving insulin formulations and pump systems can provide automated dosing and monitoring, these only treat a symptom of the disease, not the underlying pathophysiology. This is underscored by the fact that individuals with T1D still have a reduced life expectancy compared to the general population (1) and are not relieved of their disease management.

Non-antigen-specific immunotherapies, including cytokine blockade, inhibition of T-cell co-stimulation, selective immune cell depletion, and induction of polyclonal regulatory T-cells, have targeted features of T1D-related autoimmunity, not the loss of self-tolerance (2). Although these immunotherapies have improved the management of some autoimmune diseases, none have been approved for T1D. We propose that, to achieve a significant clinical impact in T1D, we need antigen-specific immunotherapies (ASI) that work in the pre-dysglycemic stage into the early insulin-requiring

period of disease, preventing and reverting overt disease manifestations. In addition, ASI might be beneficial to individuals with long-duration T1D who have residual beta-cell function (3–5). These therapies, possibly in combination with regenerative approaches, could restore glucose homeostasis by reducing the autoinflammatory pressure. ASI include various immunoregulatory formulations of proteins or peptides +/- adjuvants; plasmid-based therapies encoding multiple antigens +/- immune modulators; and antigen-presenting cell-based and engineered antigen-specific T-cell-based therapies [reviewed in (6, 7)] and some peptide, protein, and DNA-based ASI approaches have been tested in T1D [reviewed in (8)]. ASIs require a high safety profile concomitant with exquisite target/organ specificity that does not compromise host responses to pathogens or elevate cancer risks. ASIs have been challenging to develop, there has been limited clinical success, and none are approved for any autoimmune disease. The underlying challenges required to drive success are complex and involve aspects of the translatability of pre-clinical research; the lack of robust prognostic and predictive biomarkers reflecting the heterogeneity of T1D; and the hesitancy to develop treatments to intervene with the disease prior to clinical diabetes onset (**Figure 1**). Nevertheless, ASI hold such potential to transform the treatment and prevention of T1D that we believe we are at a pivotal moment to solve these challenges and progress towards solutions for prevention and cure.

KEY CHALLENGES

Translatability of Pre-Clinical Research

The identification of autoantigens driving T1D-specific B- and T-cell responses is rooted in human data, and further defined and evaluated in pre-clinical models [reviewed by (8, 9)]. However, pre-clinical studies evaluating the prognostic, diagnostic, and therapeutic relevance of T1D-associated autoantigens have often been conducted in the absence of a strong drug development context. As such, there is no consensus on the strategies required to demonstrate the efficacy of ASI. The learnings from animal models are limited; their translatability has been a challenge due to underlying MHC differences and the diversity of autoantigen responses in different models. Additionally, no animal model provides translatable insight into the many complications that occur over time in people with life-long T1D or into the long-term benefits of ASI.

Further, the development path of ASI may differ depending on the age range of the target population due to different requirements in antigens, therapeutic combinations, and administration frequencies. And finally, understanding the similarities and differences in the immune response and treatment of pediatric- versus adult-onset T1D is critical to the long-term success of ASI but challenging to model.

Lack of Robust Prognostic and Predictive Biomarkers Reflecting the Heterogeneity of T1D

Currently, islet autoantibodies are the only prognostic biomarker for T1D. Standard tests measure glutamic acid decarboxylase 65 (GAD), insulin (IAA), protein tyrosine phosphatase islet antigen-2, and zinc transporter 8-specific antibodies. Their appearance predicts that clinical disease is almost certain within one's lifetime; however, they do not predict the timing of an individual's progression to disease (10). Autoantibodies are a hallmark of T1D, regardless of the symptomatic manifestations, yet they are not causal or informative for

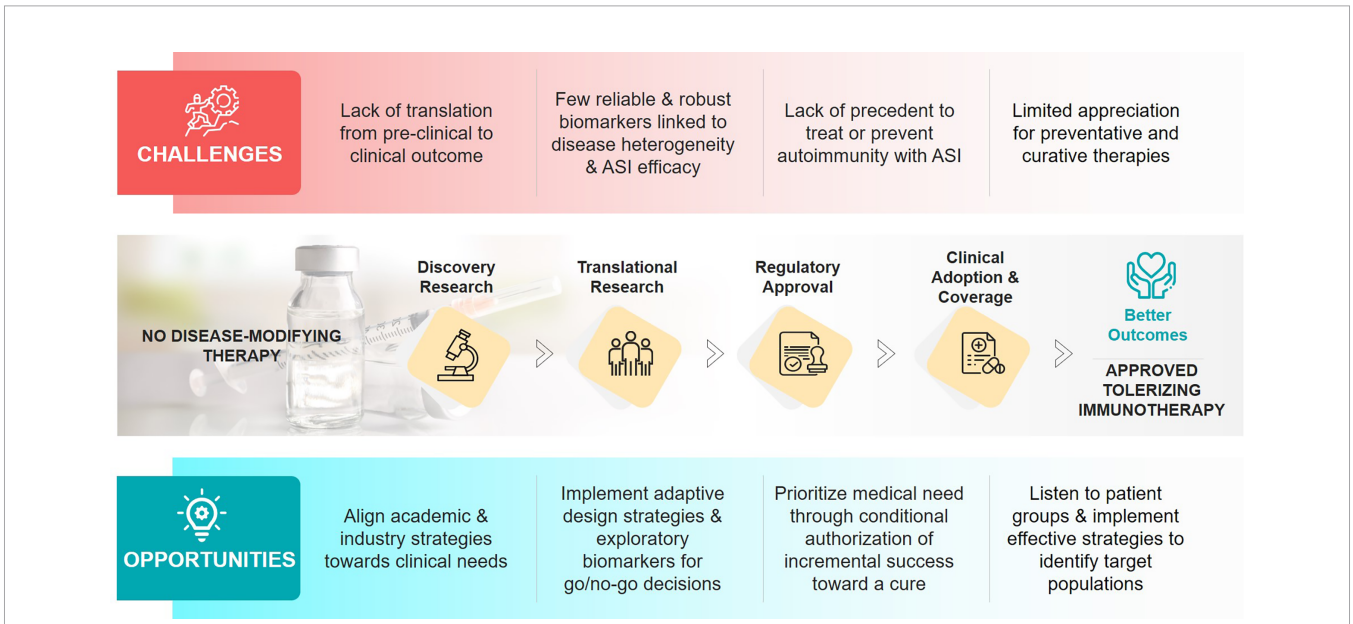

FIGURE 1 | Challenges and opportunities in the development of antigen-specific immunotherapies (ASIs) for T1D.

immediate therapeutic outcome. However, distinct T1D endotypes, driven by HLA-DRB1*03 or HLA-DRB1*04, have recently been associated with the selective appearance of GAD or IAA, respectively as the first detected autoantibody (11). These endotypes may impact the selection of tolerizing antigens and narrow patient selection for trials of peptide-based ASI, as suggested in recent trial reports (12, 13).

Autoantibody formation is preceded by pancreatic antigen-specific T-cell responses (14). However, autoreactive T-cell frequencies are highly variable in people with T1D and are also present in healthy subjects (15, 16). Though technology is rapidly advancing, especially for molecular assessment of T-cell repertoires, autoreactive T-cell-specific biomarkers suitable to predict efficacy of ASIs in T1D remain a significant roadblock. The lack of a biomarker toolkit to evaluate disease initiation and regression further challenges the design of efficient, rapid experimental studies or early clinical trials that could inform larger, phase 2 and 3 clinical trials. Although there are many reasons for this, the lack of access to the pancreas and limited data connecting peripheral immune biomarkers to beta-cell-related autoimmunity are key drivers. This challenge further hinders the identification of appropriate surrogate endpoints for clinical development of ASI in T1D (17). Ideally, predictive biomarkers for ASI should have high prognostic value for a positive clinical response outcome. For example, phenotypic changes in disease-related peripheral T cells or other surrogate markers should anticipate meaningful clinical outcomes such as reduced risk of clinical disease onset, delayed disease progression, partial or full remission, and/or reduced long-term complications (18). Without surrogate biomarkers, prevention trials may be prohibitively long (>10 years) and expensive and require that industry take on a higher risk of failure than is required with more conventional therapies. Moreover, the lack of method harmonization and of sufficient longitudinal samples to evaluate disease progression or treatment efficacy has hindered the development of a robust biomarker strategy to advance ASI for T1D.

Lack of Precedent for Treating Autoimmune Diseases With ASIs

T1D is a disease predominantly affecting children, with a notable pre-clinical asymptomatic period. The availability of life-saving insulin and more recent, novel closed loop delivery systems to treat dysglycemia has diminished the sense of urgency to find a cure or prevention for T1D. To date, no ASI approach has been approved for any autoimmune indication, and T1D is a difficult first indication in which to drive regulatory approval. ASI drug development and early phase trials are in progress in other diseases, including coeliac disease, pemphigus vulgaris, rheumatoid arthritis, and multiple sclerosis (19–21). Although it is tempting to speculate that achievements in these indications will facilitate the adoption of ASI for T1D, ASI approaches may not necessarily extrapolate from one disease to another, because antigens, disease-associated HLA, and disease mechanisms differ. Moreover, the lack of any approved disease-modifying therapy for T1D may add additional delays as there are no examples to follow.

Reluctance to Treat Prior to Overt Diabetes Onset

There are independent, notable challenges for ASIs when discussing and defining the target product profile for T1D —a key document that defines the characteristics of an innovative drug that addresses an unmet need. It seems there is agreement that intervention early in the disease carries the highest chance of success and several trials have been undertaken in people at risk of T1D, prior to overt disease (7). However, it is not broadly accepted that T1D starts earlier than the clinical diagnosis or that preventing further progression is more efficient than trying to reverse overt disease. This is compounded by the poor consensus among drug development teams regarding the desired impact of ASI on disease outcomes. For some, the goal is to permanently cure disease using tolerizing approaches in early disease stages e.g., prior to autoantibody development. For others, the goal is to establish 'functional tolerance' in pre-diagnosis (stage 2) or early onset to prevent further progression. Still others aspire to consolidate or combine ASI-induced immune tolerance with other immune-modulatory or beta-cell-preserving therapeutic strategies to quell fulminant disease. This lack of consensus is a challenge for drug developers, as this may lead to misaligned expectations of what a product might achieve in a clinical trial, with implications for trial design and outcome measures.

KEY OPPORTUNITIES

Improving Translation From Bench to Bedside

To further expand and de-risk findings from pre-clinical animal models, ASIs should be evaluated in physiologically relevant human platforms to provide greater mechanistic insight and to improve clinical translatability. With increasing access to humanized mouse models, compelling mechanism of action may be demonstrable for some ASIs, especially regarding HLA restriction of antigen and responding T cells (22). In such cases, investigational new drug (IND) applications may not need to include a demonstration of efficacy in a disease-relevant pre-clinical model (23). Furthermore, *ex vivo* tissue systems or organoids are becoming catalysts for the translation of ASIs, providing a bridge between pre-clinical and clinical studies. ASI-related researchers should take advantage of these biomimetic models whenever possible to test their therapeutic candidates. Importantly, these systems may allow assessment of drug responsiveness using representative tissue to understand disease heterogeneity. Although the availability of validated 3D systems that recapitulate *in vivo* tissue with accessible lymphatics and vascularization is not yet a reality, it is entirely plausible that the speed of technological progress will create such opportunities in the not-too-distant future.

Key learnings can be provided through small phase 0 and phase 1 clinical trials that include extensive immunologic analyses. These human studies are increasingly common (24), and may help further refine therapeutic tolerance restoration in

individuals with autoimmune disease prior to embarking on large trials. However, it is critical that such small studies are clearly presented as hypothesis- or mechanism-generating, with insufficient power to define conclusive mechanism, and not as studies evaluating efficacy, to prevent over-interpretation of study results.

Early partnering between academia and industry and across public-private consortia should become the norm to accelerate preclinical drug development by integrating core academic and industry laboratories to enable generation of strong preclinical data packages. This partnering should help validate findings in multiple orthogonal animal models, allow for confidential vetting of leads, access to GMP-grade compounds for testing, evaluation of *in vivo* mechanisms and antigens in novel *ex vivo* systems, and create public-private project teams to guide development. Such teams would include clinical experts to de-risk therapeutic candidates effectively and early. Further, pharmaceutical companies should increasingly join forces with each other, and with smaller biotech companies, to co-develop ASI programs to share the risk and financial burden during early stages of T1D drug development. These partnerships could significantly lower costs and timelines, enabling all parties to reach go/no-go decisions that allow expansion or completion of partnerships at prespecified junctures. Similarly, central topics for development, such as positions on intellectual property rights, should be discussed and agreed upon early to establish clarity for downstream activities. For real progress to occur with ASI, collaborations during the ideation and development phases of drug development programs will be critical for clinical success.

Identifying and Developing Translational Biomarkers

The need for close collaboration between academic- and industry-based groups extends to the identification and development of ASI-relevant biomarkers. These partnerships should be initiated early in the pre-clinical phase, when ASI candidates and possible predictive biomarkers are being evaluated in animal studies and biomimetic *ex vivo* model systems. With recent technological advances, such as high dimensional mass cytometry, single-cell RNA sequencing, and T-cell receptor profiling, there are increasing numbers of composite measures that hold promise as biomarkers of progression or therapeutic effect [reviewed in (25)]. For example, detailed post-hoc analyses of peripheral immune cell samples from teplizumab and alefacept trials revealed a common "partial exhaustion" transcriptomic signature of antigen-specific $CD8^+$ T-cells (26, 27). Other recent studies have identified T-cell biomarkers associated with favorable prognosis close to T1D onset (28–30). Building on these tangible advancements, promising biomarkers and methods could be optimized for costs and feasibility to be applied in large, multi-center studies and validated for clinical trial use.

Much of the ongoing biomarker identification and development work for T1D has been done by academic consortia, like the Immune Tolerance Network and TrialNet, with access to relevant patient samples and a critical mass of expertise to establish clearer assay standards and procedures. This effort should be further enhanced through expanded, early collaboration involving pharmaceutical and biotechnology company partners. Increased early-stage public-private partnerships will help drive the validation and publication of robust and feasible biomarkers and assays that are subjected to industry-level rigor to refine their context of use, clarify their limitations, and to facilitate their inclusion in clinical trials in a harmonized and standardized fashion. Early successes will then enable testing of larger panels of biomarkers for broader use, such as in patient selection or stratification, that will be critical for future ASI development programs.

Clinical Trial and Approval Path for ASI in T1D

ASI development programs will strongly benefit from early identification of regulatory challenges and development of mitigation strategies. Consultation with clinicians, trial design experts, and statisticians should begin during late preclinical activities, and feedback from regulatory agencies must be solicited as early as possible to understand their requirements (e.g., trial design elements like sample size, outcome measures, primary and secondary endpoints, and safety outputs). These early interactions can also be an opportunity to inform regulatory colleagues on the disruptive potential of ASI to prevent or cure T1D. Importantly, clinical outcomes of ASI that extend beyond delaying onset and preserving functional beta cell mass (e.g., reduced hypoglycemic events, reduction in insulin needs, and acute or long-term complications), as well as other quality-of-life measures for the patient and their caregivers, should be discussed and captured comprehensively during clinical studies and/or in post-marketing efforts. Communication of cohesive, consistent messages from academic and industry stakeholders to regulators on the rationale and strategy guiding ASI development from preclinical to clinical testing, can be a vital catalyst for progress in this field.

Consumers (i.e., people who have or are at risk of T1D, caregivers, and clinicians) should be educated on the potential of ASIs to induce permanent change in the disease processes. A more deliberate inclusion of consumer stakeholders would be a welcome addition to academic and commercial discovery and development teams. This would involve their inclusion in grant applications, in preclinical development teams during IND package generation, within clinical study design discussions, and during evaluation of clinical study results.

Further, implementation of innovative trial designs for ASI can rapidly accelerate progress. This need is underscored by the collective outcomes from traditional disease-modifying monotherapy trials conducted in T1D to date, and by consistent feedback from clinical trial sponsors and investigators.

Moving to Curative and Prevention Therapies

ASI have the potential to provide solutions for multiple stages of T1D. ASI could be highly efficacious in early phases of disease,

when there is limited autoimmunity, but ASI may also benefit people with long-term disease, especially those with residual beta-cell function. The ASI-mediated reduction of immune and metabolic stress on the beta cells could be sufficient and/or crucial, especially in combination with regenerative approaches, to restore quiescent beta-cell function. Applying the disease staging concepts of T1D could facilitate the identification of subjects who would benefit from immunotherapies intended to delay onset of or reverse T1D and to create a meaningful population health outcome (31). At-risk subjects have been successfully identified and enrolled in trials through clinical T1D consortia, such as TEDDY, TrialNet, ADDRESS, and INNODIA. Now, there is both a need and opportunity to identify large and diverse cohorts to participate in T1D trials. In fact, two general population screening programs have launched recently, one in a research setting (PLEDGE, https://research.sanfordhealth.org/fields-of-research/diabetes/pledge) and another at a patient community-level (T1Detect, https://www.jdrf.org/t1d-resources/t1detect/). Hopefully, they will pave the way for identifying at-risk subjects appropriate for clinical testing with disease-modifying therapies, including ASI.

ASI intervention should not be considered as a binary success-or-failure option in which accomplishments are compared to "one-and-done" therapies. The induction and maintenance of peripheral tolerance to pancreatic antigens may require regular therapeutic boosting in affected individuals. These could be very well received, provided they are safe, disease-specific, and patient friendly, e.g., they are administered sparsely and/or require non-invasive delivery options. Patient-focused organizations could increase discourse and information exchange amongst diverse stakeholders of the ASI development ecosystem by organizing workshops and interactive series that are designed to incentivize and facilitate drug development efforts. Such interactions could accelerate progress across the entire T1D immunotherapy field.

DISCUSSION

The abundance and speed of research witnessed during 2020 to fight COVID-19 forces us to consider whether comparable efforts could be achieved to effectively eliminate T1D. No specific remedies to fight SARS-CoV-2 infection existed before the December 2019 outbreak in Wuhan. However, the pandemic triggered an unprecedented worldwide effort to develop medicines to stop, treat, and prevent this infection. Immediate approaches aimed at repurposing existing drugs; then newly customized therapies, e.g., neutralizing monoclonal antibodies, proved more successful and, remarkably, SARS-CoV-2-specific vaccines were developed in record time (32). Interestingly, to date, there are >15 different COVID-19 vaccines authorized in different parts of the world including a few innovative options using completely novel technology platforms that could revolutionize vaccine development. The take-home message is that it is possible to develop innovative and efficacious solutions in a very short time.

The therapeutic options for T1D have seen minimal advancement in the past century, and the advances in the field have been restricted to incremental modifications of insulin formulations and delivery methods. Complementary to these treatments, the scientific community has explored a variety of "repurposing" options, mostly based on the use of non-specific immune modulators that have shown limited efficacy in clinical studies to-date (33). As in the case of COVID-19, it is time to understand the huge medical need associated with T1D and to make a concerted effort to develop curative solutions directed at the root cause of the disease: a breach in immune tolerance to pancreatic antigens. ASIs hold such potential and, like the new vaccines developed against SARS-CoV-2 infection, they promise a long-awaited transformative solution for the treatment and prevention of T1D.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

RT, JC, JW, and SA all contributed equally to the ideation, writing, and reviewing of the manuscript. All authors contributed to the article and approved the submitted version.

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Figure 1 was conceived with inspiration from JDRF's mission statement and the strategic goals of the JDRF Disease-Modifying Therapies project area, which SA helped develop during her tenure at the Foundation.

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Conflict of Interest: JC is an employee of Novartis. JW is an employee of Novo Nordisk. RT has filed provisional patents surrounding technology and is commercializing immunotherapy for targeting DCs for antigen-specific tolerance.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Oxidative Stress Leads to β-Cell Dysfunction Through Loss of β-Cell Identity

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Pancreatic β-cell failure is a critical event in the onset of both main types of diabetes mellitus but underlying mechanisms are not fully understood. β-cells have low anti-oxidant capacity, making them more susceptible to oxidative stress. In type 1 diabetes (T1D), reactive oxygen species (ROS) are associated with pro-inflammatory conditions at the onset of the disease. Here, we investigated the effects of hydrogen peroxide-induced oxidative stress on human β-cells. We show that primary human β-cell function is decreased. This reduced function is associated with an ER stress response and the shuttling of FOXO1 to the nucleus. Furthermore, oxidative stress leads to loss of β-cell maturity genes MAFA and PDX1, and to a concomitant increase in progenitor marker expression of SOX9 and HES1. Overall, we propose that oxidative stress-induced β-cell failure may result from partial dedifferentiation. Targeting antioxidant mechanisms may preserve functional β-cell mass in early stages of development of T1D.

Keywords: oxidative stress, beta-cell dysfunction, beta-cell identity, beta-cell dedifferentiation, type 1 diabetes mellitus (T1D)

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INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease caused by T cell-mediated destruction of pancreatic insulin-producing β-cells (1–3). Autoimmune recognition of β-cell antigens leads to decreased β-cell mass and the subsequent decline of insulin-mediated regulation of glucose levels in the blood eventually results in chronic hyperglycemia and T1D.

Oxidative stress has been implicated in the onset of β-cell failure in T1D (4–6). Plasma levels of oxidative stress markers such as malondialdehyde and protein carbonyl groups are increased upon early onset of T1D and are even higher by early adulthood (7). Oxidative stress occurs when reactive oxygen species (ROS) levels overcome antioxidant defenses. In physiological conditions, ROS, a by-product of mitochondrial metabolism, act as signaling molecules for glucose-stimulated insulin secretion in β-cells. β-cells have been shown to be particularly sensitive to oxidative stress when compared to other islet cell types, as they display low levels of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase (8–10).

In recent years, loss of β-cell identity has been proposed as a new mechanism underlying the β-cell failure that is central to the onset and development of diabetes mellitus (11). Alterations in β-cell identity impact their functionality, as indicated by a decreased expression of key β-cell markers such

as MAFA, and genes involved in glucose-stimulated insulin secretion such as the glucose transporter Slc2a2 (GLUT2) (12). The concept of β-cell identity loss is strongly supported by experiments performed in murine models and based on lineage-tracing of β-cells. Most of these *in vitro* and *in vivo* experiments involve genetic manipulations (13). Forced deletion of the β-cell-specific transcription factor FOXO1 converts adult murine β-cells into cells with an α-cell, δ-cell or pp-cell phenotype (11). The loss of PDX1, NKX6.1, PAX6 or NKX2.2 leads to β-cells gaining α-cell (14), δ-cell (15), ϵ-cell (16) or polyhormonal cell (17) characteristics, respectively. Additionally, inducing hyperglycemia in mice triggers β-cells to start expressing glucagon (18) or the non-endocrine peptide hormone gastrin (19). Hyperglycemia also leads to β-cell dedifferentiation in mice, as shown by the loss of key genes responsible for β-cell identity and function (11, 16). Data on human β-cell identity loss, on the other hand, remain scarce and mainly descriptive, based on histological analyses performed on donor-derived pancreatic tissue sections. In samples from T1D individuals, there is an increased frequency of hormone-negative endocrine cells (that express none of the islet hormones but that do express the endocrine marker chromogranin A) (20). We reported an eight times increased frequency of insulin-positive cells co-expressing glucagon and a five times increased frequency of NKX6.1-positive, insulin-negative cells co-expressing glucagon in donors with type 2 diabetes compared to the control group (21). We also found an increased proportion of α- and β-cells expressing the mesenchymal protein vimentin in islets from T2D individuals, indicating phenotypic plasticity in the form of an epithelial-to-mesenchymal transition (22). Furthermore, pancreatic sections of T2D donors contain insulin-depleted, degranulated β-cells (23) and show a higher portion of endocrine cells expressing none of the typical endocrine cell markers insulin, glucagon, somatostatin or pancreatic polypeptide (24). Although the concept of β-cell identity loss in diabetes is gaining ground, its underlying mechanisms remain unclear.

Here, we investigate the effects of hydrogen peroxide-induced oxidative stress on human β-cell function and identity to characterize the underlying molecular mechanisms of β-cell failure in diabetes.

MATERIALS AND METHODS

Primary Human Islets and Human β-Cell Line

Pancreata were obtained from cadaveric human organ donors. Human islet isolations from 16 non-diabetic donors (see **Supplemental Table 1**) were performed in the Good Manufacturing Practice facility of our institute (25). Islets were used for research only if they could not be used for clinical purposes and if research consent was present, according to Dutch national laws. Islets with a purity of at least 80% were cultured in regular CMRL 1066 medium (5.5 mmol/L glucose) containing 10% fetal calf serum, 20 mg/mL ciprofloxacin, 50 mg/mL gentamycin, 2 mmol/L L-glutamin, 10 mmol/L HEPES, and 1.2 mg/mL nicotinamide. Islets were maintained in culture at 37°C in 5% CO_2-humidified atmosphere and medium was refreshed the day after isolation and every two days thereafter.

EndoC-βH1 cells (26) were obtained from Univercell Biosolutions and were cultured in low glucose DMEM supplemented with 5.5 μg/ml human transferrin, 10 mM nicotinamide, 6.7 ng/ml selenit, 2% BSA fraction V, 100 units/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol. Cells were seeded in pre-coated culture plates containing ECM with fibronectin, maintained in culture at 37°C in 5% CO_2-humidified atmosphere and passaged once a week.

Hydrogen Peroxide Treatment

Hydrogen peroxide (H_2O_2, Sigma) was diluted in culture medium to prepare a stock solution of 10 M. The stock solution was then further diluted in culture medium to obtain the final concentrations. Human islets or EndoC-βH1 cells were treated with either 50 μM H_2O_2 for 24 hours or 200 μM H_2O_2 for 90 minutes (with a washing-out period of 22.5 hours afterwards) with read-outs performed 24 hours after the start of the treatment with H_2O_2, unless stated otherwise in the figure legends. A schematic overview of the experimental setup is shown in Supplementary **Figure 1A**. For inhibitory experiments, human islets or EndoC-βH1 cells were pre-incubated overnight with tauroursodeoxycholic acid (TUDCA, Millipore) prior to the H_2O_2 treatments as shown in **Supplementary Figure 1A**, as well as co-incubated together with H_2O_2 for the duration of the treatments. TUDCA was dissolved in water to prepare a stock solution, which was then further diluted in culture medium to obtain the final concentration of 1 mM.

FDA/PI Staining

An FDA/PI staining was performed to assess the viability of human islets or EndoC-βH1 cells after H_2O_2 treatment. A staining solution was prepared by diluting an FDA stock solution (5 mg/mL, consisting of fluorescein diacetate (Sigma) and aceton) and a PI stock solution (2 mg/mL, consisting of propidium iodide (Sigma) and PBS) in culture medium without fetal calf serum. Human islets or EndoC-βH1 cells were washed once with PBS and staining solution was added for approximately 5 minutes in the dark at room temperature. Thereafter, human islets or EndoC-βH1 cells were washed with PBS and imaging was done using the EVOS (Invitrogen).

Glucose-Stimulated Insulin Secretion (GSIS)

Approximately 50 IEQ per well were placed in a 96-well transwell plate. Islets were preincubated for 90 minutes in low (1.67 mmol/L) glucose-containing KRBH (Krebs-Ringer Bicarbonate Hepes) buffer (11.5 mmol/L NaCl, 0.5 mmol/L KCl, 2.4 mmol/L $NaHCO_3$, 2.2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 20 mmol/L HEPES, and 0.2% human serum albumin) at pH 7.4. Islets were subsequently transferred to low (1.67

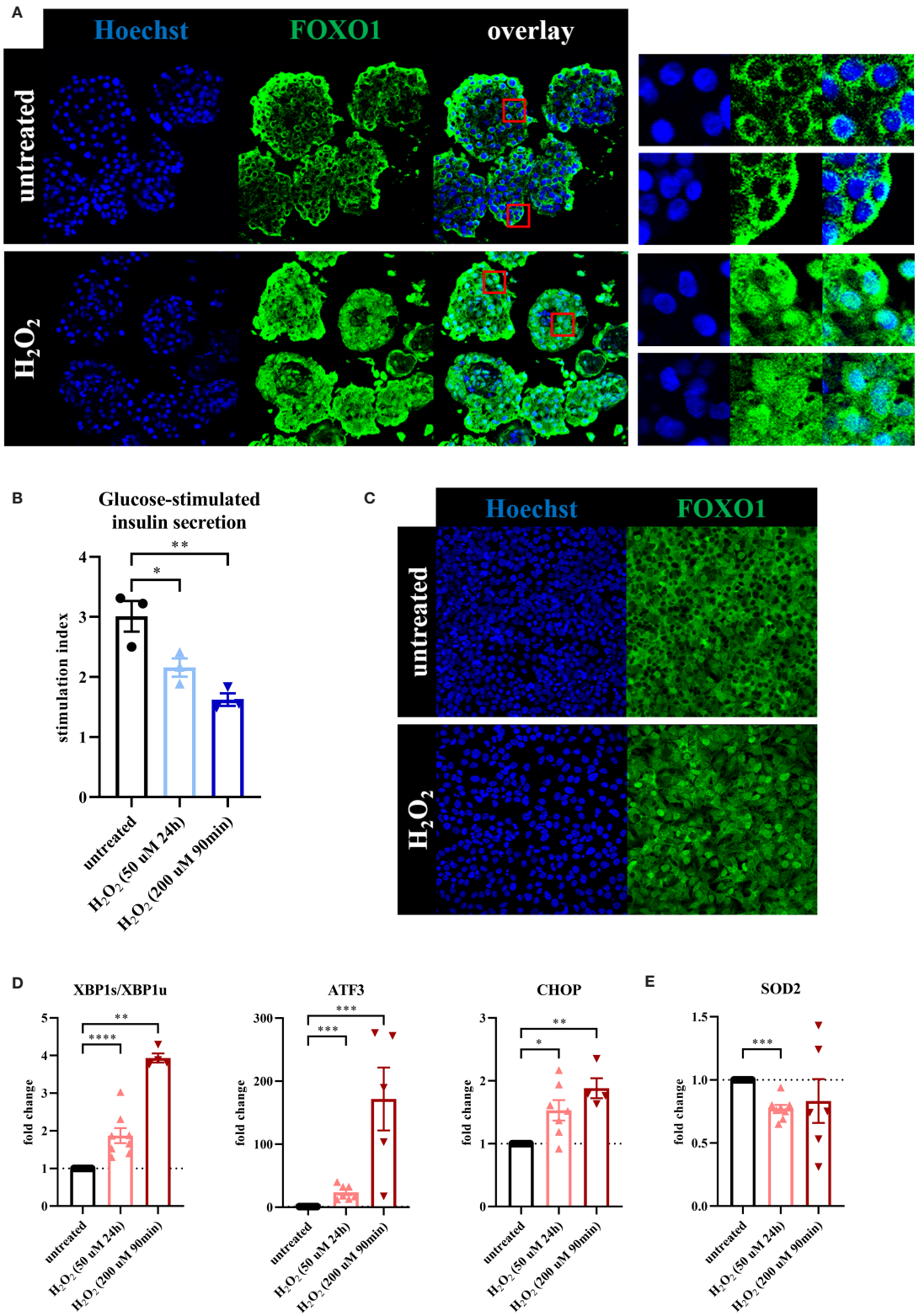

FIGURE 1 | Continued

FIGURE 1 | Oxidative stress leads to loss of β-cell function associated with a β-cell stress response. To evaluate the effect of oxidative stress on β-cell function and stress response, primary human islets (blue graphs) or EndoC-βH1 cells (red graphs) were treated with hydrogen peroxide (H_2O_2). **(A)** Treatment of human islets with 100 μM H_2O_2 for 90 minutes, assessed after 72 hours, leads to shuttling of the transcription factor FOXO1 from its normal cytoplasmic localization to the nucleus, as seen in the magnifications in the panels on the right. Nuclear FOXO1 translocation (as assessed by the overlap of FOXO1 and Hoechst staining) occurred in 94% of cells after H_2O_2 treatment, compared to 5% of cells in the untreated condition. **(B)** H_2O_2-induced oxidative stress in human islets leads to decreased glucose-stimulated insulin secretion. GSIS was performed 24 hours after the start of the 90min 200 μM H_2O_2 treatment or immediately after the 24h 50 μM H_2O_2 treatment. **(C)** Daily treatment of EndoC-βH1 cells with 100 μM H_2O_2 for 90 minutes, assessed after 72 hours, leads to shuttling of the transcription factor FOXO1 from its normal cytoplasmic localization to the nucleus. **(D)** Oxidative stress induction in EndoC-βH1 cells by treatment with 50 μM H_2O_2 for 24 hours and 200 μM H_2O_2 for 90 minutes leads to increased mRNA expression levels of the unfolded protein response-related genes XBP1s/XBP1u, ATF3 and CHOP as measured by qPCR. **(E)** Oxidative stress induction in EndoC-βH1 cells by treatment with 50 μM H_2O_2 for 24 hours leads to a decreased mRNA expression level of the mitochondrial enzyme SOD2 as measured by qPCR. Data are presented as means ± SEM of fold change over untreated control islets (blue graphs) or EndoC-βH1 cells (red graphs). n = 3-8 donors/batches; each data point represents one donor/batch. *p < 0.05, **p < 0.01, ***p < 0.0005, ****p < 0.0001 *vs.* untreated control islets/EndoC-βH1 cells as determined by an unpaired Student's t test on the stimulation indices (for the glucose-stimulated insulin secretion data) or a paired Student's t test on the dCT values (for the qPCR data). Black circles = untreated control islets/EndoC-βH1 cells, upward-pointing triangles = H_2O_2-treated islets/EndoC-βH1 cells (50 μM 24h), downward-pointing triangles = H_2O_2-treated islets/EndoC-βH1 cells (200 μM 90min). h = hours, min = minutes, H_2O_2 = hydrogen peroxide.

mmol/L) glucose-containing buffer for 1 hour and then to high (17 mmol/L) glucose-containing buffer for 1 hour. Insulin secretion was assessed using a human insulin ELISA kit (Mercodia) following the manufacturer's instructions. In addition, insulin content of the lysed human islets that were used for the glucose-stimulated insulin secretion assay was measured using the same human insulin ELISA kit. To determine basal insulin secretion levels, the insulin secretion was corrected for the amount of DNA present in the lysed human islets that were used for the glucose-stimulated insulin secretion assay. DNA content was measured using a Quant-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific) following the manufacturer's instructions.

Immunofluorescence Microscopy

Human islets and EndoC-βH1 cells were fixed with 4% paraformaldehyde for 10 minutes, permeabilized using 0.5% triton-X for 15 minutes and blocked using goat serum for 15 minutes. Primary and secondary antibodies (Alexa Fluor, Thermo Fisher Scientific) diluted in buffer containing 5% bovine serum albumin were sequentially incubated for 1 hour. After counterstaining with Hoechst (BD), samples were mounted using DABCO-glycerol on microscopy slides and confocal imaging was done using the SP8 WLL (Leica). A primary antibody against FOXO1 (Cell Signaling Technology, 2880) was used. Manual counting was performed using ImageJ to assess the overlap of nuclear FOXO1 and Hoechst.

RNA Isolation and Quantitative PCR

Human islets or EndoC-βH1 cells were washed in PBS. Total RNA was extracted using (Micro) RNeasy kit (Qiagen) according to the manufacturer's instructions and the concentration was determined using NanoDrop (Thermo Fisher Scientific). Total RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen) and oligo(dT). Quantitative PCR was performed in a CFX system (Bio-Rad). Fold change was calculated using the delta delta CT method with human β-actin or GAPDH as reference gene. Primers used are listed in **Supplemental Table 2**.

Western Blot

Human islets were washed with cold PBS and lysed using Laemmli sample buffer (60 mmol/l tris pH 6.8, 10% glycerol, 1% SDS, 0.001% blue bromophenol and 5% β-mercaptoethanol). Protein content in the supernatant was quantified using a BCA assay and immunoblotted with antibodies MAFA (Bethyl, A700-067), PDX1 (Abcam, AB47267) and SOX9 (Cell Signaling Technology, 82630S). HRP-conjugated secondary antibodies were used and the signal was developed using enhanced chemiluminescent substrate. The bands were quantified using ImageLab software (BioRad).

Statistical Analysis

All data are expressed as means ± SEM, unless stated otherwise. For analysis of qPCR data, statistical significance of differences between two groups was determined by a paired or unpaired Student's t test on the delta CT values calculated from the reference gene (β-actin or GAPDH) and the gene in question. A *P* value below 0.05 was considered statistically significant.

RESULTS

Oxidative Stress Leads to Loss of β-Cell Function Associated With a β-Cell Stress Response

To assess the effect of oxidative stress on human β-cell function, we treated primary human islets with 50 μM H_2O_2 for 24 hours, or with 200 μM H_2O_2 for 90 minutes followed by a wash-out period of 22.5 hours (**Supplementary Figure 1A**). These conditions were chosen because they have been shown to induce an oxidative stress response in pancreatic β-cells with limited cytotoxicity (27–29) and the objective of this study was to investigate the identity of the cells surviving oxidative stress. We monitored the potential toxicity of the H_2O_2 treatments on primary human islets with an FDA/PI staining performed at the end of the treatments (**Supplementary Figure 1B**) and relative RNA content was determined as indirect measurement of cell number (**Supplementary Figure 1C**). Although we found a decrease in relative RNA content in the 200 μM H_2O_2 condition in particular, we also confirmed a high viability of the remaining cells at t=24h, the main endpoint in this study.

We validated the effect of H_2O_2 in human islets by showing a shuttling of the transcription factor FOXO1 from its normal

cytoplasmic localization to the nucleus upon H_2O_2 treatment (**Figure 1A**), a known adaptation response to oxidative stress (30). Nuclear FOXO1 translocation occurred in 94% of cells after H_2O_2 treatment, compared to 5% of cells in the untreated condition. Furthermore, glucose-stimulated insulin secretion (GSIS) displayed a 30% reduction in the 50 μM H_2O_2 condition and a 45% decrease after treatment with the 200 μM H_2O_2 condition, as compared to the untreated control condition (**Figure 1B**), indicating impaired β-cell function. Basal insulin secretion of the islets that were used for the GSIS assay was not significantly altered after both H_2O_2 treatments (**Supplementary Figure 1D**).

In order to evaluate the effect of oxidative stress on β-cells specifically, we used the human β-cell line EndoC-βH1 as a model. H_2O_2-induced oxidative stress induced some significant cell death on these cells as reflected by reduced relative RNA content at the end of both treatments (**Supplementary Figure 1F**). Yet, as for human islets, we found a high viability of the remaining cells at t=24h (**Supplementary Figure 1E**). We also observed a shuttling of FOXO1 from the cytoplasm to the nucleus (**Figure 1C**). Furthermore, we found an increased expression of the typical ER stress markers XBP1s/XBP1u, ATF3 and CHOP (**Figure 1D**) that correlated with a reduced expression of the mitochondrial enzyme SOD2 (manganese superoxide dismutase) (**Figure 1E**), which may indicate a failure in the adaptive mechanism to oxidative stress.

Collectively, these data indicate that oxidative stress leads to reduced β-cell function associated with a β-cell stress response.

Oxidative Stress Leads to Loss of β-Cell Maturity Markers and a Concomitant Increase in Progenitor Marker Expression

We next hypothesized that oxidative stress could alter β-cell identity, as a potential mechanism allowing these cells to survive. We first determined the effect of oxidative stress induction on key β-cell genes in primary human islets. At 24 hours after the start of a 90min treatment with H_2O_2, gene expression of the maturity marker MAFA was strongly decreased, both at mRNA (**Figure 2A**) and protein level (**Figure 2B**). Similarly, gene and protein expression of the key transcription factor PDX1 was reduced by over 60% (**Figures 2A, B**). A similar trend was observed 24h after treatment with 50 μM H_2O_2 (**Figure 2A**), as well as for the gene expression of insulin, of the regulator of β-cell fate PAX4 and of the key β-cell transcription factor NKX6.1 (**Figure 2A**). Other β-cell-related genes that were noticeably decreased after treatment with 200 μM H_2O_2 for 90 minutes, were KIR6.2, MAFB, FOXA2, PAX6, NKX2.2 and NEUROD1 (**Figure 2A**). Gene expression of the glucose transporter GLUT1, which plays an important role in β-cell glucose metabolism, was also decreased after treatment with 200 μM H_2O_2 for 90 minutes, partly explaining the impaired function shown in **Figure 1A**.

We confirmed these findings in EndoC-βH1 cells, in which gene expression of MAFA, PDX1, PAX4, NKX6.1, insulin, KIR6.2, MAFB, FOXA2, PAX6, NKX2.2, NEUROD1 and GLUT1 was reduced upon H_2O_2 treatment (**Figure 2C**). Reduced MAFA and PDX1 expression was also validated at protein level (**Figure 2D**).

Next, we assessed the effect of oxidative stress on endocrine progenitor markers in β-cells. Strikingly, gene expression of SOX9 and HES1 was increased by 50% and 60% after 24h treatment with 50 μM H_2O_2, and up to 8- and 6-fold increased after 90min treatment with 200 μM H_2O_2, respectively (**Figure 2E**). The increased SOX9 gene expression in EndoC-βH1 cells was confirmed on the protein level in primary human islets (**Figure 2F**).

Altogether, these data show that oxidative stress results in severe alterations in β-cell maturity marker expression, associated with increased expression of progenitor markers, indicating β-cell dedifferentiation as a response to oxidative stress.

TUDCA Partially Inhibits Oxidative Stress-Induced Detrimental Effects

Finally, we hypothesized that oxidative stress-induced detrimental effects on β-cells were partly resulting from ER stress. We targeted the ER stress response by using tauroursodeoxycholic acid (TUDCA), a chemical chaperone that is known to attenuate ER stress and prevent unfolded protein dysfunction (31, 32). Primary human islets were pre-incubated overnight with 1 mM TUDCA prior to the H_2O_2 treatments, as well as co-incubated together with H_2O_2 for the duration of the treatments. As expected, oxidative stress reduced gene expression of MAFA, MAFB, KIR6.2, PAX6, NKX2.2, NEUROD1 and GLUT1 (**Figure 3A**). Interestingly, this effect was partially or completely prevented by TUDCA treatment (**Figure 3A**). This was confirmed on the protein level for MAFA (**Figure 3B**).

Collectively, these findings are in line with our proposed model that oxidative stress-induced ER stress is a key factor in altered β-cell identity, and therefore function.

DISCUSSION

Our study demonstrates that oxidative stress leads to loss of β-cell function that is associated with a stress response and evidence of dedifferentiation, as indicated by the loss of β-cell maturity markers and the upregulation of endocrine progenitor markers.

Our finding that β-cell function is decreased upon oxidative stress is supported by studies performed in animal β-cell lines, where the decreased insulin gene expression that follows supraphysiologic concentrations of glucose is prevented by antioxidant treatment, suggesting the involvement of oxidative stress in β-cell failure (33).

We observed that reduced human β-cell function is associated with shuttling of FOXO1 to the nucleus, which has been shown to protect murine β-cells from oxidative stress by increasing the expression of NeuroD and MAFA (34). On the other hand, we found the gene expression of the mitochondrial enzyme SOD2 to be downregulated upon H_2O_2 treatment. Since SOD2 functions as an antioxidant, its reduced expression could lead to excessive

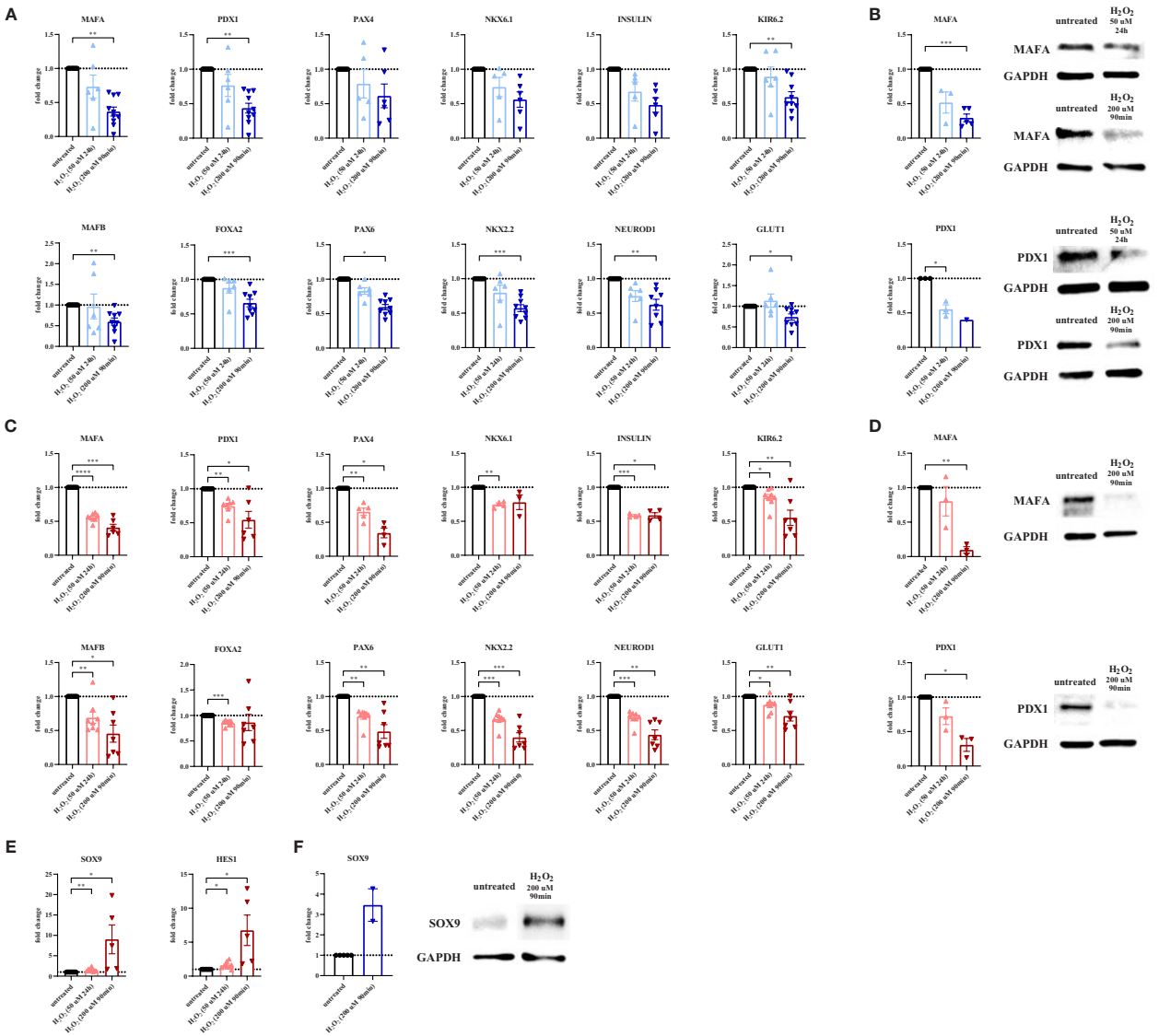

FIGURE 2 | Oxidative stress leads to loss of β-cell maturity markers and a concomitant increase in progenitor marker expression. To evaluate the effect of oxidative stress on β-cell maturity markers and progenitor markers, primary human islets (blue graphs) or EndoC-βH1 cells (red graphs) were treated with hydrogen peroxide (H_2O_2). **(A)** Oxidative stress induction in human islets by treatment with 200 μM H_2O_2 for 90 minutes leads to decreased mRNA expression levels of the β-cell-specific genes MAFA, PDX1, KIR6.2, MAFB, FOXA2, PAX6, NKX2.2, NEUROD1 and GLUT1 as measured by qPCR. **(B)** The level of β-cell-specific protein MAFA is decreased in human islets upon treatment with 200 μM H_2O_2 for 90 minutes as measured by Western blot. **(C)** Oxidative stress induction in EndoC-βH1 cells by treatment with 50 μM H_2O_2 for 24 hours and 200 μM H_2O_2 for 90 minutes leads to decreased mRNA expression levels of the β-cell-specific genes MAFA, PDX1, PAX4, NKX6.1, insulin, KIR6.2, MAFB, FOXA2, PAX6, NKX2.2, NEUROD1 and GLUT1 as measured by qPCR. **(D)** The level of β-cell-specific proteins MAFA and PDX1 is decreased in EndoC-βH1 cells upon treatment with 200 μM H_2O_2 for 90 minutes as measured by Western blot. **(E)** Oxidative stress induction in EndoC-βH1 cells by treatment with 50 μM H_2O_2 for 24 hours and 200 μM H_2O_2 for 90 minutes leads to increased mRNA expression levels of the progenitor cell-specific genes SOX9 and HES1 as measured by qPCR. **(F)** The level of progenitor cell-specific protein SOX9 is increased in human islets upon treatment with 200 μM H_2O_2 for 90 minutes as measured by Western blot. Data are presented as means ± SEM of fold change over untreated control islets (blue graphs) or EndoC-βH1 cells (red graphs). n=1-10 donors/batches; each data point represents one donor/batch. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0005$, ****$p < 0.0001$ *vs.* untreated control islets/EndoC-βH1 cells as determined by a paired Student's t test on the dCT values. Black circles = untreated control islets/EndoC-βH1 cells, upward-pointing triangles = H_2O_2-treated islets/EndoC-βH1 cells (50 μM 24h), downward-pointing triangles = H_2O_2-treated islets/EndoC-βH1 cells (200 μM 90min). h = hours, min = minutes, H_2O_2 = hydrogen peroxide.

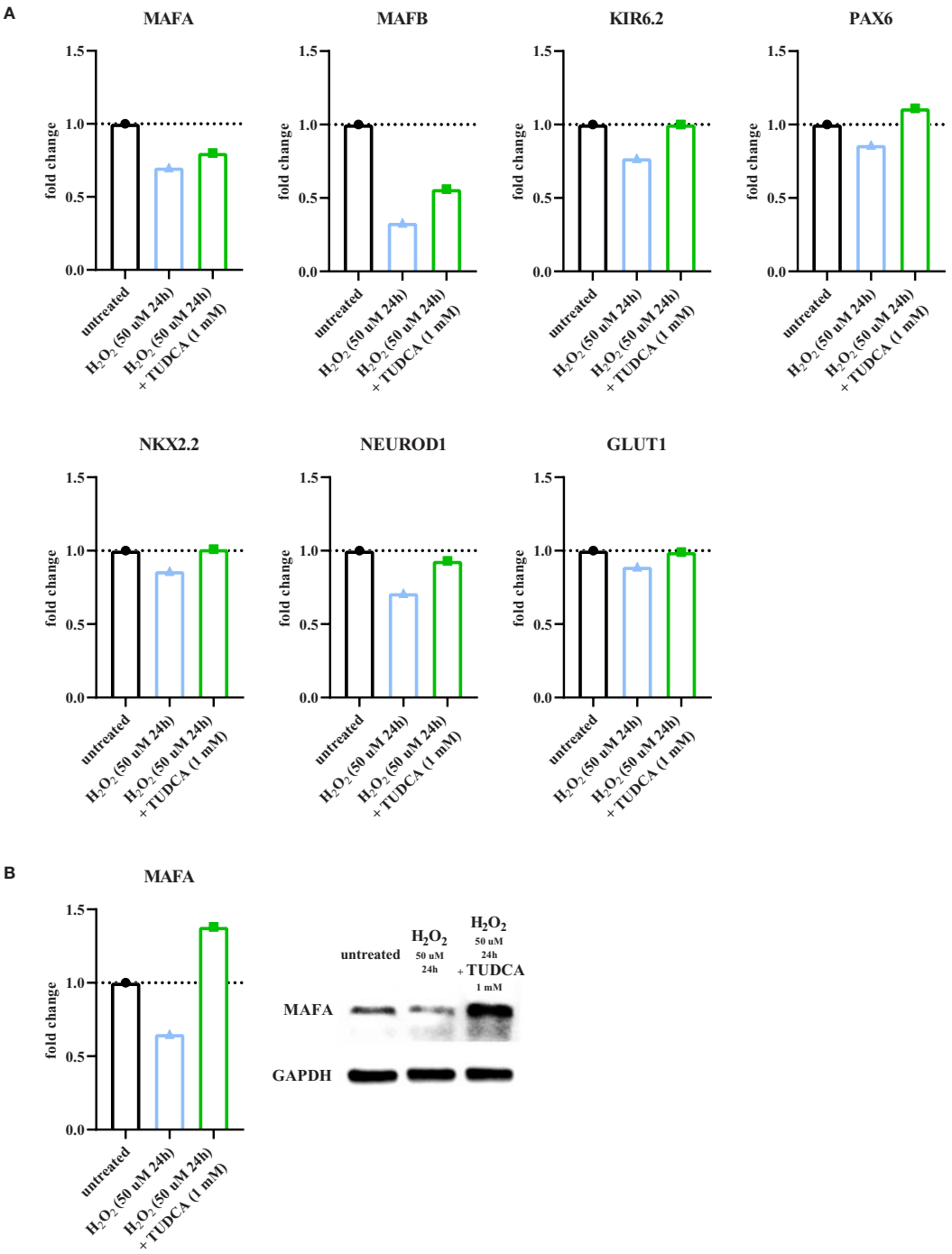

FIGURE 3 | TUDCA partially prevents oxidative stress-induced detrimental effects. To evaluate the effect of tauroursodeoxycholic acid (TUDCA) on oxidative stress-induced detrimental effects, primary human islets were pre-incubated overnight with TUDCA prior to the H_2O_2 treatments as shown in **Supplementary Figure 1A**, as well as co-incubated together with H_2O_2 for the duration of the treatments. **(A)** TUDCA partially or completely recovers the oxidative stress-induced decreased mRNA expression levels of the genes MAFA, MAFB, KIR6.2, PAX6, NKX2.2, NEUROD1 and GLUT1 in human islets as measured by qPCR. **(B)** The oxidative stress-induced decreased level of the β-cell-specific protein MAFA is recovered by TUDCA in human islets as measured by Western blot. Data are presented as means of technical triplicates as fold change over untreated control islets (blue graphs). n = 1 donor; Black circles = untreated control islets, upward-pointing triangles = H_2O_2-treated islets (50 μM 24h), downward-pointing triangles = H_2O_2-treated islets (200 μM 90min), green squares = H_2O_2 (50 μM 24h or 200 μM 90min) and TUDCA (1 mM) pre- and cotreated islets. h = hours, min = minutes, H_2O_2 = hydrogen peroxide, TUDCA = tauroursodeoxycholic acid.

ROS, which is known to suppress β-cell mitochondrial activity and other components of the insulin secretion pathway, thereby leading to β-cell dysfunction (35–37).

Oxidative stress-induced altered β-cell function was also associated with an ER stress response, as shown by the upregulation of the ER-stress-related genes XBP1s/u (38), ATF3 (39) and CHOP (40). Activation of the unfolded protein response (UPR) is involved in the preservation of β-cell survival and function (41). However, in case of persistent or severe ER stress as in T1D, UPR activation leads to the opposite cell fate, i.e. β-cell dysfunction and death (42–44). In our study, both the FOXO1 shuttling and the initial ER stress response upon H_2O_2 treatment may represent attempts to protect β-cells from oxidative stress-induced damages.

Oxidative stress leads to loss of the maturity genes MAFA and PDX1 in human β-cells. We recently described a similar adaptation in primary human islets in a model of drug-induced diabetes (45). In addition, these findings are in line with earlier studies in rodent β-cell lines or islets that link oxidative stress to alterations in β-cell identity, through reduced expression of the key β-cell transcription factors PDX1, NKX6.1 and MAFA (46–49). Additionally, reduced nuclear MAFA expression found in islets of diabetic db/db mice is restored by overexpression of the antioxidant enzyme endogenous glutathione peroxidase-1 (GPX1) (49, 50). Likewise, decreased nuclear expression of PDX1 and MAFA seen in islets of diabetic ZDF rats is prevented by treatment with ebselen, a GPX mimetic (51). Most of these studies were performed in rodent islets or cell lines, whereas our study confirms the link between oxidative stress and the decrease in maturity markers in primary human β-cells. Besides MAFA and PDX1, gene expression of other important regulators of β-cell maturity and identity, such as MAFB (52), PAX6 (16), NKX2.2 (53) and NEUROD1 (54) were also decreased upon H_2O_2 treatment, further indicating the link between oxidative stress and altered β-cell identity. Decreased expression of key regulators of glucose sensing and insulin secretion, such as GLUT1 (55), FOXA2 (56) and KIR6.2 (57) after H_2O_2 treatment could explain the impaired β-cell function upon oxidative stress.

In parallel to the downregulation of key β-cell genes, we observed a concomitant increase in SOX9 and HES1 expression in EndoC-βH1cells. Increased progenitor cell marker expression (alongside decreased β-cell markers) was previously shown in FGF2- and viral infection-induced dedifferentiation models in EndoC-βH1 cells (58, 59). Similarly, upregulated progenitor markers have been shown in rat pancreatic islets treated with hydrogen peroxide, as seen by the increased expression of C-MYC (60), a transcription factor known to inhibit β-cell differentiation (61, 62).

The decreased expression of β-cell maturity genes and the increased expression of progenitor cell markers could indicate that oxidative stress-induced β-cell failure may result from partial dedifferentiation. It is proposed that dedifferentiation might be a way for β-cells to escape from immune-mediated destruction. A study performed in the NOD mouse model indicates that under inflammatory stress, a subpopulation of β-cells decreases its characteristics of mature β-cells while displaying increased stemness-like features to escape from T-cell-mediated death (63). Furthermore, Lee et al. reported that the loss of β-cell maturity genes (induced by β-cell-specific IRE1α deletion) prevents insulitis, the autoimmune destruction of β-cells and therefore the development of diabetes in NOD mice (64).

We observed that the chemical chaperone TUDCA partially prevents the decrease of β-cell markers such as MAFA, MAFB and PAX6 upon oxidative stress. TUDCA is known to alleviate ER stress in β-cells (65, 66). Additionally, TUDCA has been shown to have anti-oxidant capacities in certain neurological disorders (67, 68). Our findings indicate that oxidative stress-induced ER stress could be a key factor in altered β-cell identity.

In this study, we focused specifically on human β-cells. The impact of oxidative stress on human α-cell identity and function remains to be elucidated, which could potentially be a future focus area. Overall, we propose that oxidative stress-induced β-cell failure may result from partial dedifferentiation, which may be an adaptive mechanism for cells to survive. Targeting antioxidant mechanisms could be an important step in preserving functional β-cell mass in T1D.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

FL designed and performed the experiments and wrote the manuscript. NG, and NdG performed the experiments. ME and TR provided infrastructure for human islet isolation. FC and EdK supervised the project. FC acquired the funding for the project and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.690379/full#supplementary-material

Supplementary Figure 1 | Schematic overview of the experimental setup and validation of the H_2O_2 treatments. **(A)** Primary human islets or EndoC-βH1 cells were treated with 50 μM H_2O_2 for 24 hours or with 200 μM H_2O_2 for 90 minutes. After 90 minutes (t = 90min), the 200 μM H_2O_2 was washed away and new medium without H_2O_2 was added to the islets or cells. At 24 hours after the start of both treatments (t = 24h), all conditions were harvested for the readouts. **(B)** Viability of human islets after H_2O_2 treatments was assessed at t = 24h by FDA/PI staining. **(C)** Relative RNA content of human islets after H_2O_2 treatments was measured at t = 24h. **(D)** The basal insulin secretion levels of the islets that were used in the glucose-stimulated insulin secretion assay are unaltered after both H_2O_2 treatments. **(E)** Viability of EndoC-βH1 cells after H_2O_2 treatments was assessed at t = 24h by FDA/PI staining. **(F)** Relative RNA content of EndoC-βH1 cells after H_2O_2 treatments was measured at t=24h. Data are presented as means ± SEM over untreated control islets (blue graphs) or EndoC-βH1 cells (red graphs). n = 3-12 donors/batches; each data point represents one donor/batch. **p < 0.01, ****p < 0.0001 *vs.* untreated control islets/EndoC-βH1 cells as determined by an unpaired Student's t test on the RNA concentrations. Black circles = untreated control islets/EndoC-βH1 cells, upward-pointing triangles = H_2O_2-treated islets/EndoC-βH1 cells (50 μM 24h), downward-pointing triangles = H_2O_2-treated islets/EndoC-βH1 cells (200 μM 90min). h = hours, min = minutes, H_2O_2 = hydrogen peroxide, FDA = fluorescein diacetate, PI = propidium iodide.

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Neutrophil Extracellular Traps Caused by Gut Leakage Trigger the Autoimmune Response in Nonobese Diabetic Mice

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Increased formation of neutrophil extracellular traps (NETs) is associated with gut leakage in type 1 diabetes (T1D). To explore the mechanism of how enteropathy exacerbated by NETs triggers pancreatic autoimmunity in T1D, we carried out a correlation analysis for NET formation with gut barrier functions and autoimmunity in nonobese diabetic (NOD) mice. Inducing chronic colitis or knocking out of peptidyl arginine deiminase type 4 (PAD4) in NOD mice were used to further study the effect of NET formation on the progression of T1D. Microbial alterations in Deferribacteres and Proteobacteria, along with the loss of gut barrier function, were found to be associated with increased endotoxin and abnormal formation of NETs in NOD mice. Both DSS-induced colitis and knockout of PAD4 in NOD mice indicated that PAD4-dependent NET formation was involved in the aggravation of gut barrier dysfunction, the production of autoantibodies, and the activation of enteric autoimmune T cells, which then migrated to pancreatic lymph nodes (PLNs) and caused self-damage. The current study thus provides evidence that PAD4-dependent NET formation is engaged in leaky gut triggering pancreatic autoimmunity and suggests that either degradation of NETs or inhibition of NET formation may be helpful for innovative therapeutic interventions in T1D.

Keywords: autoimmunity, gut barrier function, neutrophil extracellular traps, peptidyl arginine deiminase type 4, type 1 diabetes

INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by insulin-producing pancreatic beta cells destroyed by autoreactive T cells (1, 2). Adequate evidence has been provided to confirm the leaky gut-affecting pancreas pathogenic theory. For instance, the development of clinical diabetes in patients and preclinical models (diabetes-prone rats) of T1D is often preceded by increased intestinal permeability (1, 2). It has been proposed that the loss of gut barrier function caused by enteric infections (3), commensal microbiota dysbiosis (4, 5), dietary antigen exposure, and local inflammation (6) could lead to the uncontrolled invasion of bacterial

components, followed by the generation of islet-reactive CD4- or CD8-positive T cells (7) in gut-associated lymphoid tissue (GALT), and these cells possess PLN homing abilities and ultimately induce insulitis (8–10). However, in this process, the transition from innate immunity to adaptive immunity after losing gut barrier integrity has rarely been mentioned.

Neutrophils are essential innate immune cells in the recognition and elimination of invasive pathogens (11), and their contribution to the pathogenesis of T1D was demonstrated in NOD mice, in which neutrophils infiltrated the pancreas and could form NETs (12). In line with this finding, in T1D patients, a reduced circulating neutrophil number was accompanied by the rise of neutrophils or NETs in their pancreas (13, 14). Our previous clinical study suggested that NET formation is increased in new-onset T1D patients compared with healthy controls (15). Parackova et al. have recently indicated that NET-induced dendritic cell activation led to Th1 polarization in T1D patients (16). As a special way of capturing invasive bacteria, NETs contain abundant cytosolic proteins, especially those with post-translational modifications, such as PAD4 that drives citrullination, which is an important potential source of self-antigens to promote the loss of immune tolerance and autoimmunity (17) and increase the generation of antineutrophil cytoplasmic antibodies (ANCAs) (18) and anti-cyclic citrullinated peptide antibodies (ACPAs) (19). Masses of self-DNA sprayed in this process were proven to be capable of activating plasmacytoid dendritic cells (pDCs) or monocytes through cGAS-STING and triggering proinflammatory cytokines to drive autoimmune pathology in T1D patients (20). In addition, B cells activated by self-DNA produce an anti-dsDNA antibody to trigger an autoimmune response (21).

We recently reported that degradation of NETs in the gut of NOD mice by nuclease production due to recombinant *Lactococcus lactis* could protect against T1D development (22). In the current study, we aimed to investigate whether NETs participation in the T1D progression is triggered by gut leakage. First, we verified the correlation between abnormal NET formation and gut leakage in preclinical models of autoimmune T1D. Then we investigated whether aggravated gut leakage induced by 2% dextran sulfate sodium (DSS) could increase NET formation and accelerate T1D occurrence in NOD mice accordingly. Finally, we examined whether decreased NET formation and improved gut barrier function could alleviate T1D progression in PAD4 knockout mice. Thereby, data from the current study may reveal that NET mediator roles in the progression of T1D are triggered by intestinal barrier breakage and associated with both cell-mediated immunity and humoral immunity.

METHODS AND MATERIALS

Animals

Both NOD/LtJ and BALB/c mice were female and purchased from Huafukang Bioscience Co., Inc. (Beijing, China). PAD4-/- mice with the NOD/LtJ background were purchased from Animal Model Research Center of Nanjing University (Nanjing, China). All mice were maintained under specific pathogen-free conditions in Animal Laboratory Center of China Pharmaceutical University (CPU, Nanjing, China). Animal care and all experimental methods were performed following the relevant guidelines and regulations and approved by the local ethics committee in CPU.

Induction of Chronic DSS Colitis

A solution of 2% (wt/vol) DSS (molecular weight: 36,000–50,000 Da; MP Biomedicals, Solon, USA) was orally administered to 4-week-old NOD/LtJ mice in drinking water for three cycles of 7 days at intervals of 2 weeks before the mice were at 13 weeks old and an additional three cycles of 7 days at intervals of 1 week afterward.

Neutrophil Isolation and NETs Assay

Spleen neutrophils were isolated with Percoll (Solarbio, Beijing, China) gradients as described (23). NETs assay was performed as protocols published (24). Neutrophils were resuspended in RPMI-1640 containing 5-10% FBS and plated at 5×10^5 cells per well in 24-well plates (Thermo Fisher, Massachusetts, US). After incubation for 0.5-1 h, the cells were stimulated with PMA (8 μM, MedChemExpress, New Jersey, USA), ionomycin (10 μM, MedChemExpress, New Jersey, USA) or fecal microbiota (2×10^7 CFU), and the neutrophils were incubated for an additional 3 h to form NETs. Cells were stained with SYTOX green (1:1000, KeyGEN BioTech, Nanjing, China) for NETs quantification or, in some experiments, fixed in 2% paraformaldehyde, permeabilized, blocked, and stained with anti-H3cit (1:1000, Abcam, cat. no. ab5103) and anti-rabbit secondary antibodies (1:200, Servicebio, Wuhan, China). Images were acquired on an Axiovert 200 M wide-field fluorescence microscope (Nikon) with a coupled camera. The percentages of H3cit high cells and NETs were determined from five or six nonoverlapping fields per well, and the average was taken from 2-3 biological repetitions in every experiment.

Mononuclear Cell Isolation and Co-Culture With NETs

First, spleen mononuclear cells were isolated using Percoll (Solarbio, Beijing, China) gradients as described (25). After stimulating 5×10^5 neutrophils with or without ionomycin *in vitro*, the culture medium was carefully removed, the NETs/neutrophils were washed slightly 2-3 times by RPMI-1640 (10% FBS) and resuspended in 300 μL RPMI-1640 containing 5-10% FBS. NETs/neutrophils were then added to wells containing 5×10^6 mononuclear cells in 500 μL culture medium and co-cultured for 7 days, with medium added every two days.

Min6 Cell Culture and *In Vitro* Lymphocyte Cell Migration Assay

Min6 beta cells (CL-0674, Procell Life Science&Technology Co., Ltd. Wuhan, China) were cultured with RPMI-1640 (10% FBS, 50 mM β-mercaptoethanol) for 24 h (authentication and no mycoplasma contamination reports were provided by the supplier), and Min6 cell-containing medium was transferred to the bottom of 24-well Boyden chambers (NEST, Wuxi, China)

for an additional 12 h of culture. A total of 1×10^5 lymphocyte cells was seeded onto the porous permeable membrane in the upper chambers. After 12-14 h, the migrated cells in the bottom chamber were collected and counted.

Diabetes Incidence

Diabetes was weekly monitored by measuring blood glucose levels with a Boshilong blood glucose meter (Houmeide Biotechnology, Taipei, China). After two consecutive blood glucose measurements ≥13.8 mmol/L, the mice were then considered to be diabetic.

16S rRNA Microbiota Analysis

Total genomic DNA from samples was extracted by using the CTAB/SDS (Sangon Biotech, Shanghai, China) method. 16S rRNA genes were amplified using barcoded sample-specific primers: 16S V4-V5: 515F-907R. All PCRs were carried out in 30 μL reactions with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs (Beijing) LTD, Beijing, China), 0.2 μM forward and reverse primers, and approximately 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 60 s. Finally, samples were held at 72°C for 5 min. Amplicons were loaded onto a 2% agarose gel, and samples with bright main strips between 400-450 bp were chosen for further experiments. PCR products were mixed in equidensity ratios. Then, the mixed PCR products were purified with a Gene JET Gel Extraction Kit (Thermo Scientific, Waltham, USA). Sequencing libraries were generated using the NEB Next ® UltraTM DNA Library Prep Kit for Illumina (New England Biolabs (Beijing) LTD, Beijing, China) following the manufacturer's recommendations, and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific, Waltham, USA) and Agilent Bioanalyzer 2100 system (Agilent Technologies, Beijing, China). Finally, the library was sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA), and 250 bp/300 bp paired-end reads were generated. Paired-end reads were assigned to each sample according to the unique barcodes, and sequence analysis was performed by the UPARSE software package using the UPARSE-OTU and UPARSE-OTU ref algorithms.

Histopathological Analysis and Immunofluorescence

The pancreas and colon were fixed in 4% paraformaldehyde and embedded in paraffin (Servicebo, Wuhan, China). To assess histopathological signs of diabetes, consecutive sections (4 μm) were then stained with hematoxylinand eosin (H&E, Sangon Biotech, Shanghai, China) and scored for insulitis, in which 0 stood for no infiltration, 1 stood for periinsulitis, 2 stood for infiltration covering approximately half of the islet, 3 stood for infiltration covering approximately 75% of the islet and 4 stood for full insulitis. For colon sections, after H&E staining, three independent parameters were determined: the depth of injury (scores 0, 1, 2 and 3 for none, mucosal, mucosal/submucosal and transmural, respectively), muscle layer thickness, and the ratio of villus height/crypt depth. Sections were also stained with PAD4 (Proteintech, Wuhan, China), Ly6G (Servicebio, Wuhan, China), H3cit (Abcam) and insulin (Servicebio, Wuhan, China) and subsequent fluorescein-labeled secondary antibodies to display NET formation or insulin production. Histological staining of mucins and calculation of mucus thickness were conducted according to previous descriptions (26).

NET-Associated Biomarkers and T1D Autoantibody Detection

Serum samples and pancreas tissue homogenates were all stored at -80°C and thawed at 4°C for 20 min before testing. Circulating NE, MPO, PAD4, ACPA, dsDNA-Ab, IAA, ZnT8A, IA2A and GADA were measured individually by commercial enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's protocols (Tongwei Biotech, Shanghai, China). Absorbance was measured using a multifunction microporous plate detector (Spark, Tecan Group Ltd, Männedorf, Switzerland). For the detection of ANCAs in serum, first, NET formation operated just like the methods "Neutrophil isolation and NETs assay" showed. Then, the supernatant was discharged carefully by slow suction and micrococcal nuclease (1 U/mL, Solarbio, Beijing, China) was added to digest NETs at 37°C for 20 min followed by 5 mM EDTA to stop the nuclease activity. The supernatant was collected and centrifuged to eliminate cell debris. The total cytoplasmic content was diluted 1:300 in buffer (0.1 M Na2CO3, 0.1 M NaHCO3, pH=9.6) and incubated at 4°C overnight in 96-well plates. After blocking with 5% BSA (37°C, 2 h), the serum samples were diluted 1:200 in 5% BSA and incubated at 37°C for 2 h. After washing with PBST (PBS containing 0.5% Tween 20), HRP-conjugated goat anti-mouse IgG (1:3000, Servicebio, Wuhan, China) was added and incubated at 37°C for 1 h. TMB was added to all wells (Beyotime Biotechnology, Shanghai, China), and the samples were incubated in the dark for 30 min at 37°C, then 2 M H2SO4 was used to stop the reaction. The absorbance at 450 nm was measured using a multifunction microporous plate detector (Spark, Tecan Group Ltd, Männedorf, Switzerland). To assess cf-DNA levels *in vivo*, the PICOGREEN®, Quant-iT™ dsDNA Assay Kit (Thermo Fisher, Massachusetts, US) was used according to the manufacturer's protocols. To detect circulating H3cit levels, anti-H3cit (1:1000, Abcam) and anti-H3 (1:3000, Proteintech, Wuhan, China) were diluted in buffer and followed the routines mentioned above.

LPS Detection in Serum

Serum samples were all stored at -80°C and thawed at 4°C for 20 min before testing. LPS was measured individually by commercial ELISA kits, according to the manufacturer's protocols (Tongwei Biotech, Shanghai, China). Absorbance was measured using a multifunction microporous plate detector (Spark, Tecan Group Ltd, Männedorf, Switzerland).

Cross-Reaction of Insulin Autoantibody (IAA) With NETs Associated Auto-Antigens

Lysed NETs (1:300) or insulin (1:200, Yuanye, Shanghai, China, 1 mg/ml) was diluted in buffer (0.1 M Na2CO3, 0.1 M NaHCO3, pH=9.6) and incubated at 4°C overnight in 96-well plates.

After blocking these two plates with 5% BSA (37°C, 2 h), the serum samples were diluted 1:200 in 5% BSA and incubated with NET protein at 37°C for 2 h. Another uncoated plate was also blocked with 5% BSA (37°C, 2 h) and then incubated with the same diluted samples at the same time. Next, all samples from NET-coated plates or uncoated plates were collected and added to insulin-coated plates, followed by 2 h of incubation at 37°C. After subsequent routines, the differences in absorbance were compared between pretreated subjects and untreated subjects of the same sample. The cross-reaction ratio $=(OD_{(uncoated+insulin)} - OD_{(NET\ protein+insulin)})/OD_{(uncoated+insulin)}$

Western Blotting

Pancreatic samples were snap-frozen and homogenized in RIPA buffer (Sangon Biotech, Shanghai, China) on ice. After centrifugation at 12000 rpm for 10 min at 4°C, the protein content of the supernatant was mixed with loading buffer. An equal amount of protein per sample was resolved on gradient gels (15% Tris-glycine gels) and electroblotted on PVDF membranes, which were then incubated with primary antibodies (rabbit polyclonal anti-H3cit, Abcam; rabbit polyclonal anti-GAPDH, Servicebo) at 4°C overnight and subsequently with appropriate HRP-conjugated secondary antibodies (Servicebo, Wuhan, China) for 1 h at room temperature. The blots were developed with enhanced chemiluminescence substrate (Sangon Biotech, Shanghai, China). Blots were quantified using ImageJ software.

In Vivo Permeability Assay

Intestinal permeability was determined by FITC-dextran assay as previously described (8). First, 20 mL/kg of body weight PBS containing 20 mg/mL FITC-conjugated dextran (FITC-dextran; molecular mass, 4.4 kDa; FD4, Sigma-Aldrich) was administered to each mouse by oral gavage. Three hours later, blood was collected in anticoagulant tubes and centrifuged to obtain the plasma (4000 rpm, 5 min at 4°C). Then, 50 ml of plasma was added to a 96-well microplate. The concentration of fluorescein was determined by a multifunction microporous plate detector (Spark, Tecan Group Ltd, Männedorf, Switzerland) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm using serially diluted samples of the FITC-dextran marker as the standard.

Oral Glucose Tolerance Test

Nondiabetic mice in each group (n=8) were selected for the oral glucose tolerance test (OGTT). After 12 h of fasting overnight, the basal blood glucose was detected, followed by oral administration of glucose solution (2g/kg). Then, the blood glucose was monitored at the following time points: 15 min, 30 min, 60 min and 90 min.

Flow Cytometry

Analysis Single-cell suspensions from pancreatic lymph nodes (PLNs), mesenteric lymph nodes (MLNs), spleens and cultured cells were resuspended in staining buffer containing PBS and 1% FBS, stained with monoclonal antibodies against surface markers, fixed and permeabilized using the BD Cytofix/Cytoperm kit, and finally stained for intracellular transcription factors. The following antibodies were used: FITC anti-mouse CD4 (RM4-5; BD), APC anti-mouse CD25 (PC61; BioLegend), PE-cy7 anti-mouse ROR-γt (B2D; Invitrogen), APC anti-mouse T-bet (4B10; BioLegend), APC anti-mouse GATA-3 (16E10A2; BioLegend), Alexa Fluor 647 anti-mouse FoxP3 (MF23; BD), and APC anti-mouse α4β7 (DATK32; Proteintech).

RNA Isolation and RT-qPCR

Immediately after the mice were sacrificed, the colon and pancreas were cut into pieces and placed in 500 μL RNA storage preserving fluid (Tiangen Biotech Beijing CO., LTD), followed by storage at -80°C. After homogenization in 500 μL TRIzol reagent (Sangon Biotech, Shanghai, China), the RNA was extracted by adding 100 μL of chloroform and precipitating the aqueous phase with 200 μL of isopropanol. cDNA was synthesized using HiFi Script cDNA Synthesis Kit (Sangon Biotech, Shanghai, China). RT-qPCR analyses were conducted to quantitate the relative mRNA expression using Ultra SYBR Mixture (Sangon Biotech, Shanghai, China). The primer sequences are shown in the **Supplemental Table 1**. Gene expression was normalized using GAPDH as the reference gene. To analyze the relative fold change, we employed the $2^{-\triangle\triangle CT}$ method.

Statistical Analysis

SPSS 22.0 software (SPSS, Chicago, USA) was used for statistical analysis. The statistical significance of differences between two or more samples was calculated by an unpaired two-tailed Student's t test or one ANOVA test. Correlations were analyzed by the Spearman correlation test. The cumulative diabetes incidence was calculated using Kaplan–Meier estimation, whereas the statistical significance was evaluated by the log-rank test. All data are presented as means ± SEM, and $P<0.05$ was used to indicate a statistically significant difference.

RESULTS

Abnormal NET Formation Correlated With Loss of the Gut Barrier and Alterations in the Gut Microbiota in NOD Mice Before T1D Onset

Previous studies have mentioned that NOD mice exhibit prominently increased gut permeability compared to BALB/c mice at approximately 10-12 weeks old (8), and invasive microbes are indispensable for islet-reactive T cell action (27). However, little is known about the microbial changes at this time. Hence, we first conducted microbial analysis in NOD mice at four different stages (**Supplementary Figure 1A**). Interestingly, the abundance of Deferribacteres or Proteobacteria in NOD mice after 12 weeks was increased sharply when compared with mice before 12 weeks (**Supplementary Figure 1B**), and principal coordinates analysis (PCOA) exhibited the significant difference between the two groups (**Supplementary Figure 1C**). Moreover, we discovered that two gram-negative bacterial families, Desulfovibrionaceae and Deferribacteraceae, displayed significant augmentation, while the

other two families, Muribaculaceae and Lactobacillaceae, which are short-chain fatty acid producers, were markedly reduced in NOD mice after 12 weeks (**Supplementary Figures 1D, E**). The increased abundance of Desulfovibrionaceae, an essential endotoxin producer, was in line with the increased endotoxin/lipopolysaccharide (LPS) level in the serum of NOD mice after 12 weeks of age (**Figure 1E**), which indicated a high risk of subsequently abnormal neutrophil-forming NETs in NOD mice. Then, an ionomycin stimulation experiment confirmed this finding and showed that neutrophils in NOD mice were easier to form NETs *in vitro* when compared with BALB/c mice, and diabetic NOD mice released more NETs compared to prediabetic mice (**Figures 1A, B**). In line with this, the circulating levels of NET biomarkers, such as cf-DNA and H3cit in NOD mice exhibited obvious differences from BALB/c. The serum concentrations of cf-DNA (**Figure 1C**) were higher in NOD mice at all times, and highly significant increases in H3cit and LPS was observed in NOD mice at 10-12 weeks of age compared with 4-6 weeks of age (**Figures 1D, E**). More importantly, the strong correlation between LPS and H3cit only in NOD mice (**Figure 1F**) but not BALB/c mice (**Figure 1G**) suggested that the loss of gut barrier function and the invasion of booming gram-negative bacteria led to the abnormal generation of NETs before T1D onset, which could be a potential pathogenic factor.

In addition to verifying increased intestinal permeability (**Figure 1H**), obvious histological lesions, higher gut damage

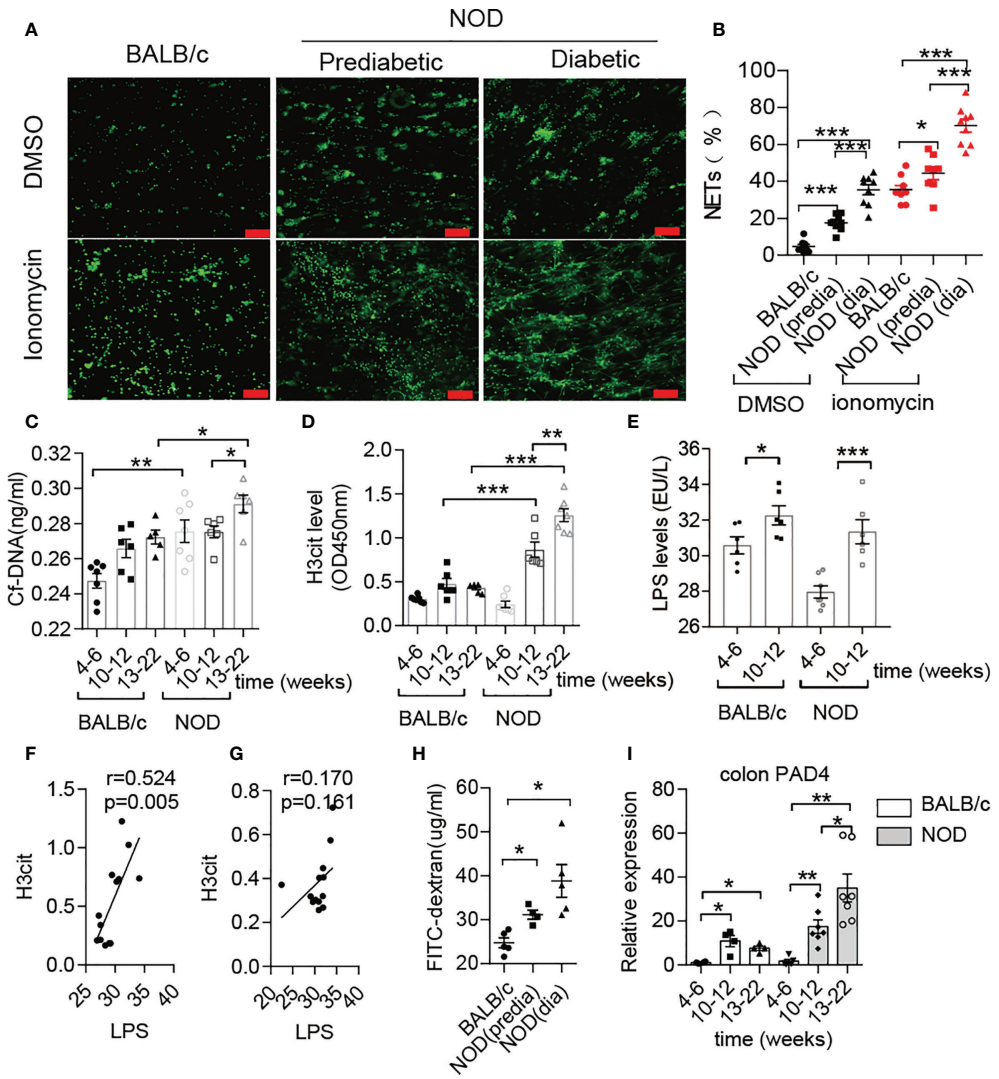

FIGURE 1 | Abnormal NET formation correlated with increased LPS levels in NOD mice. **(A, B)** NET formation ability in NOD mice *vs.* BALB/c mice (n= 4). NET quantification was conducted twice for each mouse. Circulating levels of cf-DNA **(C)**, H3cit **(D)** and LPS **(E)** in NOD mice *vs.* BALB/c mice at different ages (n= 5-7). Spearman correlation analysis of H3cit **(F, G)** with LPS in NOD mice (n= 13) compared with BALB/c mice (n= 13). **(H)** FITC-dextran *in vivo* permeability assay in female BALB/c and NOD mice (n= 5). **(I)** RT-qPCR analysis of PAD4 in colons from NOD (n= 7, respectively, at different ages) and BALB/c mice (n= 4, respectively, at different ages). The scale bars indicate 100 μm **(A)**. Data are shown as the mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.

scores and lower villus height/crypt depth ratios located in the colon of NOD mice after 10-12 weeks of age (**Supplementary Figures 2A, B**), we further observed the increased inflammatory cytokines IL-1β, IL-17 and TNF-α in colons of NOD mice (**Supplementary Figure 2C**). In contrast to BALB/c mice, continuously increased mRNA expression of PAD4 was detected in the NOD colon at different weeks (**Figure 1I**). Taken together, these results demonstrated that NET formation correlated with gut leakage and alterations in the gut microbiota in NOD mice.

NETs Caused Cellular Immunity and Humoral Immunity in NOD Mice

Increased Th1 and Th17 and decreased Treg were observed in MLNs of NOD mice compared with BALB/c mice after 12 weeks of age (**Supplementary Figures 2D, E**) as similar to the previous report (8). The latest clinical research on T1D patients has demonstrated that NETs can polarize naïve T cells to Th1 cells by activating pDCs (16). In addition, numerous studies on autoimmune diseases have also shown the role of NETs in inducing immune dysfunction (17). However, no clues have been found to test this process in NOD mice thus far. Therefore, we isolated neutrophils from diabetic NOD mice and stimulated them with ionomycin to form NETs *in vitro*, followed by coculture with autologous spleen mononuclear cells. The proliferation of memory Th1 and Th17 cells indicated that NETs in NOD mice possess the capacity to activate proinflammatory T cells, which could trigger beta cells destruction and autoimmune diabetes (**Figures 2A, B**). We then tested the migration capacity of lymphocytes from the MLN in NOD mice. Neutrophils from the spleen were stimulated to form NETs *in vitro* and then cocultured with autologous lymphocytes from MLNs. The results of the transwell migration assay showed that more lymphocytes could migrate to the insulinoma cell line MIN6 *in vitro* (**Figure 2C**). Taken together, these findings indicated that enteric lymphocytes could be activated by NETs and possessed a stronger response to beta cells.

As reported in other autoimmune diseases, such as arthritis, lupus and vasculitis, ANCAs, ACPAs or anti-dsDNA antibodies play critical roles in self-destruction. ANCA levels in serum were also elevated in NOD mice after 12 weeks of age (**Figure 2D**) compared with BALB/c mice of the same age. Moreover, ACPA was sharply increased at disease onset in NOD mice but not in BALB/c mice (**Figure 2E**). To explore whether the components of NETs and insulin shared common epitopes, the cross-reaction was performed, and the results showed that NET-associated autoantibodies could indeed cross-react with insulin at a ratio of 10-20% (**Figure 2F**). Pretreatment with the components of NETs led to a significantly lower serum level of IAA in NOD mice but not in BALB/c mice after 12 weeks of age.

DSS Triggered Increased NET Formation and Accelerated the Course of T1D

A previous report has shown that DSS-induced chronic colitis could trigger 60% BDC2.5XNOD mice developing T1D (8). In the current study, we performed similar methods for inducing loss of the gut barrier integrity through six administrations of DSS from 4 weeks old in NOD/LtJ to explore whether increased NET formation plays crosstalk roles between invasive commensal microbiota and the abnormal activation of autoimmunity. The chronic colitis was successfully induced by 2% DSS in NOD mice. During modeling, the body weight of mice decreased, and the disease activity index score significantly increased (**Figure 3A**). Increased LPS levels in the circulation and the elevated mRNA expression of NE, MPO, PAD4 and cathelicidin antimicrobial peptide (CAMP) in the colon in DSS-treated mice (**Figures 3B, C**), manifested abnormal generation of NETs after DSS treatment. Furthermore, we observed more neutrophil infiltration and H3cit catalyzed by PAD4 existed in severely damaged areas (**Figure 3D**). In line with this, the serum levels of PAD4, NE and MPO also increased significantly in DSS-treated mice (**Figure 3E**). Moreover, the neutrophils were easier to form NETs *in vitro* after DSS administration (**Figure 3F**).

After DSS treatment, the progression of T1D disease in NOD mice was obviously accelerated. In detail, T1D onset in DSS-induced colitis NOD mice started at 8 weeks old and reached maximum morbidity at 18 weeks, which was four weeks earlier than in the control group (**Figure 4A**). In addition, a significant increase in morbidity occurred at approximately 14 weeks old, just after the fourth DSS treatment. DSS-induced colitis aggravation of islet inflammation was observed in both prediabetic and diabetic NOD mice (**Figure 4B**). Subsequently, we detected phenotypes of T helper cells in the spleen, MLN and PLN of the DSS-treated group in comparison to the control group. We found that after DSS induction, the ratio of Th1/Th2 cells in the PLN increased significantly in NOD mice (**Figure 4C**), while there was little difference in the ratio of Th17/Treg cells between the two groups (**Figure 4D**). Accordingly, the mRNA expression levels of effector inflammatory cytokines of Th1 cells, such as TNF-α and IFN-γ, were increased in the pancreas (**Figure 4E**). In addition, higher circulating levels of autoantibodies such as zinc transporter 8 protein antibody (ZnT8A) and insulinoma-associated antigen 2 antibody (IA2A) in the DSS-treated group showed greater humoral autoimmunity after gut damage (**Figure 4F**). In addition, DSS treatment doubly strengthened the cross-reaction of NET-associated autoantibodies with insulin (**Figure 4G**). Moreover, increased H3cit was discovered in the pancreas (**Supplementary Figure 3A**) in accordance with increased PAD4 levels, which could trigger the activation of autoreactive T cells (28, 29) and the generation of autoantibodies and would be another powerful explanation for the aggravation of T1D after DSS-treatment.

PAD4 Knockout Diminished NET Formation in NOD Mice

Considering that PAD4 is essential for NET formation (30) and the generation of citrullinated protein *in vivo*, PAD4 knock out NOD mice were used to investigate whether it is possible to affect T1D development. Above all, we isolated neutrophils from

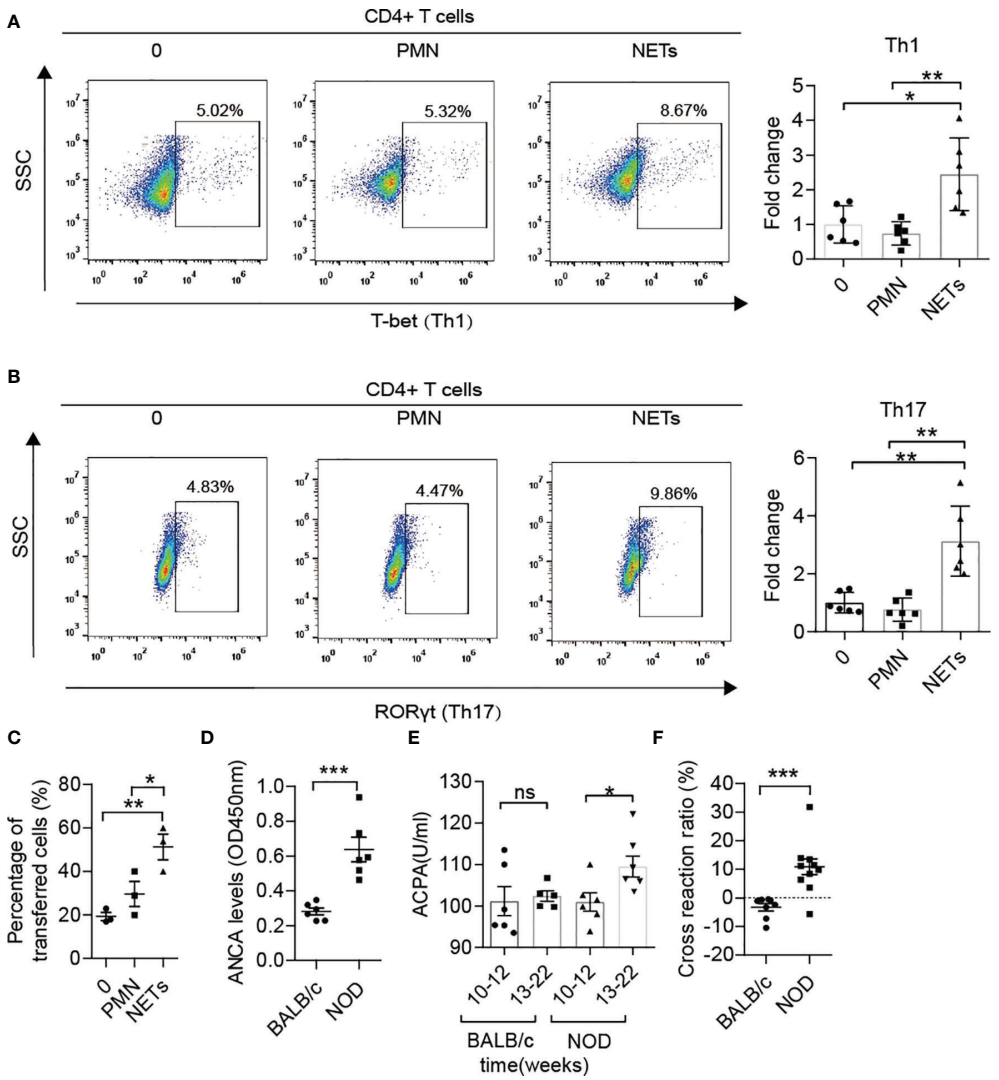

FIGURE 2 | The roles of NETs in the autoimmunity of NOD mice. **(A, B)** NETs triggered the proliferation of memory Th1 and Th17 cells from spleen mononuclear cells in diabetic NOD mice *in vitro* (n= 6). **(C)** The Transwell system showed the migration capacity of lymphocyte cells from the MLN in diabetic NOD mice (n= 3). **(D)** Increased ANCA was detected in NOD mice in comparison with BALB/c mice after 12 weeks of age (n= 6). Serum levels of ACPA **(E)** in NOD mice *vs.* BALB/c mice at different ages (n= 6). **(F)** Cross-reaction of IAA with NET-associated autoantigens in NOD mice (n= 10) but not BALB/c mice (n= 8) after 12 weeks of age. Data are shown as the mean ± SEM. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

PAD4-/- mice and PAD4+/+ mice to test their ability to form NETs under different stimulation conditions *in vitro* (**Supplementary Figure 4A**). The results came out that neutrophils from PAD4-/- mice indeed exhibited a lower ability to form NETs, especially when stimulated by ionomycin and microbes, which was reflected by the lower percentage of H3cit+ neutrophils and NETs (**Supplementary Figures 4B, C**), as well as the decreased serum levels of H3cit and NE in PAD4-/- mice (**Supplementary Figure 4D**). Furthermore, the ACPA also decreased in PAD4-/- mice (**Supplementary Figure 4E**). These results suggested that the activation of humoral autoimmunity to citrullinated proteins or other components of NETs were decreased to some extent by PAD4 knockout in NOD mice.

Blocking NET Formation Improved Gut Barrier Function and Alleviated the Development of T1D

Recently, several studies have demonstrated that PAD4 inhibitor (31) or DNase I treatment could effectively achieve remission in NETs inducing colitis (32, 33). Therefore, we compared the barrier function and NET formation in the colon and enteric autoimmune T cells in PAD4-/- mice *vs.* WT mice. In PAD4 KO mice, recovery of gut barrier function was reflected at a decrease in gut permeability through FITC-dextran permeability assay (**Figure 5A**). The thinner muscle layer, higher villus height/crypt depth ratio (**Figure 5B**), higher mucus generation (**Figure 5C**), increased tight junction protein gene expression (MUC2,

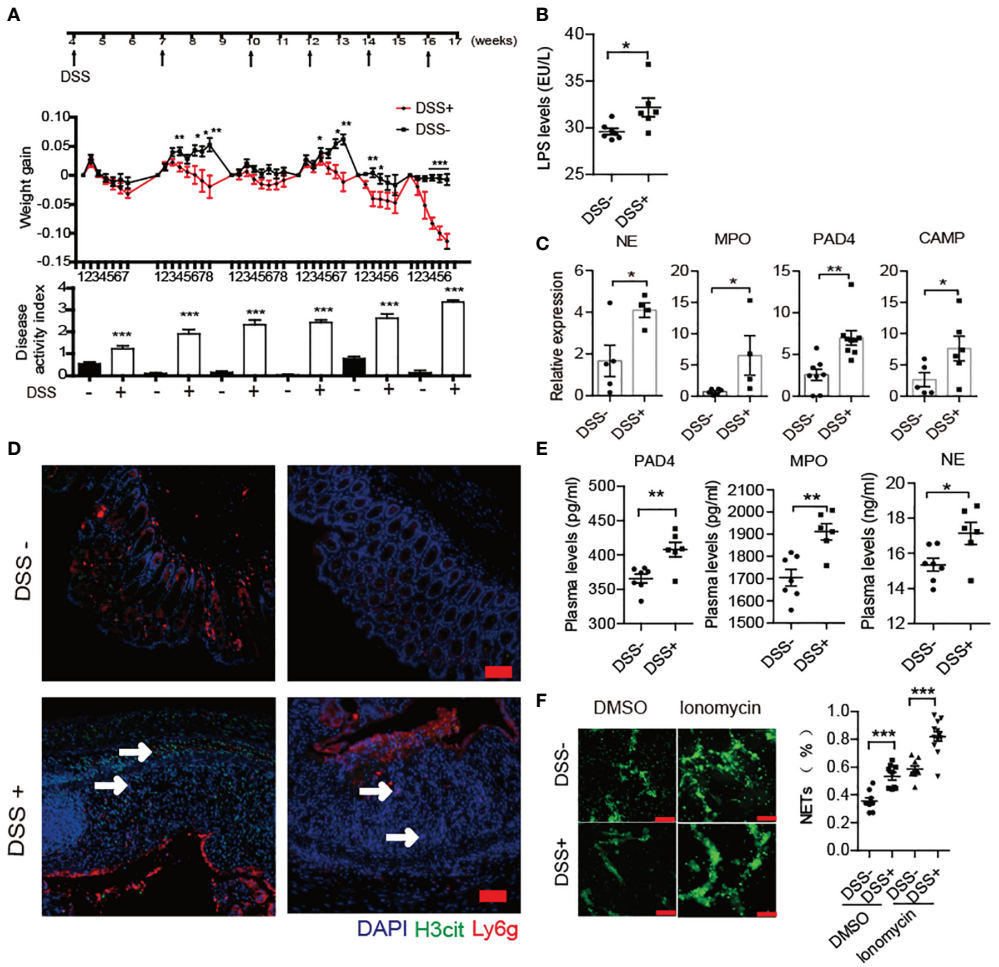

FIGURE 3 | DSS treatment triggered increased NET formation in NOD mice. **(A)** Six administrations of DSS induced chronic colitis in control (n= 14) and DSS-induced colitis NOD mice (n= 19). **(B)** Increased circulating LPS levels in DSS- induced colitis NOD mice (n= 6). **(C)** Elevated expression of NE, MPO, CAMP and PAD4 were found in colon homogenates of DSS-induced colitis NOD mice *vs.* control mice. **(D)** Immunofluorescent staining of Ly6G, H3cit and PAD4 in colon sections of DSS-induced colitis NOD *vs.* control mice. **(E)** Increased circulating NET biomarker levels in DSS-induced colitis NOD (n= 6) compared with the control mice (n= 7). **(F)** DSS administration enhanced neutrophils' capability of forming NETs *in vitro* (thrice NET quantification per mouse, n= 3). The scale bars indicate 50 μm **(D)** and 100 μm **(F)**. Data are shown as the mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.

claudin1, cadherin1 and ZO-1) (**Figure 5D**) and claudin1 protein expression (**Supplementary Figure 3B**) in the colon also indicated recovery of the intestinal tract barrier. In addition, knockout of PAD4 resulted in lower levels of colon peroxidase activity (**Figure 5E**). Then, we focused on whether NET deficiency due to PAD4 knockout could alter the balance of T cell subsets. As expected, the ratios of Th1/Th2 (**Figure 5G**) and Th17/Treg (**Figure 5H**) cells in the MLN declined in KO mice at approximately 12 weeks of age. The mRNA expression levels of Th1 cytokines, such as TNF-α and IFN-γ, and Th17 cytokines, such as IL-17, were decreased in the colon of KO mice (**Figure 5F**).

Interestingly, we also found fewer Th1/Th17 cells in the PLN of KO mice at approximately 10-12 weeks of age (**Figures 6A, B**). The KO mice displayed better glucose tolerance and lower morbidity than WT mice (**Figures 6C, D**). More importantly, the serum levels of autoantibodies such as glutamic acid decarboxylase antibody (GADA) or IA2A decreased in KO mice (**Figure 6E**). In line with this, HE staining showed less inflammation in the pancreas of KO mice, which confirmed the alleviation of autoimmunity after PAD4 knockout (**Figure 6F**). To further verify the hypothesis that NETs mediate the loss of the gut barrier and activation of autoimmune T cells, we orally administered 2.5% DSS to both KO and WT mice for 7 days to destroy the intestinal barrier function. The results showed that the expression of IFN-γ in the pancreas was significantly higher in WT mice but not KO mice (**Supplementary Figure 5A**) after colitis induction. In addition, the migration capacity of enteric lymphocytes in KO mice after DSS treatment to MIN6 cells *in vitro* was weaker than that in WT mice with colitis (**Supplementary Figure 5B**). Most importantly, we observed a significant increase in enteric CD4+ T cells (integrin α4β7

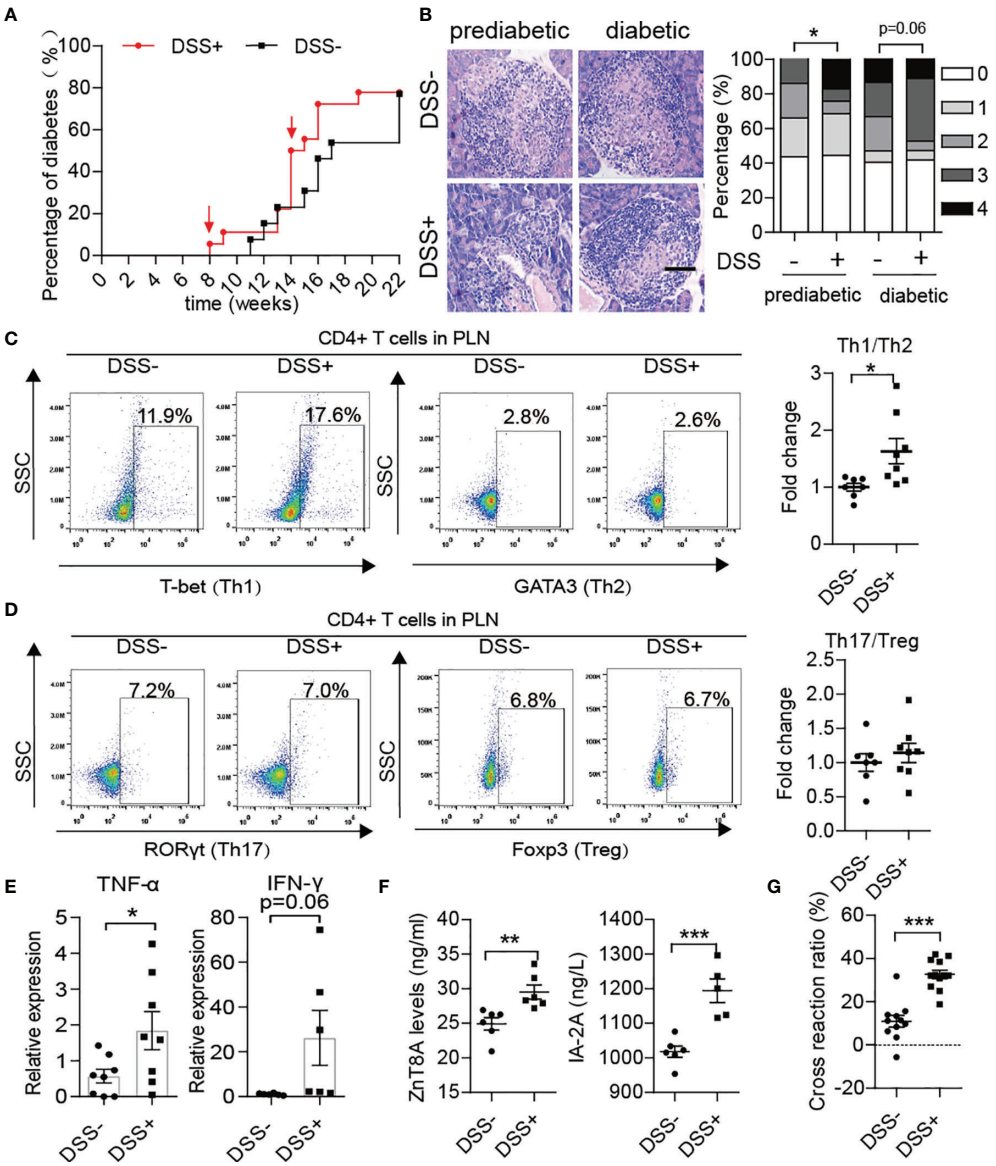

FIGURE 4 | DSS treatment aggravated autoimmunity in NOD mice and accelerated the course of type 1 diabetes. **(A)** Incidence of autoimmune diabetes in control (n= 14) and DSS-induced colitis NOD mice (n= 19). **(B)** HE staining of pancreatic tissue and insulitis scores showed more mononuclear cell infiltration in DSS-colitis NOD mice than in control mice at the prediabetic and diabetic stages, respectively (n= 6). **(C, D)** Representative flow cytometry plots (left) and bar graphs with mean percentages ± SEM (right) of Th1 and Th17 within PLN of control and DSS-induced colitis NOD mice (two independent experiments; n= 3-4 mice per group). **(E)** RT-qPCR analysis of cytokine genes encoding TNF-α and IFN-γ on tissue homogenates from the pancreas of DSS-induced colitis NOD mice and the control (n= 6-8). **(F)** Detection of circulating T1D-associated autoantibodies (ZnT8-ab, IA-2A) in DSS-induced colitis NOD mice and the control (n= 6). **(G)** Cross-reaction of IAA with NET-associated autoantigens in DSS-induced colitis NOD mice (n= 12) *vs.* control mice (n= 11). The scale bars indicate 200 μm **(B)**. Data are shown as the mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.

positive) (**Supplementary Figure 5C**), especially Th17 and Th1 cells (**Supplementary Figures 5D, E**) in the PLNs of WT mice but not KO mice with colitis. Meanwhile, the expression of integrin α4/β7 showed the same differences in the pancreas (**Supplementary Figure 5F**). Overall, our data demonstrated that NET deficiency prevents autoimmune T cells from traveling from GALT to the PLN to cause pancreatic beta cells destruction.

DISCUSSION

Accumulating evidence has demonstrated that gut barrier dysfunction and commensal microbial disorders could be critical pathogenic factors triggering T1D (34). However, knowledge of how gut leakage and invasive gut microbes in T1D patients or animal models promote beta cells

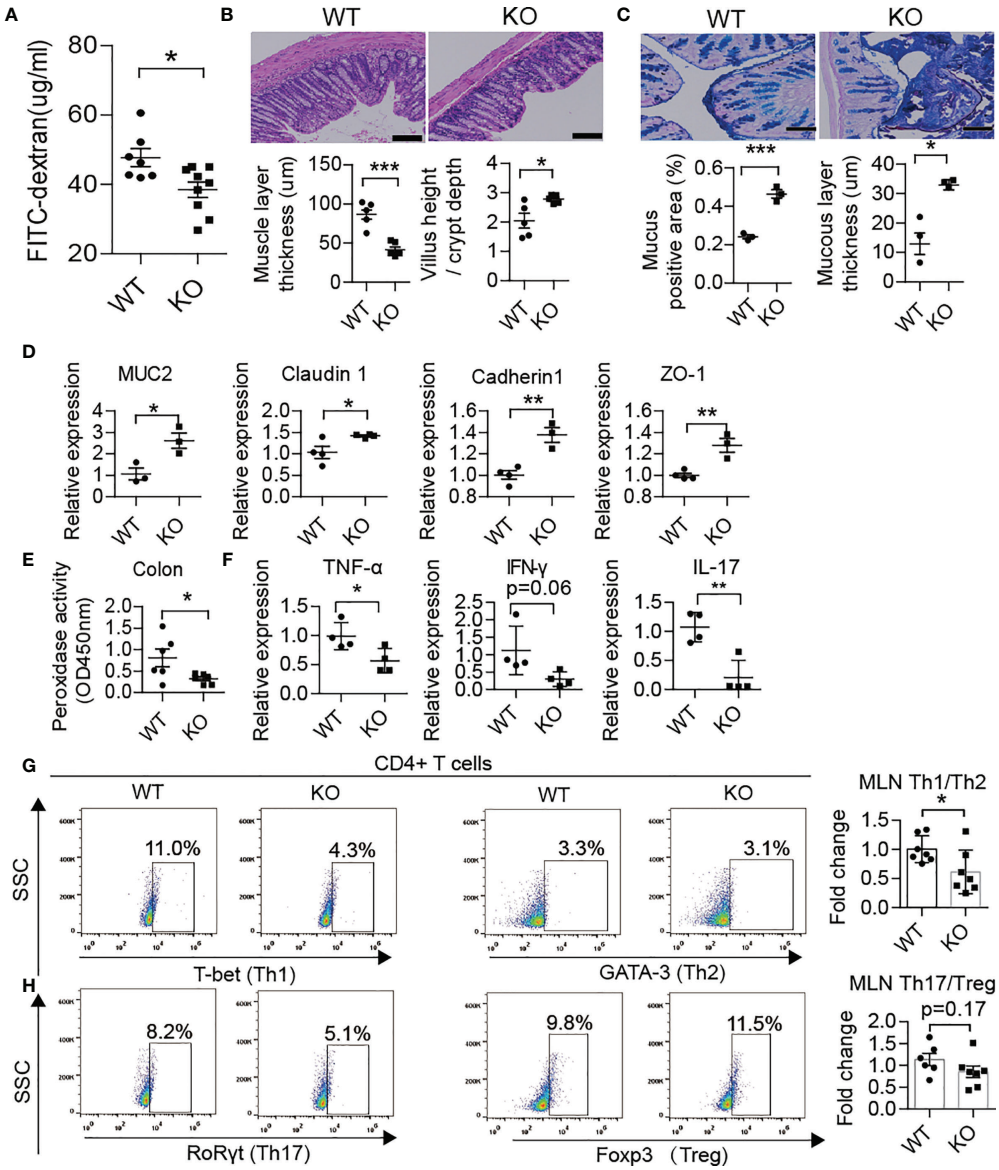

FIGURE 5 | PAD4 knockout improved gut barrier function. **(A)** FITC-dextran *in vivo* permeability assay in female WT (n= 7) and KO mice (n= 9). **(B)** HE staining of colon tissue and muscle layer thickness and the ratio of the villus height/crypt depth in female WT and KO mice (n= 5). **(C)** AB-PAS staining of colon tissue and the percentage of the positive area or mucous layer thickness in female WT (n= 3) and KO mice (n= 3). **(D)** RT-qPCR analysis of genes encoding MUC2, claudin 1, cadherin1 and ZO-1 in tissue homogenates from the colon of WT (n= 4) and KO mice (n= 3). **(E)** Decreased peroxidase activity in colon homogenates of KO mice (n= 6) compared with WT mice (n= 5). **(F)** RT-qPCR analysis of genes encoding TNF-α, IFN-γ and IL-17 in tissue homogenates from the colon of WT and KO mice (n= 4). Representative flow cytometry plots (bottom) and bar graph with mean percentages ± SEM (top) of Th1 and Th2 cells and their ratio **(G)** and Th17 and Treg cells and their ratio **(H)** within the MLN of WT and KO mice (two independent experiments; n= 6-7 mice per group). PAD4-/- or PAD4+/+ mice were all after 10-12 weeks old **(A–H)**. The scale bars indicate 100 μm **(B, C)**. Data are shown as the mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.

autoimmunity is still limited. In the current study, we have demonstrated that excessive NET formation caused by gut leakage may be one critical factor link between innate and adaptive immune responses.

In general, susceptible individuals, chronic enteric infections first activate the innate immune response and subsequently trigger autoimmunity (35). A previous clinical observation has shown that LPS exposure arising primarily from Bacteroides precludes early immune education and contributes to the development of T1D (36). Our previous clinical study has demonstrated that circulating LPS concentration is elevated in new-onset T1D patients, while the increased internal LPS levels are relate to the changes of bacteria from Deferribacteres or Proteobacteria in NOD mice after the development of diabetes, which usually

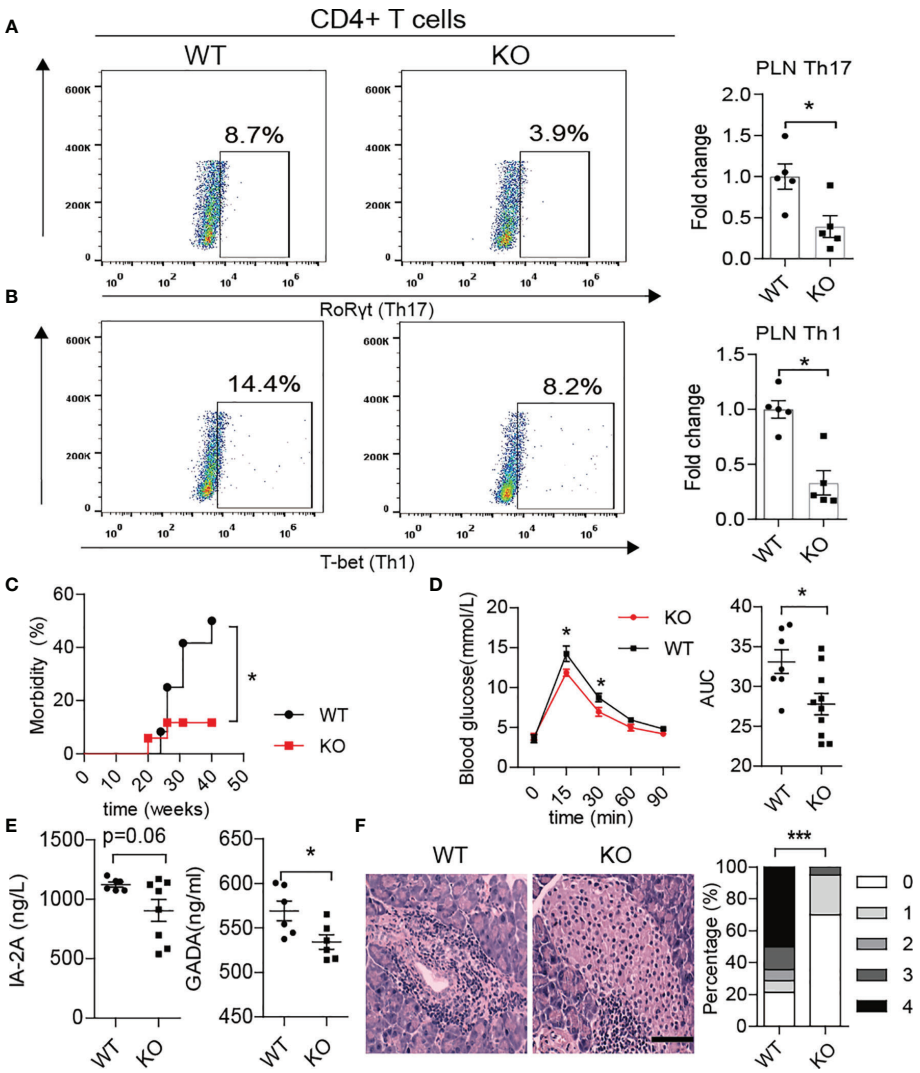

FIGURE 6 | PAD4 knockout alleviated the development of T1D in NOD mice. Representative flow cytometry plots and bar graphs with mean percentages ± SEM of Th17 **(A)** and Th1 **(B)** cells within the PLN of WT and KO mice (n= 5 mice per group). **(C)** Incidence of autoimmune diabetes in WT (n= 12) and KO mice (n=15). **(D)** OGTT of nondiabetic WT (n= 7) and KO mice (n= 10). **(E)** Serum levels of IA2 and GADA autoantibodies in WT *vs.* KO mice (n= 6-8). **(F)** HE staining of pancreatic tissue and insulitis score showing more mononuclear cells infiltration in WT mice than in KO mice (n= 3). PAD4-/- or PAD4+/+ mice were all after 12 weeks old **(A–F)**. The scale bars indicate 200 μm **(F)**. Data are shown as the mean ± SEM. *P < 0.05 and ***P < 0.001.

appears over 12 weeks of age. Similar to the previous study, neutrophils in NOD mice in the current study were also liable to form NETs and resulted in abnormal oscillation of the circulating levels of cf-DNA, H3cit and PAD4 at the prediabetic or diabetic stage compared with BALB/c mice of the same age. Importantly, a strong positive correlation between LPS and these biomarkers of NETs was found, which suggested that invasive gram-negative bacteria were potent in triggering NET formation *in vivo* after gut barrier disruption. Furthermore, we found that neutrophil infiltration and NET formation was increased in the leaky gut of NOD mice but not in BALB/c mice.

DNA and cellular contents released by NETs can be taken up by DCs (37) and can effectively induce the polarization of naïve T cells to Th1 and Th17 cells (38, 39). In the current study, we demonstrated for the first time that the population of memory inflammatory T cells were increased in mononuclear cells after coculturing with autologous NETs in NOD mice. Furthermore, after stimulation with NETs, enteric lymphocyte cells from MLNs exhibited a stronger migration ability to mouse insulinoma cells, which suggested the role of NETs in promoting self-islet reactive immune cell generation in GALT of NOD mice. In addition, increased NET-associated autoantibodies and the cross-reaction of IAA with NET contents in NOD mice revealed that NETs could trigger autoimmune processes in this T1D animal model.

The pathogenic role of NETs in DSS-induced colitis has been widely proven (32, 40), and PAD4 plays critical role in this

process (41). In the current study, our induction of chronic colitis in NOD mice also proved that gut leakage occurred together with the increased NET formation *in vivo*. Thereby, we observed aggravation of autoimmunity or insulitis and a stronger cross-reaction of IAA with NET contents in DSS-induced colitis mice, as well as the acceleration of T1D development, which suggested that the higher the exposure of NETs *in vivo*, the more serious the self-damage. According to the recent reports, PAD4-deficient mice may hardly generate NETs *in vivo* and barely affect other functions of neutrophils (30, 42, 43). Moreover, compared to healthy controls, neutrophils from T1D patients tended to form NETs after ionomycin stimulation (24), which indicated that PAD4-dependent NETs might contribute to the disease. When we knocked out PAD4 in NOD mice, neutrophils' ability to generate NETs was found to decrease significantly. Interestingly, the recovery of gut barrier function and a reduction in autoimmune T cells were also found in our PAD4-deficient mice, in terms of fewer autoimmune Th1/Th17 cells in the PLN or autoantibodies and milder insulitis, which resulted in lower disease incidence and better glucose tolerance. The heterodimeric integrin α4β7 expressed on T cells, specifies the recruitment of T cells to the intestinal mucosa upon its interaction with its ligand (26). In this study, a prominent increase in integrin α4β7-positive T cells in PLNs was discovered in WT but not in KO mice after DSS administration, in line with the elevated expression of integrin α4β7 in the pancreas. The data indicated that the improvement of gut barrier function and suppression of gut inflammation through affecting PAD4-dependent NET formation could control T1D development, which enriched the previous gut-affecting pancreas theory. Taking together, we have represented a schematic diagram to explain this mechanism in **Figure 7**.

In conclusion, the current study provides evidence that PAD4-dependent NET formation plays an essential mediating role in the

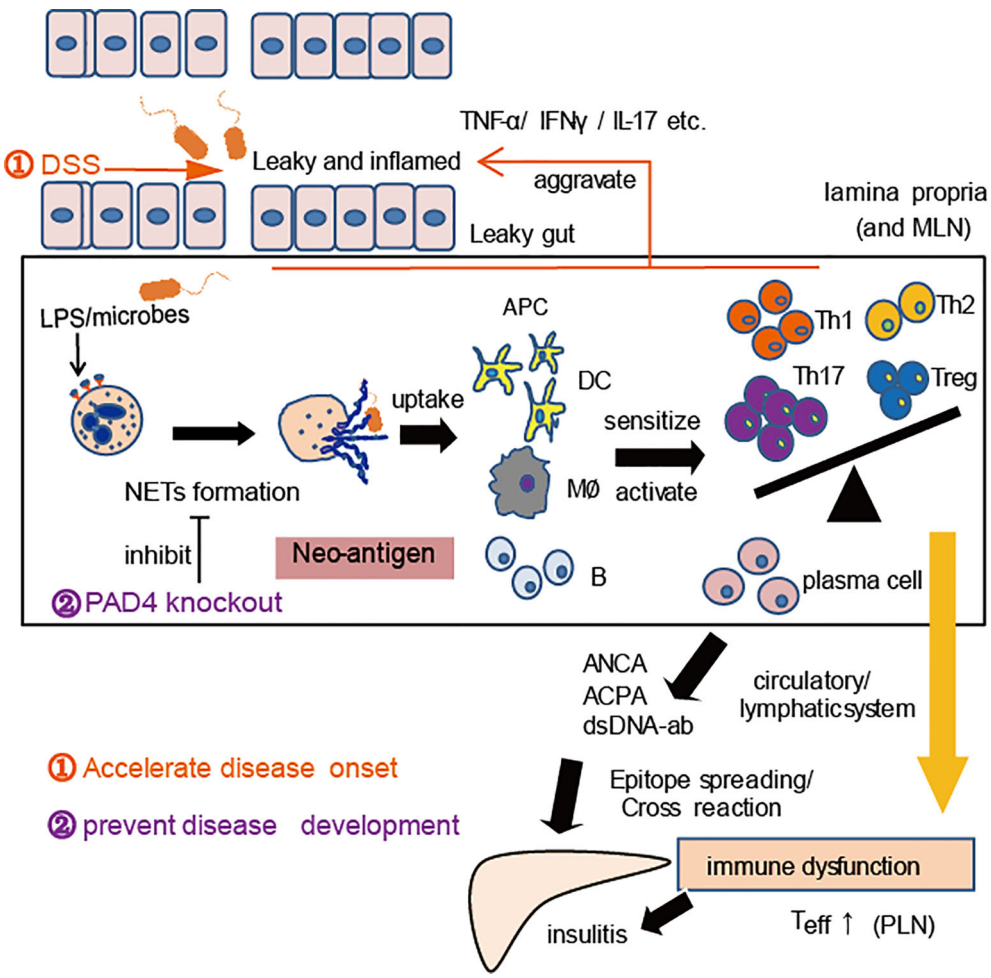

FIGURE 7 | A proposed mechanism for NET to mediate gut leakage, triggering T1D by activating the autoimmune response in NOD mice. Abnormal NET formation caused by gut barrier dysfunction and abnormal microbiota could trigger the activation of autoimmune responses and ultimately induce islet destruction. DSS treatment worsens the situation and accelerates disease onset, while PAD4 deficiency leading to a decrease of NET formation results in restoring gut barrier integrity and showing the potential of T1D prevention.

gut-pancreas axis by modulating the migration of enteric autoimmune T cells and activating autoantibodies, suggesting that clearing abnormal NETs in the gut with nuclease or blocking NET formation with PAD4 inhibitors may be a new therapy approach for T1D prevention and treatment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI [accession: PRJNA766442].

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Laboratory Center of China Pharmaceutical University.

AUTHOR CONTRIBUTIONS

JW conceived the study. QY and YW designed and performed experiments, analyzed experimental data, and prepared the manuscript. YS contributed to the cells experiment. JW, YL, CL, and FH supervised the research. JW, HG, and YS interpreted the data and revised the manuscript. QY and YS contributed equally to this work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.711423/full#supplementary-material

Supplementary Figure 1 | Commensal microbial disorders in NOD mice after 12 weeks of age. **(A)** Phylum-level phylogenetic classification of 16S rRNA frequencies in the fecal pellets of NOD/LtJ mice at 4, 8 and 12 weeks old and at the disease onset stage (n=5-6). Relative abundance of *Deferribacteres* and *Proteobacteria* **(B)**, microbial principal coordinate analysis **(C)** and 16S sequencing comparisons **(D)** in NOD/LtJ mice before 12 weeks of age *vs.* after 12 weeks of age. The bacterial families shown represent those found to be significantly different in the pairwise comparison. **(E)** Relative abundance of *Desulfovibrionaceae* and *Deferribacteraceae* in NOD/LtJ mice at 4 and 12 weeks old and at the disease onset stage. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Supplementary Figure 2 | Gut barrier dysfunction and autoimmune disorders in NOD mice compared with BALB/c mice. **(A)** Hematoxylin and eosin staining of colon tissue in female BALB/c and NOD mice. **(B)** Muscle layer thickness, gut damage score and the ratio of villus height/crypt depth in female BALB/c and NOD mice (n=3). **(C)** RT-qPCR analysis of cytokine genes encoding TNF-α, interleukin-1β (IL-1β) and IL-17A in tissue homogenates from the colons of NOD and BALB/c mice (n=5-7). Flow cytometry shows the bar graph with mean percentages ± SEM of Th17 **(D)**, Th1 **(E)** and Treg **(F)** cells within the MLNs of NOD and BALB/c mice (two independent experiments, n=3 each group every time). BALB/c and NOD mice were all after 12 weeks old **(A–C)**. The scale bars indicate 100 μm **(A)**. Data are shown as the mean ± SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$.

Supplementary Figure 3 | Western blotting analysis of h3cit in pancreas and Claudin1 in colons. **(A)** Western blotting analysis of h3cit from pancreas homogenates in DSS-colitis NOD mice (n=8) and the control (n=6). **(B)** Western blotting analysis of claudin 1 from colon tissue of WT and KO mice (n=3). Data are shown as the mean ± SEM. $^{*}P < 0.05$.

Supplementary Figure 4 | PAD4 knockout diminished neutrophil NET formation. **(A)** Detection of neutrophils from $PAD4^{-/-}$ or $PAD4^{+/+}$ mice (n= 3), forming NETs under different stimulation conditions [DMSO, ionomycin (10 μM), PMA (8 μM), fecal microbes ($2*10^{7}$)] *in vitro*. The percentage of cells that were hypercitrullinated at histone H3 **(B)** and produced NETs **(C)** in neutrophils was calculated (three quantifications for each mouse). **(D)** Lower serum levels of NET biomarkers were detected in KO mice than in WT mice (n= 6-9). **(E)** Detection of CCPA in WT and KO mice (n=6). $PAD4^{-/-}$ or $PAD4^{+/+}$ mice were all after 10-12 weeks old **(A–E)**. The scale bars indicate 100 μm **(A)**. Data are shown as the mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Supplementary Figure 5 | PAD4 deficiency diminished enteric lymphocyte T cells traveling to the pancreas. **(A)** RT-qPCR analysis of cytokine genes encoding IFN-γ in tissue homogenates from the pancreas of WT and KO mice with or without DSS treatment (n=3-4). **(B)** The Transwell system shows the migration capacity of lymphocyte cells from MLNs in WT and KO mice with or without DSS treatment (n=3). The percentage and number of enteric $CD4^{+}$ T **(C)**, Th17 **(D)** and Th1 **(E)** cells in PLNs from WT and KO mice with or without DSS treatment (n=3). **(F)** RT-qPCR analysis of integrin α4/β7 in tissue homogenates of pancreas from WT and KO mice with or without DSS treatment (n=3-4). $PAD4^{-/-}$ or $PAD4^{+/+}$ mice were all after 10-12 weeks old **(A–F)**. Data are shown as the mean ± SEM. $^{*}P < 0.05$.

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