FOOTPRINTS OF IMMUNE CELLS IN THE TYPE 1 DIABETIC PANCREAS

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FOOTPRINTS OF IMMUNE CELLS IN THE TYPE 1 DIABETIC PANCREAS

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Table of Contents

05 ***Editorial: Footprints of Immune Cells in the Type 1 Diabetic Pancreas***
Todd M. Brusko, Roberto Mallone and Teresa Rodriguez-Calvo

08 ***Immune System Remodelling by Prenatal Betamethasone: Effects on β-Cells and Type 1 Diabetes***
David Perna-Barrull, Anna Gieras, Silvia Rodriguez-Fernandez, Eva Tolosa and Marta Vives-Pi

17 ***Role of the PD-1/PD-L1 Dyad in the Maintenance of Pancreatic Immune Tolerance for Prevention of Type 1 Diabetes***
Marika Falcone and Georgia Fousteri

28 ***Molecular Footprints of the Immune Assault on Pancreatic Beta Cells in Type 1 Diabetes***
Maikel L. Colli, Florian Szymczak and Decio L. Eizirik

41 ***Pancreatic Alpha-Cells Contribute Together With Beta-Cells to CXCL10 Expression in Type 1 Diabetes***
Laura Nigi, Noemi Brusco, Giuseppina E. Grieco, Giada Licata, Lars Krogvold, Lorella Marselli, Conny Gysemans, Lut Overbergh, Piero Marchetti, Chantal Mathieu, Knut Dahl Jørgensen, Guido Sebastiani and Francesco Dotta

54 ***Chemokines as Drivers of the Autoimmune Destruction in Type 1 Diabetes: Opportunity for Therapeutic Intervention in Consideration of an Optimal Treatment Schedule***
Urs Christen and Ruta Kimmel

66 ***SARS-CoV-2 Receptor Angiotensin I-Converting Enzyme Type 2 (ACE2) Is Expressed in Human Pancreatic β-Cells and in the Human Pancreas Microvasculature***
Daniela Fignani, Giada Licata, Noemi Brusco, Laura Nigi, Giuseppina E. Grieco, Lorella Marselli, Lut Overbergh, Conny Gysemans, Maikel L. Colli, Piero Marchetti, Chantal Mathieu, Decio L. Eizirik, Guido Sebastiani and Francesco Dotta

85 ***miRNA-Mediated Immune Regulation in Islet Autoimmunity and Type 1 Diabetes***
Martin G. Scherm and Carolin Daniel

101 ***Abnormal Neutrophil Transcriptional Signature May Predict Newly Diagnosed Latent Autoimmune Diabetes in Adults of South China***
Yixuan Xing, Qiuqiu Lin, Yue Tong, Wenzhi Zhou, Juan Huang, Yanfei Wang, Gan Huang, Yanhua Li, Zhongyuan Xiang, Zhiguang Zhou, Tian Li and Yang Xiao

113 ***Gene Expression Analysis of the Pre-Diabetic Pancreas to Identify Pathogenic Mechanisms and Biomarkers of Type 1 Diabetes***
Linda Yip, Rebecca Fuhlbrigge, Reem Alkhataybeh and C. Garrison Fathman

133 ***New Insights Into the Role of Autoreactive CD8 T Cells and Cytokines in Human Type 1 Diabetes***
Christine Bender, Sakthi Rajendran and Matthias G. von Herrath

139 ***Innate Immunity Mediated Inflammation and Beta Cell Function: Neighbors or Enemies?***
Antonio Citro, Francesco Campo, Erica Dugnani and Lorenzo Piemonti

146 ***The Role of β Cell Stress and Neo-Epitopes in the Immunopathology of Type 1 Diabetes***
Jon D. Piganelli, Mark J. Mamula and Eddie A. James

156 ***Footprints of Immune Cells in the Pancreas in Type 1 Diabetes; to "B" or Not to "B": Is That Still the Question?***
Pia Leete and Noel G. Morgan

163 ***The Vbeta13 T Cell Receptor Monoclonal Antibody Reduces Hyaluronan and CD68+, CD3+, and CD8+ Cell Infiltrations to Delay Diabetes in Congenic BB DR*Lyp/Lyp *Rats***
Marika Bogdani, Linda Faxius, Malin Fex, Anita Ramelius, Anya Wernersson, John P. Mordes, Elizabeth P. Blankenhorn and Åke Lernmark

173 ***Proinsulin-Reactive CD4 T Cells in the Islets of Type 1 Diabetes Organ Donors***
Laurie G. Landry, Amanda M. Anderson, Holger A. Russ, Liping Yu, Sally C. Kent, Mark A. Atkinson, Clayton E. Mathews, Aaron W. Michels and Maki Nakayama

188 ***Islet-Resident Dendritic Cells and Macrophages in Type 1 Diabetes: In Search of Bigfoot's Print***
Henner Zirpel and Bart O. Roep

197 ***Phagocytosis and Efferocytosis by Resident Macrophages in the Mouse Pancreas***
Kristel Parv, Nestori Westerlund, Kevin Merchant, Milad Komijani, Robin S. Lindsay and Gustaf Christoffersson

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EDITORIAL
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Editorial: Footprints of Immune Cells in the Type 1 Diabetic Pancreas

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Editorial on the Research Topic

Footprints of Immune Cells in the Type 1 Diabetic Pancreas

The Footprints of Immune Cells in the Type 1 Diabetic Pancreas Research Topic represents a collection of review articles, perspective pieces, and original research articles, which together create a conceptual framework describing the interactions between cells within the islets of Langerhans and components of the innate and adaptive immune system. The cellular interactions occurring within tissues represent crucial events during the natural history and pathogenesis of the disease, and are not completely understood when studied in isolation. Access to human pancreas samples, through a number of tissue biorepositories, has dramatically improved our collective understanding of type 1 diabetes (T1D), highlighting a number of outstanding questions and key knowledge gaps within the field. Although T1D has been traditionally considered a disease of autoimmune origin, the concept of intracellular stress as a triggering event has gained considerable attention as a means by which β-cells may contribute to their own demise.

In a mini-review article, Piganelli et al. described how, under conditions of high endoplasmic reticulum (ER) stress, β-cells are prone to changes in function and immunogenicity that could lead to the formation of novel antigenic epitopes. The authors described how the unique physiology of β-cells and the extreme metabolic burden of insulin synthesis and secretion, may make them more vulnerable to certain environmental stressors. In addition, genetic risk variants expressed within β-cells may predispose susceptible individuals to increased stress and damage.

Along these lines, and with special relevance given the global coronavirus disease 2019 (COVID-19) pandemic, Fignani et al. investigated the expression of the severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) receptor Angiotensin I-Converting Enzyme Type 2 (ACE2) in β-cells and within the islet microvasculature. The authors examined human pancreas samples and reported that ACE2 could be detected in human islets. It was preferentially expressed in distinct subsets of β-cells, in addition to pericytes and some ductal cells, though β-cells primarily expressed the short-ACE2 isoform, which lacks the SARS-CoV-2 high-affinity binding sites. Proinflammatory cytokines increased the expression of ACE2 in a β-cell line and in isolated islets, indicating a potential link between inflammation and ACE2 expression. These data add an additional element to a timely question related to whether or not SARS-CoV-2 can actively infect β-cells and contribute to diabetes.

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In a systematic review article, Colli et al. evaluated selected publicly available RNA-seq datasets of pancreatic human islets or FACS-purified human β-cells exposed to: 1) pro-inflammatory stimuli (IL-1β + IFN-γ or IFN-α), 2) metabolic stressors (palmitate), or 3) the local environment present during T1D development (primary β-cells from patients). The biological processes regulated by the transcripts from β-cells exposed to IFN-α closely recapitulated those observed in β-cells from T1D subjects. Transcriptional profiles contained a gene expression signature related to responses to type I interferons (IFN-α/β), MHC class I antigen presentation, activation of tumor necrosis factor receptor subunits and ubiquitination. Conversely, biological processes regulated by the transcripts obtained from T1D subjects *vs*. β-cells exposed to IL-1β + IFNγ, included gene signatures related to immune infiltration like cell adhesion, immunoregulatory interaction between lymphoid and non-lymphoid cells, and PD-1 signaling. The authors also identified several classes of compounds with some potential to revert β-cell inflammation during the natural history of the disease, including bile acids, bromodomain inhibitors, leucine-rich repeat kinase (LRRK) inhibitors and vitamin D receptor agonists. Likewise, Yip and colleagues identified various immune pathways related to cell adhesion, insulin secretion, glucose metabolism and pancreas development that were differentially expressed in the pancreas of autoantibody positive (AAb+) individuals and partially overlapped with changes observed in pre-diabetic NOD mice. The genes included *RGS16, CLEC4D*, and *FCGR2B*, which are enriched in leukocytes and exhibited reduced expression in samples from AAb+ individuals. The authors hypothesized that reduced expression of *FCGR2B* in pre-diabetic individuals, detected in blood and pancreas, could lead to hyper-responsiveness, proliferation and maturation of autoreactive B cells contributing to a loss of tolerance and progression of disease.

PD-1 signaling represents an important negative regulator of T cell activation, with implications for controlling autoreactivity in T1D. Falcone and Fousteri reviewed the role of the PD-1/PD-L1 axis in the maintenance of immunological tolerance, described mechanisms by which this pathway is regulated, and discussed how alterations in this checkpoint could contribute to islet autoimmunity. The authors further discussed how the microbiota may alter the PD-1/PD-L1 axis, as well as recent findings in subjects treated with immune checkpoint inhibitors for cancer immunotherapy (i.e., anti-PD-1 monoclonal antibody). The authors concluded by listing strategies to target this pathway to bolster regulation and avert islet autoimmunity.

Given the interactions between islets and tissue-resident immune cells, multiple authors centered their work on the cross-talk between β-cells and innate immune subsets. Macrophages, dendritic cells (DCs), and neutrophils are often the first cells to interact with potentially abnormal β-cells and provide a link with the adaptive immune system, as suggested in a perspective article by Zirpel and Roep. They highlighted the possible roles of macrophages and DCs, and the importance of further understanding the changes between benign leukocyte residency and pathogenic infiltration. Citro et al. focused on macrophages and neutrophils, and how cytokines in the islet niche could modulate insulin secretion and β-cell function. The authors discussed the importance of their modulation for the protection and/or improvement of islet function. Xing et al. investigated the transcriptional profile of patients with latent autoimmune diabetes in adults (LADA) and identified neutrophilic dysfunction, with enhanced activation of degranulation, adhesion and migration at the transcriptional level. Parv et al. investigated the different polarization of macrophages in the pancreas using a mouse model. The authors found that endocrine-resident macrophages were more efficient at performing efferocytosis, a homeostatic process that clears endogenous cells, and phagocytosis, both *in vitro* and *in vivo*, than exocrine-resident macrophages. The authors point out the need to further understand the intrinsic differences between endocrine and exocrine innate immune cells, with respect to their ability to regulate autoimmunity, providing a potentially interesting new line of investigation.

In addition to innate subsets, B cells within the adaptive arm of the immune system are thought to participate in T1D progression. This notion emanates from their appearance as a prognostic indicator of disease and invites the hypothesis of a role as non-professional antigen presenting cells. However, the exact role of B cells in T1D pathogenesis, particularly within the islet microenvironment, remains poorly understood. Leete and Morgan discuss the preferential localization of B cells in T1D in regard to their detection in peripheral blood, secondary lymphoid organs, and islets. They highlight studies reporting a notable increase in islet B cells in individuals diagnosed with T1D in early life. The authors propose that B cells might promote the activation of autoreactive $CD8^+$ T cells in the islets and highlight therapeutic strategies for attenuating B cell function.

Autoreactive T and B cells are linked, and their collaboration can drive immune-mediated pathology. $CD4^+$ T cells have been detected at low frequencies in the islets of T1D subjects but their numbers may be more prominent at the earliest stages of the disease. Landry et al. evaluated preproinsulin reactivity among $CD4^+$ T cells isolated from islets of six organ donors with T1D. The authors identified 14 T cell receptor (TCR) clonotypes, which recognized proinsulin peptides (A- and B-chain, and C-peptide) presented by various HLA Class II molecules, and observed a trend towards dominant restriction by HLA-DQ. However, citrullination of insulin B-chain peptides did not induce stronger responses in the TCRs reactive against the native form. $CD8^+$ T cells reactive against preproinsulin have similarly been found in the pancreas of T1D subjects, and in this Research Topic, Bender et al. review the differences in autoreactive $CD8^+$ T cell frequency between peripheral blood and pancreas in individuals with and without T1D, and how cytokines secreted by stressed β-cells could attract $CD8^+$ T cells to the islets. The authors highlight the role of the chemokine CXCL10, among other chemokines, for its capacity to attract T cells, which express the CXCL10 receptor, CXCR3. They also discussed numerous studies that have investigated CXCL10 expression in α- and β-cells, with mixed results. In an attempt to provide a definitive answer, Nigi et al. investigated the expression of CXCL10 among endocrine cell subtypes in NOD mice and human tissue. CXCL10 was observed in murine α- and β-cells, but the colocalization and expression increased in α-cells of diabetic mice. Likewise, CXCL10 was preferentially expressed in

α-cells in the islets of subjects with T1D while it was absent in control donors. Christen and Kimmel reviewed the role of chemokines in T1D, focusing especially on the CXCL10/CXCR3 axis. They discussed the therapeutic potential of neutralizing chemokines as part of combination therapies with T-cell targeting drugs like anti-CD3. The authors ultimately hypothesize that the blockade of CXCR3 chemotaxis may provide a novel combinatorial strategy to prevent the migration of diabetogenic T cells from secondary lymphatics.

The enrichment of autoreactive T cells within the islets in T1D provides strong evidence for a loss of immune tolerance. Regulatory T cells (Tregs) are found in very low numbers within the islets, and their stability and function are not well understood in the context of human T1D. In their review article, Scherm and Daniel explore the regulatory potential of miRNAs, including how miRNAs regulate effector T cell function, and their use as biomarkers of islet autoreactivity, with a special section dedicated to miRNA regulation of Tregs. In addition to providing a comprehensive list of miRNAs involved in immune regulation, the authors highlight three miRNAs (miR92a-3p, miR181a-5p, miR142-3p) and describe their function in detail.

The articles noted above underscore the critical cellular interactions linking the immune effectors and targets of autoimmunity in T1D. A number of articles highlighted exciting opportunities to direct therapies not only at the immune system, but also to protect β-cells within islets. Indeed, Perna-Barrul et al. discuss the potentially protective role for betamethasone, a drug routinely administered to mothers at risk of preterm birth, on preserving β-cells function and restoring proper interactions with the immune system in early life. The authors speculate that betamethasone could be used as a protective agent shortly before birth or in the perinatal period based on its capacity to make β-cells less immunogenic. Bogdani et al. explored the therapeutic potential of a Vβ13a TCR monoclonal antibody, 17D5. They demonstrate that 17D5 delays spontaneous diabetes onset in DRLyp/Lyp rats. Some rats did not develop disease and retained a high proportion of insulin containing islets, with reductions in hyaluronan deposits, $CD68^+$, $CD3^+$ and $CD8^+$ infiltration.

Overall, the studies outlined in this Research Topic highlight the critical need for a deeper understanding of this organ-specific autoimmune disease and the exigence for therapeutic modalities that can act at the target organ to reduce cellular stress, immunogenicity, and preserve long-term immune tolerance to pancreatic β-cells in subjects with or at risk for T1D.

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All the authors have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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Immune System Remodelling by Prenatal Betamethasone: Effects on β-Cells and Type 1 Diabetes

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Type 1 diabetes (T1D) is a multifactorial disease of unknown aetiology. Studies focusing on environment-related prenatal changes, which might have an influence on the development of T1D, are still missing. Drugs, such as betamethasone, are used during this critical period without exploring possible effects later in life. Betamethasone can interact with the development and function of the two main players in T1D, the immune system and the pancreatic β-cells. Short-term or persistent changes in any of these two players may influence the initiation of the autoimmune reaction against β-cells. In this review, we focus on the ability of betamethasone to induce alterations in the immune system, impairing the recognition of autoantigens. At the same time, betamethasone affects β-cell gene expression and apoptosis rate, reducing the danger signals that will attract unwanted attention from the immune system. These effects may synergise to hinder the autoimmune attack. In this review, we compile scattered evidence to provide a better understanding of the basic relationship between betamethasone and T1D, laying the foundation for future studies on human cohorts that will help to fully grasp the role of betamethasone in the development of T1D.

Keywords: prenatal betamethasone, Type 1 diabetes, immune system, β cell, glucocorticoid

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BETAMETHASONE AS AN EMERGING ENVIRONMENTAL FACTOR IN T1D

Type 1 diabetes (T1D) is an autoimmune disease caused by the selective destruction of insulin-producing β-cells. The trigger, however, remains unknown. Postnatal environmental determinants have been thoroughly studied as risk factors (1, 2) but a crucial phase for the immune system development, the late prenatal stage, has been poorly investigated. Specifically, the interaction of drugs commonly used during late pregnancy with T1D and the pancreatic β-cells remains unexplored. Nonetheless, some studies reveal the importance of the prenatal stage and the prematurity of the newborn in the development of T1D (3–5). An indirect demonstration of how critical the *in utero* environment is in T1D development arises from the studies in twins: heterozygotic twins have an increased concordance of T1D when compared to non-twin siblings (6, 7), underlining the potential relevance of prenatal factors and their influence in the development of autoimmunity.

Synthetic glucocorticoids, most often betamethasone, are routinely given to mothers at risk of preterm birth between 24 and 34 weeks of gestation. A single course of prenatal

betamethasone reduces the occurrence and severity of respiratory distress syndrome and improves the survival chances in premature infants (8, 9). Another glucocorticoid used for lung maturation is dexamethasone and produces similar results on the newborn survivability (10). These synthetic glucocorticoids cross the placenta and accelerate foetal lung maturation, achieving maximum benefit between 24 h and 7 days after administration (11). Betamethasone is a poor substrate for the glucocorticoid inactivating enzyme 11beta-hydroxysteroid-dehydrogenase 2 (11βHSD2), therefore, its bioactivity in the foetus lasts for several days (12) and it is known to exert long-lasting effects on the hypothalamic-pituitary-adrenal (HPA) axis and cognition in children (13, 14).

Glucocorticoids exert their effects by binding nuclear receptors that are ligand-dependent transcription factors. They can regulate gene transcription, either by direct binding to DNA or by interacting with other transcription factors (15). Glucocorticoid receptors (GR) are ubiquitously expressed; however, due to the variation in the genomic location of GR binding, the transcriptional responses to glucocorticoids are cell type-specific (16). Moreover, polymorphisms of the GR result in alterations in their responsiveness to glucocorticoids and in gene expression (17, 18). In addition, human GR receptor can be a target of endoncrine disruptors such as pesticides (19) that, in combination with antenatal glucocorticoids, could increase developmental neurotoxicity (20).

The general effects of glucocorticoids administered during pregnancy have been thoroughly reviewed (21). Considering the overwhelming use of betamethasone as the treatment of choice for respiratory distress syndrome in premature infants and the cell-specific response to glucocorticoids, in this review we will dissect the specific effects of betamethasone on the main cellular players in the context of T1D, namely immune cells and their targets, the β-cells of the pancreas.

DIRECT EFFECTS OF BETAMETHASONE ON THE IMMUNE SYSTEM

Several cell types of the immune system are involved in the development of T1D, and disturbances in the activity of these cells, such as enhanced proinflammatory activity, can increase the risk to develop T1D (22). Below, the effect of betamethasone on different cell types of the immune system is detailed.

Innate Immune Cells

Prenatal administration of betamethasone can induce an anti-inflammatory status in the newborn during the first days after delivery (23), and this fact could be due to the immunomodulatory effects of betamethasone on innate immune cells.

Neutrophils

Neutrophils have gained interest in T1D aetiology due to their participation in the initial steps of autoimmunity against β-cells (24). Moreover, neutrophils are part of the islet leukocytic infiltrates of patients with T1D, and are accordingly reduced in peripheral blood at disease onset (25, 26).

A described effect of betamethasone is the increase in leukocyte counts in peripheral blood after treatment (27), similarly to the effects of natural glucocorticoids during stress (28). Accordingly, neutrophil number and percentage were increased in human blood after betamethasone treatment (29), correlating with the described neutrophil demargination into the blood vessels (30–32). Moreover, in humans, betamethasone reduces neutrophil motility and chemotaxis (33), and can affect metabolism and cytokine production, i.e., reducing interleukin (IL)-8 and macrophage inflammatory protein alpha (MIP-1α) release (34). The inflammatory capacity of neutrophils is therefore reduced, as demonstrated in a lamb model of lung inflammation after betamethasone treatment, where gene expression of *IL-1, IL-6, IL-8,* and *CCL2* was suppressed (35).

Monocytes

Monocytes are circulating innate immune cells that can become antigen-presenting cells (APCs), either macrophages, or dendritic cells (DCs). Thus, reprogramming monocytes may lead to changes in both differentiated cells. Betamethasone has an acute effect on the metabolism of monocytes, transiently reducing the production, and the secretion of IL-6 and reactive oxygen species. By contrast, the phagocytic activity of monocyte-derived APCs was not altered by betamethasone (36). In newborn children with low weight at birth, prenatal betamethasone administration induced a transient immunomodulatory effect in monocytes, causing diminished IL-6 and IL-10 release and downregulation of human leukocyte antigen DR (HLA-DR) expression (37). Moreover, the total number of monocytes was reduced by betamethasone (38). This effect was also assessed *in vitro,* demonstrating that glucocorticoids induce apoptosis in human monocytes (39). Nevertheless, these results are controversial, and other authors reported that betamethasone does not affect monocytes' IL-6 production (40).

Macrophages

Macrophages are crucial in the initial damage to β-cells in T1D. These tissue-resident APCs contribute to initiate specific immune responses (41). In macrophages, betamethasone diminishes cytokine secretion (IL-8 and TNFα) (42) and impairs their ability for antigen presentation to T cells (43). These effects point to the induction of a regulatory profile in macrophages, similar as described in M2 macrophages (44). Indeed, dexamethasone induces the polarization of the M2 phenotype (45). Moreover, it was recently reported that dexamethasone increases the migration of macrophages by CD26 overexpression, a membrane glycoprotein with enzymatic capabilities involved in inflammation, and this could contribute to the egress of macrophages from inflamed tissue (46).

Dendritic Cells

DCs are professional APCs with the ability to stimulate naïve T cells. In T1D, DCs are responsible for the presentation of β-cell autoantigens to T lymphocytes, initiating the adaptive autoimmune response against the insulin-producing cells. Similarly to the observed effect of dexamethasone on this cell type, DCs differentiated *in vitro* in the presence of betamethasone

failed to achieve a fully mature status, showing a reduced capacity to stimulate the production of IL-17, a cytokine involved in autoimmune responses, by T lymphocytes (32). Furthermore, the release of proinflammatory cytokines was reduced by this drug in DCs. Betamethasone has been reported to induce tolerogenic Langerhans DCs (LDCs) in the skin of patients with psoriasis (47) and atopic dermatitis, which in turn arrest T helper (Th) 1 and Th2 responses (48). Similarly to the effects found in monocytes, human DCs differentiated with betamethasone showed a reduction of membrane expression of costimulatory molecules, such as CD40 and CD86, accompanied by a decrease in IL-12 secretion, an important cytokine for Th1 responses. These effects resulted in tolerogenic function in DCs and impaired ability to induce T lymphocyte proliferation (39).

Natural Killer Cells

Natural Killer cells (NKc) are effector lymphocytes of the innate immune system. Their role in T1D is not completely understood, but abnormalities in this cell type may contribute to trigger autoimmune reactions against β-cells (49). Little is known about the effects of betamethasone on NKc. Betamethasone tends to increase NKc activity in very preterm newborn babies (<32 weeks of gestation), supporting the maturation of this cell type (40). However, other studies have reported that betamethasone reduces the number of NKc in newborn infants (50). In adults, NKc showed a reduced cytolytic activity after topical betamethasone administration (51).

Adaptative Immune Cells

Innate immune cells are crucial in the first phases of autoimmune diseases, but the final effector cells are the adaptative immune system cells, T and B lymphocytes. Modulation of these cells can dampen or exacerbate an autoimmune reaction.

T Lymphocytes

Betamethasone treatment results in T cell precursor apoptosis and, to a lesser extent, of mature T cells. Transient reduction in thymus weight and thymocyte numbers have been described after prenatal betamethasone administration in mice (32, 52). In humans, the thymus of the foetus of mothers that were prenatally treated with steroids showed delayed growth (53). Moreover, a reduction of 20–30% of peripheral lymphocyte counts was observed in pregnant women after treatment with betamethasone, although this effect only lasted for 3 days (27, 38, 54). Other glucocorticoids, like dexamethasone, have comparable effects on lymphocytes after prenatal treatment (55). In newborn children, a similar effect on lymphocyte counts has been described, mainly affecting CD4+ T lymphocytes (56). Data on lymphocyte counts are still controversial, since a different study reported an increase in CD3+ T lymphocytes in very preterm newborn babies (<32 gestational weeks) after betamethasone treatment (50). Prenatal betamethasone administered to the experimental model of T1D, the non-obese diabetic (NOD) mouse, resulted in long-lasting changes in the T Cell Receptor (TCR) Vβ repertoire that persisted into adulthood (32). Importantly, the TCR Vβ families that diminished in frequency after prenatal steroid treatment included pathogenic Vβ domains (57, 58), so it is reasonable to speculate that betamethasone will protect against T1D. In humans, betamethasone reduces T lymphocyte proliferation capacity (40), thus reducing clonal expansion. Overall, T cells will have an impaired capacity of interacting with β-cell antigens, thus contributing to prevent the autoimmune response.

B Lymphocytes

The role of B lymphocytes in the development of T1D is not completely understood. B cells produce autoantibodies to islet antigens that, even if extremely useful as predictive biomarkers for disease, do not appear pathogenic. Also, B cells are critical as APCs during the first stages of autoimmunity in T1D (59). Betamethasone has a deleterious effect on mature B cells of NOD mouse (32) and reduces their ability to produce antibodies, specifically IgE and IgG (60, 61). Other glucocorticoids, such as dexamethasone, show a similar deleterious effect (62), affecting early precursor B cells, whereas mature B cells –IgD positive– are resistant to glucocorticoid-induced apoptosis (63). The reduction observed in antibody production could be the result of impaired B cell receptor and Toll-like receptor 7 signalling, since without these signals B cells cannot switch their Ig isotype, thus reducing their functionality. At the same time, B cells have increased transcriptional activity of *IL-10,* amplifying the immunomodulatory capacity of these cells induced by glucocorticoids (16).

EFFECTS OF BETAMETHASONE ON THE TARGET CELLS OF AUTOIMMUNE DIABETES, THE ISLET β-CELLS

β-cells are the insulin-producing cells of the islets of Langerhans. The autoimmune destruction of these cells is the ultimate cause of T1D. β-cells also have an active role in their own destruction, facilitating the interaction with the immune system, and contributing to their own demise (64). Thus, the identification of changes induced by betamethasone may help to understand the possible outcome of this drug in the context of T1D. Studies performed in subjects with long term glucocorticoid treatment indicate that glucocorticoids can induce dysglycaemia, leading to diabetes (65, 66). Glucocorticoids increase insulin resistance (67) without affecting β-cell mass (68). On the other hand, physiological endogenous levels of glucocorticoids are necessary for maintaining the regulation of insulin secretion by β-cells (69). Moreover, prenatal glucocorticoids support the maturation of β-cells by enhancing their glucose sensitivity due to increased expression of *Glut2* and *Gck* genes and by reducing apoptosis, similarly as the overexpression of surfactant proteins induced by glucocorticoids helps with the maturation of the foetal lungs (70). In a similar way, prenatal glucocorticoids enhance insulin secretion in rats due to the overexpression of *Gck*, *Slca2,* and *Ins2* genes in β-cells, despite β-cell mass is smaller than in non-treated animals (71). Experimental data demonstrate that prenatal betamethasone reduces the risk of developing T1D in the NOD mice (57), correlating to altered

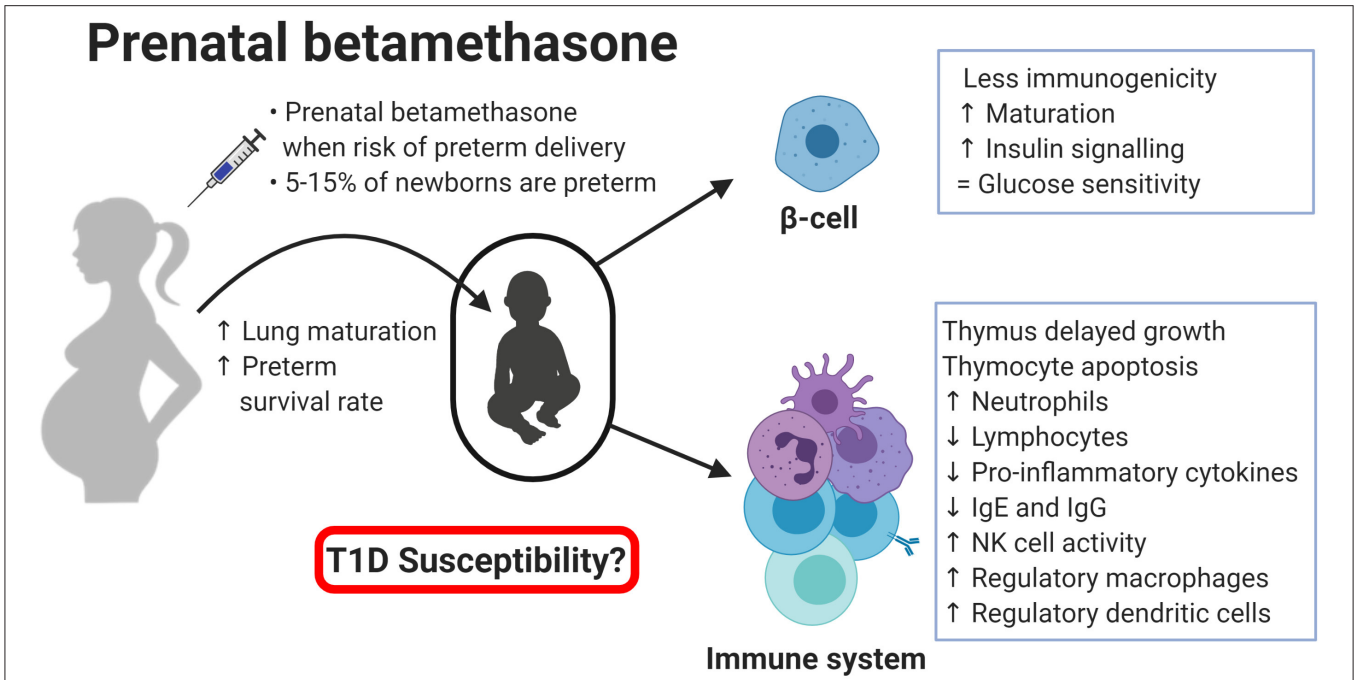

FIGURE 1 | Effects of prental betamethasone on β-cells and the immune system of the newborn. Created with BioRender.com.

expression of genes related to metabolism and autoimmunity in β-cells. In a previous study we reported a reduction of *Ccl2* gene expression in β-cells, which may lead to a reduced recruitment of macrophages and monocytes. Moreover, an increase in *Gad1* gene expression, could promote tolerance to β-cells in NOD mice (32). Recent studies in other T1D experimental models indicate that short-term treatment with betamethasone during late pregnancy does not affect β-cell metabolism in later life (72, 73). Glucocorticoid signalling can also cause epigenetic modifications in these cells. In fact, glucocorticoids impair the methylation of the DNA by altering the enzymes responsible for this process. Moreover, the prenatal period is a very sensitive phase during which the epigenome shows heightened plasticity to methylation modifications and these changes can be accumulated throughout life (74). Important β-cell functions, such as insulin secretion and islet cell mass homeostasis, are controlled by epigenetic mechanisms (75), and glucocorticoids can modify the epigenome of β-cells inducing changes that can affect their function in adults (76), altering the efficiency of glucose metabolism (77, 78).

HOW CAN BETAMETHASONE AFFECT THE INTERPLAY BETWEEN THE IMMUNE SYSTEM AND β-CELLS?

T1D is a multifactorial disease with complex interactions between the immune system and the pancreatic β-cells. Glucocorticoids are potent immune suppressors and are commonly used in patients with autoimmune diseases such as psoriasis or rheumatoid arthritis (79, 80). Betamethasone, like other synthetic glucocorticoids, can reduce cytokine production and release, thereby inhibiting specific immune responses and blocking the initiation of an autoimmune attack to β-cells (2). Dampening the autoimmune reaction can be the most efficient form of preventing T1D, and it might be a consequence of the impaired functionality of innate and adaptative immune cells (**Figure 1**). On the one side, betamethasone diminishes the proinflammatory action of innate immune cells (neutrophils, macrophages, and NK cells). On the other side, this drug induces a tolerogenic antigen presentation in macrophages and DCs, limiting the possibilities to activate autoreactive T lymphocytes in the lymph nodes. In turn, lymphocytes are also affected by betamethasone, as aforementioned. T lymphocytes reduce their proliferation capacity, decreasing the number of cells that can kill the β-cells. At the same time, prenatal treatment critically reduces the number of developing thymocytes and induces a skewed TCR repertoire towards T cells with less affinity to β-cell autoantigens (57). This fact will impair autoreactivity when APCs expose β-cell autoantigens in Major Histocompatibility Complex (MHC) molecules. Moreover, in the presence of betamethasone, T lymphocytes tend to differentiate to Th2 rather than autoimmunity-prone Th1 or Th17 cells (81), and it has been demonstrated that glucocorticoids exposure during foetal development can alter the HPA axis, impairing CD8+ T lymphocytes function later in life, making them less responsive against viral antigens (82), or blunting cortisol response against rhinovirus (83). In this sense, concerns have been raised about multiple doses of prenatal betamethasone, including an increased susceptibility to infections in children (84, 85). B cell precursors

TABLE 1 | Studies on the effect of betamethasone in the immune system and impact on T1D.

Species/substrates	Main findings	Expected effect on T1D	References
Human	↓ NKc cytolytic capacity	Protective	(49)
Human	↓ IgE synthesis by B cells	Neutral	(59)
Human	↑ Insulin resistance (long-term) No effect in T1D prevalence (long-term)	Neutral	(80)
Human	↑ T1D Hazard ratio after glucocorticoid treatment	↑ Risk	(81)
Human	↑ Neutrophils ↓ Basophils, CD3+CD4+, and CD3+CD8+ T cells	Protective	(27)
Human (adult monocytes)	↓ DCs costimulatory molecules ↓ IL-12p70 ↓ Th1 activation	Protective	(37)
Human (adult skin cells)	↓ LDCs costimulatory molecules and HLA-DR ↓ Proinflammatory cytokines No effects in IL-10 secretion or ILT3 expression	Protective	(45)
Human (cord blood of preterm babies)	↑ NKc activity (<32 weeks gestation) ↓ Lymphocyte proliferation No effect in IL-6 secretion	Protective	(38)
Human (cord blood)	↓ IL-6, IL-8 and TNFα secretion by macrophages	Protective	(40)
Human (newborn and adult)	↓ migration and motility of newborn's neutrophils No effects in adult's neutrophils	Neutral	(31)
Human (newborn)	↓ IL-8 and CCL3 secretion from neutrophils	Protective	(33)
Human (newborn)	↓ HLA-DR expression on monocytes ↓ IL-6 and IL-10 in plasma	Protective	(35)
Human (newborn)	↑ CD3+ T cells and monocytes ↓ NKc	↑ Risk	(48)
Human (newborn)	↓ $CD4^+$ and $CD25^+$ T lymphocytes	Protective	(54)
Human (pregnant women)	↑ Leukocytes and granulocytes ↓ Lymphocytes	Neutral	(25)
Human (pregnant women)	↑ Leukocytes ↓ Lymphocytes and monocytes	Neutral	(36)
Human (pregnant women)	↑ Neutrophils ↓ Lymphocytes	Neutral	(52)
Mouse (NOD)	↓ Immunogenicity ↑ Tolerance	Protective	(30)
Mouse (NOD)	↓ T1D incidence ↓ Diabetogenic Vβ TCR	Protective	(55)
Mouse	↓ Impaired antigen presentation by macrophages	Protective	(41)
Mouse	↓ Th1 and Th2 induction by LDCs	Protective	(46)
Mouse	↑ Apoptosis of thymocytes ↓ Thymus weight	↑ Risk	(50)
Sheep	↓ IL-1, IL-6, IL-8, CCL2, and TLR4 expression	Protective	(33)
Sheep	↓ IL-6 and ROS from monocytes	Protective	(34)
Sheep	No long-term effects Improves preterm delivery adverse effects	Neutral	(69)
Sheep	No impairment of insulin sensitivity ↑ Insulin signalling pathway	Neutral	(71)
Rabbit	↓ B cells IgG+	Neutral	(58)

CCL, C-C motif ligand; HLA, human leukocyte antigen; ILT3, immunoglobulin-like transcript 3; IL, interleukin; IgE, immunoglobulin E; IgG, immunoglobulin G; LDCs, Langerhans dendritic cells of the skin; NKc, Natural killer cells; ROS, reactive oxygen species; T1D; type 1 diabetes; TCR, T cell receptor; Th, T helper; TLR, toll like receptor; TNF, tumour necrosis factor.

are also affected by betamethasone, showing reduced antibody production (63). However, how these effects altogether might influence the development of T1D is not yet known. Taken together, these alterations suggest a rather positive effect leading to T1D protection, but this effect could depend, among other factors, on the concentration, and duration of the prenatal treatment. In addition, β-cell changes induced by betamethasone may enhance this protective effect. Especially, β-cell maturation and the acquisition of an apoptosis-resistant phenotype may be key factors in thwarting undesired autoimmune reactions (70). Moreover, alterations found in the expression of genes related to interactions between the immune system and β-cell reduce β-cell immunogenicity, hindering their direct interaction with immune system cells (32). This could help to avoid the activation of stray cytotoxic T cells with affinity to β-cell autoantigens. How long this effect is maintained is unknown, but it is reasonable to speculate that prenatal betamethasone could reprogramme some aspects of β-cell function until adult life, without affecting their intrinsic capacities as described for insulin secretion (86). Furthermore, glucocorticoid stimulation also induces epigenetic changes in the precursor β-cells (76), and this could be the main actor behind the long-term effects observed after prenatal

administration of betamethasone. We have summarised those studies that used betamethasone in their research (**Table 1**), focusing on their findings in the context of the immune system and β-cells, and the expected effect these changes would have on T1D. Further studies are needed to reveal the long-term effects of prenatal betamethasone treatment in the immune system and T1D (87).

FUTURE PERSPECTIVES

The effectiveness of glucocorticoids has been demonstrated for a wide range of immunologically related diseases. However, their effects during the prenatal period, both in the immune system and the target tissue of T1D, are not fully characterised. Expanding the understanding of how they can affect self-tolerance and T1D could contribute to reduce the increasing incidence of this and other autoimmune diseases. Another key point is to determine whether the effects of glucocorticoid treatment found in immune system cells could result from changes induced in hematopoietic stem cells, thus explaining the alterations found in many immune cell types. Further studies are required to dissect the exact mechanism and the magnitude of these changes in the immune system and β-cells, because other factors, such as maternal nutrition or stress during pregnancy, could also veil betamethasone effects (88). Finally, epidemiological studies are needed to explore the effect of prenatal betamethasone on T1D. Finding pieces of evidence of the precise effects of betamethasone in T1D development could lead to improved neonatal care, with special focus on children with higher genetic risk to suffer from T1D.

CONCLUSION

Knowledge about betamethasone action on the immune system is currently increasing, but it is very limited in the prenatal stage as well as in its consequences in the adulthood phase. Glucocorticoids have shown a plethora of effects, which depend on the duration of the treatment, the route of administration, the target tissue, etc. The key message of this review is that prenatal betamethasone affects the immune system cells, and these alterations may have long-term consequences. Simultaneously, betamethasone also alters β-cells towards a less immunogenic phenotype and could induce epigenetic modifications in immune cells and β-cell precursors. Considering these effects, it is tempting to speculate that betamethasone may act as a protective agent against human T1D when administered shortly before birth or in the perinatal period. Future immunological, metabolic, and epidemiological studies, together with the extrapolation of data from other glucocorticoids like dexamethasone, will shed light on the unanswered questions related to this prenatal treatment.

AUTHOR CONTRIBUTIONS

DP-B and MV-P wrote the manuscript. AG, SR-F, and ET participated in the literature review, and edited the manuscript while adding additional insights. All authors read and approved the final version of the manuscript.

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REVIEW
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Role of the PD-1/PD-L1 Dyad in the Maintenance of Pancreatic Immune Tolerance for Prevention of Type 1 Diabetes

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The human pancreas, like almost all organs in the human body, is immunologically tolerated despite the presence of innate and adaptive immune cells that promptly mediate protective immune responses against pathogens *in situ*. The PD-1/PD-L1 inhibitory pathway seems to play a key role in the maintenance of immune tolerance systemically and within the pancreatic tissue. Tissue resident memory T cells (TRM), T regulatory cells (Treg), macrophages and even β cells exhibit PD-1 or PD-L1 expression that contributes in controlling pancreatic immune homeostasis and tolerance. Dysregulation of the PD-1/PD-L1 axis as shown by animal studies and our recent experience with checkpoint inhibitory blockade in humans can lead to immune dysfunctions leading to chronic inflammatory disease and to type 1 diabetes (T1D) in genetically susceptible individuals. In this review, we discuss the role of the PD-1/PD-L1 axis in pancreatic tissue homeostasis and tolerance, speculate how genetic and environmental factors can regulate the PD-1/PD-L1 pathway, and discuss PD-1/PD-L1-based therapeutic approaches for pancreatic islet transplantation and T1D treatment.

Keywords: type 1 diabetes (T1D), programmed death 1 (PD-1), programmed death ligand 1 (PD-L1), immune tolerance, immune homeostasis, immunotherapy, pancreatic islet transplantation, pancreas

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INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease mediated by T-cell destruction of the insulin-producing β-cells in the pancreatic islets of Langerhans (1). The critical link between the Programmed death 1 (PD-1)/PD-L1 pathway and constraint of T1D has been demonstrated in numerous studies and has paved the way for novel therapeutic approaches. PD-1 is an inhibitory molecule belonging to the class of co-stimulatory molecules expressed on the surface of T cells that has been linked to immune tolerance (2). PD-1 is a member of the CD28 and CTLA-4 immunoglobulin superfamily and interacts with two B7 family ligands, PD-L1 (CD274) and PD-L2 (CD273) (3). PD-L1 is widely distributed on leukocytes and non-hematopoietic cells in lymphoid and non-lymphoid tissues, including pancreatic islets, whereas PD-L2 is expressed exclusively on dendritic cells (DCs) and monocytes (4, 5).

Upon binding to ligands PD-L1 and PD-L2, PD-1 recruits SHP2 phosphatase, which then dephosphorylates molecules downstream of the TCR and CD28, leading to a block in T cell effector function (6). Thus, PD-1 blockade can reinvigorate exhausted T cells, providing enhanced antiviral and antitumor responses (7, 8). These observations have led to the development of PD-1

immune checkpoint inhibitors (ICI), which have revolutionized cancer therapy (9). Interestingly, adverse events such as rapid autoimmunity including T1D developed following checkpoint blockade in cancer patients (10–13), possibly due to reversal of T cell exhaustion in pancreatic islets (14). Those findings suggested a key role for the PD1-PD-L1 inhibitory pathway in the maintenance of immune homeostasis and tolerance in pancreatic tissue and the prevention of T1D. Here, we discuss the role of PD-1 in pancreas immune homeostasis and tolerance and the progress made so far in exploiting the PD-1/PD-L1 dyad as a means to prevent and/or treat T1D.

THE PD-1/PD-L1 AXIS PROMOTES BETA CELL TOLERANCE AND PREVENTS T1D

Several lines of evidence indicate that the PD-1/PD-L1 axis is fundamental to maintain immune homeostasis and prevent organ-specific autoimmune diseases such as T1D. The importance of this inhibitory pathway in the pathophysiology of T1D has been demonstrated in mice and humans. The Non-obese Diabetic (NOD) mice develop spontaneous T1D and represent the most used murine pre-clinical models of T1D. For example, NOD mice deficient for PD-1 or PD-L1 develop accelerated T1D (15, 16). Using insulin tetramers, Pauken et al., quantified insulin-specific CD4 T cells in the secondary lymphoid organs (SLO) and pancreas of NOD.PD-$1^{-/-}$ mice (17). They observed significantly more insulin-specific T cells in the pancreatic LN (pancLN) of prediabetic and diabetic NOD.PD-$1^{-/-}$mice compared to WT NOD controls. Furthermore, the same group observed that selective loss of PD-1 on islet-reactive CD4 T cells enhanced their proliferation and recruitment in pancreatic islets (18). Antibody blockade experiments showed that PD-1:PD-L1 interactions, but not PD-1:PD-L2, were necessary for the maintenance of tolerance toward pancreatic islets in the NOD mice (19–25). Interestingly, genetic deletion of PD-1 in C57BL/6 and BALB/c mice led to spontaneous lupus-like disease or autoimmune cardiomyopathy, respectively, but no T1D (26, 27), thus implying that a defective PD-1/PD-L1 inhibitory pathway is not sufficient to trigger autoimmune diabetes. Even in B6.g7 mice sharing the MHCII with NOD mice and carrying high genetic risk for T1D, treatment with anti-PD-1 was not enough to induce T1D (17).

In humans, a possible role for the PD-1/PD-L1 axis in T1D pathogenesis is suggested by the observation that recent onset T1D patients have elevated gene expression levels of CD274 (PD-L1) in whole-blood RNA analysis (25). In addition, both decreased PD-1 gene expression in peripheral $CD4^+$ T cells and low frequency of circulating PD-1^+ $CD4^+$ T cells were found in T1D patients (25, 28). More recently, Granados et al., demonstrated that peripheral T cells from children with new onset T1D failed to upregulate PD-1 upon T-cell receptor stimulation (29). Also, the $CD4^+$ $CD25^+$ Treg cells of T1D patients are defective in their ability to upregulate PD-1 and to efficiently use the PD-1/PD-L pathway to mediate their immunosuppressive function (30).

The importance of the PD-1/PD-L1 pathway in maintenance of immune tolerance toward pancreatic beta cells in humans is furtherly highlighted by the observation that 0.4–2.0% of individuals undergoing treatment with ICI (anti-PD-1 and/or anti-PD-L1 mAb) develop T1D (11–13). In a recent review of the literature, 90 clinical cases of T1D induced by ICI were reported (14). In 51% of cases, T1D onset was associated with occurrence of one or more autoantibodies against islet antigens. Genotype associated with T1D susceptibility were present in 61% of cancer patients who developed T1D upon ICI treatment (11–13). These findings indicate that the PD-1/PD-L1 axis plays a key role in maintenance of immune homeostasis and tolerance to pancreatic antigens. T1D is a multifaceted disease regulated by genetic and environmental factors whose pathogenesis could be very diverse in different T1D patients. In fact, in individuals diagnosed with T1D sharing common clinical signs of the disease, the triggering pathogenic events leading to autoimmune destruction of pancreatic islets maybe very different. Hence, a defect of the PD-1/PD-L1 dyad could lead to T1D in a subgroup of patients as the anti-PD-1/PD-L1 therapy triggers T1D in a percentage of individuals and, particularly, on those who carry other T1D susceptibility genes.

How does the PD-1/PD-L1 axis control β cell autoimmunity? Expression of PD-1 on T cells controls their activation and drives them toward exhaustion. T-cell exhaustion is an important mechanism to maintain immune homeostasis and prevent autoimmune diseases including T1D (7). In support to this idea, a recent study demonstrated that slow T1D progression was associated with an exhaustion-like profile on islet-reactive T cells, with expression of multiple inhibitory receptors (including PD-1), limited cytokine production, and reduced proliferative capacity (31). Along the same line, an increase in circulating exhausted T cells predicted response to anti-CD3 therapy in T1D (32). FcR-non-binding anti-CD3 mAb immunotherapy is effective in delaying T1D occurrence in subjects with risk to develop the disease (autoantibody-positive) (33–35). Importantly, Fife et al., identified a critical role for PD-1/PD-L1 in the response of T1D patients to anti-CD3 immunotherapy (22), suggesting that PD-1–PD-L1 interactions are part of a common pathway to selectively maintain tolerance within the pancreatic tissue and the draining lymph nodes possibly through induction of T cell exhaustion.

Antigen-specific therapy is another highly promising therapeutic approach to harness the progression of T1D (36–38) that could also exploit the PD-1-PD-L1 inhibitory pathway. Using this approach, we and other groups have demonstrated disease remission, inhibition of pathogenic T cell proliferation and anergy, decreased pro-inflammatory cytokine production, and regulatory cytokine and T cell induction (39–44). Fife et al., showed that an antigen-specific therapy with insulin-coupled antigen-presenting cells was able to revert T1D in NOD mice after disease onset (22). Importantly, robust long-term tolerance following this treatment was dependent on the PD-1–PD-L1 pathway (22). Anti–PD-1 and anti–PD-L1, but not anti–PD-L2, reversed tolerance weeks after tolerogenic therapy by promoting antigen-specific T cell proliferation

and inflammatory cytokine production directly in infiltrated tissues (22), thus suggesting that the PD-1/PD-L1 blockade at pancreatic tissue level maybe important. Following a similar approach, administration of the islet antigen peptide mimic p31 coupled to chemically fixed antigen presenting cells (APCs) reversed diabetes and induced robust, long-term inactivation of islet-specific BDC2.5 T-cell receptor (TCR)–transgenic T cells (23). Here, both PD-1 and CTLA-4 interactions were critical for the induction of tolerance. However, long-term maintenance of the anergic T cell state exclusively depended on PD-1/PD-L1 pathway (23). Additional experiments indicated that PD-1 acted in a cell-intrinsic manner to maintain tolerance.

One hallmark of T1D is the presence of islet-specific autoantibodies (45, 46) whose production depends on cognate interactions between a specialized subset of CD4 T cells known as T follicular helper (Tfh) and B cells in the germinal centers (GC) (47, 48). Tfh cells express PD-1, ICOS, CXCR5, and Bcl-6 and provide IL-4, IL-21, and CD40-ligand stimulation to developing/maturing B cells, thus promoting antibody affinity maturation and somatic hypermutation (49, 50). Increases in the number of circulating Tfh cells, and, importantly, elevated expression of an activation phenotype i.e., elevation of ICOS and PD-1 expression, have been reported in patients with autoimmunity including T1D, suggesting that these cells may contribute to disease development (51–57). T follicular regulatory (Tfr) cells are a subset of FOXP3 Treg cells that also express PD-1, ICOS, CXCR5, CD25, Bcl-6, and Foxp3 and suppress Tfh–B cell interactions to limit autoimmunity (58, 59). In children with new-onset T1D, a reduction of PD-1 expression on Tfr cells was observed in a recent study (60). Additionally, children with T1D and dysregulated PD-1 expression were shown to be more susceptible to autoimmune complications of T1D, such as celiac disease and thyroiditis (29). These studies highlight that the PD-1 and PD-L1 axis plays an important role in regulating CD4 T cell–B cell crosstalk, the development of autoantibodies and the severity of T1D.

In recent work, PD-1 blockade was shown to enhance both the Tfh and Tfr CD4 T cells, but their ratio determined the final outcome of the GC response during foreign antigen immunization and in experimental autoimmune encephalomyelitis (61). In the NOD mouse model of T1D, Martinov et al., demonstrated that PD-1 or PD-L1 deficiency, as well as PD-1 but not PD-L2 blockade, increased both insulin-specific Tfh and Tfr cells and increased their survival (61). Additionally, PD-1 deficiency resulted in an increase in insulin-specific B cells and insulin autoantibodies (IAAs) in the mouse sera (61). The increase in insulin-specific Tfh/Tfr cell ratio after PD-1 blockade possibly accounted for the increased IAA production, similarly to what has been described previously for bulk Tfh/Tfr cell ratio (59). Interestingly, using an antibody that specifically disrupts TCR interactions with insulin peptide:MHC II complex, reduced the effects of PD-1 blockade on insulin-reactive B cell expansion but did not impact T1D incidence (61).

THE PD-1/PD-1L PATHWAY IS FUNDAMENTAL TO MAINTAIN IMMUNE HOMEOSTASIS IN THE PANCREATIC TISSUE

The PD-1/PD-L1 axis is instrumental for maintenance of immune homeostasis in several organs including the pancreatic tissue as suggested by the observation that blockade of the PD-1/PD-L1 pathway in 1.8% of cancer patients treated with anti-PD-1 antibodies results in acute or chronic pancreatitis (62). Furthermore, several lines of evidence indicate that this inhibitory pathway is particularly important to maintain immune tolerance against insulin-producing pancreatic β cells for prevention of T1D. Pancreatic β cells express very low levels of PD-L1 in basal conditions, however inflammation triggers higher expression mostly through the action of cytokines such interferons (63, 64). Osum et al., found that IFN-γ and, to a lesser extent, IFN-α, promoted increased frequency of PD-L1+ β cells, and increased expression of PD-L1 on a per cell basis (63). The fact that PD-L1 expression is upregulated in inflamed islets and, specifically, in the presence of CD8+ T-cell infiltration suggests that this could represent a key mechanism to control T cell activation and promote T cell exhaustion in pancreatic tissues (63).

CD8 T cells, most likely islet-specific, are found within islets and the insulitic lesions as well as in the exocrine pancreas of T1D patients (5, 16, 65–69), hence the PD-1/PD-L1-mediated control of CD8 T cell infiltration may play an important role in prevention of T1D. In support to this hypothesis, autoantibody positive patients without clinically overt T1D showed a slight increase in PD-L1 expression on residual pancreatic islets, thus suggesting that PD-L1 expression maybe protective (63). Along the same line, PD-L1 expression was absent from insulin-deficient islets where β cells had been destroyed by the autoimmune process (63).

The mechanism through which the PD-1 inhibitory pathway regulates T1D development within pancreatic tissues was elegantly addressed *in vivo* in pre-clinical models of T1D by multiphoton imaging techniques. Those experiments showed that PD-1 suppressed TCR-driven stop signals in the pancreatic islets. Moreover, they showed that blockade of PD-1 or PD-L1 inhibited T cell migration, prolonged T cell–DC engagement, enhanced T cell cytokine production, boosted TCR signaling and abrogated peripheral tolerance (23).

Recent studies indicated that the PD-1/PD-L1 dyad could be important in regulating activation of tissue-resident memory T cells (TRMs), a subset of T cells residing in the pancreatic tissue under steady-state conditions (70–72), that play a key role in pancreas immune surveillance and immunopathology in health and disease (66, 67, 73, 74). TRM cells exhibit site-specific functional and transcriptional adaptations in certain tissues including the pancreas (75, 76), playing an important role in mediating tissue homeostasis. Functionally, TRM cells rapidly release interleukin-2 (IL-2) and pro-inflammatory cytokines to mediate immediate protective responses against multiple

types of pathogens and they can also participate in tissue immunopathology (77–79). Importantly, TRM cells normally express molecules that attenuate their activation such as the inhibitory molecules PD-1 and CD103 and the regulatory cytokine IL-10 (80, 81). In the human healthy pancreas TRM cells express elevated PD-1 levels compared to the intestinal mucosa (jejunum) and pancreatic draining lymph nodes (82), thus suggesting that their activation is tightly regulated by the PD-1/PD-L1 pathway. Pancreas TRM cells exhibited tissue-specific phenotypes and transcriptional programs controlling T cell activation and metabolism (82). All together these findings indicate that in steady-state conditions, PD-1/PD-L1 triggering on TRM cells is fundamental to halt their activation and maintain immune homeostasis within pancreatic tissues. Polyclonal TRM cells are present in T1D patients, particularly in the exocrine pancreas, but their exact role in T1D development is unknown. Another major immune cell type in the exocrine pancreas are macrophages that enhance TRM cells' functions (82). Interestingly, in studies conducted on pancreatic tissues of patients with chronic pancreatitis, TRM cells exhibited reduced PD-1 expression concomitant with a marked decrease in pancreas macrophages (82). Together, these findings suggest that TRM cells, macrophages, and the PD-1 pathway contribute to *in situ* immune regulation in the pancreas and a dysregulation of this immune regulatory pathway could contribute to the pathogenesis of T1D (**Figure 1**). Multiple factors could lead to breakage of central or peripheral immune tolerance and onset of β cell autoimmunity in T1D susceptible individuals. The expression of PD-1/PD-L1 molecules on β cells and tissue-resident immune cells could represent the ultimate safety mechanism to prevent autoimmune destruction of the pancreatic islets in individuals with β cell autoimmunity whose islet-reactive T cells are activated and recruited within the pancreatic tissue. Hence, in some individuals with high genetic risk of T1D and β cell autoimmunity, a dysregulation of the PD-1/PD-L1 inhibitory pathway could be an additional mechanism leading to T1D.

GENETIC AND ENVIRONMENTAL FACTORS CONTROLLING THE PD-1/PD-L1 PATHWAY

The aforementioned studies suggest that the PD-1/PD-L1 pathway maybe fundamental to control immune activation of islet-reactive T cells and TRM cells and to maintain immune homeostasis and tolerance in the pancreas. The onset of T1D induced in some individuals treated with ICI (i.e., anti-PD-1 mAb) indicates that a failure of these control mechanisms could be one of the mechanisms leading to β cell autoimmunity also in patients with "classical" T1D. Altered PD-1 expression on islet-reactive T cells and/or polyclonal TRM cells as well as defective PD-L1 expression on pancreatic islets could lead to failure of PD-1/PD-L1-mediated tolerance and immune homeostasis, ultimately leading to T1D. How is the PD-1-PD-L1 pathway regulated and how it contributes to T1D development? Both genetic and environmental factors modulate the PD-1/PD-L1 pathway and maybe involved in its dysregulation in T1D. Polymorphisms of the PD-1 gene (*PDCD1*) have been found in different autoimmune diseases and confers genetic susceptibility also to T1D (83). In humans, several studies have been performed to assess the effects of *PD-1* gene polymorphisms on T1D (84) and few single nucleotide polymorphisms associated with T1D were identified such as rs2227981 (PD-1.5), rs2227982 (PD-1.9) (85). Importantly, a recent study demonstrated that rs2227982 had a significant association with clinical signs of T1D (i.e., hyperglycemia), thus suggesting that the *PD-1* gene polymorphisms participate in increasing T1D risk (85).

Recently, a key role for the microbiota in controlling the PD-1/PD-L1 pathway expression and function has been identified. This finding has important implication for disease prevention as diet, antibiotic assumption and others environmental factors could affect the PD-1/PD-L1 function indirectly by altering the microbiota profile. Specifically, it was demonstrated that primary resistance to anti-PD-1 immune-checkpoint immunotherapy (ICI) in cancer patients is related to abnormal gut microbiome composition (86). Importantly, transfer of gut microbiota (fecal material transfer) from ICI responders into ICI resistant patients, increased the response to the anti-PD-1 treatment indicating that the components of the microbial strains could directly or indirectly act on the PD-1/PD-L1 axis (87). Also, modification of the microbiota induced by antibiotic treatment reduced the response to ICI suggesting that antibiotics could affect the inhibitory PD-1/PD-L1 axis by acting on the microbiota (86).

The mechanism underlying microbiota-induced modulation of PD-1/PD-L1 was analyzed in a murine model. Strikingly, it was found that administration of the anti-PD-1 mAb unleashed activation and recruitment of central memory T cells (T_{CM}) into draining lymph nodes and within the tumor and increased the Teff/Treg cell ratio but only if specific bacterial strains (*A. muciniphila* and *E. hirae*) were present in the intestine of the tumor-bearing mice (86). These bacterial species may restore gut barrier integrity and reduced bacterial translocation that could induce immunosuppression of anti-tumor immunity. Alternatively, some microbiota strains could regulate PD-1 expression on T cells and/or PD-L1 expression on tumor cells thus increasing the therapeutic response to anti-PD-1.

The interaction between the microbiota and the PD-1-PD-L1 pathway is bi-directional. In fact, important evidence exists that the PD-1-PD-L1 axis regulate the gut microbiota composition. Kawamoto et al., (88) demonstrated that PD-$1^{-/-}$ mice have an altered microbiota profile. Importantly, they showed that PD-1 modulated the gut bacterial communities through selection of IgA plasmacell repertoires. PD-1 deficiency generated an excess number of Tfh cells with altered phenotypes resulting in dysregulated selection of IgA-secreting B cells in the GCs of Peyer's patches. The IgA produced in PD-$1^{-/-}$ mice have reduced bacteria-binding capacity, which causes alterations of the gut microbiota composition.

Considering the important role of the gut microbiota in modulating T1D pathogenesis (89–93), it is possible to speculate that dysregulation of the PD-1/PD-L1 pathway could affect diabetogenesis also by modifying the microbiome profiles. On the other hand, since some commensal bacterial strains modulate the response to anti-PD-1 therapy, the alteration

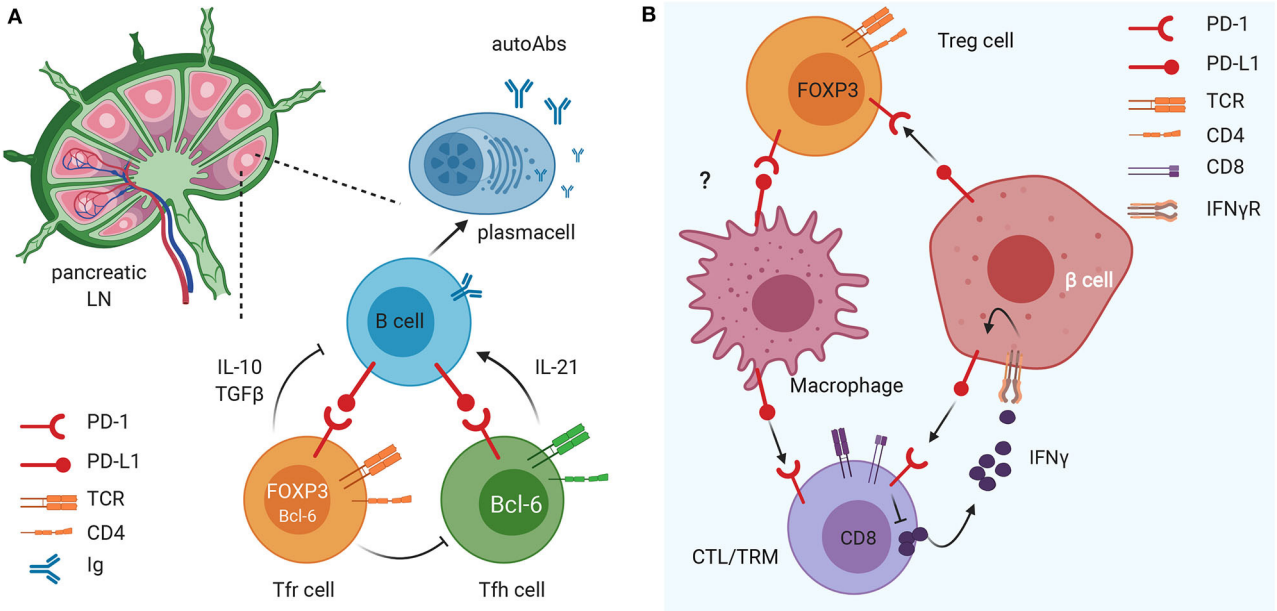

FIGURE 1 | The immunomodulatory role of the PD1/PD-L1 axis on different innate and adaptive immune cell subsets in pancreatic lymph nodes (LN) and tissue. **(A)** The PD1/PD-L1 dyad is crucial in the crosstalk between B cells and Tfollicular helper (Tfh) and follicular regulatory (Tfr) cells in the draining LN. This mechanism favors maturation of B cells and their release of islet-specific autoAbs and could have important implications in T1D pathogenesis. **(B)** PD-L1 expression on insulin-secreting 13 cells of the pancreatic islets of Langerhans down-regulates the activation and promotes exhaustion of autoreactive CD8 T cells and enhances suppressive function of FoxP3+ Treg cells. In parallel, PD-L1 expression on tissue resident memory (TRM) cells (and, possibly, Treg cells) regulates macrophage recruitment and function within pancreatic tissues.

of microbiota composition found in T1D patients could be directly or indirectly responsible for a defect of the PD-1-PD-L1 inhibitory pathway leading to reduced islet-reactive T cell exhaustion, enhanced activation of TRM cells in pancreatic tissue and β cell damage. This process could be triggered by components of the gut commensal microbiota translocating from the intestine to the pancreatic tissue. However, recent evidence indicates that not only the gut microbiota but also organ-specific commensal strains, i.e., skin-resident bacteria, play immunoregulatory function modulating skin graft rejection (94). Hence, future studies are necessary to clarify whether tissue-resident microbiota also exists in pancreatic tissues and, importantly, whether they are involved in maintenance of immune homeostasis and tolerance toward β cells possibly through modulation of the inhibitory PD-1/PD-L1 pathway.

HOW COULD THE PD-1/PD-1L PATHWAY BE THERAPEUTICALLY EXPLOITED IN T1D?

Considering the important role of the PD-1/PD-L1 pathway in controlling β cell autoimmunity and in maintaining immune homeostasis in pancreatic tissues is possible to envision several therapeutic approaches that target this inhibitory pathway for T1D prevention and/or treatment (**Figure 2**). In particular, Adoptive cell therapy (ACT) with tolerogenic dendritic cells (DCs) and Tregs is explored as a promising standalone or combination therapy to counter-regulate β cell autoimmunity in T1D (95). At this moment, there is one completed (96) and one ongoing phase I clinical trial led by Dr. Roep with autologous tolerogenic DCs in patients with new onset T1D (CT No: NTR5542). Over the years, several protocols of tolerogenic DCs have been developed, with and without *in vitro* supplied antigen [reviewed here (95)]. Tolerogenic DCs are thought to act via Treg expansion and induction, T-cell deletion, T-cell anergy and hyporesponsiveness. DCs can also be genetically modified with viral vectors to acquire stable immunogenic or tolerogenic properties (97). Li et al., reported genetically modified DCs expressing T-cell co-inhibitory receptor BTLA that induced CD8 T-cell tolerance and decreased diabetes in NOD mice (98). More recently, Gudi et al., showed that DCs can be efficiently engineered to simultaneously express multiple T cell repressor receptor-selective ligands (among them PD-L1) using a lentiviral transduction approach. These engineered DCs induced profound inhibition of T cell proliferation, modulation of cytokine response, and Treg cell induction, and prevented experimental autoimmune thyroiditis (99). Thus, DCs could be genetically modified to express PD-L1 (**Figure 2**). These DCs could in turn give rise to more Treg cells that express elevated levels of PD-1. Alternatively, Treg cells can be genetically modified to express PD-1 together with a desired, preferably islet-specific, antigen-specificity (100) (**Figure 2**). Further preclinical development and research will be required to address the

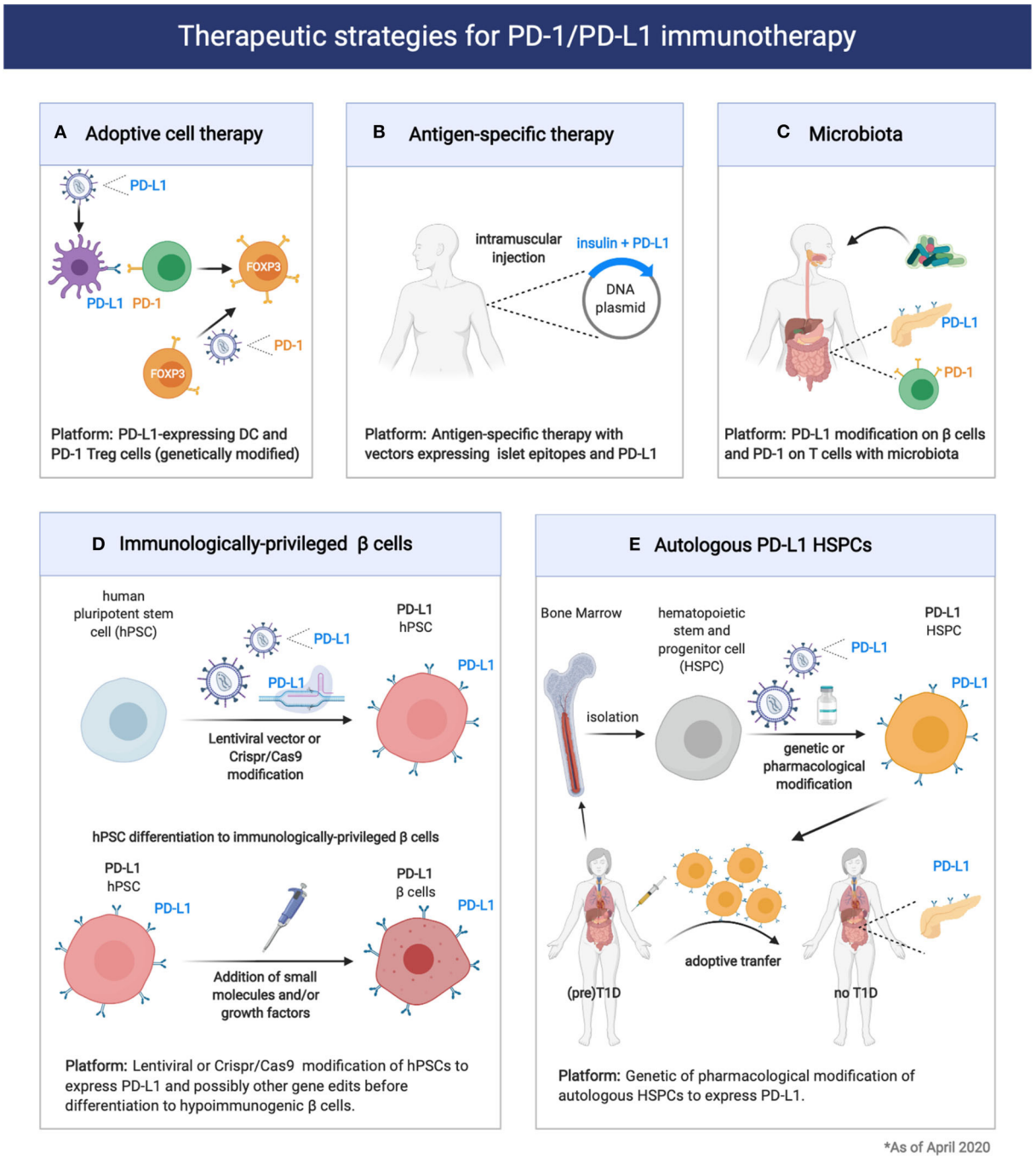

FIGURE 2 | Therapeutic strategies for exploitation of the PD1/PD-L1 pathway in prevention of Type 1 Diabetes and pancreatic islet transplantation. **(A,B)** Expression of PD-1 or PD-L1 on engineered immune cells (DC, Treg cells) and tissues could enhance immune tolerance and therapeutic efficacy of adoptive cell therapy or antigen-specific therapy in T1D. **(C)** Modification of the microbiota composition through probiotic or prebiotic administration can modulate T1D pathogenesis acting on the PD1/PD-L1 axis. **(D,E)** Induction of PD-L1 expression on engineered 13 cells or HSPCs could enhance transplantation tolerance in T1D patients.

effectiveness of PD-L1-expressing DC and PD-1-expressing Treg for the treatment of T1D. These approaches could be exploited to preserve residual β cell mass in newly diagnosed T1D patients (stage 3 T1D) as well as to prevent occurrence of clinical T1D in autoantibodies positive individuals (stage 1/2 T1D).

The antigen-specific response that characterizes T1D has been extensively studied and remains a "hot" area of investigation. Thus far, the primary antigenic drivers of the autoimmune damage are antigens which are expressed exclusively in the β cells. Proinsulin is the major antigen of the immune response and

often the first adaptive immune response to be detected (insulin autoantibodies) (101, 102). Other β cell antigens are also targeted including preproinsulin (PPI), glutamic acid decarboxylase (GAD), tyrosine phosphatase-like insulinoma antigen (IA2, also called ICA512), zinc transporter ZnT8, and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (103). Antigen-specific therapies are tolerogenic approaches that rely on the administration of islet antigens as peptides, whole proteins or DNA vaccines (36, 43, 104). Antigen-specific therapies in general have not been successful so far in inducing durable protection from T1D in autoantibodies positive individuals (stage 1/2 T1D), and current studies focus on potentiating the tolerogenic outcome with combination or more than one epitopes or combination of epitopes with other tolerogenic molecules (105). DNA-based antigen-specific therapies present several unique advantages, as DNA vectors (plasmids) are easy and cheap to be produced and can guarantee prolonged expression of the encoded antigens and co-expressing factors. A possible tolerogenic approach would include the incorporation in the same DNA vector proinsulin or the major epitopes from multiple β-cell antigens and PD-L1 (**Figure 2**). This approach, although not yet tested, might prove more effective at delaying T1D in individuals with autoantibodies positivity (stage 1/2 T1D) but also genetically "at risk" individuals.

Another way to therapeutically exploit the PD-1/PD-L1 axis for T1D prevention in genetically "at risk" and autoantibodies positive individuals would be through the modification of the microbiota. As we previously mentioned, a key role for the microbiota in controlling the PD-1/PD-L1 pathway expression and function was recently identified (85). Thus, modulation of the microbiota composition through administration of probiotics or dietary approaches could enhance PD-L1 expression on pancreatic β cells as well as PD-1 on Treg cells (**Figure 2**).

So far, we discussed how the PD-1/PD-L1 axis could be exploited to control β cell autoimmunity and preserve β cell mass in individuals with newly diagnosed T1D (stage 3 T1D) or with high genetic risk to develop disease with or without autoantibodies positivity (stage 0 and stage 1/2 T1D). For T1D patients with established disease (stage 4 T1D) pancreatic islet grafts is the main therapeutic option to restore insulin independence. Importantly, the PD-1/PDL1 pathway can also be exploited to enhance immune tolerance and promote survival of transplanted islets. The most significant limitations of clinical islet transplantation include the paucity of pancreas organ donors and the adverse effects of chronic immunosuppression. Thus, significant effort is devoted to the generation of a replenishable supply of insulin-producing cells, such as porcine pancreatic islets (106) or β cells derived from stem cells (107). Regardless of the β cell source, immunomodulatory approaches that control alloreactivity and the recurrence of autoimmunity are required. The PD-1 pathway seems to regulate autoreactive, as we previously discussed, but also alloreactive immune responses. PD-L1 blockade was shown to enhance alloreactive T cell responses and accelerated MHC class II–mismatched skin graft rejection in mice (108). A dimeric form of PD-L1 and Ig fusion protein (PD-L1.Ig) in combination with anti-CD154 blockade prevented cardiac, corneal and pancreatic islet allograft rejection, providing direct evidence for the potential of this pathway to induce allograft tolerance (109–111). Recently, a recombinant form of PD-L1 chimeric with core streptavidin (SA) (SA-PD-L1) engineered islets approach was evaluated in a preclinical model of allogeneic islet transplantation (112). SA-PDL1–engineered islets survived indefinitely in allogeneic hosts under a short course of rapamycin regimen, demonstrating the significant potential of PD-1 pathway for modulating alloreactive responses to overcome graft rejection.

Given the importance of the PD-1/PD-L1 pathway in islet-specific T cell tolerance, some investigators are using techniques of genetic engineering to generate β cells that would be immunologically "privileged" to be used for β cell replacement in T1D patients (113, 114). There are several efforts placed for generating a replenishable supply of hypoimmunogenic β cells from human pluripotent stem cells (hPSCs) using state-of the-art genome editing technologies (115). Among the multiple key genome edits that are being tried, elimination of HLA Class I and II as well as inducible overexpression of CTLA4Ig and PD-L1 are included (**Figure 2**). The successful generation of functional, immunologically "privileged" β cells would pave the way for a "universal off-the-shelf" transplantation platform avoiding the risks of immunosuppression and/or encapsulation and could be a "game-changer" in the race to cure T1D (5, 16).

Recently, normoglycemia in patients with recently diagnosed T1D (stage 3 T1D) was obtained with the Voltarelli trial (35, 116, 117). In this trial (117), autologous hematopoietic stem and progenitor cell (HSPC) transplantation in combination with thymoglobulin plus cyclophosphamide as induction therapy in 65 patients with newly diagnosed T1D showed to achieve insulin independence in nearly 60% of treated patients (117). This important finding suggested that HSPCs may be a therapeutic option for new onset T1D patients. Interestingly, the immunoregulatory properties of HSPCs in T1D appear to be linked to the expression of the immune checkpoint PD-L1. Recently, Ben Nasr et al., evaluated the levels of PD-L1 expression in HSPCs in both NOD mice and T1D patients (118). By means of transcriptomic profiling, flow cytometric analysis, RT-PCR, and direct analysis of bone marrow, they found a defect in the expression of PD-L1 expression in HSPCs in both NOD mice and T1D patients. To overcome the PD-L1 defect, they developed genetic (generation of PD-L1.Tg HSPC) and pharmacological approaches (treatment with IFN-β, IFN-γ, and polyinosinic-polycytidylic acid [poly(I:C)]), which successfully abrogated the autoimmune response in NOD mice (118). Tracking studies suggested that PD-L1.Tg HSPCs preferentially homed to the inflamed pancreas (119). Pharmacologically modulated HSPCs also markedly abrogated CD4- and CD8-restricted autoimmune responses and reverted diabetes in nearly 40% of newly hyperglycemic NOD mice (118). Thus, PD-L1-expressing HSPCs hold great promise for the treatment of T1D in humans (**Figure 2**).

SUMMARY

The PD1/PD-L1 dyad is very important to maintain immune homeostasis and to promote tolerance in peripheral tissues. Recent evidence indicates that the PD1/PD-L1 pathway is fundamental to prevent autoimmune diabetes so that, in some patients undergoing treatment with ICI, blocking this inhibitory pathway is sufficient to unleash islet-reactive T cells and trigger T1D. The PD1-PD-L1 axis could affect islet autoimmunity through different mechanisms involving innate and adaptive immune cells and taking place in draining lymph nodes as well as in the pancreatic tissue. The important therapeutic implication of those findings is that restoring the PD-1/PD-L1 function could represent a valid strategy to treat T1D at different stages: to counter-regulate β cell autoimmunity and prevent T1D in individuals genetically at-risk or autoantibodies positive (Stage 1/2), to promote immune tolerance and preserve residual β cell mass in new onset T1D patients (Stage 3) and, finally, to reduce alloreactive responses and favor survival of transplanted islets in T1D patients with established disease (Stage 4). Targeting the PD-1/PD-L1 has been already proven as an effective approach to promote immune tolerance in T1D and islet transplantation. Additional knowledge about the factors that regulate the PD1/PD-L1 pathway will pave the way to more effective treatments for T1D and islet transplantation.

AUTHOR CONTRIBUTIONS

MF reviewed the literature and wrote the manuscript. GF reviewed the literature, wrote the manuscript, and prepared the figures. All authors contributed to the article and approved the submitted version.

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Molecular Footprints of the Immune Assault on Pancreatic Beta Cells in Type 1 Diabetes

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Type 1 diabetes (T1D) is a chronic disease caused by the selective destruction of the insulin-producing pancreatic beta cells by infiltrating immune cells. We presently evaluated the transcriptomic signature observed in beta cells in early T1D and compared it with the signatures observed following *in vitro* exposure of human islets to inflammatory or metabolic stresses, with the aim of identifying "footprints" of the immune assault in the target beta cells. We detected similarities between the beta cell signatures induced by cytokines present at different moments of the disease, i.e., interferon-α (early disease) and interleukin-1β plus interferon-γ (later stages) and the beta cells from T1D patients, identifying biological process and signaling pathways activated during early and late stages of the disease. Among the first responses triggered on beta cells was an enrichment in antiviral responses, pattern recognition receptors activation, protein modification and MHC class I antigen presentation. During putative later stages of insulitis the processes were dominated by T-cell recruitment and activation and attempts of beta cells to defend themselves through the activation of anti-inflammatory pathways (i.e., IL10, IL4/13) and immune check-point proteins (i.e., PDL1 and HLA-E). Finally, we mined the beta cell signature in islets from T1D patients using the Connectivity Map, a large database of chemical compounds/drugs, and identified interesting candidates to potentially revert the effects of insulitis on beta cells.

Keywords: type 1 diabetes, beta cells, pancreatic islets, insulitis, inflammation, therapeutics, interferon, RNA-sequencing

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INTRODUCTION

Pancreatic beta cell stress and death are central components of type 1 diabetes (T1D) and also contribute in a decisive way to T2D (1, 2). Gertrude Stein once wrote in her poem "Sacred Emily" that a "rose is a rose is a rose is a rose." But is "beta cell stress a beta cell stress a beta cell stress?" In other words, are all forms of beta cell stress the same and, more importantly, does it matter, as the outcome may be the same, namely relative or absolute reduction in insulin production?

Pancreatic beta cells are highly specialized endocrine cells that have as central tasks to sense circulating nutrients and respond to their circulating levels by releasing insulin in adequate amounts to assure their proper uptake and use by different organs; this maintains circulating levels of nutrients, such as glucose, inside narrow limits in spite of the wide variations in food intake observed in most mammalian species. Pancreatic beta cells in humans are very long lived, and

our "beta cell patrimony" is probably established by early adulthood (3). These cells will thus need to cope with decades of varying insulin production – not a trivial task, considering that under stimulated conditions insulin synthesis increases 10-fold compared to basal level, approaching 50% of the total protein synthesis.

An important question is how beta cells react when exposed to mediators of autoimmune damage, such as pro-inflammatory cytokines released at the islet vicinity during the early [e.g., type 1 interferons (IFNs), such as IFNα] or late [e.g., interleukin-1β (IL1β) and interferon-γ (IFNγ)] stages of insulitis, or to saturated free fatty acids, such as palmitate, that contribute to metabolic stress in T2D (1, 2)? Available data suggest that beta cells trigger different adaptive responses that involve a decrease in its most differentiated functions, i.e., insulin synthesis and release, and the up-regulation of complex cellular responses, such as the unfolded protein response [UPR; (4)]. These adaptive responses are at least in part determined by the stress inducing them – for instance, beta cells exposed to pro-inflammatory cytokines trigger branches of the UPR that are different from the ones triggered in response to palmitate (2, 5, 6). These responses to diverse stresses will thus leave gene expression/molecular footprints that can be detected by omics techniques such as global RNA sequencing. Exam of these footprints may allow us to detect the nature of the beta cell stress causing them and, by comparing the molecular footprints induced by *in vitro* stresses with those present in beta cells isolated from patients affected by T1D, enable us to define the best experimental models to study the human disease. Furthermore, and of particular relevance for the discovery of novel therapies for T1D, comparisons of the different beta cells molecular footprints against large databases of cells exposed to different drugs, such as the recently updated Connectivity MAP database of cellular signatures, including > 1.3M profiles of human cells responses to chemical and genetic perturbations (7), can identify agents that antagonize particular gene signatures that may contribute to beta cell demise. Some of these agents, such as for instance the JAK inhibitor baricitinib, are already in use for other autoimmune diseases (8, 9) and can then be re-purposed for T1D therapy (10) (see below). We have recently published two comprehensive review articles focusing on beta cell fate in T1D (2, 11), and will focus here on the available studies characterizing the footprints left by immune or metabolic stresses on human beta cells.

In recent years RNA sequencing analysis has been done by us and others on human islets exposed to IL1β + IFNγ (12), IFNα (10) and palmitate (13) and of purified human beta cells or whole islets obtained from the pancreata of patients with T1D (14) or T2D (15); all these valuable datasets have been deposited on public access sites, such as the Gene Expression Omnibus repository (GEO). We have presently re-analyzed the most informative of these datasets, using the same pipeline [i.e., Salmon, GENCODE v31, DESeq2 (16–18)] to allow adequate comparisons between them, aiming to answer the following questions:

- How similar are the molecular footprints left on human islets by IL1β + IFNγ (12), IFNα (10) and palmitate (13)?
- Are these footprints representative of the patterns observed in beta cells obtained from patients affected by T1D?
- Can we obtain relevant indications for new therapies by mining these molecular footprints against available drug-induced footprints in other cell types?

METHODS

For the present review and analysis we have selected available RNA-seq datasets of pancreatic human islets or FACS-purified human beta cells exposed to different pro-inflammatory stimuli (10, 12), metabolic stressors (13) or to the local environment present during T1D development (insulitis) (14) that are publicly available from the GEO repository (www.ncbi.nlm.nih.gov/geo). For the search we have used the following terms combinations: (1) "pancreatic endocrine cells" [All Fields] OR "pancreatic beta cells" [All Fields] OR "human islets" [All Fields] AND "type 1 diabetes" [All Fields] AND ("Homo sapiens" [Organism] AND "Expression profiling by high throughput sequencing"[Filter]); (2) "pancreatic endocrine cells" [All Fields] OR "pancreatic beta cells" [All Fields] OR "human islets" [All Fields] AND "cytokines" [All Fields] AND ("Homo sapiens" [Organism] AND "Expression profiling by high throughput sequencing" [Filter]); (3) "pancreatic endocrine cells" [All Fields] OR "pancreatic beta cells" [All Fields] OR "human islets" [All Fields] AND "palmitate" [All Fields] AND ("Homo sapiens" [Organism] AND "Expression profiling by high throughput sequencing" [Filter]). We also searched the Pubmed using the same criteria and mined online sources for unpublished data. Since the present analysis focus on beta cell transcript (isoforms) expression, we excluded articles having insufficient reads coverage (<20 million reads per sample, $n = 3$) and depleted of beta cells (<500 transcripts per million (TPM) of insulin, $n = 1$). The PRISM flow diagram (19) describing the search strategies is represented in **Figure 1**. **Table 1** provides a detailed description of each dataset including their GEO reference number.

After downloading the raw data, we used Salmon version 0.13.2 (16) to map the reads to the human reference transcriptome [GENCODE version 31 (GRCh38) (17)] using the quasi-alignment model. The transcript abundance is represented in Transcripts Per Million (TPM).

Differential analysis was performed using the R package DESeq2 version 1.24.0 (18). The estimated number of reads obtained from Salmon were used as input for DESeq2. Briefly, DESeq2 normalizes samples according to per-sample sequencing depth and accounting for intra-sample variability. Then, it fits data to a negative binomial generalized linear model (GLM) and calculates the Wald statistic. Finally, the raw *p*-values are corrected using the false discovery rate (FDR) for multiple testing by the Benjamini-Hochberg method. Transcripts with an FDR < 0.05 were considered differentially modified.

To compare the different signatures present in each dataset, we performed Rank-Rank Hypergeometric Overlap (RRHO)

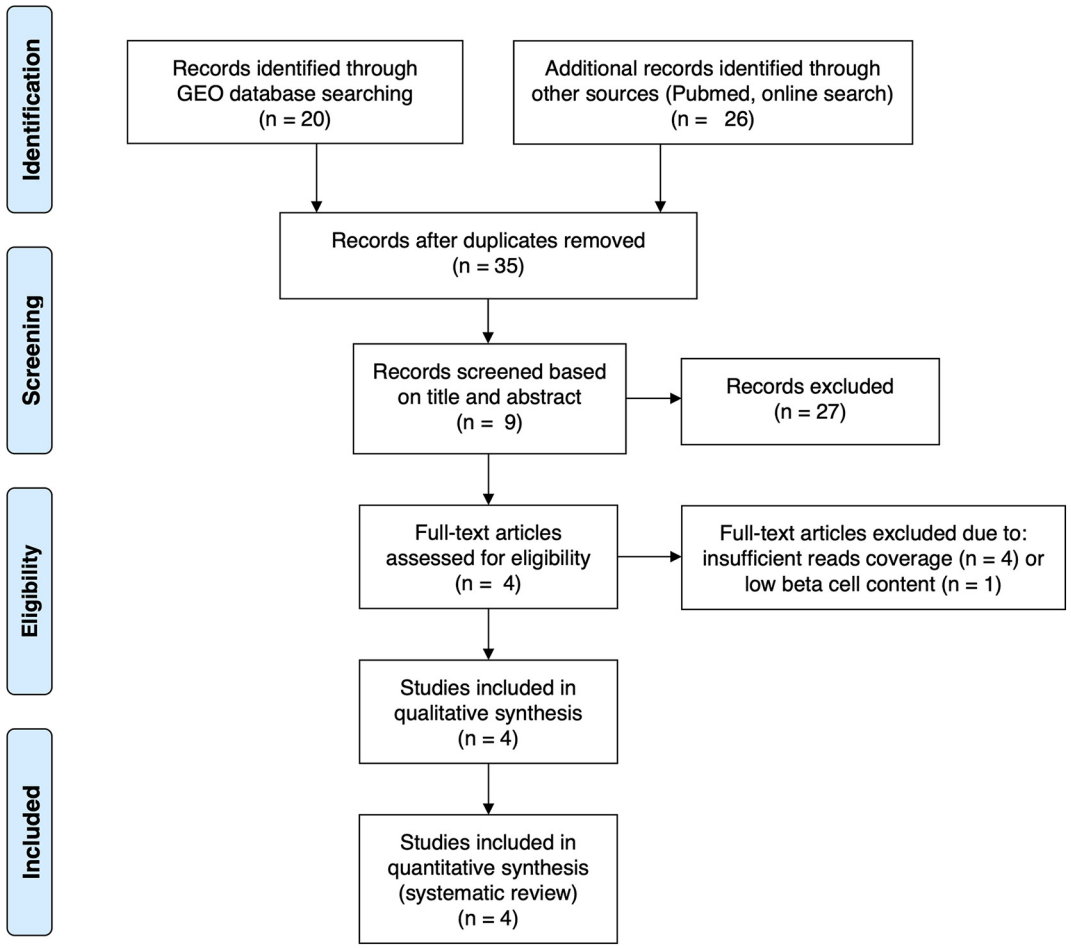

FIGURE 1 | PRISMA flow diagram (19) describing the search strategy used to identify the analyzed studies.

mapping (20). For this goal, we have generated lists of transcripts ranked by their -$\log_{10}$ *p*-values from the differential expression analysis. In a RRHO map the hypergeometric *p*-value for enrichment of *k* overlapping genes is calculated for all possible threshold pairs for each condition, generating a matrix where the indices are the current rank in each condition. The log-transformed hypergeometric *p*-values are then plotted in a heatmap indicating the degree of statistically significant overlap between the two ranked lists on that position of the map. We have applied adjustment for multiple comparison using the Benjamini-Yekutieli correction.

To evaluate the similarities between datasets we used the R package FactoExtra version 1.0.6 (https://github.com/kassambara/factoextra) considering as (dis)similarity (distance) measure the Pearson correlation between samples (1 – correlation) using the 300 most variable transcripts (i.e., median absolute deviation). Next, hierarchical clustering was performed based on the average of the pairwise (dis)similarities (distances) between samples.

For functional enrichment analysis the R/ Bioconductor package ClusterProfiler version 3.12 (21) was used in combination with gene sets from the Molecular Signatures Database (22). All the transcripts presenting a TPM > 1 in at least half of the samples were considered as background and a Benjamini–Hochberg FDR threshold of 0.05 was defined as significant enrichment.

The top 150 up-regulated transcripts in the RNA-seq of FACS-purified beta cells from T1D individuals (14) were identified from the differential expression analysis. This list of transcripts was used to query the Connectivity Map dataset of L1000 cellular signatures, which has transcriptional responses of human cells to different chemical and genetic perturbations, using the CLUE platform (https://clue.io) (7). To identify compounds potentially reverting the effects induced by insulitis on beta cells we focused on perturbagens promoting signatures that were opposite (negative tau score) to our query list. Only perturbagens having a median tau score < -60 were considered for further evaluation.

TABLE 1 | Description of the datasets of pancreatic human islets and purified beta cells exposed to different stimuli and conditions presently evaluated.

Treatment	Tissue	Donors			Duration	*n*	GEO
		Age (years)	Gender (M/F)	BMI			
IL1β + IFNγ	Human islets	50.6 ± 22.8	2/3	25.1 ± 3.4	48 h	5	GSE108413
IFNα	Human islets	71.3 ± 17.7	1/5	26.4 ± 5.4	18 h	6	GSE133221
Palmitate	Human islets	55.2 ± 20.2	3/2	24.8 ± 1.6	48 h	5	GSE53949
Condition							
Control	FACS-purified beta cells	16.1 ± 5.8	8/4	NA	–	12	GSE121863
T1D	FACS-purified beta cells	19.7 ± 5.4	3/1	NA	3.1 ± 2.8 years	4	GSE121863

GEO, Gene Expression Omnibus repository (https://www.ncbi.nlm.nih.gov/geo/); NA, non-available; M, male; F, female; BMI, body mass index. Data are mean ± standard deviation (10, 12–14).

RESULTS

The Footprints of *in vitro* Cytokine, but Not Palmitate, Exposure Are Similar to the Ones Observed in Beta Cells From T1D Patients

In order to obtain *in vitro* inflammatory and metabolic footprints of beta cells, we analyzed previously generated RNA-sequencing of pancreatic human islets exposed to pro-inflammatory cytokines [IL1β + IFNγ (12) or IFNα (10)] or to a metabolic stressor [palmitate (13)] (**Figure 1**). Furthermore, recent advances in techniques to purify beta cells and the establishment of collaborative networks between different research groups have allowed for the first time the generation of RNA-sequencing data of human beta cells from T1D individuals (14). This database offers a unique opportunity to validate the *in vitro* models by comparing them against the *in vivo* situation present during T1D development. For this purpose, we first evaluated the similarities between the signatures of transcripts induced by the different stimuli (inflammatory and metabolic) in pancreatic islets and by the local environment of insulitis that beta cells are exposed to in T1D (**Figure 2**). The analysis was performed at the transcript (isoform) level, since we have previously observed that exposure of beta cell to pro-inflammatory cytokines promotes major changes in alternative splicing (AS), leading to a high number of different splicing events (10, 23–25). This is particularly relevant since AS is a cell-type- and context-dependent mechanism. In line with this, several RNA-binding proteins that regulate gene splicing are significantly modified in beta cells isolated from individuals affected by T1D (**Supplementary Table 1**). This analysis indicated that the pro-inflammatory cytokines trigger a profile of transcripts that generate clusters of similar samples (positive Pearson correlation), while the metabolic stressor palmitate generates different groups of transcripts that cluster separately and with an opposite profile as compared to cytokines (negative Pearson correlation) (**Figure 2**). Interestingly, the samples obtained from T1D individuals (indicated by red color) clustered together with the two *in vitro* models of pancreatic islet inflammation; this similarity was slightly higher (represented by darker red color boxes) with the signature of the pro-inflammatory cytokines IL1β + IFNγ (orange) than with IFNα (yellow) (**Figure 2**). This is probably due to the fact that the beta cell samples were obtained from four patients 5 months, 2, 3, and 7 years after diagnosis of T1D, a period when a full adaptive immune response against the beta cells is in place, including exposure of islets to the cytokines IL1β + IFNγ, while IFNα may play a more relevant role during the early and more "innate-immunity related" phases of the disease (26).

Next, to identify the transcripts that are analogously modulated in beta cells from individuals affected by T1D and in pancreatic islets exposed to the different stressful stimuli, we performed a Rank-Rank hypergeometric overlap (RRHO) analysis (20) which evaluates the (dis)similarities between two ranked lists. For this comparison, ranked lists of transcripts based on the -$\log_{10}$ *p*-values from the differential expression analysis (T1D or stimuli vs. controls) were generated. The RRHO mapping demonstrated a significant intersection of similarly up-regulated transcripts in T1D beta cells and in human islets exposed to both IFNα (**Figure 3A**) and IL1β + IFNγ (**Figure 3B**). In agreement with the distance matrix findings (**Figure 2**), the significance of this intersection was more pronounced for the late cytokines, i.e., IL1β + IFNγ (**Figure 3B**), particularly related to down-regulated genes. This concordance in down-regulated genes may be due to the fact that IL1β + IFNγ but not IFNα, trigger a more severe beta cell stress, eventually leading to apoptosis (10, 23, 27). On the other hand, there was no statistically significant correlation between the T1D beta cells signature and the one induced by the metabolic stressor palmitate in human islets (**Figure 3C**). In line with this, we have previously shown that there is no clear correlation between human islets exposed to IFNα (10) or to IL1β + IFNγ (2) and human islets obtained from patients affected by type 2 diabetes.

To gain further insight into the biological processes and pathways triggered at the different stages of T1D development, we next performed enrichment analysis of the up-regulated transcripts present in the areas of significant intersection between the RNA-seq datasets from islets obtained from patients affected by T1D as compared to cytokine-treated human islets

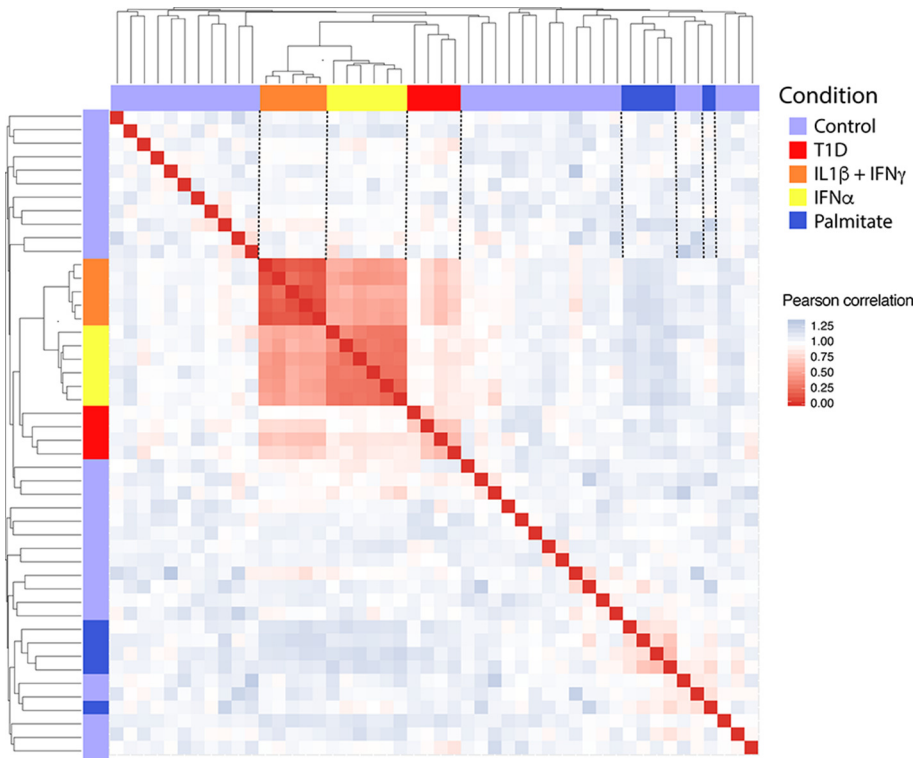

FIGURE 2 | Correlation between the transcripts expressed in human islets exposed to IL1β + IFNγ, IFNα or palmitate and the transcripts expressed in human beta cells of individuals affected by T1D. Data were obtained by RNA sequencing (10, 12–14). The Pearson correlation (1 – correlation) was used to evaluate the (dis)similarities (distance) among the 300 most variable transcripts in the RNA-seq datasets. Red squares represent a positive correlation (similarity), blue squares a negative correlation (dissimilarity) and white squares an absence of correlation between each pair of observations. Next, the hierarchical clustering was performed considering the average of the dissimilarity (distance) between samples. The resulting dendrogram is shown in the upper and lateral part of the matrix.

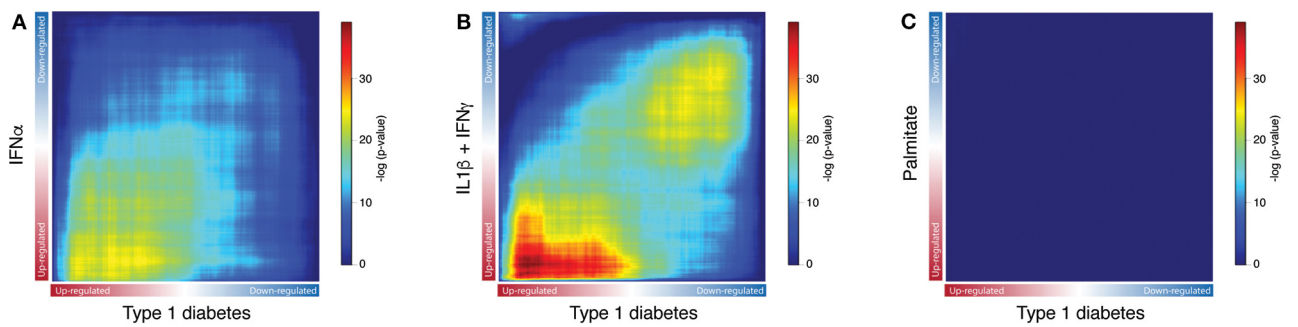

FIGURE 3 | Exposure of human islets to pro-inflammatory cytokines, but not to palmitate, induce a similar transcriptomic profile as observed in islets isolated from patients affected by type 1 diabetes. **(A–C)** Rank-Rank Hypergeometric Overlap (RRHO) map comparing the transcriptional expression profile of human islets exposed to IFNα (**A**), IL1β + IFNγ (**B**) or palmitate (**C**) to the one present in primary beta cells from individuals affected by T1D, as identified by RNA-seq. Ranked lists of transcripts based on the -$\log_{10}$ *p*-values from the differential expression analysis of human islets exposed to IFNα (**A**), IL1β + IFNγ (**B**) or palmitate (**C**) were compared to a similarly ranked-list from beta cells obtained from patients with T1D.

(**Figure 4**). **Figure 4A** outlines the three intersection areas evaluated, while **Figure 4B** shows transcripts up-regulated only in the intersection between the INFα and T1D datasets, reflecting most likely the early changes induced in beta cells during the evolution of diabetes. **Figure 4C** shows transcripts up-regulated only in the intersection between the IL1β + INFγ and T1D datasets, probably mirroring the changes present at later stage of T1D progression. Finally, **Figure 4D** shows transcripts up-regulated in the intersection between all datasets (INFα, IL1β + IFNγ, and T1D), which may represent alterations common to different phases of the disease. These putative early and late changes observed in

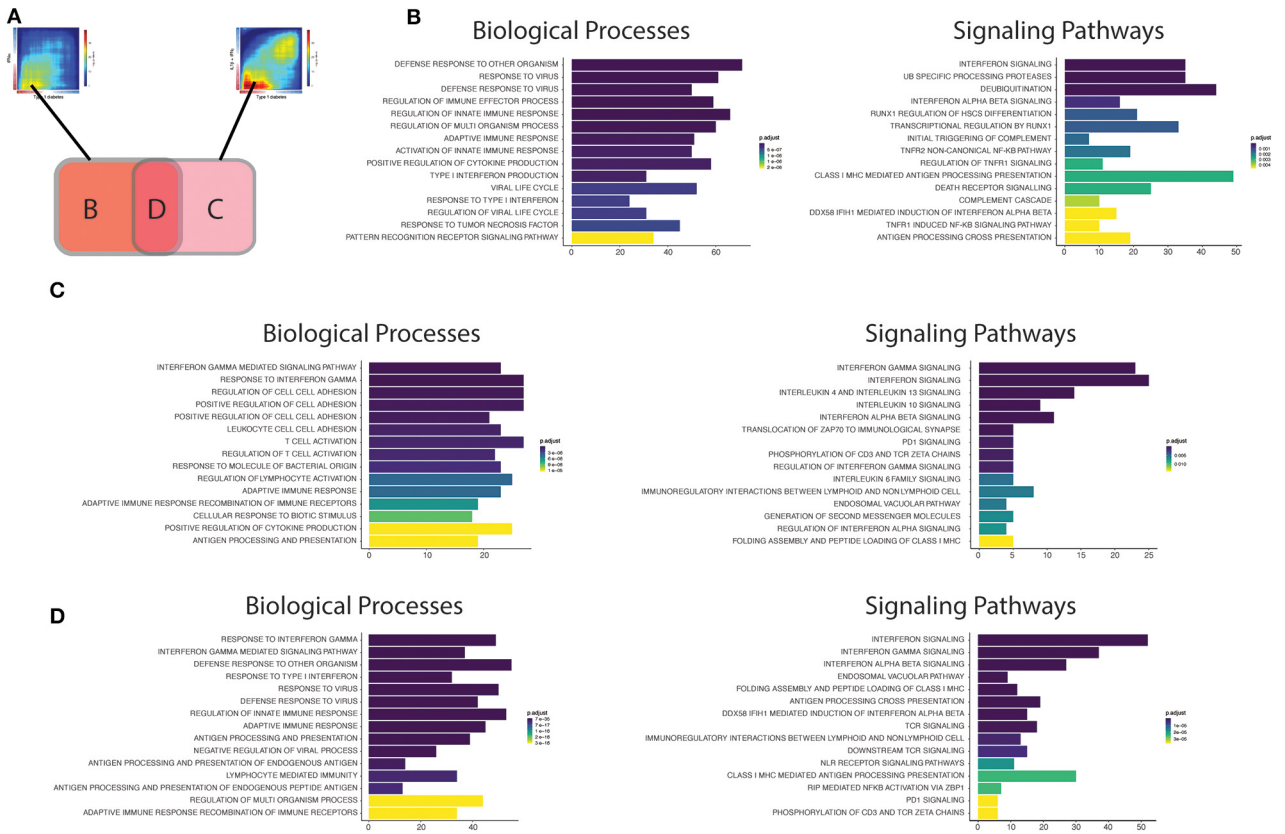

FIGURE 4 | Functional analysis of the transcripts overlapping beta cell datasets from T1D patients' islets or cytokine-exposed human islets. **(A)** First, the up-regulated transcripts present in the intersection of RRHO maps comparing the T1D signature in beta cells against the IFNα (left) and IL1β + IFNγ (right) signatures in human islets were identified. Next, these transcripts were divided in three different groups: (**B**) transcripts only present in the intersection between T1D vs. IFNα, (**C**) transcripts only present in the intersection between T1D vs IL1β + IFNγ, (**D**) transcripts present in the intersection of all the three datasets (T1D, IFNα, IL1β + IFNγ). (**B–D**) Biological processes (Gene Ontology) and signaling pathways (Reactome) enriched among the overlapping regions of the RRHO maps described above [**(B)** only T1D vs IFNα; **(C)** only T1D vs. IL1β + IFNγ; **(D)** T1D, IFNα and IL1β + IFNγ]. The y axis shows the 15 most significant genesets, while the x axis represents the total number of transcripts identified in each geneset.

the islets in the course of T1D are discussed in more detail below.

Early Pancreatic Islets Changes

The biological processes (Gene Ontology) regulated by the transcripts present in area **B** (**Figure 4B**; only T1D vs. IFNα) recapitulate well the initial events putatively present during T1D development (26). In brief, innate immune responses are probably triggered after activation of pattern recognition receptors (PRRs) (including Toll-like receptors (TLRs), and RIG-I-like receptors) by endogenous "danger signals" or exogenous ligands, such as nucleic acids (e.g., double stranded RNA) produced during viral infections, which are putative environmental factors associated with T1D (28). The activation of PRRs on beta cells promotes an increased local production of antiviral type I interferons, such as IFNα, by the beta cells themselves and by other cells present in the islets, and the recruitment of immune cells to the pancreatic islets. In support to this model, rare variants in the RIG-I-like receptor *MDA5* that lead to decreased function are associated with protection against T1D (29). In contrast, a genetic variant of *MDA5* that leads to a partial gain-of-function significantly increases the risk for T1D (30). In both cases, the impact on T1D development is dependent on, respectively, a decreased or an exacerbated production of type I IFNs. These locally produced cytokines activate several signaling pathways that include key pathways observed in early human T1D, such as MHC class I antigen presentation (31) and beta cell death (26) (**Figure 4B**). We have recently demonstrated that IFNα up-regulates different mechanisms involved in post-transcriptional regulation of gene expression, especially alternative splicing (AS) (10) and endoplasmic reticulum (ER) stress (10, 32), which can potentially generate beta cells neoantigens. This, combined with the overexpression of HLA class I, may facilitate auto-immunity progression by activation of autoreactive T-cells that may have escaped thymic selection (11). Of note, a similar phenomenon has been observed for the pro-inflammatory cytokines IL1β + IFNγ which increase the expression of an isoform of secretogranin V, SCG55-009, that is recognized by auto-reactive $CD8^+$ T-cells present in the pancreas of T1D individuals (12). It has also

been described that beta cells undergoing endoplasmic reticulum stress can modify the insulin translation initiation site, producing a highly immunogenic polypeptide capable of activating T-cells from T1D individuals with the potential for killing human beta cells (33).

Macrophages are among the main immune cell populations present in the human pancreas (34), and their density is increased in the vicinity of the pancreatic islets ($< 20\,\mu M$) of individuals with recent-onset T1D in comparison with non-diabetic individuals (34). This is compatible with findings from different animal models showing that macrophages are among the first activated immune cells present in the pancreas in autoimmune diabetes (35, 36) and the main cell type responsible for the TNF production in pancreatic islets (37). Furthermore, we identified "activation of TNF receptor subunits" and its main downstream transcription factor nuclear factor-κB (NF- κB) as important pathways activated in early human T1D (**Figure 4B**). This is particularly relevant, since differently from other cell types in which NF-κB has mainly a pro-survival role (38), NF-κB activation in beta cells is mostly pro-apoptotic (39).

Finally, pathways involved in the regulation of protein ubiquitination were also overrepresented in our analysis of common effects observed in early T1D and following human islet exposure to IFNα (**Figure 4B**). The signal transduction downstream of the PRRs relies on their association with specific adaptors, which in many cases require ubiquitin-specific modifications to became active (40). Ubiquitination is counteracted by deubiquitinases, a group of enzymes that remove these modifications and thus provide a negative feedback on the signaling cascade (41). Interestingly, single-nucleotide polymorphisms (SNPs) that decrease activity of the ubiquitin-modifying enzyme *TNFAIP3* (A20) (A20 provides a negative feedback on NF-κB activation by stimulating degradation of some of its components) are associated with a higher risk for development of T1D and other autoimmune diseases (42). Moreover, rare mutations leading to TNFAIP3 loss-of-function cause a systemic autoinflammatory disease (43). The affected individuals present a type I interferon (IFN) signature which correlates with the disease activity and predicts their response to treatment with janus kinase (JAK) inhibitors (JAK1 is a key kinase for type I interferon signaling) (44). In agreement with this, we have shown that the JAK 1 and 2 inhibitors ruxolitinib (45) and baricitinib (10) prevent IFNα-induced MHC class I and chemokine up-regulation in human islets, besides inhibiting IFNα + IL1β-induced beta cell apoptosis; another drug from this family was shown to prevent diabetes in NOD mice (46). Baricitinib is already in clinical use for other autoimmune diseases (8, 9) and may be eventually re-purposed for the early therapy of T1D. This demonstrates the utility of beta cell signature characterizations for the identification of new therapeutic targets in T1D (see below).

Advanced Pancreatic Islets Changes

In the case the local pro-inflammatory environment described above is maintained, the increased homing of different immune cells to the pancreatic islets promotes the transition to a scenario dominated by adaptive immune responses. The intersection area **C** may be representative of the findings observed during these late stages of insulitis (**Figure 4C**). The biological processes (GO) are now enriched in IFNγ responses, reflecting the increased number of T-cells present in the islets. A critical step for the immune cells to reach the inflamed tissue is their adhesion and crawling on the endothelium (47). In line with this, several processes (GO) involved in cell adhesion are induced in pancreatic islets from T1D individuals (**Figure 4C**). During this process, activated T-cells expressing high-affinity integrins bind to the endothelial cells via cellular adhesion molecules (CAMs) (48). Deficiency of the vascular adhesion molecule adhesion intercellular adhesion molecule 1 (ICAM1) (49) or its receptor (50), lymphocyte function-associated antigen 1 (LFA1), prevents the development/progression of autoimmune diabetes in NOD mice. Of interest, a genetic risk variant associated to T1D (rs657152) (51) is also associated with the circulating levels of soluble ICAM1 (52).

Other potentially important pathways identified by the present analysis were the "Immunoregulatory interactions between lymphoid and non-lymphoid cells" and "PD1 signaling" (**Figure 4C**). We have recently shown that during insulitis, in addition to pro-inflammatory stimuli, beta cells also express immune checkpoint proteins, including programmed death-ligand 1 (PDL1) and HLA-E, possibly in an attempt to down-regulate the immune responses and thus avoid further tissue damage (10, 53). In line with these observations, individuals receiving immunotherapy based on PDL1/PD1 blockers for cancer treatment have a higher risk of developing T1D (54) and other autoimmune diseases (55). The induction of these checkpoint proteins in human beta cells is mainly mediated by type I and II IFNs under the control of the transcription factor interferon regulatory factor 1 (IRF1) (53). The protective role for these co-inhibitory molecules in beta cells is reinforced by the facts that beta cells surviving the immune assault in NOD mice express high levels of PDL1 (56), that transgenic PDL1 overexpression in beta cells decreases diabetes prevalence in NOD mice (57), and that that both PDL1 (53) and HLA-E (10) are absent in pancreatic islets from T1D individuals depleted of insulin.

Another group of immunomodulatory molecules presenting activation of their signaling pathways were anti-inflammatory cytokines, including interleukin-10 (IL10) and interleukin 4/13 (IL4/13) (**Figure 4D**). In line with this, systemic delivery of IL10 via adenovirus-associated gene therapy prevented diabetes recurrence after syngeneic islets transplantation in NOD mice (58). In the same animal model, oral administration of a probiotic (*Lactococcus lactis*) expressing IL10 and the autoantigen GAD65, in combination with low dose of anti-CD3, reversed autoimmune diabetes (59). The second class of cytokines include IL4 and IL13, which exerts their actions through three different combinations of shared receptors (60). These cytokines can trigger phenotypes that range from allergy, including asthma, to anti-helminthic responses. Interestingly, helminthic infections are associated with protection against immune-mediated diseases, such as T1D (61). The systemic administration of IL4 (62) or IL13 (63) was shown to prevent the development of diabetes in NOD mice. This effect is at least in part mediated via their direct action on beta cells,

since beta cells express all the required IL4/13 receptor subunits (64) and their *in vitro* exposure to IL4 or IL13 protects them against pro-inflammatory cytokine-induced apoptosis (64, 65). This cytoprotection is associated with the activation of signal transducer and activator of transcription 6 (STAT6) in beta cells, leading to the up-regulation of anti-apoptotic proteins such as myeloid leukemia-1 (MCL-1) and B cell lymphoma-extra large (BCL-XL) (65).

Finally, analysis of the biological processes and the signaling pathways controlled by genes commonly up-regulated in all the three datasets (IFNα, IL1β + IFNγ and T1D) (**Figure 4D**) indicate that they summarize many findings present at the different stages of the disease, including regulation of antiviral responses, responses to type I (IFNα/β) and II (IFNγ) interferons, MHC class I antigen presentation, lymphocyte activation, interaction between lymphoid and non-lymphoid cells and PD1 signaling. This suggest that signatures of both innate and adaptive immunity remain present in the islets as the disease evolves, which is supported by histological and RNA-seq findings in whole islets from T1D patients (66, 67). Analysis of transcripts that are only modified in beta cells of T1D individuals, but not after exposure of these cells to cytokines (**Supplementary Figure 1A**), indicates pathways that are less dependent on these inflammatory mediators (**Supplementary Figure 1B**). Changes in genes involved in digestion and absorption were upregulated in samples from T1D only, and among these genes we identified CTRB1 and CTRB2. Polymorphisms in these genes have been associated to higher risk for T1D development (68, 69), and mild hyperglycemia induces their upregulation and that of other genes identified in the same pathway (70). There was also activation of matrix metalloproteinases, which is a potentially relevant mechanism in T1D since matrix metalloproteinases can cleave membrane-bound PDL1 present on the cell surface and thus regulate T-cell responses (71).

Mining Beta Cell Molecular Footprints for Drug Re-purposing in T1D

Up to now there is no treatment available to prevent the development of T1D in individuals at risk. This, and the worldwide increase in T1D incidence observed in recent decades (72, 73), makes T1D a major area of interest for drug discovery. We have presently mined the RNA-sequencing of FACS-purified beta of T1D individuals (14) using the recently updated version of the Connectivity Map (CMap) database (7). To avoid potential off-target findings caused by focusing on individual compounds in the analysis, we focused instead on classes of drugs that promote an opposite signature to the one present in beta cells of T1D individuals. We identified several classes of drugs/compounds that could potentially revert the inflammatory signatures present in beta cells during T1D, including bile acids, bromodomain inhibitors, leucine-rich repeat kinase (LRRK) inhibitors and vitamin D receptor agonists (**Figure 5**).

Among the top classes of compounds identified were bile acids. This is an interesting finding, since TUDCA - a conjugated bile acid with chaperone properties - has been shown to prevent autoimmune diabetes in NOD mice (74). This protection was mediated via the restoration of a defective unfolded protein response (UPR) observed in beta cells during insulitis. *In vitro* studies demonstrated that TUDCA also inhibits IFNα-induced ER stress and its subsequent UPR activation in human beta cells (75). Finally, there is an ongoing phase 2 clinical trial evaluating the potential translational impact of these findings in individuals with recent-onset T1D (ClinicalTrials.gov Identifier: NCT02218619).

Another class of compounds identified were bromodomain inhibitors. The bromodomain (BRD) proteins are "readers" of histone acetylation that, associated with other components of chromatin-remodeling complexes, promote transcriptional activation. Bromodomain and extra-terminal domain (BET) proteins are the most studied members of the BRD family. Inhibitors of BET proteins have protective effects in different animal models of autoimmunity (76, 77), including in the NOD mouse model of autoimmune diabetes (78). We have recently demonstrated that two bromodomain inhibitors, namely JQ1 and I-BET-151, partially prevent the deleterious effects of IFNα on human beta cells (10).

We also identified leucine-rich repeat kinase inhibitors (LRRK) as a potential perturbagen in T1D (**Figure 5**). The currently available LRRK inhibitors mainly target LRRK2. The LRRK2 protein has two domains with catalytic activity; a GTPase domain of the Ras of complex (ROC) protein family, and a kinase domain of the tyrosine kinase like (TKL) family (79). Genome-wide association studies have linked kinase-activating mutations in LRRK2 with an increased risk for both Parkinson disease (80) and inflammatory bowel diseases (IBD) (81). *In vivo* and *in vitro* studies indicate that these LRRK2 risk variants act mainly by promoting exacerbated responses to pro-inflammatory stimuli (82–84). In line with this, LRRK2 inhibition prevented microglial inflammatory responses triggered by TLR activation (85). Of concern, systemic LRRK2 knockout (KO) prevented the phosphorylation of Rab10, a crucial step for insulin-mediated glucose transporter type 4 (GLUT4) translocation to cell surface (86). GLUT4 is a key glucose transporter in peripheral tissues, and this inhibitory impact on its cellular localization may lead to insulin resistance. This highlights the importance of reviewing the whole body of evidence before selecting the best potential therapeutic targets.

Several studies have demonstrated the role of vitamin D and its analogs as anti-inflammatory and immunomodulatory agents. In line with this, exposure of human pancreatic islets to the active form of vitamin D, calcitriol, increases expression of the protective candidate gene *TNFAIP3* and reduces pro-inflammatory cytokine (IL1β + TNF + IFNγ)-induced MHC class I expression, IL6 production and nitric oxide synthesis (87, 88). *In vivo* studies on NOD mice support these findings, with a decrease in insulitis and diabetes prevalence observed when calcitriol was administrated early in life (89). Furthermore, two large birth cohorts of genetically at risk individuals demonstrated that lower levels of 25(OH)-vitamin D in early infancy are associated with a higher incidence of islet autoimmunity (90, 91) and T1D development (90). This effect was modified by polymorphisms in the vitamin D receptor (VDR) (91). However,

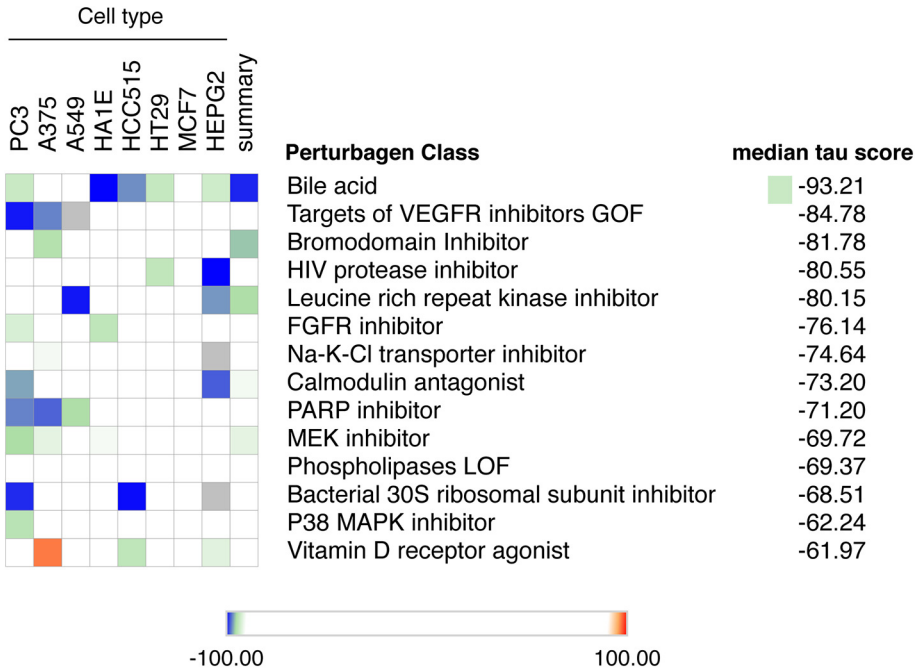

FIGURE 5 | Mining the beta cell signature in T1D to identify potential new therapeutic targets. The top 150 up-regulated transcripts identified in the RNA-seq of beta cells of T1D individuals (14) were used to query the Connectivity Map database of cellular signatures (7). The top Connectivity Map classes of perturbagens that promote an opposite signature (negative tau scores) to the one present in beta cells of T1D individuals are represented.

data from clinical trials using vitamin D and its analogies in humans have provided conflicting results. This may be caused by different factors: (1) the genetic background of the affected individuals (such as genetic variants in the candidate gene *TNFAIP3* and polymorphisms in the VDR) may modify their responses to vitamin D; (2) insufficient doses of vitamin D to modulate the immune system are often used, due to concerns regarding hypercalcemia; (3) too late introduction of the treatment; (4) the need to use vitamin D analogs in combination with other agents to address the complexity of T1D pathogenesis.

DISCUSSION

Despite the continued work performed by the research community there is still no treatment capable of preventing T1D development in individuals at risk or reverting disease after its outbreak. At the very best, use of anti-CD3, a class of drugs targeting exclusively the immune system, delayed by around 2 years the progression to T1D in individuals at high-risk for the disease (92). A similarly transient benefit on beta cell function was observed when treating patients with clinical T1D using anti-CD3 (93) or anti-CD20 (94).

In order to better understand and treat T1D, we may need to move on from an immune cell-centered view of the disease to a scenario that considers the disease as the product of a dynamic interaction between the killing immune cells and the target beta cells (2, 11, 26). To add information on the impact of insulitis on the target beta cells, we have presently characterized the human beta cell responses during T1D and after exposure to different immune-mediated stimuli. We observed that the *in vivo* beta cell responses can be closely recapitulated using an *in vitro* system biology approach that combines exposure to cytokines putatively present at different stages of the disease and high coverage RNA-sequencing. This may be further improved by the parallel evaluation of cytokine-induced changes in human beta cells protein expression (10, 95, 96) and chromatin status (10, 95). The validation of these models is an important finding, as the access to high quality beta cells from patients affected by T1D for multi-omics analysis is extremely difficult. Finally, the present integrated analysis identified important biological processes and signaling pathways activated during T1D progression, leading to the identification of potentially new therapeutic targets to be considered for future clinical trials.

Recent studies have suggested a role for aging and senescence-associated secretory profile (SASP) in beta cell disfunction and death in experimental models for both T1D and T2D (97, 98). We have searched for eventual changes in SASP-related genes (i.e., ARNTL, CDKN1A, ICAM1, ID2, LIMS1, MAP2K1, MAPK14, MIF, PRKCD, SERPINE1, TBX2, ULK3, ZMIZ1 etc.) in the present RNA-seq of beta cells from T1D individuals, but did not observe significant changes in any of these transcripts as compared to non-diabetic donors (data not shown).

Weakness of the present study, which may hamper extrapolations to most T1D patients, include the limited number of samples analyzed (there were only 4 preparations

from T1D patients available in the literature that reached the proposed criteria (see Methods) for inclusion in our analysis) and the disparate age of islet/beta cell donors when comparing for instance T1D patients (mean age 19.7 years) and IFNα-treated islets (mean age 71.3 years) (**Table 1**). It is remarkable that, in spite of these limitations, there remained major and consistent similarities between the gene expression observed in beta cells from different patients affected by T1D and the gene expression present in human islets exposed to pro-inflammatory cytokines.

To further expand the present information and better define the role of each cell type present in the islets of Langerhans or in its vicinity during insulitis, single cells analysis will be required (99); up to now, however, this has been technically challenging due to the very limited number of beta cells retrieved from diabetic individuals (100). This approach has been already performed in another autoimmune disease, systemic lupus erythematosus, in which single cell analysis of lupus nephritis identified cell specific signatures that led to the recognition of key signaling pathways suitable for specific therapeutic targeting (101).

The recent technical and scientific advances allowing the generation of beta cells derived from inducible pluripotent stem cells (iPSC) that show similar responses to pro-inflammatory cytokines as adult human beta cells (102), opens the possibility to differentiate beta cells from individuals with specific genetic variants that modulate the different stages of insulitis (2). Notably, these iPSC-derived cells could also be used to generate organoids simulating the pancreas environment, which would allow co-culture with relevant immune cells to better define beta cell-immune system crosstalk in the context of specific genetic backgrounds, and then to use this system for drug screening.

T1D is a complex disease, and a sustained therapeutic response will only be achieved by combining compounds that contribute to "re-educate" the immune system and to protect/regenerate beta cells. An interesting approach to be pursued at the beta cell level would be to down-regulate HLA class I antigen presentation while increasing signaling of the immune check-point proteins (PDL1 and HLA-E); this may be feasible, as suggested by the observation that inhibiting the transcription factor STAT2 decreases HLA class I expression while preserving/increasing PDL1 expression (53). Importantly, these interventions should be safe and started as early as possible to prevent irreversible loss of functional beta cell mass.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/) under the accession numbers: GSE108413, GSE133221, GSE53949, GSE121863, GSE121863.

AUTHOR CONTRIBUTIONS

MC and DE conceived, designed, supervised the study, wrote the manuscript, and all authors revised it. MC retrieved data, performed analysis, as such have full access to all the data in the study, take responsibility for the integrity of the data, and the accuracy of the data analysis. MC and FS performed bioinformatic analyses. 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/fendo.2020.568446/full#supplementary-material

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Pancreatic Alpha-Cells Contribute Together With Beta-Cells to CXCL10 Expression in Type 1 Diabetes

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C-X-C Motif Chemokine Ligand 10 (CXCL10) is a pro-inflammatory chemokine specifically recognized by the ligand receptor CXCR3 which is mostly expressed in T-lymphocytes. Although CXCL10 expression and secretion have been widely associated to pancreatic islets both in non-obese diabetic (NOD) mice and in human type 1 diabetic (T1D) donors, the specific expression pattern among pancreatic endocrine cell subtypes has not been clarified yet. Therefore, the purpose of this study was to shed light on the pancreatic islet expression of CXCL10 in NOD, in C57Bl/6J and in NOD-SCID mice as well as in human T1D pancreata from new-onset T1D patients (DiViD study) compared to non-diabetic multiorgan donors from the INNODIA European Network for Pancreatic Organ Donors with Diabetes (EUnPOD). CXCL10 was expressed in pancreatic islets of normoglycaemic and new-onset diabetic NOD mice but not in C57Bl/6J and NOD-SCID mice. CXCL10 expression was increased in pancreatic islets of new-onset diabetic NOD mice compared to normoglycaemic NOD mice. In NOD mice, CXCL10 colocalized both with insulin and glucagon. Interestingly, CXCL10-glucagon colocalization rate was significantly increased in diabetic vs. normoglycaemic NOD mouse islets, indicating an increased expression of CXCL10 also in alpha-cells. CXCL10 was expressed in pancreatic islets of T1D patients but not in non-diabetic donors. The analysis of the expression pattern of CXCL10 in human T1D pancreata from DiViD study, revealed an increased colocalization rate with glucagon compared to insulin. Of note, CXCL10 was also expressed in alpha-cells residing in insulin-deficient islets (IDI), suggesting that CXCL10 expression in alpha cells is not driven by residual beta-cells and therefore may represent an independent phenomenon. In conclusion, we show that in T1D CXCL10 is expressed by alpha-cells both in NOD mice and in T1D patients, thus pointing to an additional novel role for alpha-cells in T1D pathogenesis and progression.

Keywords: type 1 diabetes, pancreas, alpha-cells, chemokines, CXCL10

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease, characterized by a progressive destruction of pancreatic insulin-producing beta-cells driven by autoreactive T-lymphocytes (1) and leading to chronic hyperglycemia and to the development of chronic complications (2).

C-X-C Motif Chemokine Ligand 10 (CXCL10) is a pro-inflammatory chemokine secreted by a wide spectrum of cells. It is involved in multiple mechanisms and reported to have pleiotropic effects, including immune cell migration and attraction to inflammation sites, angiogenesis, and cancer cell growth (3, 4). CXCL10 is produced by pancreatic islet cells upon inflammatory stress (5) and is specifically recognized by C-X-C Motif Chemokine Receptor 3 (CXCR3) which is expressed by activated T-lymphocytes and other immune cells (4, 6). Several reports demonstrated that CXCL10 plays an important role in the natural history of T1D mainly through the attraction of autoreactive T-lymphocytes to the islets, thus leading to the subsequent destruction of pancreatic beta-cells (6–12). Of note, although still debated, CXCL10 has been proposed as a possible therapeutic target, supported by several studies showing the beneficial effects of CXCL10 inhibition (13).

In animal models, transgenic overexpression of CXCL10 in beta-cells, coupled to the induction of T1D through lymphocytic choriomeningitis virus (LCMV) infection, accelerated the autoimmune process by enhancing the migration of antigen-specific lymphocytes (6, 7, 10). Of note, neutralization of CXCL10 reduced the occurrence of the disease by affecting lymphocyte migration to the pancreatic islets and by enhancing beta-cell proliferation (14–18). Similar results, although more controversial, were reported in CXCR3-deficient mouse models or upon antagonistic blockade of CXCR3, leading to delayed insulitis and diabetes onset (19). Therefore, CXCL10:CXCR3-based pancreatic immune cell trafficking has been reported as an important component in the natural history of T1D.

In man, increased CXCL10 levels have been detected in serum of T1D patients compared to non-diabetic subjects and to type 2 diabetic (T2D) individuals, thus indicating a relationship between this phenomenon and autoimmune diabetes (20–23). Of note, CXCL10 levels were positively correlated with the numbers of autoreactive CD4 T cells and negatively associated with T1D duration and age at disease onset, suggesting important implications for CXCL10 in autoimmune diabetes progression and severity (21).

Using immunohistochemistry and immunofluorescence, we and others have previously demonstrated that CXCL10 expression is increased in pancreatic islets of T1D vs. non-diabetic donors (24–26). However, the exact CXCL10 expression pattern in pancreatic islet endocrine cell subsets has not been addressed.

Here, by using immunohistochemical fluorescence and confocal imaging, we aimed at elucidating the CXCL10 expression pattern among pancreatic islet endocrine cells in T1D through the analysis of pancreas samples from NOD, C57Bl/6J and NOD-SCID mice as well as from new-onset T1D patients (DiViD study) and non-diabetic multiorgan donors from the INNODIA European Network for Pancreatic Organ Donors with Diabetes (EUnPOD).

MATERIALS AND METHODS

Animals

C57Bl/6J, NOD-SCID, and NOD mice were housed and inbred in the animal facility of Katholieke Universiteit Leuven (KU Leuven, Leuven, Belgium) as previously described (27). All animal procedures were performed in accordance with the NIH guidelines for the care and use of laboratory animals and protocols were approved by the Ethics Committees of the KU Leuven. NOD female mice used in this study were screened for the onset of diabetes by evaluating urine glucose levels (Diastix Reagent Strips; Bayer, Leverkusen, Germany) and venous blood glucose levels (Accu-Chek; Roche Diagnostics, Vilvoorde, Belgium). Mice were diagnosed as diabetic when they had glycosuria and two consecutive blood glucose measurements exceeding 200 mg/mL. Pancreatic sections used for histological analysis were collected from 8-week-old C57Bl/6J ($n = 4$), 15- to 20-week-old C57Bl/6J ($n = 3$), 2- to 3-week-old NOD (Non-Obese Diabetic)-SCID (severe combined immunodeficient) ($n = 4$), 20-week-old NOD-SCID ($n = 3$), 20- to 22-week-old normoglycaemic NOD ($n = 4$) and 12- to 21-week-old new-onset diabetic NOD mice ($n = 4$).

Human Donors

Human pancreatic sections analyzed in this study were collected from two different cohorts of subjects.

INNODIA EUnPOD Cohort

Following acquisition of informed research consent, pancreata ($n = 3$) were obtained from brain-dead multiorgan donors within the European Network for Pancreatic Organ Donors with Diabetes (EUnPOD) (2), a project launched in the context of the INNODIA consortium (www.innodia.eu). Whole pancreata were processed following standardized procedures at University of Pisa. Formalin fixed paraffin embedded (FFPE) pancreatic tissue sections were obtained from $n = 3$ non-diabetic and islet-autoantibodies negative donors.

DiViD Cohort

Following the acquisition of appropriate consents, 6 new-onset T1D patients underwent pancreatic biopsy by adopting laparoscopic pancreatic tail resection, in the context of the Diabetes Virus Detection (DiViD) study (28). The pancreatic tissue was processed for multiple purposes including FFPE processing (28). From each patient included in the DiViD study, we analyzed two pancreatic sections from separate parts of the pancreas tail for CXCL10-INS-GCG staining and one pancreatic section for CXCL10-INS-CD45 staining. Collection of pancreatic tissue in the DiViD study was approved by the Norwegian Governments Regional Ethics Committee. Written informed consent was obtained from all individuals with type 1 diabetes after they had received oral and written information from the diabetologist and the surgeon separately (28). INNODIA

EUnPOD multiorgan donors' pancreata were obtained with approval of the local Ethics Committee at the University of Pisa.

Clinical characteristics of EUnPOD donors and of DiViD T1D subjects are reported in **Table 1**.

Immunofluorescence Analysis of Mouse and Human Pancreatic Sections

Formalin-Fixed Paraffin Embedded (FFPE) pancreatic sections obtained from mouse and human pancreata were analyzed using single or triple immunofluorescence and confocal imaging analysis in order to simultaneously evaluate the expression of CXCL10, insulin, glucagon or CXCL10, insulin, and CD45.

Mouse Pancreatic Sections

After deparaffinization and rehydration through alcohol series, pancreatic sections (5 μm thickness) were incubated with Tris-buffered saline (TBS) supplemented with 3% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) to reduce non-specific reactions. Antigen retrieval was performed using 10 mM citrate buffer pH 6.0 in microwave (600 W) for 10 min. Sections were incubated with polyclonal rabbit anti-murine CXCL10 [dilution 1:25, cat. 500-P129, Peprotech, Rocky Hill, NJ, USA; purified by affinity chromatography employing immobilized mCXCL10 matrix from sera of rabbits pre-immunized with highly pure (>98%) recombinant mCXCL10] (29), polyclonal guinea pig anti-insulin (dilution 1:500, cat. A0564, Dako-Agilent Technologies, Santa Clara, CA, USA) and monoclonal mouse anti-glucagon (dilution 1:300, cat. MAB1249 clone 181402, R&D Systems, Minneapolis, MN, USA) as primary antibodies; subsequently with goat anti-guinea pig Alexa-Fluor 488 conjugate (dilution 1:500, cat. A11073, Molecular Probe, Thermofisher, Waltham, MA, USA), goat anti-rabbit Alexa-Fluor 594 conjugate (dilution 1:500, cat. A11037, Molecular Probe, Thermofisher), goat anti-mouse 647 conjugate (dilution 1:500, cat. A21236, Molecular Probe, Thermofisher) as secondary antibodies. DNA was counterstained with DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride, dilution 1:3,000, cat. D8517, Sigma Aldrich). Sections were mounted with VECTASHIELD (cat. H-1000, Vector Laboratories, Burlingame, CA, USA) antifade medium and analyzed immediately or stored at 4°C until ready for confocal image analysis. Potential non-specific binding of goat anti-mouse-647 conjugate secondary antibody to mouse endogenous immunoglobulins was evaluated through a negative control staining with secondary antibody (without mouse anti-glucagon primary antibody), demonstrating the specificity of the reaction (**Supplementary Figure 1**).

Human Pancreatic Sections

FFPE Pancreatic sections obtained from DiViD and EUnPOD collections were analyzed by triple immunofluorescence using different combinations of antibodies. After deparaffinization and rehydration through alcohol series, 5 μm thick sections were incubated with methanol supplemented with 3% H_2O_2 to block endogenous peroxidases and with phosphate-buffered saline (PBS) supplemented with 3% BSA to reduce non-specific reactions. Antigen retrieval was performed with 10 mM citrate buffer pH 6.0 for 10 min. Sections were incubated with polyclonal guinea pig anti-insulin (dilution 1:500, cat. A0564, Dako), monoclonal mouse anti-glucagon (dilution 1:300, cat. MAB1249, clone #181402, R&D Systems), polyclonal rabbit anti-human CXCL10 [dilution 1:300, cat. 500-P93, Peprotech; specific antibody was purified by affinity chromatography employing immobilized hCXCL10 matrix from sera of rabbits pre-immunized with highly pure (>98%) *Escherichia coli* recombinant hCXCL10] (30–32) and/or monoclonal mouse anti-human CD45 (pre-diluted, cat. IR751, clone #2B11 + PD7/26, Dako) as primary antibodies. Subsequently, the following secondary antibodies were adopted: goat anti-guinea pig Alexa-Fluor 488 conjugate (dilution 1:500, cat. A11073, Molecular Probes, Thermofisher), goat anti-mouse Alexa-Fluor 647 conjugate (dilution 1:500, cat. A21236, Molecular Probes, Thermofisher), swine anti-rabbit HRP (dilution 1:100, cat. P0217, Dako), goat anti-guinea pig Alexa-Fluor 647 conjugate (dilution 1:500, cat. A21450, Molecular Probes, Thermofisher), goat anti-mouse Alexa-Fluor 488 conjugate (dilution 1:500, cat. A21236, Molecular Probes, Thermofisher). TSA Fluorescein system (dilution 1:50, cat. NEL742001KT, Perkin Elmer) was used to amplify CXCL10 signal. DNA was counterstained with DAPI. Sections were finally mounted with VECTASHIELD Antifade Medium (Vector Laboratories). Antibodies details

TABLE 1 | Demographics and main clinical parameters of T1D and non-diabetic donors.

	Case ID	Gender	Age	Disease duration (weeks)	Cause of death	IA	GADA	IA−2A	ZnT8A
Non-diabetic (EUnPOD)	20171031	F	54	n/a	Cardiovascular disease	n/a	neg	neg	neg
	20171114	M	49	n/a	Cardiovascular disease	n/a	neg	neg	neg
	20171118	M	39	n/a	Trauma	n/a	neg	neg	neg
Type 1 diabetic (DiViD)	DiViD-1	F	25	4	n/a	pos	pos	pos	pos
	DiViD-2	M	24	3	n/a	pos	neg	pos	pos
	DiViD-3	F	34	9	n/a	pos	neg	pos	pos
	DiViD-4	M	31	5	n/a	pos	pos	neg	pos
	DiViD-5	F	24	5	n/a	pos	pos	neg	pos
	DiViD-6	M	35	5	n/a	pos	neg	neg	neg

and main reagents used in the study are reported in **Supplementary Table 1**.

Image Acquisition and Analysis

Images were acquired using Leica TCS SP5 confocal laser scanning microscope system (Leica Microsystems, Wetzlar, Germany). Images were acquired as a single stack focal plane or in *z*-stack mode capturing multiple focal planes ($n = 5$) for each identified islet. Sections were scanned and images acquired at 40× or 63× magnification. The same confocal microscope setting parameters (laser power, photomultiplier voltage gain and offset values, pinhole value) were applied to all stained sections before image acquisition in order to uniformly collect detected signal related to each channel.

The analysis of CXCL10, insulin, and glucagon positive signal were performed using Volocity 6.3 software (Perkin Elmer, Waltham, MA, USA). A background threshold filter was uniformly applied to all processed images before the evaluation of specific parameters. A 3D model reconstruction adopting voxels quantification method or pixels, was used to compute single channel signals and to evaluate single channel volumes or the percentage of colocalization coefficient M_1 (Mander's coefficient) (33) between CXCL10 and insulin and between CXCL10 and glucagon. Colocalization Coefficient M_1 considers the percentage of pixels (or voxels in case of volume) of a given channel which overlaps to total pixels (or voxels) related to the other channel. Of note, Mander's coefficient is independent of absolute signal as it measures the fraction of one protein that colocalizes with a second protein where *M* represents the fraction (reported in percentage) of colocalizing pixels channel-1/channel-2 on total channel-1 pixels.

Colocalization coefficient M was analyzed for each identified mouse and human islet. In mouse islets, the following parameters were collected for each islet: islet/endocrine volume, insulin volume, glucagon volume, and CXCL10 volume.

Statistics

Results were expressed as mean ± SD. Statistical analyses were performed using Graph Pad Prism 8 software. Comparisons between two groups were carried out using Mann–Whitney *U* test (for non-parametric data) or Wilcoxon matched signed rank test. Multiple comparisons were analyzed using ordinary one-way ANOVA. Differences were considered significant with p-values < 0.05.

RESULTS

CXCL10 Expression in Pancreas of NOD Mice

CXCL10 expression was not detectable in pancreatic samples from 8-week-old and 20-week-old control C57Bl/6J mice, and absent or barely visible in pancreatic islets of 3-week-old and 20-week-old NOD-SCID mice, which showed no sign of immune cell infiltration or islet inflammation (**Supplementary Figure 2**).

In NOD mice pancreata, CXCL10 expression was not observed in exocrine tissue. In pancreatic islets of 20-22-week-old NOD normoglycaemic mice, CXCL10 expression was weak and localized in few cells within islet parenchyma (**Figure 1a,** panels A,B). In contrast, in new-onset diabetic NOD mice (12- to 21-week-old) the expression of CXCL10 was higher compared to NOD normoglycaemic mice, as shown by confocal z-stack imaging analysis of pancreatic islets (**Figure 1a**, panels C,D), revealing an absolute increase of CXCL10 positive volume (**Figure 1b**), as well as increased CXCL10 signal normalized per total islet volume (**Figure 1c**). Of note, CXCL10 volume normalization based on beta-cell content, similarly showed an increase of CXCL10 positive signal in pancreatic islets of new-onset diabetic NOD mice (**Supplementary Figure 3a**). Collectively, these results corroborated previous findings regarding the increase of CXCL10 in pancreatic islets of NOD mice in autoimmune diabetes.

CXCL10 Is Expressed in Beta- and in Alpha-Cells of NOD Mice

In order to verify whether the increase of CXCL10 expression observed in islets of new-onset diabetic NOD mice was exclusively dependent on its hyperexpression in beta-cells, we performed triple immunofluorescence staining for CXCL10, insulin and glucagon in pancreas sections of normoglycaemic and new-onset diabetic NOD mice. Interestingly, CXCL10 was expressed both in beta- and in alpha-cells (**Figure 2a**), suggesting that its expression is not an exclusive feature of beta-cells. Of note, in NOD normoglycaemic mice, colocalization analysis between CXCL10-insulin and CXCL10-glucagon revealed that the proportion of beta- and alpha-cells positive for CXCL10 was similar [CXCL10-INS 24.3 ± 15.3% vs. CXCL10-GCG 18.7 ± 15.2% (mean ± SD), respectively] (**Figure 2b**). In new-onset diabetic NOD mice, CXCL10-glucagon colocalization was significantly higher compared to CXCL10-insulin [40.6 ± 15.7% vs. 21.3 ± 16.0% (mean ± SD) $p < 0.0001$] (**Figures 2b,c**). Moreover, CXCL10-insulin colocalization did not differ between normoglycaemic and new-onset diabetic NOD mice [24.3 ± 15.3% vs. 21.3 ± 16.0% (mean ± SD)], suggesting that alpha-cells significantly contribute to the increase of CXCL10 in pancreatic islets of new-onset diabetic NOD mice. Importantly, such increase was not dependent on changes in islets volume (**Supplementary Figure 3b).**

Four Different Islet Subsets Can Be Identified in Pancreata of New-Onset T1D Patients Based on CXCL10 Expression Pattern

Results obtained in NOD mice demonstrated a peculiar CXCL10 expression pattern, pointing out to a significant involvement of alpha-cells in chemokine secretion in T1D. These findings prompted us to investigate its distribution in pancreatic islets of T1D donors.

Firstly, we further confirmed that CXCL10 was absent in pancreatic islets of non-diabetic donors. We analyzed pancreatic sections derived from 3 EUnPOD-INNODIA non-diabetic multiorgan donor (subjects characteristics reported in **Table 1**) using triple immunofluorescence analysis for CXCL10, insulin and glucagon. Results demonstrated that CXCL10 was not expressed in non-diabetic pancreatic islets (**Supplementary Figure 4**). Then, we analyzed CXCL10

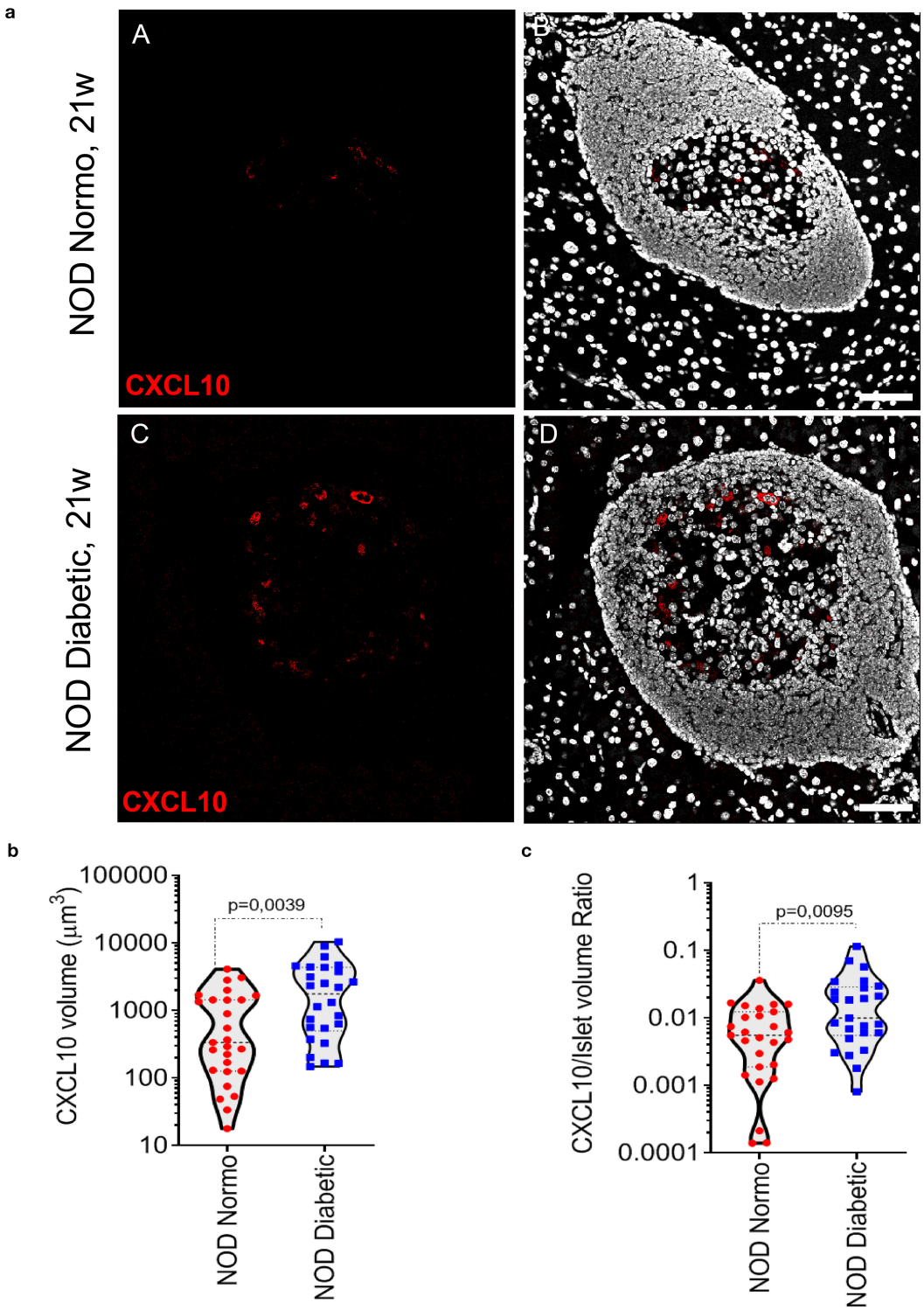

FIGURE 1 | CXCL10 is increased in pancreatic islets of new-onset diabetic NOD mice. **(a)** Immunofluorescence staining of CXCL10 in pancreatic tissue sections of $n = 4$ normoglycaemic NOD mice and in $n = 4$ new-onset diabetic NOD mice. Representative images of normoglycaemic (panels A,B) and new-onset diabetic NOD mice (panels C,D) are reported. CXCL10 is reported in red; nuclei in white/gray. Scale bar is 50 µm. Analysis of CXCL10 total voxels absolute volume **(b)** and normalized per total islet volume **(c)**, in pancreatic islets of $n = 4$ normoglycaemic and $n = 4$ NOD new-onset diabetic NOD mice. A total of $n = 27$ and $n = 25$ pancreatic islets were individually analyzed in normoglycaemic and new-onset diabetic mice, respectively; individual values for each islet are reported in μm^3 **(b)** or as a volumetric ratio **(c)**. Exact *p*-values were analyzed using non-parametric Mann–Whitney *U* test ($p < 0.05$).

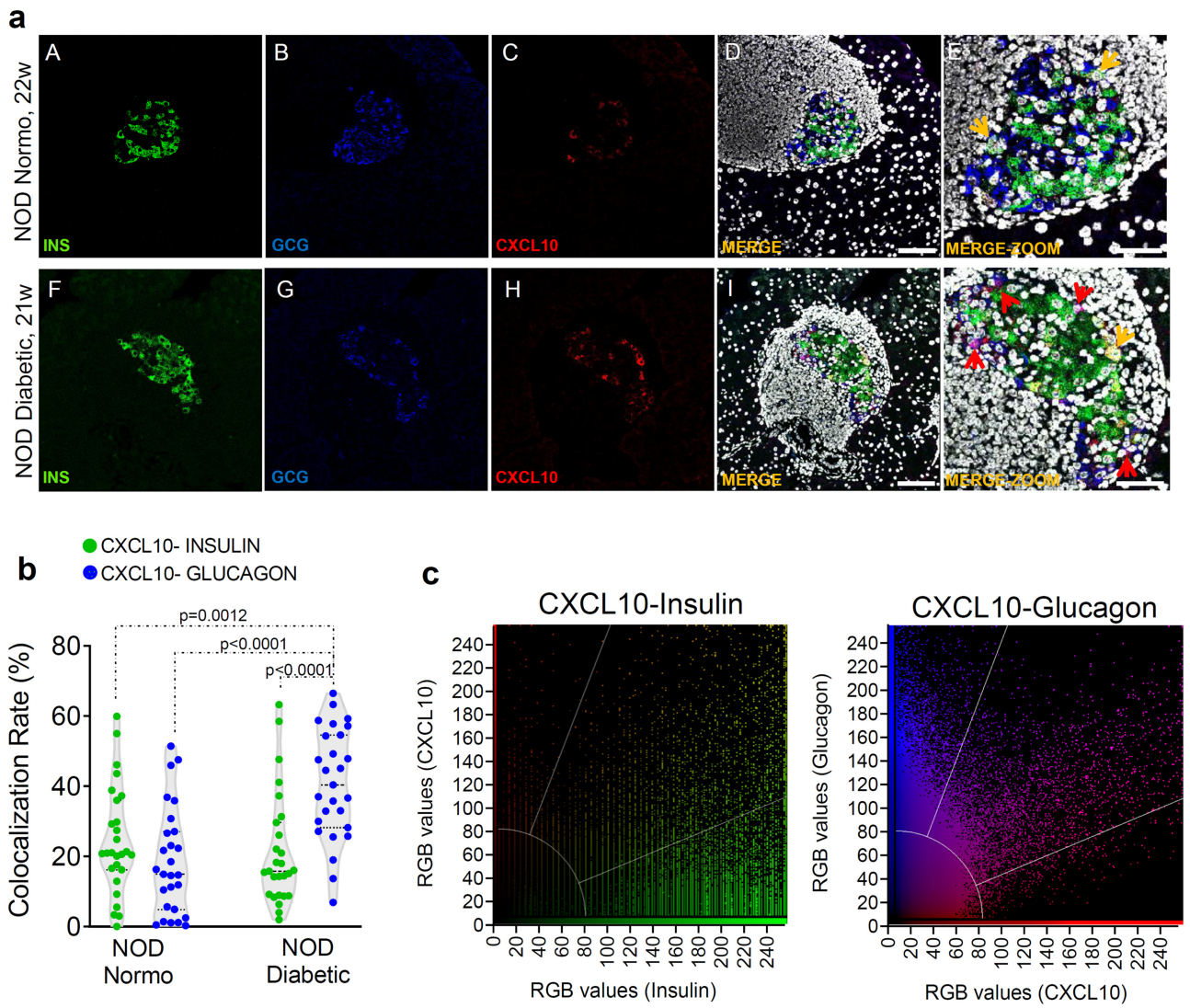

FIGURE 2 | CXCL10 is increased in alpha-cells of new-onset diabetic NOD mice. **(a)** Representative images of triple immunofluorescence reporting the expression of insulin (INS, green), glucagon (GCG, blue), and CXCL10 (red) in pancreatic islets of normoglycaemic (panels A–E) and of new-onset diabetic NOD mice (panels F–J). In panels E,K, zoom-in of pancreatic islets are shown. Colocalization between insulin and CXCL10 is shown in yellow and indicated by yellow arrows; colocalization between glucagon and CXCL10 is reported in magenta and indicated by red arrows. Scale bar in panels D,I = 50 μm. Scale bar panels E,J = 20 μm. **(b)** Colocalization rate are reported as the results of Manders's coefficient evaluation between CXCL10 and insulin (CXCL10-INS) (green dots) and CXCL10 and glucagon (CXCL10-GCG) (blue dots) in individual pancreatic islets of $n = 4$ normoglycaemic NOD mice and $n = 4$ new-onset diabetic NOD mice. Each dot represents an individual islet. A total of $n = 27$ pancreatic islets of normoglycaemic and new-onset diabetic NOD mice are reported. Values are reported as the percentage of CXCL10 signal overlapping with total INS or GCG signal. Exact p-values were analyzed using multiple comparison ordinary one-way ANOVA test ($p < 0.05$). Dotted lines represent mean ± SD. **(c)** Colocalization plots of CXCL10-Insulin (left) and CXCL10-glucagon (right) of a new-onset diabetic NOD mouse pancreatic islet. Positive pixels for CXCL10 (red), insulin (green), and glucagon (blue), alongside with colocalizing pixels (CXCL10-insulin: yellow; CXCL10-glucagon: magenta), are reported in the plots. Significant colocalizing pixels are within the area delimited by white lines, representing background and threshold levels relative to each channel. Each pixel is reported as a gray-scale RGB intensity value (0–255).

expression pattern distribution in pancreatic sections of 6 new-onset (≤9 weeks from diagnosis) T1D subjects from DiViD study (**Table 1**).

CXCL10 expression was not observed in exocrine tissue nor in CD45^{+} cells surrounding or infiltrating pancreatic islets or scattered in acinar tissue (**Supplementary Figure 5**). In line with previous reports, we confirmed that CXCL10 was expressed in pancreatic islets but not in exocrine/acinar tissue. Of note, a heterogeneous pattern of CXCL10 expression among pancreatic islets of T1D DiViD cases was clearly observed. Indeed, based on CXCL10 positivity and on the presence or absence of insulin [insulin-containing islets (ICIs) and insulin-deficient islets (IDIs)], four different islet subsets can be readily distinguished: ICIs with CXCL10 expression (ICI-CXCL10^{pos}) and ICIs without

any sign of CXCL10 positivity (ICI-CXCL10^{neg}); IDIs containing CXCL10 positive cells (IDI-CXCL10^{pos}) and IDIs without any positivity for the chemokine (IDI-CXCL10^{neg}) (**Figure 3**).

For each T1D DiViD case, we analyzed two non-consecutive sections derived from two different paraffin blocks of the pancreas tail. Overall, using fluorescent confocal microscopy, we manually screened a total of 1,148 pancreatic islets from 6 new-onset T1D DiViD subjects. Of 1,148 islets, 343 were ICIs and 805 were IDIs, in line to what has been previously observed from multiple histological analyses of the same DiViD cases, showing a higher number of IDIs vs. ICIs. Of 343 ICIs, the majority (n = 332, 96.7% of the total ICIs) were positive for CXCL10, while 11 (3.3%) were negative for the chemokine. Interestingly, among 805 IDIs, 514 (63.8%) contained CXCL10 positive cells, while 291 (36.1%) were negative (**Supplementary File 1**).

A case-by-case analysis showed a heterogeneous CXCL10 staining pattern and distribution of islet subsets, confirming a substantial heterogeneity among T1D individuals (**Figure 4** and **Table 2**). In all cases, regardless of the section analyzed, almost all ICIs were positive for CXCL10.

In both sections analyzed, Case-1, Case-2, and Case-5 showed a consistent and significant higher proportion of IDI-CXCL10^{pos} compared to IDI-CXCL10^{neg} (**Figure 4**). Of note, in these cases, IDIs represent the major source of CXCL10, being higher compared to ICI-CXCL10^{pos} (**Figure 4**, **Table 2**, and **Supplementary File 1**).

In contrast, in Case-3, Case-4, and Case-6, we found a striking heterogeneity between the two sections analyzed, mainly due to the different rate of IDIs positive for CXCL10 (**Figure 4**). Notable, in Case-6, the high heterogeneity observed in terms of ICIs and

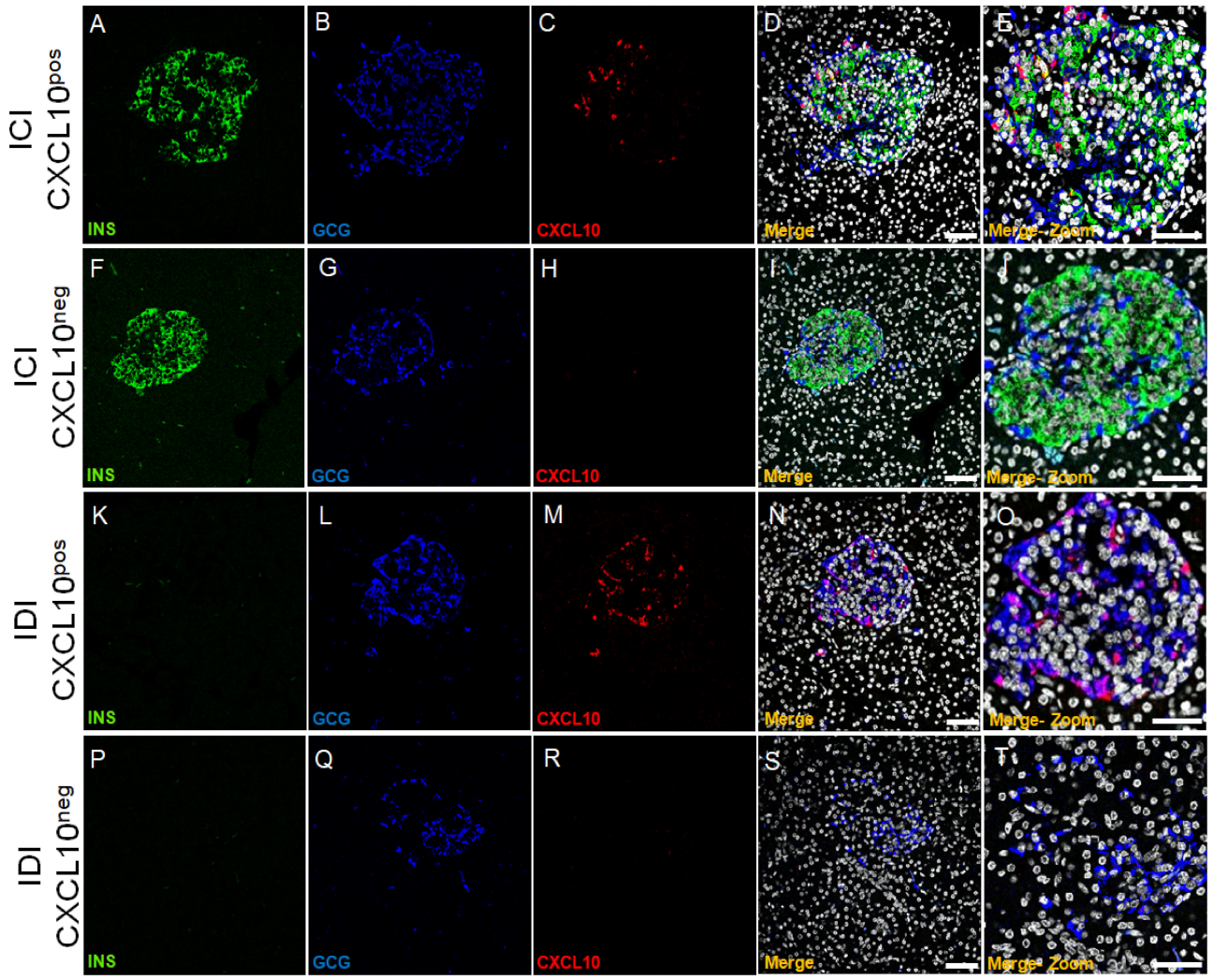

FIGURE 3 | CXCL10 is expressed in ICIs and IDIs of new-onset diabetic individuals and distinguished four pancreatic islet subsets. Triple immunofluorescence analysis of insulin (INS, green), glucagon (GCG, blue), and CXCL10 (red) in pancreatic sections of new-onset T1D DiViD cases. Representative pancreatic islet 40× confocal microscope images are shown for each channel, alongside with digital zoom-in for each set of panel. **(A–E)** ICI showing positivity for CXCL10. **(F–J)** ICI without CXCL10 positivity. **(K–O)** IDI showing CXCL10 positivity. **(P–T)** IDI without positivity for CXCL10. Scale bar =100 μm. Scale bar zoom-in = 40 μm.

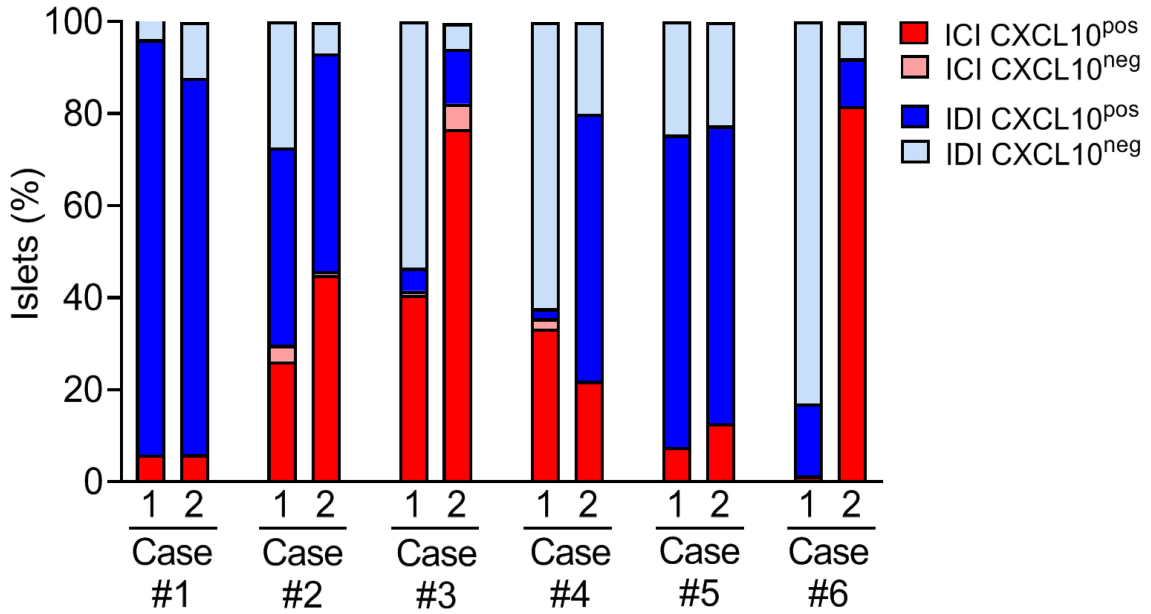

FIGURE 4 | Distribution of islet subsets in T1D DiViD individuals based on CXCL10 expression and ICI/IDI classification. Histological evaluation of islet subsets distribution in each of the six recent-onset DiViD individuals, based on the analysis of two non-consecutive pancreatic sections/case. Distribution of islets is reported as percentage value of total islets identified per section.

TABLE 2 | Table reporting the percentage values and the absolute number (in parentheses) of ICIs and IDIs positive or negative for CXCL10 in two non-consecutive pancreatic sections derived from two different formalin-fixed paraffin-embedded pancreatic tissue histological blocks of the same DiViD case.

	Section #	ICI CXCL10^{pos} % (absolute)	ICI CXCL10^{neg} % (absolute)	IDI CXCL10^{pos} % (absolute)	IDI CXCL10^{neg} % (absolute)
Case 1	Section #1	5.9 (3)	0 (0)	90.2 (46)	3.9 (2)
	Section #2	6.0 (5)	0 (0)	81.7 (67)	12.2 (10)
Case 2	Section #1	26.2 (22)	3.5 (3)	42.9 (36)	27.4 (23)
	Section #2	44.9 (53)	0.8 (1)	47.4 (56)	6.77 (8)
Case 3	Section #1	40.6 (41)	0.9 (1)	5 (5)	53.5 (54)
	Section #2	76.6 (69)	5.5 (5)	12.0 (11)	5.5 (5)
Case 4	Section #1	33.3 (15)	2.2 (1)	2.2 (1)	62.2 (28)
	Section #2	21.9 (23)	0 (0)	58.0 (61)	20 (21)
Case 5	Section #1	7.6 (16)	0 (0)	67.8 (143)	24.6 (52)
	Section #2	12.7(13)	0 (0)	64.7 (66)	22.5 (23)
Case 6	Section #1	1.4 (1)	0 (0)	15.7 (11)	82.9 (58)
	Section #2	81.6 (71)	0 (0)	10.3 (9)	8.0 (7)

*See **Supplementary File 1** for an extended version of this table.*

IDIs presence (section#1: 1.5% ICIs vs. 98.5% IDIs; section#2: 81.6% ICIs vs. 18.4% IDIs) between the two sections is paralleled by strong differences in CXCL10 islets positivity (**Figure 4**, **Table 2**, and **Supplementary File 1)** being more frequent in section#2 within ICIs (100% of ICIs CXCL10^{pos}) compared to IDIs in section#1 (15.7% of IDIs CXCL10^{pos}).

Alpha-Cells Contribute to CXCL10 Expression in Pancreatic Islets of New-Onset T1D Patients

The relevant presence of IDIs showing positivity for CXCL10 strongly suggests that also in human context, CXCL10 expression is not exclusively expressed by beta-cells. Indeed, triple immunofluorescence staining aimed at detecting insulin, glucagon, and CXCL10 expression in pancreatic sections of 6 new-onset T1D subjects from DiViD study, demonstrated that: (a) in ICIs, both beta- and alpha-cells stained positive for CXCL10 (**Figure 5a** and **Supplementary Figure 6**); (b) in IDIs, CXCL10 was expressed only in alpha-cells, since the (whole) signal of the chemokine perfectly overlapped with glucagon (**Figures 3K–O** and **Supplementary Figure 7**).

In order to quantify the contribution of beta- and alpha-cells to the overall expression of CXCL10 in pancreatic islets of T1D subjects, we analyzed the colocalization rate of CXCL10-insulin and CXCL10-glucagon in ICIs detected in all DiViD cases. Such analysis demonstrated that CXCL10-glucagon colocalization rate was significantly higher compared to CXCL10-insulin [CXCL10-GCG 36.5 ± 17.1% vs. CXCL10-INS 23.6 ± 18.9% (mean ± SD) (**Figures 5b,c**)], thus demonstrating that alpha-cells significantly contribute, together with beta-cells, to CXCL10 expression in pancreatic islets of T1D subjects.

DISCUSSION

Several studies reported that CXCL10 expression is increased in *in-vitro* cultured pancreatic islets upon inflammatory stresses (34, 35), as well as in pancreatic islets of animal models of

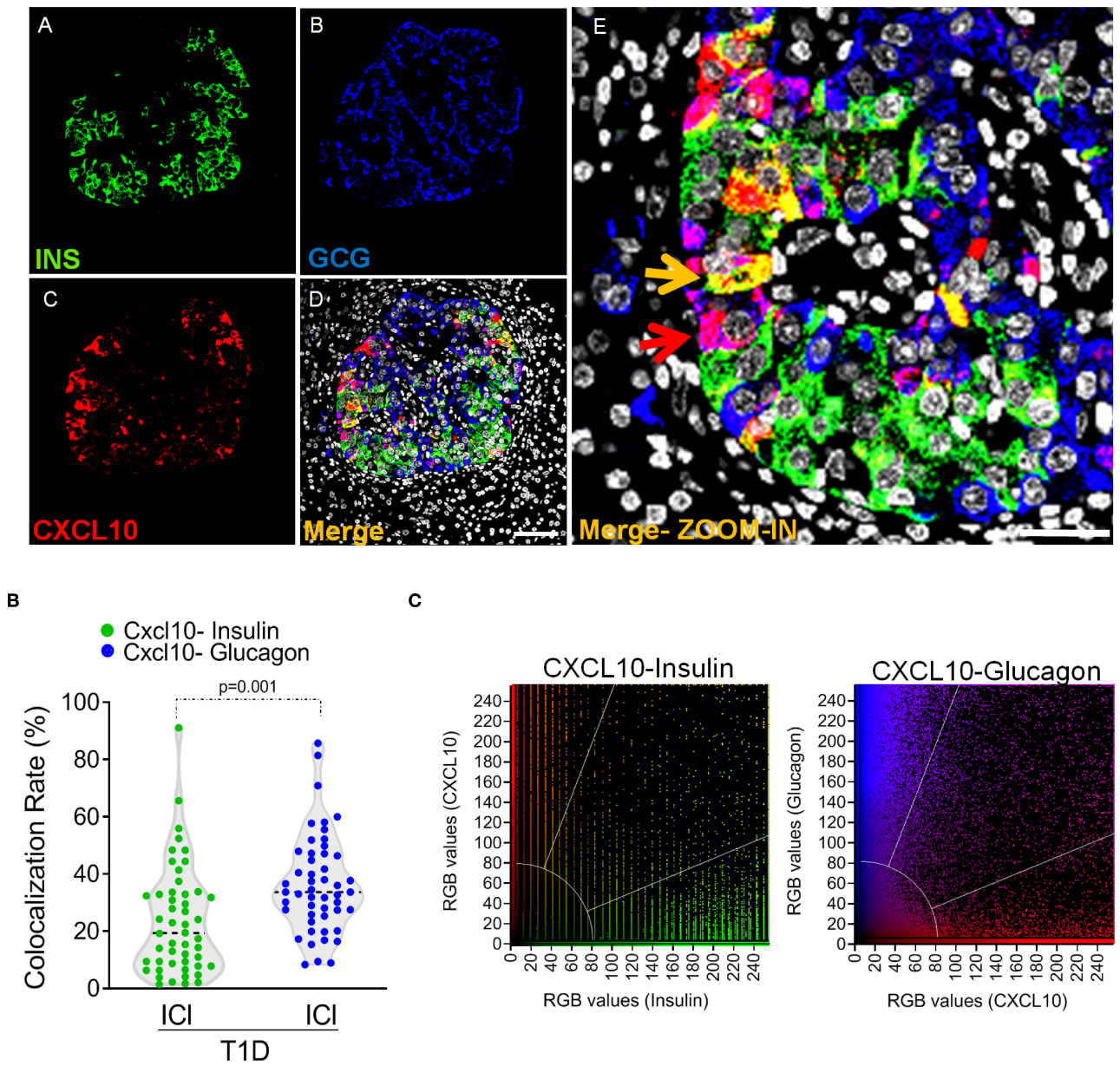

FIGURE 5 | Alpha-cells contribute to CXCL10 expression in islets of new-onset T1D patients. **(a)** Triple immunofluorescence analysis of insulin (INS, green, panel A), glucagon (GCG, blue, panel B), and CXCL10 (red, panel C) of pancreatic islets in T1D DiViD cases. Panel E: digital zoom-in of overlapping (merge) channels, showing colocalization of CXCL10 and insulin (yellow pixels) indicated by yellow arrow and of CXCL10 and glucagon (magenta pixels) indicated by red arrow. Scale bar = 50 μm. Scale bar zoom-in = 20 μm. **(b)** Colocalization analysis of CXCL10 and insulin (green dots) and CXCL10 and glucagon (blue dots) in pancreatic islets of T1D DiViD cases. A total of $n = 50$ ICIs from 6 DiViD cases were analyzed for both CXCL10-insulin and CXCL10-glucagon colocalization rate. Values are reported as the percentage of overlapping CXCL10-insulin or CXCL10-glucagon pixels over total insulin or glucagon positive pixels, according to Mander's Coefficient calculation. Exact *p*-value was calculated using Wilcoxon matched-pairs signed rank test. **(c)** Colocalization plots of CXCL10-insulin (left) and CXCL10-glucagon (right) of a recent-onset diabetic DiViD individual ICI (Case-1). Positive pixels for CXCL10 (red), insulin (green), and glucagon (blue), alongside with colocalizing pixels (CXCL10-insulin: yellow; CXCL10-glucagon: magenta), are reported in the plots. Significant colocalizing pixels are within the area delimited by white lines, representing background and threshold levels relative to each channel. Each pixel is reported as a gray-scale RGB intensity value (0–255).

autoimmune diabetes (6, 7) and in donors with T1D (24–26). However, data are lacking regarding CXCL10 intra-islet expression pattern in T1D. Such context prompted us to further investigate CXCL10 expression in pancreas sections of NOD mice and of T1D subjects from DiViD study, in order to better define CXCL10 intra-islet distribution.

In the present study, we confirmed that CXCL10 was expressed in pancreatic islets but not in exocrine tissue in T1D, while its expression was not observed in pancreas of healthy donors. Our data are in line with previous reports showing increased expression of CXCL10 in pancreatic islets in T1D (23–25).

Interestingly, our data suggest that both beta- and alpha-cells contribute to CXCL10 expression in T1D pancreatic islets, both in diabetic NOD mice and in DiViD T1D subjects.

In 12- to 21-week-old new-onset diabetic NOD mice, CXCL10 was expressed in pancreatic islets, but not in exocrine tissue, and significantly increased vs. age-matched normoglycaemic NOD mice.

Our results show a significant increase in the proportion of alpha-cells expressing CXCL10 in new-onset diabetic vs. normoglycaemic NOD mice, thus potentially suggesting that a higher rate of alpha-cells are subjected to inflammatory stresses and respond by activating CXCL10 transcription.

These findings are mirrored in pancreata of T1D DiViD subjects compared to healthy multiorgan donors collected within the EUnPOD network of INNODIA consortium. In line with previous studies (24–26), we confirmed that CXCL10 was specifically expressed in pancreatic islets of T1D subjects and absent in non-diabetic controls. In ICIs, CXCL10 expression was observed both in beta- and in alpha-cells. As expected, in all DiViD cases analyzed, most of the ICIs (95%) showed positivity for CXCL10, in line with previous observations which attributed a more aggressive insulitis and inflammation to those islets containing residual beta-cells (36). Of interest, in ICIs we observed a higher proportion of CXCL10 positive alpha-cells compared to beta-cells, suggesting a critical contribution of alpha-cells to the pancreatic islet expression of CXCL10. To this regard, it should be underlined that Mander's colocalization coefficient is independent of absolute signal as it measures the fraction of one protein that colocalizes with a second protein; therefore, it is unlikely that the differences observed in the colocalization rates are dependent on beta- or alpha-cell mass modifications.

Strikingly, the expression of CXCL10 was also clearly observed in alpha-cells of IDIs where beta-cells were absent and inflammation was lower or not present, as shown previously (37–40) and in the present manuscript as well (**Supplementary Figures 4a,b**). Based on manual counting of IDI-CXCL10^{pos} in each DiViD case, we observed that Case-1, Case-2, and Case-5 revealed a higher fraction of IDI-CXCL10^{pos} among all IDIs detected; this result is consistent between the two non-consecutive pancreatic sections analyzed. Conversely, a substantial heterogeneity between the two sections was observed in Case-3, Case-4, and Case-6, mainly due to the different rate of ICI-CXCL10^{pos}, clearly evident in Case-6. Despite the high heterogeneity, overall, Case-3, Case-4, and Case-6 showed the lowest proportion of IDI-CXCL10^{pos} (**Supplementary File 1**). In an effort aimed at looking for specific characteristics correlated with CXCL10-based DiViD cases patterning, we found that Case-6, showing the lowest rate of CXCL10^{pos} islets (considering both sections and independently of its cellular distribution) (**Supplementary File 1**), also exhibited the lowest expression of *HLA-ABC* genes among DiViD cases, as previously reported by Richardson S and colleagues (38). Additionally, in Case-3, classified by having high residual beta-cell content, severe insulitis and high expression of HLA Class-I (37, 38), we observed the highest proportion of ICI-CXCL10^{pos} among all DiViD cases.

Collectively, these results suggest that, although residual beta-cells drive severe pancreatic islet inflammation leading to a global CXCL10 increase, the expression of this chemokine in alpha cells could represent a phenomenon not strictly dependent on beta-cell content. Of note, a very high level of heterogeneity was observed among cases analyzed and among different paraffin blocks of the same case, in line with the heterogeneous nature of the disease, previously highlighted by several studies assaying the same cases (28, 37, 38, 41).

In support of our data, CXCL10 hyperexpression in DiViD cases was also previously observed at the mRNA level, being its expression significantly increased in laser-captured microdissected islets of T1D donors compared to non-diabetic controls (42); of note, CXCL10 hyperexpression was reported to be significantly associated to peri-islet insulitis microdissected tissue rather than to pancreatic islets core. Such results are in line with our data; indeed, it is likely that CXCL10 hyperexpression observed in peri-islet/insulitic microdissected tissue from T1D donors was mostly derived from alpha-cells clusters which are more closely associated to the peri-islets basement membrane (43). In addition, our results exclude an overlapping between insulitic immune cells and CXCL10 expression as shown by CD45-CXCL10 immunofluorescence staining in T1D DiViD sections (**Supplementary Figure 4a**).

In support to our findings, CXCL10 expression in alpha-cells was previously reported by Tanaka et al. in Japanese fulminant diabetes cases (26) and, more recently, by Moin et al. (44) in pancreatic islets of multiorgan donors with chronic pancreatitis, thus confirming and extending the observation of CXCL10 expression in alpha-cells in autoimmune diabetes.

Of interest, our data corroborate the increasing importance attributed to alpha-cells in the pathogenesis and progression of T1D. Alterations of several genes alongside with functional defects have been observed in alpha-cells obtained from T1D donors. These include alterations of alpha-cells phenotypic-maintenance genes and defects in glucagon secretion (45). We can speculate that inflammation may contribute to the activation of several signaling pathways, which alter alpha-cells phenotype and activate innate inflammatory responses leading to CXCL10 expression. As a matter of fact, CXCL10 is not the only pro-inflammatory molecule expressed by alpha-cells; indeed, it has been reported that alpha-cells can express also IL-1β (46) as well as IL-6 (47), thus potentially contributing to the pro-inflammatory islet microenvironment causing preferential homing of T-lymphocytes in pancreas in T1D (48). In turn, increased immune cell migration and then inflammation could enhance beta-cell antigenicity through higher HLA Class-I expression and novel peptides exposure to the immune system (49), thus generating a critical positive feedback loop.

An additional layer of evidence, supporting the expression of CXCL10 by alpha-cells, is given by their molecular equipment needed to induce those signaling pathways which lead to CXCL10 transcriptional activation. Indeed, analysis of transcriptome datasets comparing beta- and alpha-cells gene expression, showed an almost equal expression levels of those receptors and intracellular molecules which initiate the signaling cascades leading to CXCL10 transcriptional activation, such as IFNAR1, IFNAR2, IFNGR, TYK2, TNFRSF1A, and IL-1R (50–53), thus demonstrating the potential ability of alpha-cells to respond to the inflammatory milieu and potentially activate CXCL10 pathway.

However, several open questions remain. Firstly, the potential role of CXCL10 beside its effects on immune cells recruitment needs to be clarified; several reports attributed a role for CXCL10 in proliferation and angiogenesis (54). Particularly, it has been reported that CXCL10 can modulate vascular angiogenesis (55), also through the inhibition of VEGF-A (56). Angiogenesis has been linked to beta-cell regeneration through the re-arrangement of islet microenvironment, thus hypothesizing a role for islet CXCL10 as a factor involved in the modulation of beta-cell regeneration (57).

Secondly, the presence of CXCL10 in IDIs with no sign of inflammation may suggest that CXCL10 transcriptional activation is not only induced by cytokines and inflammatory mediators but may be caused by the exposure to additional factors. In this regard, alternative signaling pathways and receptors (e.g., TLR4) have been reported for the induction of CXCL10 (58).

Thirdly, the co-existence of IDI-CXCL10^{pos} and IDI-CXCL10^{neg} indicates a high level of heterogeneity involving also pancreatic islets alpha-cells expressing CXCL10; the identification of those factors determining the expression of CXCL10 in alpha-cells and how these correlate with individual islet phenotype would be of major importance to understand the role of this chemokine in T1D.

In conclusion, we have shown that chemokine CXCL10 is expressed also by alpha-cells which represent important contributors to the expression of CXCL10 in pancreatic islets. These results further underline the role of alpha-cells in T1D pathogenesis and progression and suggest the need to advance our knowledge regarding function and dysfunction of these cells in pancreatic islet autoimmunity.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Norwegian Governments Regional Ethics Committee. Written informed consent was obtained from all individuals with type 1 diabetes after they had received oral and written information from the diabetologist and the surgeon separately. EUnPOD multiorgan donors' pancreata not suitable for clinical purposes were obtained with informed written consent by organ donors' next-of-kin and processed with the approval of the local ethics committee of the Pisa University. Specific consent to the publication of the data was obtained from the participants or by organ donors' next-of-kin. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Ethics Committee of the KU Leuven. All animal procedures were performed in accordance with the NIH guidelines for the care and use of laboratory animals. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LN, NB, and GS performed the experiments, analyzed the data, and wrote the manuscript. GG analyzed the data and contributed to the scientific discussion. GL performed the experiments during the revision stage and contributed to the scientific discussion. LN, GS, and FD reviewed the manuscript and designed experiments. LK and KD provided support for DiViD cohort and contributed to the scientific discussion. CG and LO reviewed the manuscript, provided support for animal models, and contributed to the scientific discussion. CM reviewed the manuscript and contributed to the scientific discussion. LM and PM reviewed the manuscript, provided support for EUnPOD donors, and contributed to the scientific discussion. 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/fendo.2020.00630/full#supplementary-material

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Chemokines as Drivers of the Autoimmune Destruction in Type 1 Diabetes: Opportunity for Therapeutic Intervention in Consideration of an Optimal Treatment Schedule

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Type 1 diabetes (T1D) is mainly precipitated by the destruction of insulin-producing β-cells in the pancreatic islets of Langerhans by autoaggressive T cells. The etiology of the disease is still not clear, but besides genetic predisposition the exposure to environmental triggers seems to play a major role. Virus infection of islets has been demonstrated in biopsies of T1D patients, but there is still no firm proof that such an infection indeed results in islet-specific autoimmunity. However, virus infection results in a local inflammation with expression of inflammatory factors, such as cytokines and chemokines that attract and activate immune cells, including potential autoreactive T cells. Many chemokines have been found to be elevated in the serum and expressed by islet cells of T1D patients. In mouse models, it has been demonstrated that β-cells express chemokines involved in the initial recruitment of immune cells to the islets. The bulk load of chemokines is however released by the infiltrating immune cells that also express multiple chemokine receptors. The result is a mutual attraction of antigen-presenting cells and effector immune cells in the local islet microenvironment. Although there is a considerable redundancy within the chemokine ligand-receptor network, a few chemokines, such as CXCL10, seem to play a key role in the T1D pathogenesis. Studies with neutralizing antibodies and investigations in chemokine-deficient mice demonstrated that interfering with certain chemokine ligand-receptor axes might also ameliorate human T1D. However, one important aspect of such a treatment is the time of administration. Blockade of the recruitment of immune cells to the site of autoimmune destruction might not be effective when the disease process is already ongoing. By that time, autoaggressive cells have already arrived in the islet microenvironment and a blockade of migration might even hold them in place leading to accelerated destruction. Thus, an anti-chemokine therapy makes most sense in situations where the cells have not yet migrated to the islets. Such situations include treatment of patients at risk already carrying islet-antigen autoantibodies but are not yet diabetic, islet transplantation recipients, and patients that have undergone a T cell reset as occurring after anti-CD3 antibody treatment.

Keywords: CD3, CXCR3, CXCL10, combination therapy, migration, insulitis

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TYPE 1 DIABETES

It has become clear in the last decades that the predominant destructive force responsible for β-cell death in type 1 diabetes (T1D) are autoaggressive CD8 T cells. Although there is still debate on how the autoimmune response against islet autoantigens is initiated, it seems clear that local inflammation in the islets participates in drawing a broad variety of leukocytes to the islet microenvironment (1). Of course, virus infection has been associated with the etiology of T1D and there is ample evidence to support this hypothesis (2). For example, enterovirus proteins and RNA have been found in islets of T1D patients (3). A large meta-analysis confirmed a significant clinical association between enterovirus infection and T1D (4). However, there is yet no firm, causative proof that would directly demonstrate that virus infection results in immunopathogenic events that would result in the destruction of β-cells and the development of T1D. One problem is the temporal relation between infection and disease onset. Environmental triggers such as virus infection might have occurred long before clinical diagnosis. Further, it is also feasible that more than one triggering event might be required to finally precipitate the disease (5). Thereby, enterovirus infection might as well accelerate a pre-existing autoimmune condition rather than initiate it. Further investigations with more pancreas material, as available from the network for Pancreatic Organ Donors with Diabetes (nPOD), will hopefully shed some more light on the role of virus infection in the etiology of T1D in the future.

For many autoimmune diseases, including T1D, a mechanism termed "molecular mimicry" has been suggested to play a critical role. Molecular mimicry describes a sequential and/or conformational similarity between components of an invading pathogen and the host (6). Cross-reactive antibodies and/or T cells that have been generated during the anti-pathogen defense would thereby also target the similar self-structures of the host and may cause autoimmune damage resulting in clinical disease. Thereby, depending on the strength of the immune tolerance, molecular similarity between pathogen and natural occurring structures of the host is more likely to break tolerance than molecular identity. Many host proteins are expressed in the thymus and central tolerance established to identical molecules might be too strong to be broken. Indeed, in a mouse model for autoimmune hepatitis (7), an infection with a triggering antigen that is similar but not identical to the host target autoantigen was more effective in breaking tolerance and inducing disease as infection of mice that also carry the triggering antigen as a transgenic self-component (8). Interestingly, the immune response to the triggering antigen was focused on epitopes that share an intermediate homology to the host autoantigen, whereas no reactivity was found to regions with high or low homology (8).

The RIP-LCMV-GP mouse model for rapid-onset T1D is based on the concept of molecular mimicry. Such mice express the glycoprotein (GP) of the lymphocytic choriomeningitis virus (LCMV) controlled by the rat insulin promoter (RIP) specifically in the β-cells of the islets of Langerhans in the pancreas (9, 10). T1D is initiated by infecting the mice with LCMV resulting in overt T1D within 10–14 days. LCMV-GP is not expressed in the thymus of RIP-LCMV-GP mice. Thus, although the viral GP is identical with the transgenic GP in the β-cells, LCMV-GP specific T cells are generated that effectively eliminate the virus and attack the β-cells in an autoaggressive manner (10, 11). In contrast to spontaneous models, like the non-obese diabetic (NOD) mouse (12), the RIP-LCMV-GP model allows for a detailed analysis of pathogenic events at defined times after disease initiation, starting from the pathogen-induced acute damage and inflammation to the events driving insulitis and finally the destruction of the β-cells. Thus, besides the valuable information obtained from serum samples as well as pancreas sections of patients with T1D, many insights on crucial inflammatory factors involved in the recruitment of autoaggressive lymphocytes to the islets of Langerhans have been gained from inducible animal models.

CHEMOKINES AS INFLAMMATORY MEDIATORS

Cellular infiltrations into inflamed tissues as occurring in acute or chronic infections as well as autoimmune diseases are orchestrated by chemokines. These chemoattractant inflammatory mediators are mainly released by the local endothelium and by invading immune cells. There is a large, partially redundant, network of chemokines all of which attract a distinct set of migratory leukocytes expressing the corresponding chemokine receptors. Chemokines are classified according to the arrangement of their cysteins in the N-terminal region into the groups CC, CXC, CX_3C, and XC (13, 14). Specific patterns of chemokines are often found in the serum of patients with autoimmune diseases, but also in those with acute or chronic inflammatory diseases. In order to lure leukocytes to the site of inflammation, many chemokines form a gradient along the endothelial layer by binding to glycosaminoglycans, like heparan sulfate (15). In response to the chemokine gradient migratory leukocytes start rolling along the endothelial layer with the help of selectins that interact with low-affinity (16). In a second step, surface receptors of the integrin family e.g. lymphocyte function-associated antigen 1 (LFA-1) and of the immunoglobulin superfamily of adhesion molecules such as platelet endothelial cell adhesion molecule (PECAM-1) get upregulated and activated, allowing for firm adhesion and finally transmigration of leukocytes through the endothelial cell layer (17). For more detailed information about leukocyte extravasation and the role of adhesion molecules during trafficking to the islets of Langerhans I suggest a recent review by Sandor et al. (18). In general, chemokines are released by local endothelial cells as well as by leukocytes in response to inflammatory factors, including cytokines, such as interferons, interleukins and TNFα. Thereby specific patterns of chemokines seem to emerge locally depending on the corresponding inflammatory disease. Even though glycosaminoglycan-bound chemokines are forming a concentration gradient surrounding the inflamed tissue, many can also be detected in the serum of patients. Due to their partial redundancy and often widespread appearance chemokines are not considered *bona fide* biomarkers for certain diseases. Nevertheless, elevated chemokine serum levels are often associated with autoimmune diseases, including rheumatoid

arthritis, multiple sclerosis, or T1D and, together with adhesion molecules, chemokine ligands and their receptors have been and still are considered major drug targets for novel anti-inflammatory therapies (19–21).

ROLE OF CHEMOKINES IN TYPE 1 DIABETES

The term "insulitis" refers to the local inflammatory milieu with cellular infiltrations and the release of inflammatory factors including chemokines in and around the islets of Langerhans. There are considerable differences in the degree and composition of insulitis between T1D patients and animal models (22). In recent-onset T1D up to one year after diagnosis children (0–14 years) and young adults (14–39 years) still bear 38 and 56% functional islets, respectively. Thereafter, the fraction of functional islets declines to approximately 13% (22). In NOD mice the majority of females develop spontaneous T1D characterized by four stages: Early infiltration at week 4–7 of age, increased insulitis and activation of infiltrating cells at week 8–11 of age, cytotoxicity development with beginning destruction of β-cells at week 12–18 of age, and finally clinical T1D at an age of more than 18 weeks (22). However, the onset of clinical T1D for an individual female NOD mouse can range from 15 to >30 weeks of age. NOD mice display a massive insulitis that is at least one order of magnitude higher than in T1D patients and, in contrast to T1D patients, over time insulitis affects almost all islets in NOD mice. Interestingly, at early stages NOD mice show peri-insulitis, which despite the presence of large clusters of infiltrating cells that show growing similarities with tertiary lymphoid structures (TLO), remains in a surrounding position outside a barrier composed of peri-islet Schwann cells (23) and a peri-islet basement membrane (24). In the fast-onset RIP-LCMV-GP model the events leading to the destruction of the β-cells are more coordinated between individual mice. Cellular infiltration into the islets starts already after about 3 days after LCMV-infection, when neutrophils, macrophages, and dendritic cells enter. By day 7, the first CD4 and CD8 T cells appear and their numbers further increase through days 10 and 14. In contrast to the NOD model, insulitis in the RIP-LCMV-GP model has no clear peri-insulitis stage and cells infiltrate in between β-cells even at an early stage. By days 14–28 post-infection TLO-like clusters of infiltrates are apparent in and around many islets. However, due to the rapid destruction of β-cells and the decline of islet mass after day 28 these clusters get smaller in size and often only islet scars are remaining (25, 26). Overall, in both induced and spontaneous mouse models, insulitis is far more pronounced and the destruction of β-cells occurs faster than in T1D patients. However, the composition of islet-infiltrating cells is similar in T1D patients and NOD as well as RIP-LCMV mice. In particular, insulitis is dominated by T cells that include both islet-antigen specific and non-specific CD8 T cells (27–31). A recent study in human pancreas sections by Bender et al. demonstrated that islet antigen-specific CD8 T cells are found in the islet microenvironment as well as in remote regions of the exocrine pancreas (32). Interestingly, such preproinsulin-specific CD8 T cells are already abundant in the exocrine pancreas of non-diabetic donors. However, during T1D they migrate to and accumulate around and in the islets (32). These data suggest that certain triggering factors, such as local MHC class I upregulation and islet-specific chemokine production, might activate islet antigen-specific CD8 T cells in the exocrine pancreas and guide them to the islets of Langerhans.

Due to the limited access to human pancreas material, the bulk part of collected information about inflammatory factors, such as chemokines, that might be involved in driving the progressing human insulitis is derived from serum assessments. Thus, many chemokines, in particular those associated with a type 1 (Th1/Tc1-associated) T cell response, like CXCL9 and CXCL10, have been found to be elevated in the serum of T1D patients in comparison to healthy donors. For example, elevated serum levels of the type 1 chemokine CXCL10, but not the type 2 (Th2/Tc2-associated) chemokine CCL2, have been found in children with T1D (33). Another study with individuals at high risk for T1D (i.e. 1st degree relatives with multiple autoantibodies) demonstrated increased CCL3 and CCL4 levels, but no change in CXCL10 (34). The problem with all these studies is that on the one hand only small numbers of patients have been analyzed and on the other hand the individual cohorts were in different stages of the disease, ranging from individuals at risk to patients with long established T1D. Anyhow, even if there is no large meta study that would integrate the observations made, the type 1 T cell chemokine CXCL10 seems to be one of the most critical inflammatory mediators that has been associated with the pathogenesis of T1D.

Besides the analysis of serum/plasma samples, additional information has been obtained from isolated islets. The expression of chemokine ligands CCL5, CCL8, CCL22, CXCL9, CXCL10, and CX_3CL1 has been found in purified human and mouse islets after stimulation with pro-inflammatory cytokines, such as IFNγ and TNFα (35). Further, a transcriptome analysis from islets isolated from NOD and NOD.Rag1-/- control mice at different weeks of age revealed an NOD-specific upregulation of several chemokine ligands (CCL2, CCL4, CCL5, CCL19, CCL22, CXCL9, CXCL10, CXCL11, CXCL13, and XCL1) as well as the presence of chemokine receptors, including CCR2, CXCR4, and CXCR6 on all major leukocyte populations (30). However, there are two problems that arise when working with isolated islets: First, the isolation process itself is often inducing the release of chemokines and other inflammatory factors (36–38) and second, peri-insulitis leukocytes that are mostly only loosely attached to the islets are often lost during the isolation process. Thus, when analyzing the local inflammatory milieu, the expression of chemokine receptors as present on the infiltrating leukocytes might be underestimated. An alternative source for gene expression profiling would be laser-capture micro-dissected islets. Thereby, RNA can be isolated from the entire islet microenvironment, selected regions containing exclusively intact β-cells or infiltrating cells, or even from single β-cells (39). Indeed, laser-capture micro-dissection has been performed in pancreas section of healthy controls, patients with T1D, as well as of several mouse and rat models (37, 40, 41). Although some of these studies have identified proteins involved in immune cell migration to be upregulated in T1D (42), the focus of these

studies was often on other topics and therefore a clear conclusion on the role of chemokines is still missing. One reason for this lack of solid data might be the broad time frame in which T1D is manifesting in patients as well as in spontaneous models, such as the NOD mouse, that prevents a detailed analysis of inflammatory events playing a role in the various phases of T1D immunopathology. In contrast, inducible models with a defined starting point and a tightly synchronized schedule of pathogenic events allow a detailed mapping of the expression pattern over time with only little inter-individual variations. Several studies in the inducible RIP-LCMV model using RNA from whole pancreas or from isolated islets as well as performing immunohistochemistry of pancreas sections at several defined times after initiation of T1D by LCMV infection demonstrates that during the immunopathogenesis of T1D a broad variety of chemokines are released with distinct kinetic patterns (26, 35, 43). As in the study by Carrero et al. with NOD-islets (30), an increase in chemokine receptors occurs after the expression of chemokine ligands has been upregulated, indicating the presence of chemokine receptors on infiltrating cells. A selection of studies that identified chemokines in patients with T1D and/or experimental animal models for T1D is displayed in **Table 1**.

THE CXCL10/CXCR3 CHEMOKINE AXIS IN TYPE 1 DIABETES

The chemokine CXCL10 has been identified quite a while ago to have a dominant role in the attraction of effector T cells bearing the corresponding receptor CXCR3. It has been reported that CXCL10 is elevated in the serum of long-standing (44) as well as newly diagnosed (45) T1D patients. However, the serum concentration was lower in patients with long-standing T1D. Interestingly, the mean serum CXCL10 level was also found to be elevated in patients at high risk for developing T1D who are carrying antibodies against islet autoantigens indicating that CXCL10 is released during the initial β-cell destructive process (44, 45). Although the observed elevation has not been confirmed in two other similar studies (34, 52), it is important to note that in the study by Rotondi et al. the range of serum CXCL10 concentrations was much broader in newly-diagnosed T1D patients than in healthy controls and the highest concentration found in patients was more than five times higher than in controls (52). A more detailed study has been performed by Antonelli et al. who analyzed CXCL10 serum levels in 96 newly diagnosed children with T1D at the time of diagnosis and at a median follow-up time of 16 months (33). Indeed, almost half of the newly diagnosed children had elevated CXCL10 levels compared to a healthy control group. In addition, the study confirmed the earlier finding that the CXCL10 serum levels decline over time but are still elevated even 16 months after diagnosis when compared to levels found in control individuals (33).

It has been found that CXCL10 as well as its main receptor CXCR3 are expressed directly in the islet microenvironment of T1D patients (35, 46, 47). Roep et al. stained pancreas sections from three new-onset T1D patients and found CXCL10 expression by β-cells and CXCR3 expression by infiltrating

TABLE 1 | Chemokine expression in T1D patients and experimental animals.

Species	Location	Chemokine	Reference
Human			
Children/adults with T1D	Serum	**CXCL10**	(44, 45)
Individuals at high risk for T1D	Serum	CCL3, CCL4	(34)
Newly diagnosed T1D patients	Serum	**CXCL10**	(33)
Recent onset T1D patients	Islets (IHC)	**CXCL10**	(46)
Recent onset T1D patients	β-cells (IHC)	**CXCL10**	(47)
T1D patients	Stimulated isolated islets (mRNA)	CCL5, CCL8, CCL22, CXCL9, **CXCL10**, CX_3CL1	(35)
T1D patients	Islets (IHC)	CCL5, CCL8, CXCL9, **CXCL10** >> CX_3CL1	(35)
Newly diagnosed T1D patients	Serum	CCL2, CXCL8, CXCL9, **CXCL10**	(48)
Spontaneous T1D models			
NOD mice	Specific BDC T cells (mRNA)	CCL2, CCL3, CCL4, XCL1 >> CCL5, **CXCL10**	(49)
NOD mice	β-cells (IHC)	**CXCL10**	(35)
NOD mice	Islets (mRNA transcriptome),	CCL2, CCL4, CCL5, CCL19, CCL22 CXCL9, **CXCL10**, CXCL11, CXCL13, XCL1	(30)
BB rat	Islets (mRNA transcriptome),	CCL2, CCL3, CCL19, CCL20, CCL21 CXCL1, **CXCL10**	(50)
Inducible T1D models			
RIP-LCMV mice	Pancreas (mRNA)	CCL5, CXCL9, **CXCL10** >> CCL11, XCL1	(26)
RIP-LCMV mice	Islets (mRNA)	CXCL9, **CXCL10** >> CCL2, CCL5, CXCL2	(43)
RIP-LCMV mice	Islets (IHC)	α-cells: CXCL9; β-cells: **CXCL10**	(43)
Prediabetic RIP-LCMV mice	Islets (IHC)	α-cells: CX3CL1; β-cells: CCL8, **CXCL10**	(35)
RIP-LCMV mice (islet transplantation)	Islets (IHC)	**CXCL10**	(38)
STZ-islet transplantation model	Serum	CCL2, CCL22, **CXCL10**	(51)

This table lists a selection of publications reporting chemokine expression in patients with T1D and/or experimental animal models for T1D. Note that most studies have identified CXCL10 as one of the most apparent chemokines expressed.
BB rat, Biobreed rat; IHC, Immunohistochemistry; STZ, Streptozotocin.

lymphocytes (46). This observation was confirmed by Uno et al. who performed double-immunofluorescence staining of pancreas sections of five recent-onset T1D patients. They clearly identified β-cells as a main source of CXCL10 and T-cells as the main cell type expressing CXCR3 in the islet microenvironment (47). Further, an extensive study by Sarkar et al. using RNA obtained from isolated human islets of four independent donors identified CXCL10 as the dominant chemokine expressed in islets of T1D patients (35). Since the peak of CXCL10 expression seems to be at the time of diagnosis or maybe even before clinical manifestation in islet autoantibody positive individuals rather than during the chronic phase of established T1D the question for the trigger of CXCL10 release arises. In this context an intriguing study by Tanaka et al. revealed enterovirus capsid protein VP1 expression in pancreata of three patients with fulminant T1D (FT1D) and ketoacidosis together with CXCL10 expression in α- and β-cells as well as strong islet-infiltration by CXCR3-positive T cells (53). The group recently further confirmed these data in a more detailed study using pancreas tissue of three FT1D patients and 17 healthy controls. They found that close association between VP1 and CXCL10 expression is not only detected in islet cells, but also in pancreatic exocrine ductal cells and acinar cells and concluded that enterovirus infection induced CXCL10 expression in both exocrine pancreas and islets (54).

Since access to human pancreas tissue and experimental evaluations of immunopathological mechanism in T1D patients are limited, research has focused on experimental animal models for T1D. Thereby, CXCL10 has also been identified as one of the key chemokines expressed during experimental T1D. In particular, in model systems using virus-infection to induce the autoimmune destruction of β-cells, such as the RIP-LCMV mouse model, CXCL10 was among the first chemokines upregulated in the pancreas upon LCMV-infection (26). However, CXCL10 was also found to be upregulated during the pathogenesis of T1D in NOD mice (49). Stimulation of isolated human, rat, and mouse islets with pro-inflammatory cytokines (TNFα, IFNγ) induced the expression of CXCL10, demonstrating the capacity of the islets themselves to be able to express CXCL10 (55). Indeed, immunohistochemical analysis of mouse pancreas sections has indicated that similar to the findings in patients, CXCL10 is mainly generated by β-cells and CXCR3 is present on infiltrating leukocytes, including CD8 T cells (38, 43, 46).

It has also been shown that T1D is milder in mice lacking CXCL10 or CXCR3 (43, 56). In contrast, T1D was accelerated in mice overexpressing CXCL10 in the β-cells (28). Interestingly, such RIP-LCMV x RIP-CXCL10 double transgenic mice displayed large clusters of infiltrating cells around and inside the islets of Langerhans but did not develop T1D spontaneously. Nevertheless, islet stress seemed to be present since such mice needed much longer to return to normoglycemia after glucose challenge (28). Thus, in these double-transgenic mice CXCL10 recruited leukocytes to the islets, but without activation and further expansion of islet antigen-specific T cells by LCMV-infection, β-cells were not actively destroyed. Collectively, these data suggest that CXCL10 orchestrates the migration of islet antigen-specific as well as non-specific T cells to the site of inflammation. In consequence, when a high concentration of CXCL10 is present at an auxiliary site responsive T cells might be recruited away from the islets. Indeed, when LCMV-infected RIP-LCMV mice receive an additional virus infection that grows to high titers in the lymph nodes causing a high local concentration of CXCL10, islet antigen-specific T cells accumulate in the infected lymph nodes and are driven to apoptosis by hyperactivation, resulting in an abrogation of T1D (57).

From a therapeutic perspective, it is important that a blockade of the CXCL10/CXCR3 axis with a neutralizing antibody reduced the T1D incidence in mice (26, 58). In the RIP-LCMV model the incidence of T1D was found to be reduced by about 70% after administration of an anti-CXCL10 antibody (26). Thereby the reduction of T1D went together with a reduced insulitis, maintained insulin production, and a reduced frequency of islet antigen-specific T cells. Nevertheless, in a follow-up study, it has been shown that there is a certain redundancy in the CXCL10/CXCR3 axis since the reduction of T1D in CXCR3-deficient RIP-LCMV mice and in anti-CXCL10 antibody treated RIP-LCMV mice was not as pronounced as in earlier studies (Coppieters et al., 2013). Importantly, it should be noted that the anti-CXCL10 antibody treatment started before initiation of the disease by LCMV-infection and therefore like using CXCL10-deficient mice the CXCL10 neutralization constituted a preventive rather than a therapeutic intervention. An anti-CXCL10 antibody treatment of already diabetic RIP-LCMV mice starting at day 13 after LCMV-infection resulted only in a slight, non-significant reduction by about 25% (56). One reason for this lack of efficacy is that at the start of the therapy the autoaggressive T cells have already assembled in the islet microenvironment and have started to progressively destroy β-cells. By that time, CXCR3-positive cells might even be trapped in the pancreatic lymph nodes or the TLO-like inflammatory clusters in and around the islets. Thus, the question about the perfect time for treatment arises. Whereas an answer to this question might be found in inducible models, such as the RIP-LCMV model, it is much more of a central problem for a therapy of T1D patients. Insulitis is already detected in new-onset T1D patients and, like in new-onset diabetic mice, CXCL10 neutralization might come too late. Should the hypothesis (2, 59) that one or more pathogens are involved in the initiation and/or propagation of the β-cell destructive process be correct, a CXCL10 neutralization would have to begin immediately after the critical infectious event. Thus, as long as there is no firm proof for (a) particular pathogen(s) to directly induce T1D, such a therapeutic scenario is highly unlikely.

There are however other occasions that would allow for a more reasonable therapeutic intervention. One occasion would be islet or whole pancreas transplantation. Here, the precise time of transplantation is of course known and ideally the new tissue should be devoid of autoaggressive T cells by the time of transplantation. In theory, a neutralization of CXCL10 would therefore not come too late and would reduce the *de novo*

infiltration of the transplant. Indeed, the majority of diabetic RIP-LCMV mice transplanted with CXCL10-deficient islets under the kidney capsule did not reject the islets. In addition, neutralization with an anti-CXCL10 antibody significantly delayed the islet rejection in such a setting (38). Similar data have been obtained in mice administered streptozotocin (STZ) to induce T1D (51). The second occasion would be a T cell reset situation as occurring after a partial T cell depletion caused by an anti-CD3 antibody therapy. Thus, chemokine neutralization might be better suited as part of a combination therapy.

CHEMOKINE NEUTRALIZATION AS PART OF A COMBINATION THERAPY

Chemokine ligands and especially their receptors have been used as targets for immune intervention for decades (20, 60). However, most of the clinical trials have been terminated after phase II or even earlier indicating an overall lack of efficacy. Maraviroc, which targets CCR5 and is used after infections with human immunodeficiency virus (HIV), is one of the few exceptions (61). Though, interfering with HIV entry is mechanistically completely different from blocking chemokine guided immune cell migration. One of the reasons for the lack of efficacy of drugs interfering with any chemokine axes might be that, as described above for mice with already established T1D, even a treatment of patients just recently diagnosed with any autoimmune-related disease might come too late. A possible solution to this impediment might be to set a fresh starting point for the autoimmune destruction process by temporarily depleting the culprit cells responsible for the destruction. In the case of T1D, the major destructive force are the autoaggressive T cells. T cell depletion/reprogramming has been performed using several variations of anti-CD3 antibody treatment since more than twenty years. Initially, experiments in NOD mice had demonstrated that anti-CD3 antibody administration induces a remission of T1D by re-establishing self-tolerance to islet autoantigens (62, 63). Mechanistically, an anti-CD3 antibody treatment causes on the one hand an inactivation of aggressive T cells and on the other hand results in an expansion of regulatory T cells (64).

The efficacy of anti-CD3 antibodies in T1D, such as teplizumab (hOKT3g1) and otelixizumab (ChAglyCD3), has been evaluated in several clinical trials, including the DEFEND-1 (otelixizumab), Protégé (teplizumab), and AbATE (teplizumab) studies (65–68). In such trails, patients with new-onset and recently diagnosed T1D were infused for a short period of time of only 6 to 14 days with an anti-CD3 antibody. In the Protégé study the patients received a second treatment cycle at week 26 after the start of the first cycle (66, 69). Overall, the therapeutic success lasted for about one to two years. Thereafter, the C-peptide levels and the insulin need approached those values of the placebo treated arm (66, 70, 71). Interestingly, in the AbATE study more than 50% of the patients did not respond to the anti-CD3 antibody treatment, whereas responders showed restored C-peptide levels for up to two years after treatment (71). However, a seven-year follow-up study revealed that even in drug responders the C-peptide levels declined massively in the time between two and seven years post-treatment (72). Thus, in order to uphold the protection for an extended period of time, the anti-CD3 treatment should be repeated or be combined with a secondary treatment. Whereas a repetitive administration of anti-CD3 antibodies has not been considered due to a high probability of severe side effects of the rather unspecific immune suppression, several combination therapies have been evaluated in animal models. They included administration of nasal proinsulin (73), Lactococcus lactis secreting IL-10/proinsulin (74), cyclosporine A and vitamin D3 analog (TX527) (75), anti-CD20 antibody (76), fingolimod (FTY720) (77), or the selective sphinogosine-1 phosphate-1 (S1P1) modulator ponesimod (78), just to name a few. Most of these combination therapies improved the outcome compared to the monotherapies. Similar to the therapies with anti-CD3 antibody/fingolimod (77) and anti-CD3 antibody/ponesimod (78) the combination therapy with anti-CD3 antibody and CXCL10 neutralization targeted the migration of the regenerated T cells into the islets of Langerhans (56).

As mentioned above, an anti-CD3 antibody therapy provides a "reset" situation regarding the presence of aggressive T cells in the islets of Langerhans. The subsequent neutralization of the CXCL10/CXCR3 axis might prevent the re-infiltration of the islets by autoaggressive T cells that have regenerated in spleen and lymph nodes (**Figure 1**). Indeed, in both new-onset diabetic RIP-LCMV as well as NOD mice an administration of three daily doses of a non-Fc-binding anti-CD3ϵ F(ab')2 fragment [clone 145-2C11] (63) followed by a treatment with a neutralizing anti-CXCL10 antibody [clone 1F11] (79) resulted in profound remission of T1D (56). Thereby the combination therapy was superior to the corresponding monotherapies with anti-CD3 antibody or anti-CXCL10 antibody alone. Importantly, the observed remission was long lasting, since none of the cured mice relapsed until the end of the observation period of six months. As to be expected, insulitis was strongly reduced in both models. However, whereas after combination therapy the frequency of islet antigen-specific CD8 T cells was greatly reduced in the pancreas of remitting RIP-LCMV mice, there was no further reduction in NOD mice receiving the combination therapy over those that were administered with anti-CD3 antibody only. In contrast, the frequency of regulatory T cells was further elevated after combination treatment in NOD, but not RIP-LCMV mice. Interestingly, the local ratio of regulatory T cells to islet antigen-specific effector CD8 T cells was strongly enhanced after combination therapy of both RIP-LCMV and NOD mice, indicating a vital shift in the immune balance locally in the islets (56). The crucial role of CXCL10 in the islet re-infiltration process is also underlined by the fact that T1D was completely abolished in CXCL10-deficient RIP-LCMV mice after treatment with anti-CD3 antibody (56). Thus, as outlined in **Figure 1**, a well-timed partial deletion and reprogramming of T cells through short-term administration of anti-CD3 antibodies followed by the inhibition of T cell

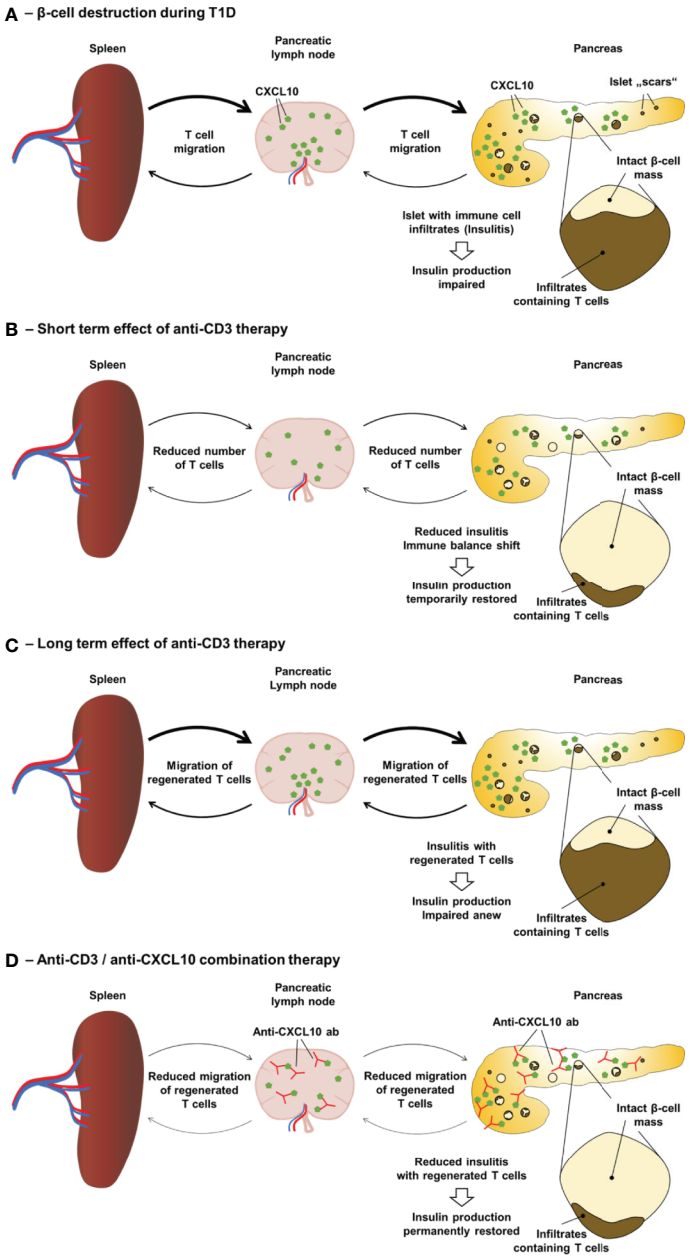

FIGURE 1 | Anti-CD3/anti-CXCL10 combination therapy for type 1 diabetes. **(A)** Local expression of CXCL10 and other chemokines in the islets of Langerhans as well as in the pancreatic lymph nodes drive the migration of leukocytes, including autoaggressive T cells to the islets. Due to β-cell destruction and stress the insulin production is insufficient to control the blood glucose level. **(B)** Anti-CD3 therapy causes a partial depletion of T cells and induces an immune balance shift resulting in a reduced insulitis and a temporarily restored insulin production. **(C)** However, in T1D patients anti-CD3 therapy only lasts for 1–2 years and in diabetic mice only about 30% go into remission. Regenerated T cells migrate to the islets and the self-destructive process start anew resulting in an impaired insulin production. **(D)** Administration of neutralizing anti-CXCL10 antibodies after the anti-CD3 therapy inhibits the migration of regenerated T cells and thereby prevents the re-infiltration of the islets resulting in a permanent T1D remission.

migration and islet re-infiltration *via* neutralization of the key inflammatory chemokine CXCL10 induces a persistent remission of T1D.

Persistent LCMV infection is causing a functional exhaustion of specific CD8 T cells (80, 81). Thereby, the expression of inhibitory receptors, such as PD-1 (programmed cell death protein 1), is considered a hallmark of T cell exhaustion (82). Indeed, blockade of PD-1 restores the cytotoxic function of exhausted CD8 T cells and reduces the viral titer (83). It has also been recently shown that the TcR signalling is strongly inhibited in such exhausted T cells (84). Although LCMV infection in the RIP-LCMV model for T1D is not persistent,

the chronic transgenic β-cell expression of LCMV-GP seems to result in a similar phenomenon of functional T cell exhaustion. During the destruction of β-cells in the RIP-LCMV model many T cells in the pancreas show an exhausted phenotype (85). Importantly, treatment of T1D patients with teplizumab as conducted in the AbATE trail resulted in an increased frequency of T cells with an exhaustion phenotype (86). A seven-year follow-up study even revealed that T cell exhaustion may serve a biomarker for response (72). Therefore, besides the observed shift in the immune balance towards a more regulatory milieu (56), another possible factor involved in the long-lasting effect of the anti-CD3/anti CXCL10 antibody combination therapy might be a predominance of exhausted T cells in the islet microenvironment. Thereby, the anti-CD3 antibody treatment would cause an accumulation of exhausted T cells and the subsequent blockade of the CXCL10-CXCR3 axis would

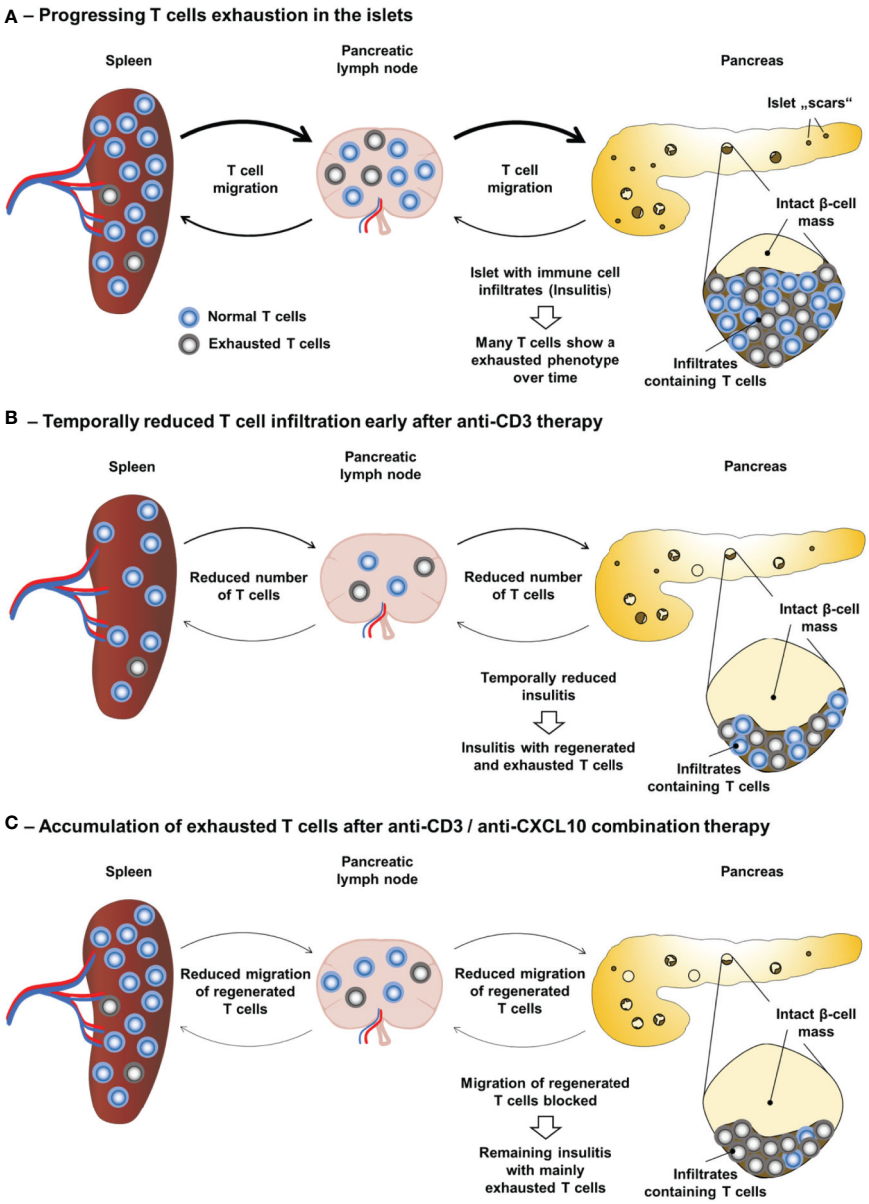

FIGURE 2 | Accumulation of exhausted T cells as possible explanation for the long-lasting effect of the anti-CD3/anti-CXCL10 combination therapy. **(A)** During the progressive destruction of β-cells in the RIP-LCMV-GP model the majority of islet autoantigen (LCMV-GP) specific T cells enter a state of exhaustion. **(B)** Upon anti-CD3 therapy the frequency of T cells is temporarily reduced and the frequency of exhausted T cells is increased. However, upon termination of the anti-CD3 antibody treatment, newly regenerated T cells are not prevented from invading the islet microenvironment. **(C)** In contrast, anti-CD3/anti-CXCL10 combination therapy prevents the migration of newly regenerated, functionally active T cells to the islets and results in an increased frequency of exhausted islet autoantigen-specific T cells in the islet microenvironment.

prevent the re-infiltration of newly regenerated, functionally active T cells (**Figure 2**).

SUMMARY AND FUTURE PERSPECTIVES

It seems clear that chemokines are important for the attraction of autoaggressive immune cells, including islet-autoantigen-specific T cells, to the islet of Langerhans. Several chemokines have been identified to be elevated in the serum of T1D patients or expressed by the islet microenvironment of mice. The CXCL10/CXCR3 chemokine axis is of particular interest, since patients with T1D display enhanced serum levels of CXCL10, both CXCL10 as well as CXCR3 have been found in pancreas tissue sections from T1D patients, and neutralization studies in mouse models demonstrated a reduction of T1D incidence and severity. However, most of the studies done in mice are not applicable to the human situation, since the precise moment of disease initiation in humans is not known and treatment might therefore come too late. Even if treatment would start in individuals at risk that have already generated autoantibodies against two or more islet autoantigens a migration blockade might be behind schedule since insulitis has very likely already started. Thus, neutralization of chemokines seems more appropriate in situations with no or only mild insulitis. The first situation would be whole pancreas or islet transplantation. Even though the transplantation process as such is initiating inflammatory responses, the islets at that point of time are largely devoid of insulitis. The second situation is a partial T cell depletion as occurring after short-term anti-CD3 antibody treatment. In patients, a monotherapy with anti-CD3 antibodies has proven to be beneficial, still many patients did not respond and even in responders the remission lasted only for one to two years. In two independent mouse models, a well-timed combination therapy of anti-CD3 antibody administration followed by an additional blockade of the CXCL10-CXCR3 axis prevented the re-infiltration of the islets by regenerating T cells and thereby induced a persistent remission in the majority of treated mice. No severe adverse effects have been reported for patients receiving two cycles of anti-CD3 antibody treatment within 26 weeks (66, 69). Nevertheless, a repetitive treatment with cycles of anti-CD3 antibody infusions over several years or even decades has not yet been considered, due to possible long term adverse effects, such as reduced pathogen defense, viral reactivation, and enhanced risk for tumor development. Due to the partial redundancy of the chemokine ligand-receptor network the blockade of a single chemokine axis, that plays a crucial role in the pathogenesis of a particular autoimmune disease, is likely to have a lower impact on the general immune defense than a partial T cell depletion. Thus, there is still need for alternative combination therapies that abstain from anti-CD3 antibody administration. Ideally such combinations should include targets that are mechanistically involved in separate steps of the immunopathogenesis. Thus, a parallel or sequential neutralization of several chemokine axes involved in the attraction of distinct leukocyte populations such as aggressive T cells and dendritic cells might resolve already existing leukocyte clusters in TLO-like structures around and in the islets of Langerhans without prior anti-CD3 antibody treatment. In any case, to achieve persistent T1D remission, it is important to find novel combination therapies that can be administered in a well-timed regimen in patients with newly diagnosed or even established T1D.

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SARS-CoV-2 Receptor Angiotensin I-Converting Enzyme Type 2 (ACE2) Is Expressed in Human Pancreatic β-Cells and in the Human Pancreas Microvasculature

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Increasing evidence demonstrated that the expression of Angiotensin I-Converting Enzyme type 2 (ACE2) is a necessary step for SARS-CoV-2 infection permissiveness. In light of the recent data highlighting an association between COVID-19 and diabetes, a detailed analysis aimed at evaluating ACE2 expression pattern distribution in human pancreas is still lacking. Here, we took advantage of INNODIA network EUnPOD biobank collection to thoroughly analyze ACE2, both at mRNA and protein level, in multiple human pancreatic tissues and using several methodologies. Using multiple reagents and antibodies, we showed that ACE2 is expressed in human pancreatic islets, where it is preferentially expressed in subsets of insulin producing β-cells. ACE2 is also highly expressed in pancreas microvasculature pericytes and moderately expressed in rare scattered ductal cells. By using different ACE2 antibodies we showed that a recently described short-ACE2 isoform is also prevalently expressed in human β-cells. Finally, using RT-qPCR, RNA-seq and High-Content imaging screening analysis, we demonstrated that pro-inflammatory cytokines, but not palmitate, increase ACE2 expression in the β-cell line EndoC-βH1 and in primary human pancreatic islets. Taken together, our data indicate a potential link between SARS-CoV-2 and diabetes through putative infection of pancreatic microvasculature and/or ductal cells and/or through direct β-cell virus tropism.

Keywords: diabetes, COVID-19, angiotensin I-converting enzyme type 2 (ACE2), beta-cell, human pancreatic islets, SARS-CoV-2, inflammation

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INTRODUCTION

The expression of molecules that act as receptors for viruses determines tissue-specific tropism. SARS-coronavirus 2 (SARS-CoV-2), that leads to the respiratory illness coronavirus disease 2019 (COVID-19), uses its surface envelope Spike glycoprotein (S-protein) to interact and gain access to host cells through the Angiotensin-I converting enzyme-2 (ACE2) receptor. As such, S-protein-ACE2 binding is the key determinant for virus entry, propagation, and transmissibility of COVID-19-related disease (1, 2).

Artificially induced ACE2 *de-novo* expression in ACE2-negative cell lines is a necessary step to SARS-CoV and SARS-CoV-2 infection (3, 4). SARS-CoV-2 does not enter cells that do not express ACE2 and does not use other coronavirus receptors, such as aminopeptidase N (APN) and dipeptidyl peptidase 4 (DPP4), thus being fully dependent on ACE2 presence in host cells (5). Additional host co-factors, such as transmembrane protease TMPRSS2, cathepsin B/L, and furin protease, have been shown to enhance efficiency of SARS-CoV-2 cell entry by processing the S-protein and eliciting membrane fusion and syncytia formation (6). The central role played by ACE2 in SARS-CoV-2 infection has been further supported by evidence that SARS-CoV-2 infection is driven by ACE2 expression level (6). SARS-CoV-2 mainly targets cells of the nasal, bronchial, and lung epithelium, causing respiratory-related symptoms; however, growing evidence shows that other tissues can also be infected.

Several reports indicate a wide, although variable, distribution of ACE2 expression patterns among different tissues (7–9), thus underlining a potential different virus infection susceptibility among cell types. The fact that COVID-19 disease may lead to multiple organ failure (10, 11) shows the crucial relevance for understanding the molecular mechanisms of host cell factors used by SARS-CoV-2 to infect their target tissues.

Recent studies showed that older adults and those with chronic medical conditions like heart and lung disease and/or diabetes mellitus are at the highest risk for complications from SARS-CoV-2 infection. Of importance, a yet unresolved conundrum relies on the recently hypothesized bidirectional relationship between COVID-19 and diabetes mellitus (12, 13). This concept is supported by reports in which impaired glycemic control is associated with increased risk of severe COVID-19. Indeed, elevated blood glucose concentrations and deterioration of glycemic control may contribute to increased inflammatory response, to abnormalities in the coagulation system, and to impairment of ventilatory function, thus leading to severe COVID-19 disease and to a worse prognosis (14). Interestingly, acute hyperglycemia has been observed at admission in a substantial percentage of SARS-CoV-2 infected subjects, regardless of the past medical history of diabetes (15–18). The same observations were previously made in SARS-CoV-1 pneumonia during 2003 SARS epidemic (19).

A recently published case report described autoantibody-negative insulin-dependent diabetes onset in a young patient who was infected with SARS-CoV-2 seven weeks before diabetes symptom occurrence (20). Additional previous studies further support such observation (21, 22). This indicates the possibility of a link between SARS-CoV-2 infection and new-onset diabetes through potential direct infection of pancreatic islets or additional indirect mechanisms. Indeed, an *in-vitro* infection model of human pluripotent stem cells derived β-cells exposed to SARS-CoV-2 (23) showed permissiveness of these pre-β-cells to the virus. However, whether fully mature primary beta-cells or other cells of the human pancreas are indeed permissive to SARS-CoV-2, infection remains to be clarified.

To address this question, we screened the ACE2 expression pattern in human pancreata obtained from adult non-diabetic multiorgan donors and in the insulin-producing human β-cell line EndoC-βH1 using different methodologies, multiple reagents, and publicly available or in-house generated RNA sequencing datasets. Our data indicate that ACE2 is expressed by pancreas microvasculature, by scattered ductal cells, and by a subset of human β-cells. These different cell types are thus potentially prone to SARS-CoV-2 infection. We also identified a differential distribution of the two recently discovered ACE2 isoforms (24, 25). Exposure of EndoC-βH1 human beta-cell line and human pancreatic islets to pro-inflammatory cytokines significantly increased ACE2 expression. Taken together, our data suggest a potential link between SARS-CoV-2 infection and new onset diabetes, which deserves further investigation based on long-term follow-up of patients recovered from COVID-19 disease.

MATERIALS AND METHODS

Human Donors

Human pancreatic sections analyzed in this study were obtained from pancreata of brain-dead adult non-diabetic multiorgan donors within the European Network for Pancreatic Organ Donors with Diabetes (EUnPOD), a project launched in the context of the INNODIA consortium (www.innodia.eu). Whole pancreata were processed following standardized procedures at the University of Pisa. Formalin fixed paraffin embedded (FFPE) pancreatic tissue sections and frozen OCT pancreatic tissue sections were obtained from n = 7 adult non-diabetic multiorgan donors and from n = 1 longstanding T1D donor pancreas (**Table S1**). In INNODIA EUnPOD network, pancreata not suitable for organ transplantation were obtained with informed written consent by organ donors' next-of -kin and processed with the approval of the local ethics committee of the Pisa University.

Human Pancreatic Islets

Human pancreatic islets were obtained from n = 4 non-diabetic multiorgan donors (**Table S1**). Briefly, purified islets were prepared by intraductal collagenase solution injection and density gradient purification, as previously described (26). At the end of the isolation procedure, fresh human pancreatic islet preparations were resuspended in CMRL culture medium (cat. 11-530-037, ThermoFisher Scientific, Waltham, MA, USA) supplemented with L-Glutamine 1% (cat.G7513-100ML), Antibiotic/Antimicotic 1% (A5955-100ML, Sigma Aldrich, St.

Louis, MO, USA), FBS 10% and cultured at 28°C in a 5% CO_2 incubator.

Cell Culture

EndoC-βH1 human β-cell line (27, 28) was obtained by UniverCell-Biosolutions (Toulouse-France) and used for all experiments between passages 78 and 88. EndoC-βH1 was cultured at 37°C with 5% CO_2 in coated flask (coating medium composition: DMEM high-glucose cat. 51441C, Penicillin/Streptomycin 1% cat. P0781, ECM 1% cat. E1270 and Fibronectin from bovine plasma 0.2% cat. F1141—all from Sigma Aldrich, St. Louis, MO, USA) and maintained in culture in low-glucose DMEM (cat. D6046) supplemented with 2% BSA fraction V (cat. 10775835001), β-Mercaptoethanol 50 µM (cat. M7522), L-Glutamine 1% (cat. G7513), Penicillin/Streptomycin 1% (cat. P0781), Nicotinamide 10 mM (cat. N0636), Transferrin 5.5 µg/ml (cat. T8158) and Sodium selenite 6.7 ng/ml (cat. S5261) (all from Sigma Aldrich, St. Louis, MO, USA).

HeLa cells (ATCC CCL-2), passages 33–34, were cultured at 37°C with 5% CO_2 in a 100 mm petri plate and maintained in culture in high glucose DMEM (cat. 51441C) supplemented with L-Glutamine 1% (cat. G7513), Antibiotic/Antimycotic 1% (A5955-100ML, Sigma Aldrich, St. Louis, MO, USA) and FBS 10%.

In order to evaluate ACE2 expression in human β-cells under diabetogenic stress conditions, EndoC-βH1 cell line was subjected to palmitate-induced lipotoxic and inflammatory stress. Briefly, EndoC-βH1 cells were plated at a density of 2.5×10^5/well in 24-well plates or 5×10^4/well in 96-well plates. After 48 h, palmitate and inflammatory stimuli were performed as previously described (29, 30). In details, palmitate and inflammatory stresses have been induced respectively by 2 mM of Sodium Palmitate (cat. P9767-5G-Sigma Aldrich, St. Louis, MO, USA) or 0.5% EtOH (as control treatment) for 24 h, or cytokines mix IL-1β (50 U/ml) (cat. #201-LB-005-R&D System, Minneapolis, MN, USA), TNFα (1,000 U/ml) (cat. T7539-Sigma Aldrich, St. Louis, MO, USA) and IFNγ(1,000 U/ml) (cat. 11040596001-Roche, Basilea, Switzerland) for 24 h.

Laser Capture Microdissection

Pancreatic human tissue samples (n = 5) from EUnPOD multiorgan donors (**Table S1**) were frozen in Tissue-Tek OCT compound, and then 7-µm thick sections were cut from frozen O.C.T. blocks. Sections were fixed in 70% ethanol for 30 s, dehydrated in 100% ethanol for 1 min, in 100% ethanol for 1 min, in xylene for 5 min and finally air-dried for 5 min. Laser capture microdissection (LCM) was performed using an Arcturus XT Laser-Capture Microdissection system (Arcturus Engineering, Mountain View, CA, USA) by melting thermoplastic films mounted on transparent LCM caps (cat.LCM0214-ThermoFisher Scientific, Waltham, MA, USA) on specific islet areas. Human pancreatic islets were subsequently visualized through islet autofluorescence for LCM procedure. Thermoplastic films containing microdissected cells were incubated with 10 µl of extraction buffer (cat. kit0204-ThermoFisher Scientific, Waltham, MA, USA) for 30 min at 42°C and kept at −80°C until RNA extraction. Each microdissection was performed within 30 min from the staining procedure. Overall n = 50 microdissected pancreatic islets from each case were analyzed.

RNA Extraction From LCM Isolated Human Pancreatic Islets

Total RNA was extracted from each LCM sample using PicoPure RNA isolation kit Arcturus (cat. kit0204-ThermoFisher Scientific, Waltham, MA, USA) following manufacturer's procedure. Briefly, the cellular extracts were mixed with 12.5 µl of EtOH (100%) and transferred to the purification column filter membrane. DNase treatment was performed using RNase-Free DNase Set (cat. 79254-Qiagen, Hilden, Germany). Total RNA was eluted in 11 µl of DNase/RNase-Free Water, and LCM captures deriving from human sample were pooled and subjected to a subsequent concentration through Savant SpeedVac SC100 centrifugal evaporator. Agilent 2100 Bioanalyzer technology with RNA Pico chips (cat. 5067-1513 Agilent Technologies, Santa Clara, CA, USA) was performed for each RNA sample, in order to analyze RNA integrity (RIN) and concentration.

RNA Extraction From Cells and Tissues

For gene expression evaluation, total RNA was extracted from approximately 3.0×10^5 EndoC-βH1 or from fresh lung tissue ($0.5 \times 0.5 \times 0.5$ cm) obtained from a lung tumor surgery donor by dissecting a not affected portion of the tissue (obtained with informed written consent of the patient and approved by the local Ethics Committee at the University of Siena). Direct-zol RNA Miniprep Kit (cat. R202-Zymo Research, Irvine, CA, US) was adopted following manufacturer's instructions. Briefly, the pelleted cells were resuspended in QIAzol (cat. 79306, Qiagen), mixed with equal volume of ethanol 100% and transferred to Zymo-Spin™ IICR Column. DNase digestion was performed using RNase-Free DNase Set (cat. 79254). RNA was eluted in 30 µl of DNase/RNase-Free Water. Fresh lung tissue was maintained in PBS1X on ice immediately after surgery until ready for the RNA extraction. Homogenization of the tissue was performed using 600 µl of QIAzol and Lysing Matrix latex beads (MP Biomedicals, cat. 6913-100) in FastPrep-24 automated homogenizer (1 min, full speed). The homogenate was diluted with vol/vol ethanol 100% and then transferred in Zymo-Spin™ IICR Column. RNA extraction was performed following manufacturer's instructions.

RT-Real Time PCR Analysis

Total RNA extracted from EndoC-βH1 and collagenase-isolated human pancreatic islet samples were quantified using Qubit 3000 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA), while those extracted from LCM-islets were quantified using 2100 Bioanalyzer-RNA 6000 Pico Kit (cat. 50671513, Agilent Technologies, Santa Clara, CA, USA) as well as RNA integrity (RIN). Samples with RIN<5.0 were excluded. Reverse transcriptase reaction was performed using SuperScript™ VILO™ cDNA Synthesis Kit (cat. 11754050-ThermoFisher Scientific, Waltham, MA, USA).

cDNA derived from LCM human pancreatic islets was then amplified using TaqMan PreAmp Master Mix (cat. 4488593,

ThermoFisher Scientific, Waltham, MA, USA) following manufacturer's instructions.

Real-Time PCR analysis was performed using TaqMan gene expression assays using primers (see *Resources Table*) and SensiFast Probe Lo-ROX Kit (cat.# BIO-84020, Bioline) following manufacturer's recommendation. Data were collected and analyzed through Expression Suite software 1.0.1 (ThermoFisher Scientific, Waltham, MA, USA) using $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ method. ViiA7 Real-Time PCR thermalcycler instrument (ThermoFisher Scientific, Waltham, MA, USA) was used to perform Real-Time PCR reactions.

ACE2 Immunohistochemistry Analysis of Human Pancreatic Sections

In order to evaluate the staining pattern of ACE2 in human pancreatic tissues, we analyzed FFPE sections (7-μm thickness) prepared by using a microtome (cat. RM2125 RTS-Leica Microsystems, Wetzlar, Germany) and baked overnight at 37°C from two different portions of pancreatic tissue for each multiorgan donor (listed in **Table S1**).

After deparaffinization and rehydration through decreasing alcohol series (Xylene-I 20 min, Xylene-II 20 min, EtOH 100% 5 min, EtOH 95% 5 min, EtOH 80% 5 min, EtOH 75% 5 min) pancreatic sections were incubated with 1× Phosphate-Buffered Saline with Ca^{2+} and Mg^{2+} (PBS 1×) supplemented with 3% H_2O_2 (cat. H1009-Sigma Aldrich, St. Louis, MO, USA) for 30 min to block endogenous peroxidases. Heat-induced antigen retrieval was performed using 10 mM citrate buffer pH 6.0 in microwave (600 W) for 10 min, maintaining boiling conditions. Sections were incubated with PBS 1× supplemented with 5% rabbit serum (cat. SCBD33ISV-Sigma Aldrich, St. Louis, MO, USA) to reduce non-specific reactions. Then, sections were incubated overnight at +4°C with primary antibody monoclonal mouse anti-Human ACE2 (cat.MAB933, R&D System, Minneapolis, MN, USA) diluted 1:33 (15 μg/ml) in PBS 1× supplemented with 5% rabbit serum. The next day, sections were incubated with secondary antibody polyclonal rabbit anti-mouse HRP-conjugate (cat. P0260, Dako, Agilent Technologies, Santa Clara, CA, USA) diluted 1:100 in PBS 1× for 1 h at room temperature (RT). Subsequently, the sections were incubated with one drop of 3,3′ Diaminobenzidine (DAB) chromogen solution (cat. RE7270-K, Novolink MAX DAB, Leica Microsystems, Wetzlar, Germany) for 5 min to trigger the chromatic reaction. Stained sections were then counterstained with hematoxylin (cat.MHS31, Sigma Aldrich, St. Louis, MO, USA) for 4 min for better visualization of the tissue morphology. After the dehydration through increasing alcohol series, the pancreatic sections were mounted with Eukitt mounting medium (cat. S9-25-37, Bio Optica, Milan, Italy) and covered with a coverslip allowing them to dry. A negative control with only secondary antibody incubation (no primary antibody control sample) was also included in order to exclude potential background artifacts generated by the secondary antibody or the enzymatic detection reaction (**Figure S1A**).

In order to further evaluate ACE2 expression in pancreas sections, the same ACE2 IHC protocol was applied to other two primary antibodies anti-Human ACE2: monoclonal rabbit anti-Human ACE2 (cat. Ab108252, Abcam, Cambridge, UK) diluted 1:100 in PBS 1× supplemented with 5% goat serum (cat. G9023, Sigma Aldrich, St. Louis, MO, USA); and polyclonal rabbit anti-Human ACE2 (cat. Ab15348, Abcam, Cambridge, UK) diluted 1:2,000 in PBS 1× supplemented with 5% goat serum. The secondary antibody in both cases was polyclonal goat anti-rabbit HRP-conjugate (cat. 111-036-003, Jackson ImmunoResearch, Philadelphia, PA, USA), diluted 1:1000 in PBS 1× for 1 h at room temperature (RT).

All the three primary antibodies anti-Human ACE2, with respective secondary antibodies, were also used to perform a positive control staining in FFPE human lung sections (7-μm thickness) in order to double check the specificity of the primary antibodies.

Immunofluorescence Staining for ACE2-Insulin-Glucagon and ACE2-CD31

FFPE pancreatic sections (see above) were analyzed by triple immunofluorescence in order to simultaneously evaluate the expression pattern of ACE2, insulin, and glucagon. Briefly, after deparaffinization and rehydration through decreasing alcohol series (see above), pancreatic sections were subjected to heat induced antigen retrieval using 10 mM citrate buffer pH 6.0 in microwave (600 W) for 10 min. Sections were incubated with PBS 1× supplemented with 3% Bovine Serum Albumin (BSA, cat. A1470-25G, Sigma Aldrich, St. Louis, MO, USA) to reduce non-specific reactions. Then, sections were incubated with primary antibody monoclonal mouse anti-human ACE2 (cat. MAB933, R&D System, Minneapolis, MS, USA) diluted 1:33 in PBS 1× supplemented with 3% BSA, overnight at +4°C, followed by polyclonal Rabbit anti-human Glucagon (cat. A0565, Agilent Technologies, Santa Clara, CA, USA) diluted 1:500 in PBS 1× supplemented with 3% BSA, and prediluted polyclonal Guinea Pig anti-human Insulin (cat. IR002-Agilent Technologies, Santa Clara, CA, USA) as second and third primary antibodies for 1 h at room temperature (RT). Subsequently, sections were incubated with goat anti-guinea pig Alexa-Fluor 555 conjugate (cat. A21435, Molecular Probe, ThermoFisher Scientific, Waltham, MA, USA) diluted 1:500 in PBS 1×, goat anti-rabbit Alexa-Fluor 647 conjugate (cat. A21245, Molecular Probe, ThermoFisher Scientific, Waltham, MA, USA) diluted 1:500 in PBS 1× and goat anti-mouse 488 conjugate (cat.A11029-Molecular Probe, ThermoFisher Scientific, Waltham, MA, USA) diluted 1:500 in PBS 1×, as secondary antibodies for 1 h. Sections were counterstained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, cat. D8517, Sigma-Aldrich) diluted 1:3,000 in PBS 1×, and then mounted with Vectashield antifade medium (cat. H-1000, Vector Laboratories, Burlingame, CA, USA) and analyzed immediately or stored at +4°C until ready for confocal image analysis. A negative control with only secondary antibody incubation (no MAB933 primary antibody control sample) was also included in order to exclude any background artifacts or fluorochrome overlaps (**Figures S1B, C**).

For ACE2-CD31 double immunofluorescence staining, FFPE pancreas sections were incubated overnight at +4°C with

primary antibody monoclonal mouse anti-human ACE2 (cat. MAB933, R&D System, Minneapolis, MS, USA) diluted 1:33 in PBS 1× supplemented with 3% BSA and with polyclonal rabbit anti-CD31 (cat. Ab28364, Abcam, Cambridge, UK) diluted 1:50 in the same blocking buffer. Subsequently, sections were incubated with goat-anti rabbit Alexa-Fluor 594 conjugate (cat. A11037, Molecular Probe, ThermoFisher Scientific, Waltham, MA, USA) diluted 1:500 in PBS 1×, and goat anti-mouse 488 conjugate (cat. A11029-Molecular Probe, ThermoFisher Scientific, Waltham, MA, USA) diluted 1:500 in PBS 1×, as secondary antibodies for 1 h. Sections were counterstained with DAPI and then mounted as described above.

Cultured Cell Immunofluorescence

Cultured EndoC-βH1 cells were immunostained for ACE2 and insulin as follows. Cytokine-treated or untreated cells were fixed in 4% PFA for 10 min, washed for 10 min in 0.1 mol/L glycine, permeabilized in 0.25% Triton-X-100 for 5 min and blocked in 3% BSA+0.05% Triton-X100 in PBS without Ca^{2+} and Mg^{2+} for 30 min. EndoC-βH1 cells were incubated with prediluted antibody polyclonal Guinea Pig anti-Human Insulin (cat. IR002-Agilent Technologies, Santa Clara, CA, USA) for 1 h at RT. Then, the cells were washed with PBS without Ca^{2+} and $Mg^{2+}$and incubated with monoclonal mouse anti-Human ACE2 (cat. MAB933, R&D System, Minneapolis, MS, USA) diluted 1:33 in BSA 1% in PBS without Ca^{2+} and Mg^{2+} or with monoclonal rabbit anti-Human ACE2 (cat. ab108252, Abcam, Cambridge, UK) diluted 1:100 in BSA 1% in PBS without Ca^{2+} and Mg^{2+} or with polyclonal rabbit anti-Human ACE2 (cat. ab15348, Abcam, Cambridge, UK) diluted 1:2,000 in BSA 1% in PBS without Ca^{2+} and Mg^{2+}, and then incubated with primary or with negative isotype control mouse IgG2a (cat. X0943-Agilent Technologies, Santa Clara, CA, USA) or negative isotype control rabbit IgG (cat. Ab199376, Abcam, Cambridge, UK) for 60 min. Next, EndoC-βH1 cells were rinsed with PBS without Ca^{2+} and Mg^{2+} and incubated with goat anti-mouse-488 1:500 in 1% BSA in PBS without Ca^{2+} and Mg^{2+} or with goat anti-rabbit-488 diluted 1:500 in 1% BSA in PBS without Ca^{2+} and $Mg^{2+}$for 30 min and with goat-anti rabbit Alexa-Fluor 594 conjugate (cat. A11037, Molecular Probe, ThermoFisher Scientific, Waltham, MA, USA). Finally, the cells were incubated with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, cat. D8517, Sigma Aldrich, St. Louis, MO, USA) diluted 1:3,000 in PBS 1× and then mounted with Vectashield antifade medium (cat. H-1000-Vector Laboratories, Burlingame, CA, USA) and analyzed immediately or stored at +4°C until ready for confocal image analysis.

HeLa cells were immunostained for ACE2 as follows. The cells were first fixed in 4% PFA for 10 min, washed for 10 min in 0.1 mol/L glycine, permeabilized in 0.25% Triton-X-100 for 5 min and blocked in 3% BSA+0.05% Triton-X-100 in PBS without Ca^{2+} and Mg^{2+} for 30 min. The cells were subsequently washed with PBS without Ca^{2+} and Mg^{2+} and then incubated with monoclonal mouse anti-Human ACE2 (cat. MAB933, R&D System, Minneapolis, MS, USA) diluted 1:33 in BSA 1% in PBS without Ca^{2+} and Mg^{2} or with negative isotype control mouse IgG2a (cat. X0943-Agilent Technologies, Santa Clara, CA, USA). Then, HeLa cells were rinsed with PBS without Ca^{2+} and Mg^{2+} and were incubated with goat anti-mouse-488 1:500 in 1% BSA in PBS 1× without Ca^{2+} and Mg^{2+} or with goat anti-rabbit-488 diluted 1:500 in 1% BSA in PBS 1× without Ca^{2+} and Mg^{2+}. Cells were counterstained with DAPI and then mounted as described above.

Image Analysis

Images were acquired using Leica TCS SP5 confocal laser scanning microscope system (Leica Microsystems, Wetzlar, Germany). Images were acquired as a single stack focal plane or in z-stack mode capturing multiple focal planes (n = 40) for each identified islet or selected representative islets. Sections were scanned and images acquired at 40× or 63× magnification. The same confocal microscope setting parameters were applied to all stained sections before image acquisition in order to uniformly collect detected signal related to each channel.

Colocalization analyses between ACE2 and insulin and between ACE2 and glucagon were performed using LasAF software (Leica Microsystems, Wetzlar, Germany). The region of interest (ROI) was drawn to calculate the *colocalization rate* (which indicates the extent of colocalization between two different channels and reported as a percentage) as a ratio between the colocalization area and the image foreground. Evaluation of the signal intensity of ACE2 expression in human pancreatic islets of EUnPOD donors was performed using the LasAf software (www.leica-microsystem.com). This software calculates the ratio between intensity sum ROI (which indicates the sum ROI of the gray-scale value of pixels within a region of interest) of ACE2 channel and Area ROI (μm^2) of human pancreatic islets. In both colocalization and intensity measurement analysis, a specific threshold was assigned based on the fluorescence background. The same threshold was maintained for all the images in all the cases analyzed.

Micro-Confocal High-Content Screening Analysis

Cultured EndoC-βH1 cells were immunostained for ACE2 and insulin as reported above. Cytokine-treated or untreated cells were fixed in 4% PFA for 10 min, washed for 10 min in 0.1 mol/L glycine, permeabilized in 0.25% Triton-X-100 for 5 min and blocked in 3% BSA+0.05% Triton-X100 in PBS without Ca^{2+} and Mg^{2+} for 30 min. EndoC-βH1 cells were incubated with antibody polyclonal Guinea Pig anti-Human Insulin (cat. A0564-Agilent Technologies, Santa Clara, CA, USA) diluted 1:1740 in BSA 1% in PBS without Ca^{2+} and $Mg^{2+}$and with monoclonal mouse anti-Human ACE2 (cat. MAB933, R&D System, Minneapolis, MS, USA) diluted 1:33 in BSA 1% in PBS without Ca^{2+} and $Mg^{2+}$for 1 h at RT or with negative isotype control mouse IgG2a (cat. X0943-Agilent Technologies, Santa Clara, CA, USA). Then, EndoC-βH1 cells were washed with PBS without Ca^{2+} and $Mg^{2+}$and incubated with goat anti-mouse-488 (cat.A11029-Molecular Probe, ThermoFisher Scientific, Waltham, MA, USA) and goat anti-guinea pig-555 (cat. A21435, Molecular Probe, ThermoFisher Scientific, Waltham, MA, USA) 1:500 in 1% BSA in PBS without Ca^{2+} and Mg^{2+}. EndoC-βH1 cells were incubated with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, cat. D8517, Sigma Aldrich, St. Louis, MO, USA) diluted

1:3,000 in PBS 1×; then washed with PBS without Ca^{2+} and $Mg^{2+}$and analyzed immediately. Fluorescence images of EndoC-βH1 cells were analyzed using Opera Phoenix High Content Screening System (PerkinElmer, Waltham, MA, USA) acquiring multiple images using 63× magnification; nine microscopic areas per well were automatically selected. Automated image analysis was performed using Harmony® High-Content Imaging (PerkinElmer, Waltham, MA, USA), and fluorescence intensity of treated or untreated cells was measured based on Alexa-555 (insulin) and Alexa-488 (ACE2) fluorochromes. Images were first segmented into nuclei and cytoplasm using the Find Nuclei building block on the DAPI channel and the Find Cytoplasm on the 488 (ACE2) channel. To detect ACE2 signals, Find spots building block was applied to the 488 fluorescence channel inside the cytoplasm area previously detected. The intensity rate was obtained from the average of the nine areas and values reported as Corrected Spot Intensity (which is the "Mean Spot Intensity" minus "Spot Background Intensity") (31).

RNA Sequencing Processing and Analysis

Total RNA of EndoC-βH1 cells and of pancreatic human islets exposed or not to IFNα or to IL-1β + IFNγ for the indicated time points was obtained and prepared for RNA sequencing as described (32–34). Bioanalyzer System 2100 (Agilent Technologies, Wokingham, UK) was used to evaluate samples' quality by determining RNA integrity number (RIN) values. Only samples presenting RIN values >9 were analyzed. The obtained libraries were submitted to a second quality control before sequencing on an Illumina HiSeq 2500. The Salmon software version 0.13.2 (35) was used to re-analyze our original RNA-seq data (32–34) by mapping the sequenced reads to the human reference transcriptome from GENCODE version 31 (GRCh38) (36) using the quasi-alignment model. Gene expression is represented in Transcripts Per Million (TPM).

Differentially expressed genes were identified with DESeq2 version 1.24.0 (37). The estimated number of reads obtained from Salmon was used to run the DESeq2 pipeline. In summary, in this approach the DESeq2 normalizes samples based on per-sample sequencing depth and accounting for the presence of intra-sample variability. Next, data were fitted into a negative binomial generalized linear model (GLM) and computed using the Wald statistic. Finally, obtained p-values were adjusted for multiple comparisons using the false discovery rate (FDR) by the Benjamini–Hochberg method (38). Genes were considered significantly modified with a FDR <0.05.

Western Blot Analysis

Total proteins from EndoC-βH1 cells were extracted using a lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 1% (w/v) NP-40, 2 mM EDTA) supplemented with 1× protease inhibitors (Roche). Total proteins were quantified using Bradford assay, and 50–100 µg protein/lane was separated using SDS-PAGE Tris-Glycine gradient Bis-Acrylamide gel 4–20%. Proteins were then transferred to Nitrocellulose 0.2 µm membrane using wet electrophoresis system. Upon transfer onto nitrocellulose, membranes were washed three times with TBST 1× (Tris-HCl 25 mM, NaCl 150 mM, Tween 20 0.1%, pH 7.4) and then incubated 2 h with 5% non-fat dry milk in TBST 1×. To identify ACE2, three different antibodies were used: #Ab108252, #Ab15348 (Abcam), and #MAB933 (R&D system) were respectively diluted 1:1,000, 1:500, and 1:250 in 5% non-fat dry milk in TBST 1× and incubated o/n at +4°C and then with Goat anti-rabbit (#111-036-003, Jackson Laboratories) or Goat anti-Mouse (#115-036-003, Jackson Laboratories) diluted 1:5,000 in 2% non-fat dry milk in TBST 1× 1 h RT. After three washes with TBST 1× and one wash in TBS 1×, chemiluminescent signal was detected by using ECL solution (GE Healthcare, Little Chalfont, Buckinghamshire, UK-RPN2232). Chemiluminescent analysis of immunoblot results was performed by using LAS400 analyzer (GE Healthcare, Little Chalfont, Buckinghamshire, UK-RPN2232).

ACE2 Targeted Mass Spectrometric-Shotgun Proteomics Analysis

To perform ACE2 targeted MS analysis, EndoC-βH1 cells were lysed with RIPA buffer 1× and protein lysate concentration quantified through BCA assay. Then the protein lysate was mixed with 400 µl of urea 8 M in Tris-HCl 100 nM pH 8.5 (UA), with the addition of 100 mM DTT. The mixture was charged on a filter 10 K Pall, incubated 30 min RT and centrifuged 13,800×g 30 min. The filter was washed twice with 400 µl of UA and centrifuged 13,800×g 30 min, then incubated with 100 µl of 50 mM of iodoacetamide (IAC) solution in a thermo-mixer for 1 min 150 rpm and without mixing for 20 min, then centrifuged at 13,800×g for 20 min. After these steps, the filter was washed twice with 400 µl of UA and centrifuged at 13,800×g 30 min, twice with 400 µl of 50 mM ammonium bicarbonate (AMBIC), and then centrifuged twice, the first time at 13,800×g for 30 min and the second time at 13,800×g for 20 min. Next, 40 µl of 50 mM AMBIC was added to the filter together with trypsin (ratio trypsin/proteins 1:25) and incubated O/N 37°C. The sample was then transferred into a new collecting tube and centrifuged at 13,800×g for 10 min. Subsequently, 100 µl of 0.1% formic acid was added on the filter and centrifuged 13,800×g 10 min. Finally, the filter was discarded, and the solution was desalted with OASIS cartridges according to the manufacturers' instructions. The retrieved peptides were concentrated through SpeedVac, and the sample was resuspended in a solution of 3% acetonitrile, 96.9% H_2O, and 0.1% formic acid. The analyses were performed on a Q-Exactive Plus mass spectrometer (Thermofisher Scientific), equipped with electrospray (ESI) ion source operating in positive ion mode. The instrument is coupled to an UHPLC Ultimate 3000 (Thermofisher Scientific). The chromatographic analysis was performed on a column Acquity UPLC Waters CSH C18 130Å (1 mm × 100 mm, 1.7 µm, Waters) using a linear gradient and the eluents were 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B). The flow rate was maintained at 100 µl/min and column oven temperature at 50°C. The mass spectra were recorded in the mass to charge (m/z) range 200–2,000 at resolution 35 K at m/z 200. The mass spectra were acquired using a "data dependent scan", able to acquire both the full mass spectra in high resolution and to "isolate and fragment" the ten ions with

highest intensity present in the full mass spectrum. The raw data obtained were analyzed using the Biopharma Finder 2.1 software from ThermoFisher Scientific.

The elaboration process consisted in the comparison between the peak list obtained "*in silico*" considering the expected aminoacidic sequence of human ACE2 protein (Uniprot ID: Q9BYF1), trypsin as digestion enzyme and eventual modifications (carbamidomethylation, oxidation, *etc.*).

ACE2 Promoter Transcription Factors Binding Motif Analysis

ACE2 proximal promoter sequence was retrieved from Ensembl Genome browser database (http://www.ensembl.org/index.html) [Release 100 (April 2020)]. ACE2 gene (ENSG00000130234) was searched in Human genome GCRh38.p13 assembly. The sequence of interest was retrieved using "Export Data" function by selecting 1,000 bp upstream 5′ Flanking Sequence (GRCh38:X:15602149:15603148:-1) and downloaded in FASTA format. The analysis of TF binding motifs was performed using Transcription factor Affinity Prediction (TRAP) Web Tool (http://trap.molgen.mpg.de/cgi-bin/trap_receiver.cgi) (39). In TRAP, ACE2 promoter sequence was analyzed by using both TRANSFAC.2010.1 and JASPAR vertebrate databases and *human_promoters* as background model.

Data and Code Availability

The NCBI GEO accession numbers for RNA sequencing data reported in this paper are: GSE133221, GSE108413, GSE137136.

Statistical Analysis

Results were expressed as mean ± SD. Statistical analyses were performed using Graph Pad Prism 8 software. Comparisons between two groups were carried out using Mann–Whitney U test (for non-parametric data) or Wilcoxon matched-pairs signed rank test. Differences were considered significant with p values less than 0.05.

RESULTS

ACE2 Expression Pattern in Human Pancreas

To determine the ACE2 protein expression pattern in human pancreatic tissue, we first performed a colorimetric immunohistochemistry analysis to detect ACE2 on formalin-fixed paraffin embedded (FFPE) pancreatic sections obtained from seven (n = 7) adult non-diabetic multiorgan donors collected by the INNODIA EUnPOD biobank (**Table S1**). To specifically detect ACE2 protein in such context, we initially used a previously validated monoclonal anti-human ACE2 antibody (R&D MAB933) (7) which passed the validation criteria suggested by the International Working Group for Antibody Validation (IWGAV) (7, 40) (see **Resources Table in Supplementary Material**). For each pancreas, two sections derived from two different FFPE tissue blocks belonging to different parts of the organ (head, body, or tail) were analyzed. Based on pancreas morphometry and histological composition, we identified three main cell types positive for ACE2 (**Figures 1A–F**). In the exocrine pancreas there was a marked and intense staining in a subset of vascular components (endothelial cells or pericytes) found in inter-acini septa (**Figures 1A, B**). We also identified ACE2 positive cells in the pancreatic ducts even though only some scattered cells with a clear ACE2 signal were detected (**Figures 1C, D**). Of interest, we observed a peculiar ACE2 staining pattern in the endocrine pancreatic islets showing a diffuse ACE2 signal in a subset of cells within the islet parenchyma (**Figures 1E, F**, **Figure S2**). However, the observed ACE2 expression in the islets was lower than the expression observed in the microvasculature, the latter representing the main site for ACE2 expression in the pancreas.

In all cases analyzed, including different blocks of the same case, a similar expression pattern of ACE2 was observed even though a certain degree of variability in terms of ACE2 staining intensity within the islets was noted (**Figure S2**).

The highest signal of ACE2 within the pancreas was observed in putative association with the microvasculature (**Figures 1A, B**). Of note, in such context, a lobular staining pattern of the microvasculature associated ACE2 was evident, as demonstrated by the presence of positive cells in certain lobules and low or null expression in other lobules of the same pancreas section (**Figure 2A**). ACE2 staining pattern in inter-acini septa suggested an overlap with cells associated with the microvasculature, most likely endothelial cells. In order to explore such possibility, we performed a double immunofluorescent staining on the pancreas FFPE sections for ACE2 and the endothelial cell specific marker CD31. The results showed that ACE2 signal is associated, but not superimposed, with the CD31-specific one, thus resembling the tight association of pericytes with endothelial cells and strongly suggesting the presence of ACE2 in microvasculature pericytes (**Figure 2B**).

In order to confirm the ACE2 cellular distribution observed in the pancreas using MAB933 antibody, we tested two additional anti-ACE2 antibodies from Abcam: Ab15348 and Ab108252 (see **Resources Table**). According to the information obtained by R&D and Abcam, while MAB933 and Ab15348 are reported to recognize the C-terminus portion of ACE2 (18-740aa and 788-805aa, respectively), Ab108252 is specifically designed to react with a linear peptide located in the N-terminal ACE2 protein sequence (200–300aa). It has been recently reported that a 459 aa short-ACE2 isoform (357–805aa of ACE2 + 10aa at N-terminus) can be co-expressed alongside the full-length ACE2 protein (1–805aa) (**Figure 3A**) (24, 25); the short-ACE2 misses part of the N-terminal region targeted by Ab108252 antibody. We observed ACE2 islet-related signal using both MAB933 (**Figure 3B**) and Ab15348 (**Figure 3C**), but the Ab108252 antibody did not show any positivity within the islet parenchyma (**Figure 3D**), raising the possibility that the most prevalent ACE2 isoform within pancreatic islets is the short one. Of note, the three antibodies tested showed ACE2 positivity in the microvasculature, thus suggesting a putative differential distribution of the two ACE2-isoforms in the human pancreas.

As a positive control for our immunohistochemistry method and ACE2 antibodies adopted, we evaluated FFPE lung tissue

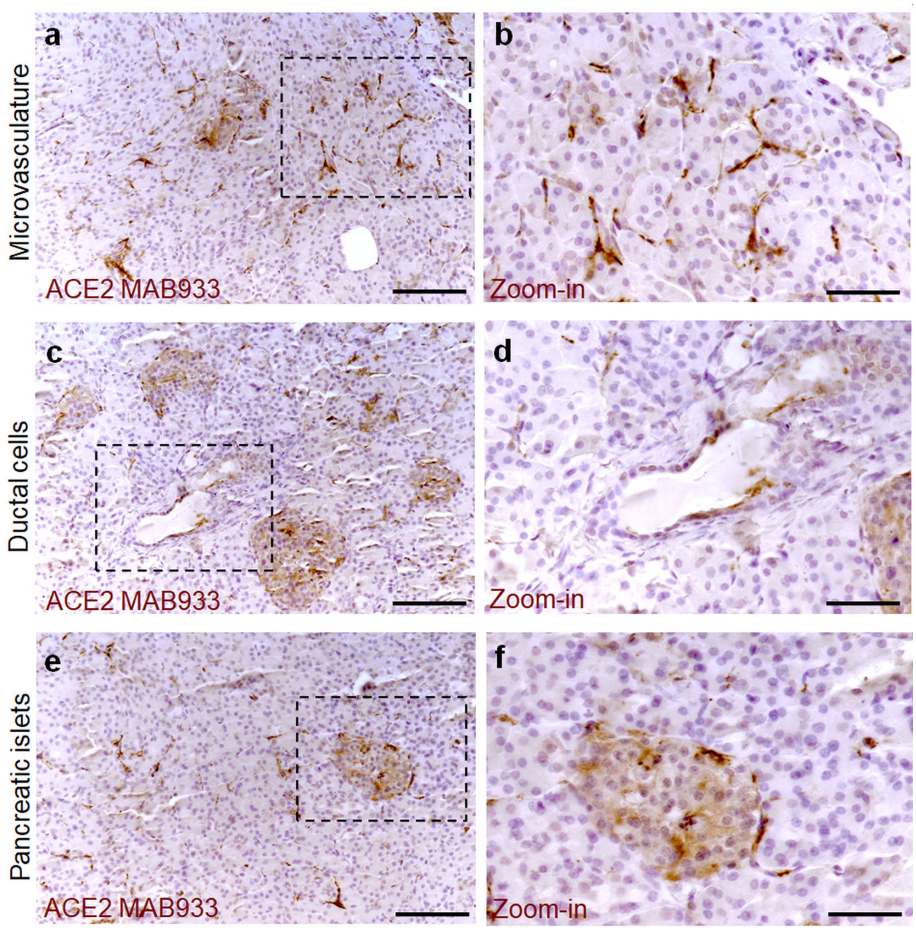

FIGURE 1 | ACE2 staining pattern in human pancreas. Immunohistochemistry for ACE2 in human pancreatic tissue sections (case #110118) using R&D MAB933 antibody. ACE2 is markedly expressed in microvasculature associated cells **(A, B)** in some rare ductal cells **(C, D)** and in a subset of endocrine cells within pancreatic islets **(E, F)**. Scale bars in **(A, C, E)** 150 µm. Scale bars in **(B, D, F)** 70 µm. Zoom-in images are reported in **(B, D, F)**.

sections. As previously shown (7, 41, 42), we observed scattered positive cells (putatively AT2 pneumocytes) in the alveolar epithelium both using MAB933 and Ab15348 (**Figures S3,** panels-a and -b. In contrast, we did not observe any signal using Ab108252 (**Figure S3,** panel-c).

Collectively, these results indicate that the same staining pattern were obtained by using two out of three antibodies that may recognize both ACE2 isoform (short-ACE2 and long-ACE2) thus confirming: (*i*) a high ACE2 expression in microvasculature pericytes; (*ii*) rare scattered ACE2 positive ductal cells; (*iii*) diffuse though weak ACE2 positive staining in a subset of cells within human pancreatic islets.

In Human Pancreatic Islets ACE2 Is Preferentially Expressed in β-Cells

Using both MAB933 and Ab15348 we observed ACE2 signal in pancreatic islets which suggests that ACE2 is expressed in endocrine cells. Therefore, we sought to determine which pancreatic islet cell subset contributes to ACE2 signal in such context. To do so, we performed a triple immunofluorescence analysis on the same set of FFPE pancreatic sections of non-diabetic multiorgan donors, aimed at detecting glucagon-positive α-cells, insulin-positive β-cells, and ACE2 signals (**Figure 4** and **Figure S4**).

Using R&D MAB933, ACE2 preferentially overlapped with the insulin-positive β-cells (**Figure 4A**, panels-a to -m), being mostly colocalized with insulin and low/not detectable in α-cells (**Figure 4A**, panels-e, -f, -l, -m). Such staining pattern was observed in all cases and was consistent between two different FFPE pancreas blocks of the same case (**Table S2**). As expected, ACE2-only positive cells within or around pancreatic islets were also observed (**Figure 4A**, panel g), potentially indicating the presence of ACE2-positive pericytes interspersed in the islet parenchyma or surrounding it.

Intriguingly, in the β-cells a major fraction of ACE2 was observed in the cytoplasm and partially overlapped with insulin positive signal, while in a subsets of them only a minor fraction of the ACE2 signal was attributable to several spots located on

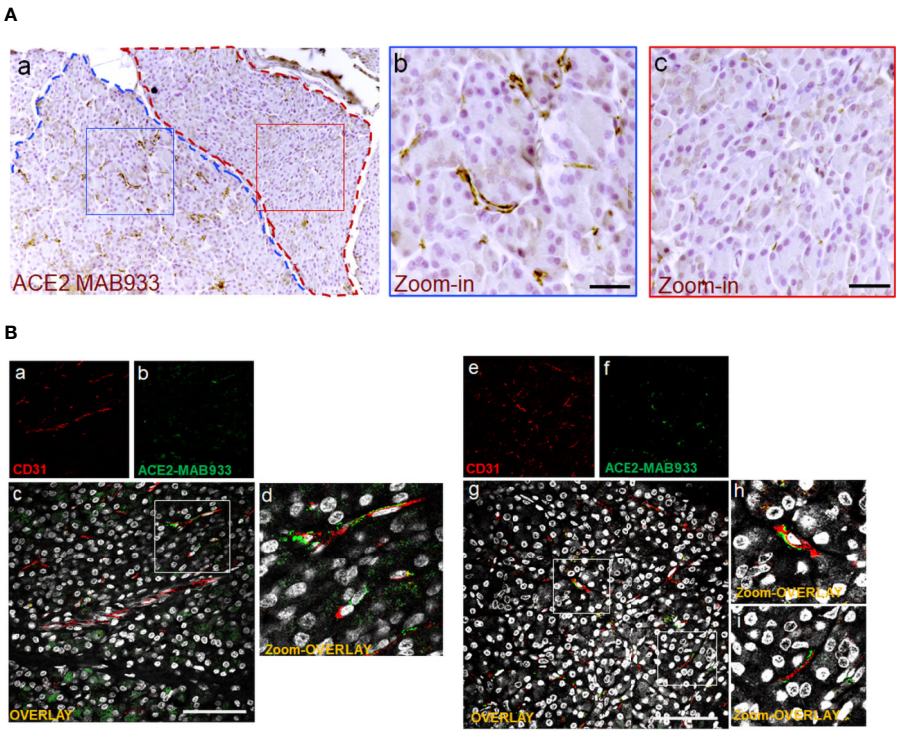

FIGURE 2 | In microvasculature, ACE2 is putatively expressed in pericytes. **(A)** Representative image of human pancreatic Formalin-Fixed Paraffin Embedded (FFPE) section stained for ACE2 in case #301118. In panel-a, a representative image of a pancreatic section showing two adjacent lobules (blue and red dotted lines) with different staining for ACE2 in endothelial cells/pericytes. A specific segmentation of the two lobules with high (blue) (zoom-in, panel-b) and low or null expression of ACE2 (red) (zoom-in, panel-c) is shown, suggesting lobularity of ACE2 expression in exocrine endothelial cells/pericytes of human pancreas. Scale bar in panel-a: 100 µm. Scale bar in panels-b and -c: 30 µm. **(B)** Double immunofluorescence staining of ACE2 (green) and CD31 (red) in FFPE pancreas sections from Body01A of Case #110118 (panels-a to -d) and of Body01B of Case #141117 (panels-e to -i). Digital zoom-in overlay images are shown in panels-d, -h and -i. Scale bar in panels-d and -g: 100 µm.

plasma membrane (**Figure 4A; Figure S5A red arrow, and Figure S5B**). In microvasculature pericytes, the ACE2-positive signal was mainly observed in plasma membrane (**Figure 4A**, panel-g; **Figure S5A green arrow)** as previously described (43). There were also some ACE2-negative β-cells (**Figure S5A, white arrow**).

Colocalization rate analysis between ACE2-insulin and ACE2-glucagon, performed on a total of 128 single pancreatic islets from seven different adult non-diabetic cases, confirmed the significant preferential expression of ACE2 in β-cells compared to α-cells (colocalization rate: ACE2-INS 57.6 ± 19.3% *vs.* ACE2-GCG 6.8 ± 5.4% $p < 0. \pm 0001$) (**Figure 4B** and **Figure S6A**). The comparison of colocalization rates between ACE2-insulin and ACE2-glucagon among all cases analyzed, confirmed the consistent preferential expression of ACE2 in β-cells in comparison to α-cells (**Figure 4B**). These results were confirmed when comparing different blocks of the same case (**Table S2**). There was however heterogeneity in terms of the ACE2-insulin colocalization rate among different islets (ACE2-INS colocalization rate range: 0.6–91.4%). Such heterogeneity was also highlighted by the presence of rare ACE2-negative pancreatic islets in the same pancreas section.

Inter-islets heterogeneity was also clearly observed regarding ACE2 islet-related signal intensity analysis (**Figure 4C**).

Of note, some cases showed a lower ACE2-insulin mean colocalization rate and islet-ACE2 signal intensity compared to the other ones (**Figure 4D**), thus suggesting a high degree of heterogeneity among cases also in terms of islet-ACE2 expression. No significant correlation between ACE2-islets signal intensity and age, BMI or cold-ischemia time were observed in our donors cohort (**Figure S6B**).

A Short ACE2 Isoform Is Prevalently Expressed in the Human β-cell Line EndoC-βH1

Using Western Blot (WB) and immunofluorescence analysis, we explored the expression of ACE2 in the human β-cell line EndoC-βH1, a model of functional β-cells for diabetes research (27, 44). To do so we used R&D MAB933, Abcam Ab15348 and Ab108252 antibodies, as previously done in the above described pancreas immunohistochemistry experiments. In WB analysis, MAB933 revealed the presence of a prevalent 50 kDa band corresponding to the short-ACE2 isoform; use of this Ab showed the brightest signal in immunofluorescence staining among the three antibodies tested (**Figure 5A**). Abcam Ab15348 worked better in WB for the recognition of both ACE2 isoforms and indicated that the most prevalent ACE2

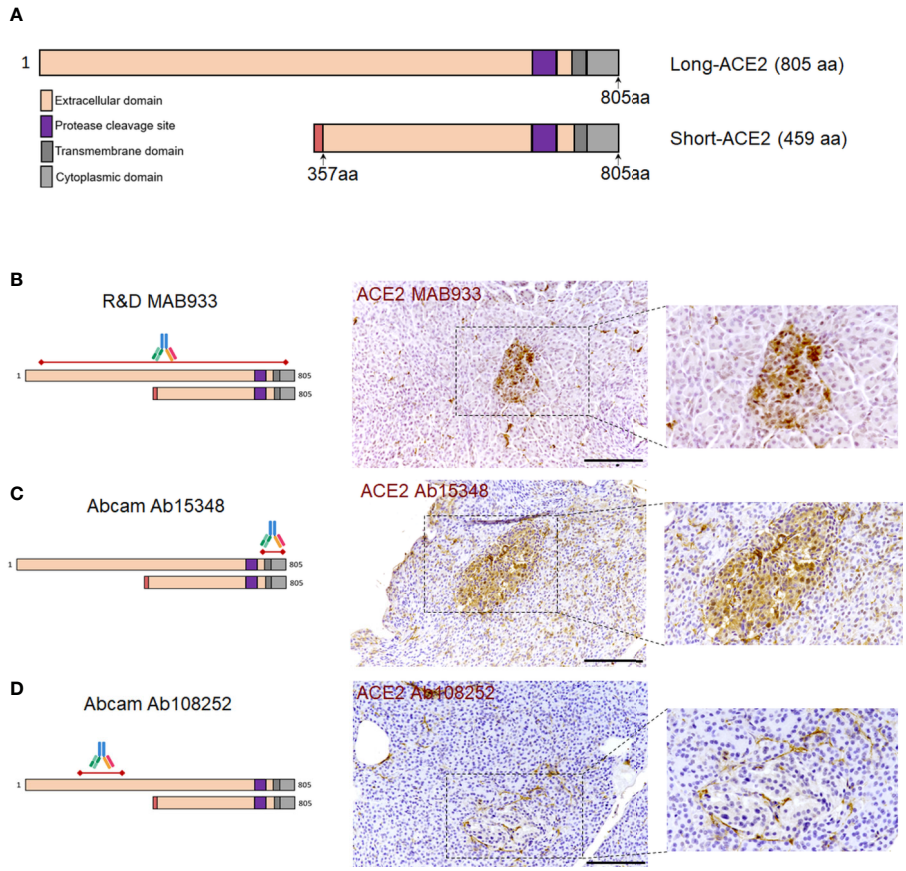

FIGURE 3 | ACE2 immunohistochemistry staining pattern in human pancreatic islets using MAB933, Ab15348, and Ab108252 antibodies. **(A)** Aligned sequences and structures of recently described ACE2 isoforms, long-ACE2 (805aa, ~110 kDa) and short-ACE2 (459aa, ~50kDa). Main ACE2 protein domains are reported with different colors. **(B)** R&D MAB933, **(C)** Abcam Ab15348 and **(D)** Ab108252 antibody predicted target sequence within the two ACE2 isoforms, alongside with immunohistochemistry staining distribution in pancreatic islets. Scale bars in **(B–D)** are 100 μm.

isoform present in human β-cells is the short-ACE2 (50 kDa, blue arrow) (**Figure 5B**). In contrast, Ab108252 recognized only the long-ACE2 isoform (>110 kDa- red arrow) (**Figure 5C**). Of note, the results obtained through WB analysis are in line with the immunofluorescence signal which revealed that Ab108252 only stained a minor fraction of EndoC-βH1 and the obtained signal was mainly found on the plasma membrane (**Figures 5C**, panel-B). Conversely, Ab15348 and MAB933, which recognized both ACE2 isoforms, showed a higher signal and a different subcellular localization with respect to Ab108252 (**Figures 5A, B**, panel-B). MAB933 ACE2-insulin double immunofluorescence staining confirmed the main punctuate and likely granular cytoplasmic ACE2 signal which also partially overlapped with insulin-positive secretory granules (**Figures 5D**, panel-A-H). In addition, we also observed some spots putatively localized on the plasma membrane (**Figure 5D**). Of note, the specificity of ACE2 MAB933 signal observed in EndoC-βH1 was orthogonally tested in comparison to HeLa cells which showed very low/absent ACE2 mRNA expression (**Figure S7A**) and resulted indeed negative for ACE2 in immunofluorescence (**Figure S7B**).

An additional evidence of the presence of ACE2 in EndoC-βH1 was provided by the shotgun proteomic analysis, aimed at detecting specific peptides derived from ACE2 protein independently of the use of specific antibodies. By this independent approach, we observed the presence of both N-terminal and C-terminal unique ACE2-derived peptides (**Figure S8**), which further confirmed the presence of the ACE-2 protein in human β-cells (**Supplementary File 1a, 1b**).

Total ACE2 mRNA Is Expressed in Human Pancreatic Islets and in the Human Beta-Cell Line EndoC-βH1

To confirm the ACE2 expression in human islets, we also evaluated its transcriptional activity both in collagenase-isolated and in Laser-Capture Microdissected (LCM) human pancreatic islets by measuring its mRNA expression using TaqMan RT-Real Time PCR. In order to avoid detection of genomic DNA, we used specific primers set generating an amplicon spanning the exons 17–18 junction of ACE2 gene, thus uniquely identifying its mRNA (**Figure S9A**). Of note, the

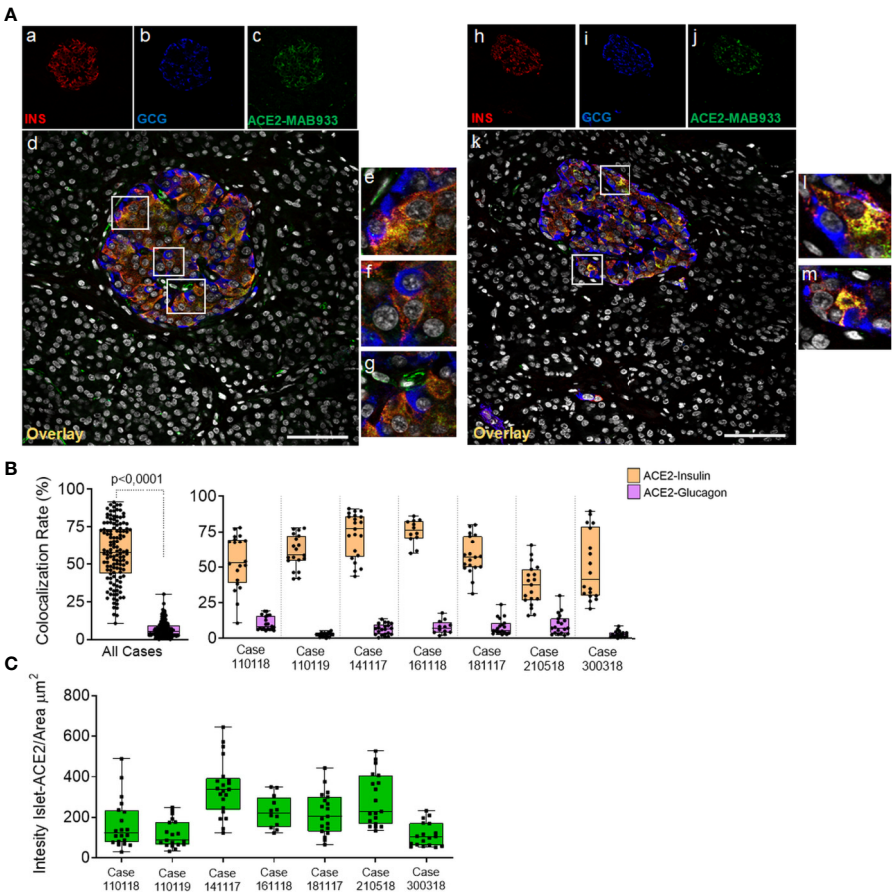

FIGURE 4 | In human pancreatic islets, ACE2 is preferentially expressed in insulin-producing β-cells. Triple immunofluorescence staining and image analysis of FFPE human pancreatic section stained for insulin (red), glucagon (blue) and ACE2 (green). **(A)** Representative islets of two different cases. Panels-a to -g: representative pancreatic islet of FFPE pancreas block Body01A of case #110118. Panels-h to -m: representative pancreatic islet of FFPE pancreas block Body01B of case #141117. Panels-e to -g: digital zoom in images of the pancreatic islet shown in panel-d. Panels-l and -m: digital zoom in images of the pancreatic islet shown in panel-k. Scale bar in panels-d and -k: 100 µm. **(B)** Colocalization rate analysis of overlapping ACE2-insulin and ACE2-glucagon in 128 single pancreatic islets of seven different cases. p-value was calculated using Wilcoxon matched-pairs signed rank test. On the right: colocalization rate analysis of ACE2-insulin and ACE2-glucagon in each of the seven cases analyzed. For each case, a total of 7–11 islets/section were analyzed. **(C)** Analysis of the intensity of ACE2 islet-related signals in the cases analyzed. Values are shown as fluorescence intensity of each islet reported as the sum of gray-scale values for each pixel normalized for the islets area (ROI, μm^2).

selected amplicon is shared between short- and long-ACE2 isoforms thus identifying total ACE2 mRNA.

First, as a positive control we analyzed total ACE2 expression in RNA extracted from a lung parenchyma biopsy tissue (**Figures 6A, E**). Collagenase-isolated human pancreatic islets obtained from four different non-diabetic donors' pancreata (**Table S1**) showed ACE2 mRNA expression, as demonstrated by RT-Real-Time PCR raw cycle threshold (Ct) values, reporting a Ct range between 28 and 29 (**Figures 6B, F**). Since human pancreatic islet enzymatic isolation procedures may induce some changes in gene expression (45), we microdissected human islets from frozen pancreatic tissues obtained from five non-diabetic multiorgan donors recruited within INNODIA EUnPOD network (46) and evaluated ACE2 mRNA levels. The LCM procedure (**Figure S9B**) allowed us to extract high quality total RNA (**Figure S9C**) from human pancreatic islets directly obtained from their native microenvironment, thus maintaining transcriptional architecture. ACE2 mRNA expression in LCM-human pancreatic islets showed a consistent expression among cases, similar to isolated islets, as shown by ACE2 mRNA raw Ct and normalized values (**Figures 6C, G**).

Finally, we analyzed total ACE2 mRNA expression in the human β-cell line EndoC-βH1. Analysis of ACE2 mRNA expression in these cells demonstrated a similar expression level in comparison to human pancreatic islets (**Figures 6D, H**), with raw Ct values ranging from 28 to 30.

ACE2 Expression Is Increased in EndoC-βH1 Cells and in Human Pancreatic Islets by Pro-Inflammatory Cytokines

In order to determine whether metabolic or inflammatory stress conditions modify pancreatic endocrine β-cell expression of

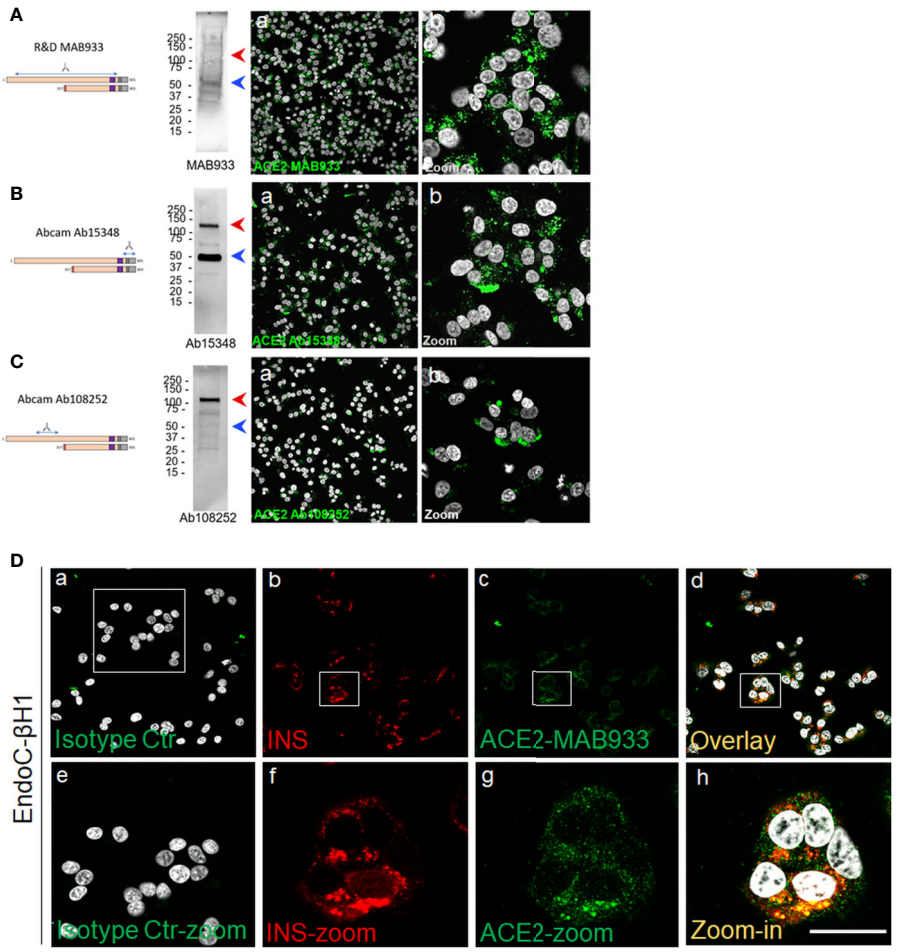

FIGURE 5 | A short-ACE2 isoform is prevalently expressed in human β-cell line EndoC-βH1. Western Blot and Immunofluorescence analysis of EndoC-βH1 using **(A)** R&D monoclonal MAB933, **(B)** Abcam polyclonal Ab15348 and **(C)** Abcam monoclonal Ab108252 anti-ACE2 antibody. For each antibody adopted, specific target sequence is reported within the aligned ACE2 isoforms. In western blot analysis, molecular weight markers (from 15 kDa to 250 kDa) are reported; red arrows indicate long-ACE2 isoform (expected band of ~110kDa), while blue arrows indicate short-ACE2 isoform (~50kDa). **(D)** ACE2 (R&D MAB933) and insulin double immunofluorescence analysis in EndoC-βH1 cultured cells. Negative isotype primary antibody control (relative to ACE2 primary antibody) is shown in panel-a. Insulin (red) and ACE2 (green) are reported in panels-b and -c, while overlay is reported in panel-d. Digital zoom-in images are reported from panels-e to -h. Scale bar in panel-h = 15 µm.

ACE2, we exposed the human β-cell line EndoC-βH1 and isolated human pancreatic islets to metabolic or inflammatory stressors and subsequently evaluated ACE2 expression levels.

Exposure to palmitate (2 mM palmitate for 24 h) did not significantly modulate ACE2 expression (**Figure 7A**). In line with these observations, neither primary human islets exposed to palmitate (47) nor human islets isolated from patients affected by type 2 diabetes (48) and evaluated by RNA sequencing showed any increase in ACE2 mRNA expression as compared to the respective controls [respectively 0.9 control *vs* 0.5 palmitate and 2.4 control *vs* 3.5 T2D; data reported as Trimmed Means of M values (TMM); not significant]. On the other hand, upon 24 h exposure to a pro-inflammatory cytokines mix (IL-1β – IFNγ and TNFα), EndoC-βH1 cells significantly upregulated ACE2 mRNA levels (fold change: 12.3 *vs.* not-treated control, $p = 0.031$) (**Figure 7B**). The same results were confirmed through immunofluorescence analysis aimed at measuring ACE2 protein levels and subcellular localization in EndoC-βH1 exposed or not to the same pro-inflammatory condition (**Figure 7C**). Indeed, we observed a significant increase in ACE2 mean intensity values upon cytokine treatment, confirming the upregulation of ACE2 protein as well (**Figure 7E**). These results were confirmed using an automated micro-confocal high content images screening system (31) which allowed us to measure ACE2 intensity in cytokine-treated *vs* not-treated EndoC-βH1 cells (**Figures 7D, F**).

In support of the observed increase of ACE2 upon pro-inflammatory stress, RNA sequencing data analysis of EndoC-βH1 cells exposed to IL-1β + IFNγ (48 h) or to IFNα (18 h) further confirmed such increase (**Table S3**). Indeed, we observed a 24.5 and 55.2 fold-increase ($p < 0.0001$) in total ACE2 mRNA [transcript (ENST00000252519.8)] (**Table S3** and **Figure S10A**) expression in EndoC-βH1 cells treated with IL-1β + IFNγ or with IFNα, respectively. Importantly, the same expression pattern was observed also in human pancreatic islets exposed to the same

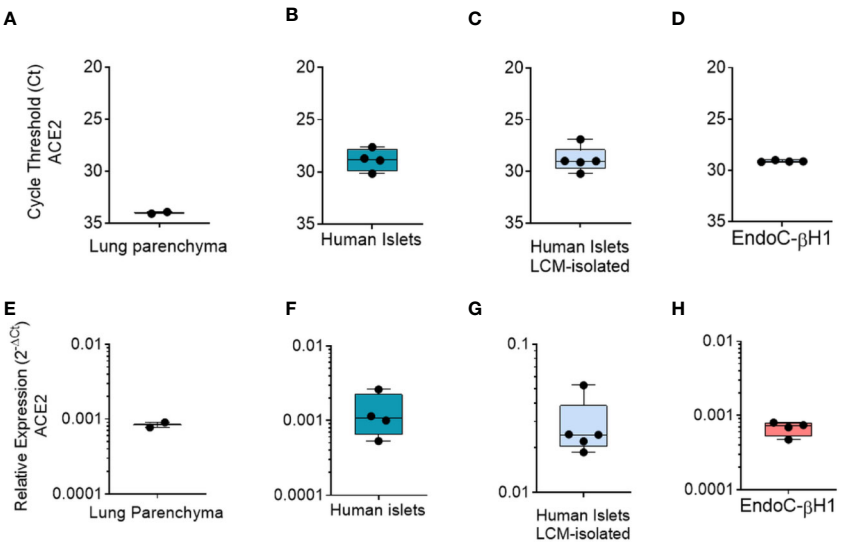

FIGURE 6 | qRT-Real Time PCR analysis of ACE2 mRNA. **(A–D)** ACE2 raw Ct values results in lung tissue (n = 1, in duplicate), in enzymatic-isolated human pancreatic islets samples (n = 4), in LCM-microdissected islets (n = 5) and in EndoC-βH1 (n = 4). **(E–H)** ACE2 expression values normalized using GAPDH and β2-microglobulin of the samples analysed in A–D. Values are reported as 2^{-dCt}. Mean ± S.D. values are shown.

cytokine mix, as demonstrated by a 2.4 and 5.1 fold-increase in ACE2 mRNA expression following IL-1β + IFNγ or IFNα treatment respectively ($p < 0.0001$) (**Table S3** and **Figure S10B**).

Additionally, in order to strengthen such observations, we focussed on the ACE2 gene promoter by analyzing its upstream sequence (−1 000 bp) from ACE2 transcriptional start site (TSS). Using two different transcription factors (TF) binding motif databases, we found several binding sites for TFs related to cytokine signaling pathways such as STAT1 or STAT3 (**Figure S11**), thus reinforcing our results of an association between inflammation and ACE2 expression and confirming what was previously reported (49). However, the analysis of ACE2 expression distribution in FFPE pancreas sections from a T1D longstanding donor (see **Table S1**) did not show remarkable changes in the levels or distribution of ACE2 in infiltrated islets (**Figure S12**). This is in line with RNAseq analysis of whole islets from two T1D patients and four controls, which indicated similar ACE2 expression (RPKM 1.2–1.7 in all cases) (50). Analysis of additional recent-onset T1D donors is needed to evaluate potential changes in ACE2 expression in β-cells of highly infiltrated pancreatic islets.

Collectively these results demonstrate that ACE2 is upregulated upon *in vitro* exposure to early and acute inflammatory, but not metabolic, stressors both in EndoC-βH1 and in human pancreatic islets.

DISCUSSION

In COVID-19 disease, clinical complications involving the metabolic/endocrine system are frequently observed. These include critical alterations of glycemic control in diabetic patients and new-onset hyperglycemia at admission in individuals without previous clinical history of diabetes. Although multiple causes have been indicated for COVID-19-related hyperglycemia, a recently published case report described autoantibody-negative insulin-dependent diabetes onset in a young patient who was infected by SARS-CoV-2 seven weeks before occurrence of diabetes symptoms. This suggests a potential effect (direct or indirect) of the SARS-CoV-2 infection on the pancreatic islet insulin producing β-cells, but additional evidence is needed to allow solid conclusions.

Previous studies suggested that ACE2, the human host cell receptor for SARS-CoV-2 and SARS-CoV, which in other tissues has been shown to be a necessary component for infection permissiveness (6, 51) is expressed in pancreatic tissue (19). However, an in-depth analysis aimed at evaluating ACE2 expression pattern distribution in human pancreas is still lacking. Here, we adopted multiple technologies and reagents to thoroughly analyze the presence of ACE2, both at mRNA and protein level, in order to evaluate its expression and localization in pancreatic tissue samples obtained from adult non-diabetic multiorgan donors from the INNODIA EUnPOD biobank collection, in enzymatic- and LCM-isolated primary adult human pancreatic islets and in human β-cell line EndoC-βH1.

In human adult pancreas, we primarily observed ACE2 expression in microvasculature component (endothelial cell-associated pericytes, both in endocrine and exocrine compartments). The expression of ACE2 in the pancreatic microvasculature compartment was associated, but not superimposed, with the endothelial cells specific marker CD31 (or PECAM-1). Such staining pattern strongly suggests the presence of ACE2 in pancreatic vascular pericytes which are tightly associated with endothelial cells.

Of interest, although the exocrine pancreas and the pancreatic islets are highly vascularized (52), only a subset of pancreatic

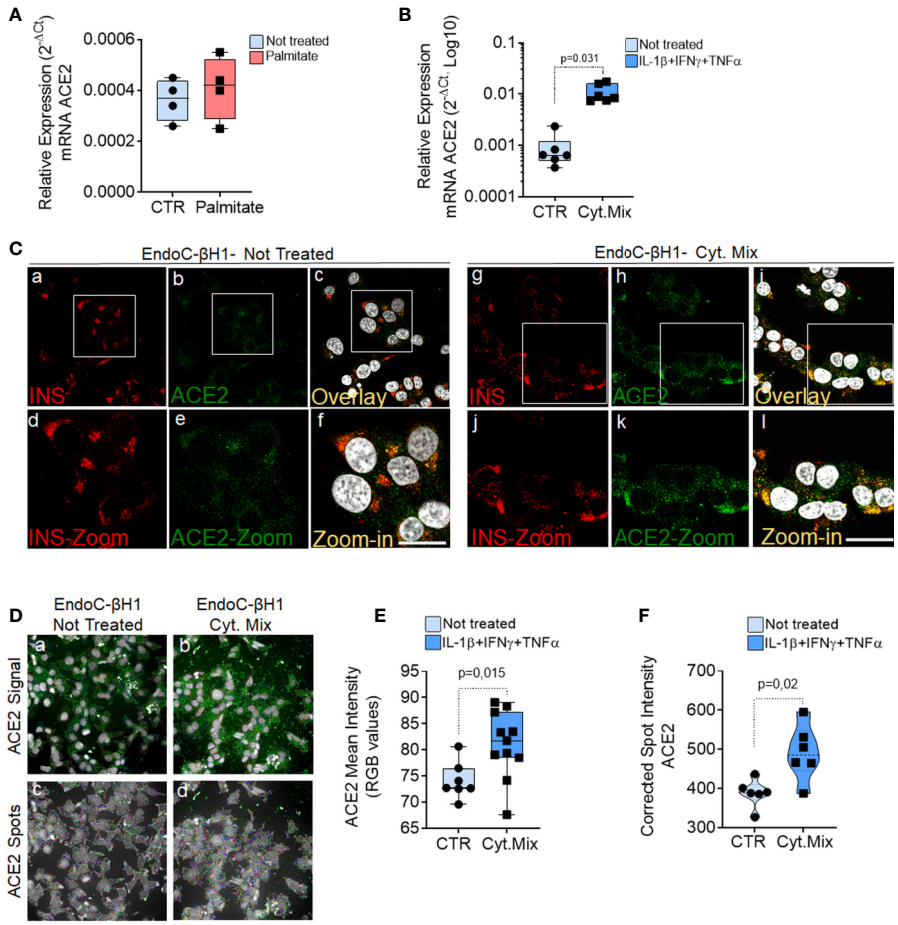

FIGURE 7 | ACE2 expression is increased by inflammatory stress. ACE2 mRNA RT-Real-Time PCR analysis in EndoC-βH1 treated or not (CTR) with Palmitate (2.0 mM) **(A)** or with cytokines (IL-1β + IFNγ+ TNFα) (Cyt. Mix) **(B)** for 24 h. Results are reported as mean ± S.D of 2^{-dCT} normalized values. p-values were calculated using Wilcoxon matched-pairs signed rank test. **(C)** Immunofluorescence analysis of insulin (red, panels-a and -g) and ACE2 (green, panels-b and -h) in EndoC-βH1 not-treated (panels-a to -f) or treated (panels-g to -l) with cytokines for 24 h. Digital zoom-in images are reported in panels-d to -f and in panels-j to -l. Scale bar in panel-f: 10 µm. Scale bar in panel-l: 15 µm. **(D)** Representative images of ACE2 staining (green) and analysis using micro-confocal High-content screening in EndoC-βH1 treated or not with cytokines (IL-1β + IFNγ+TNFα). Panels-a and -b: ACE2 signal (green) and automated cell cytoplasm segmentation and identification. Panels-c and -d: identification of ACE2 granular spots within segmented cytoplasm in panels-a and -b. Each ACE2 granular spot intensity was measured and analyzed. **(E)** Mean intensity imaging analysis related to data reported in **(C)** of EndoC-βH1 treated or not with cytokines. Data are reported as individual values alongside with mean ± S.D of RGB gray-intensity measures of 6–11 different experimental points related to two different independent experiments. Individual values alongside with mean ± S.D are reported. P-value was calculated using Mann–Whitney U test. **(F)** High content screening analysis of Corrected Median Spot intensity of ACE2 signal in EndoC-βH1 treated or not with cytokines. Median intensity values of six different experimental points are reported. P-value was calculated using Mann–Whitney U test ($p < 0.05$).

pericyte cells markedly express ACE2. Additionally, ACE2 expression in microvascular compartment is surprisingly lobular, resembling the heterogeneous staining pattern of several inflammatory markers. Additional observations on multiple pancreatic lobules are required to confirm such heterogeneous lobular patterning observed.

The presence of ACE2 in pancreatic pericytes is of sure interest. As a matter of fact, the vascular leakage and endothelitis were reported as a typical sign of SARS-CoV-2 infection in various organs, driving early local inflammation and the subsequent exacerbation of immune responses (53, 54). Of note, multiple studies addressed the importance of an intact islet microvasculature in order to render pancreatic islets fully functional (55, 56). Therefore, a pancreatic islet local vascular damage and inflammation due to SARS-CoV-2 direct infection of $ACE2^+$ pancreatic pericyte cells is a potential contributory factor for islet dysfunction. Of note, two recent preprint manuscripts also indicated the presence of ACE2 expression in pancreatic microvasculature (57, 58).

Our results indicate that ACE2 is expressed also in the pancreatic islets, and this expression is mostly located in β-cells as compared to α-cells. These results are in contrast with the two recent preprint manuscripts (57, 58) which failed to observe ACE2 expression in pancreatic islet endocrine cells. Such discrepancies may be explained by a resulting sum of differences in primary antibodies sensitivity, different epitopes targeted, tissue sections preparation and pre-treatment, as well as immunodetection methodology sensitivity (immunohistochemistry *vs.* immunofluorescence). Such variables may be of critical importance when detecting a low-expressed

protein and may generate different results. Furthermore, it should be taken into consideration that ACE2 expression may vary greatly among individuals due to genetic or environmental factors (59). Such intrinsic ACE2 variability has been previously observed also in other cellular contexts, with some authors reporting high ACE2 levels and others low or absent (60).

In our study, localization of ACE2 in pancreatic islet endocrine cells was observed using two out of three different antibodies tested. Surprisingly, we were not able to observe ACE2 pancreatic islets positive signal using Abcam monoclonal antibody Ab108252, while signal was clearly evident in microvasculature and scattered ductal cells. However, these results are in line with Kusmartseva et al., 2020 (58). Of note, Ab108252 monoclonal antibody specifically targets an epitope located in the N-terminal domain of ACE2 protein (200–300aa) which is missing in the recently discovered truncated ACE2 isoform (short-ACE2, 357–805aa) (24, 25), thus being capable to recognize only the long-ACE2 isoform. On the contrary, by using two different antibodies (MAB933 and Ab15348)—which can recognize the C-terminal domain shared between short- and long-ACE2—we obtained clear and concordant results, with identification of ACE2 in pancreatic islet endocrine cells in addition to the microvasculature. As a positive control for the antibodies and our IHC procedure, MAB933 and Ab15348 were also tested in FFPE lung tissue sections, showing overlapping results with previously published studies (7, 41, 42).

Overall, these results suggest that the short-ACE2 isoform may be the prevalent one expressed in β-cells. Indeed, ACE2 western blot analyses of the human β-cell line EndoC-βH1 support this hypothesis by confirming that: (*i*) short-ACE2 is prevalent over long-ACE2, the latter being present but low expressed in β-cells; (*ii*) Abcam Ab108252 cannot recognize the short-ACE2 isoform. Based on these results we suggest that in human β-cells both ACE2 isoforms are present, with a predominance of the short-ACE2.

Although the presence of the short-ACE2 isoform, alongside with long-ACE2, is clearly evident in human β-cells, its functional role remains to be established. A previous study suggested the ability of the short-ACE2 isoform to form homodimers or heterodimers with the long-ACE2 isoform, thus potentially being able to modulate both the activity and structural protein domain conformation of the long-ACE2 (22). Significantly, short-ACE2 is missing the aminoacidic residues reported as fundamental for virus binding; however, due to the lack of detailed data regarding its function we cannot exclude that the short-ACE2 may modulate SARS-CoV-2 susceptibility by interacting with long-ACE2 or additional membrane proteins which may mediate the binding to SARS-CoV-2 spike protein [*e.g.* ITGA5, as previously observed (61)]. Of interest, nasal epithelium, reported to represent the main reservoir of SARS-CoV-2 (62), showed higher levels of short-ACE2 *vs.* long-ACE2 (24).

As described above, we report that in human pancreatic islets, ACE2 is enriched in insulin-producing β-cells. This *in-situ* ACE2 expression pattern data are in line with three different bulk RNA-seq datasets analyzing human β- and α-cells' transcriptome, reporting higher expression of ACE2 mRNA in β-cells as compared to α-cells (63–65) (**Figure S10D–F**). Of note, such datasets rely on β- or α-cells bulk RNA-seq and do not suffer from common limitations present in single-cell RNA-seq (49), which usually detects only 1,000–3,000 genes/cell, thus representing a minor fraction (25–30%) of the total number of genes identified by bulk cells RNA sequencing (>20,000 genes). Another recent study analyzed SARS-CoV-2 host receptors expression using two different methods (microarray and bulk RNA-seq) and further confirmed that ACE2 is indeed expressed in human pancreatic islets and also demonstrated that ACE2 expression was higher in sorted pancreatic β-cells relative to other endocrine cells (66). Additional evidence of ACE2 expression in endocrine pancreas and in β-cells derive also from mouse studies, which demonstrated ACE2 expression in insulin-producing cells as well as its critical role in the regulation of β-cell phenotype and function (67–70).

Collectively, our *in-situ* expression data alongside with multiple published datasets and reports both in human and mouse show that ACE2 is expressed in pancreatic islets, albeit at relatively low levels. ACE2 expression in human β-cells may render these cells sensitive to SARS-CoV-2 entry (23). Such hypothesis is consistent with the known sensitivity of these cells to infection by several enterovirus serotypes. Indeed, multiple evidence from our and other groups (71–74) showed that enteroviruses are capable to competently infect β-cells but less so α-cells (75, 76); these viruses are thus being considered as one of the potential triggering causes of type 1 diabetes (T1D) (77). Of note, it has been previously demonstrated that human β-cells exclusively express virus receptor isoform Coxsackie and adenovirus receptor-SIV (CAR-SIV), making them prone to infection by certain viruses (78). Therefore, it would not be surprising that, under particular conditions, human β-cells could be directly infected by SARS-CoV-2. Importantly, a recent report showed that human pancreatic islets can be infected *in vitro* by SARS-CoV-2 (23), supporting our observations of a specific tropism of the virus due to ACE2 expression.

Noteworthy, the subcellular localization of ACE2 in β-cells recapitulates what was previously found for the virus receptor CAR-SIV (78). In our dataset, ACE2 protein signal is mostly cytoplasmic/granular and partially overlaps with insulin granules. Additional spots are also localized close to the plasma membrane, thus suggesting the existence of ACE2 isoforms in multiple compartments within β-cells. Such subcellular localization was observed both *in-vitro* in EndoC-βH1 and *ex vivo* in β-cells of primary human pancreatic tissues. Although ACE2 has been primarily observed on cell surfaces (42, 79), some studies also described ACE2 granular localization in other cell types of epithelial origin (9, 80). A similar intracellular localization and putative trafficking were observed for the viral receptor CAR-SIV (78), also expected to be mainly localized on the cell membrane. Against this background, we speculate that: (i) upon activation, ACE2 can be internalized through endosome/lysosome pathway (81); (ii) in β-cells, ACE2 trafficking to cell membrane may be mediated by insulin granules; (iii) in β-cells the short-ACE2 isoform could be mainly localized in cytoplasm, while long-ACE2 in the plasma

membrane; (iii) ACE2 can be secreted and found in a soluble form or within exosomes (82, 83). Additional studies are needed to fully ascertain the subcellular localization and trafficking of ACE2 isoforms in human β-cells and to determine whether ACE2 is indeed present in the secretome of β-cells.

Our data also indicate that in β-cells, total ACE2 mRNA expression is upregulated upon different pro-inflammatory conditions, but not following exposure to the metabolic stressor palmitate or to the T2D environment. Importantly, these observations were obtained both in the human β-cell line EndoC-βH1 and in human primary pancreatic islet cells, as shown by qRT-PCR, RNA-seq datasets and immunofluorescence. As a matter of fact, ACE2 has been previously indicated as an interferon-stimulated gene (ISG) in a variety of cells (59). Of interest, a previous report suggested that the short-ACE2 may represent the prevalent ACE2 isoform upregulated upon inflammatory stress (25). However, whether this is the case also in human β-cells it should be examined in depth in future studies.

Although total ACE2 expression is increased upon inflammatory stress, *in-situ* analysis of ACE2 in infiltrated pancreatic islets derived from FFPE sections of a pancreas from a longstanding T1D donor did not reveal significant changes of SARS-CoV2 receptor expression. However, we recognize that a high number of pancreatic islets with different degrees of inflammation and pancreata from recent-onset T1D donors showing highly infiltrated islets are needed to adequately characterize ACE2 expression in the early stages of the disease and to determine whether changes in ACE2 expression contribute to: (*i*) the observed alteration of glycaemic control at admission in SARS-CoV-2 individuals without previous clinical history of diabetes; (*ii*) the increased severity of COVID-19 in those subjects with previous inflammatory-based diseases.

In conclusion, the presently described preferential expression of ACE2 isoforms in human β-cells, alongside with ACE2 upregulation under pro-inflammatory conditions *in-vitro* identifies the putative molecular basis of SARS-CoV-2 tropism for pancreatic islets and uncovers a link between inflammation and ACE2 expression levels in islet β-cells. Further mechanistic studies and epidemiological evaluation of individuals affected by COVID-19, including long-term clinical follow-up and determination of islet autoantibodies, may help to clarify whether human β-cells are among the target cells of SARS-CoV-2 virus and whether β-cell damage, with the potential induction of autoimmunity in genetically susceptible individuals, occurs during and/or after infection.

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**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by local ethics committee of the University of Pisa (Italy). Pancreata not suitable for organ transplantation were obtained with informed written consent by organ donors' next-of-kin and processed with the approval of the local ethics committee. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

GS, FD, DF, GL, and NB conceived and designed the experiments. GS, DF, GL, and NB performed RT-real time PCR, laser capture microdissection and immunohistochemical/confocal fluorescence microscopy analysis experiments. DF and GG performed endoC-βH1 cell culture experiments. PM and LM isolated human pancreatic islets and contributed to the collection and processing of human pancreata. LN and NB curated FFPE processing and data analysis of INNODIA EUnPOD pancreata biobank collection. DE and MC conceived and performed RNA-seq on EndoC-βH1 and human pancreatic islets and contributed to the scientific discussion. CM, CG, and LO conceived the experiments and contributed to the scientific discussion. 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/fendo.2020.596898/full#supplementary-material

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miRNA-Mediated Immune Regulation in Islet Autoimmunity and Type 1 Diabetes

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The important role of microRNAs as major modulators of various physiological processes, including immune regulation and homeostasis, has been increasingly recognized. Consequently, aberrant miRNA expression contributes to the defective regulation of T cell development, differentiation, and function. This can result in immune activation and impaired tolerance mechanisms, which exert a cardinal function for the onset of islet autoimmunity and the progression to T1D. The specific impact of miRNAs for immune regulation and how miRNAs and their downstream targets are involved in the pathogenesis of islet autoimmunity and T1D has been investigated recently. These studies revealed that increased expression of individual miRNAs is involved in several layers of tolerance impairments, such as inefficient Treg induction and Treg instability. The targeted modulation of miRNAs using specific inhibitors, resulting in improved immune homeostasis, as well as improved methods for the targeting of miRNAs, suggest that miRNAs, especially in T cells, are a promising target for the reestablishment of immune tolerance.

Keywords: immune regulation, islet autoimmunity, type 1 diabetes, miRNA, regulatory T cell, biomarker

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INTRODUCTION

In type 1 diabetes (T1D), the loss of immune tolerance to beta cells in the pancreatic islets of Langerhans leads to an immune cell-mediated destruction of these insulin-producing cells. This progressive loss of beta cell mass is associated with insufficient insulin secretion, resulting in hyperglycemia and the risk of severe acute and chronic complications (1). The autoimmune attack in T1D is mainly driven by the infiltration of the pancreas by autoreactive T cells. These cells are normally repressed by Foxp3$^+$ regulatory T cells (Tregs), which are critical mediators of immune tolerance in the periphery. Impaired tolerance and consequently autoimmune activation, which are major drivers of T1D pathogenesis, are, among others, caused by impairments in Treg induction, stability, and function. microRNAs (miRNAs) are small non-coding RNAs, which have been recently shown to fine-tune the expression of important genes in various immune cell types, including Tregs and thereby critically add to immune regulation. The broad regulatory potential of miRNAs in the immune system indicates the potential of specific miRNA targeting to interfere with

aberrant immune reactions and autoimmunity. In this review, we discuss the complex role of miRNA regulatory networks contributing to the pathogenesis of autoimmune T1D, with a particular focus on Tregs. Furthermore, we address three studies, which reported a direct relationship between the upregulation of T cell-specific miRNAs during the onset of islet autoimmunity and impairments in Treg induction, function, and stability, as well as the therapeutic potential of these recent findings.

TYPE 1 DIABETES

Immune Tolerance

Diseases with an autoimmune etiology are characterized by impaired immune tolerance, which results in an overshooting immune reaction directed against the body's own healthy cells and tissues. The steady state of the immune system is a complex and precisely regulated balance between immunity and tolerance, which requires the accurate control of various immune mechanisms and cell types. One hallmark of proper immune function is the discrimination between the organism's own structures and pathogens, enabling the elimination of potentially harmful invaders without affecting own cells and tissues.

Due to its crucial role for proper immune function, this discrimination between self and non-self and the control of tolerance is mediated by two distinct mechanisms: During their development in the thymus, lymphocytes with a high affinity for self-antigens are negatively selected by deletion (apoptotic cell death) or functional inactivation (anergy). This mechanism is called recessive tolerance, and it was first proposed in the clonal selection theory.

Some autoreactive immune cells can circumvent the recessive tolerance and exit the thymus. To prevent fatal autoimmune attacks of these cells in the periphery, a second control mechanism maintains self-tolerance in the periphery. This peripheral tolerance is termed dominant tolerance and it is carried out by a specific T cell lineage called regulatory T (Treg) cells that can actively suppress other immune cells, including autoreactive T cells.

The dysregulation of these important control processes in the periphery promotes the maintenance and activation of autoreactive lymphocytes, which critically drives the development of autoimmune disorders.

Islet Autoimmunity and Type 1 Diabetes

To date, more than 80 autoimmune diseases have been described. They can be classified into systemic diseases, such as systemic lupus erythematosus and Sjogren's syndrome, and organ-specific diseases, such as multiple sclerosis and T1D. T1D shows a rising incidence worldwide and is the most common autoimmune disease in children (2).

T1D is characterized by the infiltration of the pancreas by immune cells and the destruction of the beta cells in the islets of Langerhans. The major drivers of the pathogenesis are impaired immune tolerance mechanisms. The beta cells in the islets of Langerhans are crucial for the maintenance of blood glucose homeostasis, by sensing the level of glucose and releasing insulin as required. Once the beta cell mass is reduced, the scarce supply of insulin prevents the body's tissues from taking up glucose from the blood stream, which is essential for the maintenance of proper tissue functioning and homeostasis. This loss of blood glucose control has been generally lethal until the establishment of insulin replacement therapy. To date, this remains the only way of controlling the disease, as no curative treatments are available. However, even with lifelong insulin supply, precise glycemic control remains challenging, and secondary complications like kidney failure and heart diseases occur regularly (3).

Multiple factors have been shown to be involved in the pathogenesis of T1D, in particular genetic and environmental factors such as diet and exposure to microbes and certain viruses influence the risk of developing the disease. The genetic predisposition is well studied and is, besides others, indicated by an increased risk of up to 10 times in children with a first-degree relative with T1D (4–7). The most robust predictor of genetic risk to develop the disease is the human leucocyte antigen (HLA) class II, with the genotype HLA-DR4, HLA-DQ8, conveying a risk of around 5% of developing T1D, even without a family history of the diseases (7, 8).

Multiple autoantibodies against islet autoantigens, such as insulin (9), the tyrosin phosphatase IA2 (10), glutamic acid decarboxylase (GAD) (11), and zinc transporter 8 (ZNT8) (12), appear before clinical symptoms of T1D arise. This presymptomatic phase of autoimmunity is termed islet autoimmunity and its duration is highly variable (13, 14). The time of progression from the first appearance of islet autoantibodies to clinically overt T1D can range from only several months to more than two decades. This heterogeneity in progression of islet autoimmunity indicates plasticity in immune activation and multiple layers of immune tolerance impairments. A multitude of recent studies focused on drivers of T1D pathogenesis; however, the precise cellular and molecular mechanisms underlying the loss of immune tolerance and their contribution to the highly heterogeneous progression from islet autoimmunity to symptomatic diabetes remain insufficiently investigated.

Mediators of Beta Cell Destruction

In T1D, immune cell infiltration into the pancreatic islets, termed insulitis, initiates the destruction of the beta cells (15). Interestingly, both the architecture of the islets and the level of insulitis differ remarkably between the human disease and mouse models of T1D like the non-obese diabetic (NOD) mouse, which exhibits much higher numbers of infiltrating cells (16). During insulitis, several immune cell types infiltrate the islets, including T cells, B cells, macrophages, dendritic cells (DCs), and natural killer (NK) cells, with $CD8^+$ T cells being the most abundant cell type (17). Besides the islet infiltration by immune cells, insulitis is characterized by an increased expression of HLA-I molecules in the islet cells (15, 18). This hyperexpression is accompanied by the production of interferon and is thought to contribute to the high abundance of $CD8^+$ T cells in the pancreatic infiltrates.

Despite this high abundance of $CD8^+$ T cells, $CD4^+$ T cells are critically involved in the pathogenesis of several autoimmune diseases including T1D, multiple sclerosis (19), rheumatoid arthritis (20), and Crohn's disease (21). In T1D, several subsets of autoreactive, islet-infiltrating $CD4^+$ T cells have been identified, including Th1, Th2 (22), Th17 (23, 24), Th9, Th22 (25), and TFH cells (26, 27).

The contribution of multiple immune cell types, including T cells, with both effector and regulatory characteristics, highlights the complex pathogenesis of T1D. Many of the molecular mechanisms underlying aberrant T cell activation and the multiple layers of impaired immune tolerance remain largely unexplored, not least because a multitude of mechanisms are involved in the regulation of T cells. One of these mechanisms are miRNAs, which have been recently highlighted as important regulators in various biological settings, including the immune system (**Table 1**).

miRNAs REGULATORS OF THE IMMUNE SYSTEM

miRNA Basics

miRNAs are small single-stranded non-coding RNAs, which are involved in almost all physiological processes, by precisely fine-tuning the expression of regulatory genes. They are a member of the family of small non-coding RNAs (sncRNAs), which are 20–30 nucleotides long and function via Argonaute (AGO) proteins. sncRNAs can be subdivided in three distinct regulatory families: miRNA, which are most abundant in human tissues, siRNA (small interfering RNA), and piRNA (PIWI-interacting RNA). In the tissues, miRNAs are directly involved in the regulation of tissue homeostasis and function, which is reflected by their distinct tissue-specific expression (68, 69). Besides their high abundance, several other characteristics of miRNAs highlight their broad regulatory potential. To date, the miRNA database MirBase contains about 2600 validated human miRNA sequences (70), but based on the constant identification of new miRNA sequences, the actual number of human miRNAs is predicted to be significantly higher (71). Furthermore, the miRNA-mediated gene regulation is a complex interplay of many miRNAs, regulating the expression of the same mRNA. Vice versa, the majority of target genes contain a multitude of miRNA binding sites, and some of them are highly conserved between species (68, 72, 73).

miRNAs are commonly 22 nucleotides in length, and their biogenesis involves several processing steps, including transcription, nuclear processing, export from the nucleus, and cytoplasmic processing. First, RNA polymerase II binds to the promoter of a miRNA gene and transcribes a stem-loop-shaped miRNA precursor, which is much longer than the mature miRNA and termed primary miRNA (pri-miRNA) (74). pri-miRNAs can contain up to six miRNA precursors (75), which are flanked by specific sequences facilitating their processing by a complex of Drosha (76) and DGCR8 (77, 78). The resulting pre-miRNA is exported into the cytoplasm where it is further processed by Dicer (79), yielding the final miRNA duplex. For gene silencing, one strand is discarded while the other one remains in contact with Dicer and associates to several additional proteins such as AGO. In line with its mode of action, the resulting complex is termed RNA-induced silencing complex (RISC) (80).

The recognition of the respective target is facilitated by complementary base pairing between the miRNA seed sequence and the corresponding binding site of the mRNA. The seed sequence is only six nucleotides long, comprising nucleotides 2 to 7 of the mRNA. While the short seed sequence facilitates the precise binding of the mRNA target, this is not sufficient to induce miRNA mediated silencing of the mRNA. For this, the complementary binding of additional miRNA nucleotides, usually 8 and 13–16, to the mRNA target is required (68). Some miRNAs form clusters, also called families, which are characterized by almost identical seed sequences and consequently similar target genes (72). The majority of miRNA binding sites is conserved between species, and they can be commonly found in the 3' untranslated region (UTR) or the coding region of the mRNA; however, their abundance in the 3' UTR is slightly increased (54, 73, 81). Mechanistically, the regulation of gene expression is mediated by the formation of the RISC, which induces translational repression or mRNA degradation (82).

In line with the broad regulatory potential of miRNAs and the high numbers of miRNA targets, the dysregulation of miRNA expression is involved in multiple human diseases, such as autoimmunity, cancer, and neurological diseases (83, 84).

miRNAs as Biomarkers for Islet Autoimmunity and T1D

Based on the high numbers of miRNAs and their involvement in virtually all biological processes, including immune regulation, multiple studies investigated their biomarker potential in the context of islet autoimmunity and T1D (42, 56, 66, 85–91). In contrast to the majority of disease symptoms, biomarkers enable the objectively quantifiable characterization of a disease and its progression. This is critical for early and precise diagnosis and treatment and facilitates strategies of personalized precision medicine aiming at the maximum benefit for the patient.

Cell-Free Circulating miRNAs

Recent studies have investigated levels of circulating miRNAs in blood or serum from T1D patients in order to evaluate their biomarker potential for the prediction of T1D onset and progression. Circulating miRNAs are particularly suitable as biomarkers in clinical practice because their analysis is feasible in small volumes of blood or serum. Several differentially expressed miRNAs regulate both pancreatic beta cells and immune cells (42, 66, 85). For example, the increased abundance of circulating miR25 correlated with glycemic control and residual beta cell function in patients with newly diagnosed T1D (42). Similarly, levels of the miR23~27~24 cluster were increased during disease progression in children with T1D and correlated with osteoprotegerin abundance. Importantly,

TABLE 1 | Selected miRNAs involved in immune regulation.

miRNA	miRNA regulation	miRNA target	Effect	Cell type	Organism	Reference
let7i	Up	IGF1R	Decreased induction	Treg	Human	(28)
miR10a	Down	BCL6, NCOR2	Reduced expression of Treg genes	Tregs	Mouse	(29)
miR15a/16	Up	Foxp3	Decreased Foxp3 abundance	Treg	Human	(30)
miR15b/16	Up	Rictor, mTor	Increased induction	Treg	Mouse	(31)
miR17	Up	IKZF4	Decreased frequencies	Treg	Mouse	(32)
miR17	Up	CREB1, TGFBRII	Decreased induction	naive T cells	Mouse	(33)
miR19b	Up	PTEN	Decreased frequencies	Treg	Mouse	(32)
miR21	Up	unknown	Increased Foxp3 abundance	Treg	Human	(34)
miR21	Down	STAT3	Decreased frequencies	Treg	Human	(35)
miR21-3p	Up	–	Correlation islet autoimmunity, progression to T1D	serum	Human	(36)
miR23b	Up	Trail, Trail-R2, Fas, Faslg	Proliferation	CD8+ T cells	Human	(37)
miR23/miR27/miR24	Up	TGFB signaling	Decreased induction	naive T cells	Mouse	(38)
miR23a-3p	Down	DP5, PUMA	Apoptosis	beta cells	Human	(39)
miR23b-3p	Down	DP5, PUMA	Apoptosis	beta cells	Human	(39)
miR24	Down	Foxp3	Increased Foxp3 abundance	Treg	Human	(40)
miR25	Up	TGFB signaling	Decreased suppression	Treg	Human	(41)
miR25	Up	–	Correlation with glycemic control, residual beta cell function	PBMCs	Human	(42)
miR29	Up	Mc11	Reduced insulin mRNA levels, impaired insulin secretion, and induced beta cell apoptosis	beta cells	Human/mouse	(43)
miR29a-3p	Up	–	Correlation islet autoimmunity, progression to T1D	serum	Human	(36)
miR31	Down	Foxp3	Increased Foxp3 abundance	Treg	Human	(34)
miR34a	Up	insulin, proinsulin	Reduction of insulin and proinsulin	beta cells	Human/mouse	(43, 44)
miR92a-3p	Up	KLF2	Decreased induction	Treg	Human/mouse	(45)
miR95	Up	unknown	Increased Foxp3 abundance	Treg	Human	(40)
miR98	Up	Trail, Fas	Proliferation	CD8+ T cells	Human	(46)
miR99a	Up	mTor	Increased induction	Treg	Mouse	(47)
miR100	Up	SMAD2/3	Decreased induction	Treg	Human	(48)
miR101	Up	Ezh2	Autoimmune activation	naive CD4+ T cells	Human/mouse	(49, 50)
miR125a-5p	Down	CXCL13	Decreased frequencies	Treg	Human	(51)
miR125-5p	Up	CCR2	Impaired migration	Treg	Human	(52)
miR126	Down	p85B	Decreased induction	Treg	Human/mouse	(53)
miR142-3p	Up	Tet2	Decreased induction and stability	Treg	Human/mouse	(54)
miR142-3p	Up	Ccl2, Ccl17, Cxcl10	Immune infiltration, beta cell death	beta cells	Human/mouse	(55)
miR142-5p	Up	Ccl2, Ccl17, Cxcl10	Immune infiltration, beta cell death	beta cells	Human/mouse	(55)
miR146a	Up	insulin, proinsulin	Reduction of insulin and proinsulin	beta cells	Human/mouse	(43, 44)
miR146a	Up	anti-apoptotic genes	Apoptosis	beta cells	Human/mouse	(43, 44)
miR146a	Down	–	Correlation with GAD and IA2 antibody levels	PBMCs	Human	(56)
miR146a	Down	STAT1	Decreased suppression	Treg	Human	(57)
miR146a	Down	STAT1	Decreased suppression	Treg	Mouse	(58)
miR146b	Up	TRAF6	Decreased suppression	Treg	Human	(59)
miR149-5p	Down	DP5, PUMA	Apoptosis	beta cells	Human	(39)
miR150	Up	mTor	Increased induction	Treg	Mouse	(47)
miR155	Up	Ccl2, Ccl17, Cxcl10	Immune infiltration, beta cell death	beta cells	Human/mouse	(55)
miR181a-5p	Up	PI3K signaling	Decreased induction	Treg	Human/mouse	(60)
miR182	Up	Foxo1	Decreased frequencies	Treg	Mouse	(61)
miR200a	Up	unknown	Decreased frequencies	Treg	Human	(62)
miR202-3p	Up	Cd247, Ccr7	Immune infiltration	autoreactive T cells	Mouse	(46)
miR210	Down	Foxp3	Increased Foxp3 abundance	Treg	Human	(40)

(Continued)

TABLE 1 | Continued

miRNA	miRNA regulation	miRNA target	Effect	Cell type	Organism	Reference
miR210	Up	Foxp3	Decreased frequencies	Treg	Human	(63)
miR214	Up	PTEN	Increased frequencies	Treg	Mouse	(64)
miR326	Up	Ets-1	Decreased frequencies	Treg	Human	(65)
miR326	Up	–	Correlation with GAD and IA2 antibody levels	PBMCs	Human	(66)
miR425-5p	Up	–	Correlation islet autoimmunity, progression to T1D	serum	Human	(36)
miR590-5p	Up	Trail, Fas	Proliferation	CD8+ T cells	Human	(37)
miR663	Up	TGFB1	Decreased frequencies	Treg	Human/ mouse	(67)

combining the levels of osteoprotegerin and the miR23~27~24 cluster in plasma of newly diagnosed T1D patients enabled the prediction of insulin secretion 12 months after diagnosis (86). Another study analyzed plasma miRNAs, immune cell subsets, and specific features of T1D and revealed correlations between miRNAs and T1D onset (let7c-5p, let7d-5p, let7f-5p, let7i-5p, miR146a-5p, miR423-3p, miR423-5p), C-peptide levels in the serum (miR142-5p, miR29c-3p), glycated hemoglobin (miR26a-5p, miR223-3p), and ketoacidosis (miR29c-3p). Furthermore, the analysis pointed towards a link between plasma miRNAs and certain immune cell subsets, which was limited to T1D patients (87). In diabetic NOD mice, miR409-3p was reduced in the plasma as well as in islet infiltrates, and miR409-3p levels were associated with insulitis severity. In human patients with recent onset of T1D, plasma levels of miR409-3p were comparably reduced and correlated inversely with the levels of HbA1c (88). A recent work systematically reviewed and analyzed profiles of circulating miRNAs in T1D patients and suggested a combination of 11 miRNAs (miR21-5p, miR24-3p, miR100-5p, miR146a-5p, miR148a-3p, miR150-5p, miR181a-5p, miR210-5p, miR342-3p, miR375, miR1275), which are involved in several facets of immune regulation and beta cell function, as biomarkers for T1D (89).

miRNAs in Blood-Circulating Cells

In peripheral blood mononuclear cells (PBMCs), several miRNAs were found to be correlated with T1D. miR21a and miR93, two miRNAs involved in the regulation of apoptosis and inflammation by targeting NF-κB signaling, were significantly downregulated in patients with recently diagnosed T1D (85), while other miRNAs such as miR20a and miR326 were upregulated (90). The analysis of miRNA signatures in PBMCs also revealed associations with autoantibodies in T1D patients, with increased levels of miR326 correlating with GAD and IA2 antibodies (66) and reduced levels of miR146a correlating with antibodies against GAD (56).

miRNAs as Biomarkers During Islet Autoimmunity

So far, most studies analyzed miRNA signatures in individuals with established T1D. However, the relevance of islet autoimmunity for the understanding of T1D pathogenesis as well as for the development of intervention strategies suggests the analysis of miRNA expression in this critical presymptomatic phase. The analysis of miRNA profiles in the presymptomatic phase of T1D was conducted in a recent study that analyzed miRNA signatures in the serum of individuals with a high genetic risk for developing T1D and ongoing islet autoimmunity, as indicated by the presence of multiple islet autoantibodies (91). However, the resulting miRNA expression patterns could not distinguish the islet autoimmunity group from individuals with newly diagnosed T1D or healthy individuals and was unable to predict the progression to clinical T1D. Despite these limitations, a set of differentially expressed miRNAs exhibited significant correlations with glycemic status and antibody titers in individuals with islet autoimmunity. Another study investigated signatures of circulating miRNAs in the serum of autoantibody-positive children vs. their autoantibody-negative siblings. In this dataset, several miRNAs, in particular miR21-3p, miR29a-3p, and miR424-5p, correlated with islet autoimmunity and the progression to T1D (36). Despite these important insights, additional evidence is needed to support the concept that circulating miRNAs are a valuable tool for human T1D risk assessment.

Limitations and Next Steps

Whole blood or serum samples of T1D patients are readily available, but the potential of miRNA profiles in such samples to reveal the underlying mechanisms of pathogenesis and progression of organ-specific autoimmune diseases is limited by several aspects. Firstly, profiles of circulating miRNAs most likely do not accurately reflect the environment in the organ, which is the target of the autoimmune attack. Secondly, whole blood and PBMCs represent a highly diverse mixture of various immune cell types. It appears likely that changes in the composition of these immune cell subsets or their miRNA expression have a more profound impact on miRNA profiles than global changes in miRNA expression in the blood or serum. Therefore, the analysis of relevant miRNAs in distinct immune cell subsets, with a particular focus on mediators of autoimmunity, such as effector T cells or Tregs, will provide critical advantages in the analysis of mechanisms underlying T1D pathogenesis. Furthermore, the dissection of miRNA signatures in relevant immune cell subsets, directly in the affected organs, appears to be crucial for the validation of miRNA biomarker signatures with the goal to better understand their contribution to immune activation and the progression to clinical T1D. However, the analysis of miRNA profiles in the respective target organ is often impeded by the limited sample availability, especially during the important pre-

symptomatic phase. Furthermore, the affected organs generally contain only very low numbers of the relevant immune cells additionally hindering the broad applicability of these in principle promising approaches.

miRNAs Involved in Immune-Mediated Beta Cell Destruction

To understand the specific contribution of miRNAs to the onset of islet autoimmunity and the progression to T1D, it is crucial to shift the focus from miRNAs as biomarkers to a more mechanistic dissection of their role for the upstream regulation of autoimmune activation and tolerance impairments.

In addition to the broad regulatory impact of miRNAs in various T cell subsets, modulating immune activation and impaired tolerance, their potential to directly drive the destruction of the pancreatic beta cells has been suggested as an additional layer of regulation. In T1D, the beta cells respond to the inflammatory milieu created by immune cell invasion with the activation of several pathways, which can intensify the immune reaction by inducing beta cell dysfunction, apoptosis, and the secretion of proinflammatory cytokines, attracting more immune cells into the islets (92). Several recent studies suggest that these responses are among others mediated by miRNAs (93).

Cytokines that are typically secreted by pancreas infiltrating immune cells directly modulated miRNA expression in a murine beta cell line (94). Beta cells were exposed to IL-1β, TNF-α, IFN-γ, or a combination of these cytokines for 24 h, and the assessment of miRNA expression showed increased expression of miR21, miR34a, and miR146a. Interestingly, a similar miRNA expression pattern was observed in pancreatic beta cells of NOD mice with considerable immune cell infiltration, while these miRNAs were not upregulated in beta cells of mice that did not show any infiltration in the pancreas (43). This indicates that the expression of these three miRNAs is indeed modulated by cytokines released by infiltrating immune cells. Furthermore, the exposure of cultured human islets to IL-1β resulted a comparable increase in expression of miR21, miR34a, and miR146a. The analysis of gene expression revealed that this short-term exposure to cytokines resulted in the miRNA-mediated reduction of insulin and proinsulin mRNA, which is also seen in T1D. In contrast, the sustained exposure to a cytokine-induced inflammatory environment induces apoptosis in human and murine beta cells (44). miR34a and miR146a could be directly linked to cytokine-mediated cell death, while the role of miR21 remains controversial. Inhibition of miR34a and miR146a resulted in higher survival rates in murine beta cell cultures exposed to proinflammatory cytokines, while the inhibition of miR21 had the opposite effect. The dissection of this effect demonstrated that during the exposure to inflammatory cytokines, miR21 triggers a protective response, which is mediated by the downregulation of cell death inducer PDCD4 (95). In addition, the exposure to IL-1β and IFN-γ resulted in differential regulation of 57 miRNAs in cultured human islets. The reduced expression of three miRNAs—miR23a-3p, miR23b-3p, and miR149-5p—upregulated the pro-apoptotic Bcl-2 family members DP5 and PUMA and consequently promoted apoptosis of beta cells (39).

Another study demonstrated a gradual upregulation of the miR29 family during the course of insulitis in NOD mice (96), and this effect was also observed in cultured murine and human islets when exposed to proinflammatory cytokines (43). This upregulation resulted in reduced insulin mRNA levels, impaired insulin secretion, and induced beta cell apoptosis by targeting Mcl1, which is an anti-apoptotic protein and a confirmed target of miR29.

Another mechanism of miRNA-mediated promotion of beta cell death and its contribution to T1D have been described recently. Both murine and human T cells release exosomes containing miR142-3p, miR142-5p, and miR155-5p, which can be transferred to beta cells and induce their apoptosis. The inhibition of these miRNAs in beta cells prevented apoptosis and protected NOD mice from diabetes development accompanied by higher insulin levels, lower insulitis scores, and reduced inflammation in these mice. Mechanistically, the exosomal miRNAs increase the expression of several chemokine genes, including Ccl2, Ccl7, and Cxcl10 in beta cells, promoting immune infiltration and beta cell death (55).

In sum, these studies indicate that miRNAs are important mediators of cytokine-induced beta cell destruction and dysfunction by modulating several different pathways in response to cytokine exposure and are in line with the concept that they can function as communicators between cells of the immune system and pancreatic beta cells.

miRNA Regulation in T Cells

T Cell Development

Dynamic changes of miRNA expression in hematopoietic precursors indicate their importance for the development and differentiation of various subsets of hematopoietic cells, including T cells. Multiple miRNAs can be linked to T cell differentiation, e.g., miR125b whose upregulation correlates with the specification of progenitor cells into the lymphocyte lineage and in later stages contributes to survival and maintenance of these cells in mice (97). Furthermore, miR181a upregulation was shown to be crucial for the development of both murine T and B cells (98, 99).

The involvement of miRNAs in the development of T cells was initially highlighted in mice by the deletion of Dicer, which resulted in impaired CD8 T cell development in the thymus (100, 101). Furthermore, dynamic miRNA profiles could be linked to distinct stages of murine T cell differentiation, suggesting miRNA regulation of thymic T cell development (102). One example is miR181a, which is highly abundant during the $CD4^+CD8^+$ double-positive stage of T cell differentiation. miR181a regulates TCR signaling by increasing the sensitivity to antigenic stimulation (103), and it targets, among others, Bcl2 and CD69, which are involved in positive selection in the thymus, highlighting the critical regulatory role of this miRNA for T cell development.

T Cell Function

The activation and proliferation of T cells in response to antigen exposure is a crucial facet of the immune system, which depends on signaling via the TCR and co-stimulatory molecules such as

CD28. These signals lead to an upregulation of miR214, which targets PTEN, a negative regulator of T cell activation, resulting in enhanced T cell proliferation in mice (104). Similarly, IL2 signaling upregulates miR182, which, in turn, downregulates Foxo1 in human and murine activated T cells thereby promoting their clonal expansion (105). In contrast, high levels of miR155 and miR221 collectively downregulate PIK3R1, which inhibits human $CD4^+$ T cell proliferation and cytokine production (106).

The transcription factor c-Myc, an important regulator of T cell proliferation and apoptosis, executes its function by modulating the miR17~92 cluster in humans and mice (107). C-Myc binds the miR17~92 locus and induces its expression. Two members of this cluster, miR17-5p and miR20a, regulate the expression E2F1 and thereby facilitate precise control of T cell proliferation. In murine Th1 cells, members of the miR17~92 cluster drive the immune response of these cells by targeting PTEN and CREB1, enhancing proliferation and cytokine production, while inhibiting apoptosis (33).

While the miR17~92 cluster is important for the fine-tuning of T cell activation and proliferation, it can also have deleterious consequences when expressed at very high levels. In mice, the high abundance of this cluster is associated with lymphoproliferative disease and autoimmunity, presumably by inhibiting PTEN and the proapoptotic protein Bim (108). Furthermore, by targeting members of important apoptosis pathways, including Bcl2 and Akt/p53, miRNAs can directly regulate T cell apoptosis in the context of human autoimmune diseases and cancer (109, 110).

Peripheral T Cell Subsets

Besides their importance for T cell development in the thymus and T cell function, miRNAs are furthermore involved in the differentiation of distinct T cell subsets.

Naive, effector, and memory $CD8^+$ T cells exhibit distinct miRNA signatures, both during and after their differentiation. In murine effector T cells, let7f, miR15b, miR142-3p, miR142-5p, miR150, and miR16 are expressed at low levels when compared to the other subsets, while miR21 is upregulated (99). *In vitro* differentiation experiments showed that miR150, miR155, and miRNAs of the let7 family are involved in memory T cell differentiation in mice, among others by targeting KCNIP1 (111). Similarly, human T cell subsets exhibit distinct miRNA patterns. For example, the naive state of human $CD4^+$ T cells is maintained by miR125b, which regulates the expression of genes involved in the differentiation to effector cells, such as IFNG, IL2RB, IL10RA, and PRDM1 (112).

Regarding the differentiation into Th subsets, Dicer deficiency in murine $CD4^+$ T cells results in a strong bias toward Th1 differentiation, the inability to develop into the Th2 phenotype, and increased IFN-γ production (100). In contrast, miR155-deficient mice exhibit a $CD4^+$ T cell compartment, which is strongly shifted towards the Th2 phenotype, including a high abundance of Th2 cytokines, while the function of Th1 cells is altered (113).

Especially the regulation of Th17 cells in autoimmunity has been extensively studied. To date, more than 30 miRNAs have been identified as contributors to function and plasticity of these cells and the Th17/Treg balance in human and murine autoimmunity (114).

Autoreactive T Cells

As described above, autoreactive $CD4^+$ and $CD8^+$ T cells are major drivers of immune infiltration and beta cell damage.

A recent study investigated alterations in T cell gene expression during the development of T1D in NOD mice and revealed the upregulation of several genes involved in autoreactivity, inducing the infiltration of the pancreas by autoreactive T cell clones. Furthermore, the analysis of miRNA signatures in these cells demonstrated that these changes in gene expression are mediated by differential regulation of miRNAs. For example, miR202-3p targets the Ccr7 chemokine receptor and Cd247, which have been shown to control autoimmunity in NOD mice (46).

Furthermore, a set of miRNAs (miR23b, miR98, and miR590-5p) drives the proliferation of diabetogenic $CD8^+$ T cells in T1D patients by downregulating apoptotic genes such as Trail, Trail-R2, Fas, and Faslg (37). Importantly, the forced expression of this set of miRNAs in T cells induced rapid expansion of diabetogenic T cells, indicating that the observed effect is indeed miRNA-mediated.

In islet autoimmunity and T1D naive $CD4^+$ T cells can exhibit dysregulated miRNA signatures, which can alter T cell function and bias them toward autoimmune activation. For example, miR101 is upregulated in naive T cells during islet autoimmunity. This miRNA targets Ezh2, leading to a shift toward the Th1 lineage in humans and mice (49, 50).

TREGS AND THEIR REGULATION BY miRNAs

Characterization of Tregs

Although the destruction of the beta cells is mediated by immune cells, in particular T cells, the contribution of T cells to the development of islet autoimmunity is not limited to these autoreactive processes. One major driver of the activation of autoreactive T cells and their invasion into the pancreas are impaired tolerance mechanisms in the periphery. These impairments are particularly the result of aberrations in Tregs, which can directly suppress various immune cells, including autoreactive T cells, making them the main mediators of peripheral immune tolerance (115).

Tregs express the surface markers CD4 and CD25, which is the interleukin 2 receptor α chain. However, since effector T cells also express these surface proteins, the transcription factor Foxp3 is of particular importance for their identification. The high expression of this lineage defining factor is indispensable for differentiation, maintenance, and function of Tregs. (115, 116). Mutations in the Foxp3 gene lead to fatal autoimmune disorders in both humans (IPEX—immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) and mice (scurfy mice), highlighting the critical role of Foxp3 for

Tregs and consequently immune homeostasis (117, 118). Similarly, the depletion of Tregs in newborn mice results in impaired immune regulation and severe autoimmune disease (119, 120).

Since its discovery, the major impact of Foxp3 on Tregs has been studied extensively, revealing a complex network of regulatory interactions termed the Foxp3 interactome (121). Specifically, these studies identified the structure of Foxp3 including its functional domains (122–124) and described multiple target genes (125–127) and interactions partners of Foxp3 (128–132). In addition, several studies demonstrated regulatory regions in the Foxp3 promoter and other noncoding sequences of the Foxp3 gene. These regions control the expression of Foxp3 and are regulated by various mechanisms including epigenetic modifications (133, 134) and transcription factor binding (135, 136).

Tregs in Islet Autoimmunity and T1D

Importantly, several studies analyzing longitudinal samples of children at different stages of T1D showed that these impairments occur during the phase of islet autoimmunity, before the onset of clinical symptoms, indicating that they are critical triggers of autoimmune activation rather than a consequence. Long-term autoimmunity without progressing to symptomatic T1D is associated with increased frequencies of insulin-specific Tregs (45, 137). Furthermore, *in vitro* Treg induction potential of naive T cells from individuals with islet autoimmunity is reduced both at the insulin-specific and polyclonal level (60). These findings illustrate the crucial role of Tregs for the maintenance of immune homeostasis and the potential of boosting Tregs to interfere with autoimmune progression.

The complex regulatory network in Tregs, which is cardinal for immune tolerance, consists of various components, which could be regulated by miRNAs. In fact, several studies have shown miRNA-mediated regulation of Tregs in different stetting, including autoimmunity.

miRNA Regulation of Tregs

The major impact of miRNAs on Tregs and consequently immune tolerance was indicated by studying the lineage-specific deletion of Dicer and Drosha. The deficiency of these enzymes involved in miRNA processing leads to reduced numbers of thymic and peripheral Tregs and reduced suppressive function resulting in fatal systemic autoimmunity in mice (101, 138–140). Additional studies identified individual miRNAs, which contribute to these defects, by affecting virtually all aspects of Treg biology, including their development, induction, stability, suppressive function, as well as the expression of critical genes.

In T1D patients, high levels of miR125a-5p were found in Tregs isolated from pancreatic lymph nodes, resulting in decreased expression of CCR2 and consequently impaired Treg migration to the pancreas (52). Furthermore, miR510 was shown to be upregulated in Tregs from individuals with T1D, while the levels of miR342 and miR191 were reduced (141). In mice, miR26a levels were reduced in Tregs during T1D progression and the forced expression of this miRNA promoted Treg expansion and suppressed T1D in NOD mice (142).

In line with the complex regulatory network of Foxp3, there are miRNAs, which are involved in Foxp3-mediated regulatory loops. For example, miR155, which is highly expressed in Tregs, is induced by Foxp3 and suppresses signaling pathways, which would interfere with Treg homeostasis. The ablation of this miRNA in mice leads to impairments in Treg development, including decreased levels of Foxp3, which result in reduced frequencies of thymic and splenic Tregs (143, 144). During Treg development in mice, miR155 downregulates SOCS1 (suppressor of cytokine signaling 1) consequently promoting the activity of STAT5, which is critically involved in IL2 signaling and consequently Treg homeostasis (145).

However, the absence of miR155 does not affect the *in vitro* induction or suppressive function of Tregs, as indicated by the ability of miR155-deficient Tregs to prevent autoimmunity in murine transfer models (143).

Retinoic acid promotes the development of Tregs through several mechanisms, some of which are mediated by miRNAs. For example, miR10a-5p, whose expression is restricted to Tregs, is induced by retinoic acid. In mice, miR10a-5p downregulates several effector T cell genes, such as BCL6 and NCOR2, which promotes the expression of Treg-specific genes (29, 146). Although high levels of miR10a-5p correlate with reduced susceptibility to autoimmune diseases in mice, miR10a-5p deficiency does not lead to autoimmunity, suggesting a compensatory effect of other miRNAs with overlapping targets (29, 146).

The importance of miRNAs for efficient Treg induction *in vitro* was shown by impaired Foxp3 expression in murine Tregs induced from Dicer or Drosha deficient naive T cells (101, 138). The investigation of individual miRNAs revealed both inhibitory and promoting effects on *in vitro* Treg induction, and several miRNAs form complex regulatory networks to regulate human and murine Treg induction (47). For example, miR150 and miR99a cooperatively promote Treg induction by targeting mTOR (47), and likewise, miR15a-16 improves Treg induction only in presence of 15b-16 and vice versa (31). In line with the important role of the PI3K/Akt/mTOR signaling for regulating Treg induction vs. T cell activation, this pathway is regulated by several miRNAs, such as miR126 (53). This miRNA contributes to efficient Treg induction in humans and mice by downregulating p85β, which is a regulatory subunit of PI3K, reducing PI3K/Akt/mTOR pathway activity. Accordingly, the inhibition of miR126 impairs Treg induction by increasing PI3K/Akt/mTOR signaling (53). As described above, miR155 targets SOCS1 to promote thymic Treg development. The same mechanisms, promoting STAT5 activity, also support efficient murine Treg induction *in vitro* (147). Besides these miRNAs promoting Treg induction, there are also miRNAs with a negative effect on Treg induction *in vitro* (47). In mice, high levels of two miRNAs of the miR17~92 cluster, miR17 and miR19, interfere with efficient Treg induction while thymic Treg development is unaffected (33). The effect of miR17 is mediated by the targeting of two proteins, which are important

for efficient Treg induction: the cAMP-responsive element binding protein 1 (CREB1) and the TGFβ-receptor II. The latter pathway is additionally regulated by the miR23-miR27-miR24 family, which downregulates important components of TGF signaling in mice and consequently impairs Treg induction (38).

IN-DEPTH DISSECTION OF miRNA MEDIATED TOLERANCE IMPAIRMENTS

As indicated above, several recent studies have investigated the role of specific miRNA in various immune cell subsets, which has significantly driven our understanding of the importance of miRNA-mediated regulation of immune cell differentiation and function as well as immune homeostasis. However, aiming at the ultimate goal of developing future intervention strategies to interfere with aberrant immune activation and delay or even prevent T1D autoimmunity, it is of major importance to understand the underlying mechanisms in more detail. Therefore, the dissection of pathways interfering with immune tolerance and triggering the onset of islet autoimmunity is an important next step following the identification of miRNAs differentially expressed in islet autoimmunity. On this account and given the importance of Treg impairments for insufficient tolerance induction and autoimmune activation, our laboratory has recently investigated the association of individual miRNAs in T cells during the onset of islet autoimmunity and Treg impairments. In three studies, we reported a direct link between dysregulated miRNA expression and defects in Tregs (**Figure 1**).

miR92a-3p

The first study investigated the role of miR92a-3p in humans and mice, revealing that the T cell-specific increased expression of this miRNA during islet autoimmunity favors the development of T follicular helper (TFH) cell precursors and simultaneously impairs the efficient Treg induction, two mechanisms that can critically contribute to the onset and progression of islet autoimmunity (**Figure 1**) (45).

TFH cells are a subset of $CD4^+$ T cells, and their ability to provide support to B cells for the production of high-affinity antibodies makes them an essential part of the humoral immune response (148). TFH cell precursors circulate in the blood where they mediate the induction of antibody responses, suggesting that these cells are an important effector T cell subset involved in the development of autoimmune diseases, which can be mediated by autoantibodies (149, 150). This provided the rationale for the analysis of the role of these cells for the development and progression of islet autoimmunity, which showed increased levels of insulin-specific and polyclonal TFH precursor cells during islet autoimmunity (45). The analysis of differential miRNA expression identified miR92a-3p to be upregulated in T cells from children with recent onset of islet autoimmunity compared to T cells from children with long-term autoimmunity or healthy controls. Furthermore, miR92a-3p expression in T cells directly correlated with the TFH precursor abundance in the peripheral blood. During TFH induction *in vitro*, the specific inhibition of miR92a-3p resulted in decreased TFH cell induction, while a miR92a-3p mimic had the opposite effect. The analysis of known targets of miR92a-3p revealed that high levels of the miRNA resulted in the reduced expression of several negative regulators of T cell activation, including PTEN, PHLPP2, FOXO1, and CTLA4.

Besides the effect on TFH cells, the modulation of miR92a-3p also limits efficient Treg induction by downregulating PTEN and consequently activating the PI3K pathway. These findings indicate that miR92a-3p functions as a shared signaling mediator by controlling negative regulators of T cell activation such as PTEN, which is involved in the control of TFH cells as well as Tregs and their induction. Consequently, *in vivo* high levels of miR92a-3p during the onset of islet autoimmunity were accompanied by reduced frequencies of insulin-specific Tregs and *in vitro* a miR92a-3p mimic impaired efficient Treg induction. The effect of the mimic was reduced upon PI3K inhibition and increased upon PTEN blockade, indicating that these two pathways control the regulation of TFH cell induction vs. Treg induction. Furthermore, KLF2 was identified as a previously unknown target of miR92a-3p. KLF2 promotes S1pr1 expression and BLIMP1 upregulation, which consequently inhibits the TFH master regulator BCL6 (151), thus offering a second mode of action of miR92a-3p to regulate TFH differentiation.

miR181a-5p

In a second study, we showed that high levels of a specific miRNA in T cells from individuals with recent onset of islet autoimmunity diminish Treg induction capacity in naive $CD4^+$ T cells (**Figure 1**). The defective Treg induction occurred in individuals with recent onset but not with long-term autoimmunity without progression to clinical T1D or in the absence of islet autoimmunity.

The impaired Treg induction was accompanied by excessive T cell activation as indicated by higher frequencies and increased proliferation of $CD4^+Foxp3^{int}$ T cells, which in turn interfered with Treg induction (60).

This enhanced T cell activation could be linked to increased levels of miR181a-5p, which has been identified as a modulator of T cell signaling by fine-tuning the thresholds of antigenic stimulation (152). miRNA modulation *in vitro* demonstrated that high levels of miR181a-5p, induced by a miR181a-5p mimic, resembled Treg impairments during the onset of human and murine islet autoimmunity. Conversely, miR181a-5p inhibition could correct these defects and resulted in higher Treg induction efficacy. The increased expression of miR181a-5p in T cells was accompanied by reduced expression of its direct target PTEN. Low levels of PTEN contribute to excessive T cell activation by enhancing PI3K signaling and increasing abundance and activity of nuclear factor of activated T cells 5 (NFAT5) (153). In addition, high expression of miR181a-5p was accompanied by increased levels of CD28, which is a costimulatory molecule involved in PI3K activation (154), NFAT5 upregulation, and T cell activation. These findings show that miR181a-5p is involved

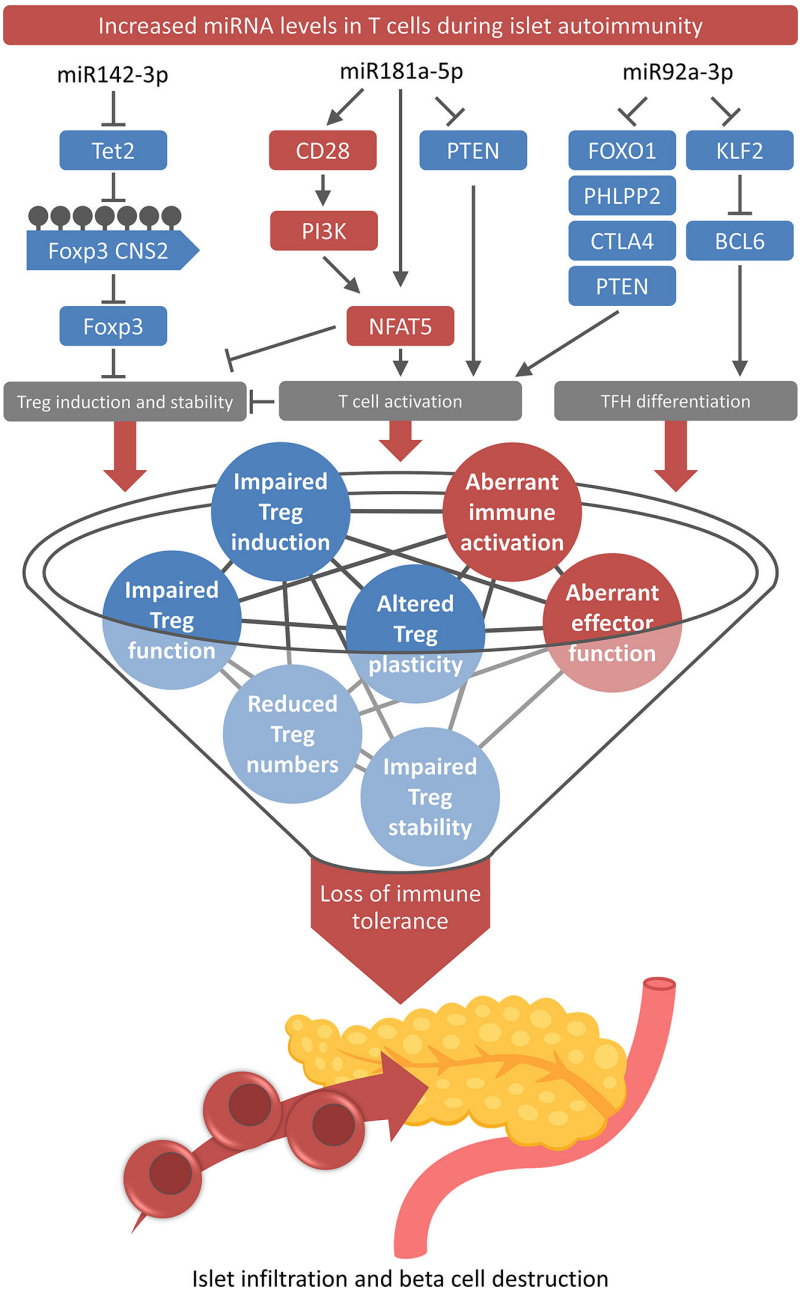

FIGURE 1 | Role of T cell-specific miRNAs for the loss of immune tolerance. High levels of miR-142-3p, miR-181a-5p, and miR-92a-3p and their downstream pathways contribute to multiple layers of tolerance impairments and aberrant immune activation during onset and progression of islet autoimmunity.

in the regulation of T cell activation vs. Treg induction during islet autoimmunity. High levels of the miRNA downregulate PTEN, which results in higher NFAT5 and CD28 expression, consequently favoring T cell activation and interfering with Treg induction.

In line with these findings, miR181a-5p levels were increased in NOD mice with islet autoimmunity, accompanied by elevated NFAT5 and low PTEN levels, resulting in reduced Treg induction capacity of naive $CD4^+$ T cells. The *in vivo* inhibition of miR181a-5p was able to improve islet autoimmunity in these mice, as indicated by reduced levels of pancreas-infiltrating immune cells. This improvement was mediated by increased PTEN levels and decreased expression of NFAT5 and CD28. The important role of NFAT5 was confirmed using NFAT5 deficient mice as well as a NFAT5 inhibitor. Both approaches resulted in improved Treg induction efficiency by upregulating levels of PTEN and of Foxo1, another positive regulator of Treg induction (155).

Given the broad regulatory potential of individual miRNAs, it is most likely that the effect of the miRNAs described above is not limited to islet autoimmunity. In line with this hypothesis, several studies revealed a contribution of miR92a-3p and miR181a-5p to other autoimmune disorders such as neuroinflammation, Th17-mediated inflammation, and lymphoproliferation (32, 108, 156).

miR142-3p

The third study demonstrated that miRNA-mediated dysregulation of DNA methylation drives islet autoimmunity by interfering with Treg homeostasis in humans and mice (**Figure 1**) (54). Using unbiased approaches including miRNA sequencing and high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP), we identified a set of miRNAs that could contribute to the onset of islet autoimmunity. In this study, we investigated the role of miR142-3p as well as its direct targets and downstream pathways. The reported increased expression of miR142-3p during the onset of islet autoimmunity and its high abundance in the RISC complex, suggesting a critical role of this miRNA in the regulation of $CD4^+$ T cells. During early islet autoimmunity, high levels of miR142-3p reduced the expression of an important mediator of DNA demethylation: the methylcytosine dioxygenase Tet2, which was confirmed as a direct target of miR142-3p using a combination of molecular and cellular approaches.

Tet2 belongs to the family of ten-eleven translocation (Tet) methylcytosine dioxygenases that are involved in the regulation of various cellular processes, including the differentiation of $CD4^+$ T cells in humans and mice. Tet2 catalyzes the conversion of methylcytosine in the DNA to 5-hydroxymethylcytosine, which is the first intermediate step of DNA demethylation. The demethylation of regulatory regions of the genome facilitates increased binding of transcription factors and consequently the regulation of gene expression. In Tregs, the stable expression of Foxp3 is maintained by the demethylated state of the conserved non-coding sequence 2 (CNS2) within the Foxp3 gene (157–159). As a direct mediator of DNA demethylation, Tet2 is of critical importance for sustained Foxp3 expression and consequently the Treg phenotype. The miR142-3p mediated silencing of Tet2 resulted in higher levels of DNA methylation at the Foxp3 CNS2, which was accompanied by decreased abundance of pancreatic Tregs in islet autoantibody positive NOD mice.

Importantly, the inhibition of miR142-3p *in vitro* and *in vivo* was able to correct the defects resulting from elevated levels of miR142-3p during islet autoimmunity. *In vitro*, miR142-3p inhibition restored Tet2 levels, resulting in improved Treg induction and stability. The application of the miR142-3p inhibitor to islet autoantibody positive NOD mice resulted in increased levels of Tet2, proper DNA demethylation of the Foxp3 CNS2 locus, higher Treg frequencies in the pancreas, and reduced islet autoimmunity. Furthermore, similar patterns were observed in preliminary experiments using humanized mouse models, indicating the relevance of these findings for established human T1D.

In addition to Tet2, the analysis of potential miR142-3p targets revealed several genes that are involved in Treg homeostasis, such as Smad3, TGFβ receptors, and Stat5. TGFβ plays an important role for immune regulation and Treg homeostasis: by phosphorylating Smad proteins, it ensures the expression of Foxp3 (160). Stat5 is induced by IL-2 signaling and is an important regulator of Treg development (161). Like Tet2

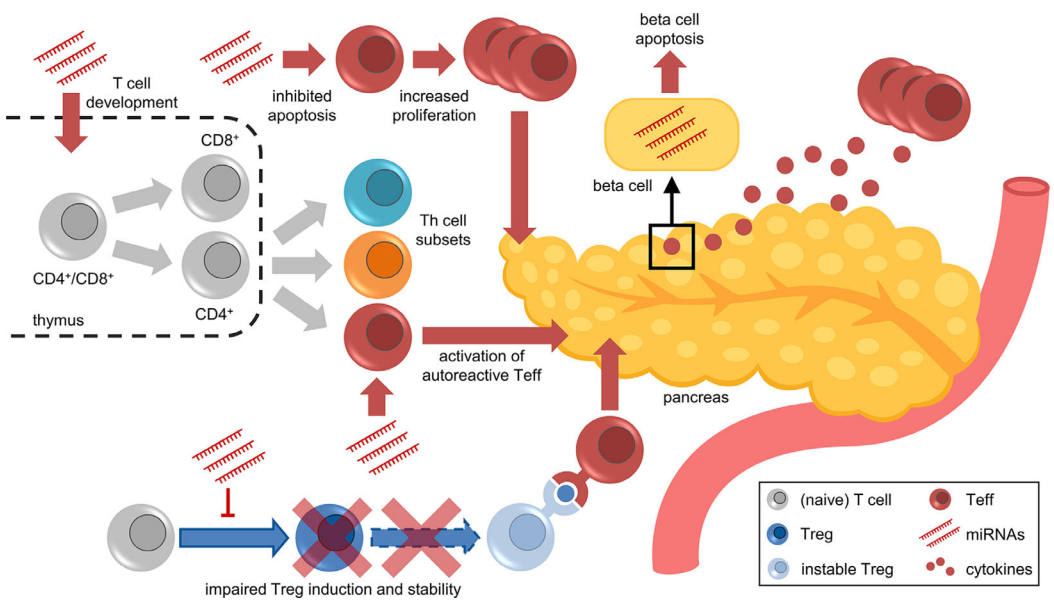

FIGURE 2 | Facets of miRNA-mediated immune regulation in islet autoimmunity and T1D. Aberrant miRNA expression contributes to various aspects of impaired immune regulation, including T cell development in the thymus, differentiation of Th subsets, apoptosis/proliferation of effector T cells, activation of autoreactive T cells, impaired Treg induction and stability, and cytokine-induced beta cell dysfunction and apoptosis.

signaling, both pathways are involved in the maintenance of Treg homeostasis, by regulating Foxp3 induction and stability, indicating that miR142-3p regulates a complex network of important regulators of Treg differentiation and function.

The results of this study highlight that miR142-3p levels are increased during islet autoimmunity, which, via Tet2 downregulation and aberrant CNS2 demethylation, interferes with Treg induction and stability and consequently drives autoimmune activation. Therefore, the modulation of the miR142-3p/Tet2 signaling pathway, by targeted miR142-3p inhibition or enhancing Tet2 abundance or activity, could be a promising strategy in order to improve Treg induction and stability to interfere with the onset of islet autoimmunity.

DISCUSSION

The highly complex pathogenesis of T1D, which is driven by several immune cell types, including T cells, with both effector and regulatory characteristics, hinders the development of efficient prevention and treatment strategies. Most of the molecular mechanisms underlying aberrant T cell activation and the multiple layers of impaired immune tolerance remain largely unexplored, not least because a multitude of mechanisms are involved in the regulation of T cells. A better understanding of the molecular and cellular mechanisms triggering autoimmune activation and promoting the progression to clinical T1D requires deeper insights into the molecular regulation of important mediators of autoimmunity, such as specific T cell subsets. One of these regulatory mechanisms are miRNAs, which are critically involved in the regulation of proper functioning of the immune system, including T cell development, differentiation, and in particular Treg induction and function (**Figure 2**). For this reason, recent studies investigated T cell-specific miRNAs during ongoing islet autoimmunity and how these miRNAs modulate T cell function and consequently contribute to both activation and progression of autoimmunity. These studies demonstrated that the targeting of individual miRNAs can induce relevant changes in expression of their target genes and modulate downstream signaling pathways, resulting in reduced islet autoimmunity in mouse models.

AUTHOR CONTRIBUTIONS

MGS reviewed the literature wrote the manuscript, and prepared the figures. CD reviewed the literature and contributed to the conceptualization of the manuscript. All authors contributed to the article and approved the submitted version.

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Abnormal Neutrophil Transcriptional Signature May Predict Newly Diagnosed Latent Autoimmune Diabetes in Adults of South China

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Objective: Latent autoimmune diabetes in adults (LADA) is an autoimmune diabetes characterized by slowly progressive of β-cell function deterioration. Our previous finding demonstrated that neutrophil numbers and migration abilities display distinct levels in different types of diabetes, including LADA, whereas its pathological alterations in the development of LADA remain unknown. We aimed to investigate the changes in transcriptional levels of peripheral neutrophils in newly diagnosed LADA.

Methods: Peripheral blood neutrophils were isolated from newly diagnosed LADA patients (n = 5) and age-and sex-matched healthy controls (n = 5). The Transcriptomic signature was determined by RNA sequencing (RNA-seq). Differentially expressed genes (DEG) were screened, followed by analyzing downstream Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. Real-time polymerase chain reaction (qPCR) was applied for validation in LADA patients (n = 9) and age-and sex-matched healthy controls (n = 18), including sequencing samples.

Results: Compared with controls, 4105 DEG were screened in LADA patients, including 2661 upregulated and 1444 downregulated DEG. In GO analysis, DEG are mainly involved in leukocyte degranulation, myeloid cell differentiation, and immune response-regulating signaling. The top enriched KEGG pathways included cytokine-cytokine receptor interaction, adhesion molecule signaling, nuclear factor-κB (NF-κB) signaling and Th17 cell differentiation. Consistent with RNA-seq results, *SELL*, *ITGA4*, *ITGAM*, *NCF4*, *ARHGAP3*, and *CLDN15* are upregulated in neutrophils by qPCR.

Conclusion: The present study results provided a profile of DEG in the newly diagnosed LADA of south China. Our study reveals an abnormality in neutrophil disposition at the transcriptional level in LADA. Several essential genes may be involved in of LADA's

pathological process, which may be useful to guide prediction for LADA and further investigation into the pathogenesis for this disease.

Keywords: latent autoimmune diabetes in adults, type 2 diabetes mellitus, neutrophils, RNA-seq, transcriptome, data mining

INTRODUCTION

Diabetes mellitus is a global concern that causes an enormous burden to society and individuals. Latent autoimmune diabetes in adults (LADA) is a form of autoimmune diabetes with an older mean age at onset, slower rate of β-cell loss and longer period of insulin independence after onset when compared with type 1 diabetes (T1DM). In addition, early clinical manifestations overlap with those of type 2 diabetes (T2DM). Unlike T1DM, LADA does not initially require insulin for at least 6 months; however, deterioration of β-cell function in LADA patients is three times faster than that in T2DM (1). Global epidemiological surveys have indicated that LADA accounts for 2%–12% of patients with diabetes mellitus, who have more severe diabetic complications and a worse prognosis (2). Compared to T1DM, less intense autoimmune attack on β-cells and a relatively long window period from onset to β-cell depletion are usually found in LADA. This delayed progression period provides a valuable opportunity for endocrinologists to understand the pathological mechanisms of autoimmune destruction of β-cells.

Neutrophils are the most abundant white blood cells (WBCs) in the circulation (3) and are recruited to inflammatory sites (4) to eliminate extracellular pathogens after activation (5). NETosis, which is characterized by neutrophil degradation and successive release of lytic enzymes and neutrophil extracellular traps (NETs), is a unique process activated by neutrophils (6). However, aberrant activation of neutrophils during autoimmunity may aggravate inflammatory responses and tissue damage. Neutrophils also play a crucial role in several autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and autoimmune diabetes (3).

A longitudinal study from Battaglia's group demonstrated that that neutrophil reduction is greatest in individuals with the highest risk of developing T1D, suggesting a closed correlation between reduced circulating neutrophils and destructive β-cell-specific autoimmunity in T1DM (7). Our group also showed a decrease in circulating neutrophil counts in patients with T1DM but not in those with T2DM (6), and additionally the circulating neutrophil counts in LADA is lower than those in T2DM and higher than those in T1DM (8). Moreover, levels of circulating protein and the activity of neutrophil serine protease 3 (PR3) and neutrophil elastase (NE) stored in primary neutrophil granules are significantly increased in T1DM (6) and LADA (9), and increased circulating levels of NE and PR3 exhibit a progressively positive correlation with the positive numbers and titres of islet autoantibodies (6). Recently, Battaglia et al. revealed an unexpected abnormal neutrophil signature both in the circulation and in the pancreas of presymptomatic and symptomatic T1DM subjects, implying that neutrophils might be involved in the pathogenesis of T1DM (10). However, the transcriptional profiling of neutrophils in LADA patients remains unclarified. In this study, we aimed to explore changes in peripheral neutrophils in the pathogenesis of LADA at the transcriptional level by comparing patients newly diagnosed LADA with healthy controls to provide a scientific basis for the screening of potential intervention targets.

MATERIALS AND METHODS

Protocol

The study protocol, conforming to the Declaration of Helsinki (as revised in Seoul, South Korea, 2008), was reviewed and approved by the Human Ethics Committee of The Second Xiangya Hospital of Central South University (approval number 2019-Research-40). Bioinformatic data are acquired from public databases online.

Subjects

Nine LADA patients diagnosed within one year were enrolled from the Second Hospital of Central South University, Changsha, China, and LADA were diagnosed according to the Chinese Diabetes Society (CDS) Consensus on diagnosis and treatment of LADA in 2012 (11): 1) diabetes diagnosed according to the 1999 World Health Organization (WHO) criteria for diabetes (12); 2) age at onset of diabetes of >18 years; 3) with one or more positive autoantibodies against β cell antigens including glutamic acid decarboxylase (GAD), insulinoma-associated protein 2 (IA2), or zinc transporter-8 (ZnT8); 4) insulin independence within the first six months after diagnosis. We recruited eighteen age-and sex-matched healthy controls who showed euglycemia in a standardized 75g oral glucose tolerance test (OGTT). The exclusion criteria were as follows: 1) acute infection, trauma, or surgery within one month; 2) treatments with glucocorticoids or other immune regulators within one month; 3) severe cardiocerebrovascular, liver, kidney, malignant disease; 4) pregnancy or lactation; 5) with other autoimmune diseases; 6) other types of diabetes (13–17). Five LADA patients and five controls were selected *via* random number table method for RNA-sequencing (RNA-seq), and all subjects were used as validation samples for Real-time polymerase chain reaction (qPCR).

Measurements

Height, weight, blood pressure, waist circumference, hip circumference, body mass index (BMI), and weight/height ratio (WHR) were calculated for all participants. Fasting venous blood samples were collected at 8:00 am. Biomedical measurements were tested for serum or plasma separation upon blood collection. Fasting blood glucose (FBG), total cholesterol (TC), and triglyceride (TG) were measured by a Hitachi 7170

analyzer (Boehringer Mannheim, Mannheim, Germany). Circulating cell counts were measured by the Sysmex XE-2100 automated hematology analyzer (Sysmex Corporation, Kobe, Japan). Serum C-peptide was measured by the Advia Centaur System (Siemens Corporation, Munich, Germany). Glycosylated hemoglobin (HbA1c) was quantified by liquid chromatography using a Bio-Rad VARIANT II Hemoglobin Testing System (Hercules, CA, USA). Glutamic acid decarboxylase autoantibody (GADA), insulinoma-associated protein 2 autoantibody (IA-2A), and zinc transporter-8 autoantibody (ZnT8A) were detected in duplicate by radio ligand assays as previously described (18, 19).

Neutrophil Isolation and RNA Extraction

According to the manufacturer, human neutrophils were isolated by density gradient centrifugation from the venous blood of both LADA patients and healthy controls using Ficoll-Paque Plus (GE Healthcare, Madison, USA)'s protocol. Further purification was completed by positive magnetic separation using human CD16 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cell pellet was dissolved in TRIzol (Roche, Basel, Switzerland) in 5–10 × 10^6 cells/1 ml and stored at -80°C. Total RNA was extracted, and corresponding concentration and purity were evaluated on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Standard OD260/OD280 value of extracted RNA is 1.8–2.1. The High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) subsequently constructed the cDNA library.

RNA Sequencing

In this study, we sequenced five samples from LADA patients and five samples from healthy controls on the BGISEQ-500 platform, the first desktop high-throughput gene sequencer independently developed by BGI, and applies DNA nanoball technology. A total of 15,255 genes were detected, averaging approximately 24.04 million reads per sample.

Data Cleaning and Bio Information Analysis Obtained From the Public Database

We carried out quality control of the raw data before downstream analysis, and filter out the clean reads through the steps of low quality, adapter pollution and unknow base (N) reads. Then, clean reads were mapped to the reference genome using HISAT (20) and Bowtie2 (21), and the gene expression calculated using a software package called RSEM (University of Wisconsin-Madison, Madison, USA) (22). We identified differentially expressed genes (DEG) between LADA patients and healthy controls by DEG-seq algorithms (23). An adjusted P-value ≤ 0.001 and an absolute value of the $\log_2$ fold change (FC) > 1 were set as the default threshold to judge the significance of gene expression differences. According to DEG, we used Metascape online website to perform GO functional enrichment, including three ontologies: molecular biological function, cellular composition, and biological process (24). Further, we performed the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway functional enrichment, using hyper package in R software. Then we calculated the false discovery rate (FDR) for each P-value, and the terms for which the FDR was not greater than 0.01 were defined as significantly enriched.

Real-Time PCR Analysis

To validate the accuracy of the RNA-seq analysis, the expression of mRNAs was measured by real-time polymerase chain reaction (qPCR) using the SYBR-Green method (Go Taq® qPCR, Promega Corporation, USA) and a MiniOpticon real-time PCR detection system (ViiATM 7 Real-Time PCR System containing the OptiflexTM Optics System). All the primers used for real-time PCR were designed and synthesized by TSINGKE (TSINGKE Biological Technology, China). Real-time PCR reactions were performed under the following conditions: 10 min at 95°C and 40 cycles of the one-step thermal cycling of 15 s at 95°C and 60 s at 60°C in a 384-well reaction plate. The expression of each gene was determined in duplicates based on the basis of the comparative $2^{-\Delta\Delta Ct}$ method. Results were normalized to the expression of reference gene β-actin. Primer sequences of genes were shown in **Table 1**.

Statistical Analysis

All data were analyzed with SPSS version 25 (IBM Corp, Armonk, NY, USA) and GraphPad Prism 5 (Graphpad Corporation, San Diego, CA, USA). Normality was tested using the Kolmogorov-Smirnov test. Data were logarithmically transformed before the Mann-Whitney U test if they were not normally distributed. Student's t-test was applied to identify differences between groups. Univariate general linear model was used to exclude the potential influence of confounding factors, regarded as covariates. Data are expressed as the mean ± SD or median with interquartile range.

RESULTS

The clinical and metabolic characteristics of LADA patients (n=9) and age- and sex-matched control subjects (n=18) are presented in **Tables 2** and **3**. Five patients with LADA and five healthy controls were randomly selected for RNA-seq, and qPCR validation was applied to the whole group. Levels of HbA1c ($P < 0.05$), FBG ($P < 0.01$), and 2-h postprandial blood glucose ($P < 0.05$) were higher in LADA patients than in healthy controls. In the validation group, the 2-h postprandial C-peptide in patients with LADA was lower than that in healthy controls ($P < 0.001$). However, no statistical significance in BMI, systolic blood

TABLE 1 | Primer sequences of forward and reverse primers.

Gene	Sense (5' to 3')	Antisense (3' to 5')
CXCR1	TCAAGTGCCCTCTAGCTGTT	TGATCTAACTGAAGCACCGGC
SELL	TCTGTTGTGATTTCCTGGCAC	CCCACCCACGTCCATATTCC
ITGA4	CGGTGATGCTGTTGCTGTG	CTAGGAGCCATCGGTTCGCC
ITGAM	GGTGGCAGTGTGATGCTGT	CATTTACGTCCCCCAGCACT
NCF4	GGCTGGAGGAAGTGAGAGGT	TGTTCAAAGTCACTCTCGGC
ARHGAP35	TAAACAAGGTCAGCCACAACA	TCAGGTCTCATCAAGGTGGG
CLDN15	TTACTCCGTTCTTTGGCCCC	GCGCGGCAGCCTGGA
β-Actin	GCATCCCCCAAAGTTCACAA	AGGACTGGGCCATTCTCCTT

TABLE 2 | Clinical and biochemical characteristics of the study participants for RNA-seq.

	HC (n=5)	LADA (n=5)	P
Sex (male/female)	5 (4/1)	5 (3/2)	0.490
Age (years)	43.40 ± 13.22	34.4 ± 4.98	0.212
BMI (kg/m²)	23.18 ± 2.21	22.95 ± 2.21	0.872
WHR	0.89 (0.80 ~ 0.91)	0.81 (0.80~0.87)	0.841
DBP (mmHg)	74.40 ± 5.32	71.8 ± 5.12	0.860
SBP (mmHg)	106.20 ± 8.95	107.8 ± 6.94	0.760
TG (mmol/L)	1.03 ± 0.56	1.25 ± 0.68	0.588
TC (mmol/L)	4.22 ± 0.49	3.82 ± 0.56	0.263
HDL-C (mmol/L)	1.47 ± 0.44	1.29 ± 0.31	0.480
LDL-C (mmol/L)	2.34 ± 0.54	2.1 ± 0.6	0.522
HbA1C (%)	5.52 ± 0.46	6.62 ± 0.89*	0.039
Fasting BS (mmol/L)	5.12 ± 0.29	6.27 ± 0.58**	0.004
2h postprandial BS (mmol/L)	4.88 ± 1.64	12.05 ± 4.88*	0.027
Fasting C-peptide (pmol/L)	350.36 ± 90.08	290.34 ± 80.39	0.299
2h postprandial C-peptide (pmol/L) a	1672.8 (1117.7~1885.15)	654.8 (503.1~1033.1)	0.095
White cell count (10^9/L) a	6.34(5.27~7.50)	4.74(4.24~6.19)	0.150
Lymphocyte count (10^9/L) a	1.83(1.38~2.20)	1.44(1.24~1.89)	0.363
Neutrophil count (10^9/L) a	3.45 (3.25~5.40)	2.88(2.47~3.92)	0.190
Mononuclear count (10^9/L) a	0.27(0.22~0.46)	0.25(0.22~0.38)	0.608

*Data are expressed by mean ± SD, or median (25th–75th percentile). LADA, Latent autoimmune diabetes in adults; HC, Healthy controls; BMI, Body mass; WHR, Waist to hip ratio; DBP, diastolic blood pressure; SBP, systolic blood pressure; TG, Triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; a compared by the Mann-Whitney U test. *P < 0.05 compared with HC. **P < 0.01 compared with HC.*

pressure, LDL-C, or TC was found between the LADA and control subjects (P>0.05).

RNA-seq analysis of peripheral blood neutrophils from 5 patients with LADA and 5 healthy controls revealed an average of 24.04 million sequences in each sample, with a reading mapping rate of 93.24% (See **Supplementary Table 1** and **2**). According to the DEG-seq algorithm, among the 15,255 transcripts that could detect differences in expression, 2,661 DEG were upregulated and 1,444 DEG downregulated in patients with LADA compared with healthy controls. The DEG were defined at levels of FC ≥ 2 or fold change ≤ 0.5 and adjusted P-value ≤ 0.001 (**Figures 1**, **2**, and **Supplementary Data 1**).

Based on the aforementioned DEG, GO classification and functional enrichment analyses were performed to determine the molecular functions (MM), cellular components (CC), and biological processes (BP) involving the proteins encoded by these genes. Among the upregulated and downregulated genes, we selected the first 1,000 DEGs as the background. Terms with a p-value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 were collected and grouped into clusters based on similarities. As expected, most identified pathways are important to neutrophil functioning. Among them, the top 10 upregulated biological processes were as follows: 1) leukocyte degranulation (Log P = -43.90); 2) myeloid cell differentiation (Log P = -14.74); 3) immune response-regulating signaling (Log P = -11.80); 4) regulation of cytoskeleton organization (Log P = -11.22); 5) covalent chromatin modification (Log P = -10.72); 6) regulation of innate immune response (Log P = -10.54; 7) dephosphorylation (Log P = -10.20; 8) positive regulation of hydrolase activity (Log P = -10.08); 9) regulation of protein kinase activity and response to peptide (Log P = -9.86); and 10) response to peptide (Log P = -9.656) (**Figure 3A** and **Supplementary Data 2**). The corresponding GO categories of the top 10 upregulated biological functions were 1) cytokine-mediated signaling pathway (Log P = -15.55); 2) response to molecule of bacterial origin (Log P =-15.43); 3) cytokine production (Log P = -15.26); 4) cellular response to lipid (Log P = -13.56); 5) apoptotic signaling pathway (Log P = -11.88); 6) lymphocyte activation (Log P = -10.61); 7) leukocyte

TABLE 3 | Clinical and biochemical characteristics of the study participants for validation.

	HC (n = 18)	LADA (n = 9)	P	HC (N = 5)	T2DM (N = 5)	P
Sex (male/female)	18 (8/10)	9 (5/4)	0.586	5(3/2)	5(3/2)	1.000
Age (years)	33.94 ± 10.34	34.56 ± 6	0.891	32.60 ± 5.94	50.8 ± 8.98**	0.005
BMI (kg/m²)	21.59 ± 1.9	21.87 ± 2.94	0.804	23.26 ± 2.32	23.54 ± 5.61	0.919
WHR	0.83 (0.80~0.89)	0.81 (0.81~0.87)	0.743	0.90 ± 0.05	0.91 ± 0.06	0.849
DBP (mmhg)	72.33 ± 6.91	71 ± 5.57	0.620	77.20 ± 11.78	78.60 ± 14.22	0.870
SBP (mmhg)	108.94 ± 9.53	109 ± 7.53	0.988	123.80 ± 14.87	127.00 ± 28.36	0.829
TG (mmol/l)	0.93 ± 0.41	0.98 ± 0.61	0.804	1.67 ± 1.09	2.61 ± 1.28	0.244
TC (mmol/l)	3.99 ± 0.54	3.84 ± 0.77	0.572	5.41 ± 0.81	5.50 ± 0.71	0.853
HDL-C (mmol/l)	1.41 ± 0.29	1.3 ± 0.26	0.372	1.34 ± 0.26	1.22 ± 0.12	0.351
LDL-C (mmol/l)	2.35 ± 0.86	2.13 ± 0.66	0.513	3.71(2.86~3.89)	3.22(2.64~3.40)	0.151
HbA1c (%)	5.31 ± 0.33	6.67 ± 1.33*	0.015	5.36 ± 0.29	9.34 ± 2.58*	0.026
Fasting bs (mmol/l)	4.7 ± 0.49	5.89 ± 0.89***	<0.001	4.85 ± 0.53	12.79 ± 5.51*	0.032
2h postprandial bs (mmol/l)	5 ± 1.13	10.38 ± 4.38**	0.006	7.59 ± 1.83	16.16 ± 3.14***	<0.001
Fasting c-peptide (pmol/l)	345.98 ± 96.51	275.20 ± 64.06	0.058	1067.60 ± 402.76	599.40 ± 335.93	0.081
2h postprandial c-peptide(pmol/l)	1687.25 ± 657.2	654.80 (590.40~895.15) ***	<0.001	3779.55 ± 1018.38	2217.78 ± 1675.14	0.113
White cell count (10^9/l) a	5.83 (4.90~6.38)	4.68 (4.39~6.52)	0.180	6.42 ± 1.31	5.36 ± 1.14	0.211
Lymphocyte count (10^9/l) a	1.67 (1.21~1.93)	1.43 (1.22~1.87)	0.9112	1.88(1.80~2.65)	1.86(1.44~3.28)	1.000
Neutrophil count (10^9/l) a	3.58 (3.27~4.18)	2.86 (2.61~4.37)	0.090	3.78 ± 1.07	2.80 ± 0.21	0.080
Mononuclear count (10^9/l) a	0.30 (0.25~0.45)	0.25 (0.20~0.33)	0.160	0.37 ± 0.08	0.28 ± 0.08	0.123

*The study participants for validation included the testing samples for RNA-seq. Data are expressed by mean ± SD, or median (25th-75th percentile). LADA, Latent autoimmune diabetes in adults; T2DM, Type 2 diabetes mellitus; HC, Healthy controls; BMI, Body mass; WHR, Waist to hip ratio; DBP, diastolic blood pressure; SBP, systolic blood pressure; TG, Triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; a compared by the Mann-Whitney U test. *P< 0.05 compared with HC. ** P< 0.01 compared with HC. *** P <0.001 compared with HC.*

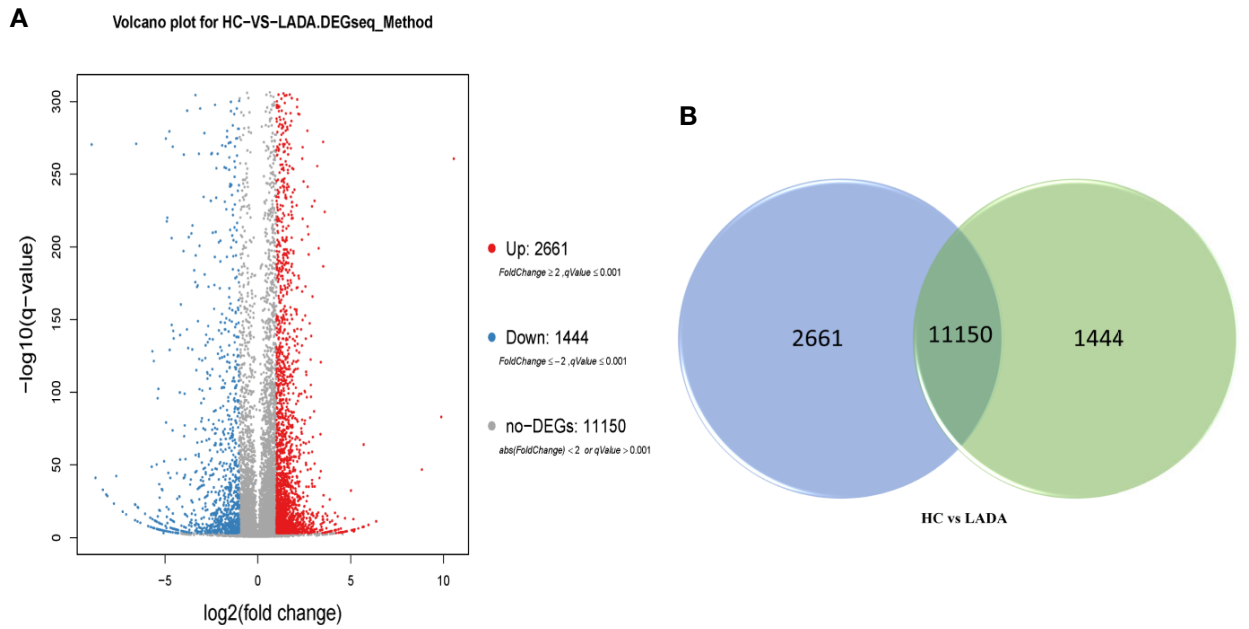

FIGURE 1 | **(A)** Volcano plot of differentially expressed genes (DEG) in peripheral neutrophils obtained from newly diagnosed LADA patients vs. healthy controls. Red points represent up-regulated DEG. Blue points represent down-regulated DEG. Gray points represent non-DEG. A total of 2,661 upregulated DEG and 1,444 downregulated DEG were found. The X-axis represents log2 transformed fold change, and Y-axis represents -log10 transformed significance. The BGISEQ-500 platform was used to compare to the reference sequence to screen the differential genes. **(B)** Veen diagram of differentially expressed genes (DEG). The numbers of DEG in peripheral neutrophil obtained from newly diagnosed LADA patients vs healthy controls. The blue pie represents 2,661 up-regulated DEG. The green pie represents 1,444 down-regulated DEG. The transposition section represents 11150 non-DEG.

differentiation (Log P = -10.39); 8) leukocyte activation involved in immune response (Log P = -9.65); 9) negative regulation of phosphate metabolic process (Log P = -9.62); and 10) positive regulation of organelle organization (Log P = -9.41) (**Figure 3B** and **Supplementary Data 2**). At the same time, we generated a network diagram of GO terms with significant enrichment; the network was visualized using Cytoscape5, where each node represents an enriched term and is coloured first by its term (**Figure 3C**) and then by its P-value (**Figure 3D**).

KEGG pathway enrichment analysis of the DEG suggested that a wide range of biological pathways are altered in neutrophils from LADA patients compared to controls. In KEGG pathway enrichment analysis, a total of 321 pathways were identified based on all detected genetic backgrounds. The top 20 differential pathways are mainly related to the NF-κB signaling pathway ($P = 2.56\times10^{-8}$), Th17 cell differentiation ($P = 1.75\times10^{-4}$), antigen processing and presentation ($P = 3.54\times10^{-4}$), Th1 and Th2 cell differentiation ($P = 7.40\times10^{-4}$), NOD-like receptor signaling pathway ($P = 9.91\times10^{-4}$), Cytokine-cytokine receptor interaction ($P=1.00\times10^{-3}$), TNF signaling pathway ($P = 1.13\times10^{-3}$), and Cell adhesion molecules (CAMs) ($P = 1.96\times10^{-3}$). (**Figure 4A** and **Supplementary Data 3**, Q value in **Supplementary Table 3** and in the text are adjusted P-value). We drew a network map between the top 20 pathways with the highest enrichment degree and the corresponding DEG (**Figures 4B, C**). Among the most enriched pathways mentioned above, especially cytokine-cytokine receptor interactions and CAM signal transduction pathways, most of the genes related to these two pathways were found to be upregulated in LADA, such as chemokine (C-X-C subfamily) ligand (CXCL7) and C-X-C chemokine receptor type (CXCR1), also known as interleukin-8 receptor (IL8RA, IL8RB), cell adhesion molecules L-selectin (*SELL*) and integrin subunit alpha M (*ITGAM*). The paths of cytokine-cytokine receptor interaction, cell adhesion molecules and leukocyte transendothelial migration are illustrated in **Figure 5**.

We next confirmed the differential expression of 7 prominent DEG closely related to neutrophil activation, rolling and migration using qPCR. These verified genes showed the same direction as in the RNA-seq data (**Figure 6A**). The expression levels of adhesion molecules *SELL, ITGAM, ITGA4*, and neutrophil cytoplasmic factor *NCF4, ARHGAP35, CLDN15* were significantly increased in neutrophils from LADA patients compared with those from controls, except for *CXCR1* (**Figure 6B**). We further measured and compared the gene expression of *CXCR1, SELL, ITGA4, ITGAM, NCF4, ARHGAP3*, and *CLDN15* in neutrophils from 5 type 2 diabetes patients within 1 year from diagnosis and 5 age- and sex-matched healthy control subjects. There was no significant difference in gene expression of *CXCR1, ITGA4, ITGAM, ARHGAP3*, and *CLDN15* between the two groups (**Figure 6C**). The relative mRNA expression of above genes was also compared among all healthy controls, LADA and T2DM (**Figure 6D**). To further exclude the potential confounder of hyperglycemia, HbA1c

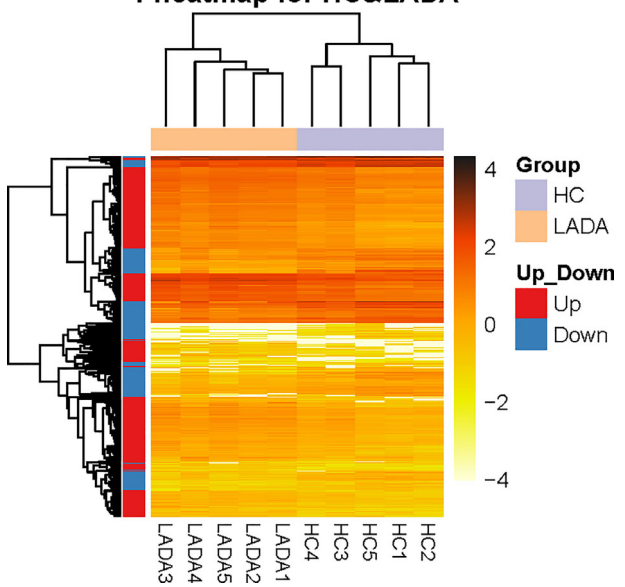

FIGURE 2 | The distinct peripheral neutrophil transcriptome of patients with newly diagnosed latent autoimmune diabetes in adults (LADA) vs healthy controls. Heatmap shows differentially expressed genes (DEG) in neutrophils isolated from healthy controls (purple, n = 5) and patients with newly diagnosed LADA (orange, n = 5). DEGs are defined as levels of fold change ≥ 2 for up-regulated and ≤ 0.5 for down-regulated. The X-axis indicated different LADA patients and healthy controls, and the Y-axis shows DEG. The orange color scale bar represents the log10 transformed gene expression level; dark orange represents high expression level, and light orange represents low expression.

was adjusted by univariate general linear model, and the expression of *SELL, ITGA4, ITGAM, NCF4, ARHGAP3*, and *CLDN15* remained significantly higher in neutrophils from LADA, but not T2DM, compared with healthy controls; and the expression of *SELL, ITGA4, ARHGAP3*, and *CLDN15* remained significantly higher in neutrophils from LADA compared with those from T2DM. Taken together, these data suggest that *SELL, ITGA4, ARHGAP35, CLDN15* may be closely associated with β-cell autoimmunity in patients with LADA, and the increase of other genes in LADA may only reflect the hyperglycemia status.

DISCUSSION

In this study, the transcriptional signature of circulating neutrophils in newly diagnosed LADA patients was found to be different from that of healthy controls without diabetes, suggesting that neutrophils may play a role in the pathogenesis of human autoimmune diabetes. In addition, levels of *SELL, NCF4, ITGAM, ITGA4, ARHGAP35*, and *CLDN* were significantly increased in neutrophils from LADA patients compared to those from healthy controls.

Emerging evidence have demonstrated the role of neutrophils involved in autoimmune diabetes. Battaglia et al. have reported reduction of circulating neutrophil numbers and infiltration of neutrophils in the pancreas before the onset of T1DM, suggesting that pathogenic role of neutrophils in human T1D is crucial for a better understanding of the disease and to open new therapeutic opportunities (7, 25). Our previous findings have demonstrated that in newly diagnosed T1DM patients, a significantly decreased number of circulating neutrophils and increased circulating neutrophil PR3 and NE levels are related to decreased β-cell function and the number and titres of islet autoantibodies (6). The level of circulating PR3 was also significantly increased in patients with LADA, which is considered a milder form of T1DM. In this study, we prove that the transcriptional signatures of circulating neutrophils change significantly in the early stage of LADA.

In the current study, DEG and related biological functions were investigated in peripheral neutrophils of patients with LADA and healthy controls. The results showed that pathways involving migration, adhesion, and degranulation were activated in circulating neutrophils at the transcriptional level in LADA. Increasing evidence has demonstrated that in autoimmune diabetes, the migration of neutrophils from circulation to islets is involved in the early stage of β-cell injury (3). Circulating neutrophil counts have been observed to decrease in presymptomatic autoantibody-positive donors and T1DM patients, and neutrophil infiltration was also found in human islet sections (10). Recruitment of neutrophils also occurred in 3-week-old nonobese diabetic (NOD) mice (26). The first step of local tissue infiltration of neutrophils is passage through vascular endothelial cells. We found that a series of adhesion molecules were upregulated in the neutrophils of LADA patients. Adhesion molecules not only play essential roles in the ability of neutrophils to pass through endothelial cells into local tissue but also participate in regulation of the immune system (3). Our RNA-seq and qPCR results both showed that *SELL*, the gene encoding the adhesion molecule L-selectin, was significantly increased in neutrophils from LADA patients. A previous study found that soluble L-selectin (sL-selectin) was altered in patients with T1DM and preclinical T1DM (27). Subsequent studies have shown that increased expression of sL-selectin may promote the destructive process of islet inflammation during the development of T1DM (28, 29). In addition, elevation of sL-selectin has been related to the positive serum conversion of islet autoantibodies, indicating that the activation of leukocytes is consistent with the occurrence of β-cell autoimmunity (30). Moreover, our results showed that *ITGA4* and *ITGAM*, which encode very late antigen 4 (VLA4) and CD11b, respectively, were significantly upregulated. Antibodies against L-selectin and VLA-4 can delay insulitis by inhibiting leukocyte adhesion to the inflamed vessels within pancreatic sections (31). Neutrophils of T1DM patients showed higher expression of the CD11b receptor than that of healthy controls, independent of diabetes duration and blood glucose level (32). These studies have shown that adhesion molecules, especially L-selectin and VLA-4, play significant roles in the development of T1DM, and the development can be halted by blocking these adhesion pathways. Therefore, further studies are required to elucidate the preventive effects of neutrophil-specific anti-adhesion therapies in the progression of LADA.

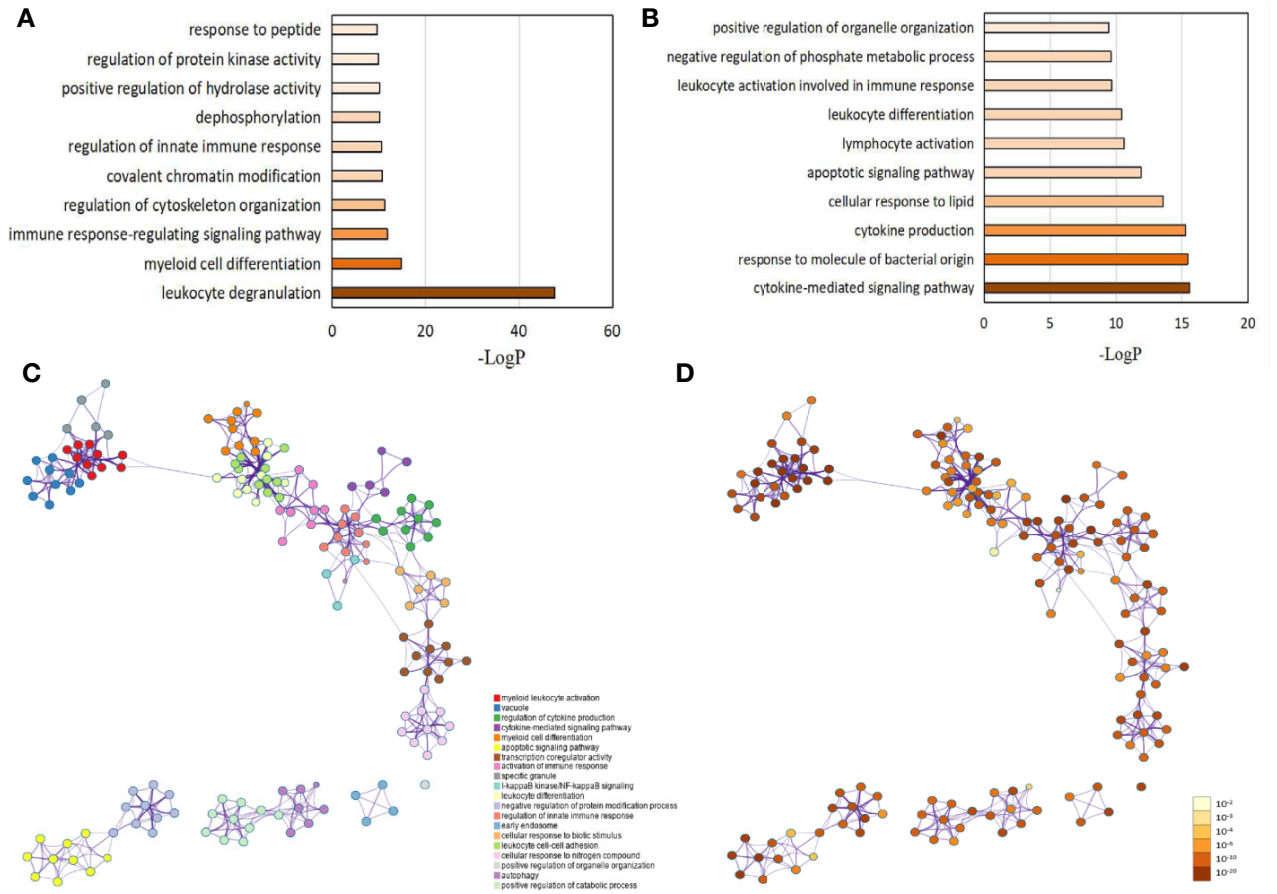

FIGURE 3 | Gene ontology (GO) analysis for differentially expressed genes (DEG) between LADA patients and healthy controls. DEGs are defined as levels of fold change ≥ 2 for upregulated and ≤ 0.5 for downregulated. GO analysis is carried out using more than 40 independent knowledge bases used by the Metascape online website. **(A)** The top 10 GO categories for up-regulated genes. **(B)** The top 10 GO categories for down-regulated genes. The network of enriched GO terms: **(C)** Colored by the term, where nodes that share the same term are typically close to each other; **(D)** Colored by p-value, where terms containing more genes tend to have a more significant p-value. Each term is represented by a circle node, where its size is proportional to the number of input genes that fall into term, and its color represent its cluster identity. Terms with a similarity score > 0.3 are linked by an edge (the edge's thickness represents the similarity score). The darker the color, the greater the statistical significance of the node. The GO network is also analyzed using the Metascape online website.

Our GO analysis showed that leukocyte degranulation was the most conspicuous biological process. Consistent with our previous results indicating increased serum PR3 in LADA patients, neutrophil degranulation is accompanied by the release of serine proteases in azurophilic granules, including PR3 and NE (33, 34), and an increase in PR3 gene expression (35). During the degranulation of neutrophils, neutrophil-induced ROS production is increased in both patients and rat models of T1DM (36, 37). These toxic substances not only damage pancreatic β-cells but also destroy the antioxidant defence of neutrophils (38–40). However, the specific molecular mechanism by which NADPH is activated in autoimmune diabetes remains unknown. According to our results, expression of *NCF4* and *ARHGAP35* in patients with LADA was upregulated compared with in healthy controls, and qPCR validated this result. The protein P40phox encoded by *NCF4* is the cytoplasmic regulator of NADPH oxidase in superoxide phagocytes; and *p190RhoGAP* encoded by *ARHGAP35* can also increase NADPH oxidase by inhibiting intracellular rac1. Thus, we speculate that neutrophils increase utilization of NADPH and the level of reactive oxygen species (ROS) in LADA through upregulation of *NCF4* and *ARHGAP35*, contributing to direct damage to β-cells. Overall, further research is needed to determine whether *NCF4* and *ARHGAP35* mediate ROS damage to β-cells *via* neutrophils and whether they can be used as intervention targets.

This study has potential limitations. First, the sample size was relatively small, and only newly diagnosed cases from a single center were included. Larger sample sizes and multicenter research need to be carried out. Second, due to the lack of deep basic research, we were unable to elucidate the precise role of the key genes we identified in LADA. Thus, it is necessary to further explore the specific molecular mechanisms of these genes in the pathogenesis of LADA. Third, the cross-sectional design cannot suggest a causal relationship between neutrophils abnormalities and the

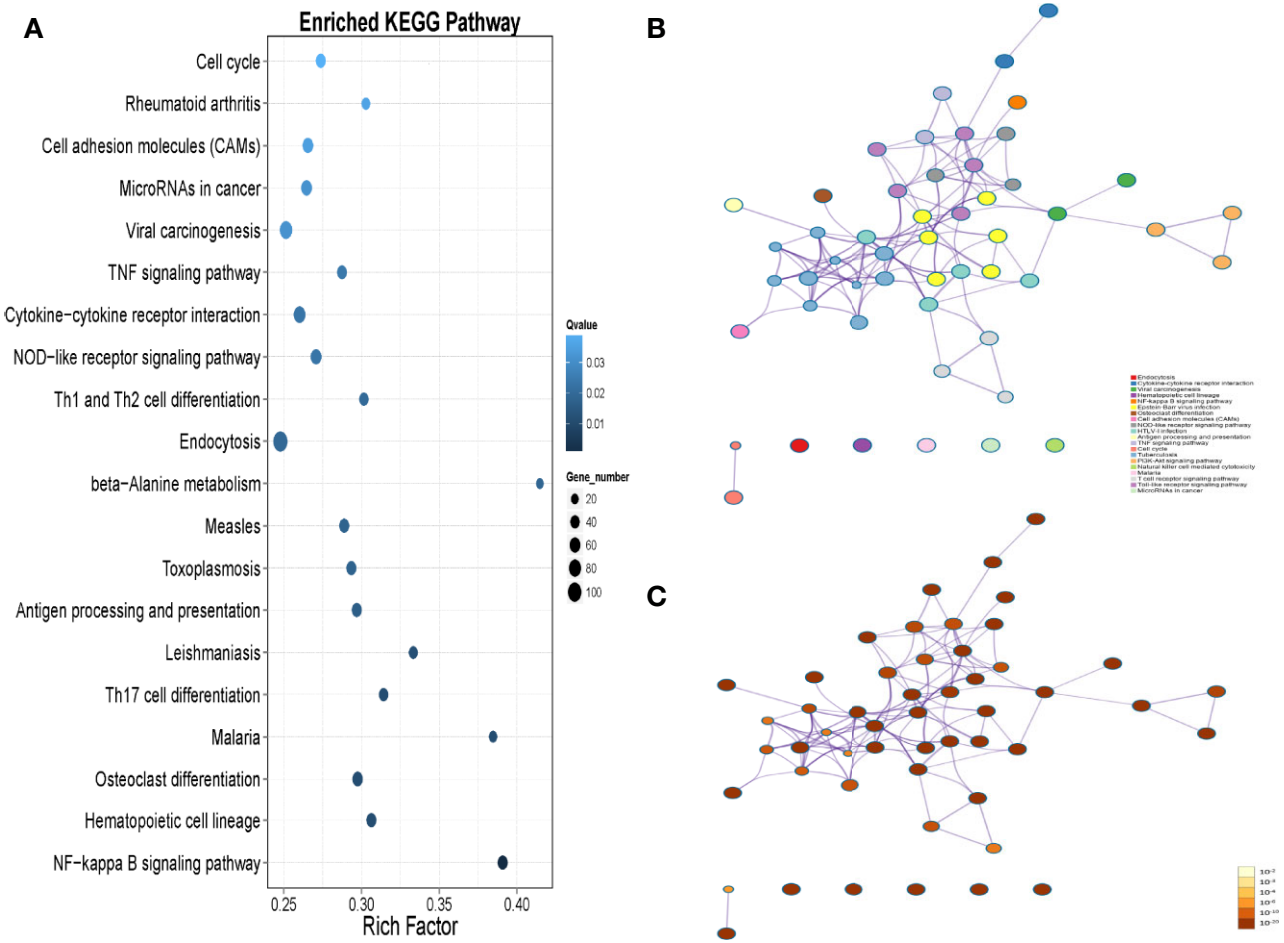

FIGURE 4 | **(A)** The top 20 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways are based on all the differentially expressed genes (DEGs) in peripheral neutrophils between latent autoimmune diabetes in adults (LADA) patients and healthy controls. DEG is defined as levels of fold change ≥ 2 for up-regulated and ≤ 0.5 for down-regulated. The enrichment results of the KEGG pathway are obtained from the KEGG database. The X-axis represents enrichment factors, and the Y-axis represents pathway names. The blue color scale bar indicates the q-value (high: light, low: dark), and the lower Q-value indicates the more significant enrichment. Point size indicates DEG numbers (bigger dots refer to more massive amounts). Rich Factor refers to the enrichment factor's value, which is the quotient of foreground value (the number of DEG) and background value (total Gene amount). The larger the value, the more significant enrichment. **(B)** KEGG-DEG relationship network colored by the pathway, where nodes that share the same pathway are typically close to each other; **(C)** KEGG-DEG relationship network colored by p-value, where pathway containing more genes tend to have a more significant p-value.

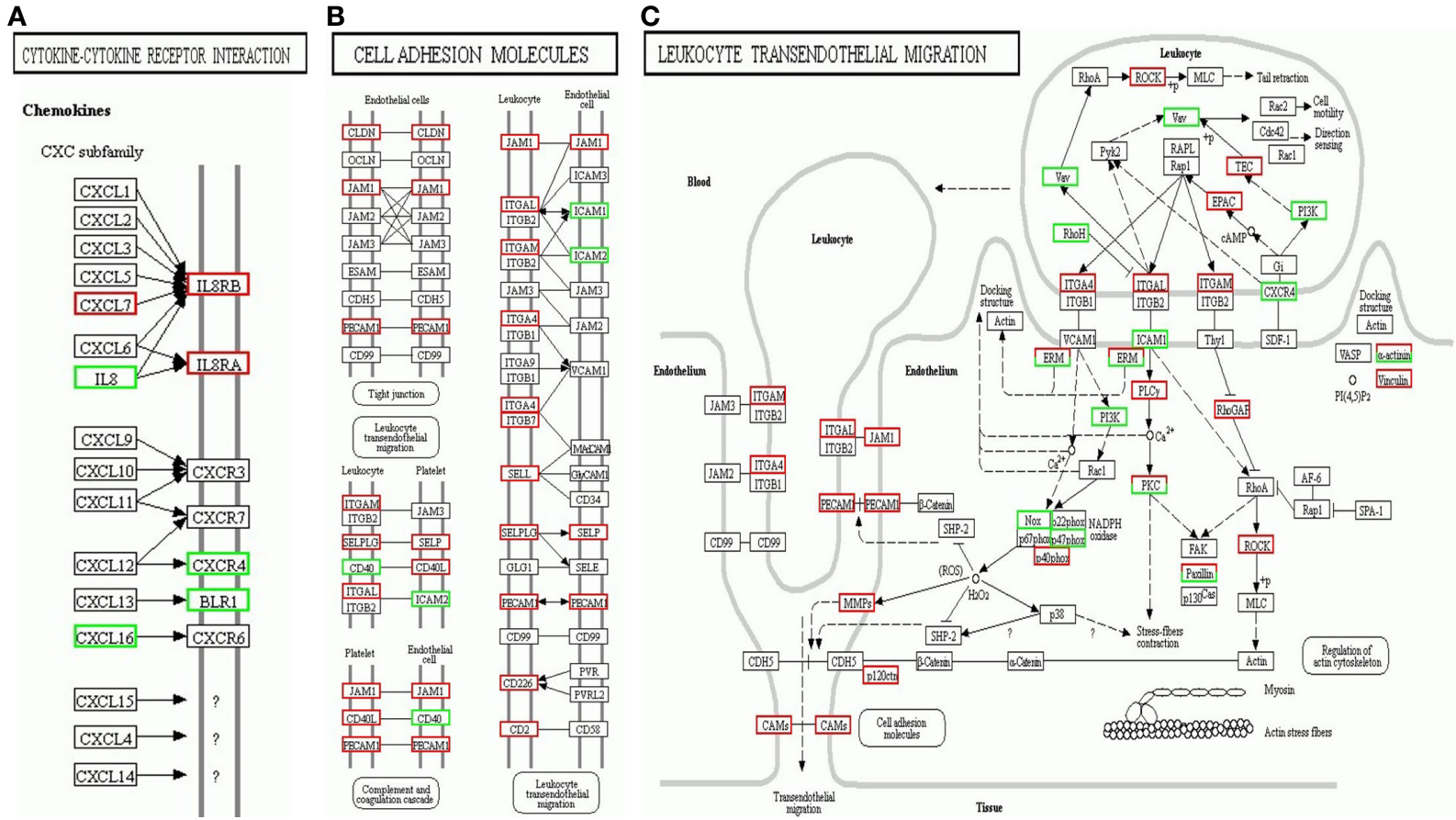

FIGURE 5 | Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway map enriched by differentially expressed genes (DEG) of neutrophils from latent autoimmune diabetes in adults (LADA) patients compared with healthy controls. **(A)** Cytokine-cytokine receptor interaction; **(B)** Cell adhesion molecules; **(C)** Leukocyte transendothelial migration. Red represents upregulated genes, and the green represents downregulated genes.

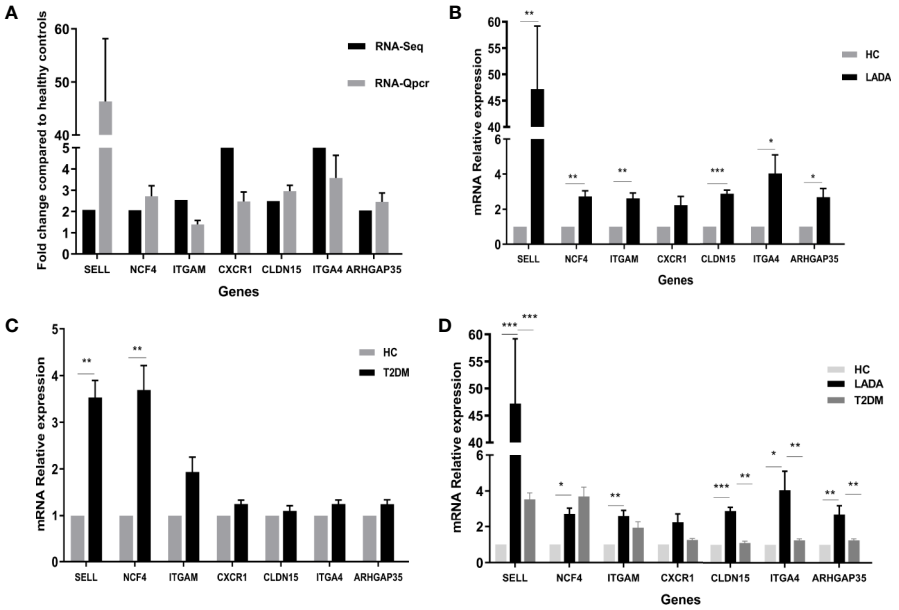

FIGURE 6 | qPCR validation for genes identified by RNA-Seq. **(A)** Black bars denote the RNA-Seq fold change values of genes identified by RNA-seq compared to healthy controls, while gray bars represent real-time polymerase chain reaction (qPCR) fold change values calculated using the $2^{-\Delta Ct}$ method. The $2^{-\Delta Ct}$ of gene expression from latent autoimmune diabetes in adults (LADA) patients and healthy controls were analyzed, and then the fold change were calculated by $2^{-\Delta Ct}$ of gene expression from LADA patients divided by $2^{-\Delta Ct}$ of gene expression from controls. Data are presented as the mean ± SD. **(B)** Expression of seven genes in neutrophils from patients with LADA and healthy controls (HC). The ordinate represents the relative mRNA expression, calculated using the $2^{-\Delta\Delta Ct}$ method. The black bar represents the gene expression in neutrophils from LADA, while the gray bar represents the gene expression in neutrophils from healthy control (HC). *P < 0.05, **P < 0.01, ***P < 0.001. **(C)** Expression of seven genes in neutrophils from patients with type 2 diabetes (T2DM) and HC. The ordinate represents the relative mRNA expression, calculated using the $2^{-\Delta\Delta Ct}$ method. The black bar represents the gene expression in neutrophils from T2DM, while the gray bar represents the gene expression in neutrophils from HC. *P < 0.05, **P < 0.01, ***P < 0.001. **(D)** Expression of seven genes in neutrophils from all HC, LADA and T2DM. The ordinate represents the relative mRNA expression, calculated using the $2^{-\Delta\Delta Ct}$ method. The black bar, dark gray bar and light gray bar represents the gene expression in neutrophils from LADA, T2DM, and HC, respectively. *P < 0.05, **P < 0.01, ***P < 0.001.

development of LADA, thus the role of neutrophils in LADA remains to be confirmed by larger long-term follow-up studies.

CONCLUSION

Our study firstly investigated the DEG in circulating neutrophils of LADA patients as well as corresponding biological functions. In patients with LADA, neutrophils showed activation of degranulation, adhesion, and migration at the transcriptional level. Four essential genes, such as *SELL, ITGA4, ARHGAP35*, and *CLDN15*, may be involved in the pathological process of LADA. These results suggest that neutrophilic dysfunction may play a role in LADA's pathological process, providing a possible prospect for predicting the onset of LADA and exploring the pathogenesis of LADA.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The datasets are deposited to Figshare and available at: https://figshare.com/articles/dataset/Abnormal_neutrophil_transcriptional_signature_may_predict_newly_diagnosed_latent_autoimmune_diabetes_in_adults_of_South_China/12651740.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Ethics Committee of The Second Xiangya Hospital of Central South University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YXX wrote the manuscript. QL, WZ, JH, YL, and ZX collected the samples. YXX, QL, and YT performed the experiment and analyzed the data. YX, YT, and TL revised the manuscript. YX, ZZ, and GH designed the study. 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/fendo.2020.581902/full#supplementary-material

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Gene Expression Analysis of the Pre-Diabetic Pancreas to Identify Pathogenic Mechanisms and Biomarkers of Type 1 Diabetes

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Type 1 Diabetes (T1D) occurs as a result of the autoimmune destruction of pancreatic β-cells by self-reactive T cells. The etiology of this disease is complex and difficult to study due to a lack of disease-relevant tissues from pre-diabetic individuals. In this study, we performed gene expression analysis on human pancreas tissues obtained from the Network of Pancreatic Organ Donors with Diabetes (nPOD), and showed that 155 genes were differentially expressed by ≥2-fold in the pancreata of autoantibody-positive (AA+) at-risk individuals compared to healthy controls. Only 48 of these genes remained changed by ≥2-fold in the pancreata of established T1D patients. Pathway analysis of these genes showed a significant association with various immune pathways. We were able to validate the differential expression of eight disease-relevant genes by QPCR analysis: A significant upregulation of *CADM2*, and downregulation of *TRPM5, CRH, PDK4, ANGPL4, CLEC4D, RSG16*, and *FCGR2B* was confirmed in the pancreata of AA+ individuals versus controls. Studies have already implicated *FCGR2B* in the pathogenesis of disease in non-obese diabetic (NOD) mice. Here we showed that *CADM2, TRPM5, PDK4*, and *ANGPL4* were similarly changed in the pancreata of pre-diabetic 12-week-old NOD mice compared to NOD.B10 controls, suggesting a possible role for these genes in the pathogenesis of both T1D and NOD disease. The loss of the leukocyte-specific gene, *FCGR2B*, in the pancreata of AA+ individuals, is particularly interesting, as it may serve as a potential whole blood biomarker of disease progression. To test this, we quantified *FCGR2B* expression in peripheral blood samples of T1D patients, and AA+ and AA- first-degree relatives of T1D patients enrolled in the TrialNet Pathway to Prevention study. We showed that *FCGR2B* was significantly reduced in the peripheral blood of AA+ individuals compared to AA- controls. Together, these findings demonstrate that gene expression analysis of pancreatic tissue and peripheral blood samples can be used to identify disease-relevant genes and pathways and potential biomarkers of disease progression in T1D.

Keywords: gene expression, type 1 diabetes, auto-antibody positive, non-obese diabetic mice, pancreas, biomarker, FCGR2B

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INTRODUCTION

Type 1 Diabetes (T1D) results from the autoimmune and chronic destruction of pancreatic β-cells by self-reactive T cells. It is the most common autoimmune disease in children, affecting approximately 1 in 500 in the United States, and the incidence is growing each year (1). The etiology of this disease is complex, and involves a strong genetic component and a number of possible environmental triggers. Genome-wide association studies (GWAS) have identified >60 polymorphisms that contribute to the genetic susceptibility of T1D (2, 3) and various environmental triggers have been shown to play a role in disease onset (4–6). The pathogenesis of this disease, however, remains unclear due to the difficulty in identifying pre-diabetic individuals and in obtaining biological specimens from such individuals.

Animal models of T1D have been crucial in understanding the mechanisms involved in the pathogenesis of T1D, but there are notable differences in human disease development. In humans, β-cell destruction occurs months to years before the onset of hyperglycemia. Individuals are asymptomatic, and develop autoantibodies (AAs) against various islet antigens including glutamic acid decarboxylase (GAD), zinc transporter 8 (ZnT8), insulin, and islet antigen 2 (IA-2) during the pre-hyperglycemic stage of disease. The presence and number of AAs detected in the serum is currently the most reliable biomarker for assessing the disease risk. However, there is a need for additional early, robust and reproducible biomarkers of disease progression.

The pancreata of at-risk AA+ individuals are significantly smaller than those of healthy individuals (7, 8), and analysis of pancreas sections demonstrate increased CD4+ and CD11c+ immune cell infiltration in the exocrine pancreas of these individuals (9). The islets of at-risk individuals differ functionally and morphologically from healthy controls (10), but insulitis is only detected in a small percentage of AA+ individuals (11, 12). In contrast, the islets of non-obese diabetic (NOD) mice, a well-established mouse model of T1D, develop large peri-insulitic lesions beginning at 4 weeks of age. Destructive insulitis occurs at ~12 weeks of age, and hyperglycemia follows at ~16 to 25 weeks of age (13).

We and others have shown that the pancreata and islets of rodent models of T1D differ from those of healthy controls, and that these differences are reflected in the gene expression profile during the progression of disease (13–15). Gene expression data support the upregulation of glucagon and insulin secretion, β-cell hyperactivation and/or ER stress playing a role during different stages of disease development (13–15). Currently, it is unclear if similar changes in gene expression are observed in the pancreata of pre-diabetic humans after seroconversion preceding hyperglycemia.

Human pancreatic tissues from healthy controls, AA+ and T1D patients are available through the Juvenile Diabetes Research Foundation Network for Pancreatic Organ Donors with Diabetes (JDRF nPOD; www.jdrfnpod.org). These samples are collected from deceased donors and processed under well-defined standard operating procedures. However, due to the high level of pancreatic ribonucleases and tissue recovery time, RNA degradation occurs (16–18). Nevertheless, studies have shown that the impact of RNA degradation on the assessment of gene expression by microarray analysis is minimal, and largely offset by biological differences between samples (19).

In this study, we obtained pancreas samples of at-risk AA+ individuals, T1D patients, and healthy controls from nPOD and performed gene expression analyses. We then compared the genes that were differentially expressed in pre-diabetic AA+ individuals with those that were differentially expressed in the pancreata of NOD mice. The overlapping genes may be pathogenic and play a role in the development of T1D and NOD disease. We identified a number of biologically relevant genes that were changed in the pancreata of AA+ individuals. One gene, in particular, is expressed in leukocytes and could serve as a potential biomarker of disease progression that may be monitored in blood samples of individuals with a genetic risk for developing T1D. This gene, *FCGR2B*, encodes the CD32B receptor, an inhibitory low affinity receptor for the Fc region of IgG complexes (20, 21). Changes to *FCGR2B*/CD32B expression have been associated with multiple autoimmune diseases in humans (22–25), and have been suggested to underlie the autoimmune susceptibility of several strains of mice, including NOD mice (26, 27).

In this study, we also examined the potential of *FCGR2B* as a candidate biomarker of disease progression, by comparing *FCGR2B* expression in peripheral blood RNA samples of T1D patients, AA+ individuals who later developed T1D (AA+ progressors), and AA- first-degree relatives of T1D patients (AA- FDRs) obtained from the National Institute of Health TrialNet repository.

MATERIALS AND METHODS

Human Samples

Pancreas

Human pancreas samples were obtained through the JDRF network for Pancreatic Organ Donors with Diabetes (nPOD). Patient information for the final samples used for this study is shown on **Tables 1A** and **B**, and standard operating procedures for tissue processing are available at https://www.jdrfnpod.org/for-investigators/standard-operating-procedures/. Tissues were obtained from cadaveric donors that died of various causes, and AA+ individuals were identified by autoantibody screening after death. Thus, the length of islet autoimmunity in AA+ individuals is unknown. The pancreatic tissues used for this study were minced, placed in cryovials containing RNAlater, incubated at room temperature for 15 min and then snap frozen and stored at -80°C until shipment. RNA was extracted as described below.

Whole Blood Samples

Whole blood RNA samples were provided by TrialNet. Samples from AA- FDRs (n=15) and AA+ FDRs who later progressed to hyperglycemia (AA+ progressors, n=20) were from individuals enrolled in the TrialNet Pathway to Prevention Study. Samples from T1D patients (n=10) were obtained from individuals

TABLE 1A | Patient information for nPOD pancreata samples.

nPOD ID	Serum AA*	Region	Age†	Sex	BMI	Ethnicity	Cause of Death††	C-peptide (ng/ml)	Pancreas Weight (g)	RNA RIN
Normal Controls										
6102	Negative	body	45	F	35.1	Caucasian	CVS	0.55	87.32	4.6
6172	Negative	tail	19	F	32.4	Caucasian	CVS	8.02	59.7	5.8
6179	Negative	tail	21	F	20.7	Caucasian	HT	2.74	72.4	5.2
6227	Negative	body	17	F	26.4	Caucasian	CVS	2.75	60.4	5.6
6229	Negative	body	31	F	26.9	Caucasian	HT	6.23	45.6	7
6234	Negative	body	20	F	25.6	Caucasian	HT	6.89	49.86	3.7
6253	Negative	body	19	F	34.3	African American	HT	7.22	102.31	5.3
AA+ subjects										
6027	ZnT8A+	tail	18	M	19.9	Caucasian	n/a	n/a	52.9	4.4
6090	GADA+	head	2	M	18.8	Hispanic/Latino	HT	5.34	19.3	5.5
6123	GADA+	head	23	F	17.6	Caucasian	HT	2.01	48.34	7.6
6170	GADA+	head	34	F	36.9	African American	A	4.29	110.3	7.6
6184	GADA+	body	47	F	27	Hispanic/Latino	HT	3.42	71.8	4.4
6197	GADA+ IA-2A+	tail	22	M	28.2	African American	HT	17.48	73.3	8.1
Established T1D subjects										
6088	GADA+ IA-2A+ ZnT8A+ mIAA+	head	31 (T1D: 5)	M	27	Caucasian	HT	<0.05	32	5.5
6143	IA-2A+ mIAA+	head	32 (T1D: 7)	F	26.1	Caucasian	A	<0.05	35.7	8.5
6148	GADA+ mIAA+	head	17 (T1D: 7)	M	23.9	Caucasian	A	0.06	26.36	5.0
6161	IA-2A+ mIAA+	head	19 (T1D: 7)	F	36.1	Caucasian	CVS	<0.05	39.8	5.0
6180	GADA+ IA-2A+ ZnT8A+ mIAA+	body	27 (T1D: 11)	M	25.9	Caucasian	HT	<0.05	36.7	4.0
6241	mIAA+	body	33 (T1D: 31)	M	18.4	Caucasian	CVS	<0.05	24.92	8.0
6245	GADA+ IA-2A+	body	22 (T1D: 7)	M	23.2	Caucasian	HT	<0.05	31.58	7.2
6258	mIAA+	body	39 (T1D: 37)	F	28.7	Caucasian	HT	<0.05	32.56	5.4
6263	Negative	body	34 (T1D: 21)	M	23.5	Hispanic/Latino	CVS	3.18	51.59	7.8
6266	GADA+ IA-2A+ mIAA+ ZnT8A+	body	30 (T1D: 23)	M	27.1	Caucasian	A	<0.05	51.66	6.6

**GAD, Glutamic acid decarboxylase; IA2+, Insulinoma-associated protein 2; mIAA+, micro-Insulin auto-antibody; ZnT8, Zinc transporter 8 as determined by nPOD. Methods used for AA+ detection are available at: http://www.jdrfnpod.org/for-investigators/standard-operating-procedures/.*

†For T1D samples, years with T1D are indicated.

††Cause of Death: HT, Head trauma; CVS, Cerebrovascular/Stroke; A, Anoxia.

enrolled in the Glutamic Acid Decarboxylase (GAD) Vaccine trial. IRB approval was obtained at the institution where samples were collected. All participants provided written informed consent. Before use, all RNA samples were repurified using the Qiagen RNeasy mini kit. Briefly, 100 µl of RNA (<100 µg) was combined with 350 µl of buffer RLT, mixed, added to 250 µl ethanol, and applied to a RNeasy mini column to bind the RNA. The RNA was washed and eluted according to manufacturer's instructions. Re-extracted RNA concentrations were measured by Nanodrop and RNA quality was determined by Bioanalyzer analysis (Agilent) and listed in **Table 2**.

Animals

Female NOD/LtJ (NOD) and NOD.B10Sn-*H2*^{b}/J (NOD.B10) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were maintained under pathogen-free conditions at the Stanford School of Medicine Animal facility, according to institutional guidelines under approved protocols. Pancreas tissues were isolated from 12-week-old NOD and NOD.B10 mice (8 animals per group) and immediately homogenized in Trizol reagent on ice, and stored at -80°C. RNA was extracted as described below. See **Supplementary Figure 1** for blood glucose levels, weight of mice, and bioanalyzer traces of RNA samples.

RNA Extraction From Pancreas Tissue

Total RNA was extracted using Trizol reagent and the Qiagen RNeasy mini kit, as previously described (28, 29). For human pancreas tissues, RNA was extracted from at least 14 samples per group (controls, AA+ and T1D). Pancreatic samples were thawed on ice, removed from RNAlater, and homogenized in Trizol reagent (100 mg tissue/ml Trizol) on ice. For human and NOD mouse samples, RNA was extracted from 1 ml of the tissue/Trizol homogenate. 0.2 ml chloroform was added to the homogenate, shaken vigorously and centrifuged at 12,000*xg* for 15 min at 4°C.

TABLE 1B | HLA Information for nPOD pancreata samples.

nPOD ID	HLA Information
	Normal Controls
6102	DRB1*03:01, 04:01 DQA1*03:01, 05:01 DQB1*02:01, 03:01 (DR3, DR4)
6172	DRB1*01:01, 13:03 DQA1*01:01, 05:01 DQB1*03:01, 05:01
HLA information not available for 6179, 6227, 6229, 6234 and 6253	
	AA+ subjects
6027	DRB1*03:01/15:01 DQA1*01:02/05:01 DQB1*02:01/06:02 (DR3)
6090	DRB1*04:04/15:01 DQA1*01:02/03:01 DQB1*03:02/06:02 (DR4)
6123	DRB1*08:01/11:01 DQA1*04:01/05:01 DQB1*03:01/04:02
6170	DRB1*04:01/13:03 DQA1*02:01/03:01 DQB1*02:02/03:01 (DR4)
6184	DRB1*04:07/04:07 DQA1*03:01/03:01 DQB1*03:02/03:02 (DR4, DR4)
6197	DRB1*03:02/07:01 DQA1*02:01/04:01 DQB1*02:02/04:02(DR3)
	Established T1D subjects
6088	DRB1*01:01/03:01 DQA1*01:01/05:01 DQB1*02:01/05:01 (DR3)
6143	DRB1*03:01/04:01 DQA1*03:01/05:01 DQB1*02:01/03:01 (DR3, DR4)
6148	DRB1*03:01/04:01 DQA1*03:01/05:01 DQB1*02:01/03:02 (DR3, DR4)
6161	DRB1*04:01/07:01 DQA1*02:01/03:01 DQB1*02:02/03:02 (DR4)
6180	DRB1*01:01/03:01 DQA1*01:01/05:01 DQB1*02:01/05:01 (DR3)
HLA information not available for 6241, 6245, 6258, 6263, and 6266.	

The aqueous phase was removed, mixed with an equal volume of 70% ethanol and bound to a Qiagen RNeasy mini-column. The column was washed and RNA was eluted according to manufacturer's instructions. Total RNA concentrations were measured by Nanodrop and quality was determined by Bioanalyzer analysis (Agilent Technologies, Santa Clara, CA). Bioanalyzer traces for human and mouse samples are shown in **Figure 1** and **Supplementary Figure 1**, respectively. The human samples with the highest RNA integrity numbers (RIN; n≥6 per group) were selected for gene expression analysis studies.

Microarray Analysis

Human Samples

One-color microarrays were performed by the Stanford Human Immune Monitoring Center to measure gene expression in individual pancreas samples of seven controls, six AA+, and 10 T1D patients (**Table 1**) using the SurePrint G3 Human GE 8x60K microarray kit (Agilent Technologies). One microgram of total RNA was labeled with Cy3 using the low RNA input fluorescence linear amplification kit (Agilent Technologies). Hybridization was performed using the gene expression hybridization kit (Agilent Technologies) and the microarray hybridization chamber (Agilent Technologies), according to the manufacturer. Microarray chips were scanned using the DNA microarray scanner (Agilent Technologies) and data were processed with Feature Extraction Software (Agilent Technologies), and analyzed using GeneSpring GX 12.6.1 Software (Agilent Technologies). All microarray data have been submitted to the Gene Expression Omnibus (GEO) Database at NCBI (GEO series accession number: GSE72492), and are accessible at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72492. Samples were filtered for entities that were detected in at least six out of 23 samples, and entities with annotated gene symbols. Because of the gender imbalance between the groups, genes that were significantly changed by ≥5 fold between male and female subjects were removed from the analysis (**Supplementary Table 1**). A final list of 29,438 entities was used for further analysis. Moderated T-tests were performed to identify differentially expressed genes ($p<0.05$), and pathway analysis of differentially expressed genes was performed using Ingenuity Pathway Analysis (IPA; Qiagen Inc; https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis).

Mouse Samples

Two-color microarrays were performed to measure gene expression in the pancreas of individual 12-week-old NOD mice against a pool of age-matched NOD.B10 mice (n=8 animals per group) using the Whole Mouse Genome Microarray Kit, 4×44K 2-color arrays (Agilent Technologies) as previously described (29). Data were analyzed using GeneSpring (version 12.6.1, Agilent Technologies). Samples were filtered for entities with annotated gene symbols that are expressed (minimum raw expression ≥ 25) in at least four of the eight NOD samples, have equivalent human homologues, and are expressed in the human pancreas samples. The resultant 25,283 entities were analyzed. Data are available at GEO (Series accession number: GSE154739, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE154739). T-test and Benjamini-Hochberg multiple testing correction were performed

TABLE 2 | Patient information for TrialNet whole blood RNA samples.

AA- First Degree Relatives

ID	HLA (DRB1:DQA1:DQB1)*	Serum AA†	Age	Sex	Years to Onset	RNA RIN
1	HLAa: 1401:0101:0503 HLAb: 1102:0501:0301	Negative	13	F	–	8.1
2	HLAa: 1501:0102:0602 HLAb: 1301:0103:0603	Negative	9	F	–	8.5
3	HLAa: 0701:0201:0202 HLAb: 0401:0301:0302 (DR4)	Negative	15	M	–	8.2
4	HLAa: 1201:0501:0301 HLAb: 1302:0501:0301	Negative	18	M	–	7.8
5	HLAa: 0101:0101:0501 HLAb: 0102:0101:0501	Negative	12	F	–	7.5
6	HLAa: 0401:0301:0302 (DR4) HLAb: 0701:0201:0202	Negative	18	F	–	8.3
7	No data	Negative	24	F	–	5.6
8	HLAa: 0701:0201:0202 HLAb: 0401:0301:0301 (DR4)	Negative	17	M	-	7.1
9	HLAa: 0701:0201:0202 HLAb: 0701:0201:0202	Negative	7	F	-	8.1
10	HLAa: 0401:0301:0302 (DR4) HLAb: 0301:0501:0201(DR3)	Negative	12	M	-	7.8
11	HLAa: 0408:0301:0301 HLAb: 0301:0501:0201 (DR3)	Negative	45	M	–	6.2
12	No data	Negative	34	M	–	7.7
13	HLAa: 1302:0102:0604 HLAb: 0101:0101:0502	Negative	9	M	–	8.4
14	HLAa: 1104:0501:0301 HLAb: 0301:0501:0201 (DR3)	Negative	43	F	–	7.4
15	HLAa: 0401:0301:0301 (DR4) HLAb: 0401:0301:0302 (DR4)	Negative	10	M	–	8.8
		Average ± SE:	**19.1 ± 3.1**		-	**7.7 ± 0.2**

AA+ First Degree Relatives that later progressed to T1D

ID		Serum AA*	Age	Sex	Years to Onset	RNA RIN
1	HLAa: 0101:0101:0501 HLAb: 0401:0301:0302 (DR4)	ICA512+	5.5	M	3.0	7.1
2	HLAa: 0301:0501:0201 (DR3) HLAb: 0404:0301:0302 (DR4)	GAD65+	43.6	F	3.9	8.5
3	HLAa: 0301:0501:0201 (DR3) HLAb: 0301:0501:0201 (DR3)	GAD65+	11.8	M	2.1	9.3
4	HLAa: 0401:0301:0302 (DR4) HLAb: 0401:0301:0302 (DR4)	ICA+	23.4	M	1.8	9.2
5	HLAa: 0401:0301:0302 (DR4) HLAb: 0404:0301:0302 (DR4)	GAD65+	40.6	F	3.7	8.8
6	HLAa: 0404:0301:0302 (DR4) HLAb: 0801:0401:0402	GAD65+ GAD65H+	10.0	M	3.3	9.4
7	HLAa: 0301:0501:0201 (DR3) HLAb: 1501:0102:0602	ICA512+	13.5	M	2.6	6.1
8	HLAa: 0301:0501:0201 (DR3) HLAb: 0301:0501:0201 (DR3)	GAD65+ ICA+	41.7	F	3.0	7.0
9	HLAa: 0901:0301:0303 HLAb: 0301:0501:0201 (DR3)	GAD65+ GAD65H+	50.0	M	1.1	7.2
10	No data	GAD65+	4.9	F	2.0	10
11	HLAa: 0401:0301:0302 (DR4) HLAb: 0801:0401:0402	GAD65+ ICA+	8.3	M	3.8	9.8
12	No data	GAD65+	12.6	F	3.4	9.7
13	HLAa: 0401:0301:0302 (DR4) HLAb: 0301:0501:0201(DR3)	GAD65+ ICA+	11.4	M	2.4	9
14	HLAa: 0301:0501:0201 (DR3) HLAb: 0404:0301:0302 (DR4)	GAD65+ ICA+	6.5	M	5.5	8.7
15	HLAa: 0301:0501:0201 (DR3) HLAb: 1302:0102:0604	GAD65+	45.5	M	3.1	8.7

(Continued)

TABLE 2 | Continued

AA+ First Degree Relatives that later progressed to T1D

ID		Serum AA*	Age	Sex	Years after Onset	RNA RIN
16	HLAa: 0103:0101:0501 HLAb: 0401:0301:0302 (DR4)	ICA512+	23.4	M	3.0	7.5
17	HLAa: 0404:0301:0302 (DR4) HLAb: 1101:0501:0301	GAD65+	41.6	F	3.0	6.9
18	HLAa: 0301:0501:0201 (DR3) HLAb: 0401:0301:0302 (DR4)	GAD65+	8.9	F	2.9	9.1
19	HLAa: 0404:0301:0302 (DR4) HLAb: 0301:0501:0201 (DR3)	GAD65+ ICA+	6.4	F	3.3	8.4
20	HLAa: 0301:0501:0201 (DR3) HLAb: 0101:0101:0501	GAD65+ ICA+	24.7	M	3.3	7.5
		Average ± SE:	**21.7 ± 3.6**		**3.2±0.2**	**8.4 ± 0.2**

T1D subjects

ID		Serum AA*	Age	Sex	Years after Onset	RNA RIN
1	HLAa: 1301:0103:0608 HLAb: 0901:0301:0303	GAD65+ ICA512+ mIAA+ ICA+	23.2	M	2.3	7.4
2	HLAa: 0401:0301:0302 (DR4) HLAb: 0103:0101:0501	GAD65+ ICA512+ mIAA+	23.8	F	2.3	8.4
3	HLAa: 0901:0301:0303 HLAb: 1201:0501:0301	GAD65+ mIAAA+	26.8	F	2.3	7.8
4	HLAa: 0404:0301:0302 (DR4) HLAb: 0404:0301:0302 (DR4)	ICA512+ mIAA+ ICA+	13.1	F	2.3	8.5
5	HLAa: 0404:0301:0302 (DR4) HLAb: 0405:0301:0201 (DR4)	GAD65+ ICA512+ mIAA+	40.8	M	2.2	8.6
6	HLAa: 0401:0301:0302 (DR4) HLAb: 0101:0101:0501	GAD65+ mIAA+	18.2	F	2.3	8.2
7	HLAa: 0401:0301:0302 (DR4) HLAb: 0301:0501:0201 (DR3)	GAD65+ ICA512+ mIAA+	18.6	M	2.2	7.6
8	HLAa: 0301:0501:0201 (DR3) HLAb: 1601:0102:0502	GAD65+ mIAA+	14.7	F	2.2	7.6
9	HLAa: 0401:0301:0302 (DR4) HLAb: 0301:0501:0201 (DR3)	ICA512+ mIAA+ ICA+	16.7	F	2.2	7.3
10	HLAa: 0401:0301:0302 (DR4) HLAb: 0301:0501:0201 (DR3)	GAD65+ ICA512+ mIAA+	16.2	M	2.3	8.3
		Average ± SE:	**20.7 ± 2.5**		**2.8 ± 0.2**	**8.2 ± 0.2**

**HLAa and HLAb, HLA Haplotype α and HLA Haplotype β shown as DRB1:DQA1:DQB1. †GAD65 - GAD65 standard (TN local) assay; Positive >0.032, ICA512 - ICA512/IA2 standard (TN local) assay; Positive >0.049, GAD65H - GAD65 harmonized assay; Positive >20, IA-2H - ICA512/IA2 harmonized assay; Positive >5, MIAA - mIAA; Positive >.01, ICA - Islet Cell Autantibody; Positive ≥10. Serum autoantibody information were provided by TrialNet.*

to identify differentially expressed genes in the pancreata of NOD vs. NOD.B10 mice with a fold change ≥2-fold (corrected $p<0.01$).

Validation by QPCR

We identified a number of biologically relevant genes among those that were changed in the pancreata of AA+ individuals vs. controls. These include genes that have previously been associated with T1D, T2D, metabolism or obesity, and genes with biological functions that may be involved in the pathogenesis of T1D. These genes and their significance are listed in **Table 3**.

QPCR was performed to validate differential gene expression. cDNA was synthesized and QPCR was performed as previously described (28, 29). Briefly, first strand cDNA was generated from 1 µg of total RNA using Superscript III (Invitrogen) and random hexamers according to manufacturer's instructions. QPCR was performed to measure human *INS*, *BCL2L15, ERAP1, CADM2, ERAP1, ETV5, KANK1, SIM1, ABCB9, PGC, CEACAM6, CALB1, S100B, SMPD3, IL33, SCIMP, SEZ6L, PLD1, GNLY, CRTAM, CD180, IL4R, PLXNA4, PDK4, FCGR2B, TRPM5, CRH, ANGPTL4, CLEC4D, RGS16, ICOSLG, CD19, CD20, CD11C*, and housekeeping genes *GAPDH, ACTB, B2M*, and 18S rRNA using commercially available Taqman gene expression assays (Applied Biosystems). cDNA was preamplified using the Taqman PreAmp Mastermix (Applied Biosystems) prior to QPCR for measurements that initially gave threshold cycle (Ct) values of >30. The 7900HT Fast Real Time PCR System (Applied Biosystems) and the Taqman Gene Expression Mastermix (Applied Biosystems) were used, according to manufacturer's instructions. The comparative Ct method for relative quantification (ΔΔCt) was used. QPCR was also performed to measure the expression of *FCGR2B, CD19, CD20, CD11C*, and *ACTB* in whole blood RNA samples obtained from TrialNet. For these experiments, cDNA was synthesized (from 100 ng of total RNA) and QPCR was performed to described above.

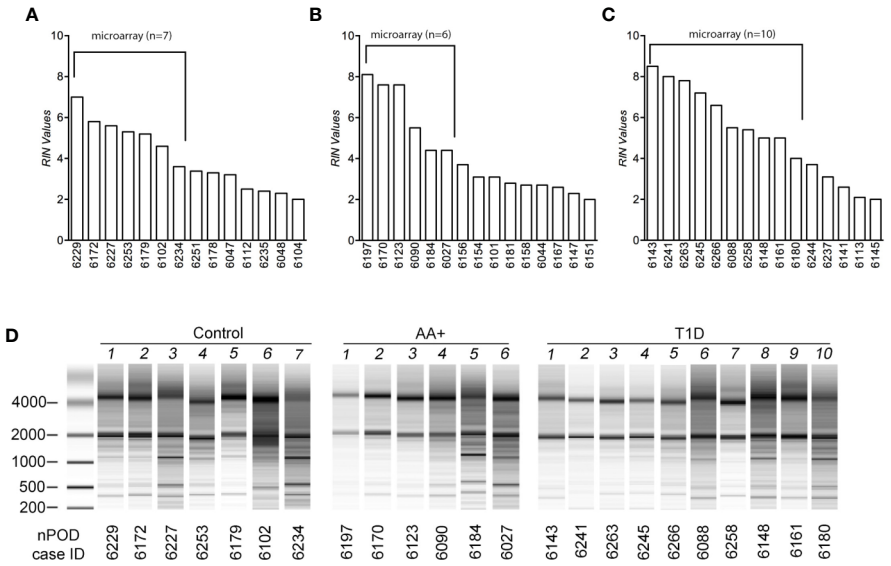

FIGURE 1 | Assessment of RNA quality of samples extracted from human pancreatic tissues. RNA was isolated from pancreatic tissues of controls **(A)**, AA+ individuals **(B)**, and T1D patients **(C)** obtained from nPOD, and bioanalyzer analysis was performed to assess RNA quality. RIN (RNA integrity numbers) values **(A–C)** and bioanalyzer traces **(D)** are shown. The samples with the highest RIN values were used for microarray analysis (as indicated).

Statistical Analysis

Statistical analyses for QPCR experiments were performed using the two-tailed Mann-Whitney test (Prism 8, GraphPad Software Inc. San Diego, CA). A P-value of ≤0.05 was considered significant.

RESULTS

Gene Expression Analysis in Human Pancreata Samples

Total RNA was initially isolated from ≥14 subjects per group (healthy controls, AA+ and T1D subjects). Bioanalyzer analysis showed considerable RNA degradation in some samples, with RIN (RNA integrity number) values ranging from 2.0 to 8.5 (**Table 1**, **Figure 1**). The majority of samples had a RIN ≥5, and samples with RIN values of ≥3.7 were used for microarray analysis (n=7 controls, 6 AA+, and 10 T1D samples). Microarray and QPCR results showed that the expression of housekeeping genes *ACTB, GAPDH* did not significantly differ between groups and did not strongly correlate with RIN values (**Figure 2**). As expected, *INS* (Insulin) expression was found to be significantly reduced in the pancreata of T1D patients compared to controls, and pre-diabetic AA+ individuals (**Figure 2**).

Microarray analysis was performed on 29,168 entities representing 20,813 genes that were expressed in at least six of the 23 human pancreata. Because of the gender imbalance between groups, we eliminated genes from our analysis that were significantly changed by >5-fold in male vs. female subjects (**Supplementary Table 1**). The expression of certain genes, however, may still be influenced by age or sex.

We showed that 155 genes (59 upregulated and 96 downregulated) were changed by ≥2-fold in the pancreata of AA+ compared to controls (**Figure 3A**; **Supplementary Table 2**), and 645 genes (252 genes upregulated and 395 downregulated) were changed by ≥2-fold in the pancreata of T1D patients compared to controls (**Supplementary Table 3**). Among the 155 genes changed by ≥2-fold in the pancreata of AA+ individuals (**Figure 3A**), 48 genes were similarly and significantly changed by ≥2-fold in T1D patients (**Figure 3B**), 19 were significantly changed by <2-fold in T1D patients (**Figure 3C**), and 88 were not significantly changed in T1D patients (**Figure 3D**). Another 28 genes were found to be significantly changed by 1.5 to 2-fold in AA+ vs. controls, but >2-fold in T1D vs. controls (**Figure 3E**). These data indicate that 107 genes may be transiently changed after seroconversion, but no longer changed after the establishment of hyperglycemia, while 76 genes may be changed after seroconversion and during T1D. Heatmaps of gene expression for the 183 genes (represented in **Figures 3A, E**) are shown in **Figure 3F** (upregulated genes) and **Figure 3G** (downregulated genes).

Pathway Analysis of Differentially Expressed Genes in At-Risk AA+ vs. Controls

IPA pathway analysis was performed on the 183 genes that are differentially expressed in the pancreata of AA+ individuals compared to controls. A significant association of genes with various immune response functions was observed (**Table 4A**), with the strongest association seen with "function of helper T lymphocytes" ($p = 1.93x10^{-6}$). Upstream regulator analysis using IPA identified several cytokines including IL-1β that may drive the observed changes in gene expression (**Table 4B**). The top upstream regulator was found to be CNR1 (canniboid receptor 1,

TABLE 3 | Biologically relevant genes that are significantly changed in the pancreata of AA+ vs. control (Microarray experiments).

Gene	Description	Significance	Reference
Genes associated with T1D, T2D, metabolism, or obesity			
CADM2	cell adhesion molecule 2	SNP associated with risk of obesity, regulates insulin sensitivity	Speliotes et al. (30); Dorajoo, 2012; Rathjen et al. (31); Yan, 2018
BCL2L15	BCL2-like 15	Gene located in a T1D-associated loci.	T1Dbase.org
ERAP1	endoplasmic reticulum aminopeptidase 1	Gene located in a T1D-associated loci.	Fung et al. (32)
ETV5	ets variant 5	SNP located near ETV5 gene linked to obesity.	Thorleifsson et al. (33)
KANK1	KN motif and ankyrin repeat domains 1	Variants associated with fasting proinsulin & insulinogenic index	Huyghe et al. (34)
SIM1	single-minded family bHLH transcription factor 1	Gene in an obesity-associated loci.	Meyre et al. (35)
PLXNA4	plexin A4	Gene associated with T2D.	Saxena et al. (36)
TRPM5	transient receptor potential cation channel, subfamily M, member 5	TRPM5 variants associated with pre-diabetic phenotypes. Regulates insulin secretion and loss of expression leads to a pre-diabetic phenotype.	Brixel et al. (37); Ketterer et al. (38)
ABCB9	ATP-binding cassette, sub-family B, member 9	Gene variant that confers susceptibility for T2D.	Harder et al. (39)
Biologically relevant to pathogenesis of T1D – Genes upregulated in AA+			
PGC	Progastricsin (pepsinogen C)	Expressed in human islets and is converted to the proteolytic enzyme pepsin in low pH.	Hassan et al. (40)
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6	Involved in insulin homeostatsis and T cell proliferation, and serves as receptor for host-specific viruses and bacteria.	Kuespert, 2006
CALB1	calbindin 1, 28kDa	Overexpression reduces glucose-stimulated insulin secretion by modulating calcium influx.	Lee et al. (41)
S100B	S100 calcium binding protein B	An inflammatory protein that serves as a ligand for the receptor of advanced glycation end products that are involved in the development of pathogenic T cells and apoptosis of beta cells.	Chen et al. (42)
SMPD3	sphingomyelin phosphodiesterase 3,neutral membrane	Can mediate cellular responses to IL-1b and TNFa.	Rutkute et al. (43)
IL33	interleukin 33	May promote the development of inflammatory autoimmune T cells in experimental models of T1D.	Milovanovic et al. (44)
ETV5	ets variant 5	Controls differentiation of TH17 cells.	Pham et al. (45)
Biologically relevant to pathogenesis of T1D—Genes downregulate in AA+			
CRH	corticotropin releasing hormone	expressed in beta cells. Plays a role in insulin secretion and beta cell proliferation.	Kanno et al. (46); Huising et al. (47)
PDK4	pyruvate dehydrogenase kinase, isozyme 4	A key modulator of glucose homeostasis. Regulates expression of the transcription factor Ptf1a.	Dateki et al. (48); Zhang, 2014
ANGPTL4	angiopoietin-like 4	Involved in glucose homeostasis, lipid metabolism, and insulin sensitivity. Regulates islet morphology.	Xu et al. (49); Kim et al. (50)
SCIMP	SLP adaptor and CSK interacting membrane protein	Expressed on B cells and involved in signal transduction after MHCII stimulation.	Draber et al. (51)
SEZ6L	seizure related 6 homolog-like	A substrate for BACE2, an inhibitor of beta cell proliferation.	Stutzer et al. (52)
ICOSLG	inducible T-cell co-stimulator ligand	Provides co-stimulation through ICOS, and may be involved in the expansion of activated Tregs	Martin-Orozco et al. (53)
PLD1	phospholipase D1, phosphatidylcholine-specific	Phosphatidic acid formation on the graunule membrane by PLD1 is essential for glucose-stimulated insulin secretion	Ma et al. (54)
TRPM5	transient receptor potential cation channel, subfamily M, member 5	TRPM5 variants associated with pre-diabetic phenotypes. Regulates insulin secretion and loss of expression leads to a pre-diabetic phenotype.	Brixel et al. (37); Ketterer et al. (38)
GNLY	granulysin	An antimicrobial peptide. Reduced *GNLY* expression has been observed in the PBMCs of T1D patients	Jin et al. (55)
CRTAM	cytotoxic and regulatory T cell molecule	Involved in establishing late phase T cell polarity, and allows increased production of IFNγ and IL22.	Yeh et al. (56)
CD180	CD180 molecule (RP105)	An important modulator of Toll-like receptor 4 signaling that is involved in activating innate and adaptive immune responses.	Divanovic et al. (57)
FCGR2B	Fc fragment of IgG, low affinity IIb, receptor	Impaired FCGR2B function leads to abberant B cell activation and the development of autoimmunity. Reduced *Fcgr2b* linked to autoimmune susceptibility in NOD mice.	Jiang et al. (26); Xiu et al. (27); Anania et al. (20)
CLEC4D	C-type lectin domain family 4, member D	Plays a non-redundant role in protecting against anti-mycobacterial and fungal infection.	Wilson et al. (58)
IL4R	interleukin 4 receptor	IL4 can prevent autoimmune diabetes in rodent models of T1D.	Ko et al. (59)
RGS16	regulator of G-protein signaling 16	Key factor in G-protein mediated activation of lymphocytes. Plays a role in beta-cell development, proliferation, and insulin secretion.	Villasenor et al. (60); Vivot et al. (61)

The following references are included in ***Table 3*** *(26, 31–37, 39–46, 48, 50–59, 61).*

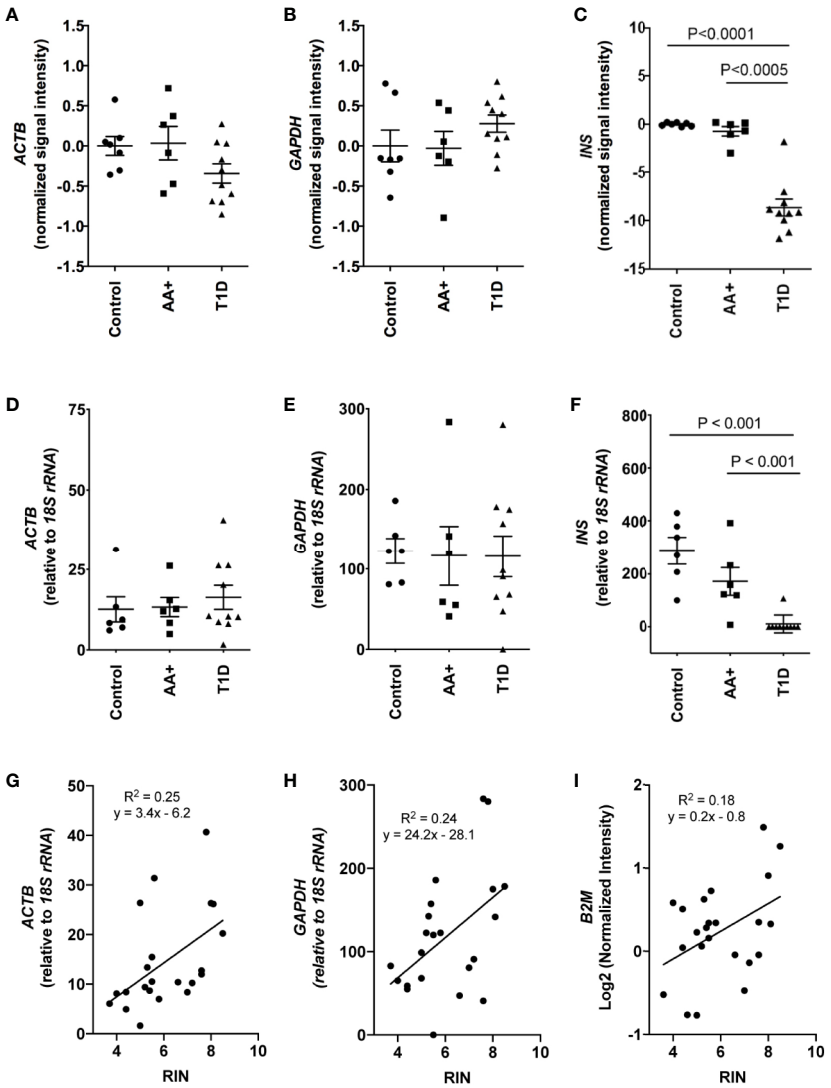

FIGURE 2 | Comparison of house-keeping gene and *INS* expression in control, AA+ and T1D samples. **(A–C)** Microarray data showing the normalized signal intensity for *ACTB* **(A)**, *GAPDH* **(B)** and *INS* **(C)** in individual control, AA+ and T1D samples. No difference in *ACTB* and *GAPDH* expression was observed between groups. *INS* expression was significantly lower in T1D patients. **(D–F)** QPCR analysis showing similar data for *ACTB* **(D)**, *GAPDH* **(E)**, and *INS* **(F)** expression. **(G–I)** Graphs showing the expression of house-keeping genes *ACTB, GAPDH*, and *B2M* against RIN values. Statistical analysis was performed using the Moderated T-test (panel A-C) or the two-tailed Mann-Whitney test (Panel **D–F**).

also known as the CB1 receptor; $p<2.55 \times 10^{-5}$), a receptor that has been linked to β-cell dysfunction and islet inflammation (62).

QPCR Validation of Biologically Relevant Genes That Are Changed in AA+ Individuals

Among the 183 genes that are significantly changed in the pancreata of AA+ individuals vs. controls, we identified 9 genes that are located in genetic loci that have previously been associated with T1D, T2D, metabolism or obesity, by GWAS studies. In addition, we identified another seven upregulated genes, and 15 downregulated genes, that have functions biologically relevant for disease pathogenesis (**Table 3**). The expression of these genes was verified by QPCR analysis. We were able to validate changes in eight out of 29 genes examined (**Figure 4**, **Supplementary Figure 2**, and **Table 5**). A significant upregulation of *CADM2*, and downregulation of *TRPM5, CRH*, *PDK4, ANGPTL4, CLEC4D, RGS16*, and *FCGR2B* was confirmed in the pancreata of AA+ vs. controls. Fold-change differences were higher when gene expression was measured by QPCR vs. microarray analysis. The QPCR cycle threshold (Ct) values for these genes are shown in **Supplementary Table 5**. RNA RIN values of the samples did not appear to influence the expression of these 8 genes when measured by microarray or QPCR analysis (**Supplementary Figures 3** and **4**).

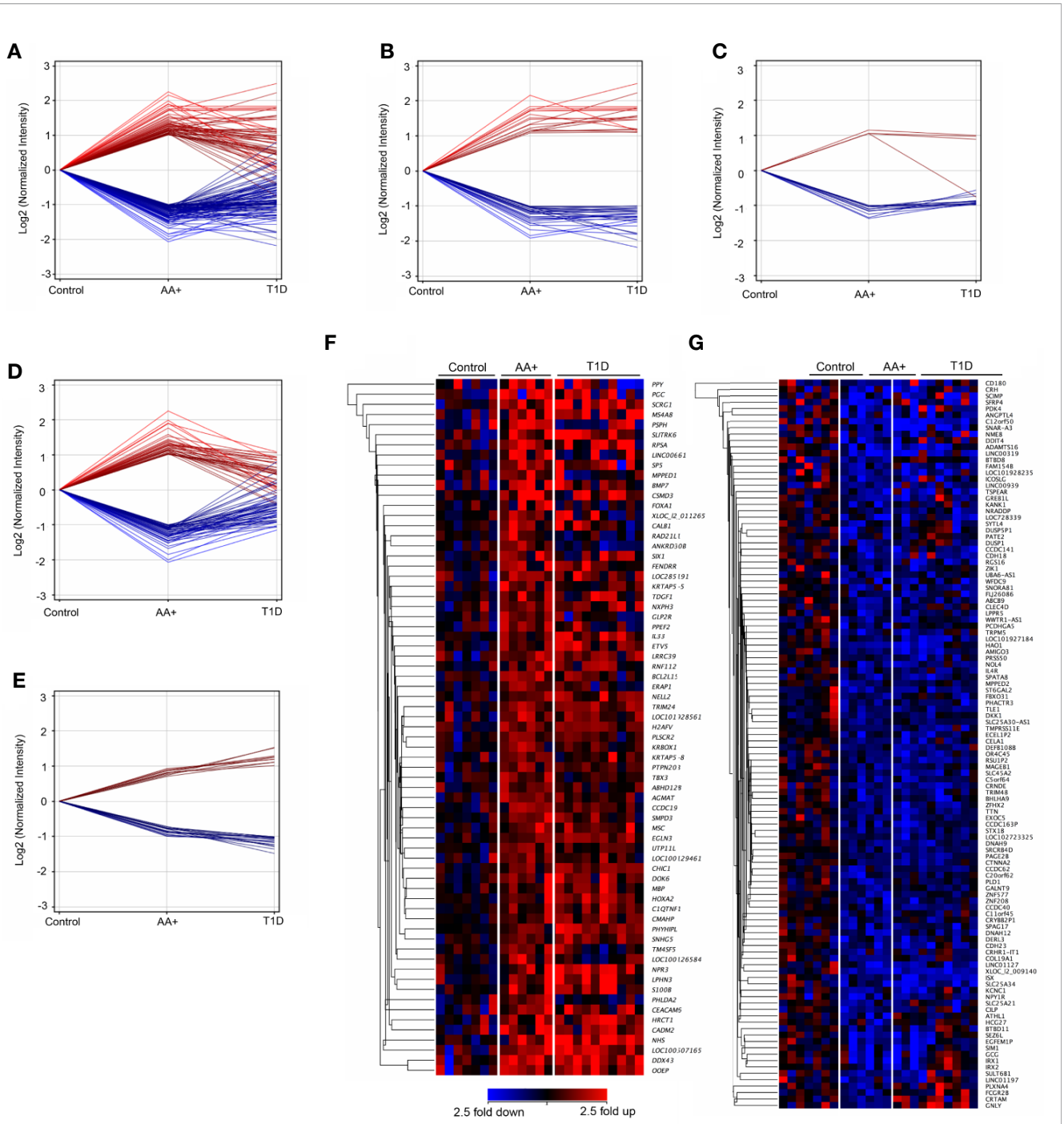

FIGURE 3 | Differentially expressed genes in the pancreata of AA+ individuals compared to controls. 155 genes (59 upregulated and 96 downregulated) were significantly changed by at least 2-fold in the pancreata of AA+ individuals vs. controls **(A).** Among these, 48 were changed by >2-fold **(B)**, 19 were changed by <2-fold **(C)**, and 88 were unchanged **(D)** in the pancreata of T1D patients vs. controls. An additional 28 genes were significantly changed by 1.5 to 2-fold in AA+ vs. controls and >2-fold in T1D vs. controls **(E)**. Gene expression heatmaps of the upregulated **(F)** and downregulated **(G)** genes from panels A and E are shown. These genes were further analyzed for biological function (See **Supplementary Table 2** and **Table 3**).

Our inability to validate additional genes may be due to inherent differences in the quantification methods used. QPCR data is normalized to house-keeping gene expression and may be affected by the choice of house-keeping gene. The Taqman assays used for QPCR may also contain primers and probes that target a different region of the gene compared to the probes on the microarray. Thus, differential expression of gene transcripts or alternative splicing of genes could result in differences between microarray and QPCR data.

TABLE 4A | Immune functions associated with differentially expressed genes in AA+ vs. control identified by IPA.

Diseases or Functions Annotation	p-Value*	Genes
function of helper T lymphocytes	1.93E-06	*CRTAM, DUSP1, ICOSLG, IL33, IL4R, NPY1R, RGS16*
proliferation of B-1 lymphocytes	1.71E-04	*FCGR2B, IL33, IL4R*
function of Th1 cells	2.89E-04	*CRTAM, DUSP1, NPY1R, RGS16*
function of Th2 cells	3.69E-04	*ICOSLG, IL33, IL4R, RGS16*
quantity of IgG2a	9.03E-04	*CD180, FCGR2B, ICOSLG, IL4R, NPY1R*
degranulation of leukocytes	9.07E-04	*FCGR2B, PLD1, PSPH, PTPN20B*
accumulation of Th2 cells	1.30E-03	*IL33, RGS16*
function of T lymphocytes	1.41E-03	*CRTAM, DUSP1, ERAP1, ICOSLG, IL33, IL4R, NPY1R, RGS16*
quantity of IgG2b	1.75E-03	*CD180, FCGR2B, ICOSLG, NPY1R*
induction of leukocytes	2.44E-03	*CRH, IL33, IL4R, MBP*
degranulation of mast cells	3.14E-03	*CRH, DUSP1, FCGR2B, IL4R, PLD1*
quantity of interleukin	3.97E-03	*CLEC4D, CRH, DUSP1, IL4R, NPY1R*
stimulation of mast cells	4.12E-03	*CRH, IL33*
Th2 immune response	4.24E-03	*ICOSLG, IL33, IL4R*
function of immune system	4.68E-03	*CLEC4D, FCGR2B, ICOSLG, IL4R, NPY1R*
activation of basophils	6.07E-03	*FCGR2B, IL33*
function of blood cells	6.67E-03	*CRH, CRTAM, DUSP1, ERAP1, FCGR2B, ICOSLG, IL33, IL4R, NPY1R, PLD1, RGS16, TRPM5*
induction of helper T cells	6.80E-03	*IL33, MBP*
accumulation of T lymphocytes	6.82E-03	*DUSP1, ICOSLG, IL33, RGS16*
induction of lymphocytes	7.28E-03	*CRH, IL33, MBP*
activation of blood cells	8.05E-03	*C1QTNF1, CD180, CEACAM6, CRH, CRTAM, DUSP1, ERAP1, FCGR2B, GNLY, ICOSLG, IL33, IL4R, MBP, NPY1R*
infiltration of cells	8.45E-03	*CRH, DUSP1, EGLN3, FCGR2B, IL33, IL4R, IRX2, MBP, S100B*
stimulation of phagocytes	8.78E-03	*CRH, IL33, IL4R*
function of leukocytes	8.83E-03	*CRH, CRTAM, DUSP1, ERAP1, FCGR2B, ICOSLG, IL33, IL4R, NPY1R, RGS16, TRPM5*
quantity of IgG	9.17E-03	*CD180, CLEC4D, FCGR2B, ICOSLG, IL4R, NPY1R*
quantity of lymph node cells	1.01E-02	*ICOSLG, IL33*
activation of leukocytes	1.08E-02	*CD180, CEACAM6, CRH, CRTAM, DUSP1, ERAP1, FCGR2B, GNLY, ICOSLG, IL33, IL4R, MBP, NPY1R*
stimulation of leukocytes	1.08E-02	*CRH, ICOSLG, IL33, IL4R, MBP*
quantity of immunoglobulin	1.09E-02	*CD180, CLEC4D, FCGR2B, ICOSLG, IL33, IL4R, NPY1R*
quantity of dendritic cells	1.25E-02	*CLEC4D, FCGR2B, GNLY, TRPM5*
hypersensitive reaction	1.26E-02	*CLEC4D, DUSP1, FCGR2B, ICOSLG, IL33, IL4R, MBP, NPY1R, PDK4*
stimulation of lymphocytes	1.35E-02	*CRH, ICOSLG, IL33, MBP*
quantity of transitional B cells	1.61E-02	*CD180, NPY1R*
quantity of lymph follicle	1.65E-02	*CD180, FCGR2B, ICOSLG, NPY1R*
inflammation of pancreas	1.72E-02	*FCGR2B, IL33*
maturation of bone marrow-derived dendritic cells	1.72E-02	*FCGR2B, NPY1R*
infiltration of leukocytes	1.77E-02	*CRH, DUSP1, EGLN3, FCGR2B, IL33, IL4R, IRX2, S100B*
delayed hypersensitive reaction	1.77E-02	*CLEC4D, FCGR2B, IL4R, NPY1R*
quantity of cytokine	1.79E-02	*CLEC4D, CRH, DUSP1, IL4R, NPY1R, SIM1*
development of Th2 cells	1.83E-02	*ICOSLG, IL4R*
proliferation of mast cells	1.83E-02	*FCGR2B, IL33*
quantity of IgA	1.88E-02	*FCGR2B, ICOSLG, NPY1R*
activation of lymphocytes	1.98E-02	*CD180, CEACAM6, CRTAM, DUSP1, FCGR2B, ICOSLG, IL33, MBP, NPY1R*

**Performed using Fisher's exact test to measure likelihood that the association between the dysregulated gene set and a related function is due to random association.*

Among the genes that we validated by QPCR, the change in *FCGR2B* expression is particularly interesting. This gene has been shown to play a role in multiple autoimmune diseases and a role in the development of NOD disease (26). *FCGR2B* encodes the inhibitory CD32B receptor that is mainly expressed in B-cells (20). To examine if the change in *FCGR2B* expression may simply reflect a change in the abundance of immune cells, we measured *CD19, CD20* and *CD11C* expression, and found no significant difference in expression between controls, AA+ and T1D patients (**Figure 5**).

Comparison of Differentially Expressed Genes in At-Risk AA+ Individuals and Pre-Diabetic NOD Mice

To determine if similar genes are altered in the pancreata of AA+ individuals and pre-diabetic NOD mice, we performed microarray analysis on pancreas samples of 12-week-old NOD mice compared to age-matched congenic non-diabetes-prone NOD.B10 mice. Female NOD mice develop insulitic lesions starting at 4 weeks of age, and the onset of destructive insulitis occurs at 12 weeks age, followed by hyperglycemia at ~16 weeks of age (13). For these experiments, pancreatic tissues of 12-week-old NOD and NOD.B10 mice were immediately isolated and homogenized in Trizol reagent. RNA prepared from these samples showed little RNA degradation, with RIN values exceeding 8 for all samples (**Figure S1**). 25,283 entities representing 15,119 genes expressed in NOD pancreas samples overlap with genes on the human microarray. Among these, 2,043 entities representing 1880 genes were differentially expressed by at least 2-fold ($p<0.01$) in the pancreata of NOD vs. NOD.B10 mice. Only 12 genes were found to be similarly

TABLE 4B | Upstream regulators of differentially expressed genes in AA+ vs. control identified by IPA analysis.

Upstream Regulator	p-Value	Targets
IL-1β	3.42E-03	*ANGPTL4, CALB1, CRH, DDIT4, DUSP1, EGLN3, FCGR2B, ICOSLG, IL33, PLD1, RGS16, RPSA, S100B*
IL-10	2.74E-02	*DUSP1, FCGR2B, IL33, IL4R, RGS16, S100B*
TNFSF13B (BAFF)	4.10E-02	*FCGR2B, ICOSLG*
TNF	2.15E-01	*ANGPTL4, CRH, DKK1, DUSP1, FCGR2B, ICOSLG, IL33, IL4R, MBP, MSC, RGS16, RPSA, UTP11L*
IL-6	4.66E-01	*CRH, DUSP1, GCG, ICOSLG, IL4R*

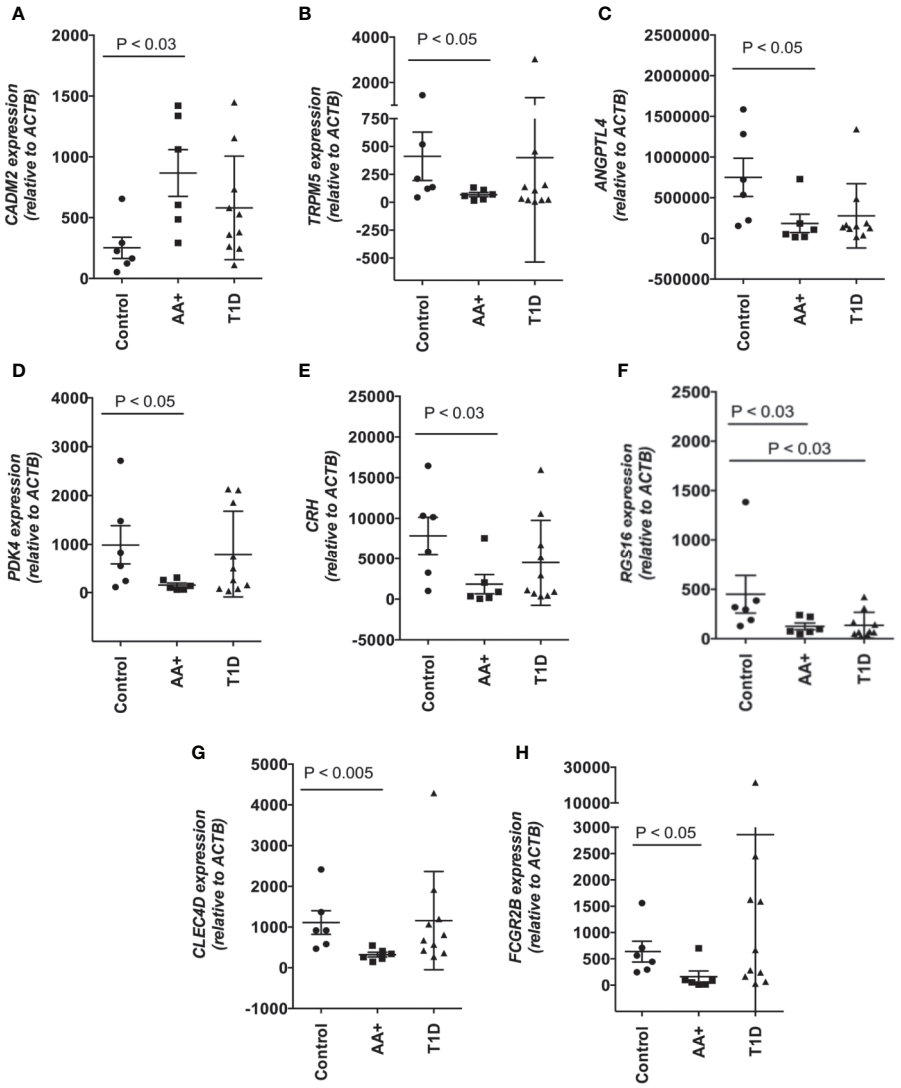

FIGURE 4 | Changes in the expression of 8 disease-relevant genes were verified by QPCR analysis. A significant upregulation of *CADM2* **(A)**, and downregulation of *TRPM5* **(B)**, *ANGPTL4* **(C)**, *PDK4* **(D)**, *CRH* **(E)**, *RGS16* **(F)**, *CLEC4D* **(G)**, and *FCGR2B* **(H)** was verified in the pancreata of AA+ vs. control individuals by QPCR analysis. Statistical analysis was performed using the two-tailed Mann-Whitney test. (See **Supplementary Figure 2** for complete set of QPCR data of all genes shown in **Table 3**).

changed in the pancreata of NOD mice and human AA+ individuals (**Figure 6A**). Interestingly 4 of the 12 genes were among those that we successfully validated by QPCR (**Figure 4**). We showed that *Cadm2* was significantly increased while *Trpm5, Pdk4* and *Angptl4* were significantly reduced in the pancreata of NOD vs. NOD.B10 mice. No changes in housekeeping genes *Actb* and *Gapdh* were observed (**Figure 6A**). *Crh* was not present in the mouse microarray. The other three genes, *Fcgr2b, Clec4d*,

TABLE 5 | Microarray and QPCR data for biologically relevant genes that are differentially expressed in the pancreata of AA+ and T1D patients vs. controls.

GWAS genes changed in AA+ vs. controls

Gene	Microarray		QPCR	
	Fold change AA+ vs. Controls	Fold change T1D vs. Controls	Fold change AA+ vs. Controls	Fold change T1D vs. Controls
CADM2	3.1*	2.3*	3.7*	2.3
BCL2L15	2.7*	1.4	1.3	1.3
ERAP1	2.1*	1.1	1.3	1.2
ETV5	1.8*	2.4*	1.4	5.0
KANK1	-2.5*	-1.4	-1.5	-1.0
SIM1	-2.4*	-2.2*	-3.2	-1.6
PLXNA4	-2.2*	1.2	-1.2	-1.1
TRPM5	-2.2*	-1.8	-6.0*	-1.0
ABCB9	-2.2*	-2.1*	-1.3	-1.4

Biologically relevant genes: upregulated in AA+ vs. controls

Gene	Microarray		QPCR	
	Fold change AA+ vs. Controls	Fold change T1D vs. Controls	Fold change AA+ vs. Controls	Fold change T1D vs. Controls
PGC	4.8*	1.8	15.8	3.5
CEACAM6	2.9*	1.9	2.6	3.9
CALB1	2.6*	1.7	3.2	4.4
S100B	2.5*	2.7*	1.4	2.1
SMPD3	2.3*	1.7	-1.2	-1.4
ETV5	1.8*	2.4*	1.4	5.0
IL33	1.7*	2.9*	-1.6	3.1

Biologically relevant genes: downregulated in AA+ vs. controls

Gene	Microarray		QPCR	
	Fold change AA+ vs. Controls	Fold change T1D vs. Controls	Fold change AA+ vs. Controls	Fold change T1D vs. Controls
CRH	-4.2*	-2.2	-4.3*	-1.7
PDK4	-4.0*	-1.6	-6.4 *	-1.2
ANGPTL4	-3.6*	-2.6*	-4.1*	-2.7
SCIMP	-3.6*	-2.0	-2.5	1.3
SEZ6L	-3.0*	-1.9	-5.0	-2.6
ICOSLG	-2.6*	-1.6	-1.4	1.3
PLD1	-2.5*	-1.4	-1.7	-1.1
TRPM5	-2.2*	-1.6	-6.0*	-1.0
GNLY	-2.2*	1.8	1.1	10.5
CRTAM	-2.2*	1.4	-3.7	-1.1
CD180	-2.2*	1.1	-2.1	-1.3
FCGR2B	-2.1*	1.2	-4.0 *	4.5
CLEC4D	-2.1*	-1.3	-3.4*	1.0
IL4R	-2.0*	-1.9*	-1.7	1.4
RGS16	-1.7*	-2.4*	-3.5*	-3.3*

$P < 0.05$ for AA+ vs. control or T1D vs. control. See Methods for statistical analysis.

and *Rgs16*, that were reduced in the pancreata of AA+ individuals, were not changed in the pancreata of NOD mice (**Figure 6B**). These genes are abundantly expressed on B-cells, T-cells, DCs, and/or macrophages (20, 63–65). Thus their expression in the pancreas is impacted by the presence of immune cells in the insulitic lesion of NOD mice. Accordingly, we found significantly elevated expression of various leukocyte markers including *Cd3e* and *CD28* (T-cell markers), *Cd20* and *B220 (*B-cell markers), *Cd11c* (*Itgax*; dendritic cell marker), and *Cd11b* (*Itgam;* macrophage marker) in the pancreata of NOD vs. NOD.B10 mice (**Figure 6C**).

FCGR2B Gene Expression in Whole Blood Samples

Since *FCGR2B* is highly expressed on B-cells, we asked whether the expression of this gene could serve as a potential biomarker of disease progression in peripheral blood cells. QPCR was performed to measure *FCGR2B* expression in whole blood samples of AA- first-degree relatives of T1D patients (AA- FDRs; n=15), AA+ FDRs who later progressed to hyperglycemia (AA+ progressors, n=20), and established T1D patients. Remarkably, we showed that *FCGR2B* expression was significantly lower in the whole blood of AA+ progressors and T1D patients compared to

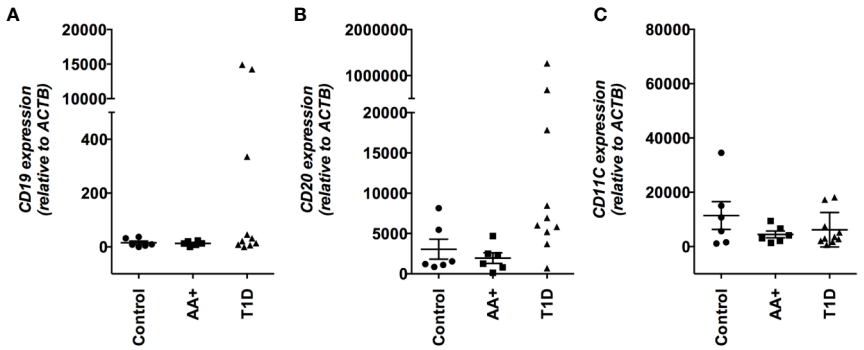

FIGURE 5 | Expression *CD19, CD20*, and *CD11c* in the pancreata of AA+ and T1D patients compared to controls. Expression levels were measured by QPCR and statistical analysis was performed using the two-tailed Mann Whitney test.

AA- FDRs (**Figure 7A**). *CD19, CD20*, and *CD11c* expression was not changed in AA+ progressors, while *CD19* and *CD20* expression was significantly elevated in peripheral blood cells of T1D patients vs. AA- FDRs (**Figures 7B–D**). When *FCGR2B* is expressed as a ratio of the B-cell markers *CD19* or *CD20*, the differences observed between AA+ progressors and T1D vs. AA- FDRs were more significant (**Figures 7E, F**). These data suggest that a loss of *FCGR2B* expression may occur in the peripheral blood, possibly in B-cells, during the development and progression of T1D.

DISCUSSION

A number of gene expression analysis studies have previously been performed to examine disease pathogenesis in T1D. The majority of studies, however, have been carried out using peripheral blood samples of AA+ at-risk and early onset T1D patients, while relatively few have been performed in the pancreata of these individuals, where insulitis, and islet destruction occur (66–72). This is partly due to the lack of pancreas samples available for study, and the difficulty in extracting high-quality RNA from these tissues. High levels of endogenous RNAses, DNases and proteases present in the pancreas result in the rapid autolysis of tissues following tissue damage, and lead to varying levels of RNA degradation (16, 73, 74). This is frequently observed in tissues collected post-mortem, when extensive tissue processing times are unavoidable. Pancreatic tissues collected from live individuals yield high quality RNA, but are difficult to obtain. In the Diabetes Virus Detection (DiViD) study, pancreatic biopsies were collected from live recent onset T1D patients, however, enrollment was halted when serious complications developed in 3 of the 6 individuals enrolled in the study (75, 76).

In this study, we utilized pancreatic tissue samples from JDRF nPOD for gene expression analysis. This repository contains one of the largest collections of tissues recovered from at-risk AA+ and T1D patients. Pancreatic tissues collected by nPOD are routinely minced and treated with RNAlater to increase RNA stability (73). The quality of the RNA extracted from pancreatic samples stored at nPOD has previously been assessed, and found to vary based on several factors including cause of death. Samples from head trauma victims had lower RIN values (18). This was also observed in our set of samples (**Table 1**). While appreciable levels of RNA degradation occurred in some of the samples, we were still able to identify a number of biologically relevant genes that were differentially expressed in AA+ and T1D patients compared to controls. Others have already shown that degraded RNA can be used successfully for transcriptome analysis and that the biological differences between groups outweigh the effects of RNA degradation on gene expression (19).

This study is the first to show that a distinct subset of genes is differentially expressed in the pancreata of at-risk AA+ individuals compared to T1D patients and to healthy controls. Of the 155 genes changed by >2-fold in the pancreata of AA+ individuals, only one-third remained changed in the pancreata of established T1D patients, reflecting the transient nature of events that are active during the early stages of disease development. After the establishment of hyperglycemia, the number of differentially expressed genes increased by ~4-fold to 645 genes. This includes genes such as *INS* that are lost as a result of β-cell death (**Figure 2**).

Changes in the gene expression profile of AA+ individuals reflect the activation of various immune pathways (**Table 4A**), and are consistent with the increased CD4+ and CD11c+ cell infiltration previously observed in the pancreata of AA+ patients (9). A number of cytokines including IL-1β, IL-10, TNSF13B (BAFF), TNF, and IL-6 were identified by IPA analysis to be upstream regulators of various differentially expressed genes (**Table 4B**). These cytokines have already been shown to play a role in the development of T1D (77–82).

To identify novel mechanisms that may contribute to disease pathogenesis, we focused on biologically relevant genes that were changed in the pancreata of AA+ individuals, and successfully validated the differential expression of 8 genes (*CADM2*, *TRPM5, PDK4, ANGPTL4, CRH*, *CLEC4D, RGS16*, and *FCGR2B*) by QPCR. We also performed gene expression analysis on 12-week-old NOD mice, during the onset of destructive insulitis,

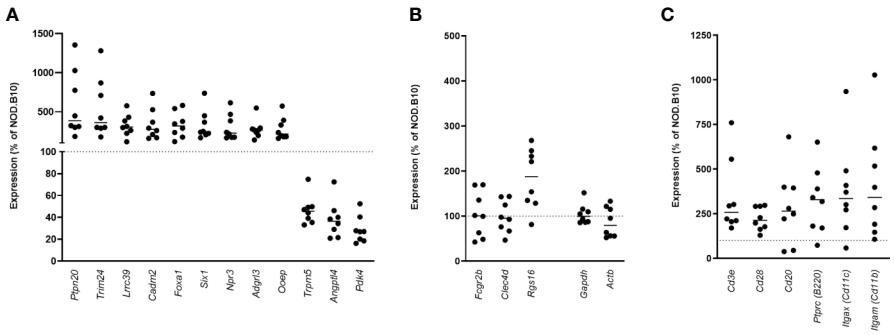

FIGURE 6 | Gene expression in the pancreata of pre-diabetic 12-week-old NOD mice compared to AA+ individuals. 12 genes were found to be similarly changed in the pre-diabetic pancreata of 12-week-old NOD vs. NOD.B10 control mice and the pancreata of AA+ individuals vs. controls **(A)** Surprisingly, 4 of these genes were among the biologically relevant genes that we verified by QPCR (**Figure 4**). *Fcgr2b, Clec4d*, and *Rgs16* were not found to be reduced in the pancreata of NOD mice **(B)**, likely due to the high level of insulitis. Abundant expression of various immune cell markers was observed in the NOD pancreas **(C)**.

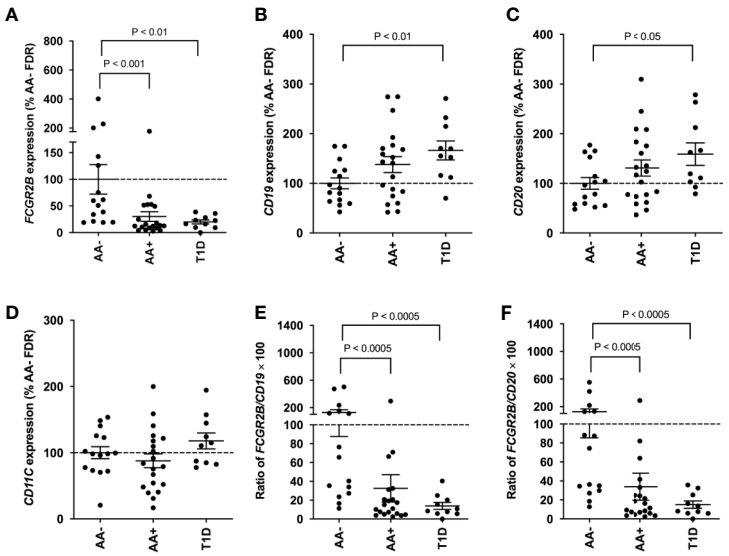

FIGURE 7 | Loss of *FCGR2B* gene expression in the peripheral blood of AA+ FDRs and T1D patients. QPCR data showing *FCGR2B* **(A)**, *CD19* **(B)**, *CD20* **(C)**, and *CD11c* **(D)** expression in the whole blood of AA- FDRs, AA+ progressors, and T1D patients. *FCGR2B* expression is also shown relative to *CD19* **(E)** and *CD20* **(F)** expression for each individual. Statistical analysis was performed using the two-tailed Mann-Whitney test.

to identify genes or disease mechanisms that may be similarly changed in the pancreata of pre-diabetic NOD mice and "pre-diabetic" AA+ at-risk individuals. NOD disease shares many similarities with human T1D, however, there are striking differences in disease progression, especially in the pancreas where relatively mild insulitis is observed in humans, and large insulitic lesions and highly infiltrated islets are observed in NOD mice (83). As a result, the most differentially expressed genes observed in the pancreata of pre-diabetic NOD mice vs. NOD.B10 control mice are those expressed in the infiltrating leukocytes (See GEO series GSE154739).

We showed that only 12 genes were similarly changed in the pancreata of NOD mice and AA+ individuals (**Figure 6A**). Surprisingly, four of these genes were among the eight disease-relevant genes that we validated to be changed in the pancreata of AA+ individuals. These four genes, *Cadm2*, *Trpm5, Pdk4*, and *Angptl*, are not highly expressed in leukocytes and thus, their expression is not significantly impacted by the presence infiltrating cells in the NOD pancreas. The other three genes validated to be reduced in the pancreata of AA+ individuals, *Clec4d, Rgs16*, and *Fcgr2b*, are abundantly expressed on leukocytes, thus, their expression in the NOD pancreas would be significantly influenced by the high level of insulitis. The *Crh* gene was not present on the mouse microarray.

Our data suggest that increased expression of *CADM2*, and reduced expression of *TRPM5, PDK4*, and/or *ANGPTL4* could

play a role in the development of both human T1D and NOD disease by altering mechanisms that control insulin sensitivity, insulin secretion, β-cell and pancreas development, and/or islet cell organization. The *CADM2* gene encodes cell adhesion molecule 2, a mediator of synaptic signaling. GWAS studies have identified SNPs near *CADM2* that are associated with higher expression of CADM2, and the development of obesity (30, 84). In animal models, increased *Cadm2* expression is associated with obesity, while knockout or loss of *Cadm2* prevents obesity, improves insulin sensitivity and protects mice from developing diabetes (31, 84). Interestingly, Cadm2 expression is reduced in obese and insulin-resistant mice by treatment with leptin (84), an adipose hormone that has been suggested as a potential treatment for T1D (85).

The *TRPM5* gene, encodes the transient receptor potential cation channel, subfamily M, member 5, a calcium-activated non-selective channel expressed in β-cells that is an indispensable regulator of insulin secretion (86, 87). In humans, genetic variation within the *TRPM5* locus associates with pre-diabetic phenotypes in subjects who are at risk for developing T2D (38). In mice, loss of *Trpm5* expression results in compromised glucose-stimulated insulin secretion, and maintained elevated blood glucose levels following a glucose challenge (37). TRPM5 has also been shown to mediate insulin secretion by L-arginine and to potentiate glucose-induced insulin secretion by glucagon-like peptide 1 (88, 89).

The *ANGPTL4* gene, encodes angiopoietin-like 4, a protein that is critically involved in maintaining normal glucose and lipid metabolism (49). In the islets, Angptl4 is expressed in glucagon-secreting alpha cells, and enhances glucose-stimulated insulin secretion. Mice deficient in *Angptl4* have impaired glucose tolerance, secreting significantly lower levels of insulin in response to glucose-stimulation, and have dysmorphic islets with abnormally distributed alpha cells (50). Interestingly, we had previously observed disorganization of alpha cell distribution within the islets of 12-week-old NOD mice (14). It is possible that this may result from the significant loss of *Angptl4* expression in the pancreas.

The *PDK4* gene encodes pyruvate dehydrogenase kinase 4. In the pancreas, PDK4 may induce the expression of the pancreas-specific transcription factor 1a (PTF1A) (48). In mice, Ptf1a is involved in pancreatic development and function. Reduced Ptf1a levels leads to pancreatic hypoplasia and glucose intolerance. Ptf1a levels are a determinant of pancreatic size (90). This is interesting, as reduced pancreatic size has been observed in T1D patients, AA+ individuals, and AA- first-degree relatives of T1D patients (7, 8, 91, 92). Ptf1a also activates expression of Pdx1 (Pancreas/duodenum homeobox protein 1) (93, 94), an important transcription factor that regulates β-cell proliferation, differentiation, and function (95). It is possible that reduced levels of PDK4 lead to loss of PTF1A, and subsequently, a loss of PDX1 expression in the pancreata of AA+ individuals and NOD mice.

The *CRH* gene encodes corticotropin-releasing hormone, an important regulator of the hypothalamic-pituitary-adrenal (HPA) axis. In islets, CRH activates corticotropin-releasing hormone receptors on β-cells to stimulate insulin secretion, and promote islet development and proliferation (46, 47, 96). Reduced levels of CRH have been observed in the plasma of T2D patients (97). Reduced pancreatic expression of *CRH* may increase the risk for developing T1D in AA+ individuals.

We observed significantly reduced expression of 3 other genes, *RGS16, CLEC4D*, and *FCGR2B*, in the pancreata of AA+ individuals, but not in that of NOD mice. These genes are enriched in leukocytes, and loss of their expression may contribute to the development of T1D by promoting inflammation, activating T cells and B-cells, and altering β-cell development. The *RGS16* gene encodes regulator of G-protein signaling 16, and is abundantly expressed in immune cells and pancreatic islets (61, 98–100). Increased RGS16 expression inhibits T cell migration in response to various chemokines, and downregulates pro-inflammatory cytokine production in monocytes (99, 100). In the pancreas, RGS16 is highly enriched in β-cells, where it is involved in the cell development and proliferation (60), and in the regulation of insulin secretion (61).

The *CLEC4D* gene encodes a C-type lectin mycobacterial receptor that is highly expressed in macrophages and myeloid cells. CLEC4D plays a crucial role in anti-mycobacterial host defense. In mice, loss of Clec4d expression results in elevated inflammation, increased mycobacterial levels, and increased mortality (58). In humans, a polymorphism in the *CLEC4D* gene that results in reduced expression, is associated with increased susceptibility to pulmonary tuberculosis (58). T1D patients have a reduced capacity to produce pro-inflammatory cytokines in response to *Mycobacterium tuberculosis* (101), and have a higher risk of developing tuberculosis (102). This may involve a loss of CLEC4D expression.

The *FCGR2B* gene encodes CD32B, an inhibitory low affinity receptor for the Fc region of IgG complexes that is highly expressed on B-cells. This receptor contains an immunoreceptor tyrosine-based inhibitory motif that negatively regulates the BCR complex and its signaling threshold (20). Activation of CD32B in B-cells ultimately leads to diminished BCR-dependent cell activation and antibody production, and loss of CD32B expression can lead to hyper-responsiveness, proliferation and maturation of B-cells. Diminished CD32B expression has already been observed in a number of autoimmune diseases. The autoimmune susceptibility of various strains of mice including NOD mice has also been attributed to polymorphisms in the *FCGR2B* gene that result in reduced CD32B expression in germinal center B-cells (26, 27). Loss of *FCGR2B* gene expression, and reduced surface expression of CD32B have been observed in activated B-cells of NOD mice, and human systemic lupus erythematosus patients (24–27), while a lower frequency of CD32B+ B-cells, and lower mean fluorescent intensity of CD32B expression have been observed in B-cells of rheumatoid arthritis patients compared to controls (23). Reduced *FCGR2B* gene expression has also been observed in the PBMCs of patients with active Graves' disease (92).

Here, we showed that *FCGR2B* gene expression was significantly reduced in the pancreas and peripheral blood of AA+ progressors and T1D patients compared to controls. *CD19,*

CD20, and *CD11c* expression was not significantly changed in AA+ progressors. However, when *FCGR2B* is expressed relative to *CD19* or *CD20* expression, differences between the AA+ progressors vs. AA-FDRs were more significant. This was also observed in T1D patients, suggesting that *FCGR2B* expression may be diminished in B-cells of "pre-diabetic" AA+ individuals and T1D patients. There is abundant evidence suggesting a role for B-cells in T1D (103–106). A loss of CD32B expression may lead to deficient negative feedback regulation of the BCR resulting in intrinsic hyper-responsiveness, proliferation and maturation of B-cells, and contribute to the onset of T1D. Since significantly reduced expression of *FCGR2B* may be seen in AA+ individuals years before the onset of T1D (**Table 2**), this gene could potentially serve as an early biomarker of disease progression, and be used to predict disease onset. Flow cytometry experiments are currently in progress at our lab to study the expression of CD32B in longitudinal blood samples collected from AA+ individuals and healthy controls.

Together, our data show that different pathological events driven by changes in *CADM2*, *TRPM5, PDK4, ANGPTL4, CRH*, *CLEC4D, RGS16*, and *FCGR2B* gene expression may contribute to the development of T1D. These genes are involved in various functions including pancreatic development, β-cell proliferation, insulin sensitivity and secretion, inflammation, and/or immune cell function. Changes in the expression of these genes were mainly observed in the pancreata of AA+ individuals, during the active development of disease, and overlapped well with changes observed in the pancreata of pre-diabetic NOD mice. These findings emphasize the importance of utilizing tissues collected from pre-diabetic individuals, rather than established T1D patients, in understanding the pathogenesis of disease and in identifying early biomarkers of disease progression. Biorepositories such as nPOD and TrialNet play a crucial role in providing access to these rare and important samples.

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: https://www.ncbi.nlm.nih.gov/geo/, GSE72492; https://www.ncbi.nlm.nih.gov/geo/, GSE154739.

ETHICS STATEMENT

The animal study was reviewed and approved by Stanford University Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

LY performed the microarray and QPCR experiments, performed the data analysis, prepared the manuscript, and composed the figures and tables. RF performed RNA extraction, bioanalyzer, and QPCR analysis. RA performed QPCR experiments. CF and LY were involved in the planning and direction of 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/fendo.2020.609271/full#supplementary-material

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MINI REVIEW
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New Insights Into the Role of Autoreactive CD8 T Cells and Cytokines in Human Type 1 Diabetes

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Since the establishment of the network for pancreatic organ donors with diabetes (nPOD), we have gained unprecedented insight into the pathology of human type 1 diabetes. Many of the pre-existing "dogmas", mostly derived from studies of animal models and sometimes limited human samples, have to be revised now. For example, we have learned that autoreactive CD8 T cells are present even in healthy individuals within the exocrine pancreas. Furthermore, their "attraction" to islets probably relies on beta-cell intrinsic events, such as the over-expression of MHC class I and resulting presentation of autoantigens such as (prepro)insulin. In addition, we are discovering other signs of beta-cell dysfunction, possibly at least in part due to stress, such as the over-expression of certain cytokines. This review summarizes the latest developments focusing on cytokines and autoreactive CD8 T cells in human type 1 diabetes pathogenesis.

Keywords: type 1 diabetes, autoreactive CD8 T cells, human pancreas, beta cells, cytokines

INTRODUCTION

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease in which the pancreatic insulin-secreting beta cells are selectively destroyed. From decades of T1D studies performed in mice, profound insulitis was thought to be a common feature of T1D. However, with more extensive recent histological studies in the human pancreata, it is evident that insulitis is not that pronounced in patients with T1D (1). Autoreactive CD8 and CD4 T cells are critical players in beta-cell destruction (2) as they recognize peptides of beta-cell antigens that include (pro)insulin and its precursor preproinsulin (PPI), tyrosine phosphatase-like insulinoma antigen (IA-2), islet-specific glucose-6-phosphate catalytic subunit-related protein (IGRP), glutamic acid decarboxylase-65 (GAD65), zinc transporter protein 8 (ZnT8), and islet amyloid polypeptide (IAPP) (3–9). While autoreactive CD8 T cells are activated through interaction with peptides presented by HLA class I (e.g., HLA-A*02:01, -A*24:02, and -B*39:06) (3, 6, 10), CD4 T cells recognize peptides presented by HLA class II molecules (e.g., HLA-DR4, -DQ8) (8, 11, 12). However, it remains unexplored how they cooperate in islet destruction during T1D development as visualizing T cell responses in the human pancreas *in vivo* remains challenging. Nevertheless, mouse studies suggest that CD4 T cells provide help to effector CD8 T cells, stimulate antibody production by B cells, and activate islet-resident macrophages (13, 14). In addition, intravital two-photon microscopy in a mouse model of autoimmune diabetes indicates a crucial role of CD4 T helper cells in sustaining effector functions of cytotoxic CD8 T cells at target sites (13).

It is not clearly known what microenvironmental cues drive autoreactive T cells near the islets in the context of T1D. It is thought that factors such as cytokines, chemokines, oxidative stress, and

altered antigen presentation may stimulate CD8 T cells, which were previously ignoring self-antigens, to activate and expand. The fact that the autoreactive T cells are also present in the pancreata of healthy donors (15) suggests a possibility of a triggering event in beta cells, which leads to the recruitment of these cells to the islets in T1D. In this review, we will discuss beta cell-intrinsic events that may increase the visibility of beta cells to the immune system and contribute to the recruitment of autoreactive T cells to the islets.

MINI-REVIEW

What Distinguishes T1D Patients From Healthy Individuals?

For several years, researchers studied T cell responses against islet and beta-cell antigens in peripheral blood to find signatures that differentiate between T1D patients and healthy individuals. Initial studies suggested that autoreactive T cells escape negative selection (16, 17) in the thymus and are released into the peripheral blood in patients with T1D, but not in healthy individuals. Then, they get activated by antigen-presenting cells in the draining lymph nodes, which present epitopes derived from islet antigens. This hypothesis was supported by the linkage of polymorphism in the *INS* gene locus that determines the level of insulin expression in the thymus (17). However, many studies showed that autoreactive T cells are present in healthy individuals' peripheral blood (4, 6), refuting the belief that autoreactive T cells escape negative selection only in T1D patients. Thus, it raises the question of whether the frequencies of such autoreactive T cells in peripheral blood can help identify patients with T1D. Indeed, higher frequencies of circulating beta-cell specific CD8 T cells were detected in HLA-A*02:01$^+$ or HLA-A*24:02$^+$ subjects with T1D compared with healthy donors (3, 7). Among them, PPI-reactive CD8 T cells were more frequently found in T1D patients than in healthy donors (4, 5, 7). On the other hand, other studies demonstrated no significant differences between T1D and control individuals in the frequencies of circulating CD8 T cells reactive to multiple A*02:01-restricted beta-cell epitopes (PPI_{14-25}, PPI_{6-14}, $InsB_{10-18}$, $GAD_{114-123}$, IA-$2_{797-805}$, IA-$2_{805-813}$, $IGRP_{265-273}$, $ZnT8_{186-194}$) (3, 4, 6, 10). However, beta cell-specific CD8 T cells are more differentiated in patients with newly diagnosed T1D compared to healthy controls (6), and memory T cell subsets were enriched within PPI_{5-12}-specific CD8 T cell populations in HLA-B*39:06$^+$ children with newly diagnosed T1D, but not in healthy control subjects (10). Overall, these studies suggest that phenotypic subset analysis of such circulating autoreactive T cells could help to identify patients with T1D.

The Presence of Autoreactive CD8 T Cells in the Target Organ

The primary critique of studies performed using peripheral blood is that the relationship between tissue-infiltrating and circulating autoreactive CD8 T cells is poorly defined. Many studies of postmortem samples of pancreata revealed the dominant presence of CD8 T cells in donors with T1D and healthy pancreas (18–20). Using HLA class-I tetramers, Coppieters et al. provided the first proof that CD8 T cells reactive against $IGRP_{265-273}$, IA-$2_{797-805}$, and PPI_{15-24} could be found *in situ* in the islets from individuals with recent-onset and long-standing T1D (21). In these cases, it appeared that early after diagnosis, the islets contained CD8 T cells specific for one particular autoantigen, whereas, in patients with long-standing T1D, those islets with insulitis showed T cells with multiple specificities. Building on these findings, Bender et al. further examined the precise localization of PPI_{15-24}-reactive CD8 T cells not only in the pancreas of nPOD donors with T1D but also in autoantibody-positive and healthy controls (15). Notably, the study showed that many PPI_{15-24}-specific CD8 T cells are present in the exocrine pancreas of healthy donors and donors with autoantibodies supporting the theory of a general leakiness of central tolerance. In T1D patients, these cells were not only enriched in the exocrine pancreas but were also present within the islets or close to the islets (15). The PPI-specific CD8 T cells significantly infiltrated insulin-containing islets suggesting a critical effector mechanism leading to beta-cell destruction. In line with this, *in-situ* staining of nPOD pancreas sections revealed similar numbers of $ZnT8_{186-194}$-positive A*02:01-restricted cells in the pancreas of healthy and autoantibody-positive donors but enriched in the pancreas of donors with T1D (4). Surprisingly, the frequencies of PPI_{15-24}-reactive CD8 T cells detected in the exocrine pancreas were similar irrespective of disease status. Remarkably, many CD8 T cells recognized the PPI_{15-24} epitopes (15). In contrast to previous studies, where less than 1% of autoreactive CD8 T cells were detected within the peripheral blood (4, 5), frequencies in the pancreas are much higher (30-40%) (15). Besides, the majority of PPI-reactive CD8 T cells in the exocrine pancreas were positive for CD45RO, suggesting an antigen-experienced phenotype.

Besides conventional peptides, CD8 T cells can also recognize post-translationally modified peptides, $IAPP_{15-17}$/$IAPP_{5-10}$, and $SCG\text{-}009_{186-194}$ generated by mRNA splicing and transpeptidation, respectively (9). Gonzalez-Duque et al. revealed an enriched presence of $IAPP_{15-17}$/$IAPP_{5-10}$, urocortin 3 $(UCN3)_{1-9}$, and transcription factor $ISL1_{276-284}$ reactive cells in the human pancreas of donors with T1D (9). In comparison, the frequency of these reactive cells was similar between T1D patients and healthy individuals (9).

Although CD8 T cells are the most predominant immune infiltrates in human insulitis [1], autoreactive CD4 T cells are also involved in the pathogenesis of T1D and CD4 T cell responses to proinsulin, GAD, and IA-2 were identified in the islets of donors with T1D (8, 22, 23).

Consequently, what determines the progression of T1D in the face of similar frequencies of autoreactive CD8 T cells? Do beta-cells respond differently to inflammation that causes the attraction of CD8 T cells? The high numbers of autoreactive CD8 T cells in the pancreas suggest that autoreactivity is physiological and that disease development, therefore, is prevented under normal circumstances by local, organ-specific control mechanisms. Thus, defective organ-specific control mechanisms and/or a pro-inflammatory islet microenvironment are key pathogenic features of T1D.

How Stressed Beta Cells Secrete Cytokines That May be Involved in Bringing in Autoreactive T Cells?

Cytokine and chemokine secretion are broadly implicated as key immune cell recruiting factors in T1D. It is believed that cytokines are typically secreted by immune cells during the pathogenesis of T1D. However, recently mounting evidence suggests that pancreatic islets per se secrete cytokines under physiological conditions or under stress. For instance, IL-6 was shown to be expressed by both beta and alpha cells in non-diabetic and autoantibody-positive individuals, but its expression was reduced in donors with T1D (24). Similarly, alpha cells were shown to express IL-1b in pancreatic tissue sections, irrespective of the diabetes status (25). Cytokines such as IL-6 act at a physiological level in islet cells to maintain glucose homeostasis (26), whereas other cytokines such as IFN-γ and CXCL10 may play a pathogenic role by contributing to immune cell recruitment and beta-cell killing. Cytokine receptors such as IL-4R, IL-13R, IL-6R were all shown to be widely expressed by islet cells (27–29), underscoring the possibility of cytokine-induced changes in islet cells that could facilitate immune attack. Here we will discuss some islet-intrinsic factors that may be involved in the recruitment of autoreactive T cells and secretion of pathogenic cytokines implicated in T1D.

Among the beta cell-intrinsic events, hyperexpression of MHC class I has been considered as a hallmark feature of T1D (30). Homing of autoreactive T cells to islets was inhibited in the absence of MHC class I expression in the NOD model of T1D (31). Treatment of human islets with IFN-γ induced MHC class I hyperexpression along with upregulation of chemokines such as CXCL10 in beta cells (32). Multiple reports identify pro-inflammatory cytokines and viral infection as major inducers of MHC class-I in islets. Although no viral infection has been shown to cause T1D directly, it may be possible that infection of beta cells may induce some islet–intrinsic changes. For instance, human islets upon infection with Coxsackie virus B (CVB) have been shown to secrete increased levels of IL-6, TNF, IP-10, and interferon-stimulated genes (33). Gallagher et al. have attempted to study CVB infection in an islet transplantation model devoid of native beta cells. Transplantation of healthy human islets in these mice reversed hyperglycemia, but this effect was abrogated upon infection of these mice with Coxsackievirus B. Signatures of viral RNA and increased levels of interferon-stimulated genes, CXCL10 and CCL5, could be observed in the transplanted islets (34). These evidence further prove that human islets could be infected with CVB, which could further drive pro-inflammatory factors and chemokines. The enrichment of autoreactive CD8 T cells near the islets suggests that they become attracted to their key antigen in insulin-containing islets during disease development (15), possibly due to the upregulation of MHC class I (30) and accumulation of target autoantigen (35). However, it is not clearly known whether MHC class I upregulation is responsible for the recruitment of autoreactive T cells or vice versa.

CXCL10 or IP-10 (IFN- inducible protein-10) is one of the most important cytokines implicated in T1D pathogenesis. CXCL10 expression could be observed in the beta cells of donors with recent-onset T1D, irrespective of their infection status with enterovirus. CXCR3, the receptor of CXCL10, could also be observed in proinsulin specific T cells of the T1D patient. In contrast, islets of non-diabetic donors were devoid of both CXCL10 and CXCR3 (36). Another independent study similarly reported that CXCL10 is expressed predominantly on beta cells and not by other endocrine cells in insulin-containing islets of T1D subjects (37). More recently, Nigi et al. have shown CXCL10 expression in alpha cells and beta cells of donors with T1D. Interestingly, insulin-containing islets in T1D had an increased percentage of CXCL10-positive alpha cells compared to CXCL-10-positive beta cells suggesting an involvement of alpha cells in chemokine secretion (38). Both CXCL9 and CXCL10 have been shown to be secreted by beta cells in insulitic islets, which drive CXCR3+ autoreactive T cells to islets in the RIP-LCMV model of T1D. The deletion of the CXCR3 gene in these mice has been beneficial in delaying the onset of T1D and insulitis (39). In addition, production of CXCL10 by autoreactive CD8 T cells was shown to be an essential factor in determining the diabetogenicity of CD8 T cell clones (40).

CCL21 has also been reported to be an essential factor for the homing of insulin-specific CD8 T cells to islets and for the interaction of islets with endothelial cells presenting the major auto-antigen, Insulin (31). Ectopic expression of CCL21 in beta cells influenced the recruitment of T cells, B cells, and dendritic cells to the islet periphery in a murine T1D model (41). Interestingly, expression of CCL21 by islets in NOD mice protected the mice from T1D; however, these mice still exhibited insulitis with T cells and fibroblastic reticular cell (FRC)–like cells expressing autoantigen, possibly inducing an antigen-specific immune tolerance (42).

In recent onset cases with T1D, laser capture microdissection studies have shown the overexpression of several interferon-stimulated genes such as *GBP1*, *TLR3*, *HLA-E*, and *STAT1* transcripts, compared to islets from non-diabetic individuals (43). Single-cell RNA sequencing of human islets revealed that chemokine transcripts were the most up-regulated among other transcripts upon exposure to pro-inflammatory cytokines. This report also confirmed the expression of cytokines and chemokines such as IL-6, IL-8, CCL2, and CXCL10 at the protein level (44). Exposure of human islets to cytokines, especially IL-1β and IFN-γ, has resulted in increased extracellular accumulation of proinsulin, suggesting its disproportionate conversion of proinsulin to insulin. This evidence points towards the role of cytokines in beta cell dysfunction (45). In contrast, the treatment of human islets with IFN-γ and IFN-α in-vitro has resulted in the upregulation of PDL1 by beta cells. PDL1 was also found to be expressed in insulin-containing islets from donors with T1D, but not in non-diabetic controls. Whether this phenomenon is a compensatory mechanism to reduce T cell activation is yet to be studied (46). Similarly, SOCS (suppressor of cytokine signaling) 1, 2, 3 were all expressed in the islets of donors with T1D, but not in non-diabetic donors. SOCS expression was up-regulated upon exposure of human islets to IFN-γ, TNFα, and IL-1β.

Additionally, the transfection of SOCS1 in beta cells has been reported to inhibit IFN-γ signaling and MHC class I hyperexpression (47). This evidence suggests that beta cells could up-regulate SOCS in an attempt to protect the islets from cytokine-induced cell damage (48).

Immunohistochemical studies of donors with T1D revealed increased expression of some components of ER stress, such as CHOP and BIP, compared to non-diabetic individuals. These components mostly co-stained with insulin (49), implying that beta cells could be undergoing cellular stress during T1D. Human beta cells pre-treated with an ER stress inhibitor, TUDCA resulted in reduced cytokine-induced JNK pathway, IRE1α (inositol-requiring enzyme 1α) activity, a key component of unfolded protein response, and apoptosis (50).

Therefore, these events, in addition to other factors leading to islet-attraction of autoreactive CD8 T cells already within the pancreas, may be a crucial mechanism in T1D development.

CONCLUDING QUESTIONS

In this review, we discussed the evidence that autoreactive CD8 T cells are an integral and large part of the pancreatic leukocyte population in healthy individuals and can be detected in large numbers in donors with T1D (**Figure 1**). Although autoreactive CD8 T cells are preferentially present in the pancreas, but not in blood of T1D patients, some key questions remain to be addressed.

1. Why are so many CD8 T cells in the exocrine pancreas PPI specific? Is it due to an abundance of this antigen in the pancreas? Could it be that beta-cells are not visible in healthy individuals under physiological conditions, a status termed immunological "ignorance" (51)? What then happens to these cells during disease development?
2. There is growing evidence from mouse and human studies suggesting insulin as the key autoantigen in T1D. But is PPI a "driver" autoantigen in T1D, and are PPI-specific T cells predominantly present in the pancreas of every individual, or does every individual have a different "driver" autoantigen?
3. Does CD8 T cell exhaustion occur in the pancreatic islets (indicated by PD-1 upregulation, and could this be a potential regulatory mechanism)? An exciting study recently demonstrated that islet-specific CD8 T cells of healthy controls and T1D patients comprise three dominant phenotypes that display characteristics of transitional memory and exhausted memory cells (52).
4. To date, less is known about post-translationally spliced peptides that can be recognized by CD8 T cells in the target organ. One study reported IAPP $_{15\text{-}17}$/IAPP $_{5\text{-}10}$ reactive cells in the human pancreas (9), but are there other neoantigens that can be recognized by CD8 T cells?
5. Is cytokine upregulation in beta cells necessarily a pathogenic factor in T1D or a compensatory mechanism to combat cellular stress?

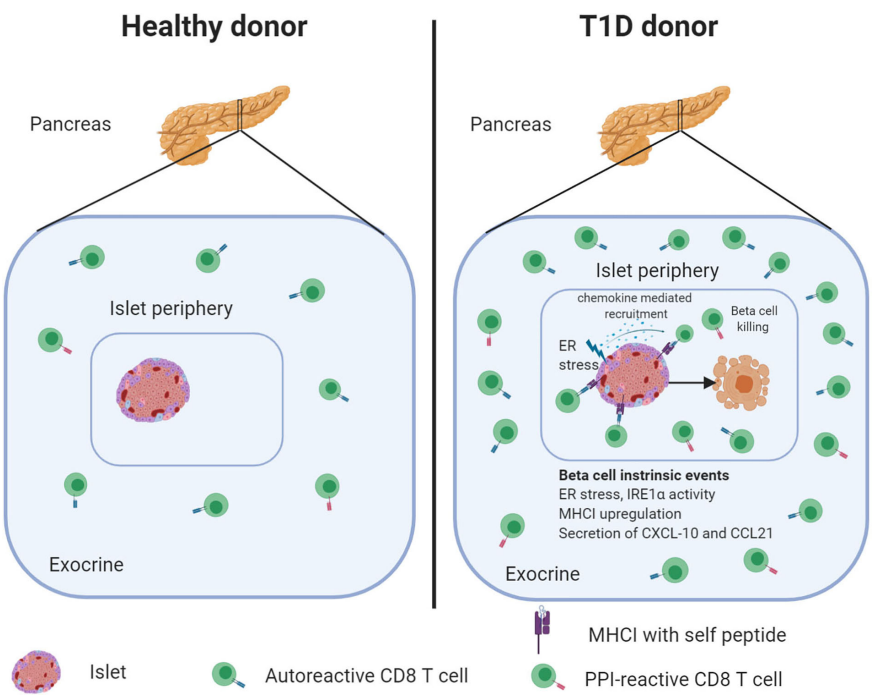

FIGURE 1 | The healthy human pancreas contains autoreactive CD8 T cells that increase in numbers during diabetes development. Frequencies of PPI-specific CD8 T cells are similar in the exocrine pancreas irrespective of disease status. However, during disease progression, PPI-reactive CD8 T cells become attracted to the islets. The secretion of cytokines, under physiological conditions or stress, may contribute to immune cell recruitment and beta-cell killing. Created with BioRender.com.

To date, antigen-specific therapy for T1D that selectively dampens T cell responses to main beta-cell proteins remains elusive. One of the major hurdles is the choice of antigens and the timing of therapeutic intervention. Therefore, to design effective and durable immunotherapies for T1D, it is imperative to identify the numbers and phenotype of islet antigen-specific T cells, specifically in the pancreas, and whether changes in specificities occur over disease progression.

AUTHOR CONTRIBUTIONS

CB and SR wrote the manuscript. MV critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Innate Immunity Mediated Inflammation and Beta Cell Function: Neighbors or Enemies?

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Type 1 diabetes (T1D) is still considered a huge burden because the available treatments are not effective in preventing the onset or progression of the disease. Recently, the idea that diabetes is an autoimmune disease mediated exclusively by T cells has been reshaped. In fact, T cells are not the only players with an active role in beta cell destruction. Macrophages and neutrophils, which physiologically reside in pancreatic tissue, can also participate in tissue homeostasis and damage by promoting innate immune responses and modulating inflammation. During the development of the pancreatic islet inflammation there is a strong interplay of both adaptive and innate immune cells, and the presence of innate immune cells has been demonstrated both in exocrine and endocrine pancreatic compartments during the earliest stages of insulitis. Innate immune cell populations secrete cytokines, which must be considered both as physiological and pathological mediators. In fact, it has been demonstrated that cytokines could regulate directly and indirectly insulin secretion and, simultaneously, trigger inflammatory reaction. Indeed, cytokines pathways could represent targets both to improve glucose metabolism and to prevent autoimmune damage. Concordantly, the combination of immunomodulatory strategies against both innate and adaptive immunity should be tested in the next future, as they can be more efficient to prevent or delay islet damage and T1D onset.

Keywords: type 1 diabetes, inflammation, cytokines, innate immunity, insulin secretion

INTRODUCTION

Although the available options of antidiabetic drugs are considerably increased and the burden of diabetes management is decreased, still we lack of therapies able to stop the pathological process that results in β cell failure and destruction. As of now, in the therapeutic clinical arsenal there are no registered treatments with indication for preventing or curing the onset or progression of type 1

diabetes (T1D). T1D was previously depicted as an exclusively T cell-mediated autoimmune disease with an inflammatory component, in which cells of specific immunity play a crucial role in β cell destruction. More recently, the immunological pathogenesis of T1D and in general of autoimmune diseases has been significantly reshaped, proposing a more complex interaction between several immunological players of the adaptive and innate immunity. In this context, the pancreatic islet inflammation present in T1D could result from an interaction between resident innate immune cells and target β cells (1). Moreover, it has been recognized that β cells proteins are released, taken up, processed, and presented by innate immune cells to islet auto-reactive T cells after their migration to the pancreatic draining lymph nodes (2). This cascade mediates the recruitment of activated T cells that increases the initial damage by mediating β cell killing and promotes further inflammation, generating a vicious loop. In this review, we will recapitulate the recent advances in understanding of the inflammation mediators and their implication in the beta cell function, paying particular attention to the relationship between innate immunity and insulin secretion both in pathological and physiological conditions.

INNATE IMMUNE AND BETA CELLS: THE FAVORITE PANCREATIC ROOMMATES

Macrophages

It is widely described that innate immune cells (i.e., neutrophils, monocytes, and macrophages) populate the pancreatic endocrine compartment (3). Indeed, macrophages reside in the healthy mouse islets beginning in the perinatal stages. They represent more than 80% of the intra islet immune cells (3–6) and some studies suggested that these macrophages are involved in islet morphogenesis (4). Endocrine resident macrophages derive from definitive hematopoiesis and are strongly self-maintained by local proliferation. Pancreatic macrophages showed different phenotype in relation to developmental stages, anatomical location, or metabolic profile. The immunoprofiling of pancreatic resident macrophage show the presence of $F4/80^{lo}CD11c^{+}$ within the islet structure whereas $F4/80^{hi}CD11c^{-}$ macrophages largely reside in the peripheral islet area. The distinct role of these two different macrophage subpopulations is still incomplete and more studies are needed to understand how pancreatic macrophages participate in tissue homeostasis maintenance and function of β cells (7). They did not reflect the classical M1 or M2 classification that is well described in other tissues.

Since their discovery, the role of these cells remains unclear. They are an essential player during early life development to establish a functional beta cell mass (6). Recent evidences structurally mapped the resident macrophages in the pancreas, showing their role as sentinel cells in the peri-islet structure. Two-photon microscopy of murine islets showed that resident macrophages are in close proximity of pancreatic blood vessels with extensive filopodial activity. Moreover, within the islet, macrophages are in intimate contact with beta cells. Through this cell contact (the beta cell–macrophage "synapse") the macrophages may take up insulin-containing vesicles. Functional studies in NOD mice showed that resident macrophages, thanks to their long filopodia, can dynamically probe whole islet volume including the vessel lumen and present insulin peptides to insulin-reactive T cells (8). Moreover, these cells may be activated from blood products and are able to monitor the islet secretory activity by detecting ATP endogenous levels through their purinergic receptors (9, 10).

Neutrophils

By combining single-cell RNA-Seq and immunophenotypic analyses to build a comprehensive view of the pancreatic mouse innate immunity cell landscape, a tissue-specific cell heterogeneity in myeloid cell (including monocytes, neutrophils, dendritic cells, and macrophages populations) was described and putative gene networks underlying the pancreatic specialization were identified (11). This is in line with past literature and confirms the presence of resident neutrophils in the healthy pancreatic tissue. As an important element of the inflammatory response, neutrophils can direct and guide the innate immune response by engaging in complex interactions with macrophages, natural killer cells, dendritic cells, and through crosstalk with most of the cellular effector mediators (12). After their activation, neutrophils can promote an innate immune response through releasing soluble pattern recognition molecules, which have the capacity to augment phagocytosis, stimulate complement, and modulate inflammation. They can also secrete a diversity of cytokines, neutrophil extracellular traps (NETs), and microorganism- and tissue-damaging molecules to participate in innate inflammatory milieu. Pancreas-infiltrating neutrophils were recently observed in T1D patients by electron microscopy and immune-histochemical analysis mainly localized at the level of very small blood vessels in the exocrine pancreas (13). Neutrophils infiltrate the pancreas prior to the onset of the T1D symptoms and they continue to do so as the disease progresses (14). Of interest, a fraction of these pancreas-infiltrating neutrophils also extrudes NETs, suggesting a tissue-specific pathogenic role (14). These findings are in line with previous evidence in the NOD mouse model. Based on flow cytometry and immunohistochemistry on pancreatic infiltrating cells, we previously described the presence of neutrophils at different stages of the autoimmune disease (15). Moreover, we showed that the inhibition of the neutrophil recruitment, mediated by the CXCL8-CXCR1/2 pathway, might prevent and revert the hyperglycemia, suggesting the relevant role of those cells in the T1D onset and progression (15). Similarly, Diana et al. confirmed that the use of neutrophil neutralizing antibody in the asymptomatic stage of the disease could improve the T1D progression in NOD mouse (16). They suggested that β cell debris form immune complexes with dsDNA-specific IgGs secreted by B-1a cells. Neutrophils produce DNA-binding peptide that potentiates these immune complexes, inducing IFN-α secretion by pancreatic pDCs through TLR9.

CAN IMMUNOLOGICAL STRESS SIGNALS AFFECT INSULIN SECRETION?

Insulin Secretion Mechanism

Although β-cells adapt well to changes in metabolic demand (whether acute or chronic, from starvation to over nutrition), persistently high insulin demand leads to progressive β cells dysfunction and loss (17). Plasma glucose concentration is the primary mediator of insulin secretion, but also a variety of other mediators (peptide hormones, ions, neurotransmitters, and pharmaceuticals) contribute in the maintenance of glucose homeostasis. Mounting evidences suggest that the components of the immune system (localized within islets or not) may also play a physiological role in β cell function. As early as 1992, the existence of immune-neuroendocrine interactions in controlling glycaemia was proposed, suggesting as mediators some specific cytokines like Interleukin-1 (IL-1), Tumor Necrosis Factor (TNF) alpha, and IL-6 (18). It was observed that administration of low doses of lipopolysaccharide, an inducer of several pro-inflammatory cytokines, caused a profound and long lasting hypoglycemia. Now it is well established that inflammatory drivers can prompt a modulation of insulin secretion sharing the same signaling mechanisms of glucose. An elevation of intracellular cytosolic Ca2+ ([Ca2+]i) is essential for glucose stimulated-insulin secretion (GSIS). In β cells, glucose increases glycolytic flux that enhances the ATP/ADP ratio and brings to closure of plasma membrane ATP-sensitive K+ channels; this leads to rapid depolarization and the opening of voltage-dependent L-type Ca2+ channels (VDCC) with consequent Ca2+ influx into the cell. The [Ca2+]i increase sustains exocytosis of readily releasable insulin secretory granules *via* SNARE and calcium-regulated proteins (priming, docking, and fusion phases) (19). A second pathway, resulting in the secretion of insulin granules, involves the activation of adenylyl cyclase and the subsequent increase of intracellular cAMP, activation of protein kinase A (PKA), and lastly the release of Ca2+ stored in endoplasmic reticulum. This cAMP/PKA-dependent pathway is the canonical signaling pathway of GLP-1 stimulated insulin secretion in the presence of glucose (20). In addition, the activation of protein kinase C (PKC)-phospholipase C (PLC) signal pathways evokes calcium mobilization and retains a role in insulin release in the presence of metabolic stimulus (20, 21).

Immunological Stress Signals

Epidemiological evidences support the role of the viral infection as a possible environmental trigger for the development of T1D (22). As of now, it is not clear the exact mechanism behind the viral infection leading to T1D onset, but numerous studies evidence multiple possible mechanisms, including the initiation of the innate immune response. For example, the enteroviruses have shown specific islet cell tropism through detection of viral RNA and viral proteins in pancreatic sections of post-mortem T1D patients (23, 24). We investigated as well the potential role of the viral infection and innate immunity pathways in animal and human pancreatic tissues, paying particular attention to influenza virus (25): H1N1 and H3N2 and avian H7N1 and H7N3 influenza virus were able to infect a selection of human pancreatic cell lines and human pancreatic islets. In this context, the cytokine activation profile indicates a significant increase of innate immune mediators such as MIG/CXCL9, IP-10/CXCL10, RANTES/CCL5, MIP1b/CCL4, Groa/CXCL1, interleukin 8 (IL-8)/CXCL8, tumor necrosis factor alpha (TNF-α), and IL-6 (25). Overall these data suggested that influenza virus may play a role as a causative agent of pancreatitis and diabetes in humans and other mammals.

Also coxsackievirus B5 (CBV-5)-DS lytic strain has a tropism for pancreatic human islets, and it has been demonstrated that the expression of pro-inflammatory cytokine genes (IL-1α, IL-1 β. TNF-α, and TRAIL) mediated cytokine-induced beta cell dysfunction is correlated with the lytic potential of the virus (26). However, as we previously discussed, the innate immune molecules are also physiologically part of the pancreatic microenvironment, and here we describe their putative relevant roles in modulating the insulin secretion and in controlling the beta cell response.

Among cytokines, IL-1β was deeply studied as an insulin secretion modulator. A short-term exposure of β cells to IL-1β is able to potentiate the glucose-dependent insulin secretion in rodent and human islets by increasing granule trafficking and SNARE complex formation without affecting Ca2+ entry and insulin content (27, 28). Islets are enriched in expression of interleukin-1 receptor type I (IL-1R) and IL-1β plays a physiological role in promoting glucose homeostasis, as demonstrated using a model of pancreatic IL-1 receptor deletion (29). IL-$1R^{Pdx1-/-}$ mice display a reduction of 25% in GSIS in isolated islets; similarly, *in vivo*, after an intraperitoneal glucose bolus, insulin secretion in IL-$1R^{Pdx1-/-}$ mice was decreased by 56% in comparison with littermate controls (29). However, a chronic exposure to IL-1β impairs insulin secretion (30) and might result in β cell exhaustion. Recently it has been demonstrated that higher levels of circulating IL-1β correlate with higher fasting plasma glucose concentrations in healthy and in hyperglycemic diabetic individuals (31, 32). Accordingly, 48 hours of exposure of EndoC-βH1, a human beta cell lines, to pro-inflammatory cytokines IL-1β and IFN-γ reduced insulin secretion in GSIS in the presence of 20 mM glucose (33). Interestingly, these pro-inflammatory cytokines remodeled the β cell regulatory landscape (induction of a subset of novel and primed regulatory regions with a predominantly induction of gene transcription rather than transcript down-regulation) both in EndoC-βH1 and in human islets (33).

Regarding the cross talk between islets and inflammatory drivers, other cytokines deserve mention too. Interleukin 6 is a pleiotropic cytokine mainly secreted by macrophages and adipocytes during inflammation but also by muscle in response to contraction, whose receptor is expressed on pancreatic endocrine cells. IL-6 has been shown to enhance insulin secretion either directly through PLC-dependent pathway in β cells (34) or indirectly by the stimulation of L and α cells-secreted GLP-1 (35). However, the regulation of glucose homeostasis by IL-6 could be context dependent and species

specific (36). In humans, acute physiological elevations of IL-6 (e.g., during exercise) delays gastric emptying (in GLP-1-independent manner) and reduces insulin secretion (in a GLP-1-dependent manner). These two actions have opposing effects on glucose tolerance, leading to an overall improvement in healthy subjects and no change in mild T2D patients (36, 37).

In 2016, Galgani et al. described that IL-1b-dependent chemokine IL-8 (physiologically secreted by macrophages, endothelial, and epithelial cells) was associated with *in vivo* insulin secretion rates during an oral glucose tolerance test in healthy humans, even if no effect of recombinant human IL-8 on GIST in isolated mice islets was observed (38). IL-8 signaling involves [Ca2+]i (39, 40), but it is not still clear if it may include a direct action on insulin secretion.

Taken together these evidences suggested that inflammation-related molecules could play not only a role in immunity but also a direct role in maintaining glucose homeostasis. A comprehensive knowledge of their physiological roles, beside the pathological ones, would help to understand better many clinical conditions associated to inflammation, as T1D, T2D, and obesity, and to select the best-targeted therapies.

CYTOKINE PATHWAYS AS POTENTIAL PHARMACOLOGIC TARGET TO AFFECT GLUCOSE HOMEOSTASIS AND AUTOIMMUNITY

Immunomodulatory strategies to target diabetes are a growing topic in both preclinical and clinical studies. Regarding the efficacy of these treatments, there is no clear common feeling, and the scientific opinions span from enthusiastic (41) to more skeptical (42). From our perspective, the topic is extremely relevant and we have also contributed something to the immunomodulation of the cytokine pathways for the protection/improvement of the pancreatic islet function within the context of prevention of autoimmune progression (15) and after islet transplantation (43–45). The basic promise is the fact that the glucose homeostasis is fine-tuned by tissue resident immune cells (e.g., macrophages, neutrophils) in most of the metabolic active tissues (e.g., adipose and liver tissues). These cells absolve a crucial role in preserving metabolic homeostasis through cross talk with metabolic tissues in the peripheral system. Alteration of this process may lead to metabolic dysregulation at large as well as many associated diseases. Tissue resident immune cells, as described above, have also been suggested to play a role within the endocrine pancreas. The modulation of their function in T1D in term of cytokines production is important to determine their double role as regulator of immunoreaction and glucose homeostasis. In fact, alongside the benefit obtained from the modulation of the immune system, an additional benefit can be obtained from the inhibition of cytokines depending on their direct role in insulin secretion. If we assume that an exposure to pro-inflammatory cytokines could lead to β cell exhaustion and death due to the direct insulin overstimulation, the honeymoon phase in T1D is a relevant time window to inhibit the acute islet inflammation and delay β cell deregulation and death. Similarly, anti-inflammatory treatment may be useful to preserve the described residual β cell function in long standing T1D by inhibiting the chronic islet inflammation (46). Since pro-inflammatory cytokines are expressed in the pancreatic islet, although the relative expression of innate and adaptive cytokines differs between models (47), the therapeutic efficacy of the immunomodulatory strategies against innate immune cells in T1D is under investigation (**Table 1**). In 2008, etanercept, an anti-TNFα compound, was clinically tested in children newly diagnosed with T1D. A randomized double blind intervention clinical trial (NCT00730392) with etanercept resulted in lower A1C and increased endogenous insulin production, suggesting preservation of beta-cell function in the T1D treated patients. Unfortunately, this study was performed in a small cohort of 18 patients and a larger study is needed to further explore safety and efficacy (48). A second candidate was IL-1. Two randomized double-blind clinical trials with different IL-1 antagonists [canakinumab (NCT00947427) and anakinra (NCT00711503)] were performed on a cohort of 69 patients with new onset of T1D for each trial. Stimulated C-peptide concentrations and percentages of HbA1c did not differ between intervention-treated and placebo-treated patients (49). Interleukin-6 inhibition based treatment was evaluated in T1D clinical trial as additional player of the T1D inflammatory milieu. The EXTEND study is a phase II, multicenter, double blind, placebo-controlled, randomized trial aimed to test the tocilizumab, an IL-6 receptor

TABLE 1 | State of the art of clinical anti-cytokine and anti-chemokine receptor compounds in T1D patients.

Drug name	Molecule type	Target	Mechanism of action	Effects on beta cell	Trial		Ref
Etanercept	Recombinant fusion protein	TNFα	Soluble TNFα Receptor fusion protein able to bind and block TNFα activity	Indirect effect: lower A1C and increased endogenous insulin production	NCT00730392 (phase II)	Closed 01/2008	(48)
Canakinumab	mAb	IL-1	Neutralization of IL-1 signaling	Direct effect: blocks direct and hyper-glycemia mediated beta cell toxicity	NCT00947427 (phase II)	Open	(49)
Anakinra	Recombinant protein	IL-1	IL-1 competitive receptor antagonist		NCT00711503 (phase II)	Closed 06/2012	(49)
Tocilizumab	mAb	IL-6R	IL-6 Receptor inhibitor	Unknown	NCT02293837 (phase II)	Closed 08/2020	(50)
Ladarixin	Small molecule	CXCR1/2	CXCR1/2 allosteric not competitive inhibitor	Indirect effect: reduction recruiting CXCR1/2+ cells and delay in the c-peptide decline compared to the placebo arm	NCT02814838 (phase II)	Closed 10/2019	(51)

Monoclonal antibody (mAb).

inhibitor, in a cohort of 30 adults with type 1 diabetes (NCT02293837). As of now no updates are available, and the EXTEND study is expected to report in 2020 (50). As an additional player, we recently investigated on the role of the IL-8-CXCR1/2 pathway in the T1D. Indeed, based on our encouraging preclinical data on the role of CXCR1/2 inhibition in NOD mice (52), we investigated the role of ladarixin, a CXCR1/2 inhibitor, in the preservation of β cell function and slow-down of the progression of T1D in a phase II, multicenter, double-blind study that involved 76 patients with new-onset (NCT02814838). Preliminary results suggest that a subgroup of patients could be beneficial from ladarixin treatment: after 26 weeks from first drug administration, they showed a delay in the c-peptide decline compared to the placebo arm. More detailed analyses are ongoing to clearly define the role of IL-8 pathway inhibition on the beta cell pathophysiology (51).

CONCLUSION

In this review, we support the idea that T1D disease should not be depicted anymore as an exclusively T cell-mediated autoimmune disease. In fact, there are increasing evidences of a strong involvement of innate immunity in T1D pathogenesis, as innate immune cells appear responsible for both the impaired islet homeostasis and the inflammatory damage. To understand the clinical potential of different anti-inflammatory treatments, we need in the future to study the role of innate immunity in the physiology of islet, paying particular attention to their impact on glucose homeostasis. Findings demonstrate that cytokines can modulate indirectly or directly insulin secretion acting on the same signaling mechanisms of glucose. Moreover, macrophages and neutrophils physiologically reside in the pancreatic endocrine compartment and participate in tissue homeostasis and beta cell function. On the other hand, innate immunity cell and mediator can trigger the inflammatory reaction promoting the immune response. As pictured in **Figure 1**, cytokines could be physiological and pathological factors. The equilibrium between these two actions is relevant for the pathogenesis of T1D, and more generally for islet damage. It is plausible that cytokines could play a role in the physiology of insulin secretion contributing to islet response to metabolic demand. However, under a strong cytokine "pressure" (as determined by long-time and/or high concentration exposure) islet can develop dysfunctional responses, which result in both impaired insulin secretion and insulitis development. In this perspective, cytokines pathways could represent potential

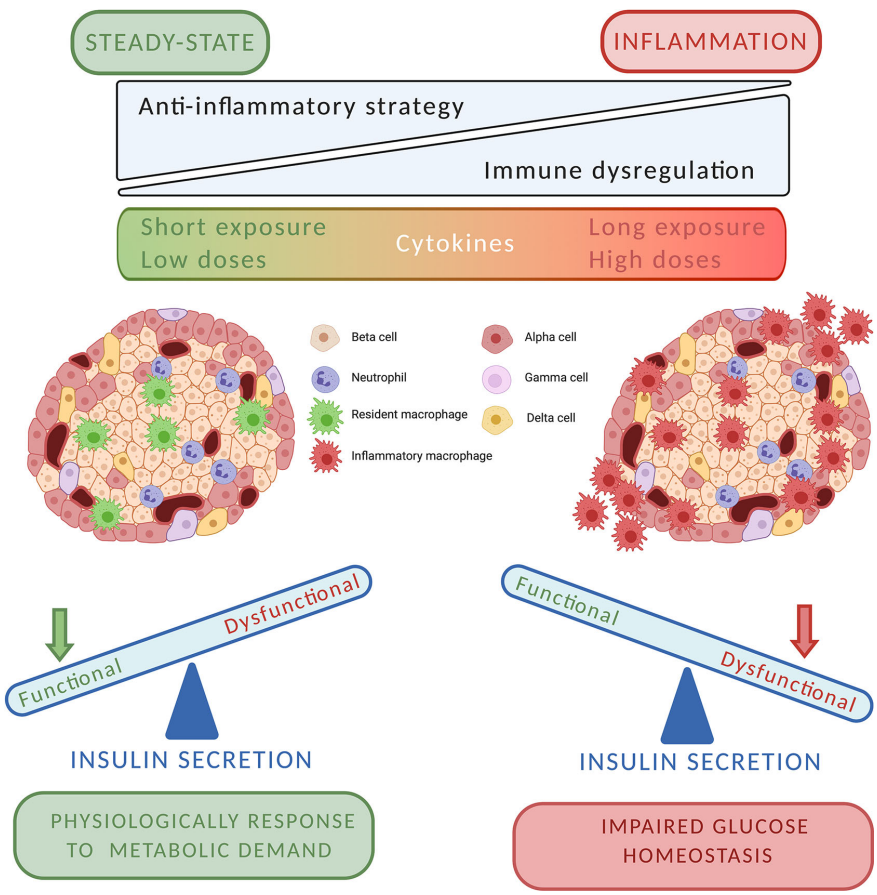

FIGURE 1 | The putative two–faces of cytokines in pancreatic islet milieu.

therapeutic targets to control efficiently glucose metabolism and to prevent autoimmune damage. Concordantly, the combination of immunomodulatory strategies against both innate and adaptive immunity should be tested in the next future, as they can be more efficient in preventing or delaying T1D.

AUTHOR CONTRIBUTIONS

AC, ED, FC, and LP conceived the study, wrote the manuscript, and contributed to the discussion. All authors contributed to the article and approved the submitted version.

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The Role of β Cell Stress and Neo-Epitopes in the Immunopathology of Type 1 Diabetes

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Due to their secretory function, β cells are predisposed to higher levels of endoplasmic reticulum (ER) stress and greater sensitivity to inflammation than other cell types. These stresses elicit changes in β cells that alter their function and immunogenicity, including defective ribosomal initiation, post-translational modifications (PTMs) of endogenous β cell proteins, and alternative splicing. Multiple published reports confirm the presence of not only CD8+ T cells, but also autoreactive CD4+ T cells within pancreatic islets. Although the specificities of T cells that infiltrate human islets are incompletely characterized, they have been confirmed to include neo-epitopes that are formed through stress-related enzymatic modifications of β cell proteins. This article summarizes emerging knowledge about stress-induced changes in β cells and data supporting a role for neo-antigen formation and cross-talk between immune cells and β cells that provokes autoimmune attack - leading to a breakdown in tissue-specific tolerance in subjects who develop type 1 diabetes.

Keywords: ER stress, neo-antigen, post-translational modification, type 1 diabetes (T1D), Beta Cell (β cell), immune cells

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INTRODUCTION

Type 1 diabetes (T1D) is a chronic immune mediated disease in which insulin-producing β cells are destroyed leading to lifelong insulin deficiency (1, 2). The autoimmune etiology of T1D is clear and both CD4+ and CD8+ T cells have been shown to recognize a wide variety of beta cell derived epitopes (3, 4). However, there is an increasing appreciation that β-cell dysfunction also plays a crucial role in disease (5–7). Emerging published work demonstrates that inflammatory cytokines and/or reactive oxygen species (ROS) can trigger ER stress, HLA Class I upregulation, and other deleterious changes in β cells (8, 9). ER stress, in turn, has been shown to promote post-translational modifications and alternative mRNA splicing, thereby generating neo-sequences that have been shown to be recognized by autoreactive T cells and autoantibodies in patients with type 1 diabetes and animal models of disease (10, 11). Importantly, such neo-epitopes are not genetically encoded and thought to be underrepresented in healthy tissue. Therefore, neo-epitope responses may be less subject to the central or peripheral tolerance mechanisms that limit autoimmunity. Clearly, native self-antigens are represented in the thymus (12), though post-translational modifications (PTMs) of

self-antigens can generate a novel autoantigenic proteome to which tolerance has not been developed by the immune system. This theme has been previously described as "autoantigenesis", a process to indicate how proteins acquire PTMs over the progression of disease and stimulate B and T cell autoimmunity (13). This phenomenon is observed with a number of autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus (SLE), and type 1 diabetes (T1D) (13, 14). This brief review will emphasize the relevance of PTMs that are generated within the insulin producing β cells of pancreatic islets. In addition, we will address how inflammatory stresses, such as cytokines and ROS, have the ability to reduce β-cell function through impaired insulin production, processing, handling, and export. Such stresses would perpetuate a continuum of immunologic epitope spreading leading to β-cell dysfunction and waves of immune attack over time (**Figure 1**).

PANCREATIC β CELLS ARE VULNERABLE TO STRESS

ER stress and activation of the UPR can occur in any human cell under increased demand for protein translation. Because of their function as professional secretory cells, β cells must carry out extremely high levels of protein translation. What this means for the β cell is nearly constant synthesis and

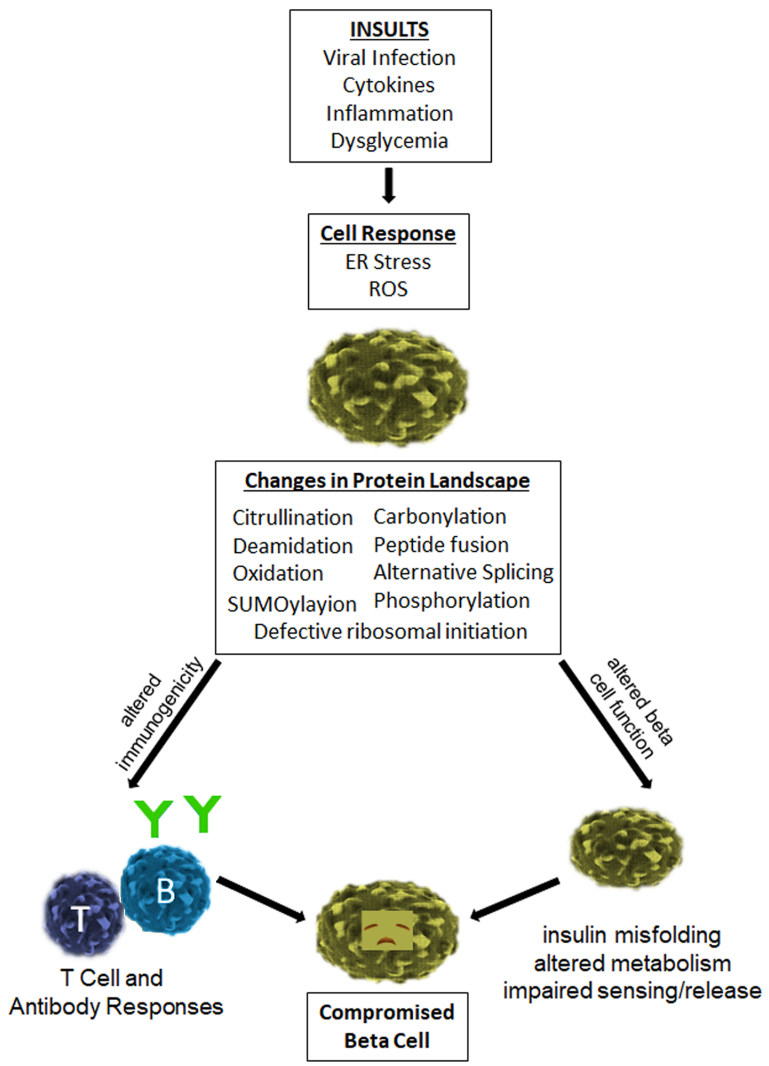

FIGURE 1 | Impact of inflammatory stresses on β-cell immunogenicity and function. Human β-cells are vulnerable to various insults that generate a cellular stress response and deleterious effects. In particular, viral infection and exposure to inflammatory cytokines elicit ER stress and ROS, which have been shown to promote enzymatic and non-enzymatic processes that lead to the generation and release of neo-epitopes. These epitopes increase the antigenicity of β-cells and provoke the activity of autoreactive T cells and B cells. These same processes elicit alternative splicing, defective ribosomal initiation, SUMOylayion, phosphorylation, and other enzymatic modifications of key β-cell proteins, all of which can have a deleterious impact on beta cell health and function. The cumulative result of these changes is a compromised beta cell.

processing of proinsulin into active insulin in the ER. The mature insulin is stored in secretory granules awaiting release upon increases in blood glucose levels (15). The insulin granules are released dynamically to maintain normal blood glucose levels (16). This dynamic glucose flux places the β cell in a constant state of secretion readiness to ensure there is a perpetual flux of insulin at the ready to maintain glucose homeostasis. To achieve this, the β cell contains a large pool of cytoplasmic proinsulin mRNA (~20% of the total mRNA), one of the most abundant mRNA species (16). One profound example of physiological fluctuation in the protein-folding load in the ER is the unique translational response of pancreatic β cells to variations in blood glucose (17). In response to increased blood glucose, β cells increase translation of preproinsulin by 50-fold (18), reaching a production rate of 1 million molecules per minute (18). The preproinsulin molecules flood the ER lumen for proper folding and disulfide bond formation, causing tremendous ER stress. The active production and packaging of insulin maintaining glucose homeostasis places the β cell in a constant state of ER stress. Although the ER of the β cell is encumbered with the major task of insulin production in the face of dynamic glucose sensing (described above), the ER has adapted a fail-safe program of intracellular signaling pathways (also active in other cell types), termed the unfolded protein response (UPR). The activation of the UPR initiates a cascade of signaling events to quell the activity of protein processing and folding in order to resolve the ER burden and restore homeostasis. The adaptive unfolded protein response (aUPR) promotes adaptation in cells experiencing increased levels of ER stress to ensure that the cellular production is regulated and manageable (19). To regulate ER stress, the chaperone GRP78 releases the protein sensors of ER stress (20). As part of this response, activated PERK phosphorylates eIF2α to generally suppress mRNA translation and reduces the protein burden on the ER. Also, active ATF6 initiates new chaperone synthesis to aid with proper protein folding in the ER, and coincident phosphorylation of IRE1 leads to splicing of XBP1 mRNA and further chaperone synthesis [recently reviewed in detail elsewhere (21, 22)]. This aUPR serves to alleviate ER stress during times of high protein load (22, 23). However, it is known that the UPR has two modes of the unfolded protein response (UPR) (19, 24) which begins with protein sensors of stress in the ER membrane (25). The aUPR (described above) occurs early to alleviate ER stress and restore normal cellular function. However, if ER stress is too great, prolonged or further induced by environmental or physiological triggers, then the aUPR-mediated recovery fails and induction of the terminal (tUPR) initiates a specialized programmed cell death pathway (19, 24, 26–28). This switch from aUPR to tUPR has been further described (24, 26, 28, 29), and may ultimately result from the unmet need in bioenergetics and reducing equivalents needed for β cell day-to-day operation leading the programmed cell death (30, 31).

Emerging work suggests that in addition to being subject to high levels of ER stress due to their function, β cells may be predisposed to increased stress and damage in subjects at high risk of developing T1D due to disease associated genetic variants. Indeed, a substantial proportion of candidate genes within T1D susceptibility loci are expressed in β cells (32). Several of these, including PTPN2, MDA5, and BACH2 have an implicated role in modulating islet inflammation, β-cell apoptosis, and responses to cytokines and viruses (33, 34). These observations support a paradigm in which genetically susceptible individuals experience higher levels of β cell stress under physiologic conditions, leading to a greater probability of β-cell dysfunction and immune attack. Indeed, a model was recently proposed in which beta-cell defects may significantly contribute to T1D (35). A key element of that paradigm is the concept that there is an intrinsic (and to some degree genetically driven) vulnerability of beta-cells to death and dysfunction, which continues to drive the loss of insulin secretion after the establishment of persistent anti-islet autoimmunity. Indeed, it there is some evidence to suggest that beta cell dysfunction is present even in the absence of overt insults. For example, recent work shows that β-cell dysfunction (evidenced by an abnormal proinsulin/insulin ratio) precedes disease and is a common feature in subjects with T1D (36, 37) and yet pronounced insulitis in human islets is rare (38). One potential consequence of such intrinsic β cell fragility and vulnerability is a continued propagation of dysfunction in even after the resolution or diminution of active immune attack.

ER STRESS IN β CELLS IS INFLUENCED BY IMMUNOLOGIC AND ENVIRONMENTAL FACTORS

It understood that cytokines play a crucial role in β cell damage. Although the precise mechanisms of responsiveness to cytokines are species specific, the induction of ER stress and/or apoptosis by cytokines in β cells is indeed important and key general aspects of cytokine-induced apoptosis are conserved in mice, rats, and humans (39). In particular, proinflammatory factors such as IL-1β, TNF-α and IFN-γ have been shown to play important roles in eliciting ER stress. Many other physiological and environmental triggers that are associated with T1D have also been shown to enhance ER stress in β cells, including viral infection (40–42), exposure to chemicals (43–46), dysglycemia (47), and the intrinsic demands of insulin secretion (as delineated above).

ER stress may be a common downstream pathway that contributes to the development of T1D. However there is some disconnect, in that the progression of immune cell infiltration leads to the deposition of cytokines at the β cell. In all likelihood, an intricate interplay between genetic predisposition, the immune system, and environmental factors precipitates T1D in humans. As described above, one feasible bridge between genetics, the immune system, and environmental factors are type 1 interferons (T1-IFNs) (48).

Type 1 interferons (T1-IFNs) are well known for inducing antiviral factors that limit infection by regulating innate and adaptive immune responses. Furthermore, as described above, several T1D genetic risk loci coincide with genes that are associated with innate and adaptive responses to T1-IFN (33, 34). Additional support that T1-IFN play a role in T1D is that these cytokines are a known constituent of the autoinflammatory milieu within the pancreas of patients with T1D. The presence of IFNα/β is correlated with characteristic MHC class I (MHC-I) hyperexpression found in the islets of patients with T1D, suggesting that T1-IFNs enhance β cell recognition by autoreactive cytotoxic CD8+ T lymphocytes and insulin-producing pancreatic β cells through increasing MHC I expression (48). Of course, cytokine induced β cell apoptosis is only one of the possible outcomes. An important scenario in which cytokine related effects could occur is through viral infection—specifically enteroviral infection with Coxsackie virus (CVB). It is well known that viral infection leads to a type I interferon response at the target tissue site (49). This serves to mobilize the immune system to the site of infection to initiate the clearance of the pathogen. Viral infection facilitates the recruitment of accessory cells and T cells to the islets (50) leading to site directed production of inflammatory cytokines, particularly INF-α, INF-β, IFN-γ, tumor necrosis factor (TNF) and IL-1β (51). The importance of these cytokines in β-cell destruction has been exhaustively demonstrated in NOD mice and rat models of diabetes mellitus (52–54). CVB infection accelerates disease onset in young non-obese diabetic (NOD) mice with established insulitis—likely acting as an accelerant to the break in tolerance as a result of type 1 and type 2 cytokines (55–57)—and elicits bot ER stress (58–60) and the release of intracellular Ca^{2+} (41, 42, 61) upon entry into β cells (62, 63). Interestingly, Ca^{2+} flux also facilitates the induction of ER stress during CVB infection (40, 62–66). While ironclad proof of direct causality has remained elusive, CVB infection is highly associated with T1D onset in humans (67–75). Furthermore, a number of studies have defined footprints of CVB infection in the islets, demonstrated through the presence of RNA and VP1 antibody staining (70, 74, 76–80). More specifically, evidence is mounting that enteroviruses such as CVB could be involved in perpetuating the break in self-tolerance by increasing islet β cell specific inflammation (28, 81–84), thereby providing a more inflammatory milieu (85, 86) that promotes the optimal activation of virus reactive and self-reactive T cells (87–94). This idea has been supported by a number of elegant studies demonstrating that CVB infection selectively activates certain pathways that allow a tunable ER-stress and unfolded protein response (UPR) that favors viral amplification (60, 75) and persistent infection (67, 73, 74, 95–98) without the induction of premature apoptosis and death. Finally, recent work demonstrates that enterovirus family members show a strong association with islet autoimmunity in human T1D patients (99), are capable of infecting islets, and show that a sizeable percentage of type 1 diabetic patients have prolonged/persistent enterovirus infection associated with gut mucosa inflammation (98, 100).

STRESSED β CELLS EXHIBIT INCREASED IMMUNOGENICITY

A key observation in murine studies that has been subsequently supported by parallel studies of human beta cell lines is that there is increased immune recognition of stressed β cells. For example, multiple studies show that endoplasmic reticulum (ER) stress in β cells increases cytosolic calcium Ca^{2+} and the activity of tissue transglutaminase 2 (tTG2), leading to the generation of deamidated neo-epitopes (10, 101–103). Our work and other published studies demonstrated increased immunogenicity of beta cell peptides following enzymatic modifications at specific residues which are crucial for HLA binding and presentation, T cell receptor recognition, or both (10, 104, 105). Indeed, the progression of T1D and beta cell dysfunction are characterized by an accumulation of autoantibodies against beta cell antigens (106) and the activation of auto-reactive T cells, which have been shown to infiltrate pancreatic islets (107, 108). Furthermore, we have shown that subjects with T1D have elevated frequencies of T cells that recognize citrullinated and deamidated epitopes from β cell antigens, and that T cells with some of these specificities can be found in the pancreatic lymph nodes of organ donors with T1D (10, 105, 108). Hence, it is plausible that T cells that recognize citrullinated and deamidated epitopes, as a result of ER stress induced Ca^{2+} flux and activation of tTG2 enzymes, become activated and expanded in subjects who progress to develop T1D, likely playing a role in the pathogenesis of the disease. Notably, many of the enzymes which are responsible for the introduction of protein modifications, including tTG2 (responsible for deamidation), peptidyl arginine deaminase (PAD) enzymes (responsible for the introduction of citrullinate into proteins and peptides), and various cysteine proteases (e.g. calpains), which may participate in peptide transpeptidation-reactions (leading to the formation of hybrid insulin peptides or "HIPs") are Ca^{2+}-dependent enzymes (109–111). HIPs belong to a new family of autoantigens in T1D, which are targeted by diabetes triggering T cells in mice, and that have been shown to be recognized by T cells in the peripheral blood of T1D patients, and by T cells identified in the residual pancreatic islets organ donors with T1D (112, 113).

A central pathway that contributes to the enhanced immunogenicity of stressed β cells is the development of neo-antigens and epitopes, which has been shown to occur by a variety of enzymatic and non-enzymatic processes that have been reviewed elsewhere (114). Several published studies illustrate the genetic risk factors associated with autoimmune diabetes, particularly the associations with susceptible HLA class II haplotypes (115). The most likely contribution of HLA class II proteins to disease is through selection of a potentially autoreactive CD4+ T cell repertoire (116). These same HLA class II molecules have been shown to have an increased capacity to bind and present peptides with post-translational modifications (10, 117). Furthermore, it has been clearly shown that autoantibodies and autoreactive T cells recognize multiple beta cell antigens, including novel stress-related specificities formed through alternative splicing and defective

ribosomal initiation that have been only recently appreciated (11, 118, 119). Therefore, mounting evidence implicates the formation of neo-epitopes as one important means of circumventing immune tolerance.

PTMs also change many other features of protein chemistry, including primary and tertiary structure, biological (and/or enzymatic) functions, and proteolytic degradation (antigen processing) that are important in creating both toleragenic and immunogenic self-peptides. Clearly, the way in which a self-protein is processed by antigen presenting cells may break immune tolerance (120, 121). The modification of amino acid(s) critical for the recognition and cleavage by certain proteases can affect the peptides generated or the rate in which they are generated. For example, the lack of *N*-glycosylation of the neuronal glutamate receptor subunit in Rasmussen's encephalitis (a severe form of pediatric epilepsy) exposes a granzyme B cleavage site that is otherwise inaccessible to the enzyme (121). Additionally, the presence of citrulline residues in peptides of myelin basic protein (MBP) increases its rate of digestion by cathepsin D (122). Tissue stress, both cytokines and ROS, amplifies the accumulation of PTMs that induce disease in the host. Several factors control the ability and rate of PTMs that occur in a given protein. Flanking residues near an epitope sequence of amino acids significantly influence how the site may be modified. Spontaneous isoaspartyl modification occurs most frequently at Asp/Asn-Ser or Asp/Asn-Gly amino acid motifs where serine or glycine adjacent sites are critical for modification (123–125). The environment of a modifying enzyme (such as in the pancreatic islet) is also important since they are compartmentalized in intracellular organelles, the endoplasmic reticulum, or in extracellular spaces. For example, protein and/or DNA methylation require both the presence and cellular proximity of methylases (DNA methyltransferases or protein methyltransferases) along with the cellular source of methyl donor groups, S-adenosylmethionine (SAM) (126). Finally, features within the beta cell protein itself, such as previous modifications, will affect how a particular residue is modified (127).

It could be said that T1D is an autoimmune disease for which evidence of how modified autoantigens contribute to pathogenesis is currently emerging. One key example of this is the evolving understanding of chromogranin A as a disease relevant antigen. Studies by Stadinski and colleagues demonstrated that chromogranin A is recognized by disease relevant T cells; specifically, the WE14 peptide (a natural cleavage product derived from chromogranin A) stimulated diabetogenic CD4 T cell clones and reactivity of those clones with islet preparations was abrogated by knocking out chromogranin A (128). However, further studies showed that the antigenicity of WE14 (chromogranin A fragment) is greatly increased by treatment with transglutaminase; this enzyme that is known to modify peptides through deamidation and also through cross-linking so either could contribute to the observed change in immunogenicity (129). Studies that are more recent strongly suggested that the most potent ligand for that T cell clone is a hybrid peptide formed between WE14 and a fragment of insulin (111, 112), implicating cross linking as the most likely mechanism.

Certain self-antigens appear to elude central and peripheral tolerance mechanisms. Previous work from our laboratory and others has identified the presence of autoreactive T and B cells even in the peripheral repertoire of normal mice and healthy human subjects (11, 130). Autoreactive cells can escape deletion because both cryptic peptides and posttranslationally modified proteins are underrepresented in the thymus, leading to impaired negative selection of these potentially self-reactive T cells (131, 132). We have shown that protein modifications alter both the antigenicity of self-proteins and the intracellular signaling properties of lymphocytes, leading to aberrant autoimmune responses (131, 133). As one example, the spontaneous conversion of an aspartic acid to an isoaspartic acid induces both T and B cell immunity to model self-antigens (14, 134). The presence of isoaspartyl modifications alter the immune processing and presentation of self peptides as indicated earlier since proteases and peptidases are not able to cleave on the carboxyl side of the isoaspartic acid modifications (135). Isoaspartyl modifications alter the structural integrity of histone H2B as well as trigger autoantibodies to H2B, characteristic of systemic lupus erythematosus (SLE) (136). Similarly, isoaspartyl modification of the SLE autoantigen Sm snRNP amplifies lupus autoimmunity and is bound by SLE patient autoantibodies (137). Finally, T lymphocytes that acquire isoaspartyl protein modifications have a hyperproliferative phenotype due to increased phosphorylation of ERK and Akt, characteristic of human SLE and murine models of disease (138–142).

PTMs often arise spontaneously, but are amplified as a consequence of cellular activation, inflammation, and cellular stress. These modifications include deamination, acetylation, glycosylation, citrullination, phosphorylation, and isoaspartylation. One unique form of modification, carbonylation, is the non-enzymatic addition of aldehydes or ketones to amino acid residues via a metal-catalyzed reaction. This PTM has not fully been studied in the initiation of T1D, although extensive oxidative carbonylation is a component of diabetic complications (143). For example, it has been demonstrated that islet lysates treated with copper and ascorbate generate new glutamic acid decarboxylase (GAD65) aggregates that react with T1DM patient sera (144). Importantly, neo-epitopes formed in peripheral tissues by these diverse mechanisms but underrepresented in the thymus could be expected to be recognized with high affinity by self-reactive T cells. As an example, certain HIP epitopes activate T cell clones at extremely low peptide doses (111, 112).

POSTTRANSLATIONAL PROTEIN MODIFICATIONS MAY ALTER THE BIOLOGIC FUNCTIONS OF CELLS

Beyond the autoimmune responses clearly defining 'biomarkers' of the onset and perpetuation of T1D, PTMs also have the potential to alter the metabolic pathways and function of the beta cell. As one example, glucose metabolism in humans is carefully regulated by the activity of glucokinase (GCK), a glucose sensor

and a protein highly expressed by pancreatic beta cells. GCK catalyzes a principle rate controlling step of glucose metabolism needed to trigger insulin release, and in the liver, where it has a role in glycogen synthesis; reviewed in (145). While not the topic of the present review, a number of genetic mutations of GCK lead to a variety of clinical manifestations including MODY (maturity onset diabetes of the young), hyperinsulinism, and loss of function (145).

Several metabolites, including glucose itself and insulin, influence the transcription of GCK. GCK regulation is a network of cellular processes that coordinate with the metabolic state of the beta cell. PTMs clearly alter the tertiary structure of proteins, particularly relevant to the metabolic properties of GCK. In particular, GCK assumes an "open" and "closed" conformational state that regulates binding to glucose and to other allosteric macromolecules. A number of PTMs of GCK that both increase and decrease metabolic activity have recently been described (145). For example, attachment of Small Ubiquitin-like Modifier proteins (SUMOylation) or S-nitrosylation can shift the conformational state of GCK to increase its catalytic activity for glucose (though only a small fraction, about 5%, of the GCK pool is SUMOylated in beta cells). Interestingly, SUMOylation has been shown to have broader effects in controlling beta cell survival and oxidative stress, such that either its overexpression or conditional ablation leads to imparted β cell function (146). Glucokinase regulatory protein (GKRP) inhibition of GCK is modulated by PTMs. Nuclear translocation of GCK is impaired by SUMOlyation, though this PTM also stabilizes GCK catalytic activity. Identification of the specific PTMs that alter the association of GCK with Ubiquitin-like domain (ULD) are a target of therapeutic intervention by several groups (145). All of these conformational states of GCK alter the downstream interactions with GKRP, phosphofructokinase biphosphatase-2, ULD, and propionyl-CoA Carboxylase β subunit. Ubiquitin-like domain (ULD) proteins also interact with and reduce GCK activity over the course of glucose metabolic pathways (145). ULD protein interactions with GCK are highly dependent on native structure. Recent studies (Yang, James, and Mamula, in preparation) have identified the presence of citrulline modifications that alter the Km and Vmax of GCK, in addition to the presence of autoantibodies and T cells specific for citrulline GCK epitopes. The repair of citrulline modifications may indeed be yet another therapeutic strategy to maintain the normal metabolic state of beta cells under inflammatory stress.

CONCLUSIONS

In this brief review, we have recounted how protein and peptide modifications, prompted by β cell stress and responsible for the formation of neo-epitopes appear to play a role in in the immunopathology of type 1 diabetes. Changes in β cell immunogenicity are easily attributable to recognition of neo-epitopes and antigens, but further research could reveal additional means through which stress-induced changes can encourage immune attack. Emerging research increasingly supports that stress and protein modification can compromise β cell function. Therefore, stress related pathways appear to elicit relevant changes in β cells altering both their immunogenicity and biological function. These effects appear to combine with genetic variants that promote β cell fragility and susceptible HLA haplotypes that are more prone to select a potentially autoreactive repertoire. This paves the way for a self-reinforcing dialogue between immune cells and β cells that provokes either sustained or recurrent autoimmune attack, eventually leading to the clinical onset of diabetes. The most elusive factor that remains to be elucidated are environmental factors such as viral infection, the footprints of which can be seen in the islet, which probably play a crucial role in initiating progression toward disease.

AUTHOR CONTRIBUTIONS

Each of the authors (JP, MM, and EJ) made contributions to this work by conceiving, writing, and editing the manuscript. All authors agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version

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Footprints of Immune Cells in the Pancreas in Type 1 Diabetes; to "B" or Not to "B": Is That Still the Question?

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Significant progress has been made in understanding the phenotypes of circulating immune cell sub-populations in human type 1 diabetes but much less is known about the equivalent populations that infiltrate the islets to cause beta-cell loss. In particular, considerable uncertainties remain about the phenotype and role of B-lymphocytes in the pancreas. This gap in understanding reflects both the difficulty in accessing the gland to study islet inflammation during disease progression and the fact that the number and proportion of islet-associated B-lymphocytes varies significantly according to the disease endotype. In very young children (especially those <7 years at onset) pancreatic islets are infiltrated by both CD8+ T- and CD20+ B-lymphocytes in roughly equal proportions but it is widely held that the CD8+ T-lymphocytes are responsible for driving beta-cell toxicity. By contrast, the role played by B-lymphocytes remains enigmatic. This is compounded by the fact that, in older children and teenagers (those ≥13 years at diagnosis) the proportion of B-lymphocytes found in association with inflamed islets is much reduced by comparison with those who are younger at diagnosis (reflecting two endotypes of disease) whereas CD8+ T-lymphocytes form the predominant population in both groups. In the present paper, we review the current state of understanding and develop a proposal to stimulate further discussion of the roles played by islet-associated B-lymphocytes in human type 1 diabetes. We cite evidence indicating that sites of direct contact can be found between CD8+ and CD20+-lymphocytes in and around inflamed islets and propose that such interactions may be important in determining the efficiency of beta cell killing.

Keywords: CD20+ B-lymphocyte, CD8+ T-lymphocyte (CTL), insulitis, islet, inflammation

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INTRODUCTION

The infiltration of immune cells into the pancreases of people with type 1 diabetes was described in graphic detail more than a century ago by pioneers such as Weichselbaum (1) and Schmidt (2), whose elegant drawings revealed the structure of the islets and their attendant infiltrate. In particular, they drew attention to the presence of unidentified groups of very small cells (almost certainly immune cells) adjacent to the islets and, as such, provided an early representation of the

process subsequently termed "insulitis" by von Meyenberg (3). Such careful observations and exquisite attention to detail paved the way for subsequent generations of investigators to apply ever more sophisticated technologies [including, for example, the latest methods of multiplexed *in situ* imaging mass cytometry (4, 5)] as a means to confirm and extend the original observations. As a consequence, it might be reasonably expected that, by now, an essentially complete picture would have emerged to describe and explain the involvement of islet-infiltrating immune cells in type 1 diabetes. However, it is salutary to reflect that, at a time when personalized immunotherapy is increasingly mooted as the means by which the progression of type 1 diabetes will soon be stalled in susceptible individuals (6, 7), we still have only a rudimentary understanding of the immune processes occurring in islets as the disease develops.

There are many reasons for this continued lack of understanding including the fact that the number of individual cases in which the events have been studied first-hand at, or very soon after, diagnosis remains extremely small (8) and that the islets of Langerhans are inaccessible in living subjects. All of which means that our grasp of the immune processes that culminate in beta cell death in type 1 diabetes are still based as much on surmise and speculation as on hard evidence. Thankfully, some progress has been made and we focus here on one particular aspect of the immunopathology of human type 1 diabetes which remains intriguing and enigmatic, namely a possible role for B-lymphocytes in driving disease. Facets of this subject have been reviewed very effectively by others (9–13), especially in relation to the differing populations of B-lymphocytes found in the circulation and, accordingly, we have concentrated on their roles in the pancreas.

It is non-contentious to argue that B-lymphocytes are involved in the progression of type 1 diabetes since the majority of subjects produce autoantibodies to one or more islet proteins as a hallmark of the disease. However, there are caveats to this scenario since it is often felt that, while B-lymphocytes are involved, they probably occupy little more than a "bit part" role. Support for this notion comes, for example, from isolated case reports such as that documenting the development of type 1 diabetes in an individual with X-linked agammaglobulinemia (14). Because of his underlying condition, the proband cited in this study had very few, if any, circulating B-lymphocytes and no islet autoantibodies but had still developed type 1 diabetes by his mid-teenage years. Hence, the secretion of islet autoantibodies by B-lymphocytes is often seen as a marker of disease rather than a causative factor [i.e., it is "smoke rather than fire" (11)] and their role is considered ancillary. This view contrasts with evidence from the NOD mouse model of type 1 diabetes where the systemic loss of B-lymphocytes has more profound effects and can prevent disease development (15). However, such evidence is easily downplayed by the understanding that disease presentation in NOD mice may not be fully representative of the human condition.

Based on such considerations, there is a temptation to dismiss any fundamental contribution of B-lymphocytes in the progression of type 1 diabetes and to consign them to largely secondary systemic actions associated with autoantibody production. However, this might prove to be an over-simplification and, as a community of type 1 diabetes researchers, we may downplay the role of B-lymphocytes at our peril.

Where Do B-Lymphocytes Exert Most Influence in Type 1 Diabetes?

As already noted, the fact that B-lymphocytes are responsible for autoantibody production in type 1 diabetes places them in one particular niche; namely the bloodstream. It is here that clonally expanded populations of B-lymphocytes produce and secrete autoantibodies which are targeted to particular epitopes located on a specific sub-group of islet proteins. However, not all B-lymphocytes are circulating, nor are they necessarily engaged actively in antibody secretion since some may play additional roles in the autoimmune process (12).

B-lymphocytes are also found within secondary lymphoid organs such as the draining lymph nodes located in most organs and tissues, and it is here that lymphocyte subpopulations typically engage with one another to prime the autoimmune response (16). The pancreas contains a relative abundance of such lymphoid tissue [more than 50 pancreatic lymph nodes have been reported in some individuals (17, 18)] but, despite this, pancreatic lymph nodes have been analyzed in detail only rarely in human subjects with type 1 diabetes. One of the few such studies examined a selection of cases held within the Network of Pancreatic Organ Donors (nPOD) Biobank and concluded that no clear structural differences exist between the pancreatic lymph nodes found in donors with type 1 diabetes and those without (19). Our own recent collaborative work has also addressed this question and we observed changes indicative of disease associated events (18). These differing conclusions may reflect the varying disease duration among the subjects studied.

In humans, lymph nodes comprise an outer capsule encompassing a cortex of B- and T-cell follicles (arranged in outer and inner zones respectively) displayed around an inner medulla and hilum; the latter serving as a conduit for the egress of lymphatic fluid (18). B-cell follicles localized within the cortex can exist in one of two principal states, being either primary or secondary, with the latter containing the germinal centers in which B-lymphocytes encounter their cognate antigen and are induced to differentiate into plasma cells. We noted that the structural organization of B-cell follicles was atypical in people newly diagnosed with type 1 diabetes when compared to similarly aged control subjects (18). The frequency of B-cell follicles in which germinal centers could be identified, was reduced by a mean of four-fold in recent-onset type 1 diabetes although, intriguingly, this difference was lost as disease duration increased. The reduction in B-cell follicle frequency did not correlate with the "intensity" of insulitis nor with the age at onset of disease, suggesting that this feature may be a characteristic of the early phase of the disease in all subjects. The precise significance of these findings has not been deduced but it is conceivable that the reduction in secondary follicle and germinal center formation may reflect the diversion of large

numbers of B-lymphocytes toward a plasma cell phenotype early in disease progression. This would be consistent with our observation that follicle numbers recover at later times since the production of autoantibodies tends to decline with disease duration.

There is an important additional site at which B-lymphocytes reside in type 1 diabetes and this is among the immune cell population associated with inflamed islets (20, 21). The weight of evidence suggests that B-lymphocytes are unlikely to accumulate here as a result of (or to facilitate) increased autoantibody production, implying an alternative role. Thus, we have explored their localization in the islet infiltrates in more detail.

Islet Infiltrates Contain Varying Proportions of B-Lymphocytes

In order to study B-lymphocyte infiltration in the pancreas in type 1 diabetes we have exploited a collection of autopsy samples held within the Exeter Archival Diabetes Biobank (EADB) which comprises a large number of samples recovered from subjects newly diagnosed with the disease (8). These represent a historical collection but they offer the advantage that, included among them, are samples from very young children (more than 50 are available from children who were under 10years at diagnosis) as well as from those who were older at onset (principally in their teenage years). The samples have been preserved effectively and they allow comparisons to be made of the profiles of insulitis according to age at diagnosis and severity of beta-cell loss in subjects with relatively short disease duration, where active disease is still evident. The collection also contains samples from people who have lived with the disease for longer periods and, in whom, beta-cell destruction is essentially complete.

An important additional feature is that many of the recent-onset pancreas samples available in the EADB contain islets at varying stages of autoimmune attack (21, 22). Thus, some islets have a relatively heavy immune cell infiltrate coupled with extensive beta-cell loss. By contrast, others appear essentially untouched, being devoid of immune infiltrates and having a full complement of beta cells. Still others display an intermediate phenotype such that varying stages of beta cell destruction can be seen in parallel with the presence of small numbers of influent immune cells. By analyzing multiple islets across a series of individual cases at each stage of the process it is possible to gain an impression of the dynamics of immune cell recruitment (and subsequent egress) and of beta cell loss by reconstructing the profile of these processes in pseudotime.

We first undertook such an analysis more than 10 years ago (21) and were surprised to find that the proportion of B-lymphocytes present within islet infiltrates varies according to the stage of islet destruction. Moreover, it also varies in parallel with the number of CD8+ T-cells but not in proportion to the numbers of CD4+ cells present. In all cases studied, and in common with earlier findings (23) it was clear that CD8+ T-cells form the predominant population as insulitis develops. We also noted that the influx of B-lymphocytes closely mirrors the CD8+ cell profile. This could be taken to imply that a similar cocktail of chemokines is involved in recruitment of both cell types but this begs the question as to why B-lymphocytes are responsive to the chemoattractant gradient and what their role at the site might be. As noted above, one possibility is that they enter the islet infiltrates as antibody producers but, arguing against this, there is little evidence of autoantibody deposition in and around infiltrated islets in type 1 diabetes. Moreover, in the majority of cases, the influent B-lymphocytes do not stain positively for the marker CD138 (21) which is recognized as being upregulated on antibody-producing plasma cells. Furthermore, the process of differentiation by which B-lymphocytes mature into plasma cells is usually associated with a loss of the surface marker CD20, but infiltrating islet-associated B-lymphocytes can be immunostained using an exogenously applied anti-CD20 antibody. Such evidence suggests very strongly that those B-lymphocytes present in and around inflamed islets are not engaged in autoantibody production. What is clear, is that the greater the number of B-lymphocytes in the islet inflammatory infiltrate, the fewer the number of beta-cells that remain at diagnosis, and the more likely a person is to have been diagnosed in the earliest years of life.

The Role of Islet Infiltrating B-Lymphocytes: A Proposal

In view of these findings, we have begun to consider possible alternative roles for islet infiltrating B-lymphocytes in type 1 diabetes and have focused on the notion that they might be involved as potential determinants of CD8+ T-cell activity. It is increasingly understood that B-cells can exert a regulatory influence on T-cell activity (24) and there is firm support for the concept that B- and T-cell interactions occur within pancreatic lymph nodes (25). However, there has been much less focus on the possibility that such interactions may also take place within the islet milieu. To begin to explore this concept, we have examined the localization and morphology of CD20+ cells within the pancreas. This has revealed that significant morphological variations occur as B-lymphocytes migrate through the pancreas and that these correlate with their localization in relation to both CD8+ T-cells and the target islets. We have studied the disposition of CD20 on these cells since on the B-lymphocyte membrane this antigen is present at all stages of B-lymphocyte differentiation in humans, except during the very earliest phase and following their ultimate transition to plasma cells.

In the pancreas CD20+ B-lymphocytes can be found most often in close proximity to islets but some also reside at more distant locations within the parenchyma of the gland (**Figure 1**). Intriguingly, the arrangement of the surface CD20 antigen varies according to their localization within the gland and those B-lymphocytes found at greatest distances from islets (at least as evidenced by examination of 2D-sections) have a relatively uniform distribution of the CD20 antigen around their periphery (**Figure 1A**). This disposition changes, however, as the cells become more closely associated with islets, where they also begin to encounter CD8+ T-cells (**Figures 1B, C**). In these situations, the CD20 immunolabeling becomes focused in discrete regions of the plasma membrane, consistent with a possible change in the activation state of the cells. Moreover, in some cases, the membrane of the CD20+ cells shows intense

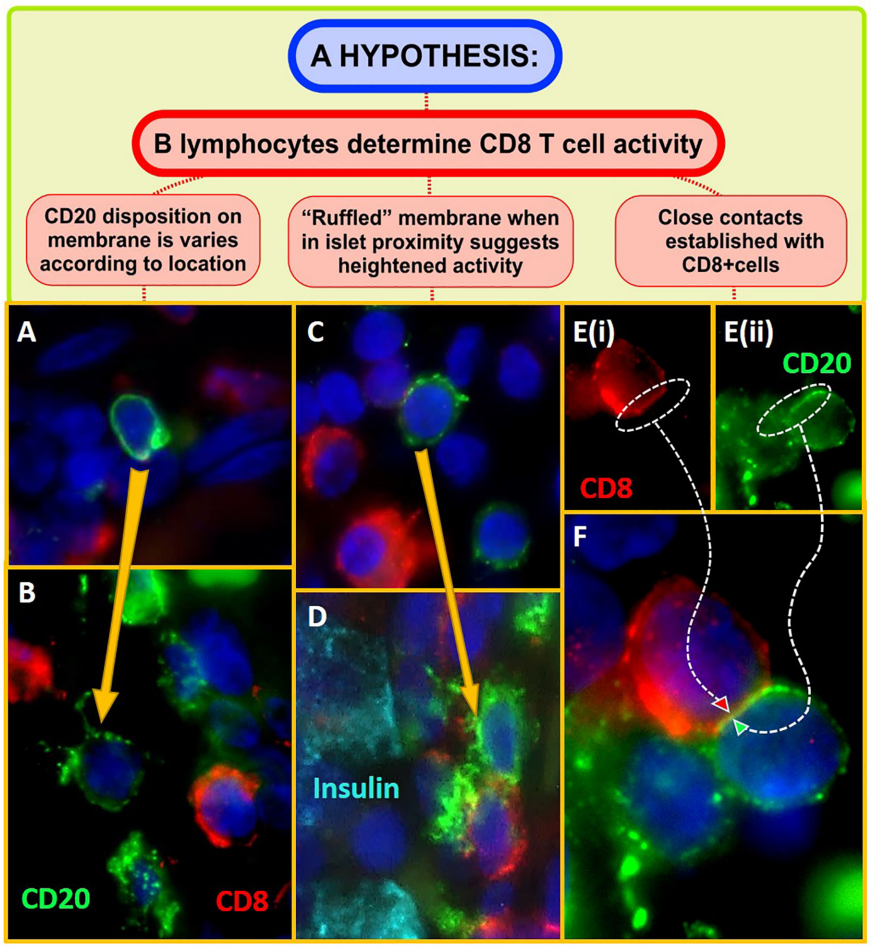

FIGURE 1 | Micrographs showing CD20+ B-lymphocytes (green) and CD8+ T cells (red) within the pancreas of individuals with recent onset Type 1 diabetes. Sections of formalin fixed, paraffin embedded pancreas were dewaxed, rehydrated in an ethanol series, and subjected to heat-induced antigen retrieval (10mM citrate pH6) to unmask antigen binding sites. Highly specific and validated antibodies targeting CD20, CD8, and insulin (Dako, UK) were applied sequentially, in various combinations, for 1 h at room temperature, using standard immunofluorescence staining techniques. Positive signal was visualized with highly cross adsorbed Alexa Fluor-labeled secondary antibodies (Invitrogen, UK) along with the nuclear stain DAPI (dark blue; Invitrogen, UK). Micrographs were captured using either a Leica Dm4000 upright or a Leica DMi8 confocal microscope. **(A)** Depicts a CD20+cell in the pancreatic paranchyma, distant from an islet. The antigen is evenly distributed around the cell and the surface appears smooth in disposition. **(B)** When in proximity to an inflamed islet (i.e., when the CD20+cell was visible within the same region of interest as an islet), anti-CD20 molecules are seen to form aggregates on the cell surface. **(C, D)** Immunolabeling of CD20 becomes increasingly ruffled on the cell surface when the cells are located in closest proximity to insulin positive beta cells (cyan) and CD8+ T-cells. **(E, F)** shows a region of close apposition between a CD20+ B and CD8+ T lymphocyte. This is only seen when the cells are found in groups in close proximity to islets.

ruffling and focusing of the antigen (**Figure 1D**). More strikingly still, as they arrive at the islet site and encounter increasing numbers of CD8+ T-cells, regions of close physical apposition can be discerned between neighboring CD8+ and CD20+ cells (**Figures 1E, F**). The formation of such sites of intimate contact may be important for sustaining and enhancing the state of CD8+ T-cell activation and could offer an explanation for the aggressive autoimmune attack and rapid loss of beta-cells, seen in certain individuals.

Evidence obtained in the NOD mouse has implied that B-cell depletion leads to reduced effector T-cell activation within inflamed islets (26) consistent with an inter-dependence in their actions and the present observations suggest that B- and CD8+ T-lymphocytes may also collaborate actively to promote beta-cell death in human type 1 diabetes.

If this proposal has validity then it might also offer predictive capability; including, for example, the suggestion that, when the number of islet infiltrating B-lymphocytes are in the minority, the rate, and extent of beta cell loss should be correspondingly reduced. Precisely this situation is seen frequently in the inflamed islets of subjects diagnosed with type 1 diabetes during their teenage years (by contrast with those who are younger at onset and often display an inflammatory profile with an elevated proportion of B-lymphocytes) consistent with the existence of two disease endotypes. These have been recently termed "type 1 diabetes endotype 1" and "2" (abbreviated to

T1DE1 and T1DE2) respectively (27). In both endotypes, the predominant immune cells are CD8+ T-cells, consistent with the view that these are likely to represent the principal effector population driving beta cell loss (**Figure 2**).

How Might B-Lymphocytes Interact With CD8+ T-Cells in Islets?

An important question arising from these observations is the nature of the interaction between B- and CD8+ T-cells (as depicted in **Figures 1E, F**) and how this then leads to altered T-cell activation. One tempting possibility is to equate the establishment of the sites of most intimate contact as possible locations where "immune synapses" might form (28, 29). Immune synapses are short lived, planar structures which are extremely difficult to visualize within the confines of two dimensional fixed sections of pancreas. Nevertheless, the close apposition of the membranes of adjacent cells is consistent with this possibility and, as imaging modalities continue to evolve, this may prove a fertile area of exploration.

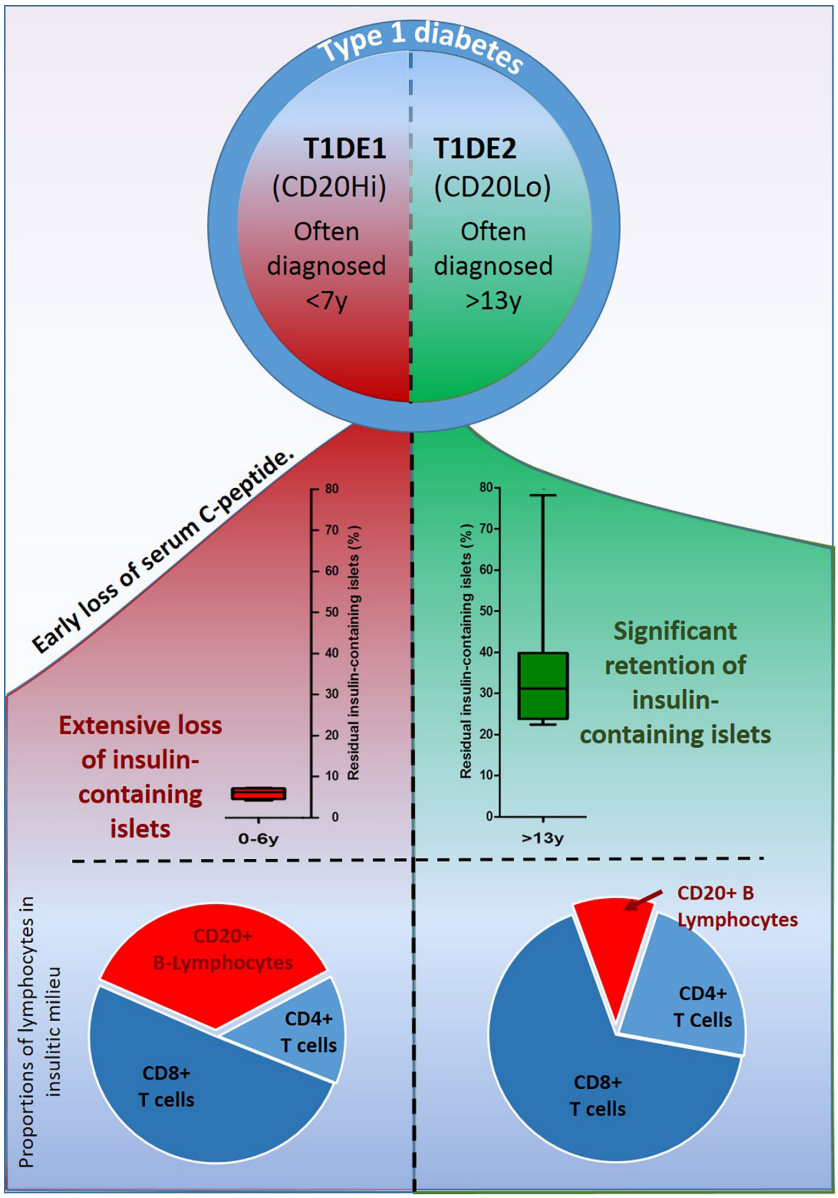

FIGURE 2 | Schematic illustration depicting the main features differentiating the two recently described endotypes of type 1 diabetes. In T1DE1 (left panel) endogenous insulin production falls rapidly, few beta cells are found at diagnosis and symptoms of disease develop in the earliest years of life. B-lymphocytes comprise more than 35% of the immune cell population infiltrating inflamed islets. In T1DE2, subjects are more likely to retain detectable C-peptide well beyond diagnosis; a greater proportion of beta cells survive and clinical symptoms often develop during the teenage years. Individuals defined as T1DE2 have relatively few B-lymphocytes in inflamed islets at the time of diagnosis.

What Are the Outcomes of Differential B-Lymphocyte Recruitment During Islet Inflammation?

Based on the proposition that CD20+ B-lymphocytes might play a role in promoting the activation state of CD8+ T-cells in and around inflamed islets, it should then be anticipated that beta cell loss would proceed least efficiently in situations where the proportion of B-lymphocytes is lowest (i.e. in subjects with T1DE2). While it cannot yet be deduced with certainty that this prediction is fulfilled (not least because the absolute number of CD8+ T-cells also varies substantially between individuals) the available evidence is consistent this possibility. This is most evident when considering two additional parameters which also differ between the two endotypes. Firstly, they segregate very strongly with age at diagnosis such that children with T1DE1 (where islet B-lymphocytes are present in greatest numbers) are diagnosed at the youngest ages and are often <7years at onset (27). By contrast, those with T1DE2 tend to be older when clinical symptoms arise and many are beyond 12 years of age. This difference does not, itself, imply that the rate of beta cell loss necessarily varies between children in each age group since the age at onset is likely to be influenced by many factors, including the specific age at which beta-cell destruction is initiated. Nevertheless, it is clear that the overall extent of beta-cell loss differs across age groups and that, at diagnosis, beta-cell loss is most profound in the youngest children (those under 7years). Not only so, but the number of residual beta cells present within the ICIs at onset is also reduced to a greater extent among the younger age group (**Figure 2**).

CONCLUSION

In summary, we conclude by noting that, while autoantibody production by plasma cells may not be an absolute requirement in type 1 diabetes, this does not mean that B-lymphocytes play only a minimal role. Rather, islet-associated B-lymphocytes may play a profound role in influencing the outcome of autoimmunity in type 1 diabetes, perhaps by regulating the cytotoxic activity of their CD8+ T-cell counterparts. If true, then this has important implications therapeutically since it suggests that maneuvers designed to reduce the proportion of B-lymphocytes available within the islet milieu, may be effective in slowing the rate of beta-cell loss. It further suggests that such maneuvers are likely to be most effective at younger ages. Consistent with this, is the observation that administration of the anti-CD20 targeted monoclonal antibody, Rituximab, delayed disease onset most effectively in the younger sub-group among a cohort of subjects newly diagnosed with type 1 diabetes (30).

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

PL conceived and performed experiments, developed experimental protocols, interpreted data, generated figures, and edited the manuscript. NM supervised the study, designed and interpreted experiments, and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Endocrinology

ORIGINAL RESEARCH
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The Vbeta13 T Cell Receptor Monoclonal Antibody Reduces Hyaluronan and CD68+, CD3+, and CD8+ Cell Infiltrations to Delay Diabetes in Congenic BB DR*Lyp/Lyp* Rats

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The depleting Vβ13a T cell receptor monoclonal antibody (mAb) 17D5 prevents both induced and spontaneous autoimmune diabetes in BB rats. Here it was tested in congenic DR*Lyp/Lyp* rats, all of which spontaneously developed diabetes. Starting at 40 days of age, rats were injected once weekly with either saline, His42 Vβ16 mAb, or 17D5 mAb and monitored for hyperglycemia. Diabetes occurred in 100% (n = 5/5) of saline-treated rats (median age, 66 days; range 55–73), and in 100% (n = 6/6) of His42-treated rats (median age, 69 days; range 59–69). Diabetes occurred in fewer (n = 8/11, 73%) 17D5-treated rats at a later age (median 76 days, range 60–92). Three (27%) of the 17D5-treated rats were killed at 101–103 days of age without diabetes (17D5 no-diabetes rats). Survival analysis demonstrated that 17D5 mAb delayed diabetes onset. Saline- and His42-treated rats had severely distorted islets with substantial loss of insulin-positive cells. These rats exhibited prominent hyaluronan (HA) staining, with the intra-islet HA+ accumulations measuring 5,000 ± 2,400 μm^2 and occupying 36 ± 12% of islet area, and severe (grade 4) insulitis with abundant infiltration by CD68+, CD3+, and CD8+ cells. The 17D5 mAb-treated rats with delayed diabetes onset exhibited less severe insulitis (predominantly grade 3). In contrast, the 17D5 no-diabetes rats had mostly normal islets, with insulin+ cells representing 76 ± 3% of islet cells. In these rats, the islet HA deposits were significantly smaller than in the diabetic rats; the intra-islet HA+ areas were 1,200 ± 300 μm^2 and accounted for 8 ± 1% of islet area. Also, islet-associated CD68+ and CD3+ cells occurred less frequently (on average in 60 and 3% of the islets, respectively) than in the diabetes rats (present in >95% of the islets). No CD8+ cells were detected in islets in all 17D5 no-diabetes rats. We conclude that mAb 17D5 delayed diabetes in DR*Lyp/Lyp* rats and markedly reduced expression of HA and concomitant

infiltration of CD68+, CD3+, and CD8+ cells. Our findings underscore the importance of refining immune suppression in prevention or intervention clinical trials to use mAb reagents that are directed against specific T cell receptors.

Keywords: autoimmune diabetes, BB rat, hyaluronan, insulitis, pancreatic islets, T-cell receptor

INTRODUCTION

The spontaneously diabetes BB rat was described in 1978 (1) and since 1980 (2) subjected to targeted breeding to dissect the genetic mechanisms underlying islet beta cell destruction [reviewed in (3–5)]. The BB rat was found to be lymphopenic (6) which was shown by marker-assisted cross-intercross breeding, to segregate with diabetes as a single locus (2). The lymphopenia gene (7, 8) has a frame-shift mutation in the *Gimap5* gene, resulting in absence of expression of the Gimap5 anti-apoptopic protein (9, 10). Successive crosses of the lymphopenic, diabetes BB-DP rat with the diabetes resistant, non-lymphopenic BB-DR rat allowed the development of the congenic DR*Lyp/Lyp* rat. In this rat, 1) spontaneous diabetes rarely occurs before 50 days of age; 2) 100% of the rats of both sexes develop diabetes before 80 days of age; and 3) a diabetes genetic susceptibility locus, *Iddm14*, was identified proximal to the *Gimap5* mutation (11). This factor was later shown to be a TCR beta chain gene, *Tcrb-V13*, specifically the *Tcrb-V13S1A1* allele. This allele encodes the Vβ13a TCR beta chain (11–13).

DR*Lyp/Lyp* rats (BB Malmö or BBM) were used first to establish that the 17D5 TCR Vβ13a mAb (14, 15) would affect development of diabetes similar to BB-DP and BB-DR rat strains from the Worcester, MA colony (15). Second, we wanted to use this monoclonal antibody to test if islet hyaluronan (HA) is a major contributor to insulitis progression. Islet-infiltrating leukocytes are therapeutic targets in type 1 diabetes since these cells and their secreted products are thought to cause beta-cell destruction. We recently found that islet leukocytic infiltration is associated with remodeling of islet extracellular matrix (ECM), specifically accumulation of hyaluronan (HA), a major islet ECM component (16). HA, a high molecular weight polysaccharide, accumulates in inflamed tissues and is a key regulator of several aspects of inflammation including leukocyte migration and the generation of inflammatory cytokines at sites of injury (17). We showed that HA accumulation in islets precedes insulitis in individuals at high risk of type 1 diabetes as well as in diabetes-prone BB rats in the pre-clinical phase of the disease, and that immune cells enter islets only in the regions that contain large amounts of HA (18). We also found that accumulation of HA in islets determines the continuum of islet immune cell infiltrates from initial peri-insulitis to severe invasive insulitis and with progressive beta-cell loss (16, 18).

The aim of the present study was to administer the 17D5 TCR Vβ13 mAb to DR*Lyp/Lyp* rats in an attempt 1) to replicate the delay in onset or prevention of diabetes reported in BB DR rats treated with virus or the viral mimetic polyinosinic:polycytidylic acid (poly I:C) as well as in BB-DP (15) and LEW.1WR1 (19) rats; 2) to compare CD3-positive (CD3+) and CD8+ T cell and CD68+ monocyte infiltration to that previously reported in the BB rat (20) and human insulitis (21–24), and 3) to examine if islet mononuclear cell infiltration affects HA deposition in islets whether the islets were protected from insulitis, or not, by the 17D5 TCR Vβ13 mAb.

MATERIALS AND METHODS

Animals

Heterozygous BB DR*Lyp*/+ rats originated at the University of Washington (UW), Seattle, WA, USA (8, 11) and were transferred to the Clinical Research Center at Lund University in Malmö, Sweden in 2008. They were re-derived by Caesarian section into a pathogen-free facility fulfilling FELASA standards. Sentinel rats were tested quarterly for known pathogens and although positive for three strains of helicobacter at UW, the re-derived BB rat line in Malmö (BBM DR*Lyp*/+) was free of these and other known bacterial strains as analyzed by the National Veterinary Institute, Uppsala, Sweden. Rats used in these studies were in their 2–4th generation of inbreeding at our facility. All rats were housed at 21–23°C with *ad libitum* access to food and water. Congenic BMM DR*Lyp/Lyp* rats were maintained in heterozygous sister-brother breeding. The project was approved by the Animal Ethical Committee in Lund University, Sweden.

Genotyping

A panel of seven SNPs was used to genotype the rats in earmarking biopsies obtained from 21-day-old pups. Each SNP marks the presence of either BB-DP or BB-DR rat DNA. SNPs above and below the flanking markers represent BB-DR rat DNA. The BB-DP rat genetic contribution is estimated to represent about 1.4 Mbp of DP-derived DNA in the BBM DR*Lyp*/+ congenic strain.

DNA was isolated by digestion for 90 min at 55°C at an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany) in 10 µl/sample of 20 mg/ml Proteinase K (recombinant, PCR grade, Thermo Scientific, Waltham, MA, USA), followed by precipitation in ice cold isopropanol. After drying, the DNA pellet was dissolved in TE buffer (AppliChem, Darmstadt, Germany) and shaken overnight at 37°C. DNA (2 µl) in 384 well plates were subjected to PCR analysis with the forward and reverse primers shown in **Table 1**. Primers and TaqMan® Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA) in a final 3–5 µl volume were added; the 384 well plates were centrifuged before sealed and then incubated (ViiA 7 RT-PCR System, Applied Biosystems, Waltham, MA, USA).

T Cell Receptor Monoclonal Antibodies

The T cell monoclonal antibodies 17D5 (14, 25) and His42 (26) directed against TCR Vβ13^a and Vβ16, respectively, were prepared in the Mordes' laboratory, University of Massachusetts, Worcester,

TABLE 1 | SNP genotyping of the *lyp* region in the DR*Lyp/Lyp* rats.

Position	Dist. Mutation	Forward Primer Seq.	Reverse Primer Seq.	Rep 1 Seq. (DR+/+)	Rep 2 Seq. (DR*Lyp/Lyp*)
77 630 116	-753 877	GCAGGAGAGGCTGCTATGTC	TCCACAGCATGCCAAGCT	CCACAGGAGAGTTGTG	CACAGGAGACTTGTG
78 170 204	-213 789	CCTCCCTTGGTATGTTGTGTATGAT	TGCCAGGGATAGCAGAGTGA	TCCCTTCTGTGTCCAGTAC	CCTTCTGCGTCCAGTAC
78 377 812	-6 181	GGTAAATACTTCACCCTGCTTCCA	GCTGGTTACCCCTTCTTTACTATTTGA	CATTATGGAGTTTCTAGCCTAT	ATGGAGTTTCTCGCCTAT
78 383 993	0	ACATGGGAGGGAAGGAGCTT	CATGTCTTGGTTCTGGATCTTTGAC	ACGCCCCCCATCTT	CACGCCCCCATCTT
78 386 587	2 594	GATACGGGAAAGTACAGGGTAGACT	TCAGTTTTTGAAAATGACATCTAAAGTTTCTGT	ATTCTCTCCGACCCCAGTT	ATTCTCTCCGAACCCAGTT
78 576 508	192 515	CCCTACTACCTGCCCTTTAGAAG	GACCTGCTTATTTCTGGAGTGGATT	TCTTCAGATGTACTTTTAG	CTTCAGATGTCCTTTTAG
79 071 296	687 303	CCATACCATCTCTATCATCCTGTTTCC	GGGCTACAAAACAAAGCAGAAAGTT	AATCTGGGAAAGGCA	TGAATCTGAGAAAGGCA

Distance mutation is the number of base pairs from the Gimap5 frame shift mutation (0). The forward and reverse primer sequences used to identify the SNP between DR+/+ (wild type) and DRLyp/Lyp are shown.

MA, USA. Two batches of 17D5 and one batch of His42 were used. The hybridoma producing 17D5 mouse anti-rat Vβ13 mAb (IgG2a) recognizes the product of the *Tcrb-V13S1A1* (Vβ13^a) allele of the *Tcrb-V13* (Vβ13) gene (14). The hybridoma producing His42 mouse anti-rat Vβ16 (IgG2b) mAb (26) was originally the gift of Dr. Thomas Hünig. Both antibodies were prepared as ascites and purified by affinity chromatography.

Treatment Protocol

The BBM DR*Lyp/Lyp* rats were weighed daily in the morning (07:30–08:30). Blood glucose was measured daily beginning at 40 days of age. Starting at 40 days of age rats were injected intraperitoneally (i.p.) once a week with either 0.1 mg 17D5 (n = 11) or His42 (n = 6) mAb in 0.5 ml saline, or with 0.5 ml saline alone (n = 5). Initially, six, three, and three, rats received either 17D5, His42, or saline, respectively. The rest of the rats, generated from additional breeding, received the treatment 2 months later, and were followed similarly to the first group. Treatment continued until the rats developed diabetes (defined as a glucose concentration >11.1 mmol/L). Six rats that did not receive any treatments were killed at 40 days of age. It was predetermined that DR*Lyp/Lyp* rats that remained diabetes-free at 95 days of age would be killed at about 100 days of age.

Immunohistochemistry

Formalin-fixed paraffin-embedded pancreas tissues were serially sectioned. Consecutive sections were stained for H-E, insulin and glucagon, HA, CD68, CD3, and CD8 in different combinations. Four to six sets of serial sections were prepared per pancreas. All islets present in a stained section, on average 71 islets (range 52–99 islets), were counted per rat. The following primary antibodies were used: insulin (Dako, Glostrup, Denmark), glucagon (Sigma-Aldrich, St Louis, MI, USA), and CD68 (clone ED1, Bio-Rad, Hercules, CA, USA), at dilutions 1:500, 1:2,000, and 1:100, respectively. Antibodies to CD3 (clone SP7, Abcam, Cambridge, UK) and CD8 (Abcam) were used at dilution 1:150. Staining for HA was performed as described (16, 18). Sections were incubated overnight with the primary antibodies and then for 1 h with the following secondary antibodies at dilution 1:400: Alexa Fluor® 488 conjugated goat anti-rabbit IgG (Invitrogen), Alexa Fluor® 568 conjugated goat anti-mouse IgG (Invitrogen), and Cy™2 donkey anti-guinea pig IgG (Jackson Immunoresearch). The stained sections were mounted with VECTASHIELD Vibrance with DAPI (a nuclear counterstain) Antifade Mounting Medium (Vector Laboratories, Burlingame, CA, USA).

Quantification of Insulin- and HA-Stained Areas

Islet insulin-stained areas were measured using ImageJ supported by the National Institutes of Health (https://imagej.nih.gov/ij/index.html). The measurement of total and intra-islet HA-positive (HA+) areas was performed using whole-section imaging as described (16, 18).

Whole-section imaging was performed using a NanoZoomer Digital Pathology slide scanner (Hamamatsu; Bridgewater, NJ, USA). Slides were scanned in bright field with a 20× objective and the digital images imported for analysis using HALO image

analysis software (Indica Labs, Albuquerque, NM, USA). Islets were identified by their staining for synaptophysin (SYN). All the islets present in the rat pancreas sections were analyzed. The intra-islet HA+ area represents the HA located within islet area bordered by the endocrine side of the peri-islet capillaries. The total islet HA area is the sum of the intra-islet HA+ and the HA+ area measured around the islets at a 5-nm distance from the endocrine side of the peri-islet capillaries (16). The outer border of this area was delineated using the HALO annotation tool. The stained areas were measured using HALO software. The percent islet HA+ area is calculated as intra-islet HA+ area/islet area × 100.

Evaluation of Insulitis

Insulitis was quantified as described (16, 18, 27). The sections were independently scored by two investigators unaware of the treatment status of the animals as follows: Grade 0, no infiltration; Grade 1, leukocytes present around islets (peri-insulitis) or ≤15 dispersed leukocytes within islets; Grade 2, leukocytes infiltrating <25% of the islet area; Grade 3, leukocytes occupying 25–50% of the islet area; Grade 4, leukocytes diffusely present throughout the islet and end-stage islets devoid of beta cells. The distribution of macrophages and cytotoxic T cells was evaluated in sections stained for the immune markers CD68, CD3, and CD8. All the islets and immune cells stained for these markers present in the sections were counted, and the prevalence of islets with CD68+, CD3+, or CD8+ cells and the number of these cells per islet were determined.

Statistical Analysis

Parametric data are given as arithmetic means ± SD. Diabetes-free survival is presented as the median and groups were compared using Kaplan-Meier methodology; equality of survival distributions was tested by log rank statistic (GraphPad®). The significance of the difference between two or more groups of data was evaluated using the Mann–Whitney U test or ANOVA. A p value of less than 0.05 was considered statistically significant. All analyses were performed using GraphPad Prism version 8.00 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Treatment With 17D5 mAb Delayed or Prevented Diabetes Onset in BBM DR*Lyp/Lyp* Rats

All saline treated DR*Lyp/Lyp* rats (n = 5/5) developed diabetes between 55 and 73 days of age (median 66 days; **Figure 1A**). The average blood glucose concentration at diagnosis was 16.4 ± 5.3 mmol/L. Administration of the His42 mAb did not affect diabetes onset; all His42-treated rats (n = 6/6) developed

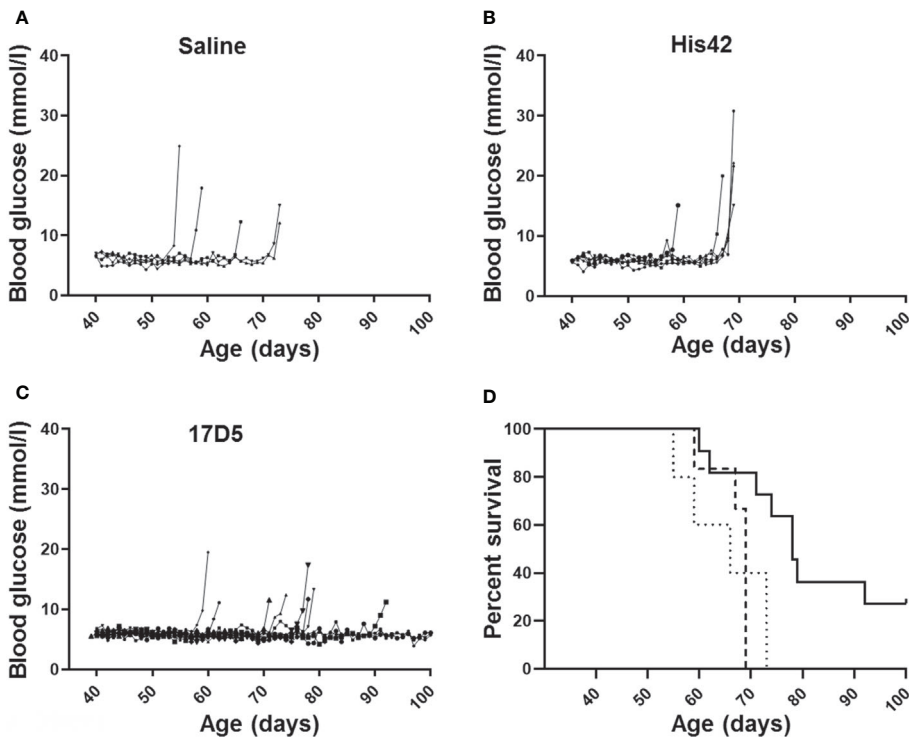

FIGURE 1 | Daily blood glucose levels measured daily and age at diagnosis of diabetes in DR*Lyp/Lyp* rats receiving either saline (**A**, n = 5), monoclonal antibody His42 (**B**, n = 6), or monoclonal antibody 17D5 (**C**, n = 11). The saline treated DR*Lyp/Lyp* rats developed diabetes between 55 and 73 days of age (median 66 days). The His42-treated rats developed hyperglycemia between 59 and 69 days of age (median 69 days). In contrast, diabetes occurred in fewer rats treated with 17D5 (n = 8/11), between 74 and 92 days of age (median 76 days). Kaplan Meier analysis in **(D)** showed that survival times in the saline (dotted line) and His42 (dashed line) groups were statistically similar whereas survival in the 17D5 group (solid line) was prolonged ($P < 0.001$).

hyperglycemia between 59 and 69 days of age (median 69 days, **Figure 1B**). The average blood glucose level at diagnosis was 20.8 ± 5.8 mmol/L. In contrast, diabetes occurred in fewer rats treated with 17D5 (n = 8/11, 73%), and the age at onset was delayed (median 76 days, range 74 to 92 days, $P < 0.05$, **Figures 1C, D**). The blood glucose concentration at diagnosis in the eight 17D5-treated rats developing diabetes (17D5 delayed group) was 13.5 ± 3.2 mmol/L, which was slightly (~3 mmol/L) lower than in the combined cohort of saline- and His42-treated rats ($P < 0.05$). Five of the eight 17D5 rats did not develop hyperglycemia until 74, 78 (n = 2), 79, and 92 days of age, respectively. Remarkably, three 17D5-treated rats maintained normal blood glucose until 101 (n = 1) and 103 (n = 2) days of age, at which time the rats were killed per protocol (17D5 no-diabetes group). The blood glucose levels at the day of killing were 5.6, 6.6, and 5.3 mmol/L, respectively. Survival analysis of the entire cohort showed that the 17D5-treated rats had a longer diabetes-free survival ($P < 0.001$) compared to the other groups ($P > 0.05$).

Growth in Saline-, His42-, and 17D5-Treated DR*Lyp/Lyp* Rats

The body weight (g) curves indicated that growth was not affected by either mAb treatment. At disease onset, the 17D5-treated female and male rats weighed 201 ± 21 g and 309 ± 18 g, respectively, which were comparable to those of the female and male rats in the combined saline and His42 groups (188 ± 9 and 292 ± 10 g). Similarly, the average growth rate (g/day) in the female and male rats did not differ significantly among the 17D5-treated (n = 11) and the combined saline and His42 groups (n = 11, data not shown).

Preservation of Islet Architecture and Beta Cells in 17D5 No-Diabetes DR*Lyp/Lyp* Rats

At 40 days of age, DR*Lyp/Lyp* rat islets typically exhibit the endocrine cell composition and organization observed in normoglycemic rodents, with the insulin+ cells being the majority of the endocrine cells (83 ± 4%) located in the center, surrounded by a halo of glucagon+ cells (**Figures 2A, E**). At the time of clinical onset, the diabetes rats in the saline, His42, and delayed diabetes groups exhibited substantial loss of beta cells (**Figures 2B, C, F, G, I**) with the remaining insulin+ cells accounting for 7 ± 8% of the islet cells. The insulin-stained areas measured 500 ± 640 μm^2 and occupied 0.03 ± 0.02% of the pancreas area. In 18 of 19 diabetes rats, the proportion of insulin+ cells varied from 0 to 15%. Interestingly, in one rat in the delayed

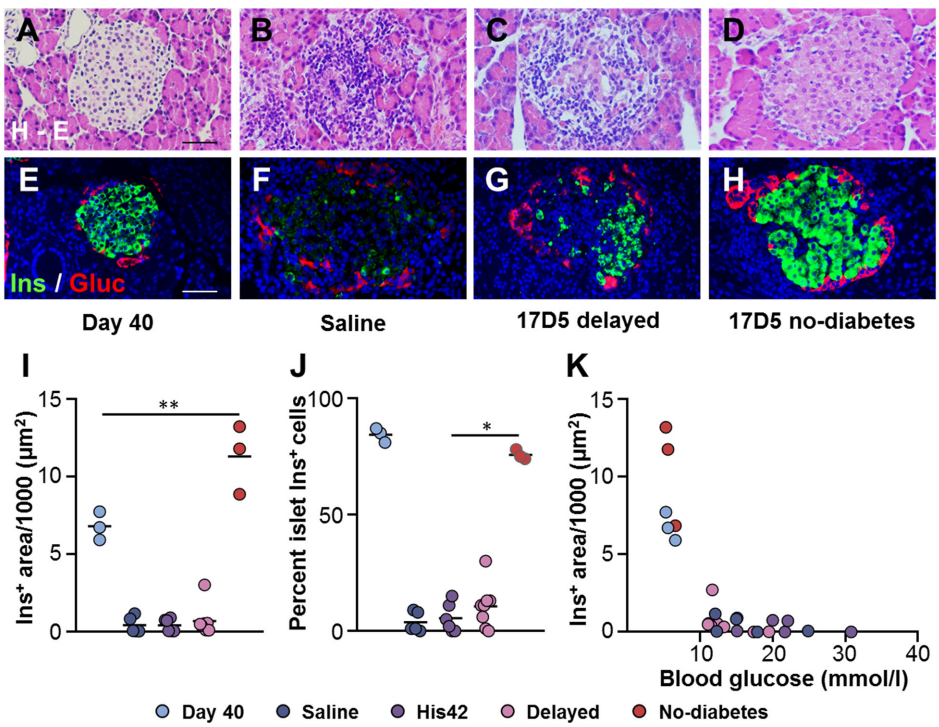

FIGURE 2 | Preservation of islet morphology in the 17D5-no diabetes DR*Lyp/Lyp* rats. **(A–D)** Hematoxylin-Eosin (H-E) staining in pancreas tissues from rats in the indicated groups. **(E–H)** Immunohistochemistry for insulin (Ins, green) and glucagon (Gluc, red). Scale bars, 50 μm. To avoid redundancy, images from His42-treated rats are not shown. **(I)** Morphometric quantification of islet Ins+ areas. **(J)** Relative proportion of islet Ins+ cells. **(K)** Ins+ areas plotted as a function of blood glucose levels. Each circle denotes an individual rat; dark slate blue, saline (n = 5); purple, His42 (n = 6); pink, 17D5 delayed (n = 8); red, 17D5 no-diabetes (n = 3); light slate blue, 40 day old (n = 3). Data are mean values of measurements obtained for each rat. The solid horizontal lines in **(I, J)** indicate the mean of the measurements in each group. *$P < 0.05$, 17D5 no-diabetes *vs* saline, His42, or 17D5 delayed; **$P < 0.01$, 17D5 no-diabetes *vs* saline, His42, 17D5 delayed, or day 40; Mann-Whitney U test.

diabetes group, which developed hyperglycemia at 78 days of age, one third of the islets still contained insulin+ cells accounting, on average, for 32% of islet cells. In addition, in this rat, the glucagon+ cells had maintained their peripheral location in islets (**Figure 2G**), indicative of some degree of preserved islet architecture. In contrast to diabetes rats, islet architecture and cell composition were maintained in the 17D5 no-diabetes rats (**Figures 2D, H**). The islet insulin+ areas in these rats were 30- and 21-fold larger than in the control (saline- and His42-treated) and 17D5 delayed diabetes rats, respectively, and the insulin+ cells represented 76 ± 3% of all islet cells (**Figures 2I–K**), which is comparable to that in the normoglycemic DR*Lyp/Lyp* rats at 40 days of age ($P > 0.05$).

Prevention of Invasive Insulitis in 17D5 No-Diabetes DR*Lyp/Lyp* Rats

The rats in the saline- and His42-treated groups exhibited severe insulitis (**Table 2**). In these rats, 94% of the islets (range 88–100%) were penetrated by dense inflammatory cell infiltrates. The rats in the 17D5 delayed group overall exhibited a lower degree of immune cell infiltration as compared to the two other diabetes groups. In the former, on average, the prevalence of islets with severe insulitis (grade 4) was 68 ± 10%, which was lower than that in the latter (94 ± 5%, $P < 0.005$). Also, in the 17D5 delayed rats, on average 18% of the islets exhibited no or limited islet infiltration, compared to only 3% of the islets in the control diabetes rats. In contrast, in the three 17D5-treated normoglycemic rats, on average, 83 ± 3% of the islets did not show any evidence of insulitis. Peri-insulitis or scarce immune cells in the islet periphery were observed in 11 ± 2% of the islets, while only 6% of the islets exhibited infiltrating immune cells that had spread throughout the islet.

Insulitic CD68+ cells were absent in the DR*Lyp/lyp* rats at 40 days of age (**Figure 3A**), while they were abundant in the diabetic rats (25 ± 16 cells/islet), where they occurred in the vast majority of the islets (**Figures 3B, C, M**). Islet-associated CD68+ cells were observed in the pancreata of the three 17D5 no-diabetes rats (**Figure 3D**). However, these cells were present in significantly fewer numbers than in the diabetic rats (1–7 cells/islet, $P < 0.05$).

Since exposure to 17D5 mAb was expected to deplete T cells, we examined the presence of CD3+ and CD8+ T cells in islets of 17D5-treated rats (**Figures 3E–L**). CD3+ cells were observed in >95% (range 88 to 100%) of the islets in all diabetes rats with a frequency that did not differ between the 17D5 delayed and saline- and His42-treated rats (18 ± 3 *vs* 20 ± 6 CD3+ cells/islet, respectively, $P > 0.05$). However, only 3% of the islets in the 17D5 no-diabetes rats exhibited CD3+ cells (**Figure 3N**) that occurred in significantly fewer numbers than in the other three groups (0.5 ± 0.6 cells/islet, $P < 0.05$). CD8+ cells were present in all the diabetes rats and were observed in 81% (range 43 to 100%) of their islets. In contrast, no CD8+ cells were detected in the vicinity or within islets in the three 17D5-treated normoglycemic rats (**Figure 3O**). Thus, the 17D5 mAb has successfully prevented immune cell infiltration and beta cell destruction in these rats.

TABLE 2 | Evaluation of insulitis in the DR*Lyp/Lyp* rats.

Group	Rat (n)	% Islets with insulitis				
		Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Saline	5	1	1	1	2	95
His42	6	0	0	3	4	93
17D5 delayed	8	2	5	8	13	71
17D5 no-diabetes	3	83	11	4	1	0

Treatment With 17D5 Prevents the Formation of Large HA Deposits in Islets in 17D5 No-Diabetes DR*Lyp/Lyp* Rats

In line with our previous findings (18), HA staining was scanty in islets of DR*Lyp/Lyp* rats at 40 days of age (**Figure 4A**). The older rats that developed diabetes exhibited substantial accumulation of HA in their islets, both within and at islet periphery (**Figures 4B, C**), which is consistent with our previous observation of the formation of large HA deposits in DR*Lyp/Lyp* rats exhibiting hyperglycemia (18). In these rats, the intra-islet HA+ accumulations measured 5,000 ± 2,400 μm^2 (**Figure 4F**), occupying on average 36 ± 12% of the islet area (**Figure 4G**). In contrast, the islet HA deposits were significantly smaller than in the 17D5-treated DR*Lyp/Lyp* rats that remained normoglycemic at the end of the period of observation. In these rats, the intra-islet HA+ areas were 1,200 ± 300 μm^2 and accounted for 8 ± 1% of the islet area (**Figures 4D–G**). However, in the 17D5-no diabetes group, the HA-stained areas were 5-fold larger than in their normoglycemic littermates at 40 days of age, while the islet size increased only 70%.

DISCUSSION

The major finding of this study is that the Vβ13 T cell receptor mAb 17D5 delayed the onset of diabetes in congenic DR*Lyp/Lyp* rats. It confirms previous observations in spontaneously diabetes BBDP rats (15) and LEW.1WR1 rats in which diabetes was induced by poly I:C or viral infection (13). While all DR*Lyp/Lyp* rats injected with saline developed diabetes between 55 and 73 days of age, remarkably, the 17D5 monoclonal mAb not only delayed the onset but also prevented diabetes up until 100 days of age in a sizable and significant proportion of the treated animals. Interestingly, 17D5-treated rats had lower blood glucose at diagnosis than those in the saline- or His42-treated groups. Diabetes still developed rapidly in some 17D5-treated rats, but the severity of hyperglycemia at onset was reduced, perhaps due to fewer beta cell-specific cytotoxic T cells. The 17D5 TCR Vβ13a mAb should therefore prove useful to dissect the mechanisms of beta-cell killing.

The three treated rats that remained diabetes-free by 100 days of age had normal blood glucose associated with a reduction in CD3+ and CD8+T cells as well as in HA staining. Some infiltration of mononuclear cells was observed in them so it cannot be excluded that, if these rats had been allowed to survive, they might have developed infiltration of CD3+ and CD8+ T cells along with CD68+ cells and expanded their islet HA deposits. The data suggest that treatment with 17D5 likely

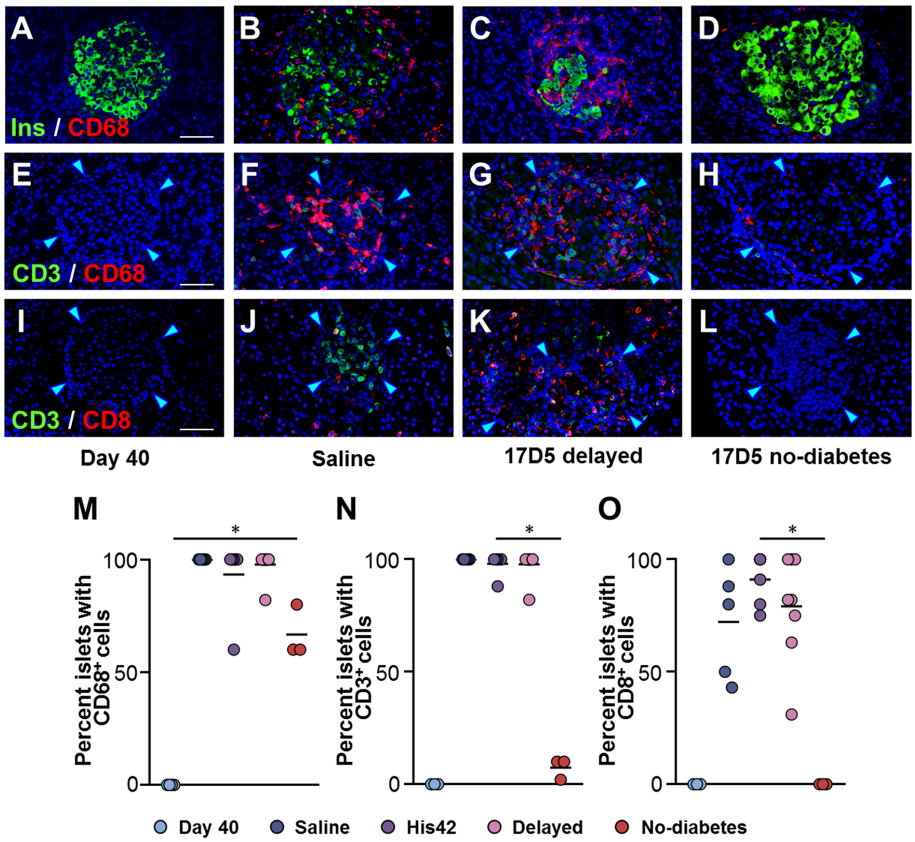

FIGURE 3 | Occurrence of insulitic macrophages and lymphocytic cells in DR*Lyp/Lyp* rats. **(A–D)** Immunohistochemistry for insulin (Ins, green) and the macrophage marker CD68 (red) in the indicated groups. Immunostaining for the T cell marker CD3 (green) and either CD68 **(E–H)**, or the cytotoxic T cell marker CD8 **(I–L)**. Scale bars, 50 µm. To avoid redundancy, images from His42-treated rats are not shown. The arrowheads point to the islet border. Prevalence of islets with insulitic CD68 **(M)**, CD3 **(N)**, and CD8 **(O)** cells. Each circle denotes an individual rat; dark slate blue, saline (n = 5); purple, His42 (n = 6); pink, 17D5 delayed (n = 8); red, 17D5 no-diabetes (n = 3); light slate blue, 40 day old (n = 3). Data are mean values of measurements obtained for each rat. The solid horizontal lines indicate the mean of the measurements in each group. *$P < 0.05$, 17D5 no-diabetes *vs* saline, His42, 17D5 delayed, or day 40; Mann-Whitney U test.

needs to be continuous as opposed to a brief treatment to prevent diabetes. It is unlikely that the 17D5 mAb treatment was curative, and further studies are warranted to explore the mechanisms by which it prevents both islet mononuclear cell infiltration and progressive accumulation of HA. It would also be important to titrate the 17D5 mAb to determine a suitable and informative dose level for future experiments.

Differences between the present study and earlier studies of the 17D5 mAb are noted. In spontaneously diabetes BBDP/Wor rats from the Worcester colony, the same mAb using the same protocol (weekly treatment from 45 to 100 days of age) completely prevented diabetes (15). However, diabetes in the BBDP/Wor rat is more indolent in onset and not 100% penetrant; not all control animals in the 17D5 treatment study developed diabetes. In studies of autoimmune diabetes that is induced in LEW1WR1 rats by viral infection or poly I:C, treatment was instituted before the induction of autoimmunity (at ~21 days of age) and given three times weekly (15). In that study most animals were protected. Hence, in regard to partial protection afforded by 17D5 in the DR*Lyp/Lyp* rat, it remains to be determined if the treatment was sufficiently intense (in terms of dose, frequency, or both), or if it was started too late. The present study protocol required killing any rat that survived 100 days of age; at that time point the blood glucose was within the normal range, and the HA deposition and insulitis scores were also markedly reduced. The variable reduction in peri-insular and islet infiltrating CD68+ cells by the 17D5 mAb suggest that the mAb was not fully effective at preventing autoimmune inflammation. Taken together, the data suggest that treatment with 17D5 mAb might have reduced the migration of inflammatory cells into islets and reduced beta cell death.

We have speculated that the migration and accumulation of CD3+ and CD8+ cells may be related to the presence of HA. This hypothesis is based on our recent work showing that, in both humans and BB rats in the preclinical phase of the disease, leukocytes that infiltrate islets do so exclusively in the regions that are rich in HA, and that progressive accumulation of HA in islets is associated with advancement to severe insulitis (18). Although the 17D5 Ab did not deplete the population of islet infiltrating CD8+ cells in the delayed-diabetes rats, it can be speculated that 17D5 might modify or inhibit their interactions with HA or other local

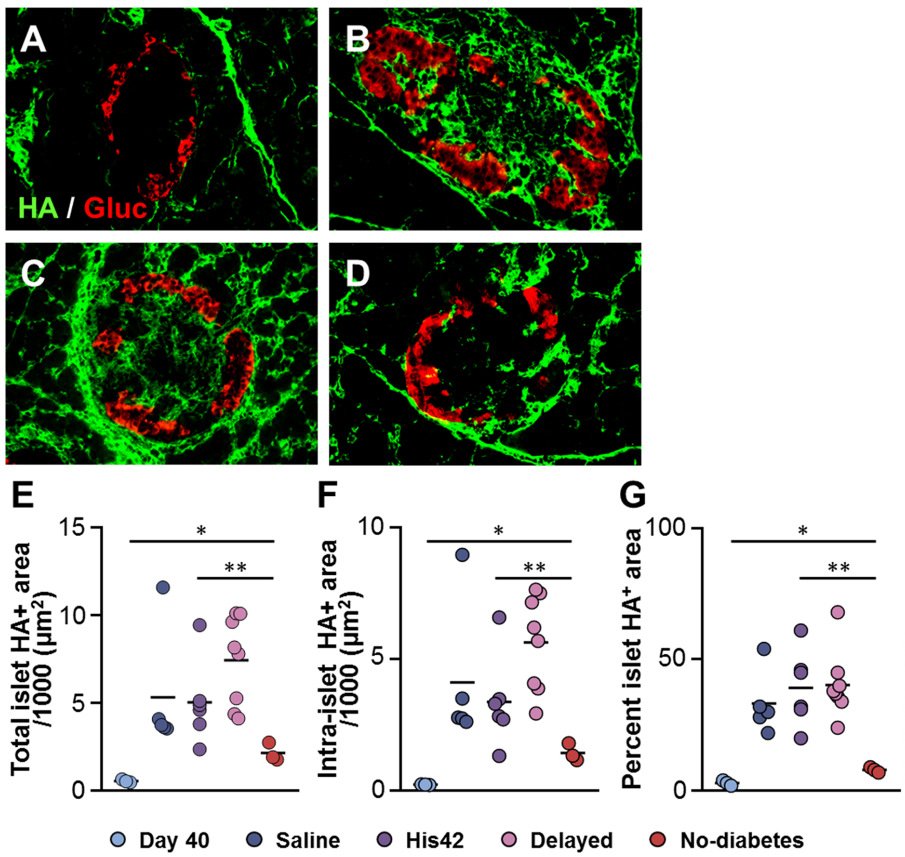

FIGURE 4 | Reduction in islet hyaluronan (HA) in 17D5 no-diabetes DRLyp/Lyp rats. **(A–D)** HA (green) and glucagon (Gluc, red) staining in islets from DRLyp/Lyp rats at 40 days of age **(A)**, and from rats in the saline **(B)**, 17D5 delayed **(C)**, and 17D5 no-diabetes **(D)** groups. Scale bar, 50 µm. To avoid redundancy, images from His42-treated rats are not shown. Morphometric quantification of total **(E)** and intra- **(F)** islet HA+ areas. **(G)** Relative proportion of islet areas stained for HA. Each circle denotes an individual rat; dark slate blue, saline (n = 5); purple, His42 (n = 6); pink, 17D5 delayed (n = 8); red, 17D5 no-diabetes (n = 3); light slate blue, 40 day old (n = 3). Data are mean values measurements obtained for each rat. The solid horizontal lines indicate the mean of the measurements in each group. *P < 0.05, 17D5 no-diabetes *vs* day 40; **P < 0.01, 17D5 no-diabetes *vs* saline, His42, or 17D5 delayed; Mann-Whitney U test.

cells, and thus reducing their capacity for migration and hence the severity of insulitis in these rats. Our recent studies indicated that in BB rats in the pre-clinical phase of the disease, the extent of the HA deposition is associated with the level of the insulitic cell infiltration, in particular that of grades 1–3. The present study reinforces this finding; the 17D5 no-diabetes rats did not develop the extensive HA deposits observed in the diabetes rats. That the size of HA deposits in the 17D5 delayed-diabetes rats was comparable to that in the saline and His42 diabetes rats, in spite of a lower degree of insulitis (grade 3 *vs* grade 4) is also in line with our previous observation that the size of the islet HA accumulations does not increase further with the progression from grade 3 to grade 4 insulitis.

The reduction in islet HA along with the attenuation of insulitis in the three normoglycemic 17D5-treated BB rats further indicates a relationship between the presence of VB13a+ T cells, invasive insulitis and the accumulation of HA.

In spite of limiting HA accumulation, the effective 17D5 mAb treatment did not fully prevent the deposition of HA in islets; the no-diabetes DR*Lyp*/*Lyp* rats still exhibited HA+ areas that were 5-fold larger than those measured in the non-diabetic 40-day-old DR*Lyp/Lyp* rats, while the beta cell areas were expanded only by 70%. We recently reported that in the diabetes prone DR*Lyp/Lyp* rats, the initial induction of HA synthesis and deposition in islets takes place prior to insulitis (18). HA synthesis may therefore not be dependent on the presence of infiltrating cells but could rather be due to other factors generated locally or reaching the islets *via* circulation (18). We also showed that the islet HA deposits expanded with time, which was associated with the progression to invasive insulitis. The fact that the treatment with a mAb expected to deplete or inhibit T cells expressing one specific particular TCR beta chain suggests that continual HA accumulation in islets could be related to the presence of insulitic T cells. It is conceivable that sufficient depletion of pathogenic T cells targeting beta cells can, directly or indirectly, limit the progressive deposition of pro-inflammatory HA in islets. Absence or reduction in the number of islet-infiltrating T cells would result in a lower concentration of locally released pro-inflammatory cytokines, most of which are reported to induce HA synthesis (28). In addition, previous work has indicated that, at least *in vitro*, activated T cells and monocytes

upregulate the expression of HA synthesizing enzymes (29). Therefore, it could be that fewer infiltrating T cells would reduce the pool of HA-producing cells in islets and subsequently the amount of islet HA. The formation of large HA deposits in the diabetes rats in the present study is in line with our previous observations (18). Whether the islet HA-rich ECM present in the 17D5-treated no-diabetes rats has proinflammatory properties remains to be determined.

A strength of the present preclinical prevention study is the robust and reliable onset of spontaneous autoimmune diabetes that is fully penetrant, at a limited time window between 55 and 73 days of age in the control DR*Lyp/Lyp* rats. Importantly, the line of the DR*Lyp/Lyp* rats used in this study is not responsive to treatments directed at preventing diabetes (27, 30–34). Also, the abrupt development of hyperglycemia over the course of 24 h provides an unambiguous end-point. The rapid progression to diabetes in the DR*Lyp/Lyp* rats is therefore useful in any attempt to delay or prevent diabetes. In addition, the depleting anti-Vbeta16 His42 mAb did not affect diabetes penetrance whereas the depleting anti-Vbeta13 17D5 mAb did.

Potential weaknesses of the study include the starting point for treatment at 40 days. Our BBM DR*Lyp/Lyp* rats subjected to optical projection tomography, morphometry and deterioration of beta cell function and mass, and intra-islet blood flow that preceded insulitis (18). It cannot be excluded that the treatment with 17D5 would have been more effective if initiated earlier than 40 days of age. Another potential weakness is the lack of a measure of invasivity of CD3+ and CD8+ T cells in relation to HA. However, this would have required killing of animals prior to the clinical onset of diabetes. For example, DR*Lyp/Lyp* rats with impaired glucose tolerance 1 day before the clinical onset would have been more informative than rats with fulminant diabetes.

The present study of spontaneously diabetes BB DR*Lyp/Lyp* rats has demonstrated that the anti-TCR Vβ13 mAb 17D5 delays the onset of diabetes in comparison with control saline- and His42-treated rats. This is the first time that we observe diabetes prevention in the congenic DR*Lyp/Lyp* rats. The effect in 30% of the rats is still important when considering individuals at high risk for autoimmune type 1 diabetes. In the recent secondary prevention trial with an anti-CD3 Ab, there were also responder and non-responder subjects observed (35). Human TCR specific mAb may have to be developed as part of a personalized medicine and used in combinatorial rather than a traditional monotherapy. Among rats without diabetes at 100 days of age there was a reduction in CD3+ and CD8+ T cells, reduction in CD68+ cells, and reduced HA deposition. DR*Lyp/Lyp* rats and type 1 diabetes patients exhibit similarities in the development of insulitis, which underscore the importance of refining immune suppression clinical trials with anti-CD3 intervention (36) or secondary prevention (35) with antibodies against specific T cell receptors.

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 authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethical Committee in Lund University, Sweden.

AUTHOR CONTRIBUTIONS

The protocol was developed by ÅL, MF, and LF in consultation with JM and EB. LF, MF, and AM carried out the treatment and collected all data while AR was responsible for all genotyping to select eligible DR*Lyp/Lyp* rats. MB planned and carried out the histological analyses together with LF. ÅL drafted the manuscript and revisions were done together with MB, JM, EB, and LF. All authors contributed to the article and approved the submitted version. ÅL is the guarantor of this work.

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Proinsulin-Reactive CD4 T Cells in the Islets of Type 1 Diabetes Organ Donors

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Proinsulin is an abundant protein that is selectively expressed by pancreatic beta cells and has been a focus for development of antigen-specific immunotherapies for type 1 diabetes (T1D). In this study, we sought to comprehensively evaluate reactivity to preproinsulin by CD4 T cells originally isolated from pancreatic islets of organ donors having T1D. We analyzed 187 T cell receptor (TCR) clonotypes expressed by CD4 T cells obtained from six T1D donors and determined their response to 99 truncated preproinsulin peptide pools, in the presence of autologous B cells. We identified 14 TCR clonotypes from four out of the six donors that responded to preproinsulin peptides. Epitopes were found across all of proinsulin (insulin B-chain, C-peptide, and A-chain) including four hot spot regions containing peptides commonly targeted by TCR clonotypes derived from multiple T1D donors. Of importance, these hot spots overlap with peptide regions to which CD4 T cell responses have previously been detected in the peripheral blood of T1D patients. The 14 TCR clonotypes recognized proinsulin peptides presented by various HLA class II molecules, but there was a trend for dominant restriction with HLA-DQ, especially T1D risk alleles DQ8, DQ2, and DQ8-trans. The characteristics of the tri-molecular complex including proinsulin peptide, HLA-DQ molecule, and TCR derived from CD4 T cells in islets, provides an essential basis for developing antigen-specific biomarkers as well as immunotherapies.

Keywords: antigens, type 1 diabetes, islets, T cell receptors, preproinsulin, epitopes

INTRODUCTION

Type 1 diabetes (T1D) is a disease resulting from dysregulation of adaptive immune responses targeting pancreatic beta cells (1, 2). A high proportion of T1D patients have HLA-DR4-DQ8 and/or DR3-DQ2 haplotypes (3–7). This strong genetic association within the HLA class II gene locus suggests a crucial role of CD4 T cells in the development of this disease. Recent studies have demonstrated that CD4 T

cells reactive to beta cell antigens are present in the islets of organ donors having T1D (8–11). Some of these T cells target peptides derived from proinsulin, providing an underlying rationale to develop proinsulin-specific immunotherapies. A number of antigen-specific immunotherapies aiming to modulate T1D-associated T cells, i.e., nanoparticle vaccination, small molecules or monoclonal antibodies to block formation of the tri-molecular complex, and infusion of antigen-specific regulatory T cells, require precise molecular information about peptide-MHC complexes targeted by T cells (12–19). Hence, identification of not only epitopes but in addition, the MHC molecules presenting these peptides, will aid in the development of antigen-specific immunotherapies targeting T cells associated with the T1D pathogenesis.

Preproinsulin is selectively and abundantly expressed by pancreatic beta cells, thereby often being highlighted as a key autoantigen for T1D. Autoantibodies directed to insulin and proinsulin are observed in sera from a majority of individuals developing T1D (20, 21). As evidence of antigens for T cells, Mallone and colleagues reported that approximately one third of peptides eluted from HLA class I molecules expressed by primary human islets were derived from preproinsulin (22). Furthermore, we and others have isolated T cells specific to preproinsulin in the islets and peripheral blood of individuals having T1D (8–10, 23–27). In particular, Mannering and colleagues identified CD4 T cell clones reactive to C-peptide, all of which recognized the peptide presented by HLA-DQ and/or DQ8-trans, in an islet sample from an organ donor having T1D (8). They subsequently demonstrated T cell responses to C-peptide in the peripheral blood of T1D patients and consistent with their islet study, the majority of C-peptide-reactive T cell clones isolated from peripheral blood samples were restricted with HLA-DQ molecules as well (27). Kent and colleagues also isolated preproinsulin-reactive T cell lines from islet samples of multiple T1D organ donors (9). In our previous study, we identified T cell receptor (TCR) clonotypes expressed by T cells in the islets of T1D organ donors and found three clonotypes specific to insulin B-chain and C-peptide presented by HLA-DQ8 and DQ8-trans (10). Thus, a number of studies have demonstrated the presence of preproinsulin-reactive T cells in the islets of individuals having T1D. In this study, we aimed to comprehensively analyze and characterize tri-molecular complexes composed of preproinsulin peptide, HLA, and TCR derived from CD4 T cells in the islets of T1D organ donors.

Recent improvements in sequencing technologies have facilitated the identification of T and B cell receptor sequences in a high-throughput manner (28, 29). It is desirable to determine antigen specificity of these immunoreceptors and indeed, there has been remarkable progress in developing high-throughput strategies to identify antigens recognized by these antigen receptors (30–37). Unlike analysis of B cell antigens, the fact that MHC molecules participate in the recognition of epitopes by TCRs makes it more complicated to identify T cell targets. Immortalized cell lines expressing TCR clonotypes of interest promoted feasibility of directly evaluating reactivity to specific peptide-MHC complexes. Hence, we recently developed a multiplex assay system that can simultaneously assess reactivity of a number of TCR transductant cell lines (38). Multiplex efforts spare samples and reagents necessary for the assay, thereby allowing analysis of reactivity to hundreds of peptides within a relatively short period of time. In this current study, we used this multiplex assay system to test responses against preproinsulin peptides in the presence of autologous B cells transformed with Epstein-Barr virus (EBV) by islet-derived TCR clonotypes obtained from the residual islets of T1D organ donors. The peptide library is composed of 12-15mers of peptides that are generated from the natural form of preproinsulin. Thus, we evaluated reactivity to preproinsulin epitopes presented by all possible HLA class II molecules in a deep and comprehensive manner. Furthermore, using antigen presenting cell lines that exclusively express a single HLA, we identified HLA class II molecules presenting peptides to individual preproinsulin-reactive TCR clonotypes. Here we report the molecular elements involved in the immune-recognition of preproinsulin peptides by islet-derived CD4 T cells.

RESULTS AND DISCUSSION

Screening of Islet CD4 T Cell-Derived T Cell Receptors for the Response to Preproinsulin Peptides

We analyzed 187 TCR clonotypes derived from 166 clonal CD4 T cells in the islets of six T1D organ donors for specificity to preproinsulin (**Table 1**, **Supplementary Table 1**). These TCR clonotypes were selected for analysis because they were detected from multiple cells or have a specific V-gene motif such as TRAV13-1, TRAV26-1, or TRAV38-2, which are preferentially used by CD4 T cells specific to amino acids 9-23 of insulin B chain (10, 26, 39). We expressed each TCR clonotype in a 5KC murine T-hybridoma cell line, which has an added activation reporter driven by the production of nuclear factor of activated T cells (NFAT) (38), to test the response to 99 peptide pools containing 12-mer to 15-mer of peptides derived from preproinsulin (**Supplementary Table 2**). To detect responses against peptides presented by any possible HLA molecules expressed by a given donor, autologous EBV-transformed B cells were used. Screening of 187 TCR clonotypes identified 14 TCRs (7.5%), derived from four donors, that responded to preproinsulin peptide pools. These 14 TCR transductants expressed a fluorescent reporter, ZsGreen-1, when cultured with particular peptide pools (**Figure 1**). As the peptide concentration used for the screening was supraphysiologic to maximize the detection of preproinsulin-reactive TCRs, some TCR transductants responded to multiple peptide pools that contain peptides sharing the same portion of amino acid sequences. To determine an optimal epitope region, we newly synthesized 15-mer peptides contained in top 4 or 5 peptide pools that most efficiently stimulated TCR transductants (**Supplementary Figure 1**, **Supplementary Table 3**) and used those peptides to evaluate responses by each TCR transductant.

TABLE 1 | HLA class II alleles of T1D organ donors and the numbers of T cells analyzed.

Donor ID	HLA-DRB1*	DQA1-DQB1*	DPA1-DPB1*	Number of clonal T cells analyzed	Number of unique TCRs analyzed	Number of PPI-reactive TCRs
nPOD 69	04:01 (DR4) 07:01 (DR7)	03:01-03:02 (DQ8) 02:01-02:02 (DQ2.2)	01:03-03:01 01:03-04:01 (DP4)	7	7	0
nPOD 6323	03:01 (DR17 [DR3]) 04:02 (DR4)	05:01-02:01 (DQ2.5) 03:01-03:02 (DQ8)	01:03-04:01 (DP4)	52	56	4
nPOD 6342	04:01 (DR4) 01:01 (DR1)	03:01-03:02 (DQ8) 01:01-05:01 (DQ5)	01:03-04:01 (DP4)	34	40	5
nPOD 6414	03:01 (DR17 [DR3]) 09:01 (DR9)	05:01-02:01 (DQ2.5) 03:03-02:02 (DQ2.3)	01:03-04:01 (DP4) 01:04-15:01	34	40	4
nPOD 6472	03:01 (DR17 [DR3]) 04:04 (DR4)	05:01-02:01 (DQ2.5) 03:01-03:02 (DQ8)	01:03-04:01 (DP4) 02:01-10:01	30	33	1
nPOD 6367	04:01 (DR4) 07:01 (DR7)	03:01-03:02 (DQ8) 02:01-02:02 (DQ2.2)	01:03-02:01 06:01-11:01	9	11	0

**Nomenclatures in parentheses indicate HLA serotypes.*

TCR transductants reacted with peptide pools in a dose-dependent manner (**Figure 2**), and thus we identified the region of peptide containing the optimal epitope for each TCR clonotype. Overall, we found seven, four, and three epitopes recognized by the 14 TCR clonotypes in insulin B-chain, C-peptide, and A-chain, respectively, including three TCRs, 6.H9, 20.D11, and 8.E3, that were previously found by screening of the response to overlapping preproinsulin peptides (10). Altogether, we identified 14 preproinsulin-reactive TCR clonotypes in the islets of four out of six T1D organ donors studied.

Identification of Peptide-MHC Complexes Targeted by Proinsulin-Reactive T Cell Receptor Clonotypes

We next aimed to identify HLA molecules presenting proinsulin peptides to the 14 TCR clonotypes. We used the newly synthesized 15-mer peptides that were identified as a region containing an optimal epitope for each TCR transductant. Our strategy was to test the response to the cognate peptide in the presence of K562 antigen presenting cells expressing HLA molecules identical to those of the TCR donor. K562 cells do not express endogenous HLA class II molecules, allowing us to examine the response restricted only by the introduced HLA. We first generated three K562 cell lines expressing HLA-DR (DRB1), DQ, or DP molecules matching each donor and used them as antigen presenting cells. Once narrowing the presenting HLA molecules to DR, DQ, or DP-derived molecules, we further examined the responses by TCR transductants in the presence of K562 cell lines that individually express any possible combination of HLA-DR, DQ, or DP molecules including cis- and trans-combinations of HLA-DQA1 and DQB1 or DPA1 and DPB1 molecules. This way, we identified one or more HLA molecules presenting the cognate peptide to each TCR. For example, the 95.A9-1 TCR clonotype was derived from donor nPOD 6472, who had the DR3 and DR4 haplotypes (**Table 1**). The 95.A9-1 transductants responded to a cognate peptide, preproinsulin 87-101, when cultured with K562 cells co-transduced with DRA1*01:01, DRB1*04:04, and DRB1*03:01, but not with those transduced with the DQ or DP alleles (**Figure 3A**, left panel). The 95.A9-1 TCR transductant cells were further examined for the response to peptides in the presence of K562 cells transduced with DRA1*01:01 along with either DRB1*04:04 or DRB1*03:01 and were activated only when co-cultured with cells expressing DRB1*04:04 (**Figure 3A**, center panel). Lastly, we confirmed that the 95.A9-1 cells responded to the cognate peptide in the presence of K562 cells expressing the determined HLA in a dose-dependent manner (**Figure 3A**, right panel). Thus, we determined that 95.A9-1 is reactive to preproinsulin 87-101 presented by DRA1*01:01-DRB1*04:04 (DR4).

Using the same strategy, we determined HLA molecules presenting a cognate peptide to all individual TCR clonotypes except one, 55.C10. **Figures 3–5** show the results (left panels: determining HLA-DR, DQ, or DP, center panels: determining HLA-alpha and -beta combinations, right panels: dose-response assessment) for five DR- (**Figure 3**), seven DQ- (**Figure 4**), and one DP (**Figure 5**)-restricted TCRs.

There was one TCR clonotype, 55.C10, that did not respond to a cognate peptide in the presence of any K562 cell lines expressing the donor's HLA-DR, DQ, and DP molecules (**Figure 6A**). To investigate which HLA genes restrict 55.C10, we tested the response by TCR transductants in the presence of autologous B cells and antibodies against HLA-DR, DQ, or DP and found that anti-HLA-DR antibodies suppressed the response (**Figure 6B**). The TCR is likely to recognize the peptide presented by HLA-DR, but K562 cells expressing molecules derived from the DRB1 allele was not able to stimulate the TCR transductant, suggesting that another DR beta chain such as those derived from the DRB3, DRB4, or DRB5 genes may be involved in the presentation of the peptide. The donor of TCR 55.C10, nPOD 6414, had DR3 and DR9 haplotypes, which are linked to functional DRB3 and DRB4 genes, respectively, but DRB5 alleles of both haplotypes are pseudogenes. After determining alleles of DRB3 and DRB4 of the donor (i.e., DRB3*01:01 and DRB4*01:01), we generated K562 cells transduced with these allelic variants and found that the 55.C10 TCR transductants were reactive to the peptide presented by HLA-DRB4*01:01 (**Figures 6C, D**). As CD4 T cells specific to another proinsulin peptide presented by DRB4*01:01 have been observed in the blood of T1D patients (40), there may be disease-specific epitopes presented by HLA-DRB3 and DRB4 alleles that are linked to the T1D-susceptible HLA-DR4-DQ8 and/or DR3-DQ2 haplotypes.

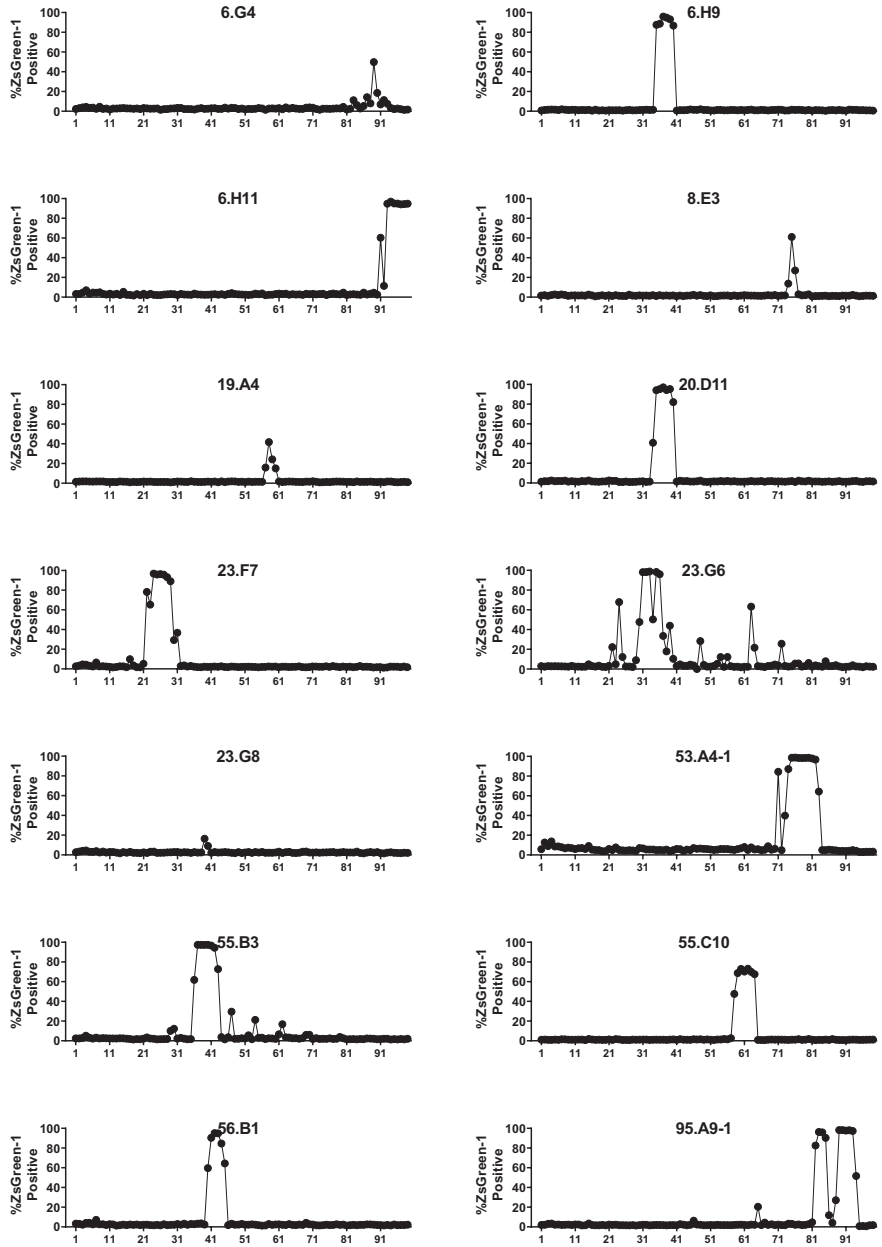

FIGURE 1 | Screening for the response to a preproinsulin truncated peptide library. 5KC T-hybridoma cells expressing ZsGreen-1 upon activation were used to express each TCR clonotype. TCR transductants were cultured with 99 truncated peptide pools containing 12-, 13-, 14-, and 15-mers of peptides ending at the same position of preproinsulin in the presence of autologous EBV-transformed B cells. After overnight culture, cells were evaluated for ZsGreen-1 expression by flow cytometry. Percentages of ZsGreen-1-positive cells in response to each peptide pool are shown for 14 TCR transductants that responded to one or more peptide pools.

Table 2 as well as **Figure 7A** summarize peptides and presenting HLA molecules targeted by individual TCR clonotypes. Of note, peptides recognized by multiple TCRs, such as preproinsulin 33-47 recognized by 6.H9 and 20.D11, preproinsulin 36-50 recognized by 23.G8 and 55.B3, and preproinsulin 72-87 recognized by 8.E3 and 53.A4-1, were presented by the same HLA gene products (i.e., DR or DQ) but sometimes different allele products. In addition, it was noted that several TCR clonotypes, 23.G8, 6.H9, 8.E3, 53.A4-1, and 6.G4, recognized cognate peptides presented by several HLA class II molecules, albeit with different levels of response, implying that the same peptides can be presented by multiple HLA molecules and induce various levels of T cell activation. These different levels of responsiveness are likely to be the combined outcome from differential peptide to HLA binding affinity, and TCR to peptide-MHC complex affinity. Intensity of

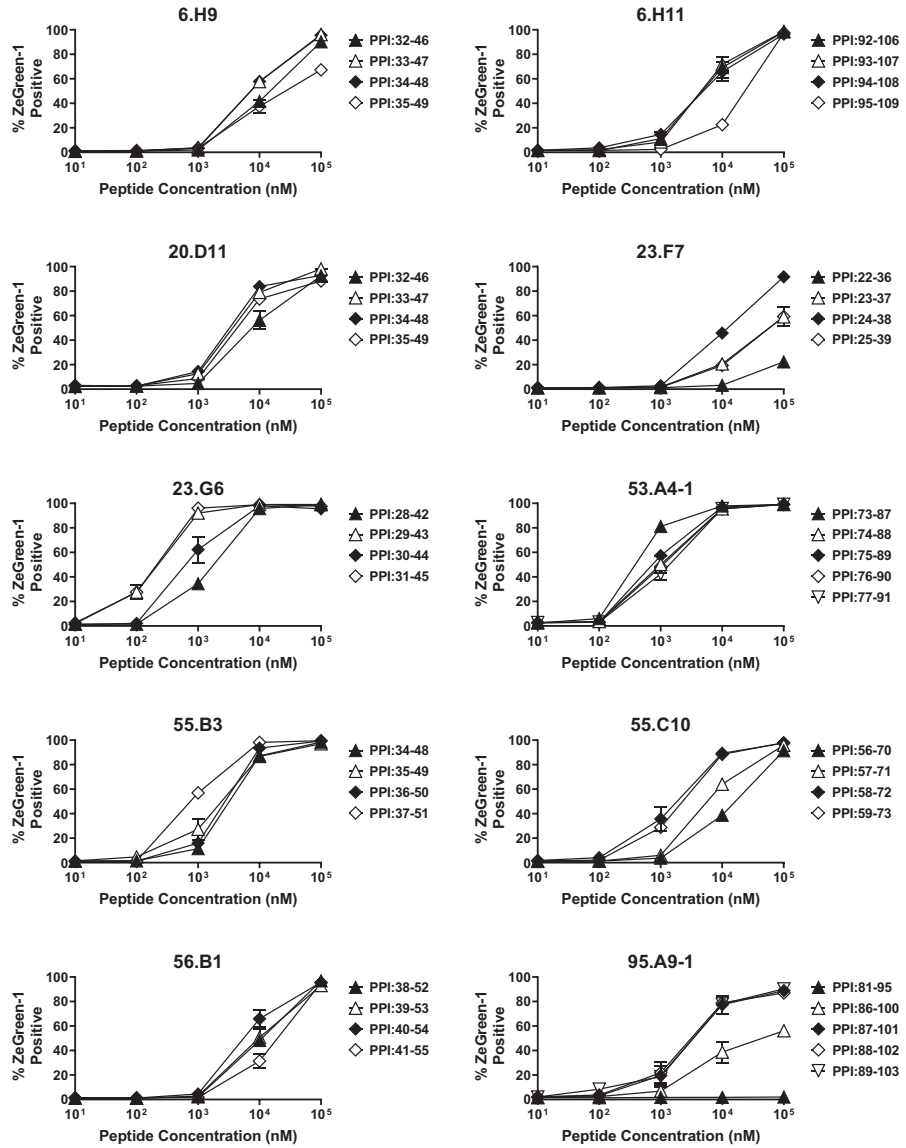

FIGURE 2 | Determining optimal peptides. TCR transductants that responded to multiple peptide pools in the screening were tested for the response to newly synthesized 15-mer peptides contained in individual peptide pools in the presence of autologous EBV-transformed B cells, and were evaluated for ZsGreen-1 expression by flow cytometry. Experiments were independently repeated three times, and mean values ± standard error of the mean are shown.

signals provided through the TCR engagement is a major factor determining T cell phenotype as well as their fate (41, 42). It will be important to elucidate how T cells determine their responses when receiving different levels of TCR signaling through epitopes presented by multiple HLA class II molecules having different affinities.

Overall, we identified five DRB1, seven DQ, one DP, and one DRB4-restricted TCR clonotypes that recognize proinsulin peptides (**Figure 7B**). It has been suggested that DRB1-restricted TCR repertoires are generally the largest among those expressed by CD4 T cells. While the number of proinsulin-specific clonotypes identified in our study is small, there was a noteworthy trend that the proportion of DR-restricted TCR clonotypes was not as high as previously reported frequencies of DR-restricted TCRs in virus-reactive repertoires (43–45), and rather DQ-restricted TCRs were more predominant. Future studies investigating HLA restriction of a large number of T cells from the islets of patients and at-risk individuals are expected to confirm this trend.

Responsiveness to Proinsulin Epitopes by Autoreactive T Cells

The responsiveness to peptides differed by individual TCRs (**Figures 3–5**, right panels). To compare response levels of

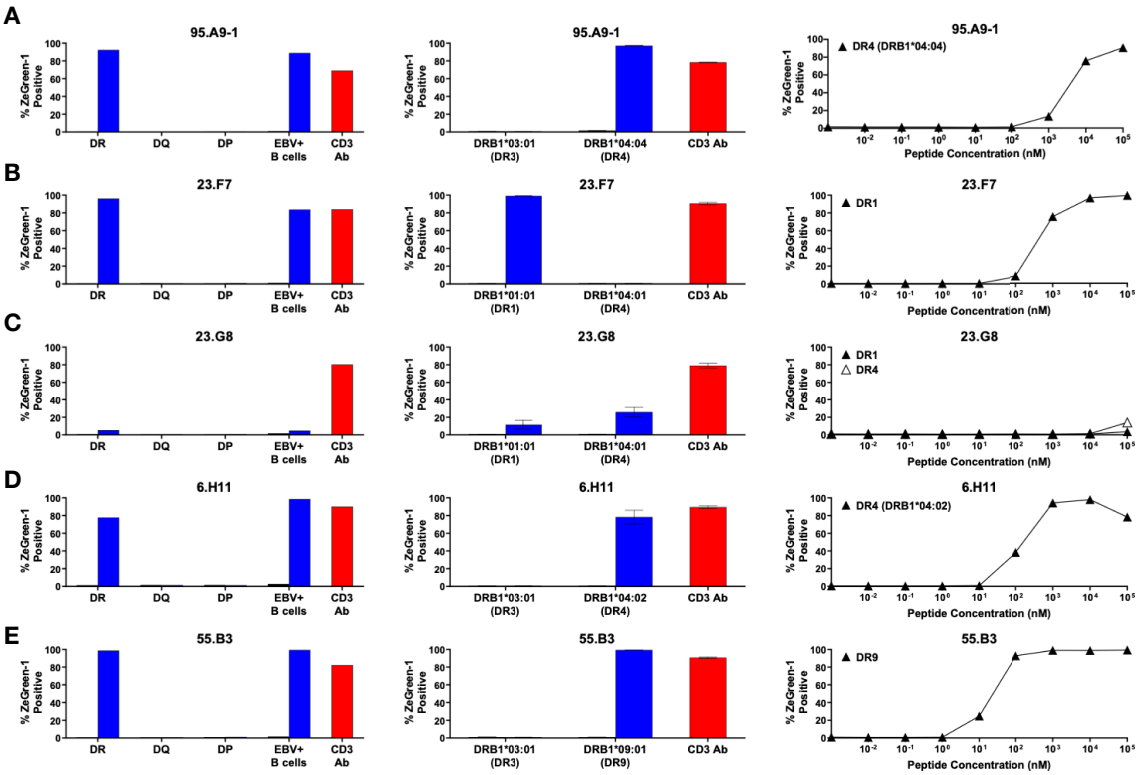

FIGURE 3 | Preproinsulin-reactive TCR clonotypes restricted by HLA-DR. TCR transductants, **(A)** 95.A9-1, **(B)** 23.F7, **(C)** 23.G8, **(D)** 6.H11, **(E)** 55.B3, were cultured with cognate peptides in the presence of designated antigen presenting cells, followed by assessment of ZsGreen-1 expression by flow cytometry. Left panels: TCR transductants were cultured with (blue bars) or without (black bars) cognate peptides in the presence of autologous EBV-transformed B cells generated from spleen cells of **(A)** nPOD 6472, **(B, C)** nPOD 6342, **(D)** nPOD 6323, or **(E)** nPOD 6414, or K562 cells transduced with DR, DQ, or DP alleles based upon their HLA genotype in **Table 1**. Culture wells containing an anti-CD3 monoclonal antibody were included as a positive control (red bars). Center panels: TCR transductants were cultured with (blue bars) or without (black bars) cognate peptides in the presence of K562 cells transduced with individual DR alleles based upon their HLA genotype in **Table 1**. Right panels: TCR transductants were cultured with different concentrations of cognate peptides in the presence of K562 cells transduced with **(A)** DRA1*01:01 and DRB1*04:04; **(B)** DRA1*01:01 and DRB1*01:01; **(C)** DRA1*01:01 and DRB1*01:01 (black triangles), or DRA1*01:01 and DRB1*04:01 (white triangles); **(D)** DRA1*01:01 and DRB1*04:02; **(E)** DRA1*01:01 and DRB1*09:01. Experiments in left panels were performed once. All remaining experiments in center and right panels were independently repeated three times, and mean values ± standard error of the mean are shown.

preproinsulin-reactive TCRs with those of TCRs unrelated to the T1D pathogenesis, we generated two TCR transductant cell lines using the same method to make islet TCR transductants. First, we used an influenza virus-reactive TCR clonotype, HA1.17. The HA1.17 TCR transductants reacted to a cognate influenza peptide presented by either HLA-DR1 or DR4 starting at only one nanomolar (**Supplementary Figure 2A**). Next, we evaluated the sensitivity of a gliadin-specific TCR, 489, to the native and deamidated forms of peptides in our transductant system. It is well-appreciated that alpha-gliadin peptides that undergo deamidation (Q→E) are target antigens for T cells involved in the pathogenesis of celiac disease, an autoimmune disease caused by gluten intake in disease-sensitive individuals (46, 47). As expected, the 489 TCR transductants responded to the deamidated peptide presented by DQ8 more sensitively than the native alpha-gliadin peptide (**Supplementary Figure 2B**). Several proinsulin-reactive TCR clonotypes including two restricted by HLA-DP and DRB4 showed comparable levels of responses to their cognate peptides compared to those of the influenza-specific HA1.17 and the gliadin-specific 489 TCRs (**Figure 7C**). The expression levels of HLA-DP and DRB4 molecules on antigen presenting cells are reported to be lower than that of HLA-DRB1 molecules (45, 48), which may influence selection and differentiation of self-reactive T cells restricted by HLA molecules with lower cell surface expression, such as HLA-DP. On the other hand, responsiveness of several preproinsulin-specific clonotypes was lower than those of the HA1.17 and 489 TCRs (**Figure 7C**). In particular, the 6.G4 and 23.G8 TCRs needed excessive amounts of peptides to be activated. There is a possibility that biological targets for these TCR clonotypes exhibiting weaker responses could be neoepitopes such as those receiving post-translational modifications and products resulting from alternative splicing. Alternatively, antigens activating T cells expressing these TCRs could be unrelated to proinsulin or even other islet antigens, such as those derived from microbes, and the TCRs may be cross-reactive to proinsulin.

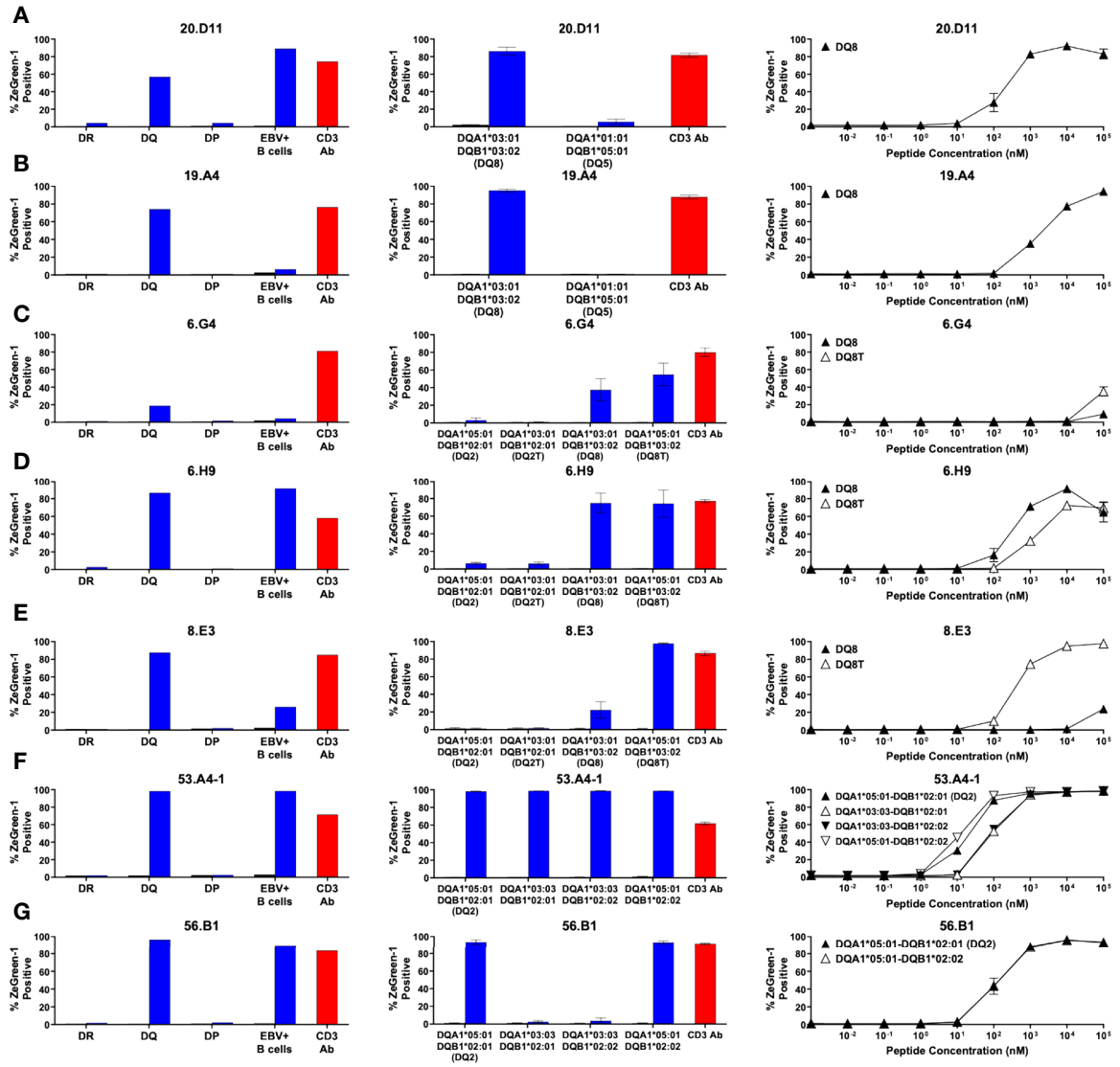

FIGURE 4 | Preproinsulin-reactive TCR clonotypes restricted by HLA-DQ. TCR transductants, **(A)** 20.D11, **(B)** 19.A4, **(C)** 6.G4, **(D)** 6.H9, **(E)** 8.E3, **(F)** 53.A4-1, **(G)** 56.B1, were cultured with cognate peptides in the presence of designated antigen presenting cells, followed by assessment of ZsGreen-1 expression by flow cytometry. Left panels: TCR transductants were cultured with (blue bars) or without (black bars) cognate peptides in the presence of autologous EBV-transformed B cells generated from spleen cells of **(A, B)** nPOD 6342, **(C–E)** nPOD 6323, or **(F, G)** nPOD 6414, or K562 cells transduced with DR, DQ, or DP alleles based upon their HLA genotype in **Table 1**. Culture wells containing an anti-CD3 monoclonal antibody were included as a positive control (red bars). Center panels: TCR transductants were cultured with (blue bars) or without (black bars) cognate peptides in the presence of K562 cells transduced with individual DQ alleles based upon their HLA genotype in **Table 1**. Right panels: TCR transductants were cultured with different concentrations of cognate peptides in the presence of K562 cells transduced with **(A, B)** DQA1*03:01 and DQB1*03:02; **(C–E)** DQA1*03:01 and DQB1*03:02 (black triangles), or DQA1*05:01 and DQB1*03:02 (white triangles); **(F)** DQA1*05:01 and DQB1*02:01 (black triangles), DQA1*03:03 and DQB1*02:01 (white triangles), DQA1*03:03 and DQB1*02:02 (black inverted triangles), or DQA1*05:01 and DQB1*02:02 (white inverted triangles); **(G)** DQA1*05:01 and DQB1*02:01 (black triangles), or DQA1*05:01 and DQB1*02:02 (white triangles). Experiments in left panels were performed once. All remaining experiments in center and right panels were independently repeated three times, and mean values ± standard error of the mean are shown.

Reactivity to Proinsulin and Islet Tissues

To validate tissue specificity, we further tested the response to whole proinsulin and tissue lysates made from primary human islets by the 14 TCR transductants reactive to proinsulin peptides. All except two TCRs, 6.G4 and 23.G8, which showed only marginal responses to their cognate peptides, responded to islet lysates (**Figure 7D**). Consistent with undetectable reactivity to islet lysates, 6.G4 but not 23.G8 weakly responded to proinsulin. These observations are compatible with the possibility of cross-reactivity to proinsulin. Intensities of responses to cognate peptides are not necessarily associated with those to whole proinsulin and islet lysates, and some TCRs such as 6.H11, 8.E3, and 95.A9-1 preferred to react with

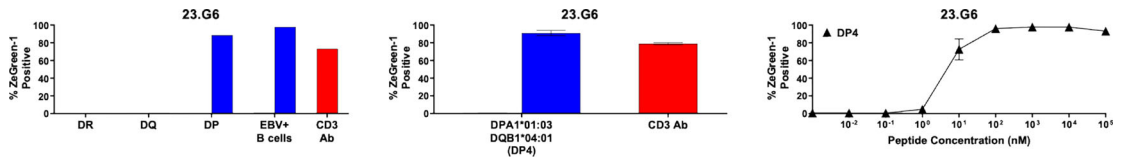

FIGURE 5 | Preproinsulin-reactive TCR clonotypes restricted by HLA-DP. The TCR 23.G6 transductant was cultured with cognate peptides in the presence of designated antigen presenting cells, followed by assessment of ZsGreen-1 expression by flow cytometry. Left panel: The 23.G6 TCR transductants were cultured with (blue bars) or without (black bars) cognate peptides in the presence of autologous EBV-transformed B cells generated from spleen cells of nPOD 6342, or K562 cells transduced with DR (DRA1*01:01, DRB1*04:01, DRB1*01:01), DQ (DQA1*03:01, DQA1*01:01, DQB1*03:02, DQB1*05:01), or DP (DPA1*01:03, DPB1*04:01) alleles. Culture wells containing an anti-CD3 monoclonal antibody were included as a positive control (red bars). Center panel: The 23.G6 TCR transductant was cultured with (blue bars) or without (black bars) cognate peptides in the presence of K562 cells transduced with a DP gene combination, DPA1*01:03 and DPB1*04:01. Right panel: The 23.G6 TCR transductant was cultured with different concentrations of cognate peptides in the presence of K562 cells transduced with DPA1*01:03 and DPB1*04:01. The experiment in the left panel was performed once. Experiments in the center and right panels were independently repeated three times, and mean values ± standard error of the mean are shown.

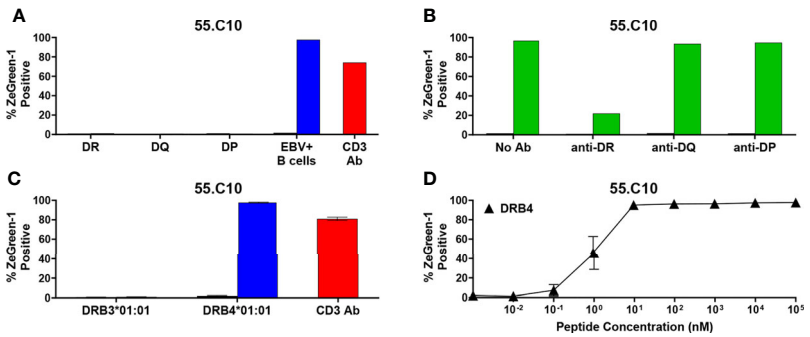

FIGURE 6 | Preproinsulin-reactive TCR clonotypes restricted by HLA-DRB4. **(A)** The 55.C10 TCR transductant was cultured with (blue bars) or without (black bars) cognate peptides in the presence of autologous EBV-transformed B cells generated from spleen cells of nPOD 6414, or K562 cells transduced with DR (DRA1*01:01, DRB1*03:01, DRB1*09:01), DQ (DQA1*05:01, DQA1*03:03, DQB1*02:01, DQB1*02:02), or DP (DPA1*01:03, DPA1*01:04, DPB1*04:01, DPB1*15:01) alleles. Culture wells containing an anti-CD3 monoclonal antibody were included as a positive control (red bars). **(B)** The 55.C10 TCR transductant was cultured with (green bars) or without (black bars) cognate peptides in the presence or absence of anti-DR, -DQ, or -DP antibodies. Autologous nPOD 6414 EBV-transformed B cells were used as antigen presenting cells. **(C)** The 55.C10 TCR transductant was cultured with (blue bars) or without (black bars) cognate peptides in the presence of K562 cells transduced with DRB3 (DRA1*01:01 and DRB3*01:01) or DRB4 (DRA1*01:01 and DRB4*01:01) alleles. **(D)** The 55.C10 TCR transductant was cultured with different concentrations of cognate peptides in the presence of K562 cells transduced with DRA1*01:01 and DRB4*01:01. Experiment in panels **(A, B)** were performed once. All remaining experiments in panels **(C, D)** were independently repeated three times, and mean values ± standard error of the mean are shown.

whole proinsulin rather than islet lysates. These discrepancies may be caused by difference between optimal epitopes and peptides formed islet cells or antigen presenting cells.

Citrullinated Insulin B-Chain Peptides Do Not Enhance T Cell Responses

Five out of 14 proinsulin-reactive TCR transductants responded to peptides located in the latter portion of insulin B-chain, and the majority of these TCRs showed relatively weak responsiveness to the native insulin peptides. Citrullination converts arginine to citrulline and is an important post-translational modification of self-antigens in autoimmune diseases such as Rheumatoid arthritis and T1D (49–51). To begin to examine the potential for neoepitopes within proinsulin, we investigated antigenicity of a citrullinated insulin B-chain peptide. We tested the five insulin B-chain-reactive TCR transductants for response to the citrullinated and the native forms of insulin B-chain peptides. All TCR transductants except 23.G8, that reacted with the cognate 15-mer peptide very weakly (**Figure 3C**), responded to the native insulin B-chain peptide, but citrullination of the peptide did not induce stronger responses in any TCRs (**Figure 8**). Further studies to identify biological epitopes, especially for TCRs with low responses to the natural form of proinsulin, such as 23.G8, will be important to understand the mechanisms of initiating and driving anti-islet autoimmunity.

In the current study, we identified 11 new proinsulin-reactive TCR clonotypes in addition to three clonotypes found in our previous study that screened reactivity to ten overlapping preproinsulin peptides (10), and thus 14 TCR clonotypes were characterized for preproinsulin specificity and HLA restriction. The use of a truncated peptide library containing 99 preproinsulin peptide pools and autologous EBV-transformed

TABLE 2 | HLA class II alleles of T1D organ donors and the numbers of T cells analyzed.

nPOD Donor	TCR ID	Peptide	Peptide Sequence	Region in Proinsulin	HLA*#
6342	23.F7	PPI:24-38	AFVNQHLCGSHLVEA	B-chain	**DRB1*01:01 (DR1)**
6342	23.G6	PPI:29-43	HLCGSHLVEALYLVC	B-chain	**DPA1*01:03-DPB1*04:01 (DP4)**
6323	6.H9	PPI:33-47	SHLVEALYLVCGERG	B-chain	**DQA1*03:01-DQB1*03:02 (DQ8)** DQA1*05:01-DQB1*03:02 (DQ8-trans)
6342	20.D11	PPI:33-47	SHLVEALYLVCGERG	B-chain	**DQA1*03:01-DQB1*03:02 (DQ8)**
6342	23.G8	PPI:36-50	VEALYLVCGERGFFY	B-chain	**DRB1*04:01 (DR4)**, DRB1*01:01 (DR1)
6414	55.B3	PPI:37-51	EALYLVCGERGFFYT	B-chain	**DRB1*09:01 (DR9)**
6414	56.B1	PPI:40-54	YLVCGERGFFYTPKT	B-chain	**DQA1*05:01-DQB1*02:01 (DQ2.5)** **DQA1*05:01-DQB1*02:02**
6342	19.A4	PPI:55-69	RREAEDLQVGQVELG	(RR) C-peptide	**DQA1*03:01-DQB1*03:02 (DQ8)**
6414	55.C10	PPI:58-72	AEDLQVGQVELGGGP	C-peptide	**DRB4*01:01 (DR53)**
6323	8.E3	PPI:72-87	PGAGSLQPLALEGSLQ	C-peptide	**DQA1*05:01-DQB1*03:02 (DQ8-trans)** DQA1*03:01-DQB1*03:02 (DQ8)
6414	53.A4-1	PPI:72-87	PGAGSLQPLALEGSLQ	C-peptide	**DQA1*05:01-DQB1*02:01 (DQ2.5)** **DQA1*05:01-DQB1*02:02** DQA1*03:03-DQB1*02:02 (DQ2.3) DQA1*03:03-DQB1*02:01
6323	6.G4	PPI:86-100	LQKRGIVEQCCTSIC	(B-KR) A-chain	**DQA1*05:01-DQB1*03:02 (DQ8-trans)** DQA1*03:01-DQB1*03:02 (DQ8)
6472	95.A9-1	PPI:87-101	QKRGIVEQCCTSICS	(B-KR) A-chain	**DRB1*04:04 (DR4)**
6323	6.H11	PPI:94-108	QCCTSICSLYQLENY	A-chain	**DRB1*04:02 (DR4)**

**Nomenclatures in parentheses indicate HLA serotypes.*

#HLA allele combinations recognized by the TCR most efficiently are shown in bold.

B cell lines facilitated our ability to identify these additional TCR clonotypes. Epitopes were found in B-chain, C-peptide, and A-chain regions, and there were regions that contained multiple epitopes recognized by TCRs derived from different donors (**Table 2**, **Figure 7A**). Some of these epitopes were identified as targets for islet-derived CD4 T cell clones observed in previous studies by others (8, 9). Overall, there are several peptide regions spanning about 20 amino acids, i.e. preproinsulin 33-53 (insulin B-chain), preproinsulin 55-71 (C-peptide), preproinsulin 72-90 (C-peptide), preproinsulin 86-101 (A-chain), that are commonly targeted by CD4 T cells in the islets of individuals having T1D. Inflammatory responses by CD4 T cells to these "hot spot" regions have previously been observed in peripheral blood samples of T1D patients (26, 27, 52–55). While it is unknown whether CD4 T cells reactive to the preproinsulin hot spots in the blood are clonally identical to those in the islets, it is noteworthy that the same antigen specificities have been confirmed in blood and islets.

It will be important to determine binding motifs as well as affinity of the epitopes to the restricting HLA molecules as these elements are essential to determine responsiveness of T cells and to develop therapies targeting peptide-MHC complexes. To begin, we conducted a computational simulation analysis to predict core epitope sequences using the Immune Epitope Database (IEDB) MHC-II Binding Prediction online tool (http://tools.iedb.org/mhcii/), which allows us to use multiple *in silico* prediction models. A number of simulations predicted identical amino acid motifs as a core epitope for each proinsulin-reactive clonotype (**Supplementary Table 4**). Further efforts validating binding affinities and T cell reactivities to these predicted epitopes are desired in the future.

All HLA-DR, DQ, and DP molecules were used to present peptides to proinsulin-reactive TCR clonotypes. However, there was a trend of restriction with HLA-DQ, particularly the T1D risk alleles, DQ8, DQ2, or DQ8-trans (**Table 2**, **Figure 7A**). Mannering and colleagues also reported that this preferred restriction with risk DQ alleles was observed in C-peptide-specific CD4 T cells in the blood (27). Our study extends this finding to islet-derived TCRs specific to other regions of proinsulin. Future studies to determine antigen specificity outside of preproinsulin (e.g., glutamic acid decarboxylase, zinc transporter 8, islet antigen 2) will clarify whether the trend of DQ restriction is a general feature of TCR clonotypes expressed by T1D-associated CD4 T cells. Additionally, it is important to elucidate the mechanisms by which HLA-DQ is preferentially used to present epitopes to T cells reactive to proinsulin and potentially other islet antigens. Whether this is a global phenomenon across patients or patient-specific will help design and personalize immune therapies to preserve endogenous beta-cell function in T1D. Notably, there is a therapy (methyldopa) being tested that specifically blocks self-antigen presentation by DQ8 and subsequent autoreactive T cell responses (12). Our results also indicate that antigen specific immunotherapies with insulin, should give strong consideration to including A-chain, B-chain, and C-peptide (e.g., all of proinsulin) as there are islet-derived CD4 T cell epitopes within all of these regions.

In conclusion, we identified 14 proinsulin-specific TCR clonotypes expressed by CD4 T cells in the islets of four out of six organ donors having T1D. These TCRs were restricted by various HLA class II molecules, but there was a trend of using T1D-risk conferring HLA-DQ molecules. There are four hot spots within proinsulin that contain epitopes preferentially targeted by the responding islet TCRs, which overlapped with antigenic regions recognized by T cells in the peripheral blood

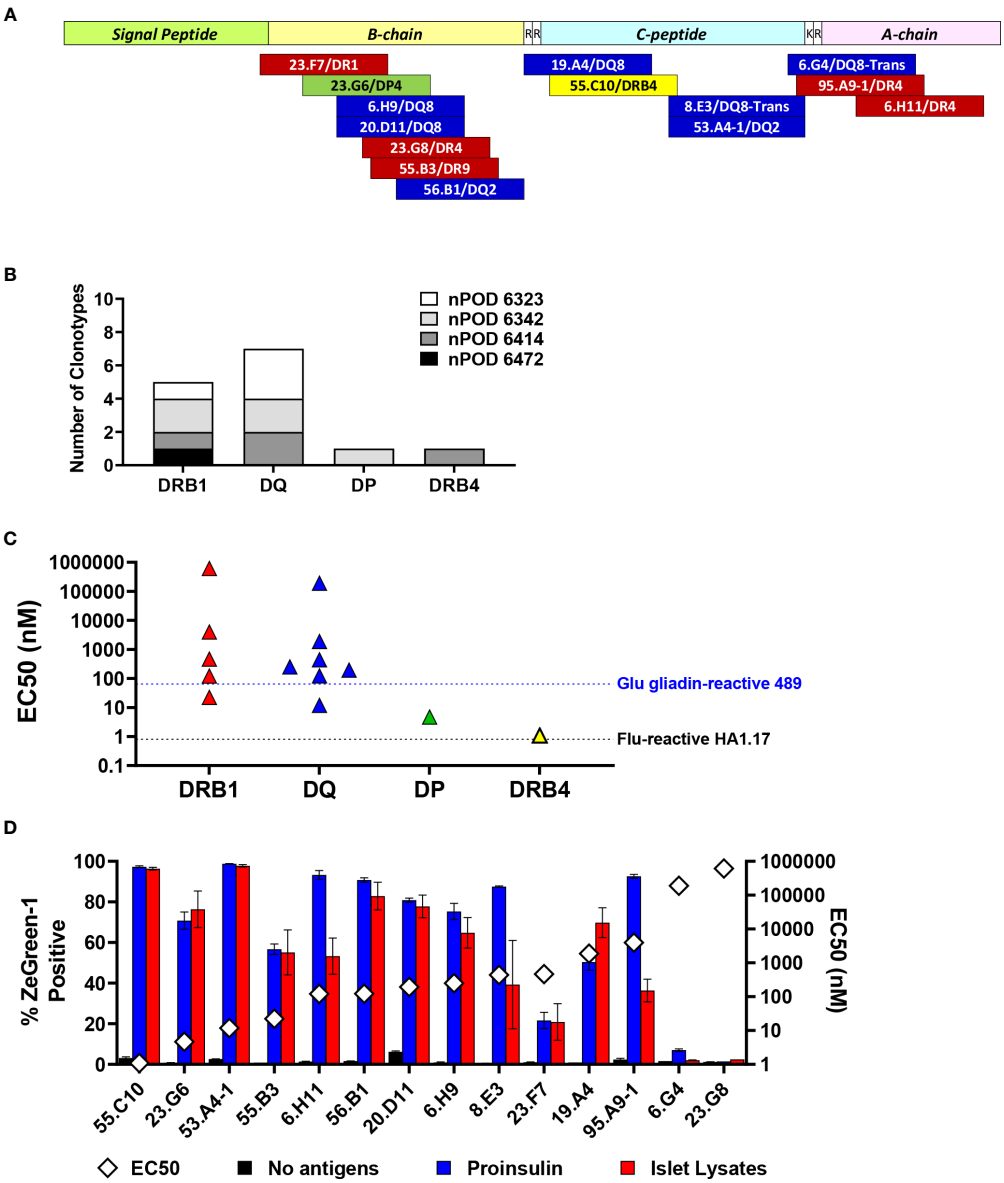

FIGURE 7 | Characteristics of proinsulin-specific TCR clonotypes. **(A)** Location of epitopes in preproinsulin and restricting HLA molecules are mapped. Red, blue, green, and yellow bars represent epitopes for TCR clonotypes restricted by HLA-DRB1, DQ, DP, and DRB4. **(B)** Numbers of preproinsulin-reactive TCR clonotypes restricted by HLA-DRB1, DQ, DP, and DRB4. Preproinsulin-reactive TCR clonotypes derived from individual nPOD donors are shown separately by HLA restriction. **(C)** EC_{50} values of responses by preproinsulin-reactive TCR clonotypes are shown separately by HLA restriction. EC_{50} values of responses by HA.1.17 to the cognate influenza peptide (black line) and 489 to the deamidated alpha-gliadin peptide (blue line) are included for reference. **(D)** Responses to proinsulin and human islet lysates by TCR transductants reactive to proinsulin peptides. TCR transductants were cultured with K562 cells expressing cognate HLA molecules pulsed with proinsulin (blue bars) or islet lysates (red bars), or without antigens (black bars). After overnight culture, ZsGreen-1 expression was evaluated by flow cytometry. Experiments were independently repeated twice, and mean values ± standard error of the mean are shown. EC_{50} values of responses to cognate peptides (white diamond symbols) are included for reference.

of T1D patients. T cell antigen specificity to these proinsulin regions provide an avenue for developing biomarkers in the peripheral blood that mirror the islets. The level of T cell response to proinsulin epitopes was lower than that observed with an influenza-specific TCR, but over half of the TCRs responded to native proinsulin peptides as comparably as a level of optimal response exhibited by a TCR specific in another autoimmune disease (celiac disease). Biological targets for the TCRs with low responses may be neoepitopes modified from the natural form of proinsulin.

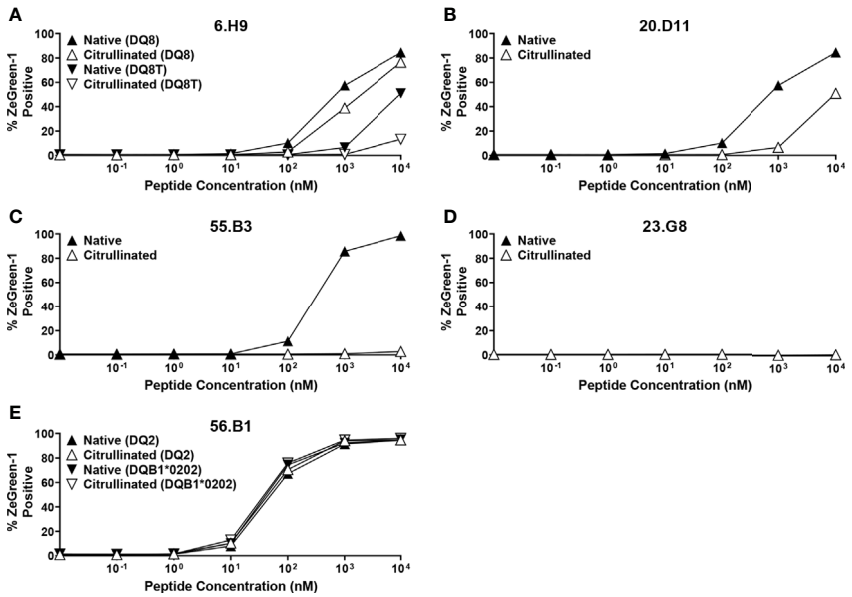

FIGURE 8 | Responses to citrullinated and native insulin B-chain peptides. TCR transductants, **(A)** 6.H9, **(B)** 20.D11, **(C)** 55.B3, **(D)** 23.G8, **(E)** 56.B1, were cultured with different concentrations of insulin B-chain peptide (SHLVEALYLVCGE-**R**-GFFYTPK) (black symbols) or that with citrullination (SHLVEALYLVCGE-**Cit**-GFFYTPK) (white symbols) in the presence of K562 cells expressing the following HLA molecules for each TCR clonotype: **(A)** DQ8 (DQA1*03:01-DQB1*03:02) (triangles) and DQ8T (DQA1*05:01-DQB1*03:02) (inverted triangles); **(B)** DQ8 (DQA1*03:01-DQB1*03:02) (triangles); **(C)** DR9 (DRA1*01:01-DRB1*09:01) (triangles); **(D)** DR4 (DRA1*01:01-DRB1*04:01) (triangles); **(E)** DQ2 (DQA1*05:01-DQB1*02:01) (triangles) and (DQA1*05:01-DQB1*02:02) (inverted triangles). All experiments were independently repeated three times, and mean values ± standard error of the mean are shown.

MATERIALS AND METHODS

T Cell Receptor Transductants

TCR sequences were identified as described previously (10). TCR transductants were generated using a recently published protocol (38). Briefly, 5KC T-hybridoma cells (56) were transduced with a NFAT-driven fluorescent reporter, ZsGreen-1, along with the human CD4 gene with two amino acid mutations at positions 40 (glutamine to tyrosine) and 45 (threonine to tryptophan) that increase binding to MHC molecules (57) (the retroviral vector is available from addgene, plasmid ID 162745) using a standard spinfection protocol with viral supernatant produced from phoenix-eco cells (ATCC CRL-3214) (58). Cells were also transduced with a combination of two fluorescent protein genes (addgene plasmid ID 153423, 153424, 153524, 153426, 153427, 153428) as an identifier of each TCR. We then transduced each 5KC cell line expressing a specific combination of fluorescent proteins with a retroviral vector encoding a chimeric TCR alpha gene followed by a porcine teschovirus-1 2A (P2A) peptide and a chimeric TCR beta gene (58).

Screening of T Cell Receptor Transductants for Reactivity to Preproinsulin

Up to eight TCR transductants (20,000 cells per line) expressing different combinations of fluorescent proteins were pooled and cultured with a preproinsulin truncated peptide pool in the presence of autologous B cells transformed with EBV (100,000 cells per well) in a well of round-bottom 96-well-plates. The preproinsulin truncated peptide pools were purchased from Mimotopes (Mulgrave, Australia) as pools of four crude peptides containing 3-7 mg peptides per pool. Each peptide pool was dissolved in 250 µl 80% dimethylsulfoxide/20% water, and 2 µl of dissolved peptide pool was added in a culture well containing total 200 µl media; thus the peptide concentration was approximately 200 µg/ml. Peptide sequences contained in each pool are shown in **Supplementary Table 2**. Autologous EBV-transformed B cell lines were made from spleen cells of individual islet donors using a standard protocol (59). Cultured cells were harvested next day and analyzed for expression of ZsGreen-1 in each TCR transductant line using a flow cytometer (Cytoflex, Beckman Coulter) and FlowJo (BD) (38). An example of gating strategy is shown in **Supplementary Figure 3**. Cells cultured in the presence or absence of 5 µg/ml of anti-mouse CD3ϵ antibody (Clone 125-2C11, BD) were included in the assay as positive and negative controls, respectively. The threshold for positive ZsGreen-1 expression was determined such that the majority of cells in negative control wells for all TCR transductants in a same pool becomes negative (**Supplementary Figure 3**). When the proportion of ZsGreen-1-positive cells in a negative control culture well exceeded 20% or in a positive control culture was lower than 50%, the TCR clonotypes were excluded from analysis, resulting in total 187 TCR clonotypes evaluated for the response to preproinsulin truncated peptide pools.

Determining Optimal Epitope Regions

For TCR transductants that responded to more than one truncated peptide pools in the screening, responses against lower amounts of truncated peptide pools (x10, x100, and x1,000 dilutions of peptide solutions used for the screening) were examined to identify the most preferred pools (**Supplementary Figure 1**). 15-mer peptides contained in top 4 or 5 peptide pools that most efficiently stimulated individual TCR transductants were newly synthesized by Genemed synthesis (San Antonio, USA) (**Supplementary Table 3**). TCR transductants (20,000 cells per well) were cultured with different concentrations (10 nM, 100 nM, 1 μM, 10 μM, and 100 μM) of peptides in the presence of autologous EBV-transformed B cell lines (100,000 cells per well). After overnight culture, cells were harvested and analyzed for ZsGreen-1 expression using a flow cytometer (Cytoflex, Beckman Coulter) and FlowJo (BD) (38).

HLA Typing

HLA-DRB1, DQA1, DQB1, DPA1, and DPB1 alleles were determined using DNA samples extracted from spleen cells of each donor by the Barbara Davis Center Autoantibody/HLA Core Facility (https://medschool.cuanschutz.edu/barbara-davis-center-for-diabetes/service-centers/autoantibody-hla-service-center). To determine DRB3 and DRB4 alleles of the donor nPOD 6414, we PCR-amplified 273-294 bp fragments in which 96-185 bp were overlapped with adjacent fragments at each end using sets of primers as shown in **Supplementary Table 5**. The amplicons were connected to Illumina adaptors by 8 cycles of PCR using a set of primers (CAAGCAGAAGACGGCATAC GAGAT-CGTGAT (Index)-GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT & AATGATACGGCGACCACCGA GATCT-ACACTCTTTCCCTACACGACGCTCTTCCG ATCT), followed by sequencing on a NovaSEQ sequencer (Illumina). Contig sequences were blasted to the IMGT/HLA database (https://www.ebi.ac.uk/Tools/services/web_ncbiblast/toolform.ebi?tool=ncbiblast&context=nucleotide&database=imgthla) to identify alleles of DRB3 and DRB4 genes.

HLA Transductants

Two types of antigen presenting cells were generated using the retroviral or lentiviral expression system. For the initial analysis to determine HLA-DR, DQ, or DP (experiments shown in left panels of **Figures 3**–**5**, and **6A**), retroviral vectors encoding a puromycin resistance gene along with a first set of DRA1-P2A-DRB1, DQA1-P2A-DQB1, or DPA1-P2A-DPB1 gene cassette were generated for each donor, and transduced into K562 cells (ATCC CCL-243) using a standard spinfection protocol with viral supernatant produced from 293T cells (ATCC CRL-3216) (58), followed by sorting of cells stained with anti-HLA-DR antibody conjugated with allophycocyanin (clone L243, BioLegend), anti-HLA-DQ antibody conjugated with phycoerythrin (clone REA303, Miltenyi BIotec), or anti-HLA-DP antibody conjugated with allophycocyanin (clone B7/21, Leinco Technologies). Retroviral vectors encoding an E2-Crimson fluorescent protein gene along with another set of DRA1-P2A-DRB1, DQA1-P2A-DQB1, or DPA1-P2A-DPB1 gene cassettes were generated for each donor, and transduced into K562 cells that have been transduced with the first set of DR, DQ, or DP gene cassette, followed by sorting of cells expressing E2-Crimson. For all subsequent experiments, lentiviral vectors encoding a DRA1-P2A-DRB1, DQA1-P2A-DQB1, DPA1-P2A-DPB1, DRA1-P2A-DRB3, or DRA1-P2A-DRB4 gene cassette were generated and transduced into K562 cells (ATCC CCL-243) using a standard spinfection protocol with viral supernatant produced from 293FT cells (Thermo Fisher Scientific). Expressions of HLA molecules were analyzed using the antibodies described above. Over 97% of cells in all cell lines except the combinations of DQA1*01:01 & DQB1*03:02 and DQA1*03:01 & DQB1*05:01 (DQ trans-combinations of donor nPOD 6342) were stained with the antibodies, and therefore no sorting was needed. K562 cells transduced with the two HLA allele combinations above failed to express HLA molecules on cell surface. This observation was consistent with a previous report characterizing preferred and failed HLA alpha and beta chain combinations (60), and therefore these two trans-combinations were excluded from analysis of TCRs derived from the nPOD 6342 donor.

Determining HLA Restrictions

TCR transductants (20,000 cells per well) were cultured with an optimal 15-mer peptide (100 μM) in the presence of K562 cells expressing appropriate HLA molecules (50,000 cells per well) in a well of round-bottom 96-well-plates. Peptide sequences used for individual TCR transductant lines are included in **Table 2**. HLA allele combinations used for individual assays are designated in figure legends. After overnight culture, cells were harvested and analyzed for ZsGreen-1 expression using a flow cytometer (Cytoflex, Beckman Coulter) and FlowJo (BD) (38). Cells cultured in the presence or absence of 5 μg/ml of anti-mouse CD3ϵ antibody (Clone 125-2C11, BD) were included in an assay as positive and negative controls, respectively. For the TCR clonotype 55.C10, TCR transductants (20,000 cells per well) were cultured with the cognate peptide (100 μM) in the presence of an EBV-transformed cell line generated from nPOD 6414 spleen cells with or without anti-HLA-DR antibody (clone L243, BD), anti-HLA-DQ antibody (generated in the Michels laboratory), or HLA-DP antibody (clone B7/21, Abcam) at 1 μM, followed by analysis of ZsGreen-1 expression next day.

Dose–Response Assessment

TCR transductants (20,000 cells per well) were cultured with a peptide designated in **Table 2** at different concentrations (10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM) in the presence of K562 cells expressing HLA molecules designated in figure legends (50,000 cells per well), followed by analysis of ZsGreen-1 expression next day. Peptide sequences tested for the response by TCRs HA1.17 and 489 are PKYVKQNTLKLAT (influenza HA:306–318), SG**Q**GSFQPSQ**Q**NP (native α-gliadin), and SG**E**GSFQPSQ**E**NP (deamidated α-gliadin p1E,p9E). EC_{50} values were calculated using Prism 8 (GraphPad), the nonlinear regression log (agonist) vs. response (three parameters) equation model. For TCR clonotypes, 6.G4 and 23.G8, EC_{50} values were

not determined due to weak responses, and therefore the nonlinear regression log (agonist) vs. normalized response equation model was used to determine EC_{50} values.

Responses to Proinsulin and Human Islet Lysates

Primary islet tissues isolated from non-diabetic organ donors were obtained from the Integrated Islet Distribution Program (IIDP). Islet lysates were generated by three steps of extractions using trifluoroacetic acid, ammonium bicarbonate, and trifluoroethanol. Extracts were lyophilized and dissolved in urea. K562 cells expressing cognate HLA molecules for each TCR clonotype (50,000 cells per well) were cultured with proinsulin (Amidebio LLC, Louisville, CO) at 200 μg/ml or lysates generated from 600-1600 IEQs of primary islets overnight. TCR transductants (20,000 cells per well) were directly added to culture wells containing K562 cells pulsed with proinsulin or islet lysates, followed by analysis of ZsGreen-1 expression next day.

Responses to Native and Citrullinated Peptides

TCR transductants (20,000 cells per well) were cultured with native insulin B-chain peptide (SHLVEALYLVCGE-**R**-GFFYTPK) or a citrullinated insulin B-chain peptide (SHLVEALYLVCGE-**Cit**-GFFYTPK) at different concentrations (100 pM, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM) in the presence of K562 cells expressing HLA molecules designated in figure legends (50,000 cells per well), followed by analysis of ZsGreen-1 expression next day.

Computational Simulation of Predicting Core Epitopes

The IEDB MHC-II Binding Prediction online tool (http://tools.iedb.org/mhcii/) was used to predict core epitopes. Up to five 15-mers of peptides that were contained in peptide pools stimulating individual TCR transductants were analyzed using the IEDB recommended prediction 2.22 method for binding to cognate HLA molecules.

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

The studies involving human participants were reviewed and approved by the University of Florida Institutional Research Board (IRB201600029). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MN and AM designed and oversaw the studies. LL and AA conducted the experiments, and LL, AA, and MN analyzed the data. HR, LY, SK, MA, and CM provided essential information and materials. MN wrote the manuscript, and all authors reviewed and edited 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/fendo.2021.622647/full#supplementary-material

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Islet-Resident Dendritic Cells and Macrophages in Type 1 Diabetes: In Search of Bigfoot's Print

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The classical view of type 1 diabetes assumes that the autoimmune mediated targeting of insulin producing ß-cells is caused by an error of the immune system. Malfunction and stress of beta cells added the target tissue at the center of action. The innate immune system, and in particular islet-resident cells of the myeloid lineage, could function as a link between stressed ß-cells and activation and recognition by the adaptive immune system. We survey the role of islet-resident macrophages and dendritic cells in healthy islet homeostasis and pathophysiology of T1D. Knowledge of islet-resident antigen presenting cells in rodents is substantial, but quite scarce in humans, in particular regarding dendritic cells. Differences in blood between healthy and diseased individuals were reported, but it remains elusive to what extend these contribute to T1D onset. Increasing our understanding of the interaction between ß-cells and innate immune cells may provide new insights into disease initiation and development that could ultimately point to future treatment options. Here we review current knowledge of islet-resident macrophages and dendritic cells, place these in context of current clinical trials, and guide future research.

Keywords: macrophage, dendritic cell, islets of Langerhans, innate immunity, beta-cell stress, autoimmune diseases

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INTRODUCTION

Type 1 diabetes is characterized by the loss of insulin-producing ß-cells in pancreatic islets of Langerhans leading to insulin shortage. This loss is caused by an autoimmune mediated attack, in which ß cell specific $CD8^+$ T-cells are the ultimate effectors. In past decades ß-cells were deemed "innocent victims" of this autoimmune attack. Consequently, intervention therapies focused to suppress the adaptive immune system, but showed limited success (1). Plausibly, the cause of T1D is not only due to an erroneous immune system and involves additional pathophysiological reasons. Research shifted toward ß-cells provoking autoimmunity, changing our view of T1D immunopathogenesis in which stressed ß-cells trigger an autoimmune attack in a predisposing genetic and immunological environment (2, 3). An important gap in knowledge is what kicks off this process and what connects the adaptive immune system and ß-cells. Pancreatic islets are complex micro-organs. Besides hormone releasing cells, resident antigen presenting cells (APCs) of the myeloid lineage and innervating neurons are present. In spite of their footprint in islets, little is still known about resident myeloid cells and whether these cells play any role in health or disease,

alike the snowprint of Bigfoot, the mystical legend that most scientist consider to be a misidentification. We propose that myeloid APCs are the missing link between distressed ß-cells and the adaptive immune system. We focused attention to islet-resident myeloid cells and investigated their possible role as connectors bridging ß-cells and adaptive immunity.

T1D AS A DISEASE OF THE ADAPTIVE IMMUNE SYSTEM

T1D is a disease of the adaptive immune system (4). The best tool to predict T1D onset is screening for islet autoantibodies. These can be directed against a range of different islet antigens, including insulin, glutamate decarboxylase, zinc transporter 8 and insulinoma antigen-2 (5). Their appearance follows activation of T-cells and depends on poorly understood interactions between the environment, genetic factors and the immune system in a process that can range from months to years before clinical manifestation of T1D. However, positive autoantibody testing does not necessarily imply onset of disease and proof of a direct role of islet autoantibodies in beta-cell destruction is still lacking (6). Islet autoreactive $CD4^+$ and $CD8^+$ T-cells are present in islets, blood and lymph nodes (7). Distressed islets of T1D patients display increased HLA class I on the surface of endocrine cells, apparently preceding insulitis and facilitating autoreactive $CD8^+$ mediated ß-cell targeting. Both islet autoantibodies and islet-autoreactive T-cells indicate a break in immune tolerance and identify the adaptive immune system as essential component in the autoimmune process leading to loss of beta-cells.

Auto-reactive T-cells evade thymic education in both healthy individuals and patients with T1D (8). Regulatory T-cells are critical in maintaining tolerance and are present in similar numbers in healthy and diseased individuals but display reduced regulatory potential in patients (9). An imbalance between immune regulation and activation in favor of islet autoimmunity is evident in T1D (8). Yet, why T-cell becomes activated and what role beta-cells and the innate immune system may play in this imbalance remains largely unknown.

T1D AS A DISEASE OF THE BETA-CELL

The idea of T1D being a disease of the adaptive immune system has become challenged in the past decade (4). Several observations point toward additional key players. Research shifted toward the 'victim' target tissue and increasing evidence places ß-cells at the center of initiation.

ß-cells are highly specialized to produce large quantities of insulin (10). This specialization is at expense of reduced defense mechanisms and pronounced stress sensibility. Cellular stress could result from constantly increased demand of insulin. Pancreas sizes seem to matter in T1D (11). A smaller pancreas implies reduced numbers of ß-cells, which subsequentially increases the metabolic burden on islets (12). Beside reduced pancreas size, other factors such as viral infections or inflammatory milieu have been suggested as stressors (13, 14). Pancreata from T1D donors showed ß-cells under increased intra-cellular stress during insulitis as indicated by markers of endoplasmic reticulum stress, such as CHOP, BIP and XBP-1 (15). ß-cell stress may trigger adaptive immunity but this requires involvement of the innate immune system, since activation of islet auto-reactive T-cells only occurs following priming by dendritic cells due to presentation of immunogenic islet peptides (**Figure 1**). Research on stressed ß-cells revealed various mechanisms for the generation of new auto-immune peptides (neoantigens) not present during thymic education, selection and formation of the immune system. Post-translational modifications add to variety of the proteome and modified peptides might be recognized as neo-epitopes (16–18). Other neoantigens include so-called hybrid peptides joining peptides fragments of two islet proteins, which stimulate T-cells found in islets of T1D patients (19, 20). Neoantigens can also occur by erroneous translation, leading to defective ribosomal proteins (DRiP), or by alternative splicing (21–23). DRiPs can be generated by ribosomal complex skipping of the canonical start codon and instead initiation at a start codon within an alternative reading frame. This whole set of ß-cell released stressors points toward T1D being a disease of the adaptive immune system as well as ß-cells, where distressed ß-cells change their faces and prime the immune system.

THE INNATE IMMUNE SYSTEM AS A CONNECTOR

Macrophages and dendritic cells are professional APCs and the most extensively studied myeloid cells. They are present in islets and accumulate there during disease progression (**Figures 1**, **2**) (24). One of two major classes of APCs are dendritic cells (DCs). Immature DCs are tolerogenic (25). Conventional dendritic cells (cDCs) are strong APCs that activate naïve T-cells once they mature upon stimulation, while plasmacytoid dendritic cells (pDCs) secrete large amounts of proinflammatory interferons. DCs play a crucial role in maintaining immune tolerance and preventing tissue-specific autoimmunity, which harbors great therapeutic potential.

Macrophages can be divided into pro-inflammatory 'M1' and anti-inflammatory 'M2' macrophages based on their phenotype (26). However, this strict classification is changing into tissue and microenvironment specific flavors. Based on their local microenvironment, monocytes can differentiate into different subtypes of macrophages and fully differentiated macrophages are able to change their phenotype when transferred into other tissues (27). Additionally, changes are observed in enhancer landscape and gene expression profiles in different tissue-resident macrophages (28).

Having auto-reactive T-cells on one side and stressed ß-cells on the other raises the question how these two players interact. APCs characteristically infiltrate and monitor different tissues. They become activated upon recognition of pathogen- or

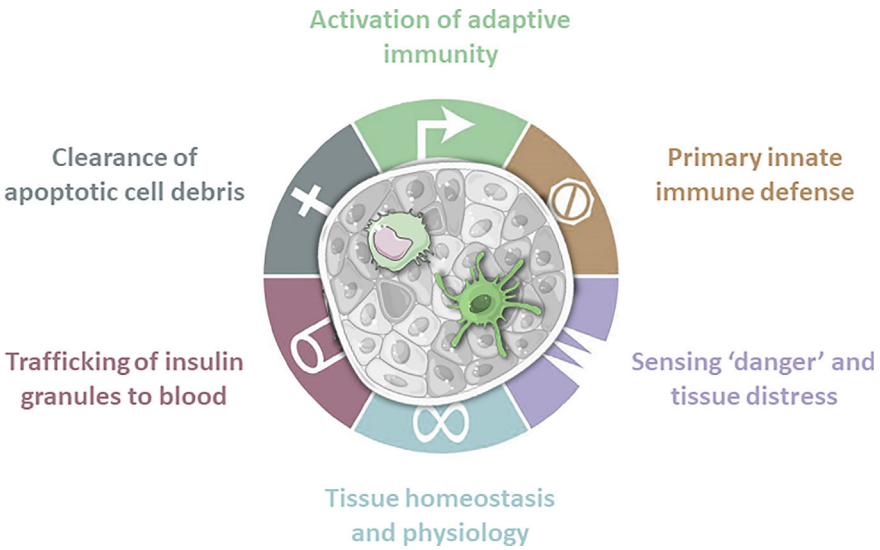

FIGURE 1 | Antigen presenting cells are present in islets of Langerhans. These innate immune cells fulfill a wide range of tasks. Macrophages play a crucial role in tissue homeostasis and physiology by expressing tissue remodeling cytokines. Due to constant sampling of the surrounding environment they clear apoptotic cell debris, but also sense danger signals and tissue distress. Obtained granules are then trafficked to the blood. In case of invading pathogens macrophages and dendritic cells are first line of defense. Dendritic cells are mainly involved in screening for danger signals and subsequent activation or regulation of the adaptive immune system.

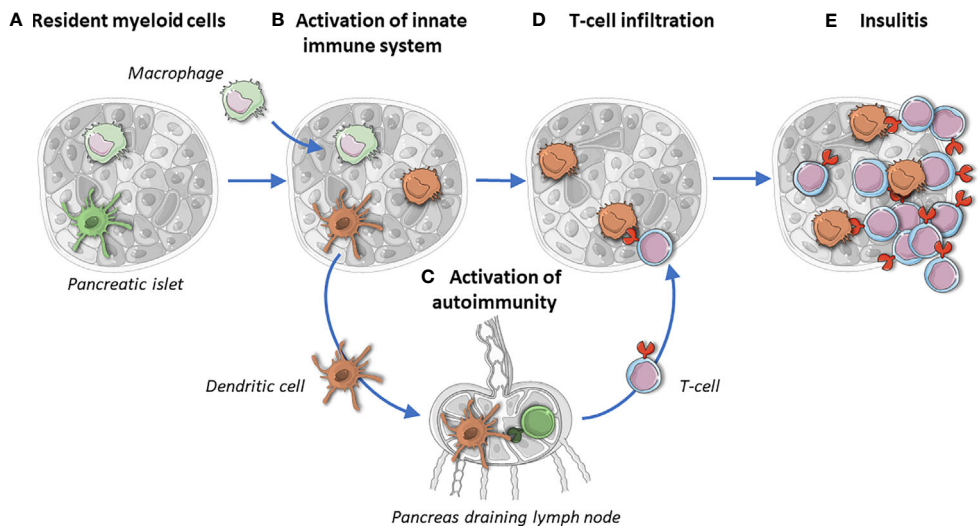

FIGURE 2 | Islets resident myeloid cells maintain tissue homeostasis and protect islets **(A)**. In T1D, macrophages infiltrate islets and their ratio changes toward a pro-inflammatory phenotype **(B)**. Upon activation, islet-resident dendritic cells migrate to pancreatic draining lymph nodes and activate naïve T-cells **(C)**. Activated T cells infiltrate islets and $CD4^+$ T cells scan for islet autoantigens taken up, processed and presented by macrophages and dendritic cells **(D)**. Insulitic auto-reactive $CD8^+$ T cells target ß-cells **(E)**. The missing link in **(D, E)** is the place of the elusive dendritic cells: are they still there, if so, how many and what do they look like? Brown stands for activated myeloid cells.

danger-associated molecular patterns, resulting in different responses, such as migration (29). DCs migrate after uptake of antigen from tissue to draining lymph nodes for antigen presentation and subsequent activation of antigen specific T-cells (**Figures 1** and **2**) (30, 31). Therefore, DCs could function as physical activators of T-cells in T1D (**Figure 2**). Besides DCs, macrophages play a crucial role in tissue homeostasis and antigen presentation toward approaching T-cells (**Figure 1**). The observation that transplantation of islets is more successful upon APC depletion strengthens the idea that

resident APCs play contribute to onset (32, 33). If APCs are the missing link between ß-cells and the innate immune system, it is necessary to determine their individual role in a spatial manner.

MYELOID CELLS IN CIRCULATION IN HEALTH AND DISEASE

Given that APCs play a crucial role in T1D onset by connecting ß-cells to the adaptive immune system, it is worth to assess differences in APCs between healthy individuals and T1D patients. Studies in NOD mice indicate altered numbers of DCs in blood and thymus compared to control mice (34, 35) and a DC subtype analysis reported an imbalance toward $CD8\alpha^-$ DCs (36, 37). Several studies claim possible variation in humans. Yet, whether DC numbers are increased, decreased, or remained stable and whether this happens before, during, or after onset is inconsistent (38–43). These inconsistences might relate to the notion that most studies focused mainly on monocyte derived DCs rather than *bona fide* DCs. Besides quantitative changes, functional differences, such as reduced phagocytic capabilities or increased HLA-DR expression in T1D have been reported (44).

Monocyte derived tolerogenic DCs (tolDcs) from patients under sub-optimal glycemic control display reduced tolerogenic capabilities compared to those from patients under optimal control (45–48). However, this glycemia dependent difference may not necessarily be a general difference between health and disease, and could be a consequence, rather than causally related to T1D immunopathogenesis. We recently showed that tolDCs generated from T1D patients' blood induce immune tolerance indifferently from those from healthy individuals, proving that they still possess their immune-regulatory capacity (47).

RESIDENT MYELOID CELLS IN HEALTH AND DISEASE

Besides circulating APCs, the role of resident APCs must be evaluated, especially since these are the first sensors of any changes in islets (**Figure 1**) (49). Islets contain macrophages as shown by staining for CD68 using imaging mass cytometry (50–52). They were present in low numbers and numbers were greater before and after onset of disease (52). However, it remains unclear whether infiltrating macrophages differ from resident ones, whether resident macrophages change, and whether they affect, or are affected by, the islet microenvironment in T1D.

Studies from NOD mice show that the vast majority (up to 98%) of APCs are macrophages, while inconsistencies exist about the presence of other APCs, such as DCs (53–55). Analysis of resident macrophages in NOD mice classifies them as cells with a mixed M1/M2 phenotype, polarizing toward M1, as indicated by transcripts of IL1b and TNFα. During pancreas development immature macrophages enter the islets and mature by week 4 of age, as measured by MHC II (53). Afterward, they are self-maintaining with low infiltration of immature macrophages or monocytes (53). The occurrence of mature APCs by week 4 is particularly interesting, since NOD mice develop insulitis soon after. NOD mice did not develop diabetes in absence of resident macrophages (56). Depletion of islet-resident APCs at 8 weeks of age resulted in a complete disappearance of lymphocytes from the pancreas. Upon reappearance of DCs and macrophages, lymphocytes reappeared (57). Ex vivo depletion resulted in a reduced release of pro-inflammatory cytokines such as IL-6, IP-10, and G-CSF (58). Interestingly, T-cells from macrophage-depleted NOD mice were unable to induce diabetes upon transfer into NOD.*scid* mice (59–61).

Beside their function as APCs, macrophages play a critical role in tissue development and remodeling (**Figure 1**), where they promote proliferation of ß-cells by creating a favorable microenvironment and upregulation of SMAD7 (62–65). During pancreas development in mice macrophages were present at increased numbers that declined until weaning (66). Curiously, lymphocyte infiltrates consisting of T-cells with some macrophages and DCs were observed in human fetal and neonatal pancreata (67). Lack of macrophages as in osteopetrotic *op/op* mice ($CSF1^{-/-}$), or due to chemical or antibody depletion, resulted in reduced pancreas size and vasculature, supporting a crucial role of resident macrophages, given that T1D patients also display reduced pancreas sizes (11, 68). With regards to vascularization, human islets from T1D patients display lower levels of vascular endothelial growth factor-A (VEGF-A) (58). VEGF-A is produced by ß-cells and seems to play a role in the development of islet vasculature, in ß-cell function, and in macrophage mediated ß-cell proliferation (69–71). Since resident macrophages are located in close contact to vasculature it seems plausible that some crosstalk between ß-cells, macrophages, and vasculature exist (72). However, this interplay remains elusive and warrants further studies. Macrophages might also directly induce beta-cell destruction by the synthesis of proinflammatory cytokines and reactive oxygen species, which lead to the so-called 'Copenhagen model' that put macrophages at the heart of islet inflammation and beta-cell destruction (73). While support of cytokine-mediated beta-cell toxicity was obtained in rodents, this did not hold for human beta-cells that proved far more resistant to cytokines (requiring a 100-fold larger dose than is not even feasible pathologically) and much better at dealing with oxygen radicals than rodents (74).

In marked contrast to mice, macrophages in humans only make for half or less of resident APCs and their phenotype was reported to be mixed M1/M2 (TNF, IL1b, IL6, IL10, with release of additional tissue remodeling cytokines MMP2, MMP9) (51, 75). Alike macrophages from other tissues that maintain tissue homeostasis by sensing hyperosmolarity, metabolic stress, hypoxia and ECM components, islet-resident macrophages sense their surrounding by detecting extracellular ATP concentrations *via* purigenic receptors, resulting in an increased concentration of intracellular Ca^{2+} levels (72, 75). Since ATP concentrations correlate with insulin levels, macrophages can sense ß-cell function.

In addition to microenvironment sensing, resident APCs constantly probe their surroundings. Islet-resident macrophages engulf vesicles released from ß-cells, a process taking place over a short distance, process and present these (76–78). Importantly,

these granules contain immunogenic peptides, which can be recognized by auto-reactive T-cells that had escaped thymic education (79–82). Such peptides can be taken up by DCs and their presence in draining lymph nodes is confirmed, which can result in activation of the innate immune system (**Figure 2**). Subsequent, targeting of immunogenic peptide presenting APCs by autoreactive $CD4^+$ T-cells supports this process of initiation and strengthens macrophages' potential role in onset (83, 84).

But where is 'Bigfoot', the dendritic cell in human islets? While mouse studies suggest that the myeloid compartment in islets is up to 98% consisting of macrophages, the rare studies on human islets pointed that 50% of leukocytes at best were macrophages, while the other 50% was ignored. We contend that islet DCs are important candidates to be identified and characterized, given their key role in regulating immune activation and modulation (**Figure 2**). Curiously, studies in both mice and men thus far have been biased to either macrophages or DCs. This leaves a significant opportunity to study the role of islet DCs in health and disease.

CHICKEN OR EGG

The above presented data point toward differences in myeloid cells between mice and men, between health and T1D, between different individuals and between neighboring islets. Even though genetic differences in the myeloid lineage exist, phenotypic alterations might not necessarily be present from the beginning (48). Instead, they might appear only in an altered microenvironment, such as in inflamed islets or hyperglycemia. The microenvironment plays a crucial role for macrophages, since these cells possess high microenvironment-dependent plasticity, which results in change of their phenotype (27). A stressed microenvironment caused by distressed ß-cells due to infection or other perturbations (metabolic, inflammatory) could lead to genetically prone malfunctioning of macrophages, or indeed be caused by these innate immune cells. Subtle changes in the microenvironment could occur over years that have skipped attention. The role of the microenvironment on macrophage phenotype is supported by recent findings showing that microenvironment alters infiltrating macrophages after diabetes onset (85). Such changes might also occur in healthy individuals but be better compensated.

Another question arising is whether APCs engulf, process, and present antigen in a different way in diabetes prone subjects. Building on our scenario, changes might even relate to healthy or inflamed microenvironment.

In summary, cells of the myeloid lineage display genetic, qualitative, and quantitative changes in T1D. Yet, it remains unclear to what extend these differences contribute to onset of T1D.

THERAPEUTIC OPPORTUNITIES

While a main goal of T1D research is to understand loss of immune tolerance, another objective is to restore tolerance in affected patients. Different therapeutic strategies aim on modulating cells of the myeloid lineage using granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF). In presence of GM-CSF, cDCs can induce T_{reg} proliferation, while G-CSF increases levels of cDC2s and shifts the cytokine profile from T_H1 toward T_H2 in healthy individuals (86–88). Furthermore, G-CSF has an immune-regulatory effect, as indicated by increased levels of tolerogenic DCs (89, 90).

Clinical trials in T1D using colony-stimulating factor focused mainly on G-CSF, based on findings that G-CSF prevents diabetes in NOD mice by recruiting pDCs and functional $CD4^+CD25^+T_{regs}$. Obtained T_{regs} protected against diabetes onset when transferred into NOD.*scid* mice (91). G-CSF treatment combined with anti-thymocyte globulin (ATG) reversed diabetes in NOD mice (92). In the clinic, ATG together with G-CSF preserved ß-cell function in T1D patients up to one year after treatment (93). However, a consecutive study indicated ATG as the main factor, because G-CSF alone increases numbers of circulating neutrophils, while C-peptide level or insulin needs remained unaffected. In addition, CD4:CD8 and naïve:memory T-cell ratios did not change upon G-CSF treatment (94). If anything, G-CSF even seemed to reduce the benefits of ATG. Patients treated either with ATG/G-CSF, or ATG alone had reduced conventional and regulatory $CD4^+$ T-cell numbers after 2 weeks, with stable $CD8^+$ T-cell numbers (95).

Since targeting myeloid hematopoiesis does not seem to offer major benefit to patients, other myeloid strategies might be more successful (96, 97). As discussed above, monocyte derived tolDCs do not differ between healthy and diseased individuals (98). Given their role as connectors and immune modulators, it seems plausible to use patients' tolDCs to restore immune tolerance (99–101). In a recent clinical trial, tolDCs generated from monocytes by vitaminD3 followed by dexamethasone and loaded with proinsulin peptide C19-A3 were tested to restore immune tolerance in long-term T1D patients, demonstrating feasibility, safety, tolerability and mechanistic efficacy of this novel therapeutic intervention strategy engaging innate immunity (47). This strategy will next be tested for its capacity to delay disease progression and preserve endogenous beta-cell function.

CONCLUSION

A myeloid footprint exists in pancreatic islets, irrespective of insulitis. Resident myeloid immune cells play a key role in islet morphology, physiology and function and are essential for tissue homeostasis and clearance of cell debris. These innate cells are intrinsic components in dialogue between islets and the immune system. Their role in diabetes seems clear in rodent models of autoimmune diabetes, but remains ignored, vague, inconsistent and inconclusive for human T1D. While limited information on residing myeloid cells in human islets is available after disease onset, our knowledge on these moderators before and during

onset is even scarcer. Genetic variation and phenotypic differences in myeloid cells have been linked to T1D, but causality remains unclear. Subtle differences between health and disease can be largely attributed to dysglycemia, and may be a consequence, rather than causative, diabetogenic feature. Islet-resident myeloid immune cells conceivably prime the adaptive immune system, but with reason, as they are equipped to sense danger and tissue distress, and play a crucial role in tissue sensing, spatial antigen presentation, and tissue remodeling, in addition to immediately responding to ß-cell stress, changes in the microenvironment, or invading pathogens. Their failure in this process could predispose or trigger T1D. Dendritic cells can function as both sensors and connectors to the adaptive immune system. Adaptive immunity needs these cells to present islet autoantigens to the immune system so it is conceivable that they are involved in propagating the autoimmune response, while they could equally contribute to restoring/repairing islet tissue homeostasis, as well as restore immune tolerance! We contend that the innate immune system and myeloid cells in particular are connecting the dots in T1D. Their footprint in healthy islets underscores their essence and warrants more investigation. Therefore, it is critically important to learn more about changes between benign leukocyte residency and infiltration into pathogenic footprint and what causes these, to turn this knowledge into novel therapeutic intervention modalities and strategies. Engaging myeloid immune cells holds great promise as future treatment options.

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

Both authors studied literature and composed this review and its figures. Both authors contributed to the article and approved the submitted version.

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Phagocytosis and Efferocytosis by Resident Macrophages in the Mouse Pancreas

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The tissue microenvironment in the mouse pancreas has been shown to promote very different polarizations of resident macrophages with islet-resident macrophages displaying an inflammatory "M1" profile and macrophages in the exocrine tissue mostly displaying an alternatively activated "M2" profile. The impact of this polarization on tissue homeostasis and diabetes development is unclear. In this study, the ability of pancreas-resident macrophages to phagocyte bacterial and endogenous debris was investigated. Mouse endocrine and exocrine tissues were separated, and tissue-resident macrophages were isolated by magnetic immunolabeling. Isolated macrophages were subjected to flow cytometry for polarization markers and qPCR for phagocytosis-related genes. Functional *in vitro* investigations included phagocytosis and efferocytosis assays using pH-sensitive fluorescent bacterial particles and dead fluorescent neutrophils, respectively. Intravital confocal imaging of *in situ* phagocytosis and efferocytosis in the pancreas was used to confirm findings *in vivo*. Gene expression analysis revealed no significant overall difference in expression of most phagocytosis-related genes in islet-resident vs. exocrine-resident macrophages included in the analysis. In this study, pancreas-resident macrophages were shown to differ in their ability to phagocyte bacterial and endogenous debris depending on their microenvironment. This difference in abilities may be one of the factors polarizing islet-resident macrophages to an inflammatory state since phagocytosis has been found to imprint macrophage heterogeneity. It remains unclear if this difference has any implications in the development of islet dysfunction or autoimmunity.

Keywords: pancreas, macrophages, phagocytosis, efferocytosis, *in vivo* imaging, type 1 diabetes

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INTRODUCTION

Type 1 diabetes is a disease with autoimmune features where insulin producing beta cells are progressively lost leading to hyperglycemia and dependence on exogenous insulin. Hallmark histopathological features include the accumulation of lymphocytes and macrophages in and around the pancreatic islets (1). The cells from the adaptive immune system have been extensively studied in this disease whereas the specific contributions and functions of innate immune cells, including macrophages, during disease onset have gathered less attention.

In work by the Unanue group, a surprising dichotomy in the phenotypes of pancreatic macrophages in mice has been uncovered (2, 3). Macrophages residing in the islets of Langerhans (endocrine-resident) are classically activated and exhibit an inflammatory phenotype (also called "M1 macrophages") whereas macrophages residing in the exocrine pancreas are mostly alternatively activated, and exhibit an anti-inflammatory phenotype (also called "M2 macrophages") (4). The complete functional impact of the very specific polarization of pancreatic macrophages in the mouse is however not fully clear.

Endocrine-resident macrophages have been shown to be in close contact with both islet capillaries and beta-cells and can take up insulin granule contents and present these on MHC-II (5). Supporting their active role in controlling the inflammatory environment, endocrine-resident macrophages have also been observed to act as "gatekeepers" for the entry of lymphocytes into islets during onset of T1D in mouse models (6). Further observations in the *op/op* mouse (inactivating mutation in the macrophage colony stimulating factor-1 gene (*Csf1*) resulting in the absence of CSF-1) lends data to suggest that the endocrine-resident macrophage also likely has homeostatic properties since lacking macrophages in islets leads to poorly developed islets (7).

An important homeostatic function of macrophages is clearance of apoptotic cells and debris from the normal turnover of cells in the body (8). A comprehensive study on efferocytosis (clearance of endogenous cells) by macrophages in different organs of mice found expression level of the mannose receptor (Mrc-1/CD206/MMR), and a general M2-phenotype, to be the main factors correlating to the efferocytic capacity of tissue-resident macrophages (9). Since endocrine-resident macrophages in mice lack the expression of MMR (2), this poses the question whether these macrophages are less prone to phagocytosis and efferocytosis, possibly resulting in aggregation of cellular debris in events of intra-islet apoptosis and in clearance of invading bacteria.

We decided to investigate the function of pancreas-resident macrophages in healthy mice with regards to their efficiency to take up bacteria by phagocytosis and apoptotic endogenous cells by efferocytosis. We found no difference in the expression of phagocytosis-related genes between endocrine-resident and exocrine-resident macrophages. Neither were endocrine-resident macrophages impaired in their ability to engulf bacteria or apoptotic cells *in vitro* or *in vivo*, showing that despite the inflammatory phenotype of endocrine-resident macrophages, they still likely contribute to homeostatic processes.

MATERIALS AND METHODS

Animals

Male C57Bl/6 (wild-type) mice were purchased from Taconic, C57Bl/6$^{Ly6g(tm2621(Cre\text{-}tdTomato)Arte}$ (also named 'Catchup') mice (10) bred in-house were used as a source of red-fluorescent neutrophils, and CX$_3$CR1$^{+/Gfp}$ (B6.129P2(Cg)-*Cx3cr1*^{tm1Litt}/J) mice (11) bred in-house were used for imaging of pancreatic macrophages. Mice used in experiments weighed 25-30 g. Mice had free access to tap water and pelleted food throughout the study. All experiments were approved by the Uppsala Region Laboratory Animal Ethics Board (number 5.9.18-03603/2018).

Tissue Staining and Human Histopathology

Mouse pancreata fixed in 4% paraformaldehyde were snap-frozen and sectioned into 20 µm sections. The tissues were then stained for MMR and F4/80 using antibodies listed in **Supplemental Table 1** and imaged using a Leica SP8 scanning confocal microscope.

Human biobanked normal pancreas tissue excised from three patients undergoing surgery for pancreatic neoplasms had been snap-frozen, and sections were fixed in 4% paraformaldehyde before being stained for MMR and CD68 using antibodies listed in **Supplemental Table 1** and imaged using a Leica SP8 scanning confocal microscope. Ethical approval for use of the biobanked material was provided by the Regional Ethics Review Board.

Neutrophil Isolation and Apoptosis Induction

Mouse bone marrow- (BM) and spleen-derived neutrophils were isolated from heterozygous Catchup mice using a positive selection kit for Ly6G$^+$ cells (Miltenyi Biotec, #130-092-332). Isolated neutrophils were cultured for 24 h at 37°C/5% CO_2 in RPMI-1640 media with 2% FBS to induce apoptosis. Neutrophil apoptosis was determined using the Annexin V Apoptosis Kit with 7-AAD (Biolegend; #640922) according to the manufacturer's instructions.

Pancreatic Macrophage Isolation

Endocrine and exocrine fractions of the pancreas were separated using a density gradient approach as described earlier (12). Single cell suspensions of the endocrine and exocrine fractions were prepared by incubating the fractions for 5 min at 37°C in 3 ml of non-enzymatic cell dissociation buffer (Thermo Fisher Scientific; #13151014), followed by rapidly pipetting the solution and further incubation for 3 min at 37°C. Thereafter 10 ml of buffer (RPMI-1640, 2% FBS, 2 mM EDTA) was added and suspensions were filtered through a 70 µm cell strainer. Following centrifugation at 300xg for 10 min, anti-F4/80 microbeads (Miltenyi Biotec; #130-110-443) were used for positive selection of macrophages from endocrine and exocrine fractions according to the manufacturer's instructions. The fractions were run twice through the magnetic columns to reach a purity of 90% for endocrine tissue and 55% for exocrine tissue, see **Supplemental Figure 1** for representative flow cytometry plots.

Gene Expression Analysis

Isolated fractions of exocrine and endocrine macrophages pooled from two pancreata per observation were subjected to mRNA isolation using a commercial kit (Single Cell RNA Purification Kit, Norgen Biotek). Synthesis to cDNA was done using the High Capacity cDNA Synthesis kit (ThermoFisher). Custom oligos

were from ThermoFisher (primer sequences in **Supplemental Table 2**). Gene amplification was detected using Fast Sybr Green (ThermoFisher) and read on a QuantStudio 5 qPCR machine (ThermoFisher).

Pancreatic Macrophage Characterization

Exocrine and endocrine macrophages were stained for various M1 and M2 macrophage markers. Briefly, after separation of the exocrine and endocrine fraction as described above, cells were stained with Violet Dead Cell Stain Kit (Invitrogen; #L34955). Thereafter cells were incubated with BD Mouse Fc Block (BD Biosciences; #553141) in RPMI-1640 containing 10% FBS for 10 minutes at room temperature, followed by adding optimal concentrations of extracellular antigen primary antibodies (**Supplemental Table 1**) for 15 minutes on ice. Cells were washed twice with buffer (RPMI-1640, 2% FBS, 2 mM EDTA), followed by secondary antibody staining (if applicable) for 15 min on ice. Thereafter cells were fixed by adding Intracellular Fixation buffer (eBioscience; #88-8824-00) for 15 min at room temperature and washed twice with Permeabilization Buffer (eBioscience; #88-8824-00). The cells were then stained with intracellular antigen antibodies (**Supplemental Table 1**) in Permeabilization buffer for 30 min on ice. The cells were then washed in Permeabilization buffer twice and resuspended in Intracellular Fixation buffer. Cells were analyzed using CytoFlex (Beckman Coulter) or Northern Lights 2000 (Cytek) flow cytometers and data was processed using FlowJo software (BD).

Phagocytosis and Efferocytosis Assays

For determining phagocytic capacity of macrophages, pHRodo Red *E.coli* BioParticles Conjugate for Phagocytosis kit (ThermoFisher) was used at a concentration of 1 mg/mL according to the manufacturer's instructions. The pHRodo Red conjugate is non-fluorescent outside the cells but will be brightly fluorescent in the low pH environment of phagosomes. Briefly, isolated macrophages pooled from the exocrine and endocrine fractions of four pancreata were cultured for 30 min with pHRodo *E.coli* BioParticles at 37°C. For determining efferocytic capacity of macrophages, isolated macrophages were cultured for 30 min with tdTomato-fluorescent apoptotic neutrophils (from Catchup mice) at 37°C in a 1:30 ratio (each tube contained approximately 2,000 macrophages and 60,000 neutrophils). These experiments were performed in 1 mL complete culture medium (RPMI1640 supplemented with 10% FCS and penicillin/streptomycin). Following phagocytosis and efferocytosis assays, cell suspensions were stained with antibodies against CD45 and F4/80 (**Supplemental Table 1**). Cells were analyzed using a CytoFlex flow cytometer with CytExpert software (Beckman Coulter).

Cytokine Analysis

Supernatants were collected from the phagocytosis and efferocytosis experiments conducted during 4 h instead of 30 min, and diluted 1:2 in Diluent 41 (Mesoscale Diagnostics). Diluted supernatants were added in triplicates on a Vplex Proinflammatory Panel 1 multiplex protein assay (Mesoscale Diagnostics) and read on a Sector S 600 instrument (Mesoscale Diagnostics).

In Vivo Confocal Imaging

$CX_3CR1^{+/Gfp}$ mice were injected intravenously with either pHrodo *E.coli* bioparticles (50 µl of the supplied stock solution) or 10^7 Catchup tdTomato$^+$ neutrophils. After 4 and 24 h, respectively, mice were anesthetized by spontaneous inhalation of isoflurane (Abbvie) and injected intravenously with anti-CD31 (390) antibody tagged with Alexa Fluor 647 (**Supplemental Table 1**). The pancreas was exposed and mounted for intravital imaging with a suction ring as previously described (13, 14). The 3D printed suction ring and imaging setup has been previously described by us (15). Images were acquired using a confocal laser scanning microscope (Leica TCS SP8).

Image Analysis

Analyses for all images were performed using Imaris (Bitplane) and FIJI/ImageJ (NIH). More specifically, for quantifying uptake of red-fluorescent pHrodo *E.coli* bioparticles or Catchup neutrophils, green-fluorescent macrophages in confocal z-stacks were transformed into volume surfaces and masked for red fluorescence. This allows for distinction of only the red-fluorescent particles present within the macrophages. To distinguish intra-islet macrophages from exocrine-resident macrophages, the reflection signal from the islet was transformed into a volume surface and masked for green fluorescence. This allows for distinction of intra-islet-resident macrophages. The total number of macrophages and macrophages with intracellular red fluorescence were counted manually.

For tissue sections stained for F4/80 (mouse) or CD68 (human) and CD206, cells staining positive for each antibody were counted using the spots-function in Imaris. Since a small fraction of the CD206 signal does not label macrophages, only double-positive cells were counted as CD206-positive. The area of islets and exocrine tissue was measured using ImageJ.

Validation of Results Using Tabula Muris Senis

Tabula Muris Senis (16) pancreatic FACS dataset processed files from figshare (https://figshare.com/articles/dataset/Tabula_Muris_Senis_Data_Objects/12654728) were utilized for this study. Only cell types identified as leukocytes in (16) were analyzed. Analysis of scRNA-seq data sets were performed with Scanpy Package (v1.6.0) (17) in Python Package (v3.6.10), with counts log-normalised using size factor normalization. Macrophages were defined with *Emr1* expression > 0.5 (log-normalised counts).

Statistics

Results are presented as mean ± standard deviation. Type of statistical test for each analysis is mentioned in the figure

legends. *P*-values of less than 0.05 were considered statistically significant.

RESULTS

Exocrine and Endocrine Pancreatic Macrophage Profile Under Steady State

The differing phenotypes of macrophages in the pancreas have been characterized previously (2), and the dichotomy between endocrine-resident and exocrine-resident macrophages becomes very evident when staining for the mannose receptor 1 (Mrc1/MMR/CD206) (**Figure 1A**). Macrophages within mouse islets do not express MMR, whereas macrophages in the exocrine tissue are largely positive for this receptor (**Figure 1B**).

For assessing the expression of MMR in the human pancreas, we stained pancreata from three human non-diabetic donors. Similar to the mouse pancreas, macrophages in the endocrine tissue expressed less MMR than macrophages in the exocrine tissue (**Figure 1C**). Macrophages in exocrine tissue were however expressing MMR to a higher extent, suggesting a similar dichotomy also in human pancreata (**Figure 1D**).

Macrophages ($CD45^+CD11b^+F4/80^+$) of the exocrine and endocrine mouse pancreas were further characterized for their phenotypic polarization by flow cytometry (**Figure 1E**). Endocrine-resident macrophages had higher expression of MHCII compared to exocrine-resident macrophages (**Figure 1F**; mean fluorescence intensity (MFI) $5.7 \pm 0.53*10^6$ *vs.* $2.4 \pm 0.17*10^6$, respectively, $P<0.0001$) as previously found (2). Also by flow cytometry, endocrine-resident macrophages expressed less MMR than exocrine-resident macrophages (**Figure 1G**; MFI $3.2 \pm 0.48*10^4$ *vs.* $8.9 \pm 0.20*10^4$, respectively, $P<0.0001$). Further, endocrine-resident macrophages expressed more CD86 (**Figure 1H**; MFI $9.0 \pm 0.23*10^3$ *vs.* $7.6 \pm 0.10*10^3$, respectively, $P=0.0008$), and less Arginase-1 by percent $MHCII^-$ macrophages (**Figure 1I**; MFI $1.0 \pm 0.020*10^3$ *vs.* $8.0 \pm 1.6*10^3$, respectively, $P=0.1$) than exocrine-resident macrophages, recapitulating the previously reported skewing of endocrine-resident macrophages to an inflammatory phenotype.

Expression of Phagocytosis-Related Genes

We isolated macrophages from the endocrine and exocrine compartments of the pancreas by first separating the islets from the exocrine fraction through enzyme digestion and density-gradient centrifugation, dissociating the tissues, and isolating resident macrophages using anti-F4/80 magnetic beads. A selection of phagocytosis-related genes was then assessed by RT-qPCR in the two different fractions. As expected, there was a great difference in expression of *Mrc1* (MMR) with endocrine-resident macrophages expressing significantly less of this gene. Also *Timd4*, which encodes Tim-4 – a receptor involved in engulfment of apoptotic cells, was significantly more expressed in endocrine-resident macrophages. None of the other assessed phagocytosis/efferocytosis-related genes (*Anxa1, Cd163, Cd300b, Mertk, Pparg, Sirpa, Stab2*) were differentially expressed through the two fractions of macrophages (**Figure 2A**). We could thus not discern any obvious skew in any of the subsets for having extraordinary phagocytic abilities on the mRNA level. Further, we validated our findings with data on exocrine and endocrine leukocytes from the open single-cell sequencing database *Tabula Muris Senis* (16). Also in this data, apart from significant differences in *Mrc1* and *Cd163* expression, no apparent pattern in the differences between these genes could be found (**Supplemental Figure 2**).

Endocrine Macrophages Phagocytose More Than Exocrine Macrophages

To test whether lack of MMR expression on endocrine macrophages predicts lower phagocytic capacity compared to exocrine macrophages, we purified exocrine- and endocrine-resident macrophages for phagocytosis and efferocytosis assays using the method described above. Isolated macrophages were incubated with *E.coli* particles loaded with the pH-sensitive probe pHrodo which will increase its fluorescence when present in the acidic endosomes following phagocytosis (**Figure 2B**). We found that whilst 58 ± 3% of the exocrine macrophages had taken up the bacterial particles, 92 ± 3% of the endocrine-resident macrophages had taken up the same (**Figures 2E, F**). For comparison, we performed the same assessment for cultured mouse bone marrow-derived macrophages (BMDMs) pushed into either M1 or M2 states. Cultured M2 BMDMs were in our hands expressing twice the amount of MMR of M1 BMDMs (MFI $2.23 \pm 0.42*10^4$ *vs.* $1.11 \pm 0.18*10^4$, respectively, $P=0.01$). We found that M1 macrophages were more efficient phagocytes than M2 macrophages (MFI $2.24 \pm 0.25*10^5$ *vs.* $1.73 \pm 0.15*10^5$, respectively, $P=0.02$).

Endocrine-Resident Macrophages Are Efficient in Efferocytosis

The paper identifying the correlation between macrophage MMR expression and phagocytic capacity primarily investigated the engulfment of dead endogenous cells, efferocytosis (9). The most prevalent cell to be subject to efferocytosis is the short-lived neutrophil. To assess the efficiency of efferocytosis in pancreatic macrophages, we used neutrophils isolated from Catchup mice (red-fluorescent tdTomato expression in neutrophils driven by Ly6G expression). As neutrophils generally will not survive prolonged *in vitro* culture, these neutrophils were incubated overnight following isolation to induce apoptosis in the main fraction of the cells. Following overnight culture at 37°C/5% CO_2, and 58.25 ± 1.35% of the neutrophils were found to be early apoptotic ($AnnexinV^+/7\text{-}AAD^-$, **Figures 2C, D**). The neutrophils were incubated with pancreatic macrophages, and in this assay, 32 ± 6% and 52 ± 4% of the exocrine- and endocrine-resident macrophages, respectively, were tdTomato-fluorescent, indicating an ongoing engulfment of an apoptotic neutrophil by a macrophage (**Figures 2G, H**). The ratios of macrophages to apoptotic neutrophils were not significantly different between the

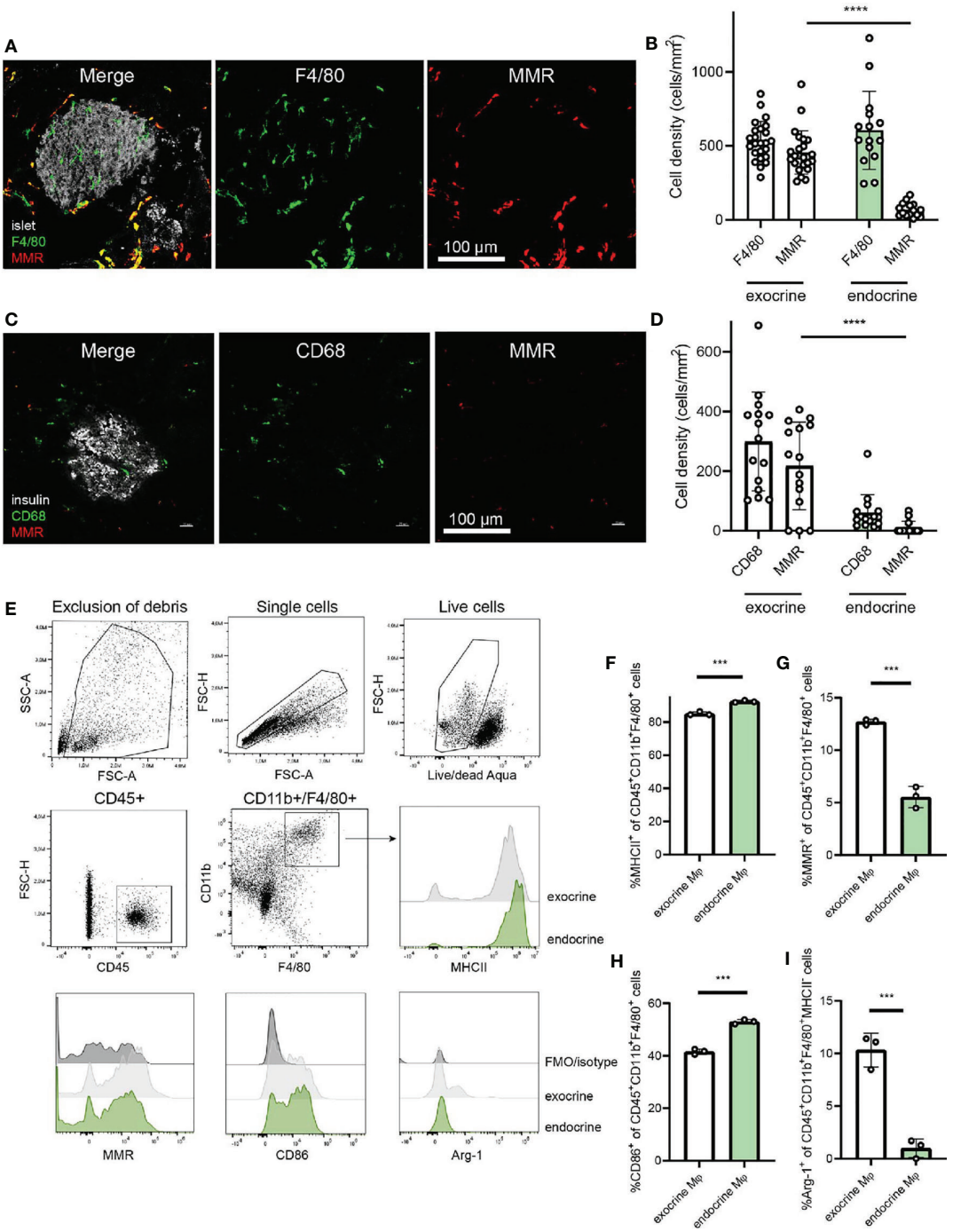

FIGURE 1 | Endocrine-resident macrophages are skewed to an inflammatory phenotype. **(A)** Immunofluorescence of the mouse pancreas reveals total absence of the mannose receptor (MMR) in endocrine-resident macrophages, whereas exocrine-resident macrophages express this receptor to a large extent (islets in grey, laser reflection, pan-macrophage marker F4/80 in green, MMR in red). **(B)** Quantification of immunofluorescence stainings for the pan-macrophage marker F4/80 and MMR in the pancreas showed that more than 90% of macrophages in the exocrine tissue expressed MMR and only a few percent of the endocrine-resident macrophages did (n=9 mice, 15 islets, 24 exocrine regions, ****$P < 0.0001$, Mann-Whitney U test). **(C)** Representative image of staining human pancreata for CD68 (green), MMR (red), and insulin (grey) **(D)** The expression of MMR in macrophages in human endocrine tissue was found to be lower than for macrophages in the exocrine tissue (n=3 donors, ****$P < 0.0001$, Mann-Whitney U test). **(E)** Gating strategy for endocrine- and exocrine-resident macrophages from mouse pancreas, including representative flow histograms of MHCII, MMR, CD86, and Arg-1 expression (representative of three independent experiments). **(F)** Endocrine-resident macrophages expressed significantly more MHCII, **(G)** significantly less MMR, **(H)** significantly more CD86, and **(I)** significantly less Arg-1 (of MHC-II$^-$ populations) than exocrine-resident macrophages (***$P < 0.001$, Mann-Whitney U test).

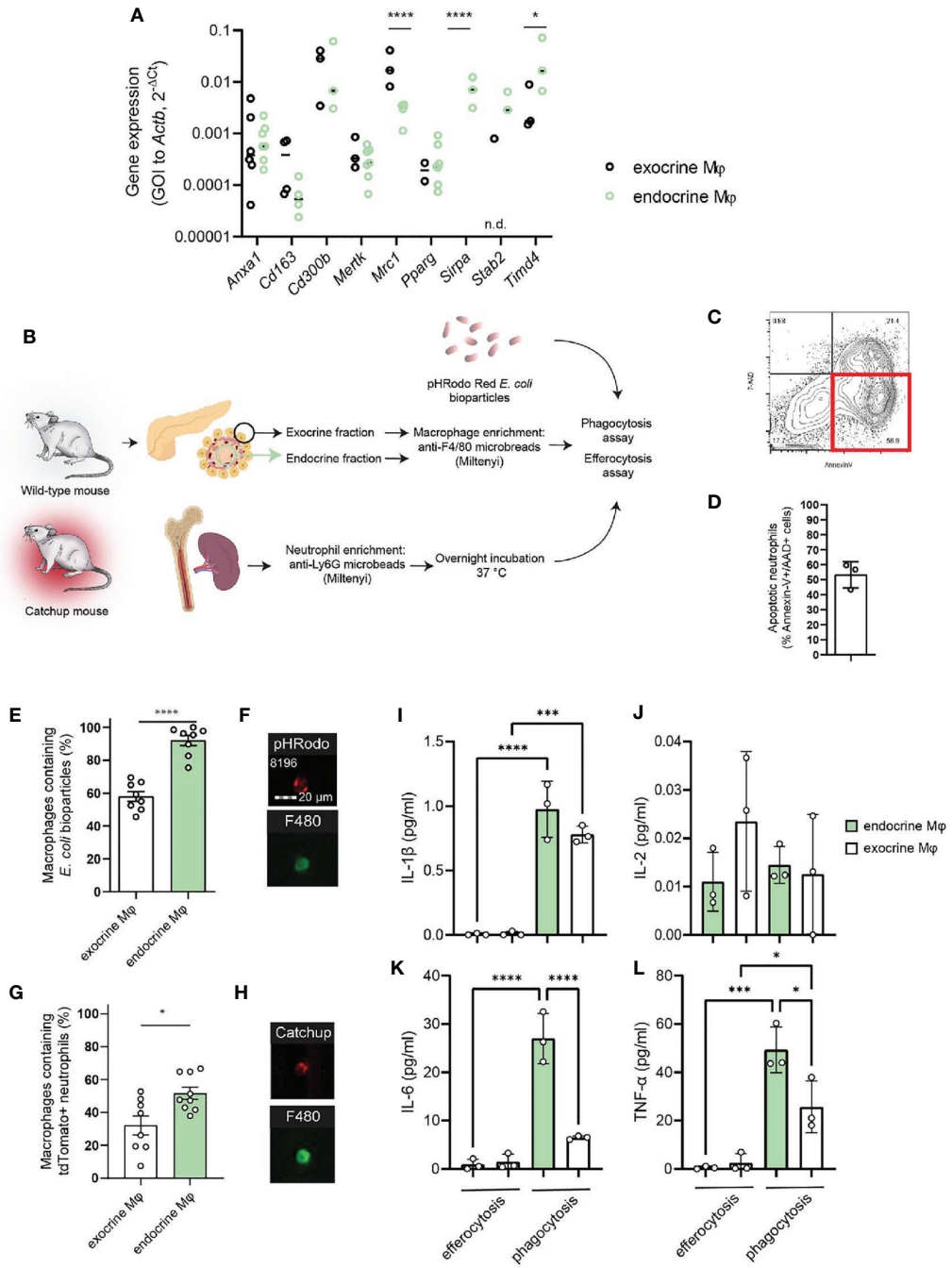

FIGURE 2 | Endocrine-resident macrophages are efficient in phagocytosis and efferocytosis *in vitro*. **(A)** A selection of phagocytosis-related genes were assessed by qPCR in macrophages from the endocrine and exocrine fractions, respectively. No distinct pattern suggesting functional impairment between macrophages from the two compartments could be observed other than the very low expression of MMR in endocrine-resident macrophages (n=3-7 mice per group, *P > 0.05, ****P ≤ 0.0001, 2-way ANOVA, n.d.=not detected). **(B)** Schematic of workflow for phagocytosis and efferocytosis assays. **(C, D)** Flow plot and quantification of the fraction of Catchup neutrophils being in a pre-apoptotic state for the efferocytosis assay. **(E)** Endocrine-resident macrophages were significantly more efficient in phagocytosis of pHrodo *E.coli* bioparticles than exocrine-resident macrophages (n=8 mice, ****P < 0.0001, Mann-Whitney U test). **(F)** Images from the FlowSight flow cytometer demonstrating the internalization of red-fluorescent bacterial particles and positivity for the pan-macrophage marker F4/80. **(G)** Endocrine-resident macrophages were also more efficient in efferocytosis of dying neutrophils than exocrine-resident macrophages (n=9 mice, *P < 0.05, Mann-Whitney U-test). **(H)** Images from the FlowSight flow cytometer demonstrating the internalization of tdTomato-red fluorescent neutrophil particles and positivity for the pan-macrophage marker F4/80. **(I–L)** Cytokine content in supernatants from efferocytosis and phagocytosis experiments for endocrine- and exocrine-resident macrophages (n=3 separate experiments in which macrophages from 2 mice were pooled per datapoint, *P < 0.05, ***P < 0.001, ****P < 0.0001, one-way ANOVA).

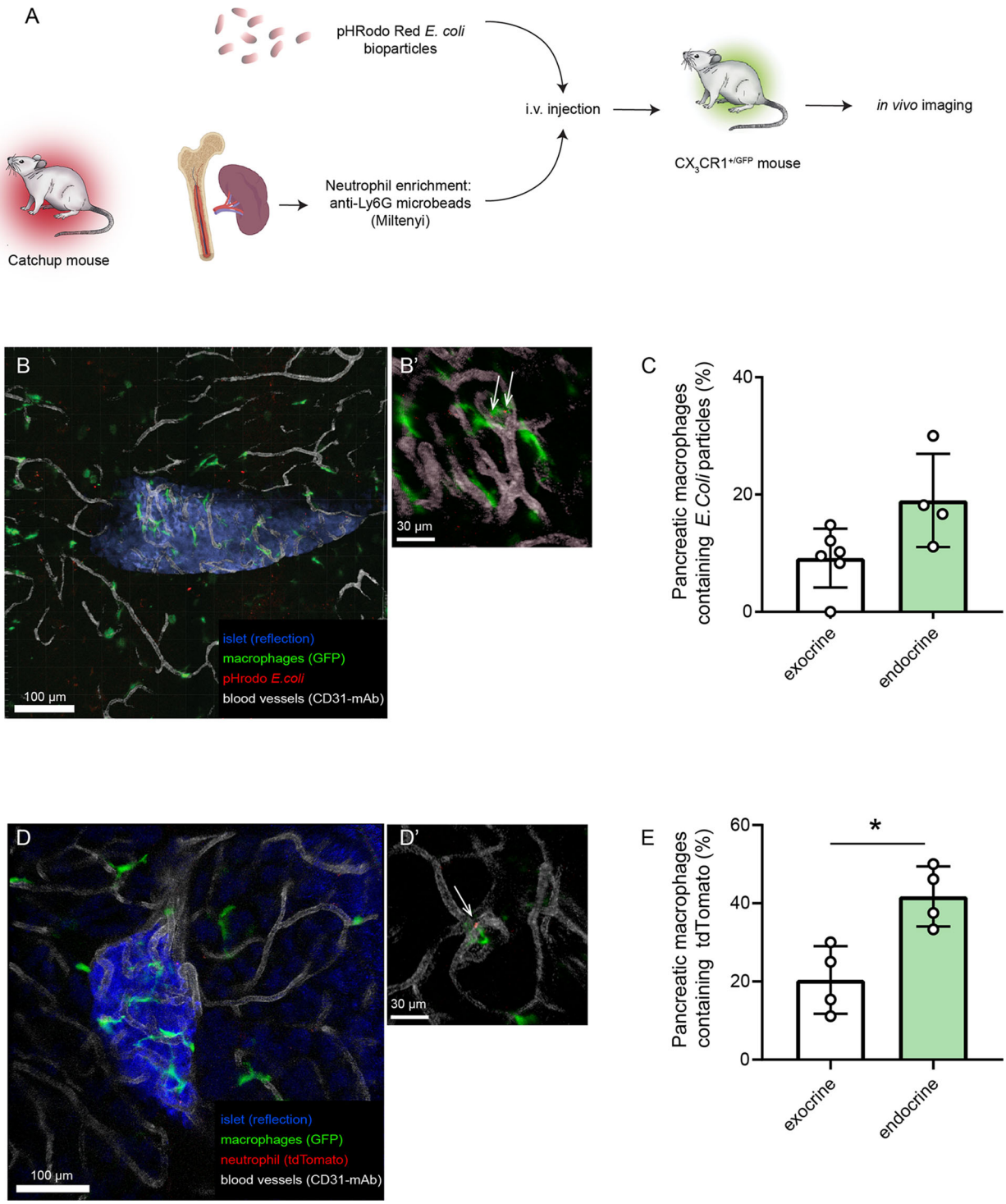

FIGURE 3 | Endocrine-resident macrophages are efficient in phagocytosis and efferocytosis *in vivo*. **(A)** Schematic of workflow for *in vivo* assessment of phagocytosis and efferocytosis in pancreatic macrophages by intravital confocal imaging. **(B)** Confocal z-projection of an intravital recording showing an islet (blue), blood vessels (white), macrophages (green), and pHrodo *E.coli* bioparticles (red). **(B')** Closeup showing pHrodo signals within macrophages (arrows). **(C)** Quantification of phagocytosis in pancreatic macrophages (n=4, Mann-Whitney U test). **(D)** Confocal z-projection of an intravital recording showing an islet (blue), blood vessels (white), macrophages (green), and tdTomato-red particles of engulfed neutrophils (red). **(D')** Closeup showing tdTomato signal within a macrophage (arrow). **(E)** Quantification of efferocytosis in pancreatic macrophages (n=4, *P < 0.05, Mann-Whitney U test).

two groups (1:21 ± 11 in the exocrine fraction *vs.* 1:42 ± 15 in the endocrine fraction).

Whether the macrophages from the two different compartments responded in different ways to *E.coli* particles or dying neutrophils was assessed by measuring cytokine release in supernatants from the phagocytosis and efferocytosis assays after 4h of incubation. Using a multiplexed assay, we found that of the detectible cytokines, the phagocytic response to bacterial particles elicited a much greater release of IL-1β, IL-6, and TNF-α than the efferocytic response to dead cells (**Figures 2I–L**). During phagocytosis, secretion of these cytokines were also higher in macrophages from the endocrine compartment than the exocrine compartment, likely reflecting their initial proinflammatory phenotype. The secretion of IL-2 was similar across the different stimuli and compartments (**Figure 2J**).

In Vivo Phagocytosis and Efferocytosis by Pancreatic Macrophages

As the microenvironment in the pancreas likely is driving the polarization of the tissue-resident macrophages, *in vitro* assessment of their function may therefore introduce phenotype shifts. In order to have an *in vivo* comparison to our *in vitro* data on pancreatic macrophage phagocytosis, we evaluated the phagocytic and efferocytic capacities of pancreatic macrophages by intravital imaging. Either pHrodo *E.coli* bioparticles or neutrophils isolated from Catchup mice were intravenously injected into different groups of $CX_3CR1^{+/Gfp}$ mice (green-fluorescent macrophages, **Figure 3A**). After 4 and 24 hours, respectively, intravital confocal imaging of the pancreas was performed, and macrophages which had engulfed bacterial particles or $tdTomato^+$ neutrophils could be observed having an intracellular red-fluorescent signal (**Figures 3B, C**). Quantification of these data using image analysis masking functions showed that also *in vivo*, endocrine-resident macrophages showed a tendency (P=0.12) to follow the same pattern seen in the *in vitro* assays regarding phagocytosis with a slightly higher fraction of endocrine-resident macrophages containing engulfed bacterial particles (**Figure 3D**). For efferocytosis of dying neutrophils, endocrine-resident macrophages had significantly more intracellular tdTomato residues than macrophages residing in the exocrine tissue (**Figure 3E**). Even though total percentages were lower than in the *in vitro* assays, these results show that the level of expression of MMR does not predict the phagocytic capacity of subsets of pancreatic macrophages.

DISCUSSION

In reaching for a better understanding of the underlying mechanisms that congregate to the onset of T1D, knowing the functions of resident immune cells in the pancreas is crucial. Macrophages, a cell type with a multitude of homeostatic and pathophysiological functions, make up a major part of the resident immune cells in mouse islets of Langerhans (2), and is present also in human islets where the leukocyte composition is somewhat more diverse (18, 19). The microenvironment in mouse islets cause resident macrophages here to take on an inflammatory phenotype whereas macrophages in the surrounding exocrine tissue are mostly of an anti-inflammatory phenotype. Previous literature had identified inflammatory macrophages to be less efficient in clearing tissue debris (9), and we thus became interested in investigating some homeostatic functions of endocrine-resident macrophages. We found that despite lacking the MMR receptor – previously shown to be the primary marker correlating to efficient phagocytosis (9) – endocrine-resident macrophages were very efficient in phagocyting bacterial particles and clearing apoptotic cells by efferocytosis both *in vitro* and *in vivo*.

In their role as a resident, antigen presenting cell in the islets of Langerhans, macrophages have been associated with the initial steps in triggering an autoimmune attack against the beta-cells. Since the primary identified susceptibility loci for T1D are in the class II human leukocyte antigen (HLA) genes in the major histocompatibility complex (MHC-II) (20), the antigen presentation capability of these cells and their proximity to the beta cells have led to hypotheses regarding their ability to induce an antigen-directed response against e.g. released insulin granule contents (21). Endocrine-resident macrophages have also been found to act as regulators in limiting access to the islet parenchyma to T cells. By intravital imaging, T cell intravascular arrest and extravasation was observed in close proximity to endocrine-resident macrophages in a chemokine-dependent manner, and the depletion of macrophages limited this extravasation (6). Macrophage depletion has also been found to protect from the onset of T1D in the NOD and LCMV-RIP-GP mouse models (22, 23). Taken together, data from mouse models of T1D point to a pivotal role of endocrine-resident macrophages in the initial steps leading up to antigen-dependent T cell targeting of beta-cells.

The past few decades have seen great developments in the detailed understanding of macrophages in different organs and different situations; in homeostasis, in tissue restoration, and in pathophysiology. From the early discovery that macrophages are a population of polarized cells; classically activated/inflammatory/M1, and alternatively activated/anti-inflammatory/M2 (24), a wide range of subsets have been identified on that spectrum. Most tissue-resident macrophages display an M2 phenotype (25, 26), and thus the finding that endocrine-resident macrophages are distinctly polarized into an M1 phenotype, and very different from tissue-resident macrophages in the exocrine pancreas was very surprising (2). The functional relevance of this dichotomy is however still unclear. A recent paper has however provided detailed data on the development of the inflammatory environment in pancreatic islets of the NOD mouse through RNA sequencing of isolated immune cells exposing activation programs in the macrophage population through the disease course (27).

An intriguing finding from a study where the phagocytic ability of tissue-resident macrophages across a range of organs in the mouse body was that the level of expression of the mannose receptor (MMR) was the factor that correlated most

positively to efferocytosis of apoptotic endogenous cells (9). In our own observation and those by others (2), we have found that endocrine-resident macrophages do not express MMR. This does also not seem to be the case for macrophages in the human pancreas as seen sections from three donated human pancreata, where the density of MMR^+ macrophages was higher in exocrine tissue than in islets. This poses the question whether the endocrine-resident macrophages have a functional impairment in phagocytosis and efferocytosis, which could in turn have implications to islet homeostasis. Endocrine-resident macrophages have previously been found to take up beta cell fragments in non-diabetic, steady-state conditions, but were not compared to resident macrophages in other tissues (28).

We thus set out to investigate phagocytosis and efferocytosis in pancreatic macrophages. We found no major differences in the level of expression of a range of phagocytosis-related genes between exocrine (high expression of MMR) and endocrine (no expression of MMR) macrophages. In the functional assessment of their phagocytic abilities, we used commercially available *E.coli* particles which will turn brightly fluorescent once internalized in phagosomes (29). Following incubation of macrophages isolated from the mouse pancreas with bacterial particles, we used imaging flow cytometry as a readout to make sure that the signal from the *E.coli* had indeed been internalized in the macrophages and was not only adhering to the outer cell membrane. A similar procedure was performed using red-fluorescent apoptotic neutrophils for assessing efferocytosis. In both of these assays, endocrine-resident macrophages were more efficient in engulfing bacteria or dying cells. To the background of the paper stating MMR expression to correlate to phagocytic efficiency, this was somewhat surprising. We did however also find that cultured macrophages derived from mouse bone marrow and pushed into M1 or M2 states that the inflammatory M1 macrophages were more efficient in phagocytosis. The different activation states of endocrine- and exocrine-resident macrophages was reflected in their secretion of cytokines in response to bacterial particles. Endocrine-resident macrophages secreted significantly more IL-6 and TNF-α than exocrine-resident macrophages. Interestingly, the response to efferocytosis of dead cells induced a much lower secretion of cytokines than the phagocytic response to bacteria. Efferocytosis is a homeostatic process which is constantly ongoing physiologically, and should thus not lead to an exaggerated inflammatory response (8).

Since the experiments above are all performed *in vitro* on isolated cells, there is a possibility that a phenotype shift may occur during the short period of the assay and thereby skewing results. We therefore used our intravital imaging model (14, 15) to investigate efferocytosis and phagocytosis *in vivo*. Following intravenous injection of bacterial particles or neutrophils we could identify macrophages in the pancreas that had engulfed red-fluorescent material. Comparing exocrine-resident macrophages to endocrine-resident macrophages, we found similar differences in efferocytosis and phagocytosis compared to the *in vitro* results. We conclude from this that despite the lack of MMR expression in endocrine-resident macrophages, there does not seem to be any impairment in functional phagocytosis by these cells. Instead, an increased level of phagocytosis may influence the phenotype of a macrophage (9), and might thus be one of the cues from the microenvironment skewing endocrine-resident macrophages into an inflammatory subset. Since the endocrine part of the pancreas is more densely vascularized and receives 10-15% of the total pancreatic blood flow despite only consisting about 1-2% of its mass (30, 31), there is a possibility that deposition of *E.coli* bioparticles and dying neutrophils would be favored here. However, we used a significant time-period for (4 h post injection for bacterial particles and 24 h for neutrophils post injection) that would likely allow for equilibration across tissues.

With this study we sought to investigate some homeostatic and immune-defensive properties of endocrine-resident macrophages in healthy mice. Some aspects in the phenotype of endocrine-resident macrophages led us to hypothesize an impaired clearance of endogenous debris and bacteria. However, we found that endocrine-resident macrophages were more efficient in efferocytosis and phagocytosis, both *in vitro* and *in vivo*, than exocrine-resident macrophages. This difference in abilities may be one of the factors polarizing endocrine-resident macrophages to an inflammatory state since phagocytosis has been found to imprint macrophage heterogeneity. It remains unclear if this difference has any implications in the development of islet dysfunction or autoimmunity.

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 use of human biobanked tissue in this study was approved by the Regional Ethics Board in Uppsala, Sweden. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Uppsala Region Laboratory Animal Ethics Board.

AUTHOR CONTRIBUTIONS

KP performed phagocytosis and efferocytosis experiments, flow cytometry, bioinformatics, statistical analysis and wrote the paper. NW performed cell isolation and qPCR. KM performed immunofluorescence staining and imaging. MK performed qPCR. RL performed flow cytometry analyses. GC conceptualized the study, performed intravital imaging experiments, statistical analysis, provided overall supervision and wrote the paper. 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/fendo.2021.606175/full#supplementary-material

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