IMMUNOPATHOLOGY OF TYPE 1 DIABETES

EDITED BY: F. Susan Wong, Li Wen, Jon D. Piganelli, Sarah J. Richardson, E. Allison Green and Anne Cooke

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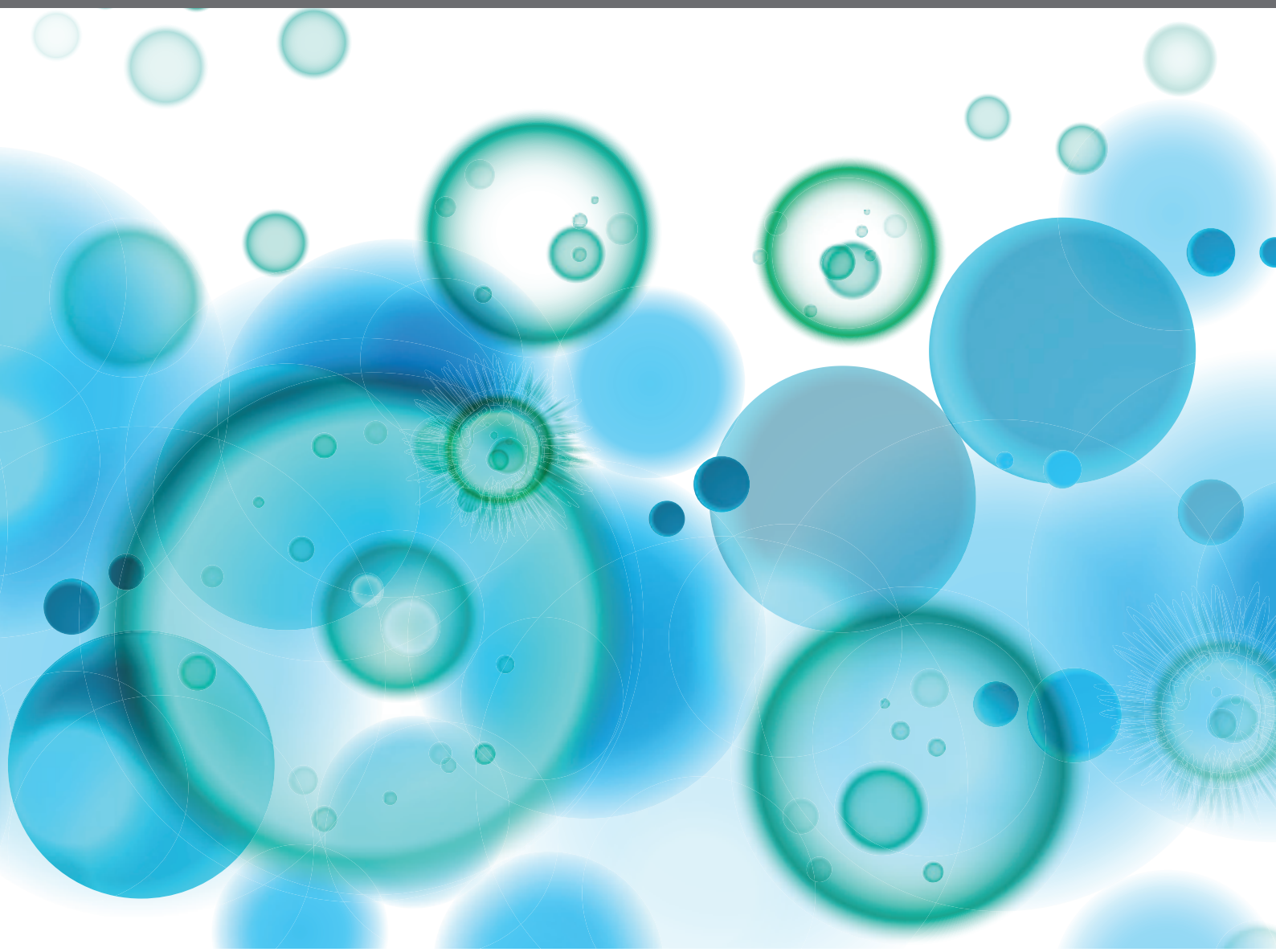

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IMMUNOPATHOLOGY OF TYPE 1 DIABETES

Topic Editors:
F. Susan Wong, Cardiff University, United Kingdom
Li Wen, Yale University, United States
Jon D. Piganelli, University of Pittsburgh, United States
Sarah J. Richardson, University of Exeter, United Kingdom
E. Allison Green, University of York, United Kingdom
Anne Cooke, University of Cambridge, United Kingdom

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EDITORIAL
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Editorial: Immunopathology of Type 1 Diabetes

E. Allison Green[1], *Anne C. Cooke*[2], *Jon D. Piganelli*[3], *Sarah J. Richardson*[4], *Li Wen*[5] *and F. Susan Wong*[6*]

[1] Hull York Medical School, University of York, York, United Kingdom, [2] Department of Pathology, University of Cambridge, Cambridge, United Kingdom, [3] Department of Pediatric Surgery, Rangos Research Center, UPMC Children's Hospital of Pittsburgh, University of Pittsburgh, Pittsburgh, PA, United States, [4] Islet Biology Group, Exeter Centre for Excellence in Diabetes (EXCEED), Institute of Biomedical and Clinical Sciences (IBCS), University of Exeter, Exeter, United Kingdom, [5] Section of Endocrinology, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT, United States, [6] Division of Infection and Immunity, Cardiff University School of Medicine, Cardiff University, Cardiff, United Kingdom

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Editorial on the Research Topic

Immunopathology of Type 1 Diabetes

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Edited by:
Lucienne Chatenoud, Université Paris Descartes, France

Reviewed by:
Jayne Danska, University of Toronto, Canada

***Correspondence:**
F. Susan Wong
wongfs@cardiff.ac.uk

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Our understanding of the islet-immune interface in autoimmune diabetes has exploded with the vast array of knowledge coming from recent works. Historically we know that genetic susceptibility and environmental influences trigger, or perpetuate gradual autoimmune destruction and damage to the insulin-producing islet beta (β) cells. However, the events leading to the breakdown in tolerance towards islet β cells, and the ensuing pro-inflammatory cytokine laden environment that perpetuates T cell-mediated β cell destruction, remain elusive.

This timely Research Topic comprises a compendium of 5 original research articles and 15 reviews focusing on recent advances in the immunology of type 1 diabetes (T1D) that build on earlier observations and provide new knowledge. These articles center on environmental drivers of β cell destruction and use new models and technologies to interrogate factors influencing the breakdown of self-tolerance, enhancing our understanding of T1D immunopathology.

In a thorough review, Quinn et al. describe data from past and present clinical trials that have assessed environmental elements associated with progression to T1D. The authors examined the likelihood of true causality of many favored initiators (beyond the well-characterized genetic predisposition, primarily at the HLA loci) activating self-reactive T cells that target β cells, in this heterogeneous condition. Environmental determinants, including enterovirus infection, rapid weight gain in early life, and the microbiome, correlated highly with T1D incidence, suggesting a 'threshold hypothesis' where genetic and environmental factors interact to promote T1D over time. The pancreatropic viruses, particularly the T1D-associated coxsackievirus B (CVB), are prominent contenders. In support of the viral induction hypothesis, Morse and Horwitz provide a compelling review describing how the antiviral response can modulate the microbiome, causing dysbiosis, and diabetes onset. This work stresses the importance of communication between the intestinal microbiota and the local immune population in dictating the outcome of the interaction. This interaction is also influenced by other predisposing factors, such as genetic predisposition, viral responses leading to dysbiosis and the background state of the host immune system. Further, Lincez et al. used elegant animal models that express altered expression of two key viral sensors-melanoma differentiation-associated protein 5 (MDA5) and toll-like receptor-3 (TLR-3). They showed that alterations in sensing of the same virus (CVB) by MDA5 and TLR3 led to unique IFN-α and IFN-β

signatures, which profoundly affected disease outcomes. Specifically, infection with islet β cell-tropic CVB4, under reduced MDA5 signaling, protected against diabetes, whereas reduced TLR3 function did not influence diabetes susceptibility. Thus, the pressure of a diabetogenic β cell-tropic virus, in conjunction with a genetic predisposition to autoimmunity, creates a perfect storm, providing the necessary inflammatory signature leading to disease onset. Interestingly, Zipris presents an alternative concept linking viruses to T1D, involving visceral adipose tissue. Zipris discusses how the Kilham Rat Virus, which causes diabetes in BioBreeding and LEW1.WR1 rats, also induces inflammation in visceral adipose tissue (VAT). Infection is associated with macrophage recruitment, pro-inflammatory cytokine and chemokine upregulation, as well as endoplasmic reticulum (ER) and oxidative stress responses. Together, these have a negative impact on insulin signaling, and may link to autoimmune diabetes progression.

However, β cells are more than innocent bystanders in the progression to diabetes. Toren et al. provide evidence that the ER in β cells is under tremendous strain to maintain euglycemia. When this metabolic demand is coupled with the highly vascularized setting needed for insulin uptake into the bloodstream, pathology may result. β cells exposed to noxious agents including pro-inflammatory cytokines and chemokines, and immune cells that extravasate into the islet, will respond to pathogen-associated molecular patterns (PAMPs) by releasing danger-associated molecular patterns (DAMPs). These, in turn, mobilize innate and adaptive immune cells to the target tissue, triggering β cell death. Thus, a focus on the role of the β cell as an active participant, rather than an unfortunate victim in T1D progression, may allow us to identify new targets in T1D. In this context, Ding et al. focus on the role of alpha-protein kinase 1 (ALPK1), a newly identified cytosolic pathogen-recognition receptor (PRR) specific for ADP-β-D-manno-heptose (ADP-heptose) associated with β cells, and how it predisposes β cells to cytokine-mediated apoptosis *via* upregulation of the TNF-α signaling pathway. Using the Min6 murine β cell line, mechanistic investigations showed that ALPK1 activation was sufficient to induce the expression of TNF-α and Fas after cytokine stimulation. It will be interesting to determine whether this translates to primary beta cells, establishing the *in vivo* significance of these findings. The PAMP ADP-heptose may also arise in the islet *via* the dysbiosis described by Morse and Horwitz, leading to the exacerbation of cytokine signaling seen in β cells.

The cells and molecules of innate immunity involved in the early T1D responses, particularly the inflammasome, have been discussed by Pearson et al. They highlight altered gut microbial composition and associated influences on inflammasome activity and T1D development. As modulation of inflammasomes has had some therapeutic success in other autoimmune diseases, a similar approach may have clinical benefits for T1D. Focusing also on innate immunity, Klocperk et al., studying children with T1D and first-degree relatives, showed that neutrophilia and neutrophil products including neutrophil extracellular traps (NETs), myeloperoxidase (MPO), neutrophil elastase (NE), proteinase 3 (PR3) and LL37, were evident before diabetes onset, but reduced over time. In addition, Huang et al. found that IL-10 deficiency in diabetogenic T cell receptor (BDC2.5) transgenic mice promoted the expansion of bone marrow and peripheral neutrophils. IL-10 deficiency enhanced neutrophil expression of IFNγ and IL-1β compared with IL-10-sufficient controls. IL-10 plays an important regulatory role systemically and in mucosal immunity, and IL-10-deficient BDC2.5 NOD mice had altered gut microbiota, which in turn modulated systemic neutrophil homeostasis. The innate lymphoid cells (ILC), another group of innate cells, classified by their cytokines and transcription factor profiles, may be considered to be the innate counterparts of the T helper subsets. Stojanovic et al. review gut-associated lymphoid tissue residing ILC3, which secrete IL-17 and GMCSF, in T1D development and therapeutic targeting. Finally, Gardner and Fraker comprehensively review the role of innate NK cells in immunopathogenesis of T1D development. β cells express NK ligand(s), which may contribute to their direct killing by NK cells. It is noteworthy that specific NK cell markers, such as NKG2D, are also expressed on $CD8^+$ T cells, especially human $CD8^+$ T cells, which are central adaptive immune cell players in β-cell destruction.

Adaptive immunity, focused on both effector and regulatory cells (Tregs), has long been implicated in the pathogenesis of T1D. Foxp3-expressing Tregs regulate autoimmune disease; humans with a mutated *FOXP3* gene develop the multiorgan autoimmune syndrome. Here, Watts et al. utilized DEREG (Depletion of REGulatory T cells) mice where a bacterial artificial chromosome (BAC)-encoded Foxp3 promoter controls fluorescent diphtheria toxin (DTR-eGFP) fusion protein expression. Using diphtheria toxin-mediated transient Treg depletion, in NOD mice, they found that T1D was induced only in the mice where pancreatic infiltration was already present. Furthermore, Treg depletion exacerbated a Th1 type response in the pancreas and associated lymph nodes, highlighting the importance of CD8+ T cells as effectors of autoimmune-mediated beta cell destruction.

Advances in techniques that can investigate the transcriptome, epigenetic and proteomic profile at the single-cell level are revolutionizing our understanding of the immune system in health and disease. In Bode et al., the history of approaches taken to assess global immune cell gene changes and the emergence of single-cell studies that assess the heterogeneity of transcriptome, epigenetics and proteasome in immune cells is reviewed. Using cancer immunology as exemplars, single-cell approaches are adaptable to investigate facets of T1D. Hanna et al. extend this theme, reviewing single-cell RNAseq studies from human T1D. RNAseq analysis of human immune cells from various tissues has facilitated novel discoveries of the cytokine profiles and exclusive gene expression that may be predictive of T1D progression. However, the use of scRNAseq is not without challenges, requiring robust computational approaches to facilitate biomarker discovery. There is a clear need for biomarkers in T1D to track progression and monitor the efficacy of therapeutic interventions, particularly T cell-based biomarkers, given that many current immunotherapies for T1D target T cells.

Nakayama and Michels review the current knowledge and potential for using the T cell receptor (TCR) as a biomarker in T1D. They focused on pancreatic infiltrating T cells and revealed proinsulin or insulin reactivity. They suggested that these cells were more reactive or had higher affinity TCRs than comparable cells in the peripheral blood. Documenting the challenges of current technical approaches to identify unique clusters of TCRs associated with T1D, newer approaches using high throughput sequencing of tens of millions of TCR clonotypes are proving more insightful. Nevertheless, the robustness of using TCR clonotypes as biomarkers will need TCR datasets from many individuals with and without T1D to elicit the best performance by machine learning and clustering algorithms.

B cells also play an important role in the pathogenesis of T1D, beyond their function as antibody-producing cells. In a review by Greaves et al., a detailed discussion of the development, phenotype and function of enigmatic thymic B cells in health and T1D is provided. Interestingly, the emergence of thymic ectopic germinal centers is a commonality in lupus, myasthenia gravis and T1D, all diseases where thymic B cells may have a proposed pathogenic role. Studying the human thymus is difficult, and the authors discuss the potential of computational modeling to evaluate key pathways by which thymic B cells may perturb central T cell tolerance. Like T cell responses, there is heterogeneity in B cell responses. Boldison and Wong have reviewed the role in T1D of distinct subsets of regulatory B cells in mice and man, highlighting their cytokine profiles, costimulatory requirements, and interactions with the innate immune system to elicit their function. In human T1D, the definitive role for regulatory B cells is controversial. Future studies on heterogeneity of regulatory B cell repertoires at defined stages of T1D and dissection of their cross-talk with other immune cells will help resolve this debate.

To what are the T cells responding? Various antigens, suggested by antibody reactivities, have been identified as targets for the autoreactive T cells in diabetes. Whilst for both humans and mice, peptides of proinsulin are targets for both $CD8^+$ and $CD4^+$ T cells; however, there are still some intriguing biological questions. Jhala et al., building on a previous observation that proinsulin-1 deficient mice are protected from autoimmune diabetes, have further investigated autoimmune responses to proinsulin-1, using a mouse model with tetracycline-regulated expression of proinsulin-1 in antigen-presenting cells (TIP-1 mice). They found that the mice had reduced proinsulin-1-specific T cells, reduced insulitis and diabetes, and the proinsulin-1 specific cells were less able to transfer diabetes to an immunodeficient recipient, all of which indicate the induction of immune tolerance. Post-translational modifications of self-proteins, such as the post-translational conversion of arginine to citrulline residues by peptidylarginine deiminase enzymes can also produce novel T cell targets in T1D. Such modified antigens would not be encountered in the thymus by developing T cells, and thus central tolerance to them would not occur. The mini-review by Reed and Kappler documents how epitopes of unique chimeric antigens are generated post-translationally following β cell-granule fusion with lysosomes, in a process called crinophagy. Secreted exosomes transport the chimeric peptides as cargo to draining lymph nodes where $CD4^+$T cell stimulation of diabetogenic T cells could occur. However, the key activation signal for these strongly stimulatory peptides is not clear and remains an important further question for consideration.

Finally, Armitage et al. provide an in-depth review on the role and function of the protein tyrosine phosphatase, non-receptor type 22 (PTPN22), a negative regulator of T and B cell receptor signaling. This review describes an expanded role for this phosphatase in controlling many cells in the immune system. It could explain why dysregulation of this molecule can profoundly impact normal immune function. The authors also describe a set of single nucleotide polymorphisms (SNPs) at the *PTPN22* locus, leading to immune defects that precipitate the loss of self-tolerance and progression to autoimmune diabetes. Since the SNP in PTPN22 (rs2476601) is associated with TCR and BCR signaling and other adaptive and innate immune cell processes, it is of major interest to further define the downstream effects of suboptimal control of its signaling in people living with T1D.

Overall, the studies outlined in this Research Topic highlight the heterogeneity of factors that may lead to the development of T1D. There is a critical need for a deeper understanding of these factors if we are to develop new immunotherapeutic strategies, which may need to be multifaceted to be most effective for both prevention, and as a therapeutic in those in whom diabetes has already developed.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript and edited the final version.

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REVIEW
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Influence of *PTPN22* Allotypes on Innate and Adaptive Immune Function in Health and Disease

Lucas H. Armitage[1], *Mark A. Wallet*[1,2] *and Clayton E. Mathews*[1]*

[1] Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL, United States,
[2] Immuno-Oncology at Century Therapeutics, LLC, Philadelphia, PA, United States

Protein tyrosine phosphatase, non-receptor type 22 (PTPN22) regulates a panoply of leukocyte signaling pathways. A single nucleotide polymorphism (SNP) in *PTPN22*, *rs2476601*, is associated with increased risk of Type 1 Diabetes (T1D) and other autoimmune diseases. Over the past decade PTPN22 has been studied intensely in T cell receptor (TCR) and B cell receptor (BCR) signaling. However, the effect of the minor allele on PTPN22 function in TCR signaling is controversial with some reports concluding it has enhanced function and blunts TCR signaling and others reporting it has reduced function and increases TCR signaling. More recently, the core function of PTPN22 as well as functional derangements imparted by the autoimmunity-associated variant allele of PTPN22 have been examined in monocytes, macrophages, dendritic cells, and neutrophils. In this review we will discuss the known functions of PTPN22 in human cells, and we will elaborate on how autoimmunity-associated variants influence these functions across the panoply of immune cells that express PTPN22. Further, we consider currently unresolved questions that require clarification on the role of PTPN22 in immune cell function.

Keywords: PTPN22, PTPN22 620Arg > Trp, type 1 diabetes, cell signaling, rs2476601, autoimmunity, leukocytes

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Edited by:
F. Susan Wong,
Cardiff University, United Kingdom

Reviewed by:
Bergithe Eikeland Oftedal,
University of Bergen, Norway
Maikel Peppelenbosch,
Erasmus Medical Center, Netherlands

***Correspondence:**
Clayton E. Mathews
clayton.mathews@pathology.ufl.edu

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INTRODUCTION

Almost 1.6 million Americans have Type 1 Diabetes (T1D), an autoimmune disease that results in destruction of the insulin producing β cells in the pancreas and eventually requires exogenous insulin (1). T1D shows familial clustering and concordance rates between monozygotic twins is over 50% indicating that T1D has a strong genetic component (2, 3). It is estimated that up to 88% of the

Abbreviations: ABCs, Age-associated B cells; APCs, Antigen presenting cells; aT_{reg}, Activated T_{reg}; BCR, B cell Receptor; BMDC, Bone marrow-derived dendritic cell; BMMΦ, Bone marrow-derived macrophage; BND cells, Naïve IgD+ B cells; CLL, Chronic B lymphocytic leukemia; CSK, C-src tyrosine kinase; DCs, Dendritic cells; fMLF, N-formyl-Methionine-Leucine-Phenylalanine; fMLP, N-formyl-Methionine-Leucine-Phenylalanine; Hep-2 cells, Human epithelial type 2 cells; HLA, Human Leukocyte Antigen; HSCs, CD34+ hematopoietic stem cells; Idd intervals, Insulin-depending diabetes intervals; IFNγR, Interferon gamma receptor; iT_{reg}, Induced T_{reg}; LAD, Leukocyte adhesion deficiency; MDM, Monocyte-derived macrophage; MDP, Muramyldipeptide; MHC, Major Histocompatibility Complex; moDC, Monocyte-derived dendritic cell; MSU, Monosodium urate; NETosis, Neutrophil extracellular trap formation; NOD2, Nucleotide-binding oligomerization domain-containing protein; NTC siRNA, Non-targeting control siRNA; PBMC, Peripheral blood mononuclear cells; pDC, Plasmacytoid dendritic cell; PRR, Pattern recognition receptor; PTPN22, Protein tyrosine phosphatase non-receptor type 22; RA, Rheumatoid arthritis; ROS, Reactive oxygen species; siRNA, Small interfering RNA; SLE, Systemic lupus erythematosus; SNP, Single nucleotide polymorphism; T1D, Type 1 Diabetes; T1-IFN, Type 1 interferon; TCR, T cell receptor; TLR, Toll-like receptor; T_{reg}, T regulatory cell; upLPS, Ultrapure LPS.

phenotypic variance is due to genetic factors such as predisposing or protective human leukocyte antigen (HLA) haplotypes and SNP-tagged variants (4–6). Of the genetic component of T1D risk, the HLA region, encoding the major histocompatibility complex (MHC) proteins, accounts for approximately 50% of heritable risk (7). The MHC class I (MHC-I) proteins are expressed on all nucleated cells and present antigenic peptides to $CD8^+$ T cells while the MHC class II (MHC-II) proteins are primarily expressed on APC subsets and present antigen only to $CD4^+$ T cells. The HLA Class II genes, encoding MHC-II, are the major contributing factor of HLA to risk with the DR3 (*DRB1*03:01*), DR4 (*DRB1*04:01/02/04/05/08*), DQ8 (*DQA1*03:01-DQB1*03:02/04*), and DQ2 (*DQA1*05:01-DQB1*02:01*) haplotypes conferring the greatest risk (7, 8). Indeed, the DR3/4 diplotype confers the greatest risk for T1D development (9, 10). These haplotypes increase risk in a synergistic manner and current research shows they have augmented ability to present T1D autoantigens to T cells, possibly due to alterations in the critical amino acids in the peptide binding pocket involved in which peptides are presented (10–12).

Although the HLA region contributes the bulk of genetic risk for T1D, there have been over 60 non-HLA genetic loci identified that have variants associated with enhanced or reduced risk of T1D (4, 13–22). Of these non-HLA loci, a non-synonymous SNP in *PTPN22* has one of the highest reported odds ratios, ~2, and has been repeatedly confirmed across multiple studies and populations (4, 13, 15, 23–25). Protein tyrosine phosphatase, non-receptor type 22 (PTPN22) is a negative regulator of T cell receptor (TCR) and B cell receptor (BCR) signaling (26, 27). The diabetes-associated SNP in *PTPN22* (*rs2476601*) affects TCR and BCR signaling as well as other adaptive and innate immune cell processes (27–39). The following sections will elaborate the known functions of PTPN22 and its autoimmune-linked/diabetogenic, missense SNP in human cells and how this might contribute to the pathogenesis of T1D. While the primary focus of this review is on human biology, we will emphasize specific areas of murine *Ptpn22* research, where relevant, to highlight key similarities and differences between species.

GENETIC VARIATION IN *PTPN22*

Protein tyrosine phosphatase, non-receptor type 22 (PTPN22) is expressed in leukocytes and is well-known as a negative regulator of TCR and BCR signaling (26, 27). In non-activated T cells PTPN22 directly complexes with C-src tyrosine kinase (Csk) (32, 40, 41). This interaction is enhanced by phosphorylation of PTPN22 on Ser^{751} by PKCa. Further, phosphorylation of this residue increases the half-life of PTPN22 by protecting the enzyme from K48-linked ubiquitination and preventing recruitment of PTPN22 to the plasma membrane (42). During leukocyte activation PTPN22 is recruited to the plasma membrane to limit proximal immune cell receptor signaling. Here PTPN22 interacts with and dephosphorylates Grb2 (43), VCP (44), Vav (32, 44), Zap70 (32, 44), Lck (26, 32, 44), TCRζ (44), CD3ϵ (44), c-CBL (45), EB1 (46), and the p85 subunit of PI3K (47) to downregulate NFAT and reduce IL-2 production and secretion. However, PTPN22 also acts a regulator of other signaling networks (i.e., interferon γ receptor signaling, LFA-1 signaling, and TLR4 signaling) in monocytes, macrophages, dendritic cells, and neutrophils (29, 32, 35). There are multiple non-synonymous SNPs in PTPN22 associated with increased risk or decreased risk of autoimmune diseases (**Table 1**). The minor allele at *rs56048322*, $PTPN22^{K750N}$, influences PTPN22 splicing and appears to cause $CD4^+$ T cell hyporesponsiveness that increases risk for T1D (48). The minor allele at *rs33996649*, $PTPN22^{R263Q}$, is a loss-of-function variant with diminished phosphatase capacity that reduces the risk of both SLE (49) and RA (50) (**Table 1**). Here we will examine *rs2476601*. The minor allele has a thymine substituted for a cytosine at nucleotide 1858, $PTPN22^{C1858T}$, and encodes a tryptophan instead of an arginine at amino acid 620, $PTPN22^{R620W}$ (**Table 1**). It was first linked to T1D by Bottini et al. in 2004 (51) and the association between *rs2476601* and T1D was quickly replicated (52). This SNP has also been associated with increased risk for multiple autoimmune diseases including rheumatoid arthritis (RA) (28), systemic lupus erythematosus (SLE) (53), Graves' disease (52, 54), myasthenia gravis (55), primary Sjogren's syndrome (56), generalized vitiligo (57), Addison's disease (58), and alopecia areata (59) strongly suggesting PTPN22 regulates immunity.

The SNP, *rs2476601*, lies in the proline-rich c-terminal domain of PTPN22 and interrupts some protein-protein interactions (e.g., interactions with CSK, TRAF3, and PAD4) (30, 35, 51). This is well illustrated in a recent review article (60). To determine the function of the common or major allotype of PTPN22, namely $PTPN22^{620R}$, diverse approaches including knock down or overexpression of *PTPN22* in primary human cells or human cell lines and knock down/out of *Ptpn22*, the mouse orthologue of *PTPN22*, in mice and mouse cell lines, have been used. To study the altered function of the minor allotype of PTPN22, $PTPN22^{620W}$, researchers have again utilized many techniques including comparative studies in primary cells from human $PTPN22^{620W}$ donors vs. $PTPN22^{620R}$ donors, overexpression of $PTPN22^{620W}$ vs. $PTPN22^{620R}$ in primary human cells and human cell lines, transgenic expression of human $PTPN22^{620W}$ vs. $PTPN22^{620R}$ in $Ptpn22^{-/-}$ mice, and introduction of a mutation that is analogous to $PTPN22^{620W}$ in the mouse orthologue, PEP^{619W}. Notably, this SNP is also associated with protection from *Mycobacterium tuberculosis*, an infection primarily controlled by T cells and T cell-activated macrophages (61–64). PTPN22 has been described as a negative regulator of multiple stages of danger signal recognition, from the process of T and B cell education, throughout initial detection of microbes, and then T and B cell effector functions. Thus, genetic variation that confers beneficial immunity to a globally-relevant pathogen (*M. tuberculosis*) might lower the threshold for danger signal responses. In murine models of T1D, lack of key macrophage/$CD4^+$ T cell effector molecules (e.g., CD154 and CD40) but not all (e.g., IFNγ and IFNγR) prevents autoimmunity in T1D-prone NOD mice (65–67).

TABLE 1 | Single nucleotide polymorphisms in human *PTPN22*, their analogous mutations in mice, and their disease associations.

SNP	Human (*PTPN22* or PTPN22)		Mouse (*Ptpn22* or PEP)		Effect	Associations
	Major Allotype	Minor Allotype	Major Allotype	Minor Allotype		
rs2476601	$PTPN22^{620R}$	$PTPN22^{620W}$	PEP^{619R}	PEP^{619W}	variable	Increased risk multiple autoimmune diseases
rs56048322	$PTPN22^{750K}$	$PTPN22^{750N}$	–	–	alternative splice variant	increased risk T1D (48)
rs33996649	$PTPN22^{263R}$	$PTPN22^{263Q}$	$PEP^{195D:227C}$	$PEP^{195A:227S}$	loss-of-function	reduced risk SLE (49) and RA (50)

We propose that the T1D-associated risk allotype of PTPN22 permits excessive innate and adaptive immune signaling in response to aseptic and/or septic stress/danger signals, in turn, driving a type IV delayed hypersensitivity response against pancreatic β cell antigens. The end result is insulin deficient diabetes mellitus. Herein we review the findings that support a pan-leukocyte role for PTPN22 in immune regulation. For the purpose of this review, we will examine the known roles for PTPN22 in innate and adaptive leukocyte signaling pathways and functions in humans as well as supporting data from mouse models. Where data is available we will also discuss how the minor allotype of PTPN22, $PTPN22^{620W}$, influences signaling pathways as well as cellular functions and how these alterations may contribute to the development of T1D.

PTPN22 EXPRESSION

PTPN22 is expressed in most types of human leukocytes, including $CD4^+$ T cells, $CD8^+$ T cells, B cells, NK cells, monocytes, macrophages, dendritic cells, and neutrophils. Of these cells, *PTPN22* has the highest expression in activated naïve $CD8^+$ and $CD4^+$ T cells, followed by NK cells and B cells, with lower levels in monocytes (28, 68). While the non-synonymous SNP at *rs2476601* changes the amino acid sequence, the allelic difference does not modify *PTPN22* expression in most lymphocyte subsets. Peripheral blood mononuclear cells (PBMCs) from $PTPN22^{620R/W}$ donors expressed *PTPN22* mRNA equally from both alleles and this did not vary with gender (69). Upon anti-CD3/anti-CD28 stimulation of PBMCs (simulated activation of the TCR/CD3/CD28 complex), *PTPN22* mRNA expression increased and this rise in expression was equally attributed to both alleles (69). Similarly, *PTPN22* expression levels in PMBC-derived DCs and PBMC are the same in $PTPN22^{620R/W}$ and $PTPN22^{620R/R}$ donors (35).

There are, however, exceptions; $PTPN22^{620W/W}$ donors had 9% lower *PTPN22* expression in naïve $CD4^+$ T cells compared to $PTPN22^{620R/R}$ donors but there were no additional differences in *PTPN22* expression in other T cell subsets (47). There is a report showing that $PTPN22^{620W}$ is more susceptible to calpain-1-mediated degradation and that the $PTPN22^{620W}$ protein is less expressed in naïve and memory T cells compared to $PTPN22^{620R}$ (70); yet, this has been disputed by later studies that observed the antibody used to detect PTPN22 had a higher affinity for $PTPN22^{620R}$ versus $PTPN22^{620W}$ (35, 40, 71). *PTPN22* mRNA and protein expression in freshly-differentiated macrophages (so-called M0 or non-polarized macrophages) from $PTPN22^{620R/W}$ and $PTPN22^{620W/W}$ donors was lower than that of $PTPN22^{620R/R}$ donors (38). After M1 polarization of these macrophages (treatment with lipopolysaccharide and IFN-γ to mimic an inflamed septic environment), mRNA and protein expression of *PTPN22* was higher in $PTPN22^{620R/W}$ and $PTPN22^{620W/W}$ donors than $PTPN22^{620R/R}$ donors but there was no difference in M2 polarized macrophages (treatment with IL-4 and IL-13 to generate so-called "alternatively activated macrophages") (38). For macrophages, these findings are suggestive of a relationship between *PTPN22* allotype and *PTPN22* expression in the context of microbial infections wherein type 1 $CD4^+$ T helper response (T_H1) typified by IFN-γ secretion occur – for example, mycobacterial infections. Overall, allelic differences at *rs2476601* have modest effect on the expression of PTPN22 in human cells that might be associated with observed immune phenomena (e.g., altered susceptibility to mycobacterial infections), but many questions remain unanswered and causality is merely speculative until more complex studies can be completed. While *PTPN22* expression is only modestly influenced by allele, the function of *PTPN22* is measurably altered by *rs2476601*.

REGULATION OF T CELL FUNCTION BY PTPN22 ALLOTYPES

The majority of studies focused on *PTPN22* have investigated how the $PTPN22^{620}$ allotypes influence the composition of the T and B cell compartments and intracellular signaling in T cells and B cells. PTPN22 allotypes have minor effects on T cell composition across immune compartments in humans; there are no differences in total T cells, total $CD4^+$ or $CD8^+$ T cells, or $CD4^+$ or $CD8^+$ effector memory T cells when comparing $PTPN22^{620R/W}$ donors to $PTPN22^{620R/R}$ donors (72). Most studies report no differences in most $CD4^+$ T cells subsets (i.e., T_H1, T_H17, T_H1T_H17, T_{FH}) (73). However, $PTPN22^{620W/W}$ donors had slightly-increased $FOXP3^+CD4^+$ regulatory T cells(T_{regs}) (7.94% vs. 6.76%) compared to donors with the common $PTPN22^{620R/R}$ allotype (74, 75). It has been reported that $PTPN22^{620R/W}$ donors have increased memory $CD4^+$ T cells when compared to $PTPN22^{620R/R}$ donors (about 50% vs. 41% respectively) with a concomitant decrease in naïve $CD4^+$ T cells (76). EOMES is a T box transcription factor that drives IFNγ secretion by $CD4^+$ T cells (73). $PTPN22^{620W/W}$ donors exhibited increased $EOMES^+CD4^+$ T cells compared to $PTPN22^{620R/R}$ donors (~7% vs. ~5%) again with an accompanying decrease in naïve $CD4^+$ T cells (73). It is unclear whether *PTPN22* genotype influences naïve $CD4^+$ T cell

frequency (72, 73, 76). Two studies have reported a trend toward decreased naïve $CD4^+$ T cells in $PTPN22^{620W/W}$ donors (73, 76) while a third study reported no difference in naïve $CD4^+$ T cells when examining *PTPN22* genotype (72). The study that reported no difference had a low number of subjects (3 in each group) and no subjects that were homozygous for the minor allele (72). The two studies that have reported a difference included more participants [13 per group (73) or ≥22 per group (76)] and included a group homozygous for the minor allele. Differences in study populations may explain the inconsistencies. A study with a larger cohort of all three genotypes (i.e., $PTPN22^{1858C/C}$, $PTPN22^{1858C/T}$, and $PTPN221858^{T/T}$) may be better powered to address whether *PTPN22* genotype influences naïve $CD4^+$ T cell frequency.

Impact of PTPN22 Allotypes on TCR Signaling

While PTPN22 allotypes have a minor impact on T cell compartment composition, a significant impact on signal transduction in human T cells has been observed. In primary T cells, $PTPN22^{620R}$ is a negative regulator of TCR (26, 28, 43, 77, 78) (**Figure 1A**) and lymphocyte function-associated antigen 1 (LFA-1) (32) signaling (**Figure 2**) while it is a positive regulator of *in vitro* T regulatory cell (T_{reg}) induction (33). In T cells, $PTPN22^{620R}$ has been shown to directly interact with Grb2 (43), VCP (44), Vav (32, 44), Zap70 (32, 44), Lck (26, 32, 44), TCRζ (44), CD3ε (44), c-CBL (45), CSK (32, 40, 41), EB1 (46), and the p85 subunit of PI3K (47). Studies do not agree whether $PTPN22^{620W}$ is a gain-of-function or loss-of-function variant in human TCR signaling but there is compelling evidence for both views (**Figures 1B, C**) (40, 41, 47, 70, 72, 76, 79–81). $PTPN22^{620W}$ is a loss-of-function variant in LFA-1 signaling (**Figure 2**) (32). $PTPN22^{620W}$ has not been studied in the context of T_{reg} induction in humans, however activated T_{regs} (aT_{regs}) from $PTPN22^{620W/W}$ donors have a reduced capacity to inhibit IFNγ secretion from other T cells compared to those from $PTPN22^{620R/R}$ donors (47).

PTPN22 is a known negative regulator of TCR signaling (**Figure 1A**) (82). To investigate the function of PTPN22 in human T cells many studies have utilized the T-cell acute lymphoblastic leukemia cell line, Jurkat (26, 28, 43). This has allowed dissection of the influence of PTPN22 on proximal TCR signaling. In Jurkat T cells, it has been shown that PTPN22 negatively regulates activation of JNK2 (26) and LCK (26), and transcriptional activity driven by NF-κB (28), CD28 response element/NF-IL2B AP-1 (43), NFAT/AP-1 (26), c-fos (26), and c-

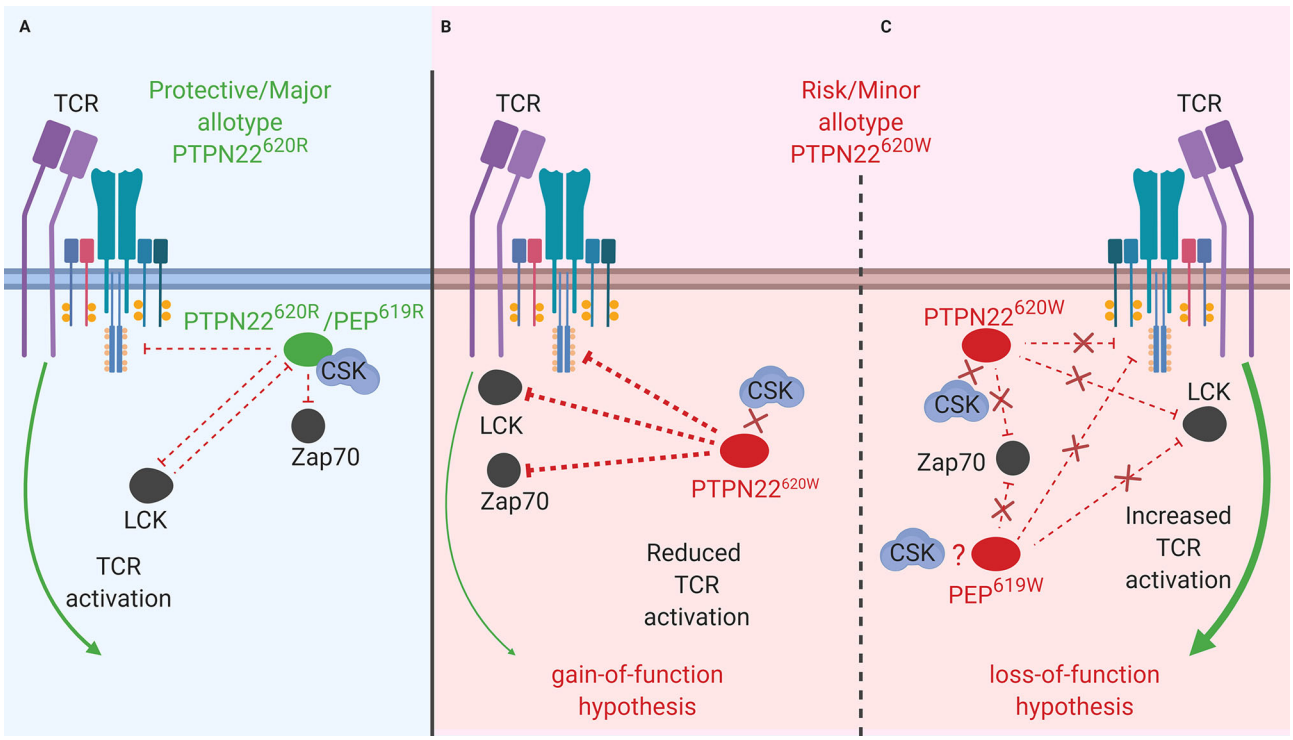

FIGURE 1 | PTPN22 function in T cells. **(A)** $PTPN22^{620R}$ and PEP^{619R} are negative regulators of TCR signaling in T cells where they dephosphorylate/deactivate signaling intermediates and reduce signaling from the TCR to the nucleus. **(B)** The $PTPN22^{620W}$ gain-of-function hypothesis. In this scenario, $PTPN22^{620W}$ is more active and dephosphorylates signaling intermediates at an increased rate compared to $PTPN22^{620R}$. This blunts TCR signaling compared to $PTPN22^{620R}$ and reduces T cell response. **(C)** The $PTPN22^{620W}$ and PEP^{619W} loss-of-function hypothesis. In this scenario, $PTPN22^{620W}/PEP^{619W}$ are less efficient at dephosphorylating TCR signaling intermediates compared to $PTPN22^{620R}/PEP^{619R}$. This allows more signal from the TCR to reach the nucleus and increases T cell response to TCR stimulation.

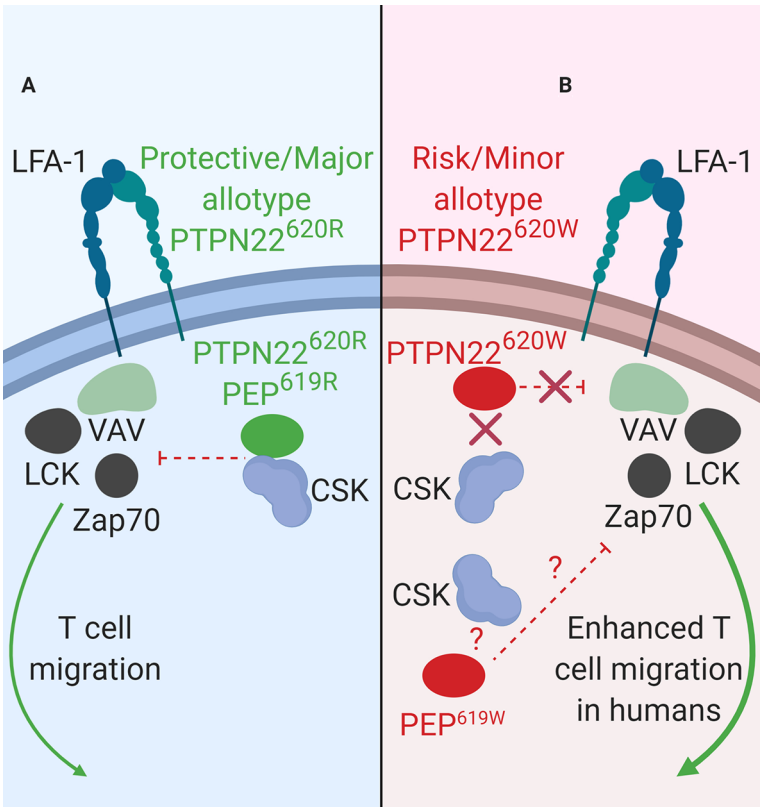

FIGURE 2 | PTPN22 function in LFA-1 signaling. **(A)** PTPN22^{620R} and PEP^{619R} are negative regulators of LFA-1 signaling in T cells. Upon LFA-1 binding of ICAM-1, PTPN22^{620R} and PEP^{619R} associate with CSK, and are recruited to the leading edge in an LCK-dependent manner where they dephosphorylate PTPN22 substrates and inhibit LFA-1 signaling. **(B)** PTPN22^{620W} is a loss-of-function variant in LFA-1 signaling. Upon LFA-1 binding of ICAM-1, PTPN22^{620W} does not associate with CSK and is not recruited to the leading edge. This prevents PTPN22^{620W} from interacting with its substrates and inhibiting LFA-1 signaling. PEP^{619W} has not been studied in this context.

jun (26) downstream of the TCR. CRISPR/Cas9 mediated knockout of *PTPN22* in Jurkat T cells revealed that PTPN22 negatively regulates TCR-driven IL-2 and CD69 expression especially in the context of weak antigen stimulation (83). CRISPR/Cas9 mediated knockout of *PTPN22* in primary CD4$^+$ T cells supports that PTPN22 is a negative regulator of TCR signaling (78). These studies also revealed how PTPN22 achieves negative regulation of TCR signaling. PTPN22 cooperates with CSK to inhibit initial TCR signaling (**Figure 1A**) (26). In resting T cells, PTPN22 is associated with CSK and upon TCR stimulation, this complex dissociates at a rate that parallels dephosphorylation of PTPN22 substrates (40).

While PTPN22^{620R} is a negative regulator of TCR signaling, the effect of the SNP on function of PTPN22^{620W} remains controversial. It is currently debated whether PTPN22^{620W} is a gain-of-function variant that reduces response to TCR stimulation (**Figure 1B**) or a loss-of-function variant that allows enhanced TCR signaling (**Figure 1C**). The most studied hypothesis is that PTPN22^{620W} is a gain-of-function variant that suppresses TCR signaling (**Figure 1B**) (40, 41, 47, 70, 72, 76, 79, 80). These studies have shown that PTPN22^{620W} reduces signaling through the TCR and is associated with significantly reduced IL-2 secretion (72, 79), calcium mobilization (72, 76, 79), and IFNγ production from CD4$^+$ T cells (**Table 2**) (79). There is also evidence that the PTPN22^{620W} allotype drives enhanced skewing of CD4$^+$ T cells to EOMES$^+$ T_H1 cells (47, 73). PTPN22^{620W} is also associated with reduced expression of CD25, lower proliferation, and decreased IL-10 secretion by CD4$^+$ memory T cells (76, 79) (**Table 2**). In concordance with this, *in vivo* TCR stimulation in the form of a trivalent influenza vaccine resulted in reduced induction of an influenza virus-specific CD4$^+$ T cell response in PTPN22$^{620R/W}$ subjects compared to PTPN22$^{620R/R}$ subjects (**Table 2**) (80). Another indication of reduced influenza virus-specific CD4$^+$ T cell induction is the impairment of anti-flu antibody affinity maturation. Antibody affinity maturation relies on activation of CD4$^+$ T follicular helper (T_{FH}) cells; PTPN22$^{620R/W}$ subjects had reduced affinity maturation compared to PTPN22$^{620R/R}$ subjects implying they had reduced activation of T_{FH} cells or reduced activation of anti-flu B cells (80).

Studies tracing the proximal events following TCR stimulation agree that PTPN22^{620W} is a gain-of-function variant in primary T cells (**Figure 1B**). Overexpression of PTPN22^{620W} decreases NFAT/AP-1-driven luciferase transcription more than

TABLE 2 | *PTPN22* genotype influence on TCR-related phenotypes.

Phenotype	Donor genotype		
	PTPN22$^{1858C/C}$	*PTPN22*$^{1858C/T}$	*PTPN22*$^{1858T/T}$
IFNγ production (fold change) (79)	4.34	*	1.35*
CD4$^+$ memory T cells that are CD25$^+$ (%) (76)	58	45	n/a
IL-10 secretion from CD4$^+$ memory T cells (pg/ml) (76)	2,200	800	n/a
CD4$^+$ T cell proliferation (CFSE MFI) (79)	1,834	1.8	n/a
Influenza virus-specific CD4$^+$ T cells (%) (80)	0.23	n/a	0.15

**The PTPN22$^{1858C/T}$ donors were included in the PTPN22$^{1858T/T}$ donor group.*

overexpression of PTPN22^{620R} (72). In primary T cells from donors with PTPN22$^{620W/W}$ TCR stimulation resulted in lower TCRζ-chain phosphorylation and increased ERK, AKT, and PI3K p85 activation compared to PTPN22$^{620R/R}$ donor T cells (**Table 3**) (47, 70). These studies offer a molecular mechanism for the difference in function of PTPN22^{620R} and PTPN22^{620W} centered on reduced interactions of CSK and LCK with PTPN22^{620W}.

As noted above, in resting T cells PTPN22^{620R} is associated with CSK and upon TCR stimulation this complex dissociates at a rate that parallels dephosphorylation of PTPN22 substrates (**Figure 1A**) (26, 40). Simultaneously, PTPN22 is phosphorylated at Ser^{751} by PKCα which enhances the CSK/PTPN22 interaction and restricts PTPN22 activity to allow appropriate TCR signaling (42). PTPN22^{620W} interacts with CSK to a lesser extent than PTPN22^{620R} (immunoprecipitation of PTPN22^{620R} pulls down 2.9 fold more CSK than PTPN22^{620W}), and is more available to dephosphorylate PTPN22 substrates at the initiation of TCR signaling (28, 40, 41, 51). Both PTPN22^{620R} and PTPN22^{620W} are subject to phosphorylation at Ser^{751} by PKCα, however this only seems to inhibit PTPN22^{620R} activity, by enhancing its association with CSK, while it does not inhibit PTPN22^{620W} or enhance PTPN22^{620W}/CSK interactions (42). Similarly, PTPN22^{620R} is associated with LCK to a greater degree than PTPN22^{620W} and this appears to be CSK-dependent (41). LCK phosphorylates PTPN22 on an inhibitory Y536 residue (41). PTPN22^{620R} has more phosphorylated Y536 residues and is less active than PTPN22^{620W} in Jurkat cells at rest and upon TCR stimulation (41). This may also explain why the *in vitro* phosphatase activity of PTPN22^{620W} is 50% higher compared to PTPN22^{620R} when the two allotypes of PTPN22 are purified from mammalian cells. When purified from insect cells, where this post-translational modification is absent, the phosphatase activity is equal among the two allotypes (41, 72). In conclusion, PTPN22^{620W} is a more potent inhibitor of TCR signaling than PTPN22^{620R} because PTPN22^{620W} is more available to interact with PTPN22 substrates due to reduced sequestration by CSK. Further, PTPN22^{620W} is more active due to reduced association with its own negative regulator, LCK, and consequent reduced phosphorylation at an inhibitory tyrosine residue (**Figure 1B**).

TABLE 3 | *PTPN22* genotype influence on proximal TCR signaling events.

Phenotype	Donor genotype	
	PTPN22$^{1858C/C}$	*PTPN22*$^{1858T/T}$
relative TCRζ-chain phosphorylation (1 min)	100%	95%
relative ERK phosphorylation (15 min)	0%	50%
relative AKT phosphorylation (15 minutes)	40%	75%

While evidence that PTPN22^{620W} is a gain-of-function variant remains compelling, sufficient results exists to argue that PTPN22^{620W} could be a loss-of-function variant (**Figure 1C**) (70, 81). These studies have observed that T cells from healthy PTPN22$^{620W/W}$ donors expand more upon TCR stimulation than those from healthy PTPN22$^{620R/R}$ donors (70). Further, when CSK is co-expressed with PTPN22^{620W} in Jurkat T Cells, higher calcium fluxes are measured than when CSK is co-expressed with PTPN22^{620R} (81). A study found that the *PTPN22*^{1858T} allele enhances expression of a dominant negative isoform, PTPN22.6, that increases signaling through the TCR (**Figure 1C**) (84). The authors offered a hypothesis that reconciles human data showing that PTPN22^{620W} is a gain-of-function; PTPN22^{620W} allows chronic signaling through the TCR that drives T cell exhaustion, causing T cells from PTPN22$^{620R/W}$ and PTPN22$^{620W/W}$ donors to be less responsive to stimulation through the TCR—a finding reported by most studies. This is supported by evidence that expression of PD-1, a marker of T cell exhaustion, is enhanced on CD4$^+$ T_{eff} and T_{regs} in healthy PTPN22$^{620W/W}$ donors compared to healthy PTPN22$^{620R/R}$ donors (74). Furthermore, the reduced calcium flux seen in PTPN22$^{620R/W}$ donors was most notable in memory CD4$^+$ T cells with no difference observed in naïve CD4$^+$ T cells; this could indicate that the experienced population is exhausted (76). While it is not certain whether PTPN22^{620W} is a gain-of-function or loss-of-function variant in human TCR signaling, it is clear that the mouse orthologue of PTPN22^{620W}, PEP^{619W}, is a loss-of-function variant in mouse TCR signaling.

Data from mouse models support the role of PTPN22/PEP as a negative regulator of TCR signaling (**Figure 1A**). Overexpression of PEP in the mouse antigen specific T cell line, BI-141, reduced TCR-mediated phosphorylation of ZAP70, c-Cbl, and the CD3 ζ-chain (77). Overexpression of PEP also reduced IL-2 secretion from these cells (77). C57BL/6J mice with a genetic ablation of *Ptpn22* (B6.Cg-*Ptpn22*^{tm2Achn}/J commonly referred to as C57BL/6-*Ptpn22*$^{-/-}$ mice) as well as NOD mice with doxycycline-induced knockdown of PEP [NOD-Tg(tetO-RNAi : *Ptpn22*,UBC-tetR,-GFP)P2Kslr commonly referred to as NOD-*Ptpn22*^{KD}] starting at birth have an accumulation of effector/memory CD4$^+$ and CD8$^+$ T cells in secondary lymphoid organs. This phenotype is thought to be a product of increased TCR signaling in the absence of PEP (85–88). Similar to humans harboring PTPN22^{620W}, PEP^{619W} knock-in C57BL/6 mice (C57BL/6-*Ptpn22*$^{tm1.1Kas}$ commonly referred to as C57BL/6-PEP^{619W}) exhibited an expansion of CD4$^+$ memory T cells compared to unaltered C57BL/6 mice that carry the PEP^{619R} allele (70, 71). In C57BL/6-PEP^{619W} mice there was also a marked expansion of the total effector/memory T cell pool and

T cells from these mice exhibited increased IL-2 secretion, increased calcium mobilization, enhanced/prolonged tyrosine-phosphorylation of ZAP-70 and Lck, and increased *ex vivo* expansion of T cells compared to C57BL/6 mice (70, 71, 86). While the R619W conversion in PEP appears to be a loss-of-function variant with respect to TCR signaling (**Figure 1C**), controversy exists regarding the human autoimmunity risk allotype, PTPN22^{620W}, with regard to gain-of-function or loss-of-function TCR signaling (**Figures 1B, C**). Despite this ongoing lack of clarity for PTPN22^{620W} in human TCR signaling, evidence clearly supports that PTPN22^{620W} is a loss-of-function variant in LFA-1 signaling in T cells.

Impact of PTPN22 Allotype on LFA-1 Signaling in T Cells

LFA-1 is fundamentally important to general leukocyte trafficking. Loss of LFA-1 causes the life-threatening disease known as leukocyte adhesion deficiency (LAD) resulting in uncontrolled microbial infections (89). LFA-1 is also critical in T cell activation and migration (90). In human T cells, PTPN22 inhibits LFA-1 signaling (**Figure 2**) (32). T cells treated with PTPN22 targeting small interfering RNA (siRNA) exhibited increased ICAM-1 (LFA-1 ligand)-induced phosphorylation of LCK, ZAP70, ERK1/2, and Vav compared to cells treated with a non-targeting control (NTC) siRNA. There was also an increase in ICAM-1-induced motility in cells treated with the PTPN22 targeting siRNA (32). The autoimmune associated variant, PTPN22^{620W}, is a loss-of-function variant in LFA-1 signaling (**Table 2B**). Similar to what was observed with knockdown of PTPN22, human T cells from PTPN22$^{620R/W}$ and PTPN22$^{620W/W}$ donors have enhanced LFA-1 induced signaling (pERK1/2 fold change over unstimulated; PTPN22$^{620W/W}$ ~35 vs. PTPN22$^{620R/W}$ ~25 vs. PTPN22$^{620R/R}$ ~20) and adhesion (mean # of T cells adhered to LFA-1 coated slide at 8 min under shear flow; PTPN22$^{620W/W}$ ~32 vs. PTPN22$^{620R/R}$~24) compared to T cells from PTPN22$^{620R/R}$ donors. At rest, PTPN22^{620R} and PTPN22^{620W} are aggregated near the plasma membrane of T cells. Upon engagement of ICAM-1 with LFA-1, PTPN22^{620R} leaves these aggregates, associates with CSK, and is recruited to the leading edge of migrating cells in an LCK-dependent manner where it dephosphorylates PTPN22 substrates to inhibit LFA-1 signaling (**Figure 2A**). In contrast, PTPN22^{620W} stays more clustered and is less recruited to the leading edge resulting in less PTPN22-mediated negative regulation of LFA-1 signaling (**Figure 2B**) (32).

As observed in human T cells, PEP negatively regulates mouse T cell responses to ICAM-1 stimulation (**Figure 2A**). T cells from C57BL/6-*Ptpn22*$^{-/-}$ mice displayed enhanced ERK1/2 phosphorylation (pERK1/2 fold change over-unstimulated; *Ptpn22*$^{-/-}$ ~12 vs. *Ptpn22*$^{+/+}$ ~8) after ICAM-1 stimulation and adhered better to ICAM-1 coated glass slides under shear flow (mean # of T cells adhered at 8 min; *Ptpn22*$^{-/-}$ ~55 vs. *Ptpn22*$^{+/+}$ ~30) compared to *Ptpn22*-intact mouse T cells (32). C57BL/6-*Ptpn22*$^{-/-}$ mouse T cells also had increased LFA-1 induced IFNγ secretion and were better at forming T cell-DC conjugates compared to *Ptpn22*-intact T cells (86). PEP and PTPN22 are both negative regulators of LFA-1 signaling in mice and humans (**Figure 2A**). PTPN22^{620W} is a loss-of-function variant in humans while it is not known how the PEP 619R to W conversion affects mouse LFA-1 signaling (**Figure 2B**). While the molecular mechanisms behind PTPN22's influence on receptor-proximal signaling in T cells (i.e., activation and mobilization) are well studied, PTPN22 has also been shown to influence T_{reg} induction and function however the mechanism is less resolved.

Treg Induction and T Cell Suppression by aTreg

PTPN22 positively regulates *in vitro* induced T_{reg} (iT_{reg}) differentiation in human T cells. Primary naive T cells ($CD4^+CD127^+CD25^-$) from PTPN22$^{620R/R}$ healthy donors and PTPN22$^{620R/R}$ donors with T1D were subjected to *PTPN22* knockdown with antisense oligonucleotides. Differentiation of iT_{regs} *via* treatment with IL-2/TGF-β1/αCD3/αCD28 was reduced with *PTPN22* knockdown compared to control oligonucleotide transfected cells (% of CD4 T cells that are $CD25^+FoxP3^+$; *PTPN22* knockdown resulted in ~20% iTreg vs. control ~40%) (33). No direct clinical studies have shown how PTPN22^{620} allotypes influence iT_{reg} differentiation; however, healthy PTPN22$^{620W/W}$ donors have increased $CD4^+$ T_{regs} compared to healthy PTPN22$^{620R/R}$ donors (7.94% vs. 6.76%) implying that PTPN22^{620W} might potentiate iT_{reg} development (74, 75). Although PTPN22$^{620W/W}$ donors have slightly more $CD4^+$ T_{regs}, these T_{regs} exhibit a reduced capacity to inhibit IFNγ secretion from conventional T cells compared to those from PTPN22$^{620R/R}$ donors (47, 76).

As observed in humans, PEP also influences T_{reg} development in mice; C57BL/6-*Ptpn22*$^{-/-}$ mice and NOD-*Ptpn22*^{KD} mice had increased numbers of T_{regs} (87, 88). Data from C57BL/6-*Ptpn22*$^{-/-}$ mice and NOD-*Ptpn22*^{KD} mice provided evidence that deficiency of PEP reduces the TCR signal strength required for *in vitro* induction of iT_{regs} (91, 92). The iT_{reg} induction can be accomplished by stimulating naïve FoxP3-$CD4^+$ T cells with a combination of agonistic anti-CD3 and anti-CD28 targeting antibodies in the presence of TGF-β (87). Lower levels of stimulation with reduced concentrations of anti-CD3 antibodies increased *in vitro* iT_{reg} induction in *Ptpn22*$^{-/-}$ cells compared to *Ptpn22*-intact cells. Increased concentrations of anti-CD3 resulted in elevated stimulation and decreased iT_{reg} induction in *Ptpn22*$^{-/-}$ cells compared to *Ptpn22*-intact cells. At levels of TCR-stimulation that drive optimal *in vitro* iT_{reg} induction in parental C57BL/6 mice, C57BL/6-*Ptpn22*$^{-/-}$ had reduced iT_{reg} induction (87). Much like PTPN22^{620W} humans, aged C57BL/6-PEP^{619W} mice had increased T_{regs} compared to C57BL/6 mice. However, young C57BL/6-PEP^{619W} mice exhibited no increase in T_{regs}. T_{regs} from young C57BL/6-PEP^{619W} mice exhibited no differences in suppressive activity when compared to C57BL/6 mice, however T_{regs} from aged mice were not assessed. This difference may be due to the age of the mice, however, it remains to be seen if T_{regs} from older C57BL/6-PEP^{619W} mice exhibit the same defect in suppression as human T_{regs} (71). It is clear that PTPN22 plays multiple roles in human

T cells and that the diabetogenic allotype of PTPN22, PTPN22^{620W}, alters these roles; how might the altered function of PTPN22^{620W} in T cells impact T1D development?

PTPN22 in T Cells and Impact on T1D

T1D is generally considered a T cell mediated disease where CD8$^+$ T cells are the major islet infiltrating immune cells (93, 94). The SNP in *PTPN22*, *rs2476601*, is associated with increased risk for T1D, reduced age at onset (95), and reduced residual β cell function at diagnosis (96). This SNP affects T cell function. PTPN22 is a negative regulator of TCR (26, 28, 43, 77) and LFA-1 (32) signaling and influences aT$_{reg}$ suppressive capacity (**Figures 1** and **2**) (47). In T cells, the effect of the T1D-risk variant PTPN22^{620W} on TCR-induced signaling is currently unresolved with data supporting both gain-of-function and loss-of-function hypotheses (40, 41, 47, 70, 72, 76, 79, 80). In contrast, PTPN22^{620W} has been characterized as a loss-of-function variant in LFA-1-induced signaling because it is not available to interact with its substrates (32). Adaptive T$_{regs}$ (aT$_{regs}$) from PTPN22$^{620W/W}$ donors have reduced capacity to suppress IFNγ secretion from conventional T cells (47). The enhanced LFA-1-induced signaling and motility, and the reduced capacity of aT$_{regs}$ to suppress IFNγ secretion from conventional T cells seen in PTPN22$^{620R/W}$ and PTPN22$^{620W/W}$ humans could help explain why *rs2476601* is associated with increased overall risk of T1D development. The seemingly small magnitudes of reported biochemical, phenotypic, and functional effects of PTPN22^{620W} in human T cells are surprising for a genetic variation that ranks near the top of the list for T1D genetic risk. We ask ourselves, "How could such minor fluctuations contribute to a life-threatening pathology?" The answer might lie in the thymus - the immune tissue where developing thymocytes (soon to be T cells) are exquisitely sensitive to the strength and duration of nascent TCR signaling. If PTPN22^{620W} is a gain-of-function variant in TCR signaling, the PTPN22^{620W} variant might impair the process of negative selection whereby autoreactive thymocytes are normally eliminated upon strong TCR signaling. Thus, effectively blinded to the fact that a given TCR is recognizing a self-antigen (e.g., insulin), autoreactive T cells might survive and escape into the periphery (72). More autoreactive T cells in the periphery would lead to increased autoreactive T cells surveying tissues, including the pancreas, and more opportunities for an autoimmune reaction to occur.

The alternate scenario postulates that thymic selection is more or less unaffected, and that the biologic effects of PTPN22^{620W} manifest in the periphery. If PTPN22^{620W} is a loss-of-function variant in TCR signaling, circulating T cells would be more sensitive to TCR ligation and this could explain the genesis of autoreactive T cell activation and thus autoimmunity. Both intrathymic and peripheral scenarios would be complicated by enhanced LFA-1-induced signaling (enhancing T cell migration) and reduced capacity of aT$_{regs}$ to suppress IFNγ secretion from activated T cells that could result in enhanced T cell infiltration into tissues (i.e., islets of Langerhans) as well as secretion of more IFNγ, thus creating a more inflammatory local environment. For T cells, additional new work will be needed to understand how thymic development and intra-islet T cell function is modulated by PTPN22 variants. Is there a single dominant mechanism at fault for autoimmune risk, or is this a case of death by a thousand cuts—multiple subtle effects which alone appear innocuous but together add up to complete destruction of a vital tissue? If the story weren't complicated enough, T cells alone might not be the culprit of T1D. Autoantibodies produced by B cells are a prevalent feature and remain the gold standard biomarker of T1D progression. While it is hypothesized that autoantibodies are not pathogenic in human T1D, B cells are thought to play an important role as antigen specific APCs. It is known that depletion of B cells with Rituximab can delay disease progression (97). Additionally, many of the other *rs2476601*-asocciated autoimmune diseases are characterized by production of autoantibodies (e.g., RA, SLE, etc.). As such, many studies have focused on the effect of the PTPN22^{620R} versus PTPN22^{620W} in human B cells.

REGULATION OF B CELL FUNCTION BY PTPN22 ALLOTYPES

PTPN22 has been studied extensively in human B cells. Unlike the minor difference observed in the T cell compartment, PTPN22^{620W} has a profound impact on B cell composition (described in detail below) (76). PTPN22 also impacts signal transduction in human B cells where it functions as a negative regulator of BCR signaling and BCR-induced apoptosis (34). Because PTPN22 influences BCR signaling and BCR-induced apoptosis, it also influences the central and peripheral B cell tolerance checkpoints (27, 76, 98–100).

Impact of PTPN22 Allotype on BCR Signaling

PTPN22 functions to dampen BCR signaling as well as BCR-induced apoptosis (**Figure 3B**). PTPN22 is overexpressed in primary chronic B lymphocytic leukemia (CLL) cells (34). CLL cells express functional BCRs and have been characterized for ligand-dependent signaling. PTPN22 depletion in CLL cells increased soluble-αIgM (simulated strong BCR signaling) induced apoptosis (34). Knockdown of PTPN22 also resulted in increased soluble αIgM-induced phosphorylation of LYN, SYK, BLNK, PKCδ, ERK, JNK, and p38 MAPK and reduced soluble-αIgM-induced phosphorylation of AKT, GSK3, and FOXO (34). PTPN22^{620W} is a gain-of-function variant that acts to further blunt BCR signaling (**Figure 3B**). In heterozygous PTPN22$^{620R/W}$ donors there is reduced BCR-induced calcium flux compared to PTPN22$^{620R/R}$ donors (27, 76, 98). Heterozygous donors also had reduced phosphorylation of the BCR-proximal signaling components, SYK, PLCγ2 (MFI phospho-PLCγ2-Y759; PTPN22$^{620R/W}$ ~700 vs. PTPN22$^{620R/R}$ ~950), and AKT compared to PTPN22$^{620R/R}$ donors (27, 76, 98). In PTPN22$^{620R/W}$ donors there is also reduced total phosphorylated tyrosine in resting (% of CD27$^+$ B cells that are phospho-tyrosine$^+$; PTPN22$^{620R/W}$ ~4% vs. PTPN22$^{620R/R}$ ~8%) and BCR-activated memory B cells compared to

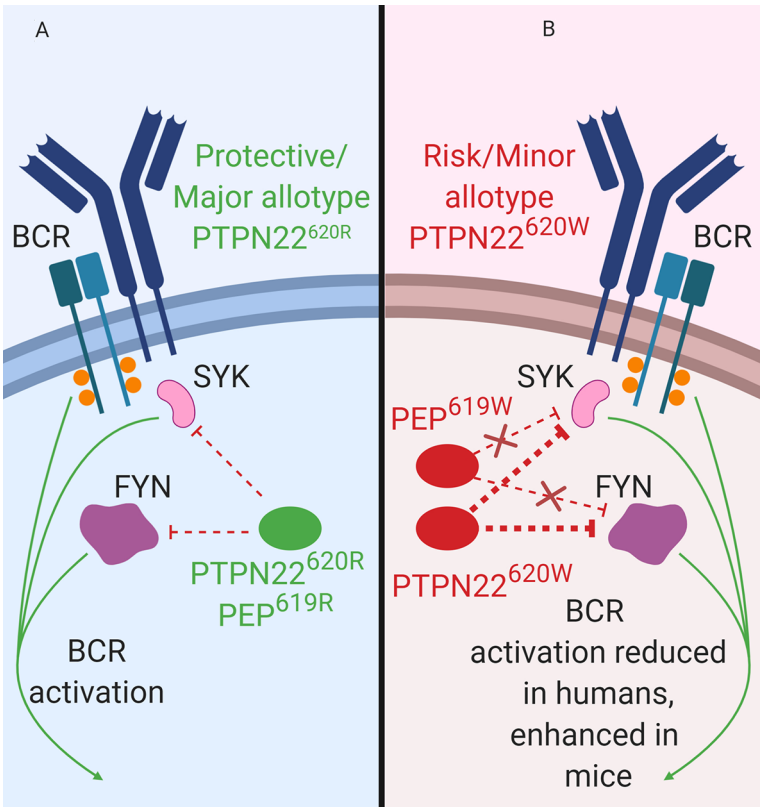

FIGURE 3 | PTPN22 function in B cells. **(A)** PTPN22^{620R} and PEP^{619R} are negative regulators of BCR signaling in B cells. **(B)** PTPN22^{620W} is a gain-of-function variant with respect to BCR signaling and leads to blunted BCR signaling. PEP^{619W} is a loss-of-function variant with respect to BCR signaling and leads to enhanced BCR signaling.

PTPN22$^{620R/R}$ donors (27). Inhibition of PTPN22 in B cells of PTPN22$^{620R/W}$ donors increased SYK, PLCγ2, and AKT phosphorylation to levels equivalent to those of B cells from PTPN22$^{620R/R}$ donors (27, 98). Signaling through the BCR can also induce B cell expansion. However, it is not clear how PTPN22^{620W} affects BCR-induced expansion of B cells; different studies have shown conflicting results (27, 70). Overall, PTPN22^{620W} is more effective at regulating BCR signaling (**Figure 3B**).

Consistent with data from human B cells, PEP is also a negative regulator of BCR signaling in mice (**Figure 3**). Silencing of PEP *via* doxycycline-induced expression of a *Ptpn22*-targeting siRNA in NOD mice (the NOD-*Ptpn22*^{KD} mice) increased B cell response to anti-IgM/anti-CD40 stimulation (88). Additionally, silencing of PEP resulted in the increased proliferation, robust expression of CD25 and CD69, and elevated phosphorylation of PLCγ2 (88). These results have been replicated *via Ptpn22* knockout in other mouse strains (81, 101, 102). Unlike PTPN22^{620W} humans, C57BL/6-PEP^{619W} mice had a phenotype similar to C57BL/6-*Ptpn22*$^{-/-}$ mice, with increased anti-IgM induced B cell activation, increased anti-IgM induced proliferation, and increased phosphorylation of PLCγ2 compared to C57BL/6 mice (70, 71, 103). While PTPN22^{620W} is a gain-of-function variant in human BCR signaling, PEP^{619W} is a loss-of-function variant with respect to BCR signaling in mice (**Figure 3B**).

Regulation of B Cell Gene Expression and B Cell Expression of Surface Receptors by PTPN22

PTPN22^{620W} alters gene expression and immune receptor levels in B cells. Naïve B cells from both PTPN22$^{620R/W}$ and PTPN22$^{620W/W}$ donors had significantly upregulated *IL4R*, *IL13R*, *IL17R*, and *IL21R* mRNA expression (genes involved in B cell proliferation/differentiation) and significantly upregulated genes in the BCR, CD40, and TLR activating pathways compared to those from PTPN22$^{620R/R}$ donors (100). PTPN22^{620W} differentially affects expression of other genes with SNPs associated with T1D and other autoimmune diseases (*BLK, PTPN2, CD40, TRAF1, CD19, SLAM, IRF5)* (100). The surface expression of BAFFR, CD40, and SLAMF6 was enhanced in PTPN22$^{620R/W}$ and PTPN22$^{620W/W}$ donors compared to PTPN22$^{620R/R}$ donors (**Table 4**) (100, 103). Naïve B cells from PTPN22$^{620R/W}$ and PTPN22$^{620W/W}$ donors were more responsive to CD40L stimulation with an increased percent of B cells expressing CD69 and CD25 than those from PTPN22$^{620R/R}$ donors (100). CpG stimulation of PBMC for 4 days resulted in greater expansion of IgM+ memory B cells

(CD19+CD27+IgM+) and IgM- Plasma cells (CD19+ $CD27^{hi}$IgM-) in $PTPN22^{620R/W}$ patients with T1D compared to $PTPN22^{620R/R}$ patients with T1D and in healthy control $PTPN22^{620R/W}$ donors compared to healthy control $PTPN22^{620R/R}$ donors (99). The combination of increased BAFFR, CD40, and SLAMF6 surface levels and the increased expression of *IL4R*, *IL13R*, *IL17R*, *IL21R*, as well as genes belonging to the CD40, TLR, and BCR activation pathways may explain the enhanced CpG-induced expansion of IgM+ memory B cells and IgM- Plasma cells in PBMCs seen in $PTPN22^{620R/W}$ and $PTPN22^{620W/W}$ donors compared to $PTPN22^{620R/R}$ donors. Importantly, this phenomenon is present in both $PTPN22^{620R/W}$ and $PTPN22^{620W/W}$ donors implying that the effects of $PTPN22^{620W}$ are either dominant or co-dominant.

Unlike humans, there was decreased CD40 and BAFFR surface expression on total splenocytes with decreased CD40 and BAFFR on immature B cells and increased CD40 on T2 B cells of C57BL/6-PEP^{619W} mice compared to C57BL/6 mice (103). *Tnfrsf13c* (*BAFFR*) mRNA levels were enhanced in immature B cells and *Cd40* mRNA levels were enhanced in T2 B cells of C57BL/6-PEP^{619W} mice compared to C57BL/6 mice (103). Taken together we see that PTPN22 and PEP affect expression of costimulatory molecules in B cells of both humans and mice however the effects of the R to W conversion are not consistent when comparing humans to mice.

B Cell Tolerance Checkpoints and Composition

$PTPN22^{620W}$ alters the central and peripheral B cell tolerance checkpoints as well as the composition of the B cell compartment in humans (76, 98–100). Central B cell tolerance is mediated *via* clonal deletion or receptor editing to remove autoreactive or polyreactive B cells from the bone marrow before they enter the periphery (e.g., spleen, blood, lymph nodes, tissues) (104). Central tolerance results in a large reduction of polyreactive and autoreactive B cells and is readily apparent when comparing the bone marrow to the spleen and blood; 40%–70% of early immature B cells are polyreactive and 50%–75% are autoreactive in the bone marrow while 5%–10% of transitional B cells are polyreactive and 30%–50% are autoreactive in the periphery (104, 105). A common method for determining if B cells are autoreactive is to assess their response to human epithelial type 2 (HEp-2) cells. HEp-2 cells express a large array of self-antigens and HEp-2 reactive B cells are considered autoreactive (106).

TABLE 4 | *PTPN22* genotype influence on B cell surface receptor expression.

Phenotype	Donor genotype					
	***PTPN22*$^{1858C/C}$**			***PTPN22*$^{1858C/T}$ and *PTPN22*$^{1858T/T}$**		
	Transitional	**Naïve**	**IgM Memory**	**Transitional**	**Naïve**	**IgM Memory**
BAFFR MFI	~90	~90	~90	~120	~100	~110
CD40 MFI		~190			~210	
SLAMF6 MFI		~190			~200	

Healthy $PTPN22^{620R/W}$ and $PTPN22^{620W/W}$ donors had an increased proportion of polyreactive and HEp-2-reactive new emigrant/transitional B cells ($CD20^{+}CD10^{+}CD21^{lo}IgM^{hi}CD27^{-}$: 25%–30% of new emigrant/transitional B cells were polyreactive and ~50% were HEp-2-reactive) compared to healthy $PTPN22^{620R/R}$ donors (8%–10% of new emigrant/transitional B cells were polyreactive and ~30% were HEp-2 reactive) (100, 107). Most studies agreed that transitional B cells ($CD19^{+}CD27^{-}CD24^{hi}CD38^{hi}$) were increased in healthy $PTPN22^{620R/W}$ and $PTPN22^{620W/W}$ donors compared to healthy $PTPN22^{620R/R}$ donors (percentage of total B cells that are transitional; $PTPN22^{620W/W}$ and $PTPN22^{620R/W}$ ~5% vs. $PTPN22^{620R/R}$ ~2.5%), although not all studies observe this effect (98, 99, 108). The increased numbers of transitional B cells and polyreactive/HEp-2-reactive new emigrant/transitional B cells in healthy $PTPN22^{620R/W}$ and $PTPN22^{620W/W}$ donors indicates that the central B cell tolerance checkpoint is altered by $PTPN22^{620W}$. Ergo, the autoimmune-linked allotype allows more polyreactive and autoreactive B cells to escape central tolerance and proceed into the periphery. B cells that enter the periphery will go through another round of selection to remove or inactivate autoreactive cells.

Peripheral B cell tolerance results in anergy or clonal deletion *via* apoptosis that is dependent on caspase-3 activation and is triggered by strong signaling though the BCR (98). This results in the reduction of autoreactive peripheral B cells. There are more autoreactive transitional B cells than autoreactive naïve mature B cells due to the peripheral B cell tolerance checkpoint; 30%–50% of transitional B cells are autoreactive while 10%–30% of naïve mature B cells are autoreactive (105). To simulate strong BCR signaling in naïve B cells, anti-IgM is used to crosslink the BCRs; this is similar to encountering a multivalent self-antigen during peripheral B cell tolerance and will cause some naïve B cells to undergo apoptosis. After 12 h of anti-IgM treatment, significantly fewer naïve B cells from $PTPN22^{620R/W}$ donors had begun the process of apoptosis by cleaving/activating caspase-3 when compared to $PTPN22^{620R/R}$ donors (% of naïve B cells with cleaved/active caspase-3; $PTPN22^{620R/W}$ ~10% vs. $PTPN22^{620R/R}$ ~18%) (98). Basal levels of the anti-apoptotic protein, Bcl-2, were higher in transitional B cells from $PTPN22^{620R/W}$ donors compared to $PTPN22^{620R/R}$ donors (Normalized BCL-2 MFI; $PTPN22^{620R/W}$ ~20 vs. $PTPN22^{620R/R}$ ~12) with no alteration in the pro-apoptotic protein, Bim (98). Healthy $PTPN22^{620W/W}$ and $PTPN22^{620R/W}$ donors had increased frequencies of polyreactive and HEp-2-reactive mature naïve B cells ($CD20^{+}CD10^{-}CD21^{+}IgM^{+}CD27^{-}$). In these donors ~30% of mature naïve B cells were polyreactive and ~45% were HEp-2-reactive. In contrast, healthy $PTPN22^{620R/R}$ donors had ~10% polyreactive mature naïve B cells were and ~20% HEp-2-reactive (100). A unique subset of autoreactive anergic B cells (naïve IgD^{+} B cells [B_{ND}]: $CD19^{+}CD27^{-}IgD^{+}IgM^{-}$) are cells in the periphery thought to be anergic due to low chronic antigen stimulation through the BCR (109). B_{ND} cells were increased in healthy $PTPN22^{620R/W}$ donors compared to healthy $PTPN22^{620R/R}$ donors (% of $CD19^{+}$ B cells that are B_{ND} cells; $PTPN22^{620R/W}$ ~3% vs. $PTPN22^{620R/R}$

~2%) (98). PTPN22$^{620R/W}$ donors had a lower percentage of memory B cells compared to PTPN22$^{620R/R}$ donors (% of CD19$^+$ B cells that are CD27$^+$; PTPN22$^{620R/W}$ ~35% vs. PTPN22$^{620R/R}$ ~45%) (76). The reduced caspase-3 activation, increased levels of Bcl-2, increased frequencies of B_{ND} cells, HEp-2-reactive mature naïve B cells, and polyreactive mature naïve B cells, and decreased frequency of mature B cells found in PTPN22$^{620R/W}$ and PTPN22$^{620W/W}$ donors indicates that PTPN22^{620W} alters the peripheral B cell tolerance checkpoint (98, 100, 107). The increase in autoreactive/polyreactive new emigrant/transition B cells, all transitional B cells, B_{ND} cells, and decrease in memory B cells was also seen when comparing T1D donors regardless of genotype to healthy PTPN22$^{620R/R}$ donors and this may represent a common B cell phenotype present in T1D patients (98, 100). Currently, it is thought that the blunting of BCR signaling by the gain-of-function PTPN22^{620W} allotype leads to reduced negative selection and is responsible for the alterations seen in central and peripheral B cell tolerance mechanisms (76, 98, 100, 107). These B cell phenotypes are observed in both patients with autoimmunity and healthy controls that encode PTPN22^{620W}.

C57BL/6-*Ptpn22*$^{-/-}$ as well as other strains of *Ptpn22* knockout mice exhibit an altered B cell compartment. Deletion of *Ptpn22* increased age-associated B cells (ABCs), plasma cells, autoantibodies, as well as germinal center activity and size when compared to *Ptpn22*-intact mice. However, germinal center size and activity appears to be partially dependent on an alteration in T follicular helper cells (81, 101, 102). Unlike humans harboring PTPN22^{620W}, alterations in the B cell compartment of the loss-of-function PEP^{619W} variant in mice is attributed to altered positive B cell selection due to enhanced BCR signaling (103). C57BL/6-PEP^{619W} mice have increased splenic transitional 1 B cells, increased age-dependent B cells (ABCs), increased class-switched B cells, increased germinal center B cells, and less mature recirculating B cells when compared to C57BL/6 mice (103). Like humans however, the enhanced positive selection leads to increased self-reactive B cells, increased autoantibody titers, and reduced apoptosis of T1 B cells in C57BL/6-PEP^{619W} when compared to C57BL/6 mice (70, 71, 103). The similarities between the B cell compartments of *Ptpn22*$^{-/-}$ mouse strains and C57BL/6-PEP^{619W} mice implies that PEP^{619W} is a loss-of-function variant in mice with respect to its effects on B cell positive selection while PTPN22^{620W} decreases human B cell negative selection.

While PEP^{619W} mice do not display the same central B cell tolerance phenotype as humans heterozygous or homozygous for PTPN22^{620W}, immunodeficient NOD.Cg-*Prkdc*^{scid}.*Il2rg*^{tm1Wjl} (NSG) mice engrafted with human CD34$^+$ hematopoietic stem cells (HSCs) from either PTPN22$^{620R/W}$, PTPN22$^{620W/W}$ donors, or with HSCs overexpressing PTPN22^{620W} phenocopy humans that are heterozygous or homozygous for PTPN22^{620W}. These PTPN22^{620W} HSC engrafted NSG mice display an increased proportion of polyreactive and HEp-2-reactive new emigrant/transitional B cells when compared to NSG mice engrafted with HSCs from PTPN22$^{620R/R}$ donors or HSCs overexpressing PTPN22^{620R} (100, 107). Importantly, inhibition of PTPN22 in NSG mice engrafted with PTPN22^{620W} HSCs reduced polyreactive and HEp2-reactive new emigrant B cells to the same levels as NSG mice engrafted with PTPN22^{620R} HSCs indicating that PTPN22 is the main driver of this difference (107). The increased numbers of transitional B cells and polyreactive/HEp-2-reactive new emigrant/transitional B cells in healthy PTPN22$^{620R/W}$ and PTPN22$^{620W/W}$ donors and in PTPN22^{620W} HSC engrafted NSG mice indicates that the central B cell tolerance checkpoint is altered by PTPN22^{620W}. This alteration allows more polyreactive and autoreactive B cells to escape central tolerance and proceed into the periphery. Overall, PEP^{619W} is a loss-of-function variant in mice with respect to its effects on B cell positive selection while PTPN22^{620W} decreases human B cell negative selection; both of these alterations result in more autoreactive B cells with increased autoantibody titers.

PTPN22 in B Cells and Impact on T1D

Autoantibodies produced by B cells are a prevalent feature of T1D and remain the gold standard biomarker of islet autoimmunity and T1D progression (110). The SNP in *PTPN22*, *rs2476601*, is associated with increased risk of persistent islet autoimmunity (i.e., autoantibodies directed against insulin, GAD65, or IA-2) (111). While the role of pathogenesis of human T1D remains controversial, the importance of B cells has been demonstrated in preclinical models and clinical trials. Depletion of B cells pauses the loss of β cell function in some patients with recent onset T1D and can prevent or reverse disease in NOD mice (97, 112). B cells are not only capable of producing antibodies, they also act as APCs to present antigen to T cells in a process called linked recognition (113). In linked recognition, B cells uptake antigen recognized by the BCR, process it, and load peptides derived from the antigen on MHC-II to present to CD4$^+$ T cells (113). These responding CD4$^+$ T cells must have already encountered antigen and been activated by other APCs in the periphery before they can provide T cell help to the B cells. The T cell help initiates class-switching in germinal centers, while the B cells provide co-stimulatory signals to the T cells capable of enhancing in-progress T cell responses (114). In NOD mice, it is thought that B cells primarily enhance autoreactive T cell function as APCs and through the production of pro-inflammatory cytokines (115). PTPN22$^{620R/W}$ and PTPN22$^{620W/W}$ donors have increased B cell surface expression of CD40, SLAMF6, and BAFFR (**Table 4**), as well as B cell mRNA expression of *IL4R*, *IL13R*, *IL17R*, *IL21R* compared to PTPN22$^{620R/R}$ donors. PTPN22^{620R} is a negative regulator of BCR signaling and PTPN22^{620W} is a gain-of-function variant that reduces signaling through the BCR. This reduction in BCR signaling alters central and peripheral B cell tolerance allowing more autoreactive and polyreactive B cells into the periphery. The increased surface expression of CD40, SLAMF6, and BAFFR (**Table 4**), as well as B cell mRNA expression of *IL4R*, *IL13R*, *IL17R*, *IL21R* could enhance clonal expansion of B cells, differentiation into plasma cells, class switching, and cell survival in PTPN22$^{620R/W}$ and PTPN22$^{620W/W}$ humans (116–121). Increased SLAMF6 and CD40 expression on B cells could also enhance/prolong B cell-

T cell interactions leading to more T cell and B cell activation in PTPN22$^{620R/W}$ and PTPN22$^{620W/W}$ humans. The combination of these phenotypes could lead to increased class switching of autoreactive B cells and increased survival of autoreactive and polyreactive B cells. These autoreactive/polyreactive B cells could go on to increase or simply sustain activation of autoreactive T cells. The increased/sustained activation of autoreactive T cells by autoreactive/polyreactive B cells could explain why *rs2476601* is associated with increased risk of persistent islet autoimmunity (111) and why treatment with a B cell depleting therapy (rituximab) can delay loss of, but not restore, the c-peptide response in patients with recent onset T1D (97).

While adaptive immune cells are integral for targeting and destroying β cells, they are not the only cells implicated in development of T1D. The innate arm of the immune system is generally required to initiate antigen-specific responses by T and B cells. Monocytes, macrophages, and dendritic cells (DCs) are all APCs capable of initiating these potent immune responses in inflammatory contexts.

PTPN22 ALLOTYPES IN MONOCYTES, MACROPHAGES, AND DENDRITIC CELLS

Monocytes, macrophages, and DCs are innate immune cells that are a part of the front-line sentinels that sense (via conserved PRRs such as TLRs and nucleic acid sensors) and eliminate invading microbes. While the function of PTPN22^{620R} and altered function of PTPN22^{620W} have been extensively examined in T and B cells, the roles of these allotypes in monocytes, macrophages, and DCs have been less studied. In human DCs and macrophages, PTPN22^{620R} is a positive regulator of TLR4-induced Type 1 interferon (T1-IFN) production while PTPN22^{620W} is less effective at driving TLR4- and TLR7/8-induced T1-IFN production (35, 122). In macrophages, PTPN22^{620R} is a positive regulator of NLRP3 inflammasome activation and PTPN22^{620W} is a gain-of-function variant leading to more NLRP3 activation and subsequent IL-1β release (36, 37). In monocytes, PTPN22^{620R} negatively regulates NOD2-induced autophagy (39) and regulates IFNγ-induced signaling (29) while PTPN22^{620W} has not been studied in the regulation of NOD2-induced autophagy or IFNγ-induced signaling. When examining the polarization of macrophages, PTPN22^{620R} is a negative regulator of IL-23/IL-12 production following M1 induction (IFNγ/LPS treatment) while PTPN22^{620W} is a gain-of-function variant that reduces IL-21/IL-12 production following M1 polarization. PTPN22^{620R} is a positive regulator of IL-10 expression following M2 induction (IL-4/IL-13 treatment) and PTPN22^{620W} does not alter this (38). As these previous studies illustrate, PTPN22 plays diverse roles in monocytes, macrophages, and DCs and the 620R to W conversion alters function in many aspects.

TLR-Induced Type 1 Interferons

PTPN22^{620R} associates with TRAF3 following LPS stimulation and promotes T1-IFN production while PTPN22^{620W} does not (**Figure 4A**) (35). This effect is not limited to TLR4 stimulation, plasmacytoid dendritic cells (pDCs) from PTPN22$^{620W/W}$ and PTPN22$^{620R/W}$ patients with SLE have reduced IFNα production following R848 (TLR7/8 agonist) stimulation compared to PTPN22$^{620R/R}$ patients (PTPN22$^{620R/W}$+PTPN22$^{620W/W}$; ~35% pDCs IFNα2+ with gMFI of ~250 vs. PTPN22$^{620R/R}$; 45% pDCs IFNα2+ with gMFI of ~500) (122). STAT1 phosphorylation, a marker of interferon receptor signaling, is significantly reduced by about 50% in PBMCs from PTPN22$^{620R/W}$ donors after LPS treatment when compared to PTPN22$^{620R/R}$ donors. T1-IFN-inducible genes (*IRF7*, *MX1*, and *ISG15*) were also significantly reduced by about 50% in PBMC-derived DCs from PTPN22$^{620R/W}$ donors compared to PTPN22$^{620R/R}$ donors, probably due to reduced production of T1-IFNs. TRAF3 is an adaptor protein that links TLR4 and TLR7/8 signaling to induction of T1-IFNs. PTPN22 co-immunoprecipitated TRAF3 from human monocyte derived DCs (moDCs). In transgenic C57BL/6-*Ptpn22*$^{-/-}$ mice expressing either human PTPN22^{620R} or PTPN22^{620W}, PTPN22^{620R} associated with TRAF3 and promoted its poly-ubiquitination and subsequent induction of *Ifnb1* while PTPN22^{620W} did not. C57BL/6-*Ptpn22*$^{-/-}$ mice expressing human PTPN22^{620W} had reduced LPS-induced T1-IFN production [~50% of *Ifnb1* from bone marrow-derived dendritic cells (BMDCs), and ~50% of *Ifnb1*/*Ifna4* from bone marrow-derived macrophages (BMMΦ)] compared to those expressing human PTPN22^{620R} (35).

Like PTPN22^{620W} in humans and transgenic mice, BMMΦ from C57BL/6-*Ptpn22*$^{-/-}$ mice had impaired TLR4-induced T1-IFN (*Ifnb1* and *Ifna4* mRNA production were ~50% less) and decreased TLR4- and TLR3-induced IFN-β production (~60% less) compared to WT BMMΦ (**Figure 4A**). BMMΦ from C57BL/6 mice reconstituted with PEP^{227S}, a phosphatase-inactive mutant, restored TLR-induced *Ifnb1* expression indicating that the phosphatase activity of PEP is not required in this process. C57BL/6-*Ptpn22*$^{-/-}$ BMMΦ have reduced K63-linked polyubiquitination of TRAF3 following LPS stimulation compared to WT BMMΦ. These data are not confined to mouse BMMΦ, pDCs from C57BL/6-*Ptpn22*$^{-/-}$ mice and BXSB/MpJ-*Ptpn22*$^{-/-}$ mice had fewer pDCs making IFNα (~50% reduction) and the pDCs that were making IFNα made less than pDCs from WT mice (again ~50% reduction) (102). Also like PTPN22^{620W} humans, C57BL/6-PEP^{619W} mice had significantly reduced TLR-7-driven T1-IFN serum levels following injection of R848 compared to C57BL/6 mice (~3 ng/ml in C57BL/6-PEP^{619W} mice vs. 5 ng/ml in C57BL/6 mice) (122). The combined data from mice and humans shows that both PTPN22^{620W} and PEP^{619W} are loss-of-function variants with respect to TLR-induced T1-IFN resulting in reduced T1-IFN following TLR stimulation (**Figure 4A**) (35). TLR stimulation does not only induce T1-IFN, it is also capable of priming the NLRP3 inflammasome for subsequent activation following an inflammatory stimulus such as murmamyldipeptide (MDP), an aganoist of nucleotide-binding oligomerization domain-containing protein (NOD2) that is a component of bacterial cell walls. The role of PEP/PTPN22 allotypes in NLRP3 inflammasome may also impact autoimmunity.

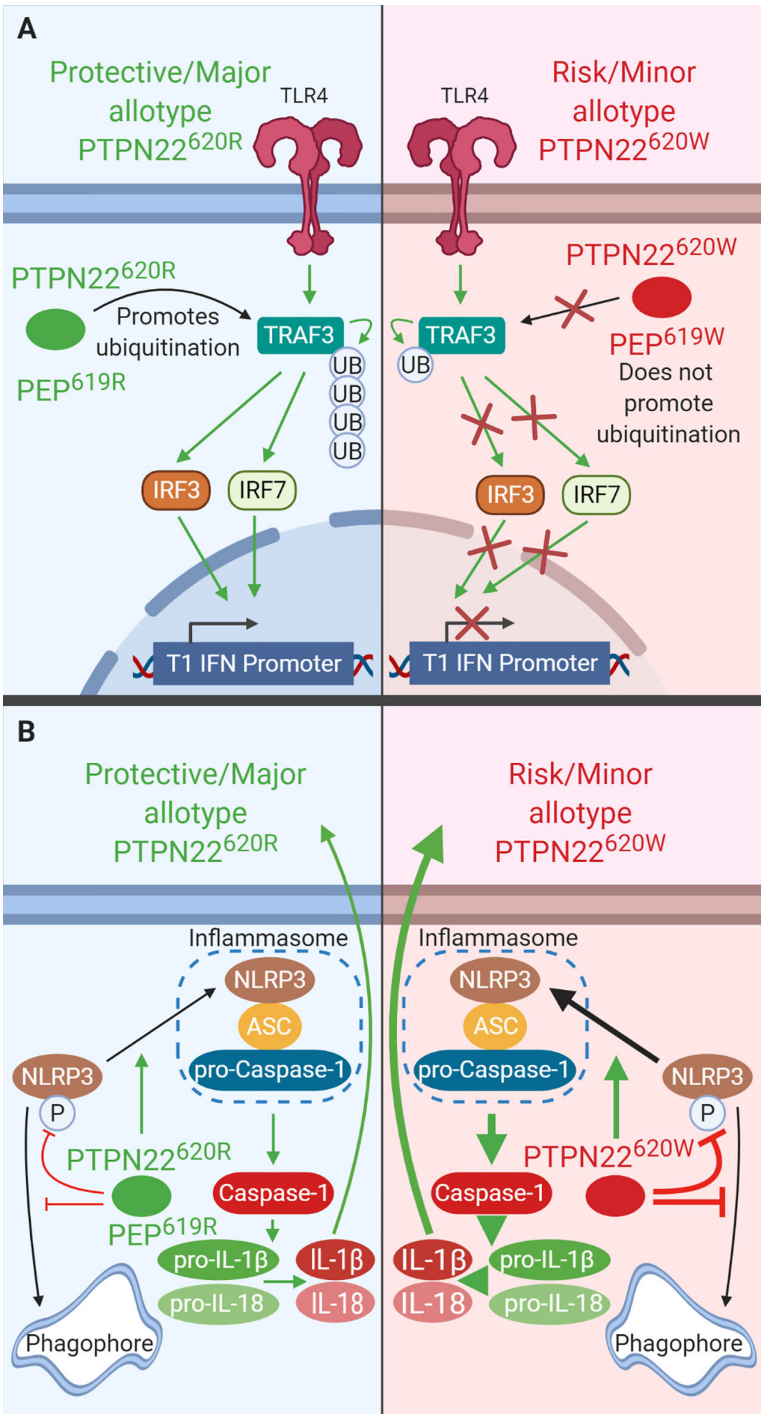

FIGURE 4 | PTPN22 regulates TLR-induced T1-IFN secretion and NLRP3 inflammasome activation in macrophages and DCs. **(A)** PTPN22^{620R} and PEP^{619R} promote T1-IFN secretion in response to TLR-agonists by interacting with TRAF3 and promoting its autoubiquitination and subsequent induction of T1-IFN. PTPN22^{620W} and PEP^{620W} do not interact with TRAF3 and fail to support TLR-induced T1-IFN production. **(B)** PTPN22^{620R} and PEP^{619R} promote NLRP3 inflammasome activation by dephosphorylating NLRP3 and preventing its sequestration into the autophagosome. PTPN22^{620W} is a gain-of-function variant that has enhanced capacity to dephosphorylate NLRP3. This leads to increased NLRP3 inflammasome activation.

NLRP3 and IL-1β

PTPN22^{620R} positively regulates activation of NLRP3 and subsequent release of IL-1β (**Figure 4B**). PTPN22^{620W} is a gain-of-function variant that potentiates NLRP3 activity (**Figure 4B**) (36, 37). PTPN22 dephosphorylates NLPR3 at Y861 which prevents it from being sequestered into phagophores and degraded *via* autophagy (36, 37). PTPN22 knockdown in THP-1 macrophages primed with ultrapure LPS (upLPS) led to increased NLRP3 phosphorylation and increased NLRP3 sequestration in autophagosomes, with a concomitant reduction in IL-1β secretion ranging from about 50% with MDP treatment and up to 80% with monosodium urate (MSU) treatment (36, 37). In support of this, inhibiting autophagy restored IL-1β secretion from PTPN22 knockdown THP-1 cells (37). PTPN22^{620W} is a gain-of-function variant and is better able to dephosphorylate NLRP3 and prevents its sequestration into phagophores and subsequent degradation (**Figure 4B**). PTPN22^{620W} has an enhanced capacity to dephosphorylate NLRP3 in a cell free system compared to PTPN22^{620R} (36). When moDCs from PTPN22$^{620R/W}$ donors were primed with ultrapure LPS and treated with monosodium urate (MSU) cleaved caspase-1 was increase by 500% and produced 300% more mature IL-1β compared to PTPN22$^{620R/R}$ donors (36).

Much like THP-1 cells with PTPN22 knockdown, C57BL/6-*Ptpn22*$^{-/-}$ mice exhibited a 50% reduction in MDP-, MSU-, and ATP-induced IL-1β secretion from BMDCs compared to those of *Ptpn22*-competent mice and this effect was abrogated by inhibition of autophagy (**Figure 4B**) (37). This is due to the catalytic activity of PTPN22. In C57BL/6-*Ptpn22*$^{-/-}$ BMDCs expressing the catalytically dead human PTPN22 (PTPN22^{263Q}) the same effect was observed. Similar to moDCs from PTPN22$^{620R/W}$ donors, BMDCs from C57BL/6-PEP^{619W} mice have less NLRP3 in autophagosomes upon upLPS/MSU treatment and over 50% increased IL-1β secretion compared to C57BL/6 mice (36, 37). The same was seen when comparing BMMΦ from C57BL/6-PEP^{619W} mice with C57BL/6 mice (36). Taken together, these data demonstrate that PTPN22^{620W} and PEP^{619W} are gain-of-function variants with respect to NLRP3 dephosphorylation and enhance NLRP3-inflammasome activation and mature IL-1β release. While signaling *via* NOD2 is capable of activating the NLRP3 inflammasome following priming with LPS, it also induces autophagy and cytokine secretion.

NOD2-Induced Autophagy and Cytokine Secretion

PTPN22 is a negative regulator of NOD2-induced autophagy (**Figure 5A**). Knockdown of PTPN22 *via* shRNA in THP-1 monocytes enhanced NOD2-induced LC3B-II, a cleaved and activated form of LC3B indicative of autophagosome formation. There was also a decrease in p62 protein levels consistent with enhanced autolysosome activity. Knockdown of PTPN22 *via* shRNA in THP-1 monocytes also led to enhanced JNK, p38, NF-κB-p65, and NF-κB-p50, activation downstream of NOD2 while reducing ERK activation. Enhanced NOD2-induced *IL-6* and *TNF* mRNA expression and IL-6, IL-8, and TNF secretion were also seen with PTPN22 knockdown (36, 39). In addition, the reduction in PTPN22 resulted in decreased NOD2-induced

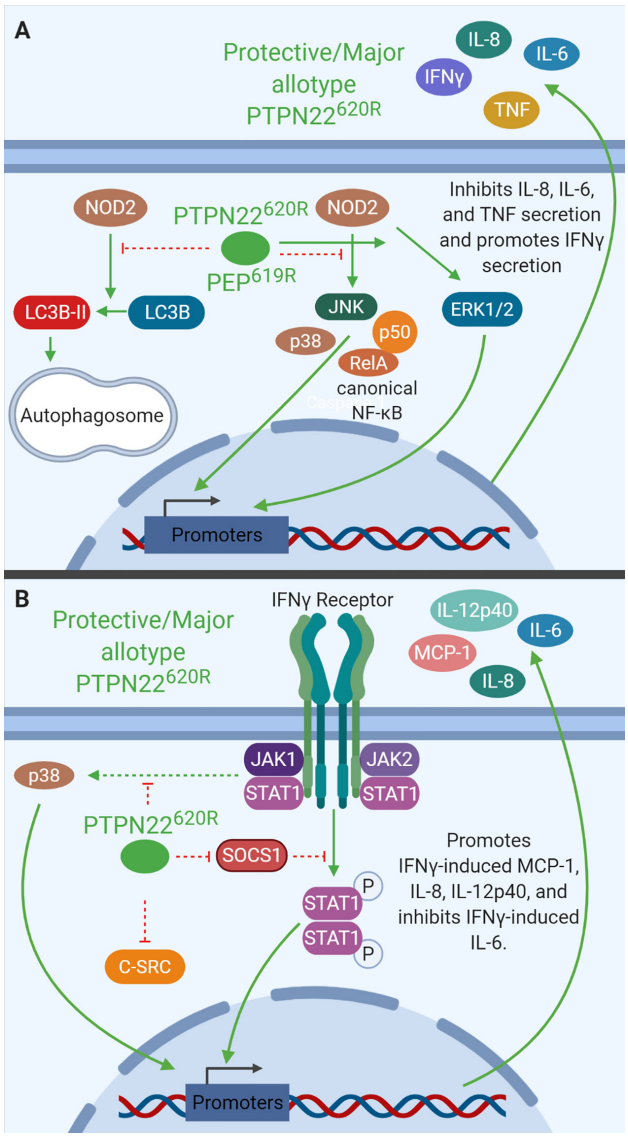

FIGURE 5 | PTPN22 regulates NOD2 and IFNγ signaling in monocytes. **(A)** PTPN22^{620R} and PEP^{619R} negatively regulate NOD2-induced autophagy and NOD2-induced IL-8, IL-6, and TNF secretion while promoting NOD2-induced IFNγ secretion. **(B)** PTPN22^{620R} negatively regulates p38, SOCS1, and C-SRC activation downstream of the IFNγR. PTPN22^{620R} promotes IFNγ-induced MCP-1, IL-8, and IL-12p40, while it inhibits IFNγ-induced IL-6.

ICAM1, NOD2, T-bet, and IFN-γ mRNA expression as well as reduced IFN-γ secretion (39). Interestingly, the variant in PTPN22 is associated with reduced risk of Crohn's disease while loss-of-function mutations in NOD2 are associated with increased risk of Crohn's disease (123, 124). This could indicate that the T1D-risk allotype (PTPN22^{620W}) enhances NOD2 activity to suppress gastrointestinal pathology, however, more studies are necessary to clarify how PTPN22^{620W} alters NOD2 response compared to PTPN22^{620R}. These PTPN22 knockdown studies indicate that PTPN22 negatively regulates NOD2-induced autophagy, IL-6,

IL-8, and TNF production while positively regulating NOD2-induced ICAM1, NOD2, and IFN-γ production.

Like PTPN22 knockdown in THP-1 cells, C57BL/6-*Ptpn22*$^{-/-}$ mice demonstrate that PEP is a negative regulator of NOD2-induced cytokine secretion in BMDCs of mice (**Figure 5A**). BMDCs from *Ptpn22*$^{-/-}$ mice treated with MDP had increased p38, NF-κB p65, and NF-κB p50 phosphorylation, and decreased ERK phosphorylation compared to *Ptpn22*-competent BMDCs. MDP-treated BMDCs from *Ptpn22*$^{-/-}$ mice had increased levels of *IL6* and *TNF* but decreased levels of *NOD2*, *ICAM-1*, and *IFNγ* mRNA compared to *Ptpn22* competent BMDCs (39). MDP-treated *Ptpn22*$^{-/-}$ BMDCs had enhanced IL-6, IL-8, and TNF secretion compared to *Ptpn22*-intact BMDCs (39). These data closely mirror data from PTPN22 knockdown THP-1 cells and demonstrate that PTPN22^{620R} and PEP^{619R} are negative regulators of NOD2-induced autophagy and cytokine secretion (**Figure 5A**). PTPN22 does not only influence signaling downstream of TLRs and other pattern recognition receptors in monocytes, macrophages, and DCs, it also influences cytokine secretion and signaling in response to IFNγ.

IFNγ Receptor Signaling

PTPN22 regulates IFN-γ receptor (IFNγR) signaling in human monocytes (**Figure 5B**). PTPN22 knockdown in THP-1 monocytes followed by treatment with IFNγ induced increased SOCS1 phosphorylation and activity and reduced protein levels of SOCS3 compared to control siRNA transfected cells. PTPN22 pulls down with SOCS1, suggesting that PTPN22 may be responsible for dephosphorylating and inactivating SOCS1 when it is present. In agreement with this, PTPN22 knockdown reduced activation (phosphorylation) of known SOCS1 targets, Jak1, STAT1, and STAT3 in response to IFNγ. It also reduced subsequent production of ICAM1 (~70% reduced), NOD2 (~15% reduced), and T-bet mRNA (~40% reduced) when compared to control siRNA transfected cells. Knockdown of PTPN22 also decreased IFNγ-induced MCP-1 (~70% reduced), IL-8 (~50% less), and IL12p40 (~75% reduced) secretion (29). These data indicate that PTPN22 is a positive regulator of STAT1 and STAT3 activation following IFNγ treatment. Activation of STAT1 and subsequent gene induction is the most well characterized portion of IFNγR signaling, however, the signaling cascade activated by the IFNγR includes many other signaling molecules. Treatment with IFNγ also induces signaling *via* p38 MAPK and Src. Upon knockdown of PTPN22 in THP-1 monocytes, IFNγ-induced p38 MAPK activation and subsequent IL-6 mRNA expression and protein production were enhanced compared to control siRNA transfected cells. This suggests that PTPN22 is negatively regulating p38 MAPK activation downstream of the IFNγR. It is unknown how PTPN22 regulates p38 MAPK activation downstream of the IFNγR, however, there are several plausible targets. Current literature indicates that p38 MAPK is activated by the IFNγR *via* a signaling cascade involving JAK2, Pyk2, MEKK4, MEK6, and finally p38 MAPK (125, 126). Pyk2, MEKK4, and p38 MAPK are attractive potential targets of PTPN22 because they are all activated by phosphorylation on a tyrosine residue. At this time, more targeted research is necessary to define the PTPN22 target(s) in this pathway. Similarly, PTPN22 knockdown induced basal Src phosphorylation that increased after IFNγ treatment; however, in control siRNA transfected cells there was no basal Src phosphorylation nor was there IFNγ-induced Src phosphorylation. This indicates that PTPN22 negatively regulates basal Src activation and IFNγR-induced Src activation (29). While PTPN22 influences response to IFNγ treatment alone it also influences macrophage cytokine secretion following polarization in response to IFNγ/LPS or IL-4/IL-13 treatment.

Macrophage Polarization

In primary MDMs, PTPN22 is a negative regulator of IL-12 and IL-23 production following M1 polarization (**Figure 6A**) and a positive regulator of IL-10 production following M2 polarization (**Figure 6B**). PTPN22 knockdown in MDMs led to increased IL-23 (~60% more) and IL-12 (~30% more) secretion upon IFNγ/LPS treatment (M1 polarization) and decreased IL-10 expression (~50% less) following IL-4/IL-13 treatment (M2 polarization). PTPN22^{620W} appears to be a gain-of-function negative regulator of IL-12 and IL-23 production following M1 polarization (**Figure 6A**). M1 polarized macrophages from PTPN22$^{620W/W}$ donors expressed significantly less IL-12, IL-1β, and IL-6 than those from PTPN22$^{620R/R}$ donors. It is thought that this gain-of-function phenotype is due to enhanced expression of PTPN22^{620W} upon M1 polarization. M1 polarized macrophages from PTPN22$^{620W/W}$ donors expressed significantly more PTPN22 than those from PTPN22$^{620R/R}$ donors. PTPN22^{620W} and PTPN22^{620R} are comparable positive regulators of M2 polarization with no differences in IL-10 expression following IL-4/IL-13 treatment (**Figure 6B**) (38).

Like PTPN22 knockdown in human MDMs, splenic macrophages from C57BL/6-*Ptpn22*$^{-/-}$ mice had increased expression of IL-23 (~200%) and IL-12 (~250%) following M1 polarization (**Figure 6A**) and decreased expression of IL-10 (~50%) following M2 polarization (**Figure 6B**) compared to those from *Ptpn22*-intact mice (38). These *Ptpn22*$^{-/-}$ splenic macrophages had increased NF-κB activity (~200%) compared to *Ptpn22*-intact macrophages and this could explain the increase in LPS/IFNγ-induced IL-12 and IL-23. Splenic macrophages from C57BL/6-*Ptpn22*$^{-/-}$ mice reconstituted *in vitro* with PEP^{619R} or PEP^{619W} and then polarized to M1 or M2 macrophages had no difference in gene expression. If the level of PEP expression is important in mouse macrophages like the level of PTPN22 expression is in human MDMs, then reconstituting macrophages with the same amount or PEP^{619R} and PEP^{619W} would not capture the effects seen in human MDMs where PTPN22^{620W} and PTPN22^{620R} expression levels are different (38). Like human PTPN22^{620W} M1 macrophages, M1 peritoneal macrophages from C57BL/6-PEP^{619W} mice had lower mRNA levels for the M1 genes, iNOS (~50 fold less) and TNF (~2 fold less), than those from WT mice (127). Overall, these data indicate that PTPN22^{620W} and PEP^{619W} are gain-of-function negative regulators of macrophage cytokine secretion following M1 polarization due to increased PTPN22 expression (**Figure 6A**). PTPN22 has multiple roles in macrophage polarization and in fact

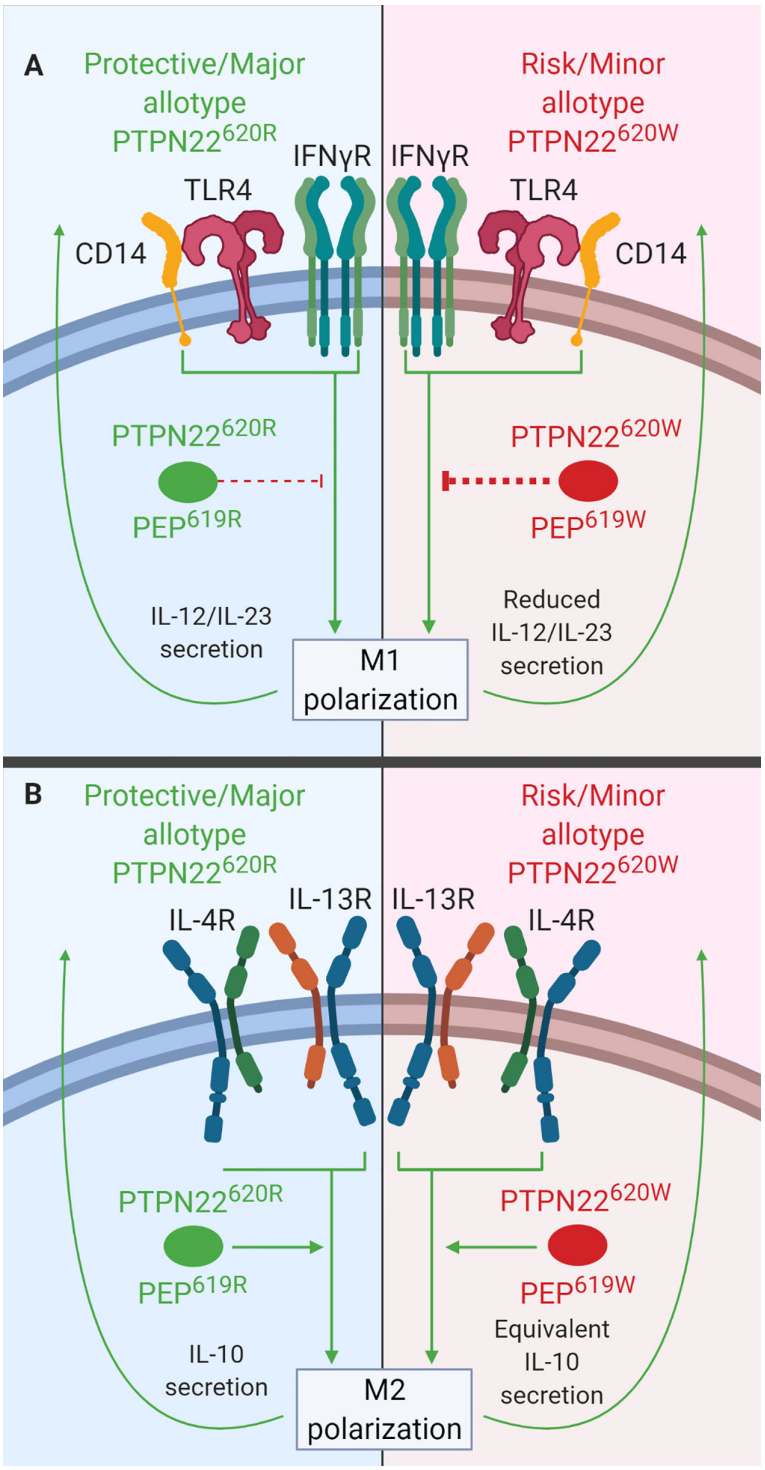

FIGURE 6 | PTPN22 regulates macrophage polarization. **(A)** PTPN22^{620R} and PEP^{619R} inhibit cytokine secretion from M1 macrophages. Upon M1 polarization of PTPN22^{620W} or PEP^{619W} macrophages, there is more PTPN22^{620W} and PEP^{619W} present and the enhanced expression leads to an increased capacity to inhibit cytokine secretion from M1 macrophages. **(B)** PTPN22^{620R} and PTPN22^{620W} promote cytokine secretion from M2 macrophages equivalently.

influences diverse functions in monocytes, macrophages, and DCs. The T1D-associated variant of PTPN22, PTPN22^{620W}, influences a large number of these functions and these cellular phenotypes could contribute to the pathogenesis of T1D.

PTPN22 in Monocytes, Macrophages, and DCs and Impact on T1D

Monocytes, macrophages, and DCs are APCs that are all capable of initiating and enhancing adaptive immune responses. The precipitating events that lead to loss of tolerance and the development of T1D are unknown; be it physiological β cell death, viral infection, bacterial infection, or some other initiating event, monocytes, macrophages, and DCs are the cells most likely to sense β cell death/inflammation and initiate the adaptive immune response. After APCs trigger the adaptive immune response, these cells enhance and support the ongoing immune response against β cells. In APCs, PTPN22^{620R} plays a role in signaling downstream of many PRRs [i.e., TLR4 (35), TLR7/8 (122), NOD2 (36, 37, 39)], cytokine receptors [i.e., IL-4R/IL-13R (38), and IFNγR (29, 128)]. PTPN22^{620W} enhances NLRP3 activation and subsequent IL-1β release following priming *via* TLR4 (LPS) and treatment with a NOD2 agonists (MDP) while dampening the T1-IFN response following TLR4/7/8 stimulation. The combination of these phenotypes renders APCs from PTPN22$^{620R/W}$ and PTPN22$^{620W/W}$ humans more sensitive to NLRP3 activation while dampening their ability to produce T1-IFNs in response to PRR signaling. IL-1β enhances naïve and memory CD4 T cell expansion and this could in turn exacerbate activation of autoreactive CD4 T cells during the initiation of T1D (129). T1-IFNs enhance CD8 T cell activation and support activated T cell survival and are considered a major feature of the diabetic islet microenvironment where they enhance expression of MHC-I on β cells and expression of T cell chemoattractants (e.g., CXCL10) (130–132). Importantly, the T1-IFN phenotype results in a reduction of T1-IFN and not a complete loss. This might reduce the induction of MHC-I and T cell chemoattractants, however, it would not ablate them and in a genetically predisposed individual this may still be more than sufficient to help initiate and sustain T1D especially in combination with enhanced IL-1β production.

NEUTROPHILS

While neutrophils are not essential for T1D pathology (133, 134), they do play a role in other *rs2476601*-associated autoimmune diseases (e.g., RA, SLE). Thus, it is paramount to consider how PTPN22 influences neutrophil function (135). Importantly, PTPN22 is expressed in neutrophils and PTPN22^{620} allotype influences neutrophil function. This section will review what is known about the function of PTPN22^{620R} and PTPN22^{620W} in human neutrophils. PTPN22 protein level does not vary when comparing neutrophils from PTPN22$^{620R/R}$ and PTPN22$^{620R/W}$ donors; however, at time of writing, PTPN22$^{620W/W}$ donors have not been assessed for neutrophil PTPN22 content (30). In human neutrophils, PTPN22 plays a role in protein citrullination (30), neutrophil extracellular trap formation (NETosis) (**Figure 7A**) (30), transmigration across inflamed endothelium (31), and response to N-formyl- Methionine-Leucine-Phenylalanine (fMLP) (**Figure 7B**) (31). PTPN22^{620R} has been shown to interact with PAD4 in human neutrophils and is a negative regulator of PAD4 activity and NETosis while PTPN22^{620W} is a loss-of-function variant in this process (**Figure 7A**) (30). PTPN22^{620W} potentiates neutrophil calcium flux and ROS production in response to fMLP stimulation (**Figure 7B**) as well as transmigration across inflamed epithelium when compared to PTPN22^{620R} (31).

Protein Citrullination and NETosis

PTPN22 is a negative regulator of protein citrullination and NETosis and PTPN22^{620W} is a loss-of-function variant (30) (**Figure 7A**). Neutrophils from heterozygous PTPN22$^{620R/W}$ donors displayed a hypercitrullinated protein profile (~4 fold more in PTPN22$^{620R/W}$ neutrophils), they had enhanced citrullination of histone H3, a marker of NETosis (~5 fold more in PTPN22$^{620R/W}$ neutrophils), and they were more prone to NETosis (3%–15% of PTPN22$^{620R/W}$ neutrophils vs. ~2% of PTPN22$^{620R/R}$ neutrophils) compared to those from PTPN22$^{620R/R}$ donors (30, 136). PAD4 co-immunoprecipitated PTPN22 in human neutrophils and PTPN22 allotype influences this interaction; there is a significantly decreased amount of PTPN22 co-immunoprecipitated with PAD4 in heterozygous PTPN22$^{620R/W}$ donors when compared to PTPN22$^{620R/R}$ donors (~66% decreased). The total PTPN22 protein level was the same between donors implying that PTPN22^{620R} interacts with PAD4 more than PTPN22^{620W}. In C57BL/6-*Ptpn22*$^{-/-}$ mouse macrophages transfected with human PTPN22^{620R} or PTPN22^{620W} expressing constructs, PTPN22^{620R} but not PTPN22^{620W} reduced protein citrullination and co-immunoprecipitated with PAD4 further supporting the lack of association of PTPN22^{620W} with PAD4 (30).

Much like in human neutrophils, PEP in C57BL/6 mouse neutrophils interacts with PAD-4. PEP co-immunoprecipitated with PAD-4. The absence of PEP in C57BL/6 mice enhanced protein citrullination by approximately 100%; however, the enhanced protein citrullination was abrogated in the presence of a catalytically dead PEP indicating that the catalytic activity of PEP is not involved in this process. Unlike in humans, PEP does not specifically impact histone H3 citrullination or NETosis in mouse neutrophils (30). Taken together, these data indicate that PTPN22^{620R} is a negative regulator of protein citrullination and NETosis in human neutrophils and PTPN22^{620W} is a loss-of-function variant (**Figure 7A**).

Transmigration, ROS Production, and Calcium Flux

PTPN22 plays a role in transmigration across inflamed endothelium, as well as the response to fMLP, a highly chemotactic n-formylated oligopeptide actively released by invading bacteria or passively released by mitochondria of dying host cells (31, 137, 138). Significantly more neutrophils from PTPN22$^{620R/W}$ donors transmigrate across inflamed (TNF treated) endothelium over 2 min than those from PTPN22$^{620R/R}$ donors (PTPN22$^{620R/W}$ = 43 ± 9% vs. PTPN22$^{620R/R}$ = 24 ± 4%). Stimulation of neutrophils from healthy PTPN22$^{620R/W}$ donors with fMLP resulted in increased calcium flux compared to

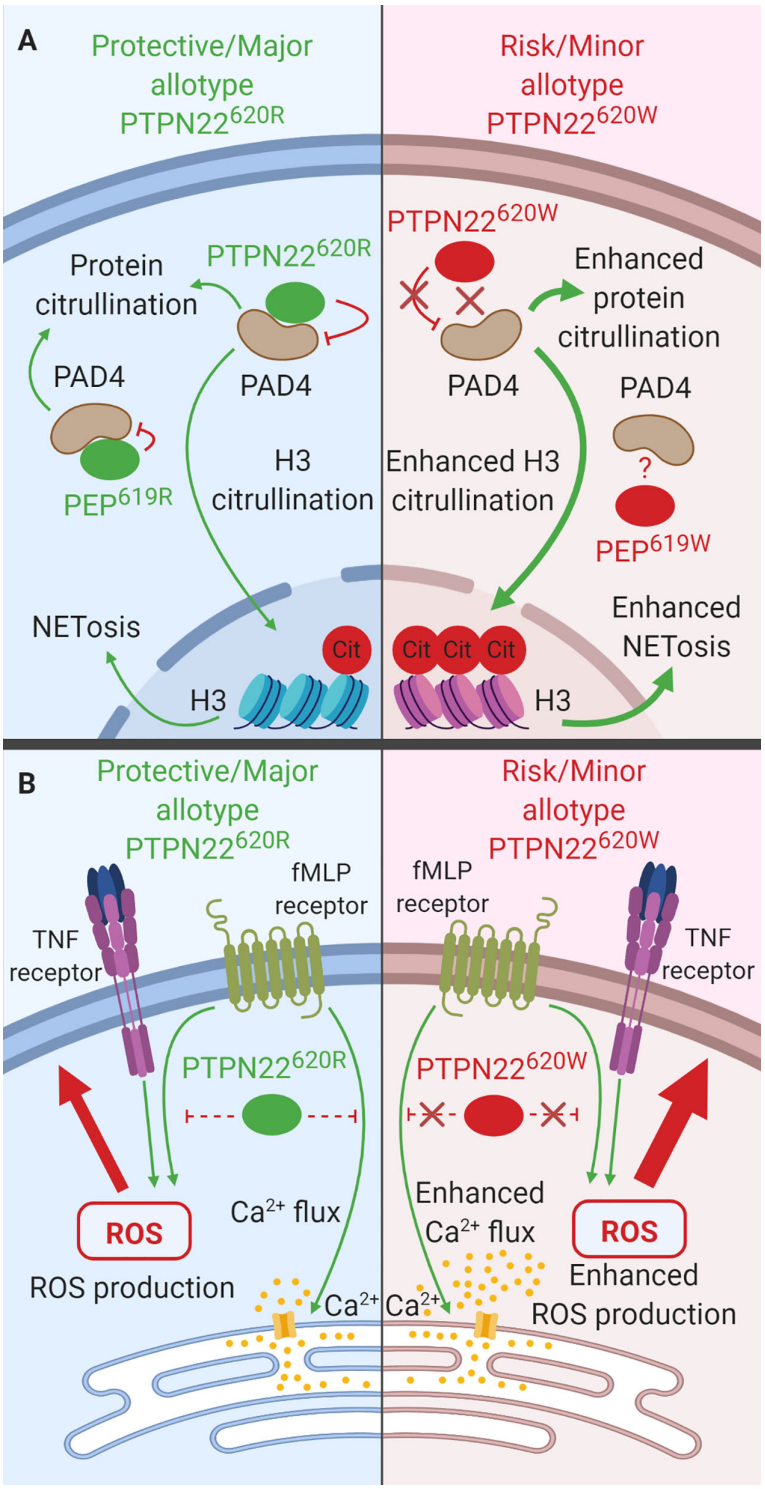

FIGURE 7 | The function of PTPN22 in Neutrophils. **(A)** PTPN22^{620R} and PEP^{619R} are negative regulators of PAD4 activation and subsequent citrullination of target proteins in neutrophils. PTPN22^{620W} is a loss-of-function variant that potentiates PAD4 activation and citrullination of PAD4 targets. **(B)** PTPN22^{620R} is a negative regulator of fMLP induced calcium flux and ROS production while PTPN22^{620W} a loss-of-function variant that results in enhanced fMLP-induced calcium flux and ROS.

neutrophils from healthy PTPN22$^{620R/R}$ donors (PTPN22$^{620R/W}$ = 0.28 ± 0.02 vs. PTPN22$^{620R/R}$ = 0.24 ± 0.02 Indo-1 ratio). Priming of neutrophils from healthy PTPN22$^{620R/W}$ donors with TNF followed by stimulation with fMLP resulted in significantly increased ROS production (4-fold increase) compared to PTPN22$^{620R/R}$ donors (**Figure 7B**) (31).

Unlike in humans, PEP does not appear to play a role in transmigration across inflamed endothelium or the response to fMLP in C57BL/6 mice. *Ptpn22*$^{-/-}$ and *Ptpn22*-intact mouse neutrophils migrated across TNF-treated endothelium at the same rate (139). *Ptpn22*$^{-/-}$ and *PTPN22*-intact neutrophils produce similar amounts of ROS in response to fMLF (also called fMLP) and PMA stimulation, however, they were not primed with TNF like the human neutrophils which may explain why there was no difference in ROS production. Neutrophils from C57BL/6-*Ptpn22*$^{-/-}$ mice did however exhibit decreased ROS production (~50% reduced) and degranulation (~25% reduced) in response to FcγR and integrin stimulation compared to neutrophils from C57BL/6 mice. These pathways have not been investigated in the context of PTPN22 in humans (139). In human neutrophils, PTPN22^{620W} enhances transmigration across inflamed endothelium, calcium flux in response to fMLP stimulation, and ROS production in response to TNF priming followed by fMLP stimulation.

PTPN22 in Neutrophils and Impact on T1D

Current data indicates that neutrophils most likely do not play a direct role in the pathogenesis of T1D in humans (133, 134) and it is apparent that they do not influence pathogenesis in NOD mice; depletion of neutrophils starting at 4 weeks of age does not impact development of T1D in NOD mice (134). While data indicate that neutrophils do not play a role in human T1D pathogenesis, many neutrophil products (e.g., ROS, NETs, cytokines) are capable of damaging tissues, including pancreatic β cells (140). Neutrophils from PTPN22$^{620R/W}$ donors had enhanced calcium flux and ROS production in response TNF priming followed by treatment with fMLP compared to those from PTPN22$^{620R/R}$ donors. These PTPN22$^{620R/W}$ neutrophils also transmigrated across TNF-inflamed epithelium faster than their PTPN22$^{620R/R}$ counterparts, displayed enhanced protein citrullination, and were more prone to NETosis (30, 31, 136). The combined effects of these phenotypes mean that PTPN22$^{620R/W}$ and PTPN22$^{620W/W}$ patients with T1D could display enhanced neutrophil accumulation in the exocrine pancreas due to enhanced transmigration across inflamed epithelium and increased frequency of these infiltrating neutrophils releasing NETs and producing high amount of ROS. More studies need to be undertaken to understand if neutrophils participate in the pathogenesis of human T1D and if the influence of PTPN22^{620} allotype effects their participation. Overall, it is clear that PTPN22 plays diverse roles in many cell types that have the potential to influence the pathogenesis of T1D.

CONCLUSIONS

PTPN22 acts as a negative regulator of TCR and BCR signaling by preventing weak TCR/BCR ligation from activating T cells or B cells. In addition, PTPN22 functions in diverse signaling pathways in leukocytes. This phosphatase downregulates signaling in the NOD2, IFNγ/LPS, IFNγR, and fMLP receptor signaling pathways. Conversely, PTPN22 positively regulates NLRP3 inflammasome activation, TLR4/7/8 induction of T1-IFN secretion, PAD4 activation, and IL-4/IL-13 signaling. There are several rare genetic variants of *PTPN22* in humans that are associated with increased or decreased risk of autoimmune diseases. Also, *rs2476601* marks the PTPN22^{R620W} variant that is associated with increased risk for T1D and many other autoimmune diseases (28, 51–59). The 620R->W conversion creates a gain-of-function variant that suppresses TCR/BCR signaling and impacts autoimmunity by increasing the number of autoreactive T cells and B cells that escape central tolerance. Similarly, *rs56048322*, marks the variant, PTPN22^{K750N}, and is associated with increased risk of T1D. The 750K->N conversion induces alternative splicing of PTPN22 that results in a novel isoform that competes with other PTPN22 isoforms for CSK binding causing T cell hyporesponsiveness and, like *rs2476601*, could allow more autoreactive T cells to escape central tolerance (48). In contrast, *rs33996649*, encodes the variant, PTPN22^{R263Q}, which has diminished phosphatase activity and reduces risk for SLE and RA possibly by enhancing T cell central tolerance (49, 50).

The mouse orthologue of *PTPN22*, *Ptpn22* encoding PEP, plays similar roles to human PTPN22 and is even included in one of the *insulin-dependent diabetes* (*Idd*) intervals, *Idd18.2* (141). While rodent models, especially the NOD mouse, have been integral to furthering our understanding of T1D, the analogous mutation to PTPN22^{R620W}, PEP^{R619W}, is not naturally present in NOD and does not induce the same phenotype as observed in humans. This is not entirely surprising, PTPN22 and PEP are two of the most divergent phosphatase orthologues between humans and mice (38, 142). PTPN22 and PEP share 70% amino acid identity overall and only 61% amino acid identity in the c-terminal domain, where *rs2476601* lies (38, 45, 51, 142).

PTPN22 is also expressed in NK cells, monocytes, macrophages, DCs, and neutrophils where it influences diverse signaling pathways (28, 68). The expression of PTPN22 in APCs adds another layer of possible confounding factors when interpretting data in TCR and BCR signaling due to the fact that APCs directly influence T cell and B cell activation. Data describing the influence of PTPN22 on interactions of APCs with T cells in humans is lacking, but there are hints in both human and mouse data that can inform future studies. T cell/macrophage interactions are largely mediated by IFNγ/IFNγR and CD40L/CD40 in an antigen-dependent context (143, 144). PTPN22 knockdown in THP-1 monocytes had diverse effects on the IFNγR signaling pathways. PTPN22 knockdown increased activation of SOCS1, and predictably led to lower activation of JAK1, STAT1, and STAT3, known SOCS1 targets, as well as lower mRNA expression of ICAM-1, NOD2, and T-bet. PTPN22 knockdown also enhanced IFNγR-induced p38 activation and subsequent IL-6 mRNA and protein expression (29). There have

not been any studies of the impact of PTPN22 on human CD40 signaling however heterozygous and homozygous PTPN22^{620W} donors have increased CD40 expression on their immature B cells compared to PTPN22^{620R} donors promoting speculation that CD40 signaling would be enhanced in these cells (100, 103). All of these data emphasize the need to elucidate how PTPN22^{620W} influences human macrophage and DC expression of CD40, MHC-II, CD80 and CD86.

At this time, there are more questions than answers pertaining to the influence of *rs2476601* on TCR signaling and the interface of the innate and adaptive arms of the immune system. More studies aimed at illucidating the impact PTPN22^{620W} has on TCR signaling and innate immune cell/adaptive immune cell interactions and crosstalk in humans need to be undertaken, especially in light of the conflicting data between mouse PEP^{619W} and human PTPN22^{620W} studies, to answer these questions.

AUTHOR CONTRIBUTIONS

LA, MW, and CM wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Tolerance to Proinsulin-1 Reduces Autoimmune Diabetes in NOD Mice

Gaurang Jhala[1,2], Claudia Selck[1†], Jonathan Chee[3,4], Chun-Ting J. Kwong[1], Evan G. Pappas[1], Helen E. Thomas[1,2], Thomas W.H. Kay[1,2‡] and Balasubramanian Krishnamurthy[1,2‡]*

[1] St. Vincent's Institute, Fitzroy, VIC, Australia, [2] Department of Medicine, The University of Melbourne, St Vincent's Hospital, Fitzroy, VIC, Australia, [3] National Centre for Asbestos Related Diseases, Institute of Respiratory Health, University of Western Australia, Perth, WA, Australia, [4] School of Biomedical Sciences, University of Western Australia, Perth, WA, Australia

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Edited by:
F. Susan Wong,
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***Correspondence:**
Thomas W.H. Kay
tkay@svi.edu.au

†Present address:
Imperial College London,
United Kingdom

‡These authors have contributed equally to this work and share senior authorship

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T-cell responses to insulin and its precursor proinsulin are central to islet autoimmunity in humans and non-obese diabetic (NOD) mice that spontaneously develop autoimmune diabetes. Mice have two proinsulin genes proinsulin -1 and 2 that are differentially expressed, with predominant proinsulin-2 expression in the thymus and proinsulin-1 in islet beta-cells. In contrast to proinsulin-2, proinsulin-1 knockout NOD mice are protected from autoimmune diabetes. This indicates that proinsulin-1 epitopes in beta-cells maybe preferentially targeted by autoreactive T cells. To study the contribution of proinsulin-1 reactive T cells in autoimmune diabetes, we generated transgenic NOD mice with tetracycline-regulated expression of proinsulin-1 in antigen presenting cells (TIP-1 mice) with an aim to induce immune tolerance. TIP-1 mice displayed a significantly reduced incidence of spontaneous diabetes, which was associated with reduced severity of insulitis and insulin autoantibody development. Antigen experienced proinsulin specific T cells were significantly reduced in in TIP-1 mice indicating immune tolerance. Moreover, T cells from TIP-1 mice expressing proinsulin-1 transferred diabetes at a significantly reduced frequency. However, proinsulin-1 expression in APCs had minimal impact on the immune responses to the downstream antigen islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) and did not prevent diabetes in NOD 8.3 mice with a pre-existing repertoire of IGRP reactive T cells. Thus, boosting immune tolerance to proinsulin-1 partially prevents islet-autoimmunity. This study further extends the previously established role of proinsulin-1 epitopes in autoimmune diabetes in NOD mice.

Keywords: type 1 diabetes, proinsulin-1, CD4+ T cells, immune tolerance, NOD mice

INTRODUCTION

Recognition of proinsulin by the immune system is a major determinant in the pathogenesis of autoimmune diabetes in both humans and non-obese diabetic (NOD) mice (1, 2). A polymorphic variable number of tandem repeats (VNTR) located in the promoter region of the insulin locus controls the transcription level of the *Ins* gene and is strongly associated with susceptibility to type 1 diabetes (T1D) in humans (3–5). Mice do not have a VNTR upstream of the insulin locus; however, they have two Insulin genes, *Ins1* and *Ins2* encoding proteins that are highly homologous with 92% identity at the

amino acid level. Proinsulin 1 and 2 proteins have identical A chains but differ by two amino acids in the B chain, three amino acids in the connecting peptide (C-peptide) and six amino acids in the leader peptide (6). The two proinsulin isoforms are differentially expressed with proinsulin 1 (PIns1) predominantly expressed in the pancreatic beta-cells and proinsulin 2 (PIns2) being the predominant isoform detected in the thymus (7–9).

Immune responses to native insulin peptides, in particular the B chain amino acids 9-23 (Ins B:9-23), are essential for autoimmune diabetes in NOD mice (10, 11). The two proinsulin isoforms differ by a single amino acid in the B: 9-23 region (PIns1: B9 proline, PIns2: B9 serine) and strong cross-reactivity of T cells for the Ins B: 9-23 epitope in both proinsulin molecules has also been reported (12). Despite the high degree of homology in the B:9-23 epitope and cross-reactivity of T cells for the Ins B: 9-23 epitope, a divergent immune response was observed when NOD mice were immunized with either Ins1 B:9-23 or Ins2 B:9-23 peptides, with Ins2 peptide conferring protection from diabetes onset, whereas Ins1 peptide did not prevent disease (13, 14). Further differences in cellular and humoral immune responses to both proinsulin isoforms have been highlighted by individual gene knockouts. NOD mice lacking *Ins2* gene develop accelerated diabetes, ascribed to loss of central tolerance to insulin peptides; however, development of insulin autoantibodies (IAA) in *Ins2* -/- mice suggests that immune responses against PIns1 epitopes are intact (15). In contrast, genetic deletion of *Ins1* or replacement of murine *Ins1* with human insulin gene (*INS*) in NOD mice provides significant protection from diabetes (16, 17). Protection from diabetes in NOD mice lacking *Ins1* is likely due to the absence of cognate antigen in the target tissue, indicating that PIns1 peptides may be primarily targeted by insulin reactive T cells. Immunogenic epitopes in the PIns1 molecule have been reported (18), and T cells recognizing PIns1 amino acids 47-64 in the C-peptide region induce diabetes in NOD.SCID recipients (19). Thus, epitopes in PIns1 molecule may contribute to islet autoimmunity.

In contrast to NOD mice, non-autoimmune strains lacking *Ins2* globally (20), or in medullary thymic epithelial cells (mTECs) did not develop pathological islet destruction however, when C57Bl/6 mice lacking Ins2 in mTECs were crossed to *Ins1* knockout mice, the progeny developed spontaneous autoimmune diabetes within 3 weeks after birth (21). These studies suggest that thymic expression of PIns1 may add to the effect of PIns2 in eliminating insulin-specific autoreactive T cells. Constitutive or temporal expression of PIns2 in APCs induces recessive tolerance to PIns2 as it provides lasting protection from autoimmune diabetes in NOD mice (22). These mice were also thought to be tolerant to PIns1 epitopes because of cross-reactivity of the T cells to the conserved Ins B: 9-23 epitope. However, the role of PIns1 specific immune responses in pathogenesis of islet autoimmunity in NOD mice remains unclear, given the differential immune response observed upon immunization with Ins1 B:9-23 or Ins2 B:9-23 peptide. To resolve this, we investigated the impact of induced PIns1 expression in APCs on the development of antigen-specific T cells as well as insulitis and diabetes in NOD mice.

MATERIALS AND METHODS

TetO-Ins1 Mice

To generate the TetO-Ins1 construct, a 411 bp cDNA fragment spanning the coding region of murine PIns1 was amplified by PCR using NOD pancreatic islet cDNA as a template and cloned into HindIII and EcoRV sites of the pTRE-tight plasmid (Clontech). A 1100 bp transgene cassette comprising of the TetO-minimal CMV promoter, followed by the PIns1 gene and a polyA signal was excised between Xho I sites and purified for injection into fertilized NOD/Lt ova using standard techniques. Founders and transgene positive offspring were screened by PCR using primers spanning the PIns1 gene (5'-TTAAGATATCTTCATTCATTATAGAACTC -3') and the tetO-CMV promoter (5'-TCAGTGATAGAGAACGTATGTCG -3').

Other Mice

NOD/Lt mice were bred and housed at the bioresources center St. Vincent's Hospital, Fitzroy. The NOD-IEα-tTA mice that drive the expression of tetracycline transactivator (tTA) under the control of MHC class II IEα promoter have been previously described (23) and were obtained from Prof. C. Benoist and Prof. D. Mathis (Dept of Pathology, Harvard Medical School, Boston, Massachusetts, USA). Generation of NOD8.3 mice, which express the TCRαβ rearrangements of the H-2Kd-restricted, β cell-reactive, CD8+ T cell clone NY8.3, was previously described in detail (24). TIP-1/8.3 mice were generated by crossing NOD-IEα-tTA-TetO-Ins1double transgenic TIP-1 mice with TCR transgenic NOD8.3 mice. All mice were bred, maintained and used under specific pathogen free conditions at St Vincent's Institute (Melbourne, Australia). All experimental procedures followed the guidelines approved by the institutional animal ethics committee.

Doxycycline Treatment

Untreated TIP-1 mice constitutively express proinsulin-1 in antigen presenting cells (APCs). To turn-off proinsulin-1 expression, doxycycline hyclate (Dox) (Sigma-Aldrich) was administered *via* drinking water at concentration of 2mg/ml. Water bottles were changed thrice weekly.

RT-PCR

For total RNA extraction, whole spleen and thymus were harvested in cold Phosphate Buffered Saline (PBS). Tissue homogenates were prepared in RNA lysis buffer RA1 (Macherey-Nagel) from a 15mg slice of tissue using a tissue homogenizer. RNA was isolated using Nucleospin RNA II-isolate kits (Macherey-Nagel), and first strand cDNA was generated from 500ng RNA using High Capacity cDNA Reverse Transcription kits (Applied Biosystem) according to the manufacturers' instructions. cDNA was diluted (1:20) and Real-time PCR analysis was performed using Rotor-Gene-RG-3000 cycler (Corbett Research, Sydney, Australia). Taqman gene expression primers murine insulin 1 (*Ins1*; Mm01950294_s1), murine β-actin (*Actb*; Mm00607939_s1) and murine Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*; Mm99999915_g1) were purchased from Applied Biosystems. To

determine relative expression, Ct values of Insulin were subtracted from Ct values of reference genes for each sample and the difference (dCt) was plotted to determine the abundance of the gene of interest.

Histology and Immunohistochemistry

For immunohistochemistry, pancreata were snap-frozen in optimal cutting temperature compound (OCT Compound; Sakura Finetek, Torrance, CA) and stored at −80°C. For histological analysis 5-µm frozen sections of pancreas were prepared from three levels (200 µm apart), acetone fixed, stained with guinea pig anti-insulin followed by horseradish peroxidase–conjugated anti–guinea pig Ig (Dako Cytomation, Carpenteria, CA) and counterstained with hematoxylin. Insulitis was graded using the following scale: 0 = no infiltrate, 1 = peri-islet infiltrate, 2 = extensive (>50%) peri-islet infiltrate, 3 = intraislet infiltrate, and 4 = extensive intraislet infiltrate (>80%) or total β-cell loss. The percentage of islets with each grade per pancreas was calculated by addition of the grades for the three sections. Individual insulitis scoring for each mouse was performed as previously described (22).

Incidence of Diabetes and Insulitis

Diabetes onset was monitored by weekly measurement of urine glucose levels using Diastix (Bayer Diagnostics). Blood glucose levels were measured in mice with glycosuria using Advantage II Glucose strips (Roche). Animals displaying two consecutive blood glucose measurements of ≥ 15mmol/L were considered diabetic. For adoptive transfer of diabetes, 2 x10^7 splenocytes from 13-17 week old pre-diabetic TIP-1 mice or control NOD mice were transferred (i.v.) into 9-12week old NOD Rag-/- recipients and diabetes development was monitored as above.

Flow Cytometry

Antibodies used were anti-CD4 (RM4-5) conjugated to PerCpCy5.5, anti-CD3 (145-2C11) conjugated to FITC or anti-CD3 (500 A2) V500, anti-CD44 (1M7) conjugated to AlexaFlour700 (all BD Biosciences), anti-CD11c (N418), anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-F4/80 (BM8) conjugated to eFlour450 and anti-FoxP3 (FJK-16S) conjugated to APC (all eBiosciences), anti-CD8a (5H10) conjugated to Pacific Orange (Invitrogen) or anti-CD8a (53-6.7) conjugated to PE-Cy7, anti-CD62L (MEL-14) conjugated to APC-Cy7 (BD Biosciences). FoxP3 was stained intracellularly using FoxP3/Transcription Factor Fixation/Permeabilization kit (eBiosciences). Data were collected on an LSR Fortessa flow-cytometer (BD) and analyzed using FlowJo (Treestar) software.

Tetramer and Magnetic Bead-Based Enrichment

The tetramer and magnetic bead-based enrichment method was previously described (25). I-Ag7 tetramers were obtained from NIH tetramer core facility (Emory University, Georgia, USA), Kd-tetramers were obtained from ImmunoID (Parkville, Victoria, Australia). To enrich insulin-specific CD4+ T cells single cell suspensions from peripheral lymphoid organs (PLO), (pooled spleen and non-draining lymph nodes), were stained with phycoerythrin (PE)-conjugated I-Ag7-$INSB_{10-23}$ (HLVERLYLVCGGEG) tetramer for 1 hour at room temperature. The Ins B_{10-23} peptide in the I-Ag7-$INSB_{10-23}$ tetramer has been mutated (Glutamic acid to Glycine (E-G) at position 20 and Arginine to Glycine (R-G) at position 21) to improve its binding to the I-Ag7 molecule, which allows for better detection of insulin-specific CD4+ T cells (26). Insulin-specific CD8+ T cells were enriched from pooled PLO by staining the cell suspensions with APC-conjugated H-2Kd- $INSB_{15-23}$ (LYLVCGGEG) tetramer for 1 hour on ice. Hen Egg Lysozyme I-Ag7-HEL (AMKRHGLDNYRGYSL) tetramer or H-2Kd-TUM (KYQAVTTTL) were used as controls. Cells were then washed and stained with anti-PE or anti-APC microbeads (Miltenyi Biotec) followed by magnetic separation using an AutoMACSpro (Miltenyi Biotec) according to manufacturer's instructions. $IGRP_{206-214}$ specific CD8+ T cells (H2-Kd, VYLKTNVFL) were stained and enriched as previously described (27). The separated fractions were stained and analyzed by flow cytometry. Gating strategy for tetramer enrichment was as follows: single cells were gated on forward and side scatter, and dead cells excluded using propidium iodide. From the live cell population, CD11c-CD11b-B220-F4/80-CD3+ cells were gated as the T cell population for analysis. Further selection of CD4+ T cells or CD8+ T cells was followed by analysis of the insulin or IGRP tetramer positive population respectively.

Insulin Autoantibody (IAA) Assay

A non-competitive IAA assay was performed in a 96 well ELISA format as previously described (28, 29). Briefly, an ELISA plate (Costar) was coated with or without human insulin (10 µg/ml, Actrapid, Novo Nordisk) overnight at 4°C. Wells were blocked with PBS containing 2% BSA for 2 hours and room-temperature and then probed with sera from 12-15 weeks old TIP mice, NOD or C57BL/6 mice (1:10 dilution) for 2 hours. Wells were washed 4 times and a biotinylated anti-mouse IgG1 (AbCam, 1:10000 dilution) antibody was added for 30 minutes. After washing, horse-radish-peroxidase conjugated streptavidin (BioLegend) was added for 15 minutes. The plate was washed five times, TMB substrate solution (BioLegend) was added and absorbance was measured at 450 nm using a Polarstar (BMG labtech) microplate reader. Each sample was run in duplicate and absorbance (450 nm) of test sample without plate bound insulin was subtracted from absorbance of test sample with plate bound insulin to calculate the actual absorbance value for each sample.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 Software (GraphPad, San Diego, CA, USA). Pooled data are shown as dot-plots with individual mice and the mean ± SEM. Data were tested for normal distribution using D'Agostino-Pearson omnibus normality test or Shapiro-Wilks test. Comparisons between two groups were performed using two-tailed unpaired student *t*-tests. Multiple comparisons were performed using One-way ANOVA with Sidak's post-hoc test. Survival curves were compared using Log-Rank (Mantel-Cox) test. Statistical significance was defined as $P < 0.05$.

RESULTS

Conditional Expression of Proinsulin-1 in NOD Mice

To test whether inducing immune tolerance to proinsulin-1 (PIns1) influenced autoimmune diabetes we generated transgenic NOD mice to facilitate conditional expression of PIns1 in the antigen presenting cells (APCs). Reporter NOD mice expressing PIns1 under the control of the tetracycline-responsive CMV promoter (TetO-Ins1 mice) were bred with previously described driver NOD mice expressing TetR-VP16 tetracycline transactivator protein (tTA) under the control of IEα-MHC-II promoter referred to as TA-NOD mice (23). Bi-transgenic progeny referred to as TIP-1 (Tet Inducible PIns1) mice (**Figure 1A**) express Pins1 in the APCs, which can be turned-off upon doxycycline (Dox) treatment. Analysis of PIns1 expression in TIP-1 mice revealed that PIns1 transgene was expressed in the thymus and spleen as measured by RT-PCR (**Figure 1B**). After one week of Dox treatment, PIns1 expression dropped to baseline levels (**Figure S1**). Thus, PIns1 expression in TIP-1 mice was conditional, and tightly regulated.

TIP-1 Mice Have Reduced Insulitis and Insulin Autoantibody Expression

We recently reported that constitutive or temporal expression of PIns2 (PIns2) in the APCs limited to the perinatal period prevented insulitis and diabetes in NOD mice (22). To test whether PIns1 expression in the APCs influenced the progression of islet autoimmunity, we examined the immune infiltrate (insulitis) in the pancreata of TIP-1 mice expressing PIns1 continuously. At 12-14 weeks of age, insulitis was significantly reduced in TIP-1 mice expressing PIns1 compared to age matched NOD mice or TIP-1 mice fed dox to suppress PIns1 expression (**Figures 2A–C**). Analysis of pancreas histology from TIP-1 mice at 20-25 weeks of age revealed that approximately 50% of the islets examined were free of insulitis, whereas more than 80% of the islets examined from non-transgenic littermates were infiltrated (**Figures 2D, E** and **Table S1**), indicating that PIns1 expression in the APCs decreased but did not completely abolish development of insulitis, which progressed over time. Production of insulin autoantibodies (IAA) indicates spontaneous anti-insulin autoimmunity and IAA are frequently detected prior to diabetes onset in both humans and NOD mice (30, 31). We examined whether induced PIns1 expression in TIP-1 mice influenced B cell mediated humoral responses against insulin by measuring IAA in TIP-1 mice. IAA was significantly reduced in 12-15 weeks old TIP-1 mice as compared to age matched non-transgenic NOD mice (**Figure 2F**). Previously described PIns2 tolerant NOD-PI mice that are protected from diabetes and non-autoimmune prone C57BL/6 mice were used to set the baseline. Collectively, these results indicate that immune tolerance to PIns1 influenced progression of insulitis and reduced the development of IAA.

PIns1 Overexpression Partially Suppresses Spontaneous Diabetes in NOD Mice

Reduced insulitis and IAA suggest that diabetes development may be altered in TIP-1 mice. A cohort of female TIP-1 mice expressing PIns1 continuously and control NOD mice were observed for incidence of spontaneous diabetes. TIP-1 mice developed diabetes but at a significantly reduced incidence compared to non-transgenic control NOD mice. By 300 days of age 40% of TIP-1 mice and 65% of the control mice developed diabetes (**Figure 3A**). In addition, we investigated whether PIns1 expression in TIP-1 mice influenced the pathogenic potential of effector T cells. Splenocytes from 15-18 weeks old TIP-1 mice with ongoing expression of PIns1 and age matched control NOD mice were transferred into NOD.Rag1 -/- recipients. All recipient mice receiving control splenocytes developed diabetes between 50-70 days post-transfer, whereas only 2 out of 6 (33%) animals that received splenocytes from TIP-1 mice developed diabetes 70-90 days post-transfer (**Figure 3B**). Taken together these results suggest that overexpression of PIns1 in APCs is able to partially dampen immune responses against insulin and reduce diabetes incidence in NOD mice.

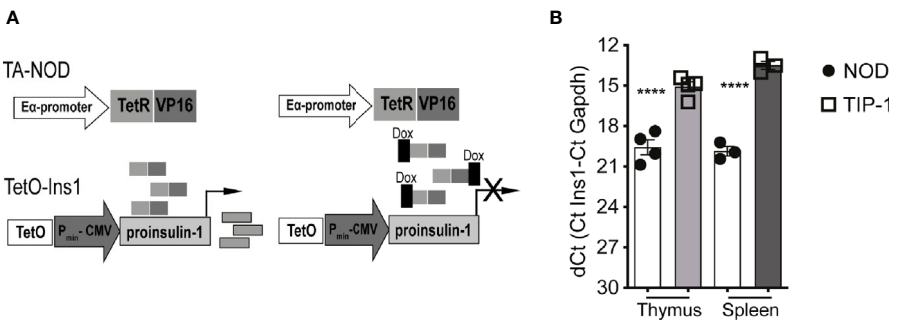

FIGURE 1 | Conditional proinsulin 1 expression in TIP-1 mice **(A)** Scheme of generation of tetracycline regulated NOD.IEα-tTA (TA-NOD) and tetO-Ins1 dual transgenic mice referred to herein as TIP-1 mice. TA-NOD mice were crossed with tetO-Ins1 mice. Bi-transgenic animals constitutively express PIns1 in APCs **(B)** Quantitative RT-PCR was performed using Taqman probes for *Ins1* and *Gapdh* in thymic and splenic lysates of WT-NOD mice TIP-1 mice. Data represent dCT values (Mean ± SEM) from 2-3 independent experiments run in duplicate for each probe. ****P < 0.0001. Data compared using One-way ANOVA with Sidak's multiple comparisons test.

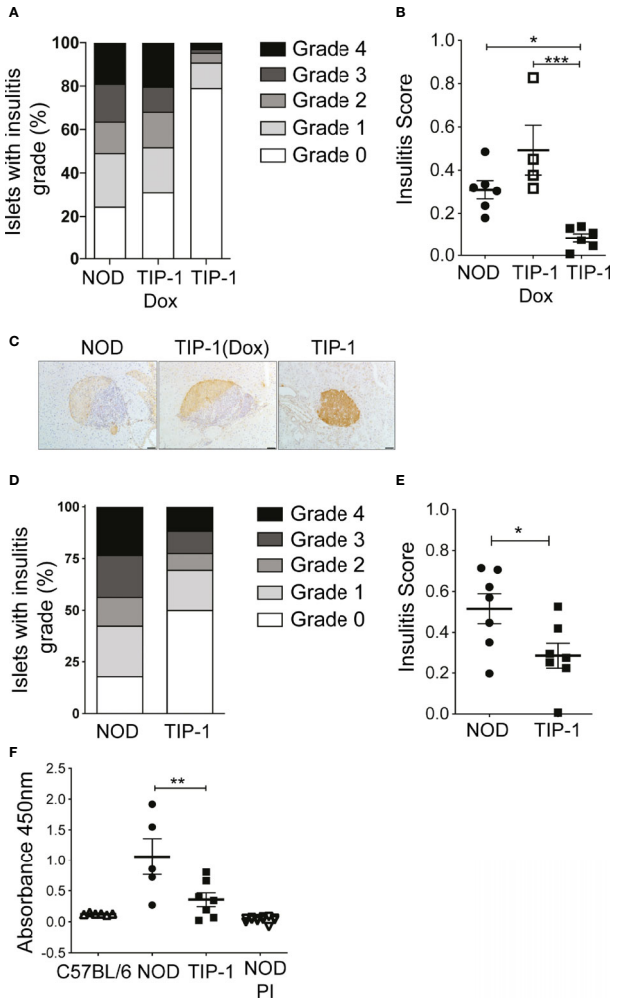

FIGURE 2 | Insulitis and insulin autoantibodies (IAA) in TIP-1 mice **(A, B)** Histological grading and individual insulitis scores in NOD mice, Doxycycline treated and untreated TIP-1 mice at 12-14 weeks of age. **(C)** Representative images of islet histology from 12-14 week old NOD, TIP-1 and Dox treated TIP-1 mice, 200x magnification, scale bar 50 µm. **(D, E)** Histological grading and individual insulitis scores at 20-25 weeks of age in NOD mice and TIP-1 mice (n=4-7, > 60 islets scored per mouse). **(F)** Sera from 12-16 weeks old C57BL/6 mice, NOD mice, TIP-1 and NOD-PI mice were tested for the presence of insulin autoantibodies (IAA) by ELISA assay. Absorbance values at 450nm are plotted. Each symbol in the scatter plot represents data from individual animals. Data plotted as Mean ± SEM, ***P < 0.001,**P < 0.01, *P < 0.05. Data compared using One-way ANOVA with Sidak's multiple comparisons test **(B, F)** and 2-tailed unpaired t-test **(E)**.

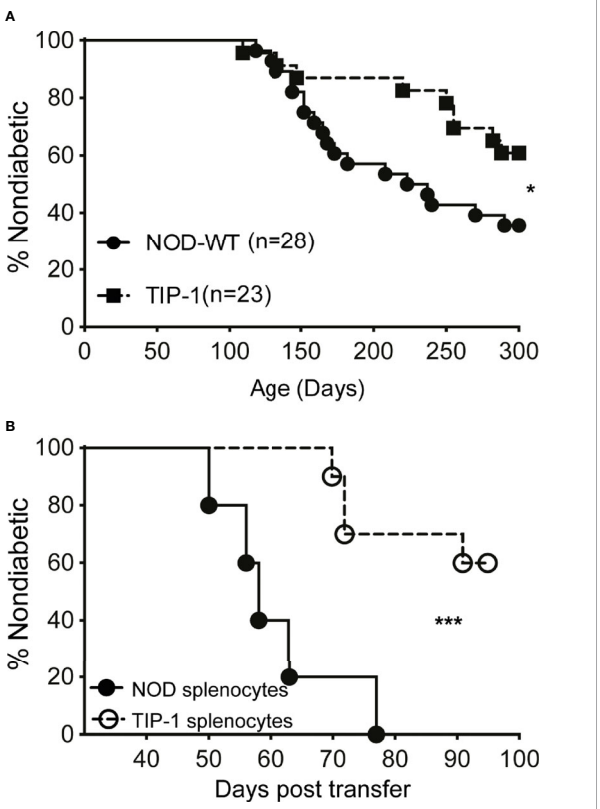

FIGURE 3 | Spontaneous diabetes incidence in TIP-1 mice **(A)** Incidence of spontaneous diabetes in female TIP-1 mice and non-transgenic littermates until 300 days of age. Numbers in parentheses indicate the number of mice analyzed. **(B)** Incidence of diabetes following transfer of splenocytes (2 x 10^7 cells/recipient) from 13-17 weeks old non-diabetic TIP-1 mice or NOD mice into 8-9 weeks old NOD.Rag1$^{-/-}$ recipients (n > 5 each). *P < 0.05; ***P < 0.0001. Survival curves were compared using log-rank (Mantel-Cox) test.

Proinsulin-Specific Tolerance in TIP-1 Mice

The partial protection from insulitis and diabetes in TIP-1 mice expressing PIns1 in the APCs could be due to immune tolerance to PIns1 epitopes. To demonstrate tolerance to PIns1, we enumerated the frequency of Insulin B $_{9\text{-}23}$ reactive CD4+ T cells and Insulin B $_{15\text{-}23}$ reactive CD8+ T cells in the peripheral lymphoid organs (PLO) (pooled spleen and non-draining lymph nodes) of 20-25 weeks old non-diabetic TIP-1 mice and age matched control mice using respective I-A (g^7) and K^d tetramers. There was a significant reduction in the absolute number of insulin B:$_{9\text{-}23}$ specific CD4+ T cells binding to insulin B:$_{10\text{-}23}$/I-A (g^7) tetramer (26) and the antigen-experienced CD44^{hi} subset of insulin B:$_{9\text{-}23}$ specific CD4+ T cells in TIP-1 mice (**Figures 4A–D**). The absolute number of CD8+T cells recognizing insulin B:$_{15\text{-}23}$ epitope (32) as well as the number of antigen-experienced CD44^{hi} subset of insulin B:$_{15\text{-}23}$ specific CD8+ T cells were comparable in both TIP-1 mice and controls (**Figures 4E–H**). While the significant reduction of insulin-specific CD4+ and CD8+ T cells in TIP-1 is suggestive of deletional tolerance, it is possible that transgenic antigen expression in APCs may induce regulatory T cells (Tregs) that confer dominant tolerance and prevent diabetes in TIP-1 mice. We examined the expression of Foxp3 on insulin B:$_{9\text{-}23}$ specific CD4+ T cells in PLO of TIP-1 mice and non-transgenic controls and did not observe any significant differences (**Figure S2A, B**), In addition we examined the frequency of Foxp3+ CD4+ Tregs in the thymus and pancreatic lymph node (PLN). The proportion of Tregs was similar in both TIP-1 and control mice (**Figure S2C, D**).Taken together our data indicate that ectopic PIns1 expression induces deletion of cognate CD4+

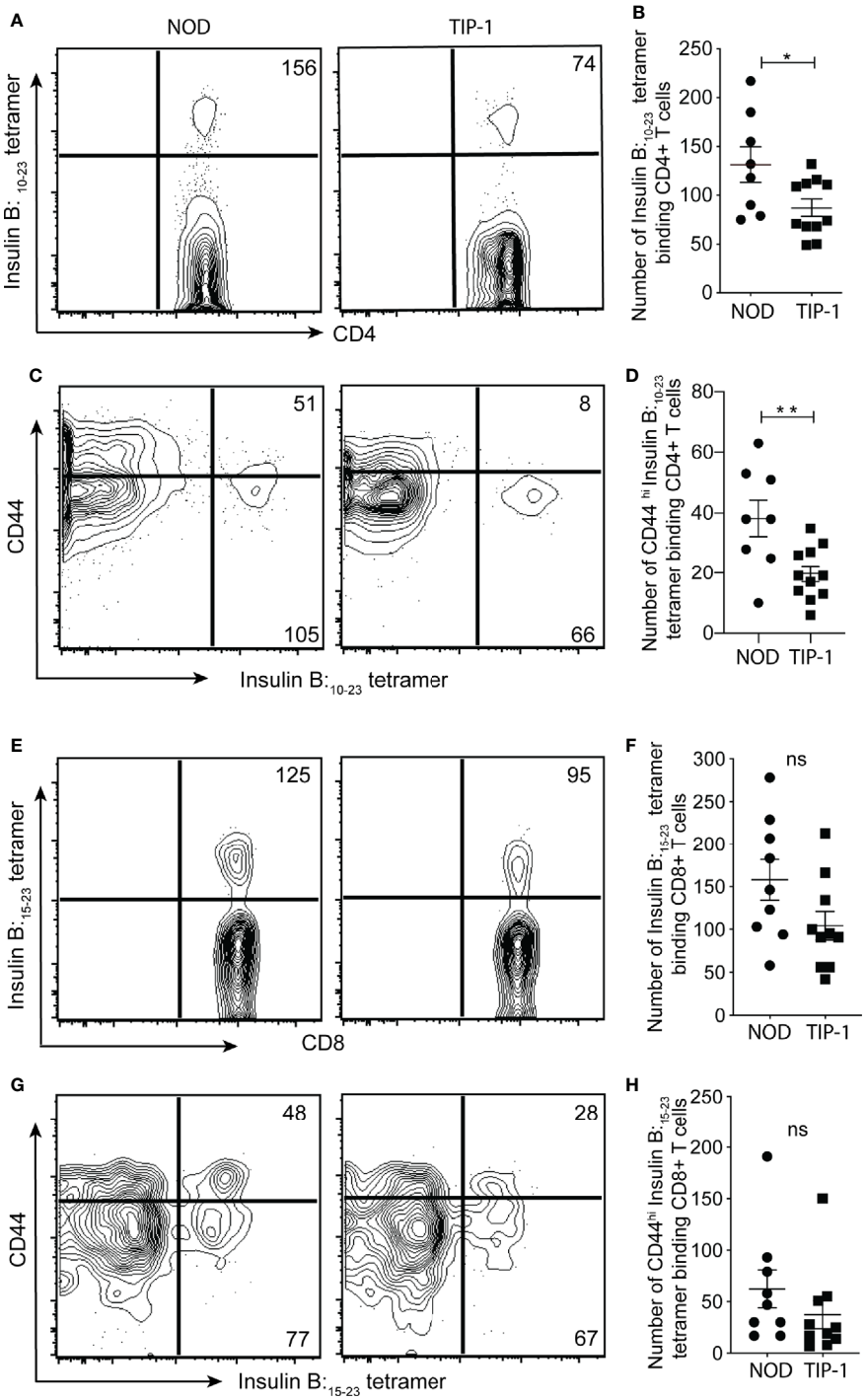

FIGURE 4 | Immune tolerance to insulin specific T cells in TIP-1 mice Insulin B:$_{10-23}$-specific CD4+ T cells or Insulin B:$_{15-23}$-specific CD8+ T cells were stained with respective tetramers and enriched from pooled peripheral lymphoid organs (PLO) of 20-25 weeks old TIP-1 mice and NOD mice using magnetic beads and enumerated by flow-cytometry. Representative FACS plots **(A, C, E, G)** and enumeration of insulin tetramer+ CD4+ T **(B)** cells, insulin tetramer+ CD8+ T cells **(F)**, CD44^{hi} Insulin tetramer + CD4+ T cells **(D)** and CD44^{hi} Insulin tetramer + CD8+ T cells **(H)** in TIP-1 and NOD mice. Values in the FACS plots indicate absolute number of tetramer binding cells. Each symbol in the scatter plots (Mean ± SEM) represents data from an individual mouse. **P < 0.01, *P < 0.05, ns= not significant. Data compared using 2-tailed unpaired t-test.

T cells, but does not induce antigen specific Tregs. The few remaining insulin reactive CD4+T cells could not be activated by the expressed antigen, whereas the low-affinity insulin B:$_{15\text{-}23}$ reactive CD8+ T cells (33) are not influenced by transgenic PIns1 expression.

Downstream Responses to IGRP Are Delayed in TIP-1 Mice

Previous work from our group has demonstrated that autoreactive responses to islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) are dependent upon immune response to PIns2 (1). To investigate if tolerance to PIns1 influenced the immune response to IGRP we examined the frequency of pathogenic IGRP $_{206\text{-}214}$ reactive CD8+ T cells in TIP-1 mice. The number of IGRP $_{206\text{-}214}$ specific CD8+ T cells was significantly reduced in 12-14 weeks old TIP-1 mice expressing PIns1 as compared to age matched controls. However, the frequency of IGRP $_{206\text{-}214}$ specific CD8+ T cells in TIP-1 mice expressing PIns1 did not differ from age-matched controls at 20-25 weeks of age (**Figures 5A, B**). This indicates that tolerance to PIns1 delays but does not prevent the spreading of immune responses to downstream antigen IGRP.

Immune Response to Proinsulin-1 Is Not Required for Diabetes in NOD 8.3 Mice

Autoreactivity to PIns2 is required for diabetes development in NOD 8.3 mice that have a pre-existing repertoire of IGRP specific T cells (34). Since we observed reduced frequency of IGRP reactive CD8+ T cells in 12-14 weeks old TIP-1 mice, we wished to know if immune responses to PIns1 were necessary for diabetes development in NOD 8.3 mice. TIP-1 mice were crossed with NOD 8.3 mice to generate offspring that were TIP-1/NOD8.3 double transgenic or NOD 8.3 transgenic alone. TIP-1/8.3 mice developed diabetes with significantly delayed kinetics (median survival 97 days) compared to NOD8.3 mice (median survival 70 days) but all mice eventually developed disease (**Figure 5C**). The frequency of insulin specific T cells is very low even in NOD mice and with a skewed T cell repertoire in NOD 8.3 transgenic mice it is not possible to detect any insulin specific T cells. We were unable to detect insulin-specific T cells

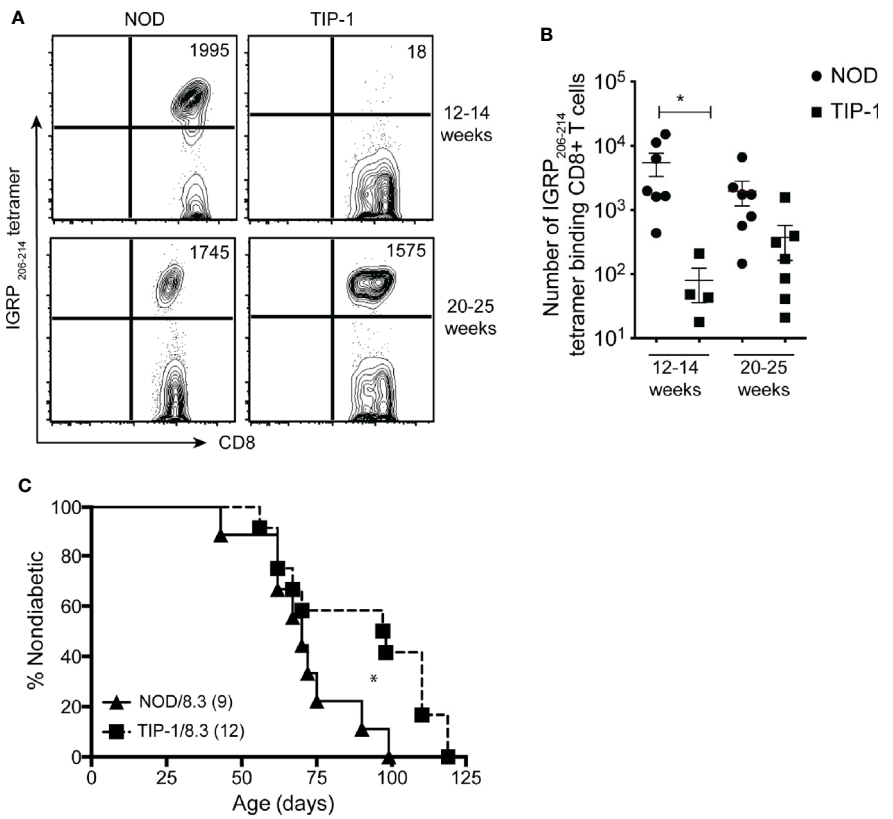

FIGURE 5 | Enumeration of IGRP specific CD8+ T cells in TIP-1 mice IGRP $_{206\text{-}214}$-specific CD8+ T cells were stained with Kd- IGRP tetramer and enriched from pooled peripheral lymphoid organs (PLO) of 12-14 weeks and 20-25 weeks old NOD mice and TIP-1 mice. Representative FACS plots **(A)** and quantification **(B)** of absolute number of IGRP $_{206\text{-}214}$ tetramer + CD8+ T cells at indicated ages. Values in the FACS plots indicate absolute number of tetramer binding cells. Each symbol in the scatter plots (Mean ± SEM) represents data from an individual mouse. *P<0.05, data in **(B)** compared using one-way ANOVA with Sidak's multiple comparisons test. **(C)** Incidence of spontaneous diabetes in female TIP-1/8.3 mice and NOD 8.3 littermates. Numbers in parentheses indicate the number of mice analyzed. *P < 0.05. Survival curves were compared using log-rank (Mantel-Cox) test.

in TIP-1/8.3 mice (data not shown). Therefore, tolerance to PIns1 significantly delays but does not prevent diabetes development in NOD 8.3 mice.

DISCUSSION

In this study we generated transgenic NOD mice to induce PIns1 expression in the APCs and examined the impact of antigen specific tolerance on autoimmune diabetes. The main findings of this study are 1) TIP-1 mice expressing PIns1 in the APCs show significantly reduced incidence of diabetes, which is associated with reduced insulitis and insulin autoantibody (IAA) expression. 2) Proinsulin specific CD4+ T cells are detectable in TIP-1 mice at a reduced frequency and are not activated. 3) Immune responses to downstream antigen IGRP are delayed but not absent in TIP-1 mice.

Given the high degree of homology between proinsulin 1 and 2 proteins, especially in the immunodominant insulin B chain epitope Ins $B:_{9\text{-}23}$ we expected to achieve robust protection from diabetes onset in TIP-1 mice, similar to previously described proinsulin-2 tolerant NOD mice (35); however, the partial protection from insulitis and diabetes observed in TIP-1 mice points to the existence of distinct pathogenic peptide epitopes in the PIns2 protein that can precipitate autoimmunity in NOD mice. A previous study characterizing immunogenic epitopes in NOD mice reported existence of multiple epitopes on both PIns1 and PIns2 molecules recognized by CD4+T cells (18). Importantly, epitopes outside of the highly homologous Ins $B:_{9\text{-}23}$ peptide were identified in the leader and A chain sequences of PIns2 molecule. Thus, it is likely that PIns2 reactive T-cells recognizing these unique epitopes may induce islet destruction and subsequent diabetes onset in TIP-1 mice.

Our data complement the previous observations that reported detection of PIns1 reactive T cells (18, 19) in NOD mice. While the previous studies did not directly demonstrate the role of PIns1-reactive T cells in spontaneous disease, the significant reduction in diabetes incidence in TIP-1 mice suggests that PIns1 specific T cells participate in autoimmune destruction of beta cells. On the other hand, development of IAA and diabetes in TIP-1 mice may be related to ongoing immune responses to PIns2 epitopes.

A drawback of our study is that we have analyzed a single transgenic founder line expressing PIns1 in the APCs. Varying levels of transgenic insulin expression in the thymus may influence the diabetes progression in NOD mice. PIns2 levels were 7-fold higher in the spleen (~140 pmol/L) as compared to thymus (~20pmol/L) in the partially protected Pins2 tolerant mice previously described by Jaeckel et al. (12), whereas in the recently described TIP mice with robust protection from autoimmune diabetes upon conditional PIns2 expression in APCs, the level of thymic Pins2 expression (100pmol/L) was 5-fold more compared to peripheral tissues(20pmol/L) (22). TIP-1 mice may have relatively reduced transgenic expression of PIns1 in the thymic APCs as compared to transgenic PIns2 expression in the previously described TIP mice, thus imparting incomplete protection from autoimmune diabetes. Chentoufi and Polychronakos previously reported that Ins2 is expressed at a level more than 3-fold higher than Ins1 in the thymus of NOD mice (9). In TIP-1 mice analyzed here, induction of PIns1 results in approximately 5-fold higher expression as compared to non-transgenic NOD mice or uninduced TIP-1 mice. Moreover, protection from insulitis in TIP-1 mice is associated with the expression of PIns1 transgene, as TIP-1 mice fed doxycycline to suppress PIns1 expression develop islet infiltration comparable to non-transgenic controls indicating that ectopic PIns1 expression in APCs influences anti-islet immunity.

Does the reduction in the incidence of spontaneous diabetes in TIP-1 mice correlate with deletion of PIns1 specific T cells? Insulin $B:_{10\text{-}23}$ and Insulin $B:_{15\text{-}23}$ specific tetramers used in our study are likely to detect both PIns1 and 2 reactive CD4+ and CD8 +T cells, due to the invariant nature of the Insulin $B:_{9\text{-}23}$ peptide between the two isoforms. Immune responses to Insulin $B:_{9\text{-}23}$ epitope are required for both priming and effector phase of islet autoimmunity in NOD mice. Moreover, Insulin $B:_{9\text{-}23}$ primed CD4+ T cells are able to induce islet autoimmunity evidenced by IAA production (36). The significant reduction in absolute number of Insulin $B:_{9\text{-}23}$ tetramer binding CD4+T cells, and the antigen-experienced subset of tetramer binding CD4+ T cells, coupled with reduced IAA production in TIP-1 mice is suggestive of antigen-specific tolerance. While Tregs are an important tolerance mechanism, we did not find any evidence to suggest that the partial protection from diabetes in TIP-1mice is due to antigen-specific Tregs. We are currently unable to conclude whether central or peripheral tolerance mechanisms regulate the insulin specific T cells in TIP-1 mice; however, future studies with ectopic antigen expression induced after the exit of antigen-specific T cells from the thymus may resolve this question.

Autoimmunity to insulin determines immune responses to other downstream antigens such as IGRP (1). IGRP $_{206\text{-}214}$ reactive CD8+ T cells were reduced in TIP-1 mice at 12-14 weeks; but ongoing tolerance to PIns1 did not prevent development of diabetes onset in TIP-1/8.3 mice. The precursor frequency of IGRP reactive CD8+ T cells is low in NOD mice (27), and the residual immune response to PIns2 in TIP-1 mice may be reduced as compared to control mice. The reduced CD4+ T cell help possibly accounts for the delayed expansion of IGRP specific T cells seen in TIP-1 mice. However, the residual immune response to PIns2 in TIP-1/8.3 mice with a pre-existing repertoire of IGRP specific T cells may be sufficient to help IGRP specific CD8+ T cells to mediate beta-cell destruction.

In summary, we find that immune tolerance to PIns1, whilst partly protective, is not sufficient to prevent spontaneous diabetes in NOD mice. Our data clarifies the role of PIns1 in the pathogenesis of autoimmune diabetes in NOD mice and extends the previously established role of PIns1 in autoimmune diabetes. The experimental model we have presented here, with its conditional gene-expression system, has the potential to delineate whether antigen-specific interventions can induce immune tolerance after islet autoimmunity is well established. Understanding this is important for development of strategies to induce antigen-specific tolerance clinically in people with stage 1 or 2 type 1 diabetes (37).

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 animal study was reviewed and approved by Animal Ethics Committee, St Vincents Hospital, Melbourne.

AUTHOR CONTRIBUTIONS

GJ performed experiments, analyzed data, and wrote the manuscript. CS, JC, C-TK, and EP performed experiments and analyzed data. HT, BK, and TK designed the study, analyzed data, and wrote the manuscript. BK and TK supervised 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/fimmu.2021.645817/full#supplementary-material

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Hidden in Plain View: Discovery of Chimeric Diabetogenic CD4 T Cell Neo-Epitopes

Brendan K. Reed[1,2,3] *and John W. Kappler*[1,2,3,4]*

[1] Research Division, Barbara Davis Center for Diabetes, University of Colorado, Aurora, CO, United States, [2] Department of Immunology and Genomic Medicine, National Jewish Health, Denver, CO, United States, [3] Department of Immunology and Microbiology, University of Colorado, Aurora, CO, United States, [4] Biochemistry and Molecular Genetics, University of Colorado, Aurora, CO, United States

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Hubert M. Tse,
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***Correspondence:**
John W. Kappler
kapplerj@njheatlh.org

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The T cell antigens driving autoimmune Type 1 Diabetes (T1D) have been pursued for more than three decades. When diabetogenic CD4 T cell clones and their relevant MHCII antigen presenting alleles were first identified in rodents and humans, the path to discovering the peptide epitopes within pancreatic beta cell proteins seemed straightforward. However, as experimental results accumulated, definitive data were often absent or controversial. Work within the last decade has helped to clear up some of the controversy by demonstrating that a number of the important MHCII presented epitopes are not encoded in the natural beta cell proteins, but in fact are fusions between peptide fragments derived from the same or different proteins. Recently, the mechanism for generating these MHCII diabetogenic chimeric epitopes has been attributed to a form of reverse proteolysis, called transpeptidation, a process that has been well-documented in the production of MHCI presented epitopes. In this mini-review we summarize these data and their implications for T1D and other autoimmune responses.

Keywords: antigen presenting cell, transpeptidation, immune tolerance, type 1 diabetes mellitus, chimeric peptide, CD4 T cell, beta cell, antigen

INTRODUCTION

In the Non-Obese Diabetic (NOD) mouse model of T1D, a variety of CD4 T cell clones or T cell hybridomas were prepared that responded to antigens within the secretory granules of the beta cells of pancreatic islets of Langerhans (1, 2). In some cases, the protein source of the stimulatory activity was identified (1, 3), but in others, no target could be identified. A particularly stringent test for the relative contribution of these T cells to the disease came from introducing the T cell clones into immunodeficient NOD-SCID mouse lacking T cells and observing whether the clone was sufficient to induce T1D (4). A number of T cell clones failed this test, but others, originally isolated by investigators at the Barbara Davis Center (BDC) (1, 2) were very active. It has now taken an effort of more than two decades to identify the functional peptide epitopes recognized by these BDC CD4 T cells. This work has now identified diabetogenic CD4 T cell epitopes derived in part from three beta cell proteins - insulin, chromogranin A (ChgA) and islet amyloid polypeptide (IAPP). In each case a

fusion to the N- or C-terminus fragment of these proteins to a peptide from another or the same protein was required to construct a fully stimulatory chimeric epitope. We begin with a short review of how these three altered antigenic epitopes were discovered.

IDENTIFICATION OF EPITOPES FOR DIABETOGENIC T CELLS

Insulin

Insulin has become recognized as a major CD4 T cell target in T1D in humans and the NOD mouse model of the disease, reviewed in (5). In the 1990's at the BDC, a series of CD4 T cells clones were produced from the NOD mouse, including the prototypical BDC-12-4.1 and BDC-12-4.4 clones, that were reactive to a peptide from the insulin B chain, B:9-23, presented by the NOD MHCII allele, IA^{g7} (2, 3). Many of these clones were diabetogenic when introduced into NOD mice. Subsequently, the BDC (6) and other institutions (7, 8) went on to produce many other T cell clones and T cell hybridomas reactive to this peptide. Later, similar T cells were identified in human T1D reactive to the same peptide presented by human DQ8 (9–11). In IA^{g7} (12, 13), HLA-DQA1*03:01/DQB1*03:02 (HLA-DQ8) (14) and other MHCII alleles, the core of the peptide binding groove accepts 9 amino acids in the p1 to p9 positions. Therefore the 15 amino acid B:9-23 peptide could theoretically bind in multiple positions or "registers" (Regs) in the MHCII groove, each with different amino side chains interacting within anchoring pockets in the binding groove versus appearing on the surface for T cell receptor (TCR) recognition. Three registers for this peptide have been studied the most. The 9 amino acid cores of these epitopes are: Reg1-B:12-20, VEALYLVCG, Reg2-B:13–21, EALYLVCGE and Reg3-B:14-22, ALYLVCGER. Several studies proposed Reg1 or Reg2 bound epitopes as the relevant peptide register for two groups of B:9-23 reactive T cells (termed Type B and Type A, respectively after the nomenclature of the Unanue laboratory) (7, 8, 15). However, we performed many experiments that have led us to conclude that the relevant register for both types of T cells is actually Reg3.

To study these registers, we made versions of the B chain peptide in which the amino acids predicted at the p1 and p9 positions in the various registers were mutated to optimize binding to IA^{g7} in that register, but to inhibit T cell recognition if bound in a different register (16). When tested with the BDC-12-4.1 T cell, as well as others reported to respond to the peptide in bound in Reg1 and Reg2 (7), only the peptide forced to bind in Reg3 stimulated these T cells. Additional experiments established that the key modification to the peptide for Reg3 binding was the mutation of B:22R to E at p9, thus changing a very unfavorable amino acid for the IA^{g7} p9 pocket for an optimal acidic one (12, 13, 17). Similar experiments with human T cells responding to B:9-23 bound to HLA-DQ8 established that the B:22R to E mutation at p9 greatly improved T cell reactivity. Eventually, crystal structures of the peptide bound to IA^{g7} or DQ8 confirmed the Reg3 binding of the mutated peptide (10, 11). This modified peptide has been used as a tolerogen for *in vivo* prevention of T1D in NOD mice (18).

We subsequently performed other experiments (19) showing that, while the peptide with p9R to E mutation strongly stimulated Type A T cells, it remained a weak antigen for Type B T cells. This was eventually tracked to interference of the Type B T cell responses by the exposed side chain of B:21E at p8 in Reg3. Combining the p9R to E with a p8E to G mutation to remove the interfering p8 side chain created a strong agonist for Type B T cells, but reduced the Type A T cell responses. Subsequently, crystal structures of these complexes and of Type A and Type B TCRs bound to them explained the Type A vs. Type B discriminating activity of the Reg3 mutations (10, 11). These studies also showed that, for a subset of Type B T cells, changing the p8E to V or L, rather than G, resulted in epitopes that were even stronger stimulators, sometimes even 100-fold better than the p8G modified version (11). Therefore, creating the appropriate CD4 T cell epitopes from the B:9-23 peptide required modifications of the peptide at B:22R (p9) to greatly improve IA^{g7} binding and sometimes also at B:21E to greatly improve TCR interaction.

Chromogranin A

A similar multi-decade effort led to the identification of the epitope for other T cells identified at the BDC, BDC-2.5 and BDC-10.1 (1, 20). These T cell clones were shown to be extremely diabetogenic in NOD mice (4, 21) and responded to pancreatic islets *in vitro*, but the source of the antigen and the target epitope of these clones eluded researchers for many years. The first clues to its nature came from the identification of stimulatory epitopes for these T cells in various types of peptide libraries (22–25). These independently discovered "mimotopes" eventually pinpointed ChgA as the likely source of the natural antigen (25), since they bore a C-terminal 5 amino acid (p5-p9) motif that was similar to a sequence in ChgA (WSRMD).

A synthetic 9 amino acid ChgA peptide KDRKWSRMD was synthesized, which placed the WSRMD in p5 to p9 positions to mimic the active library mimotope peptides, but this peptide had no activity with the T cells, which we attributed to inhibitory amino acids for T cell recognition (p3R) (25) and IA^{g7} binding (p4K) (17)within the KDRK portion of this peptide. However, we noticed that there was a conserved 14 amino peptide (WE14) (26) released from ChgA during prohormone convertase processing leaving the WSRMD sequence at its C-terminus, while removing the inhibitory amino acids. This peptide stimulated BDC-2.5 and BDC-10.1 weakly, presumably because of the missing p1 to p4 amino acids, but we found that pancreatic islets from mice lacking a functional ChgA gene failed to stimulate these T cell leading us to the conclusion that, while the WE14 peptide was in some way involved in the ChgA derived epitope, a post-translational modification was likely required to make up for the loss at the p1 to p4 positions in the epitope (25).

Delong et al. pursued the idea that the modification was due to the action of the tissue transglutaminase enzyme (TG) on the glutamine within WE14 (27, 28). However, reminiscent of our results with the insulin B:9-23 peptide, we postulated that a more likely modification was one that would change the p1 to p4 positions with optimal TCR and IA^{g7} amino acids. To test this idea, we replaced the natural amino acid extension of WE14 peptide with the N-terminal fragment (RLGL) from our library mimotope peptide (19, 25, 29). This peptide remarkably improved the stimulatory activity of the peptide nearly a million-fold. A crystal structure of this RLGL extended WE14 peptide bound to IA^{g7} confirmed the positions of these amino acids in the peptide binding groove (29). Therefore, we concluded that, in the reciprocal case to that of the insulin B:9-23 derived epitopes, the major epitope for ChgA specific T cells required replacement of the natural ChgA amino acids at the N-terminus, rather than the C-terminus, of the epitope with optimal ones.

Islet Amyloid Polypeptide (IAPP)

The BDC-6.9 T cell was produced at the BDC at about the same time as the insulin and ChgA specific clones (1). As with the BDC-2.5 and BDC-10.1 clones, it was highly diabetogenic *in vivo* (30), but the source and nature of the epitope was not known. In this case the clue to the source came from the fact that the stimulatory activity was absent in the islets of BALB/c mice (20). Genetic analyses of the stimulatory activity in backcrossed mice mapped it to a section of NOD chromosome 6 and pointed to the IAPP gene as the likely source (30). Several polymorphisms in the IAPP gene coding region between NOD and BALB/c strengthened this idea (31, 32). Disappointingly *in vitro* stimulations at the time with overlapping peptides throughout the IAPP protein failed to identify a stimulating epitope, but experiments in which NOD mice bred to carry the BALB/c genomic region were protected from T1D induction by the BDC-6.9 clone (33) leading to the conclusion that the functional epitope was probably a post-translational modified form of an IAPP peptide. It has taken several decades to confirm this idea.

CHIMERIC PEPTIDES LIKELY ACCOUNT FOR THE INSULIN, CHGA AND IAPP EPITOPES

The results of the studies above, led to the idea that the functional epitopes for these diabetogenic CD4 T cells were likely post-translational versions of the natural peptides, derived from these proteins. Post-translational modifications of CD4 T cell epitopes had been well-established in other autoimmune diseases, for example, conversion of arginines to citrullines by peptidylarginine deiminases (PADs) in rheumatoid arthritis (34–37) and of glutamines to glutamic acids by tissue transglutaminase (TG) in celiac disease (38). In fact, the presence of these modified amino acids as well as antibodies to the modification or to the modifying enzyme has become diagnostic markers of the diseases.

In T1D, neither of these two types of post translational modification has been established to be a component of the disease driven by the three CD4 T cell specificities discussed here. While Delong, et al. demonstrated an increase in the stimulatory activity of the WE14 peptide after *in vitro* TG treatment (27, 28), the active products of the treatment have not been identified nor did the simple conversion of the glutamine to glutamic acid in the peptide account for the increased activity. Furthermore, the increase in activity was orders of magnitude less than that seen with the library mimotopes (22, 24, 25).

An alternate hypothesis has arisen from studies of post-translationally modified MHCI bound epitopes generated in the proteasome. During the 2000's a series of studies documented the creation of chimeric MHCI epitopes by the fusion of peptides from the same or different proteins (39, 40) through a form of reverse proteolysis often referred to as "transpeptidation" (41–43). Subsequently, new methods developed to look for these chimeric peptides among those eluted directly from MHCI molecules revealed that they are much more frequent than previously appreciated (44, 45), raising the question that mass spectrometry methods that simply match MHCI bound peptides to sequences in naturally encoded proteins may miss many important MHCI epitopes. These results spurred us (11, 29, 46) and others (33, 47, 48) to test whether synthetic versions of chimeric peptides between pieces of beta cell proteins could create MHCII compatible chimeric epitopes for the diabetogenic CD4 T cells discussed here.

In our studies on the B:9-23 peptide, a scan of the sequence of proinsulin C-peptide revealed short sequences that when synthetically added to the C-terminus of fragments the B:9-23 peptide truncated to B:21 or to B:22 would be predicted to create chimeric peptides with the amino acids at p8 and/or p9 required for stimulation of Type A or Type B insulin reactive T cells (11). In vitro testing of synthetic versions of these chimeric epitopes showed strong activation of the appropriate Type A and Type B CD4 NOD T cells and Type A human CD4 T cells. These results are summarized in **Table 1**. For ChgA, based on the highly stimulatory activity of the RLGL when added to the N-terminus of WE14 (19, 29), we looked in well expressed beta cell granule proteins for similar sequences that could be added to WE14 to make similar complete epitopes predicted to stimulate the BDC-10.1 and/or BCD-2.5 T cell (29). When synthesized, many of these chimeric peptides stimulated BCD-10.1 and or BDC-2.5 T cells, bearing out the predictions (46). These results are summarized in **Table 1**. One of the predicted epitopes involving a fragment of C-peptide with an C-terminal TLAL added to the N-terminus of WE14 has been shown by Delong and colleagues not only to be active, but also present in pancreatic beta cell tumors and in the islets of Langerhans in mouse pancreata (47). This approach of testing candidate fused peptides also turned up the long-sought IAPP-derived epitope for the BDC-6.9 diabetogenic T cell (33). In this case, the same C-peptide fragment ending in TLAL that was used to complete

TABLE 1 | Chimeric Peptides Derived from Insulin B:9-23, ChgA-WE14 or pro-IAPP.

Acceptor		Donor		Fusion Epitope Sequence	Active T Cell Clone	Synthetic chimeric peptide active *in vitro*	Chimeric peptide found in beta cells	Fused by cathepsin L *in vitro*	Ref
Sequence	Source	Sequence	Source						
VEALYLVCGE	m/h Insulin B:9-23	EVE	mC-peptide	VEALYLVCGEEVE	12-4.1 PCR1-10 I.29 AS150	+	–	–	(11)
"	"	DLQ		VEALYLVCGEDLQ		+	–	–	(11)
"	"	EAE	hC-peptide	VEALYLVCGEEAE	T1D3 T1D4 T1D10	+	–	–	(11)
"	"	EDG		VEALYLVCGEEDQ		+	–	–	(11)
"	"	ELG		VEALYLVCGEELG		+	–	–	(11)
VEALYLVCG	"	GDLQ	mC-peptide	VEALYLVCGGDLQ	8F10 8-1.1 AS91 12-4.4	+	–	–	(11)
"	"	VEQL		VEALYLVCGVEQL	12-4.4 AS91	+	–	–	(11)
"	"	LEVA		VEALYLVCGLEVA		+	–	–	(11)
TLAL	mC-peptide	WSRMDQL	mChgA-WE14	TLALWSRMDQL	BDC-10.1 BDC-2.5 G7W-120	+	+	+	(46, 47)
QLAL	mSecretogranin2	"		QLALWSRMDQL		+	–	–	(46)
RIPV	"	"		RIPVWSRMDQL	BDC-2.5	+	–	–	(46)
TIAL	mSecretogranin3	"		TIALWSRMDQL	BDC-10.1 BDC-2.5 G7W-120	+	–	+	(46)
TLTL	"	"		TLTLWSRMDQL		+	–	+	(46)
ERIL	mChgA	"		ERILWSRMDQL	BDC-2.5	+	–	+	(46)
ILSI	"	"		ILSIWSRMDQL	BDC-10.1 BDC-2.5 G7W-120	+	–	–	(46)
DLAL	"	"		DLALWSRMDQL		+	–	+	(46)
TLAL	mC-peptide	NAARD	NOD IAPP	TLALNAARD	BDC-6.9	+	+	+	(46)
"	"	NAAGD	BALB/c IAPP	TLALNAAGD		+	–	+	(33, 46)

This is a list of chimeric peptides derived from Insulin B:9-23, ChgA-WE14 or pro-IAPP and whether these peptides are capable of stimulating a panel of Diabetogenic T cell clones in vitro, have been discovered in Beta cells, and whether they are capable of being generated by Cathepsin L in vitro. The full length, stimulatory fusion epitope and cognate T cell(s) are listed.

the ChgA WE14 epitope was fused to N-terminus of a peptide released from proIAPP during its natural processing to mature IAPP. This epitope was a very strong agonist for the BDC-6.9 T cell. Importantly the G (p8) from the donor fragment is an R in the corresponding peptide in the BALB/c proIAPP, accounting for the difference between the strains in creating the epitope. As with ChgA, this chimeric peptide has been identified in NOD beta cell tumors and in pancreatic islets (33).

Recently, numerous chimeric epitopes have been reported by others for mouse and human CD4 and CD8 T cells in T1D [reviewed in (49, 50)]. The presence of CD4 and CD8 T cells responding to fusion peptides in mouse and human have now all been described and these findings have bridged the gap in our understanding of the T cell mediated pathogenesis in both the mouse and human diseases (51–53). Additionally, the use of these hybrid peptides as therapeutics to tolerize the cognate T cells and prevent the onset of disease has gained a lot of traction (54), but significant limitations still exist in translating these findings to humans.

TRANSPEPTIDATION: THE PROCESS OF REVERSE PROTEOLYSIS AND ITS IMPLICATIONS FOR MHC I/II EPITOPES

These accumulating results with chimeric peptides make it highly likely that addition of amino acids to the N- or C-terminus of fragments of insulin B:9-23, WE14 or IAPP derived peptides create the functional epitopes for the corresponding CD4 T cells in T1D. This conclusion begs the question of what mechanism can lead to the generation of these chimeric epitopes *in vivo*. As mentioned above, the best clues comes from the expanding work on the role of proteasomal transpeptidation in creating many chimeric peptides for MHCI presentation (39, 40, 44, 45, 55–58).

Transpeptidation is an inevitable side reaction during digestion of proteins with proteases with a catalytic serine, threonine or cysteine in the protease active site [reviewed in (43, 56)] (**Figure 1**). During the protein cleavage reaction these amino acids attack the peptide bond at the cleavage site forming

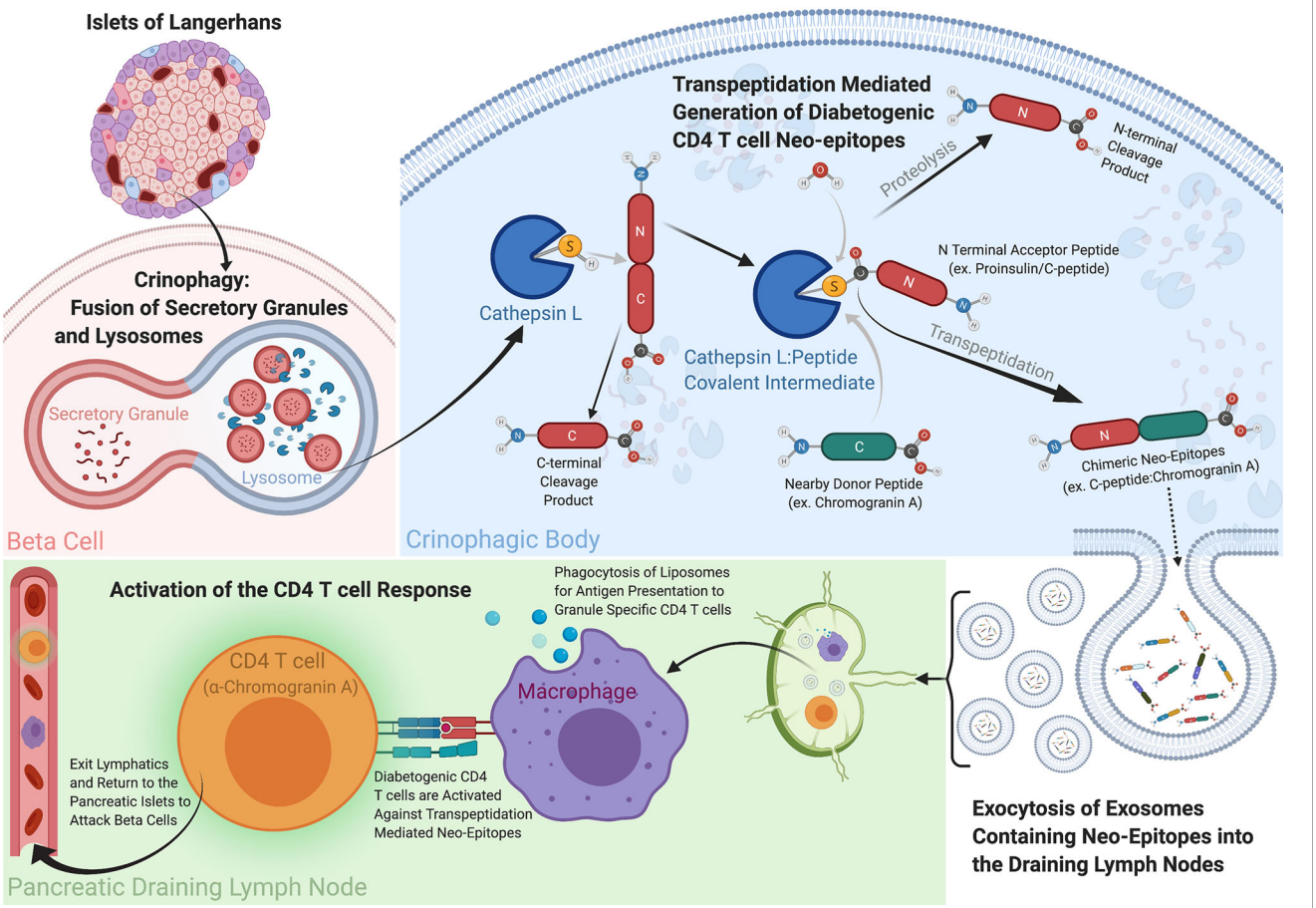

FIGURE 1 | How Transpeptidation in Crinophagic Vesicles Could Create the Chimeric Epitopes Driving T1D. Within the pancreas exist a specialized multicellular network referred to as the Islets of Langerhans. Contained within these islets are the Insulin producing beta cells responsible for maintaining stable blood glucose levels among other neuroendocrine processes. The secretory granules within the beta cells contain prohormones like Proinsulin, Chromogranin A and ProIAPP, and their levels are continually regulated through a catabolic recycling process called crinophagy, whereby secretory granules are fused with lysosomes and their contents are degraded, recycled and secreted. Due to the high concentrations of beta cell hormone donor and acceptor proteins present within these crinophagic bodies, the biochemical conditions are optimal for the reverse proteolysis reaction, transpeptidation to occur. Cathepsin L is a protease capable of cleaving the hormone acceptor peptides (ex. Proinsulin C-peptide) and creating an enzyme linked intermediate complex with the acceptor peptide. Water is generally responsible for breaking this transient bond between the carbonyl carbon of the acceptor peptide and the a sulfur or oxygen in enzyme to complete the digestion, however when high concentrations of a donor peptide with a free N-terminus are present they can outcompete water and generate a new peptide product through transpeptidation. We propose these neo-peptides can be exocytosed and secreted out of the beta cells and to be taken up antigen presenting cells to and presented to diabetogenic CD4 T cells. These peptides are considerably more active than their germline encoded parental counterparts and their presentation can lead to T cell activation and destruction of the beta cells in the islets. This figure was created with Biorender.com.

a covalent bond between the oxygen or sulfur in the protease active site and the carbonyl carbon in the peptide bond, while releasing the C-terminal fragment of the digestion. This transient covalent bond is usually broken by water to complete the cleavage by releasing the N-terminal fragment of the digestion and restoring the protease active site, but this bond can also be broken by attack with the N-terminus of a nearby donor peptide restoring a peptide bond and replacing the original C-terminal fragment with a new one to create a chimeric peptide. Transpeptidation is generally a predictable, but minor, side reaction in protease digestions, but its efficiency can be greatly improved by adjusting the conditions present during the proteolysis. Especially effective is a high concentration of the donor peptide in close proximity to the cleavage site and a relatively low concentration of the competing water during the reaction. Under ideal conditions, transpeptidation can be efficient enough to be an important mechanism for natural processing of functional proteins in various organisms (43, 59).

The proteasome has a milieu very favorable for transpeptidation. It contains threonine proteases (60) and has a steady high concentration of cytoplasmic proteins directed into the organelle for degradation [Reviewed in (61)]. It has an encapsulated interior containing low water content. It is the main source of protein digestion products destined to the

endoplasmic reticulum for further processing and MHCI loading. These ideal conditions perhaps explain the high proportion of chimeric peptides found in those eluted from surface expressed MHCI molecules (44, 58). While the conditions in the proteasome may be ideal to catalyze these reactions, the spatial constraints of the proteasome have been shown to prefer Cis-splicing events, where internal deletions are made within the same protein, instead of fusion events between two different proteins (Trans-splicing) (62).

THESE DIABETOGENIC CHIMERIC EPITOPES CAN BE PRODUCED BY LYSOSOMAL PROTEASE MEDIATED TRANSPEPTIDATION

A parallel pathway involving lysosomes exists in pancreatic islet beta cells and can be predicted to favor the generation of chimeric peptides. In beta cells, secretory granules have a high concentration of insulin and other proteins, including ChgA and IAPP [Reviewed in (63)]. Convertase proteases in the granules convert the precursor forms of these proteins into their mature, active forms, by releasing protective prohormone fragments, as well as additional active hormone fragments by internal cleavages (64). The number of granules in a beta cell is strictly regulated (63). Therefore, since new granules are constantly being formed, excess granules need to be eliminated to maintain the optimal number. This is accomplished by a form of autophagy called crinophagy (65), in which granules are fused with lysosomes and their proteins denatured and degraded by a variety of enzymes including cathepsins and other cysteine or serine proteases. Thus, ideal conditions for transpeptidation are set up – a high concentration of actively degrading proteins encapsulated in a vesicle with multiple proteases that are capable of the transpeptidation reaction. Exosomes from these crinophagic vesicles carrying antigenic fragments of insulin and other granule proteins can be released from beta cells and into circulation (66), providing a pathway for chimeric peptides to reach the pancreatic draining lymph nodes for activation of diabetogenic CD4 T cells (67) (**Figure 1**). While these findings are considered circumstantial by some, these extracellular vesicles have been shown to carry cargo relevant to T1D in the form of prohormone proteins for both CD4 and CD8 T cells, and miRNAs, all of which have been implicated in multiple facets of the disease [reviewed in (68)]. Since the lysosomal/endosomal pathway in MHCII bearing antigen presenting cells (APCs) is the primary site for proteolytic generation of peptides for MHCII presentation [reviewed (69)], a number of laboratories have studied this antigen processing reaction *in vitro*, by exposing proteins to various lysosomal proteases under lysosomal conditions and testing the products of the digestion for antigenic activity (46, 70–72). We have used this system to see if any of the active chimeric epitopes identified in the beta cells mentioned above could be generated *in vitro* during lysosomal protease digestion of a suitable acceptor protein fragment in the presence of a donor peptide that when fused to a site within the acceptor would form the active diabetogenic epitope (46).

We tested a number of cathepsin proteases, we settled on cathepsin L for these experiments, due to its ability to generate the necessary complimentary Proinsulin acceptor peptide. In looking for an active WE14 containing chimeric epitope, we used a fragment of C-peptide containing the previously documented TLAL sequence discussed above (47), as well as fragments of other granule proteins with embedded sequences that also were active when fused to the N-terminus of WE14 (46). In each case an internal cleavage at the C-terminus of the embedded fragment was required to create a site for transpeptidation fusion to an N-terminal fragment of WE14. Cathepsin L digestions were performed at lysosomal pH with a molar excess of a WE14 donor fragment to favor the transpeptidation reaction.

When the digests were used to stimulate the prototypical NOD WE14-specific CD4 T cells, BDC-10.1 and/or BCD-2.5, five (**Table 1**) were active with one or both T cells (46). Tandem mass spectrometric analysis (MS-MS) of the digests revealed the presence of the predicted chimeric peptide in the digests. Synthetic versions of the identified epitope had the same stimulating specificity as the digests. In each case the identification was further confirmed by showing that the MS-MS fractionation pattern of the synthetic peptide was virtually identical to that seen in the corresponding peptide found in the digest.

The MS-MS analyses also showed that these functional peptides were by no means the only chimeric peptides detected in the digests. Hundreds of additional chimeric peptides were identified involving many combinations of the input acceptor and donor peptides. In the case of joining of the WE14 fragment to sites within the input acceptor peptide precursor, fusions were detected at nearly every position (46), but strikingly, the positions that contained a preferred cathepsin L cleavage sequence were highly favored. A similar digestion with the C-peptide fragment containing TLAL using a donor peptide from NOD proIAPP (NAARD) generated the previously reported functional chimeric peptide for the BDC-6.9 T cell (46). Substituting the equivalent donor peptide from BALB/c IAPP (NAAGD) also generated the predicted chimeric peptide, but as expected this peptide was 10x less active than the NOD derived one.

Cysteine proteases have been implicated in disease resistance in Type 1 Diabetes (73), however further investigation by other groups determined that the effect was indirect, due in part to T cell repertoire changes resulting from Cathepsin L being absent during thymic selection. They observed a 2 fold higher incidence of regulatory T cells in the knockout mice compared to their CatL sufficient counterparts, which they attributed to the disease protection (74). Although splenocytes from NOD mice are capable of mounting a response against the CatlL-/- islets, it is not clear whether the absence of CatL has allowed for a compensatory mechanism whereby alternative proteases are utilized to generate these fusion peptides, or if another protease is responsible for it altogether. To date, we have not

discovered another protease capable of generating these fusions other than Cathepsin L.

FINAL THOUGHTS

After a decades-long struggle to understand the structures of the diabetic CD4 T cells epitopes in T1D, the door has cracked open with the discovery of the functional chimeric epitopes and their formation by transpeptidation. As more acceptor-donor pairs are tested with multiple lysosomal proteases, it seems likely that this form of post-translational modification will play an important role in epitope formation in other CD4 T cell driven autoimmune diseases, especially those of other neuro-endocrine tissues containing secretory granules. This phenomenon may also contribute to epitopes for CD4 T cells derived from foreign (viral/bacterial) and tumor antigens. As with MHCI, these MHCII results point out that existing peptides databases for MHCII bound to peptides directly encoded in the genome may be incomplete and need to be updated to include chimeric peptides found directly bound to MHCII molecules. One can hope that the computational methods for identifying chimeric epitopes bound to MHCI molecules can be adapted to those bound to MHCII. The variable lengths of MHCII bound epitopes presents a challenge in approaching this task, but the longer MHCII bound peptides may also be an advantage. They could make it easier to identify independently the N- and C-terminal components of a chimeric peptide among the MS-MS generated b-ion versus y-ion fragments. The similarities between these recent findings within the MHCI and MHCII epitope fields might also provide reason to reexamine old data sets for MHC peptide elutions and reprobe them for their presence of transpeptidation mediated fusions.

AUTHOR CONTRIBUTIONS

BR and JK both reviewed relevant literature and drafted the initial manuscript. BR and JK edited and approved the final manuscript.

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Modulation of Intestinal ILC3 for the Treatment of Type 1 Diabetes

Ivana Stojanović, Tamara Saksida, Đorđe Miljković and Nada Pejnović*

Department of Immunology, Institute for Biological Research "Siniša Stanković" – National Institute of the Republic of Serbia, University of Belgrade, Belgrade, Serbia

Gut-associated lymphoid tissue (GALT) is crucial for the maintenance of the intestinal homeostasis, but it is also the potential site of the activation of autoreactive cells and initiation/propagation of autoimmune diseases in the gut and in the distant organs. Type 3 innate lymphoid cells (ILC3) residing in the GALT integrate signals from food ingredients and gut microbiota metabolites in order to control local immunoreactivity. Notably, ILC3 secrete IL-17 and GM-CSF that activate immune cells in combating potentially pathogenic microorganisms. ILC3 also produce IL-22 that potentiates the strength and integrity of epithelial tight junctions, production of mucus and antimicrobial peptides thus enabling the proper function of the intestinal barrier. The newly discovered function of small intestine ILC3 is the secretion of IL-2 and the promotion of regulatory T cell (Treg) generation and function. Since the intestinal barrier dysfunction, together with the reduction in small intestine ILC3 and Treg numbers are associated with the pathogenesis of type 1 diabetes (T1D), the focus of this article is intestinal ILC3 modulation for the therapy of T1D. Of particular interest is free fatty acids receptor 2 (FFAR2), predominantly expressed on intestinal ILC3, that can be stimulated by available selective synthetic agonists. Thus, we propose that FFAR2-based interventions by boosting ILC3 beneficial functions may attenuate autoimmune response against pancreatic β cells during T1D. Also, it is our opinion that treatments based on ILC3 stimulation by functional foods can be used as prophylaxis in individuals that are genetically predisposed to develop T1D.

Keywords: type 3 innate lymphoid cells (ILC3), type 1 diabetes (T1D), gut-associated lymphoid tissue (GALT), regulatory T cells (Treg), interleukin-22 (IL-22), interleukin-2 (IL-2)

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****Correspondence:***
Ivana Stojanović
ivana@ibiss.bg.ac.rs

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INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease that is characterized by low insulin concentration and hyperglycemia. The autoimmune process in pancreatic islets can last for years before the clinical signs of the disease appear. This process is initiated by autoreactive effector T cells including $CD4^+$ and $CD8^+$ cells and it is characterized by high levels of proinflammatory cytokines IL-1β, TNF and IFN-γ (1). The described events are accompanied by decreased numbers and/or defective function of regulatory T cells (Treg) that have an immunosuppressive role and maintain immune tolerance by producing IL-10 and TGF-β and by other mechanisms (2). The overall outcome is the destruction of pancreatic β cells that leads to reduced or completely absent insulin production (1, 3).

Many environmental factors including food ingredients (β-casein or bovine insulin from cow's milk, gluten), exposure to infectious agents (enteroviruses), and intestinal microbiota dysbiosis (due to antibiotics, alcohol abuse, inadequate diet or chronic diseases) are believed to be the reason for the dramatic increase in T1D incidence in people under the age of 18, but also in older adults (4–6).

It is becoming increasingly clear that T1D pathogenesis is linked to the complex interaction between the gut-associated lymphoid tissue (GALT) and the gut microbiota (7). Intestinal barrier serves as an integrator of signals coming from the gut lumen and it is comprised of mucus layer leaning on tightly connected epithelial cells (physical border) and mediators secreted by epithelial cells and immune cells (functional border). GALT cells maintain immune tolerance to food constituents and commensal microbes. The reduction or improper function of GALT-residing tolerogenic dendritic cells (DC) and Treg enables the impairment of oral tolerance (8–10), that may lead to T1D initiation mediated by autoreactive T cells present in the intestinal lamina propria (11, 12). In such case, antigens sampled from the gut might activate β cell-reactive immune cells directly *via* molecular mimicry or indirectly by the bystander activation during the immune response towards gut microorganisms (13). The close link between the gut and the pancreas is exemplified in the finding that pancreatic lymph nodes can drain antigens from the duodenum that leads to Treg induction in GALT and development of oral tolerance (14). Therefore, maintaining a balance between effector T cells and Treg in the gut and pancreatic lymph nodes is essential for sustaining tolerance to islet antigens and prevention of autoreactive T lymphocytes activation and migration to the pancreas where they can initiate β-cell destruction.

GUT-PANCREAS AXIS

The impaired function of the intestinal barrier and dysbiosis precede the development of T1D both in humans and mice. The loss of gut barrier integrity and low-grade intestinal inflammation were discovered in first-degree relatives of T1D patients that are at high-risk of disease development (15–17). The same was confirmed in new-onset and long-term T1D patients (17, 18). The altered microbiota content in T1D patients were found in many studies worldwide as reviewed by Marietta et al. (19).

Increased intestinal permeability and the lack of oral tolerance to ovalbumin was found in 4-6 weeks old, insulitis-free nonobese diabetic (NOD) mice that spontaneously develop T1D (10). Also, these mice had diminished mucus production, lower levels of secretory IgA and increased Th17 and type 3 innate lymphoid cells (ILC3) numbers in the small intestine lamina propria. This coincided with the significant reduction of tolerogenic DC and Treg in the gut-draining lymph nodes during prediabetic stage (10).

There are very few studies that address the activation of autoreactive cells in GALT and their causal link to pancreas autoimmunity. Our recent study implies that activation of insulin-specific $CD4^+$ T cells can occur in the GALT as these cells are present in Peyer's patches of prediabetic NOD and healthy C57BL/6 mice (11). Also, a study that used a β cell–specific TCR-transgenic mouse model has shown that islet-specific T cells activated in the intestinal lamina propria migrated to the pancreatic lymph nodes and the islets causing autoimmune diabetes (20). Further, it was demonstrated that the infection with *Fusobacteria* activates β cell-reactive $CD8^+$ T cells by molecular mimicry within GALT of transgenic NOD mice (12). In addition to the possibility of autoreactive cell activation in the GALT, it was shown that gut microbiota can migrate to the pancreatic lymph nodes where it acts through NOD2 receptors to accelerate the onset of streptozotocin-induced T1D in mice (21). Human studies about the autoreactive cells activation within the GALT indirectly suggest that ingested food or bacterial antigens stimulate the production of β cell-specific autoantibodies *via* molecular mimicry. Examples can be found in reports of Auricchio et al. (22) and Niegowska et al. (23) where data about crossreactivity between β cell antigens and antigens derived from gluten or *Mycobacterium avium* subspecies *paratuberculosis*, a bacterium found in cow's milk, were suggested. Also, higher density of intraepithelial $CD3^+$ and γδ cells and activated $CD25^+$ in lamina propria and lower numbers of $FoxP3^+$ cells in the jejunal mucosa of T1D patients were found (22, 24, 25). In general, individuals with T1D exhibit increased markers of inflammation within GALT suggesting its association with disease development (26).

Prevention or treatment of human T1D through diet-based interventions proved to be very difficult (27). However, a forced change in microbiota content through fecal microbiota transplantation from healthy donors to early-onset T1D patients successfully halted a decline in endogenous insulin production and down-regulated colonic $CD4^+$ cell count, thus further confirming the importance of microbiota content for T1D control (28). In contrast to scarce data in humans, numerous studies provide evidence about prevention or treatment of animal T1D through diet or modulation of microbiota (29, 30). To mention a few: NOD mice fed with a fiber-rich diet had decreased T1D incidence and lower proportion of autoantigen-specific $CD8^+$ lymphocytes in the spleen (31), supplementation with bacterial metabolite butyrate decreased severity of insulitis in NOD mice and their offspring by promoting Treg proliferation in GALT and their migration to the pancreas (32, 33), administration of probiotics exerted beneficial effects in T1D in mice (34–36).

The majority of available data point to the importance of intestinal Treg and their suppressive properties in the prevention and/or treatment of T1D (8, 9). ILC3 have recently been identified as cells critical for maintenance and regulation of mucosal homeostasis in mice and humans (37), but their role in the initiation or development of T1D is largely unknown. This Perspective review will specifically discuss ILC3 biology and their hypothetical role in pancreatic autoimmunity along with possibilities of ILC3-targeted therapies.

INTESTINAL ILC3

Immature ILC develop in bone marrow from common lymphoid progenitor and they generally migrate to mucosal tissues, but can also be found in other lymphoid tissues such as spleen and lymph nodes and non-lymphoid organs skin, liver, brain and pancreas (38–41). As reviewed by Guia et al. (42), ILC3 differentiation process is similar in humans and mice. ILC3 can be identified as the innate counterpart of Th17 cells due to their mandatory expression of retinoid-related orphan receptor γt (RORγt). ILC3 exist in at least two subsets that differ developmentally, transcriptionally and functionally: lymphoid tissue inducer cells (LTi)-like ILC3 (characterized by surface expression of CCR6) and natural cytotoxicity receptor $(NCR)^+$ ILC3 that express NKp46 in mice (43) and NKp44 in humans (44). However, human ILC3 can also express NKp46 and their distribution in skin and intestine was found very similar in humans and mice (45). ILC3 are generally sedentary (46, 47), although in some human pathological conditions differentiated ILC3 were found in the bloodstream (48). Therefore, their regular divisions driven by different internal and environmental signals is essential for their maintenance in the tissues. ILC3 proliferation is stimulated by cytokines, such as IL-18 in human tonsils (49), or combination of tumor necrosis factor-like cytokine 1A, IL-1β, IL-23 and IL-2 in both human and mouse intestinal tissue (50, 51). The major environmental stimuli for murine intestinal ILC3 proliferation are short chain free fatty acids (SCFA) and vitamins A and D (52, 53).

Mature ILC3 develop in the lamina propria of the intestine due to specific differentiation factors (retinoic acid, polyphenols and microbiota) (37). Mouse studies indicate that intestinal ILC3 express integrin α4β7. Their specific signature is the expression of GPR183, a receptor for oxysterols that recruits ILC3 to the small intestine and regulates their migration to the cryptopatches and positioning in the mesenteric lymph nodes. The expression of GPR109A (a receptor for butyrate) dictates ILC3 distribution in Peyer's patches, while distinct pattern of chemokine receptors drives their migration to the specific sites in the GALT such as mesenteric lymph nodes (CCR7), microvilli (CXCR6) or lamina propria (CCR9) (reviewed in 54). In addition, intestinal ILC3 exhibit high free fatty acid receptor (FFAR) expression in contrast to spleen ILC3, for example (55).

Intestinal human and mouse ILC3 are critical for the generation of the organized lymphoid tissue in the intestinal wall during development (LTi-like cells) and they regulate microbiota content and the integrity of the intestinal barrier (46, 56). Mouse ILC3 sense environmental cues either coming from the food or microbiota metabolism products by expressing numerous receptors: retinoic acid receptor (RAR) (57), vitamin D receptor (VDR) (58), aryl hydrocarbon receptor (AhR) (56, 59), or FFAR (55). Also, gut ILC3 respond to cytokines predominantly produced by myeloid cells (IL-1β, IL-23, IL-18 and TNF). In response to these triggers, ILC3 produce several cytokines, including IL-22, IL-17A/F, GM-CSF and IL-2.

IL-22 maintains barrier integrity through stimulation of epithelial cells turnover (60, 61), induction of tight junction proteins production, anti-bacterial peptides and mucins (62, 63). Vitamins A or D are potent inducers of IL-22 production by murine ILC3 (57, 58), while human ILC3 produce IL-22 after microbial stimulation of phagocytes (64). AhR activation is mandatory for IL-22 expression in mouse ILC3 due to its protein-protein interaction with RORγt (59). For example, L-kynurenine (produced by gut epithelial cells) after ligation to AhR stimulates the proliferation of $IL\text{-}22^+$ ILC3 (65). Another stimulus for IL-22 production is the activation of G-protein-coupled receptors FFAR on murine ILC3 by the action of SCFA (66, 67). The signaling cues that come from FFAR2 can indirectly affect IL-22 through augmenting expression of the IL-1 receptor and ILC3 responsiveness to IL-1β (66). What is more, IL-23 produced by myeloid cells as a part of an anti-microbial response has the same effect on ILC3 (68).

ILC3-mediated production of IL-17A/F is important for the induction of antimicrobial peptides and tight junction proteins in epithelial cells (69). However, data obtained from both human and murine studies imply that the major role of ILC3-derived IL-17 is to attract neutrophils to the intestinal tissue in response to bacterial (*Mycobacterium tuberculosis* and *Clostridium difficile*) and fungal infections (70–72).

Secretion of GM-CSF and IL-2 from ILC3 is triggered by IL-1β from intestinal macrophages. Mouse ILC3-derived GM-CSF was shown to act upon intestinal macrophages and dendritic cells to promote their production of IL-10 and retinoic acid, that in turn stimulate the induction and enable maintenance of Treg (73). However, ILC3 in the intestine of inflammatory bowel disease patients produce large amounts of GM-CSF that causes a loss in ILC3 and exacerbation of the disease (74). Recently, a very interesting finding was published identifying a population of mouse and human ILC3 that produce IL-2 and are involved in the preservation of oral tolerance through stimulation of Treg differentiation (51). Along with cytokine-mediated activity, ILC3 can modulate adaptive immune response through antigen presentation *via* class II MHC. Namely, ILC3 have the ability to present microbial antigens and to limit $CD4^+$ cell response by inducing their cell death (75). The reduction in the specific $MHCII^+$ ILC3 population in the intestine is associated with Crohn's disease in pediatric patients (76).

ILC3 IN T1D

The precise contribution of intestinal ILC3 to the onset and progression of T1D has not been investigated, so far. However, there are some data that emphasize ILC3 as important players in shaping GALT environment for T1D initiation or progression. First, decreased frequency of ILC3 was found in the duodenum of T1D patients (77). The human data are in contrast to total ILC3 increase found in small intestine lamina propria of prediabetic NOD mice (10) and in 20 weeks old NOD mice (our unpublished data). So, the second key statement for hypothetical ILC3 relation to T1D pathology comes from the investigation of ILC3 function. Namely, our preliminary data show lower numbers of potentially protective IL-2-producing ILC3 in small intestine lamina propria in 20 weeks old NOD

mice with insulitis and in diabetic C57BL/6 mice with streptozotocin-induced T1D. This was accompanied by down-regulation of FoxP3$^+$ Treg number and IL-22 and GM-CSF mRNA expression in the intestine suggesting a causal relationship between IL-2$^+$ ILC3 and Treg (unpublished results). Higher number of ILC3 and lower of IL-2-producing ILC3 could point to the pro-inflammatory environment in GALT that is related to T1D pathogenesis. The observed ILC3 reduction in human intestinal biopsies from patients with T1D (77) could be associated with ILC3 ability to convert to IFN-γ-producing ILC1 in the inflammatory environment, a process found both in humans and mice (78, 79). That was surely the case in these T1D patients, as the numbers of ILC1 were significantly increased in the intestinal tissue (77).

The close relationship between gut microbiota and proper function of ILC3 within the pancreas in the prevention of T1D development in mice was identified by Miani et al. (41). T1D in NOD mice was found to be associated with reduced numbers of ILC3 in the pancreas and their down-regulated IL-22 production that led to compromised expression of antimicrobial proteins in the pancreas. In the same study, low IL-22-producing ILC3 were found in pancreatic and mesenteric lymph nodes of diabetic NOD mice. Instead of IL-22, they produced rather significant levels of IFN-γ and TNF. All mentioned findings indicate that the transition from prediabetes to diabetes in NOD mice is associated with impaired ILC3 function that could lead to reduced numbers of Treg and imply the protective role of IL-2$^+$ and IL-22$^+$ ILC3 against T1D. In general, there are many pathological conditions where ILC3 play a role such as inflammatory bowel disease, experimental autoimmune encephalomyelitis, Graves' and Hashimoto's thyroiditis (52, 80–82). Still, further investigation will discriminate whether ILC3 reduction precedes or is the result of ongoing inflammation during T1D pathogenesis.

PERSPECTIVES FOR ILC3 MODULATION IN T1D

There are at least three key ILC3 activities that can counteract initiation and/or progression of T1D: 1. Maintenance of gut barrier integrity; 2. Regulation of gut microbiota homeostasis; 3. Stimulation of Treg proliferation and suppressive function. Therefore, the preserved abundance and function of ILC3 within the intestine could largely aid T1D prevention. The hypothetic model of ILC3 role in protection from autoimmune process during T1D is shown in **Figure 1**.

As previously stated, there is a number of external stimuli that can be used for ILC3 modulation (**Figure 2**). In addition to stimulation of IL-22 production, vitamin A attracts specifically ILC3 to the intestinal tissue in both mouse and humans (83, 84). Although there are no data about the influence of retinoids on ILC3 during T1D pathogenesis, their effect on Treg stimulation and suppression of pro-inflammatory adaptive and innate immune cells both systemically and within the pancreas was firmly established (85, 86). Indeed, the oral or intraperitoneal application of retinoids showed a significant preventive effect in NOD and streptozotocin-treated C57BL/6 mice (85, 86). Similarly, vitamin D3 (calcitriol) supplementation led to reduced T1D incidence in NOD mice through generation of

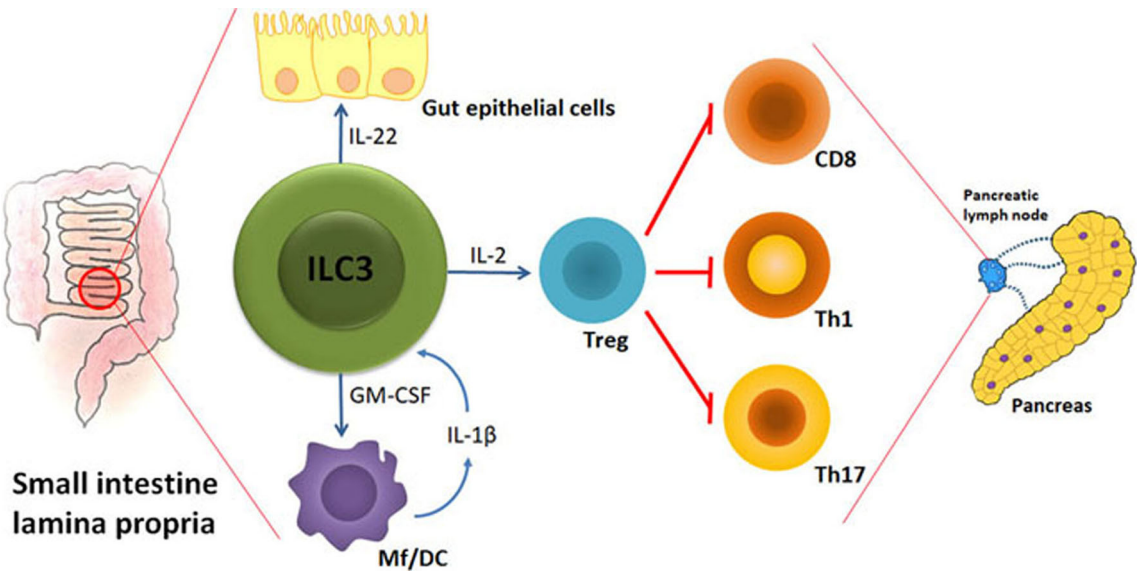

FIGURE 1 | The hypothetical model of ILC3-mediated effects on autoimmune process during T1D. Under the influence of gut microbiota, their metabolites and food ingredients, intestinal ILC3 produce IL-22 that stabilizes the gut barrier and GM-CSF that influences dendritic cells (DC) and macrophages (Mf). Upon activation by microbial cues, Mf produce IL-1β that stimulates ILC3 to increase their production of IL-2 and thus promote intestinal Treg stability and proliferation. Intestinal Treg are able to migrate to the pancreatic lymph nodes and modulate the autoimmune response by providing a suppressive environment in which cytotoxic CD8$^+$ cells, Th1 and Th17 cells are inhibited. The final outcome is the blockade of T cell-mediated autoimmune destruction of pancreatic β cells.

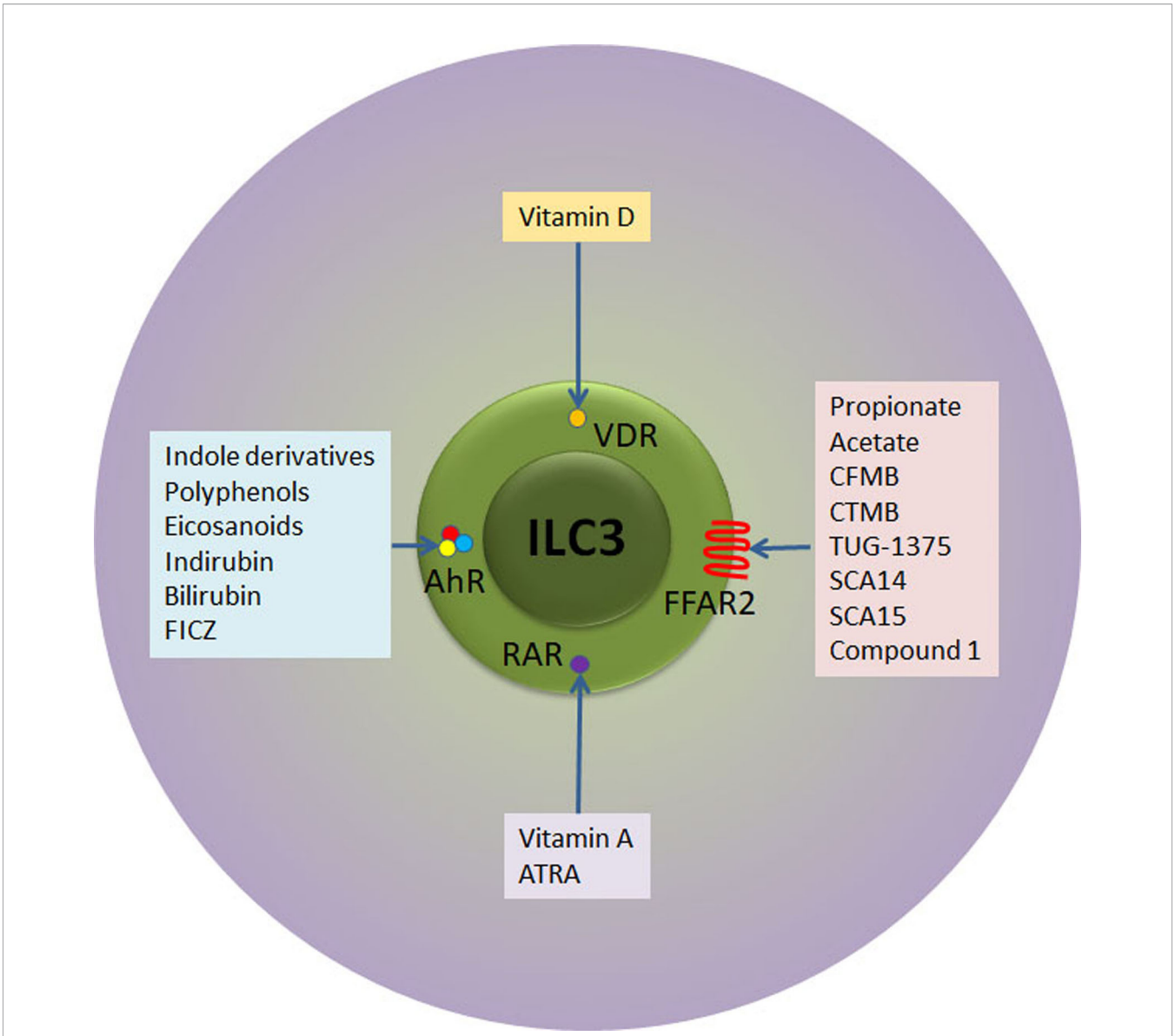

FIGURE 2 | Receptor-ligand interactions relevant for therapeutic targeting of ILC3. ILC3 express receptors for retinoic acid (RAR) and vitamin D (VDR) that upon activation with respective vitamins instigate ILC3 proliferation and/or secretion of IL-22. In addition, ILC3 express AhR transcription factor that can ligate to versatile indol-containing compounds. The activation of AhR is mandatory for the development of mature ILC3 in the intestinal lamina propria, their proliferation and IL-22 secretion. Finally, ILC3 express FFAR2 at very high levels. SCFA (propionate and acetate) as well as several synthetic compounds bind to FFAR2 with high affinity, while Compound 1 and CTMB are selective FFAR2 agonists that promote beneficial ILC3 functions. ATRA, all trans retinoic acid; FICZ, 6-formylindolo[3,2-b] carbazole; CFMB, S)-2-(4-chlorophenyl)-3,3-dimethyl- N-(5-phenylthiazol-2-yl)butamide; 4-CTMB, (S)-2-(4-chlorophenyl)-3- methyl-N-(thiazol-2-yl)butanamide; SCA14, propiolic acid; SCA15, 2-butynoic acid.

suppressive environment, including the promotion of Treg (87, 88). Again, similarly to vitamin A, it remains unknown whether the beneficial effect of vitamin D can be attributed to the modulation of ILC3.

Another way of intestinal ILC3 modulation is the application of AhR ligands. The examples of endogenous AhR ligands are eicosanoids, indirubin, bilirubin, or 6-formylindolo[3,2-b] carbazole (89), while exogenous ligands are mainly derived from cruciferous plants (indole-3-carbinol derivatives) (**Figure 2**). In addition to IL-22 stimulation, AhR ligands promote ILC3 survival and proliferation through Notch-dependent pathways (56, 59). The presence of AhR is mandatory for the development of ILC3 in the intestine as AhR-deficient mice show reduced numbers intestinal ILC3, resulting in increased susceptibility to *Citrobacter rodentium* infection (56, 59). Several studies show that AhR activation can prevent T1D and they point to either Treg-dependent mechanisms (90) or Treg-independent mechanisms (91). Again, the role of ILC3 in AhR-mediated protection from T1D remains unknown.

Finally, SCFA can be potent stimulators of ILC3 function. Acetate, propionate and butyrate, gut microbiota metabolites

that are released during the digestion of fibers, bind to FFAR2 and FFAR3 expressed on ILC3 surface. FFAR2 is predominantly expressed on intestinal ILC3, compared to other ILC in the gut (55). FFAR2, unlike FFAR3 exerts higher affinity for acetate and propionate, than for butyrate (92).

To date, there are numerous studies that explored the role of SCFA in the prevention of T1D. Oral intake of fibers or purified SCFA decreased disease severity in animal models of T1D. This specialized diet even prevented T1D initiation in the offspring of treated female NOD mice (31, 32, 93–95). In general, the mechanism of SCFA action is mainly attributed to Treg induction. Although considerably effective in animal models, administration of oral butyrate for one month did not affect autoimmune response in individuals with longstanding T1D (27). This effect might be due to the butyrate higher affinity of binding to FFAR3 (92), and its differential effect on different subsets of ILC3 (96). Specifically, butyrate stimulates $NKp46^-$ ILC3 that, in addition to IL-22, produce pro-inflammatory cytokines IFN-γ and IL-17 (96).

The fact that FFAR2 is predominantly and highly expressed in the small intestine and colon ILC3 (55) suggests that FFAR2 is the most fitted target for the specific modulation of ILC3. As the highest FFAR2 expression was detected in $CCR6^+$ ILC3 subset that predominantly produces IL-22 in response to SCFA (52), the application of FFAR2 ligands implicate even more stringent control of ILC3-mediated immune response within the GALT. The importance of stimulation of ILC3 for autoimmunity prevention or treatment resides in their FFAR2-mediated IL-22 production and proliferation, but also in the fact that this FFAR2-mediated stimulation will not initiate IFN-γ production (97). In addition to natural ligands, several synthetic FFAR2 agonists have been identified so far: class of phenylacetamides that include (S)-2-(4-chlorophenyl)-3,3-dimethyl- N-(5-phenylthiazol-2-yl)butamide (CFMB) and (S)-2-(4-chlorophenyl)-3- methyl-N-(thiazol-2-yl)butanamide (4-CTMB), TUG-1375, propiolic acid (SCA14), 2-butynoic acid (SCA15) and Compound 1 (patent no. WO 2011/076732 A1) (98) (**Figure 2**).

Application of agonists that preferentially bind FFAR2 (such as Compound 1 and 4-CTMB) would increase the probability of beneficial ILC3 activation (52). In contrast to SCFA that activate FFAR2 in such a manner that it couples to either $G_{i/o}$ or G_q proteins, Compound 1-activated FFAR2 on ILC3 binds to both proteins (52). The consequence of such FFAR2 activity is increased AKT and STAT3 phosphorylation that lead to up-regulated IL-22 expression in mouse colonic ILC3 (52). FFAR2 agonists may expand their anti-inflammatory effects by binding to FFAR2 expressed on colonic epithelial cells. Specifically, SCFA administration alleviates colonic inflammation in mice by augmenting inflammasome activation in colon epithelial cells (99). However, FFAR2 is relatively highly expressed on mouse pancreatic β cells where it controls (inhibits) glucose-stimulated insulin secretion (100) implicating the use of selective ILC3 stimulators.

Engagement of two different types of receptors on ILC3 might provide even better output, as for example, signals through AhR and FFAR2 integrate at the level of IL-22 expression (69). Another benefit of this joint treatment may be synergistic activation of Treg as they express AhR and FFAR2 as well (52). The consumption of functional foods that contain vitamins A and D, AhR and FFAR ligands may provide the beneficial activation of ILC3. In addition, some of the synthetic compounds, Compound 1 for example, exert rather selective effects on intestinal ILC3 when applied orally (52). The perspective of such compounds is immense as they can control complex cellular interaction within GALT and intestinal barrier and consolidate the anti-inflammatory environment that can lead to prevention or blockade of autoimmunity in pancreas, as well as at other distant sites.

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

IS conceptualized the paper. IS, TS and NP drafted the manuscript. DM revised the manuscript and made Figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Inflammasomes and Type 1 Diabetes

James Alexander Pearson[1]*, *F. Susan Wong*[1] *and Li Wen*[2]

[1] Diabetes Research Group, Division of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, United Kingdom, [2] Section of Endocrinology, Internal Medicine, School of Medicine, Yale University, New Haven, CT, United States

Microbiota have been identified as an important modulator of susceptibility in the development of Type 1 diabetes in both animal models and humans. Collectively these studies highlight the association of the microbiota composition with genetic risk, islet autoantibody development and modulation of the immune responses. However, the signaling pathways involved in mediating these changes are less well investigated, particularly in humans. Importantly, understanding the activation of signaling pathways in response to microbial stimulation is vital to enable further development of immunotherapeutics, which may enable enhanced tolerance to the microbiota or prevent the initiation of the autoimmune process. One such signaling pathway that has been poorly studied in the context of Type 1 diabetes is the role of the inflammasomes, which are multiprotein complexes that can initiate immune responses following detection of their microbial ligands. In this review, we discuss the roles of the inflammasomes in modulating Type 1 diabetes susceptibility, from genetic associations to the priming and activation of the inflammasomes. In addition, we also summarize the available inhibitors for therapeutically targeting the inflammasomes, which may be of future use in Type 1 diabetes.

Keywords: inflammasomes, microbiota, type 1 diabetes, NOD mice, humans

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***Correspondence:**
James Alexander Pearson
pearsonj1@cardiff.ac.uk

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INTRODUCTION

Inflammasomes, a term first coined by Dr. Jurg Tschopp in 2002, are multiprotein complexes found in the cytosol, which mediate the activation of inflammatory caspases (1). Inflammasome formation is driven ("primed") by activation of the pattern-recognition receptors (PRRs) in response to pathogen-associated molecular patterns (PAMPs) or damage signals (e.g. damage-associated molecular patterns that are also known as danger-associated molecular patterns, DAMPs) in the cytosol (2–4) (**Figure 1**). In some inflammasomes, the inflammasome adaptor protein designated as Apoptosis-associated Speck-like protein, containing a Caspase activation and recruitment domain (ASC), aids in the oligomerization of the inflammasome components and links the upstream inflammasome sensor molecules to procaspase 1 (21). In ASC-independent inflammasomes, interactions occur between inflammasome components, which can alter the protein structure e.g. NLRC4 can be activated by Neuronal apoptosis inhibitory proteins (NAIPs), resulting in the formation of the disk-like inflammasome (22, 23). In both ASC-dependent and -independent inflammasomes, procaspase 1 becomes dimerized and through autoproteolysis forms catalytically-active caspase 1, which subsequently induces IL-1β and IL-18 cytokine release, as well as inducing pyroptosis, a form of lytic cell death. There are many different types of proteins involved in the formation of the inflammasomes, including the NBD leucine-rich repeat-containing receptor (NLR) family (e.g. NLRP1) and the PYHIN protein families [e.g. absent in melanoma 2 (AIM2)]. In humans,

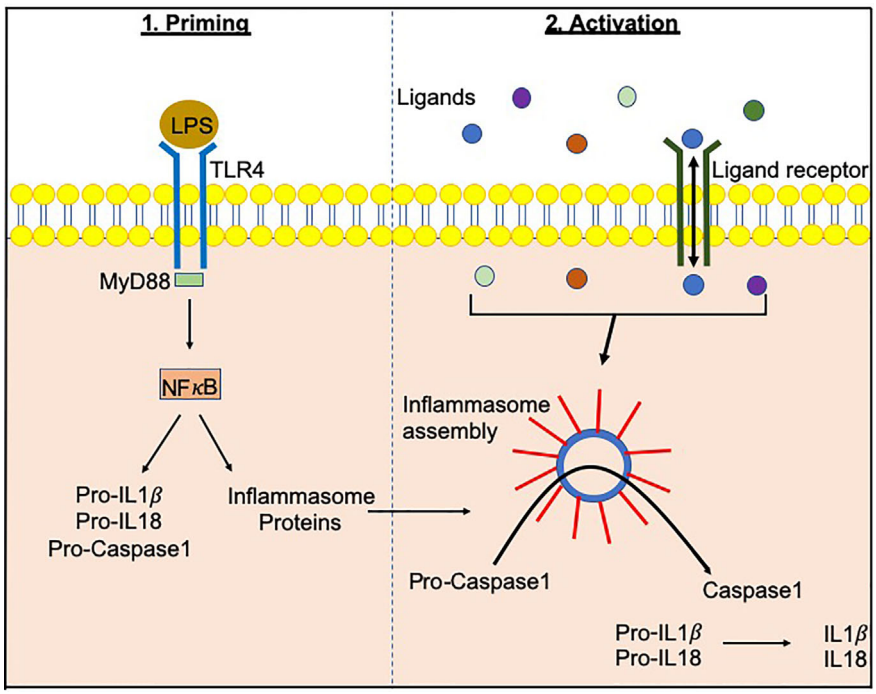

FIGURE 1 | Inflammasome priming and activationInflammasome-related genes e.g. NLRP3, NLRC4 are transcribed following PAMP/DAMP recognition by their respective receptors e.g. bacterial Lipopolysachharide (LPS) recognition by TLR4 pathogen-associated molecular patterns. This "priming" step alerts the cells to potential dangers and prepares the inflammasome machinery to be translated. Upon recognition of additional activating signals (**Figure 2**), the inflammasome proteins oligomerize and form a wheel/disk-like structure. The formation of these inflammasome complexes enables the activation of caspase 1 from its precursor form (procaspase 1), which in turn activates other cytokines including IL-1β and IL-18 (5, 6). Inflammasome-associated proteins can also activate other caspases including caspase 4, 5, 8 and 11 (7–20).

there are 22 NLRs but only NLRP1, NLRP3, NLRP6, NLRP7, NLRP12 and NLRC4 have been shown to form inflammasomes (24–30). Structural and functional differences between the inflammasome proteins result in differences in their ability to bind their respective ligands, and thus each can be activated by different mechanisms (**Figure 2**). In the case of NLRP3, multiple types of ligands can be recognized, which induce disassembly of the trans-Golgi network, leading to the recruitment and binding of NLRP3 *via* its lysine motif (between the PYRIN and NACHT domain) to the phosphatidylinositol-4-phosphate on the disassembled *trans* face of the golgi (39). However, it is unclear whether there are additional mechanisms, including the question of whether other factors contribute to the Golgi network disassembly, or protection from disassembly, or whether similar mechanisms exist for other inflammasomes.

Inflammasomes can be activated by a number of components released during cell/tissue damage, metabolism, infection or by commensal bacteria. Microbial ligands from host commensals or infectious organisms e.g. type 3 secretion system proteins, flagellin, and DNA/RNA can all activate inflammasome proteins. Furthermore, aggregates of Lipopolysaccharides (LPS; specifically, the Lipid A component), an endotoxin present in the outer membrane of gram-negative bacteria, can directly bind to and activate non-canonical inflammasome caspases 4 and 5 (humans) and 11 (mice) (40–43). Importantly, this process 1) is independent of Toll-like receptor (TLR) 4, which can also bind LPS (40, 42), and 2) promotes protection from cytosolic invading pathogens (40–43). Together, these suggest an important role for microbial modulation of inflammasome responses.

Studies using inflammasome-deficient mice have demonstrated that inflammasomes can influence disease susceptibility to inflammatory bowel disease (IBD) (27, 44), cancer (44, 45), obesity (46, 47), viral/bacterial infection (38, 48–53) and type 1 diabetes (T1D) (34, 54, 55). To date, few studies have functionally investigated the mechanistic role of inflammasomes in T1D; however, there are studies indicating a link to inflammasomes and susceptibility to T1D. As susceptibility to T1D can be modulated by microbial components, as discussed later, we highlight the role of inflammasomes as important microbial sensors in the context of T1D.

SINGLE NUCLEOTIDE POLYMORPHISMS LINK INFLAMMASOMES TO TYPE 1 DIABETES SUSCEPTIBILITY

Genetic analyses often provide important insight into genes or mutations that may be associated with disease susceptibility in

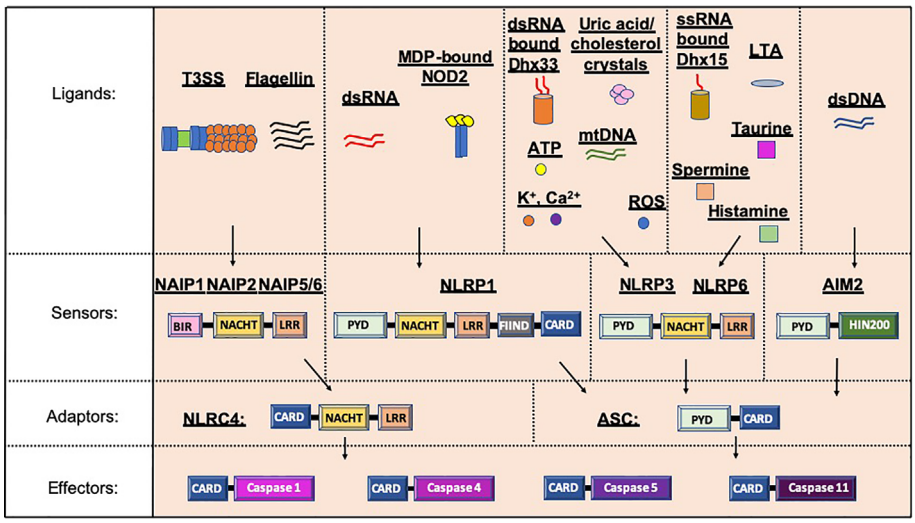

FIGURE 2 | Inflammasome protein sensors and adaptors recognize a variety of ligands, either directly or indirectly. Upon ligand binding, the sensors and adaptors interact via PYD-PYD domain interactions to form the oligomers prior to ASC-mediated recruitment of the Procaspase via CARD-CARD interactions (5–11, 21, 25, 31, 32). NAIP1, 2 and 5/6 bind bacterial-derived Type 3 Secretion system (T3SS) rod or needle proteins or flagellin respectively, prior to activation of the NLRC4 inflammasome (12, 13). NLRP1 can be activated by double stranded RNA (dsRNA; human only) or muramyl dipeptide (MDP) bound to the Nuclear oligomerization domain-containing 2 (NOD2) protein (14, 33). Numerous ligands for NLRP3 have been found including K+, Ca2+, reactive oxygen species (ROS), Adenosine triphosphate (ATP), uric acid crystals, cholesterol crystals, double-stranded RNA (dsRNA) bound by DExD/H-box helicase (Dhx) 33 and mitochondrial DNA (mtDNA) (7, 16, 34–37). Single stranded RNA (ssRNA) bound to Dhx15, lipoteichoic acid (LTA) as well as spermine, taurine and histamine can all activate the NLRP6 inflammasome (32, 35, 38). To date, double stranded DNA is the only ligand known for AIM2 (10, 19, 20). PYD, Pyrin domain; HIN200, Hematopoietic expression, interferon-inducible nature, and nuclear localization 200 domain; NACHT, Nucleotide binding and oligomerization domain; LRR, Leucine-rich repeat; FIIND, function to find domain; CARD, Caspase recruitment domain; BIR, Baculovirus IAP-repeat domains.

humans. Gene mutations in NLRP3, resulting in a gain of function and thus increased IL-1β secretion, were initially linked to a number of inherited autosomal dominant inflammatory diseases e.g. Muckle-Wells syndrome and familial cold autoinflammatory syndrome and chronic infantile neurological cutaneous articular syndrome (56). Since then, single nucleotide polymorphisms (SNPs) in *NLRP1*, *NLRP3* and *NLRC4* have been associated with many autoimmune diseases including IBD (57), celiac disease (58), multiple sclerosis (59) and autoimmune diabetes (60–64). **Table 1** summarizes the SNPs in *NLRP1*, *NLRP3* and *NLRC4* genes that have been investigated in individuals with Type 1 diabetes. Of these SNPs, only 2 are within the coding region of *NLRP1* and *NLRP3* genes (rs12150220 and rs35829419 respectively) and both have been linked to a gain of function and excessive IL-1β and IL-18 secretion in other disease settings (67, 68). The other SNPs that are located in the promoter region may influence gene regulation, but this has not yet been fully elucidated. As **Table 1** illustrates, not all populations studied show the same SNP associations in individuals with Type 1 diabetes. For example, the SNP rs12150220, located in the *NLRP1* gene region, was increased in a Norwegian population with T1D (60); however, no associations were identified in either a Polish (65) or Brazilian (62) population with T1D, compared to their controls. There may be many reasons for this, including population-based genetic differences, the presence of other comorbidities or the microbiota composition. Two studies conducted in the Han Chinese population also showed SNP associations in *NLRP3* and *NLRC4* gene regions with clinical characteristics, including the age of diabetes onset, 2-hour postprandial c-peptide and the presence of anti-glutamic acid decarboxylase (GAD) autoantibodies (63, 66). These suggest a potential link to altered immunity; however, larger scale studies are needed to help us to better understand the association of different allelic variants and combinations of haplotypes in the inflammasome-related genes and susceptibility to Type 1 diabetes. Studies using knock-in mice, in which the SNPs can be introduced into the gene, may provide valuable tools to elucidate the functional consequences of these SNPs.

ALTERED MICROBIAL COMPOSITION MAY DRIVE INFLAMMASOME ACTIVATION IN TYPE 1 DIABETES

Environmental factors, e.g. the microbiota (referring to all microorganisms including bacteria, viruses, fungi, protozoa and archaea), have gained significant traction as modulators of susceptibility to T1D. In turn, it is clear that genes involved in the genetic susceptibility to T1D are important modulators of the bacterial composition in humans and animal models (69, 70). Furthermore, altered gut bacterial composition has been found in individuals diagnosed with T1D (71–75), in Bio-breeding (BB)

TABLE 1 | SNPs in inflammasome genes that have been investigated for associations with autoimmune diabetes in humans.

Gene and location	SNP (and alleles)	Study population	Association	Reference
NLRP1 (17p13.2)	rs12150220 (T/A) rs6502867 (C/T) rs2670660 (G/A) rs878329 (C/G)	Norwegian population; T1D: n=1086 with disease onset before 17 years of age; Controls n=3273	rs12150220 increased in individuals with T1D vs controls - OR=1.16, p=0.006 No differences between individuals with T1D and controls in any of the other SNPs	(60)
	rs6502867 (G/A) rs12150220 (T/A) rs2670660 (T/C) rs878329 (C/G) rs8182352 (A/G) rs4790797 (C/T)	Polish population; T1D: n=221 with disease onset before 13 years of age; Controls: n=254	No differences between individuals with T1D and controls in any of the SNPs	(65)
	rs12150220 (A/T) rs2670660 (G/A)	Pediatric Brazilian population; T1D: n=196 (n=136 with T1D only, n=50 with T1D and Celiac disease and/or Thyroiditis); Controls n=192	No differences between individuals with T1D and controls in any of the SNPs	(62)
	rs11651270 (C/T) rs2670660 (G/A)	Chinese Han population; T1D: n=510; Sex-matched controls n=531	rs11651270 CT frequency lower in T1D population vs controls – OR=0.714 p=0.002 rs2670660 GA frequency lower in T1D population vs controls – OR=0.706 p=0.026 rs11651270 TT genotype associated with younger age at onset vs rs11651270 CT and CC genotypes in T1D cohort p=0.001	(63)
NLRP3 (1q44)	rs10754558 (C/G) rs35829419 (C/A) rs10802501 (T/A)	Pediatric Brazilian population; T1D: n=196 (n=136 with T1D only, n=50 with T1D and Celiac disease and/or Thyroiditis); Controls n=192	rs10754558 G minor allele frequency lower in T1D population vs controls p=0.004 No differences between individuals with T1D and controls in the other SNPs.	(62)
NLRC4 (2p22.3)	rs212704 (T/C) rs385076 (C/T)	Chinese Han population; T1D: n=510; Sex-matched controls n=531	No differences between individuals with T1D and controls in any of the SNPs rs212704 genotype vs 2 hour postprandial c-peptide, p=0.003 rs385076 genotype vs Onset age, p=0.031 rs385076 genotype vs GADA+ (%), p=0.041	(66)

rs12150220 and rs35829419 SNPs encode coding sequence variants. Many of the other SNPs are located within the promoter regions.OR, Odds Ratio at 95% confidence interval.

rats (76), and in Non-obese diabetic (NOD) mice (77, 78), compared to non-diabetic controls. In addition, in individuals who are at genetic risk of developing T1D, changes in gut bacteria are associated with the early development of β-cell autoimmunity (74, 75, 79–81). As mentioned, microbial ligands are one activator of the inflammasomes; changes in the microbial composition and thus the availability of microbial ligands may alter inflammasome activation (**Figure 3**), and this may be one way in which microbes influence pathogenesis of type 1 diabetes.

Viruses have also been implicated in the pathogenesis of T1D. Coxsackie viruses and Rotaviruses have been implicated in the development of T1D due to 1) their association with the development of autoantibodies (82, 83), which are predictive biomarkers for immune progression and T1D development (84); 2) viral proteins e.g. enteroviral capsid protein vp1 can be identified in the islets (85–89); 3) susceptibility to T1D in animal models can be modulated by viral infections (90–98); and 4) an oral Rotavirus vaccine has shown potential to protect individuals at risk of developing T1D from future development of the disease (99). We recently demonstrated that a mouse norovirus infection in NOD mice modulated susceptibility to T1D, mediated through changes in the gut microbiota (100), highlighting the necessity for increased understanding of

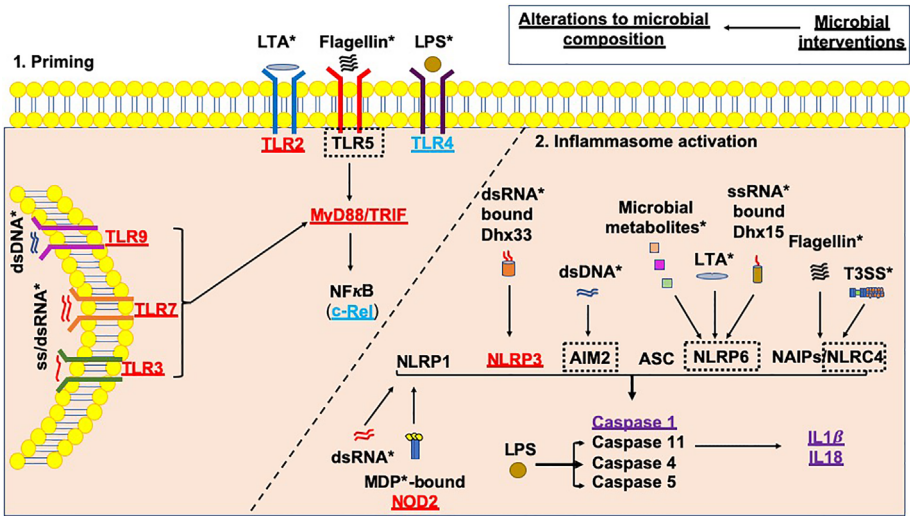

FIGURE 3 | Microbial influences on inflammasome priming and activation in type 1 diabetes. Microbial interventions e.g. fecal microbiota transplants, antibiotic, probiotic and prebiotic usage can all influence the microbial composition, subsequently altering the availability of microbial ligands involved in both the priming, and canonical and non-canonical activation of inflammasomes (as shown by *). Studies of single PRR or inflammasome (*nlrp3*) gene-deficient mice have shown that these proteins would be needed to promote the development of T1D (shown in red); however, Tlr4-deficient and c-Rel-deficient NOD mice (c-Rel is a subunit of the NFκB protein) promote tolerance and limit the development of T1D (shown in blue). In addition, some gene-deficient mice showed no significant effect on mediating susceptibility to T1D (shown in purple). A number of planned studies are currently underway using a number of gene-deficient mice to assess their ability to alter susceptibility to T1D development, as shown by the black dotted boxes. Paradoxically, the gene-deficient mice are also likely to have altered microbial composition, contributing to the protection against/susceptibility to disease. Studies of these gene-deficient mice will need to evaluate the contribution of the gene independently from any alterations to the microbial composition.

broader microbial community interactions. Changes in the viral DNA and RNA abundance, alongside any virus-induced bacterial changes, would also potentially alter inflammasome activation.

Both fungal glucans and parasite/helminth antigens can also stimulate inflammasomes and these may modulate susceptibility to T1D in animal models (101–104); however, few studies have been conducted in humans. Individuals with T1D have greater fungal species diversity compared with healthy controls (105). Others demonstrated that individuals with islet autoimmunity, who later progressed to T1D, had a higher abundance of *Sacchromyces* and *Candida*, compared to those who did not progress to T1D over the 8-9 years of follow up (106). There has been much debate about whether parasitic infection modulates autoimmunity in T1D. One study in Norwegian children showed fewer *Enterobius vermicularis* (a pinworm) infections in children at high genetic risk for T1D (107), while another study in Sweden, suggested no association with worms and the development of T1D in children (108). It is possible that parasites may contribute to the reduction in autoimmunity, as parasite-endemic areas have lower incidences of T1D in their populations, compared to non-parasite endemic areas (109). Whilst this may be because parasitic infections promote Th2 immune responses, other factors are likely to be involved including the lower genetic susceptibility to T1D of the populations living in parasite endemic areas. Thus far, although work in animal models has suggested that helminths, and other parasites like schistosomes or their antigenic products (101, 102, 110) could have a beneficial effect on autoimmunity, these have not yet been translated into therapeutics for humans with type 1 diabetes.

Most of the studies mentioned above focus on the microbiota composition and association with the development of either islet autoimmunity or T1D; however, understanding the mechanisms by which the immune system is activated by the microbiota is important. Furthermore, all of these changes in microbial composition may have profound impacts on inflammasome activation (**Figure 3**).

INFLAMMASOME PRIMING IS LINKED TO TYPE 1 DIABETES SUSCEPTIBILITY

Microbial recognition by PRRs expressed by immune cells are key to regulating crosstalk between immune cells and the microbiota. PRRs such as Toll-like receptors (TLRs), of which there are 10 in humans (TLR1-10) and 12 in mice (TLR1-9, 11-13), selectively bind to their unique microbial ligands, leading to the downstream activation of proinflammatory cytokines (111). These TLRs can be found on different immune and non-immune cells, including the islet β-cells in both humans and mice (112). Studies using TLR-deficient NOD mice have identified that signaling through TLR2, 3, 7 and 9 (97, 113–116) are important for promoting disease, while TLR4 signaling prevents disease development (117). These TLRs signal through one of two key adaptor proteins: Myeloid differentiation primary response 88 (MyD88, which all TLRs

utilize except TLR3) or TIR domain-containing adaptor inducing IFN-β (TRIF, which only TLRs 3 and 4 utilize). Deficiencies in either (118, 119), or both (120), of these two key genes results in significant protection of the NOD mice from the development of diabetes, indicating a reliance on downstream-mediated signaling to induce the proinflammatory immune response. Interestingly, only MyD88-deficient mice, but not MyD88 and TRIF double-deficient mice, were protected from immune infiltration in the islets, suggesting that TRIF-mediated signaling, most likely due to TLR4 signaling, was responsible for inducing tolerance (120). TLR4 signaling in human monocyte-derived DCs, stimulated by *E.coli* lipopolysaccharide [LPS; a TLR4 ligand (121)], induced immune tolerance, unlike the effect seen from stimulation with LPS derived from *B.dorei* (122). As Finnish children have a higher abundance of *B.dorei*, and a higher incidence of Type 1 diabetes, compared to their genetically-similar Russian neighbors, it is likely that LPS-induced tolerance is important for modulating susceptibility to T1D in humans (122). TLR activation is also important for priming the inflammasome proteins and thus, changes to the TLR stimulation highlighted above are likely to modulate inflammasome activation as well. It is unclear, at present, whether any of these studies of TLR-deficient mice, or studies of TLR stimulation of cells from individuals with Type 1 diabetes, will differentially influence the activation of the inflammasome and how the functional consequences of this could influence susceptibility to T1D.

In addition to the TLRs, there are also other microbial sensors that can prime the inflammasome complexes, including the cytosolic Nucleotide-binding oligomerization domain (NOD) proteins, NOD1 and NOD2. NOD1 and NOD2 both recognize bacterial peptidoglycan moieties (123, 124) and upon binding, oligomerize and signal through the Receptor-interacting-serine/threonine-protein kinase 2 (RIP2) resulting in the activation of NFκB and production of inflammatory cytokines (125). Using a streptozotocin (STZ)-induced type 1 diabetes model, NOD2 deficiency, but not NOD1 or RIP2 deficiency, protected the mice from disease development (126). These findings were also supported by other studies in NOD mice, demonstrating that NOD2-deficient NOD mice were protected from type 1 diabetes development, and this was dependent on the gut microbiota composition (127), whereas RIP2-deficient NOD mice were not protected (120). Interestingly, both NOD1 and NOD2 appear to have RIP2 independent functions; NOD2 binds CARD9 to mediate downstream signaling independent of RIP2 (128), while NOD1 regulates MAPK signaling independent of RIP2 (129). It is still unclear what the role, if any, NOD1 has in the immunopathogenesis of autoimmune Type 1 diabetes. Importantly, following muramyl dipeptide (ligand) binding, NOD2, complexed with NLRP1, promotes inflammasome activation (33), independent of NOD1 activation (25). Furthermore, in NOD2-deficient mice, induction of intestinal inflammation by dextran sodium sulfate (DSS) resulted in elevated NLRP3 inflammasome formation, suggesting that NOD2 may interact with and/or modulate NLRP3 inflammasome formation (130). Thus, understanding NOD2 activation and its role in modulating inflammasome formation in relation to T1D pathogenesis will need further mechanistic investigation.

It should be noted that in most studies using PRR-deficient NOD mice, the microbiome can be altered by the gene deficiency, which promotes a tolerizing influence and suppression of type 1 diabetes development, as in the case with NOD2-deficient NOD mice (127). Thus, in evaluating studies using these models, it is vital to control for environmental variables such as cage effects (i.e. comparisons between mice in different cages) and legacy effects (i.e. comparisons between mice bred from different breeders), both of which can substantially alter the bacterial composition (131, 132). Failure to consider these variables can promote non-reproducible data and thus future studies need to 1. be transparent in the reporting of these elements in their animal experiments, and 2. Control for these variables.

INFLAMMASOME PROTEIN DEFICIENCIES ALTER SUSCEPTIBILITY TO TYPE 1 DIABETES

To date, only two inflammasome-associated proteins (NLRP3 and AIM2) have been studied for their role in modulating susceptibility to T1D using gene-deficient mice (34, 54, 55). NLRP3-deficient NOD mice were protected from the development of T1D compared to wild-type littermates, as were wild-type NOD mice treated with an NLRP3 inhibitor (parthenolide; 10mg/kg body weight, twice a week for 4 weeks from 10-12 weeks of age) (54). NLRP3-deficient C57BL/6 mice were also protected from diabetes development following STZ treatment, whereas ASC-deficient C57BL/6 mice were not (34). NLRP3 deficiency in NOD mice was found to reduce T cell activation and Th1 differentiation, as well as reducing T cell expression of both the chemokines CCR5 and CXCR3, and *ccl5* and *cxcl10* gene expression from the islet β-cells, resulting in poor T cell chemotaxis into the islets and protection from T1D development (54). Furthermore, diabetic NOD mice exhibited increased *Nlrp3* and *pro-il-1β* gene expression in the pancreatic lymph nodes, compared to pre-diabetic NOD mice, suggesting an increasing role for inflammasome activation (shown to be mediated by circulating mitochondrial DNA) with disease progression (34). In contrast to NLRP3-deficient C57BL/6 mice, AIM2-deficient C57BL/6 mice had accelerated STZ-induced diabetes development, compared to wild-type control mice (55), implying that ASC regulates inflammasome activation. This acceleration in STZ-induced diabetes development in AIM2-deficient mice occurred through enhanced gut permeability and increased bacterial translocation to the pancreatic lymph nodes. These findings were similar to those from the STZ-induced NOD2-deficient mouse study (126), with the inference that NOD2 activation of inflammasomes may be ASC-dependent. In humans, *Aim2* gene expression was increased in the pancreas but not in peripheral blood mononuclear cells (PBMCs) in individuals with T1D compared to healthy controls (55); however, the data from the pancreas was only available in a small group (n=4-8) and thus needs to be confirmed in larger cohorts, ideally separating infiltrating immune cells from the islet β-cells. Another study in humans found

that *NLRP1* and *NLRP3* gene expression was reduced in PBMCs and granulocytes in individuals with newly diagnosed T1D (less than 6 months), compared to healthy controls (133). While these studies indicate an important involvement of two of the inflammasome proteins in the development of T1D, further studies are needed to evaluate the other inflammasome-related proteins and how different types of stimulation may influence their function. More studies both in animal models, particularly those developing spontaneous autoimmune diabetes, and in humans, are needed to better understand inflammasome involvement and modulation during diabetes development. Finally, identifying the role of inflammasomes in individual cell types will be pivotal for understanding the key players in inflammasome activation and regulation. Thus, cell-specific gene knock out mice may be valuable tools for such studies.

THERAPEUTIC INTERVENTION – A ROLE FOR TARGETING INFLAMMASOMES?

Inflammasome activation induces IL-1β and IL-18 cytokine release following Caspase activation. Both IL-1β and IL-18 cytokines increase with progression to diabetes and destruction of the islet β-cells (134–136). To further investigate whether blocking these pathways could be therapeutically useful, studies targeting the IL-1 pathway were conducted in individuals with recent-onset T1D. Two Phase 2a randomized, multicenter, double-blind, placebo-controlled trials were carried out in which Canakinumab (a human monoclonal anti-IL-1 antibody), or Anakinra (a human IL-1 receptor antagonist), were administered (137). Contrary to expectations, these single immunotherapy interventions failed to prevent the ongoing autoimmunity. This result was concordant with data from NOD mouse models that included IL-1 receptor- (138), Caspase-1- (139, 140), IL-1β- (140) and IL-18- (141)-deficient NOD mice, where no significant changes to diabetes protection were observed with any of these mutations. However, a study combining anti-CD3 treatment with either Anakinra or an anti-IL-1β antibody resulted in reversal of diabetes in recent-onset T1D NOD mice (142), suggesting that combined therapy may also improve clinical efficacy in humans. Given the success of Teplizumab (anti-CD3) in delaying the development of T1D in relatives at risk (143, 144), a combined study evaluating the role of Teplizumab with IL-1 blockade may further enhance clinical efficacy. It is intriguing that NLRP3-deficient NOD mice were protected from T1D, while IL-1 receptor-, Caspase-1/11-, IL-1β- and IL-18-deficient NOD mice were not. There could be multiple reasons for this including: 1. Altered microbiota caused by the gene deficiency, influencing priming/activation of inflammasomes, 2. Promotion of other inflammasome signaling when Nlpr3 is deficient, 3. Effects on other caspases, for example Caspase 8 can also regulate inflammasome activation (145, 146), 4. Effects on other proteases which can process IL-1β (147, 148), and 5. Other unknown protein interactions may be involved. It is clear that further study of multiple pathways of influence is needed to fully comprehend and understand these differences.

Modulation of inflammasomes has had some therapeutic success in autoimmune diseases. A small-molecule inhibitor (MCC950), specifically targeting NLRP3 inflammasome activation (ASC oligomerization) but not AIM2, NLRC4 or NLRP1 inflammasomes, was able to attenuate mouse models of multiple sclerosis (149) and Parkinson's disease (150). Additional NLRP3 selective inhibitors have been developed, which inhibit ATPase activity (151, 152), or oligomerization of NLRP3 (153), and these inhibitors prevented or ameliorated the development of joint inflammation in arthritis (154), metabolic perturbation in high fat diet-fed mice (151, 153), and autoinflammatory syndromes (151–153). There are also less selective natural inflammasome inhibitors including Genepin, a component of *Gardenis* fruits (155), which can inhibit NLRP3 and NLRC4 inflammasome activation *via* inhibiting autophagy, the eicosanoid 15-deoxy-Δ(12,14)-PGJ2 (15d-PGJ2) and related cyclopentenone prostaglandins (156), which inhibit the NLRP1 and NLRP3 inflammasomes and thence conversion of procaspase 1 to caspase 1. Parthenolide inhibits NLRP1, NLRP3 and NLRC4 inflammasomes (but not AIM2) (157–160), by alkylating the cysteine residues in Caspase 1 and in the ATPase domain of NLRP3 and inhibiting IκB kinase function required for NF-κB activation. As previously mentioned, Parthenolide prevented the development of T1D in 10-12-week old prediabetic NOD mice after 4 weeks of treatment (54). Thus, further investigation of inflammasome inhibitors as a potential therapeutic intervention in T1D is needed. More inflammasome regulators and inhibitors have been studied in different diseases, and which have been reviewed elsewhere (161–163). Future studies should focus on the more selective inflammasome inhibitors, as these will likely have minimal effects on other inflammasome pathways, thereby minimizing detrimental impacts on host defense. Initiating these studies will be vital to fully determine their potential clinical benefits and long-term safety.

Microbes contain multiple ligands that can promote inflammasome activation, thus, therapies targeting the microbiome may also modulate inflammasome responses. Therapies employing microbes or their metabolites have shown some promise in modulating T1D development in animal models (164–168). While supplementation with bacterial-derived short chain fatty acids (SCFAs) protected NOD mice from the development of T1D (164, 168), a human intervention study in which butyrate was administered to longstanding T1D participants was found to have minimal immunological or metabolic effects compared to placebo-treated individuals (169). The human studies were not comparable with the NOD mouse studies however, and further investigation of SCFA administration including dose, duration and timing of treatment should be conducted in those at risk of developing T1D, if the human and mouse investigations are to be compared. In children, early probiotic administration (at the age of 0-27 days) was associated with reduced islet autoimmunity (autoantibodies), compared with children receiving probiotics later than 27 days of age, or those who had never received them (170). A recent study showed that β-cell function could be preserved in newly diagnosed T1D patients,

who were recipients of an autologous fecal microbiota transplant, when compared to recipients of an allogeneic (healthy donors) fecal microbiota transplant (171). Together, these studies highlight the potential of harnessing the microbiota as a therapy to modulate ongoing immunity in T1D; however, these studies have not yet evaluated the involvement of the microbial-sensing pathways such as inflammasomes for their ability to modulate the development of diabetes or improved β-cell survival and function.

SUMMARY

Inflammasomes are important activators of the innate immune response, leading to subsequent adaptive immune responses, particularly in response to microbial ligands. There has been a clear knowledge gap in understanding these inflammasomes in the context of Type 1 diabetes, but more studies are emerging highlighting the importance of the following areas - 1) single nucleotide polymorphisms in inflammasome genes; 2) priming of the inflammasome and 3) the function of the inflammasome proteins in modulating susceptibility to Type 1 diabetes. Together these studies indicate a need to better understand the role of inflammasomes in responding to the microbiota in Type 1 diabetes. At present, to achieve this would require investigators to 1) enlarge the sample sizes for the SNP association studies and investigate the mechanisms behind their association with disease; 2) decipher TLR signaling and inflammasome crosstalk in disease development; 3) investigate how inflammasomes specifically modulate microbial composition and 4) further evaluate inflammasome inhibitors in disease development and how these may be used therapeutically. While this is a new area of investigation, the evidence suggests that studying the inflammasome may provide another possible set of involved pathways that may be amenable to therapeutic targeting to prevent or delay Type 1 diabetes development. Finally, while inflammasomes may have a role in modulating susceptibility to T1D, we should not forget that they are likely to form a part of a multi-mechanistic pathway contributing to the development of T1D. Thus, assessing inflammasome activation in conjunction with other mechanisms of immune activation and regulation may be important to determine a broader picture for clinical interventions.

AUTHOR CONTRIBUTIONS

JAP wrote the review. FSW and LW edited the review. All authors contributed to the article and approved the submitted version.

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Elevated Biomarkers of NETosis in the Serum of Pediatric Patients With Type 1 Diabetes and Their First-Degree Relatives

*Adam Klocperk[1], Jana Vcelakova[2], Petra Vrabcova[1], Irena Zentsova[1], Lenka Petruzelkova[2], Zdenek Sumnik[2], Stepanka Pruhova[2], Anna Sediva[1] and Zuzana Parackova[1]**

[1] Department of Immunology, 2nd Faculty of Medicine Charles University, University Hospital in Motol, Prague, Czechia, [2] Department of Pediatrics, 2nd Faculty of Medicine, Charles University and Motol University Hospital, Prague, Czechia

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Marion Russier,
Max Planck Institute
of Biochemistry, Germany

***Correspondence:**
Zuzana Parackova
zuzana.parackova@fnmotol.cz

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Type 1 diabetes (T1D) is an autoimmune disorder with unambiguous involvement of both innate and adaptive immune mechanisms in the destruction of pancreatic beta cells. Recent evidence demonstrated that neutrophils infiltrate the pancreas prior to disease onset and therein extrude neutrophil extracellular traps (NETs), web-like structures of DNA and nuclear proteins with a strong pro-inflammatory biologic activity. Our previous work showed that T1D NETs activate dendritic cells, which consequently induce IFNγ-producing Th1 lymphocytes. The aim of this study was to assess direct *ex vivo* biomarkers of NETosis in the serum of recent onset and long-term pediatric T1D patients, their first-degree relatives and healthy controls. To this end we evaluated serum levels of myeloperoxidase (MPO), neutrophil elastase (NE), proteinase 3 (PR3), protein arginine deiminase 4 (PAD4), LL37 and cell-free DNA-histone complexes in sex- and age-matched cohorts of T1D first-degree relatives, recent-onset T1D patients, and in patients 12 months after clinical manifestation of the disease. Our data shows that disease onset is accompanied by peripheral neutrophilia and significant elevation of MPO, NE, PR3, PAD4 and cell-free DNA-histone complexes. Most biomarkers subsequently decrease but do not always normalize in long-term patients. First-degree relatives displayed an intermediate phenotype, except for remarkably high levels of LL37. Together, this report provides evidence for the presence of ongoing NETosis in pediatric patients with T1D at time of clinical manifestation of the disease, which partly subsides in subsequent years.

Keywords: type 1 diabetes, neutrophils, NETosis, PAD4, pediatric, neutrophil extracellular trap, NET, ELISA

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease resulting from the destruction of insulin-producing beta cells in the pancreas, which involves both innate and adaptive immunity. Neutrophils in particular enjoy the interest of the scientific community, with both reduced (1–3) and elevated (4, 5) neutrophil counts being reported in T1D patients. While it has been shown that neutrophils can infiltrate the pancreas and initiate the autoimmune response driving its destruction in mice (6), their precise role in the pathogenesis of T1D is still under debate.

Neutrophils are able to extrude web-like structures called neutrophil extracellular traps (NET) in a process of specific cell death called NETosis (7). Even though NETosis is an essential part of host defense against infection, it can also be involved in the pathogenesis of autoimmune diseases. We and others have shown the involvement of NETs in the pathogenesis of autoimmune diabetes (6, 8–10), where neutrophils and NET-related products act as drivers of inflammation (11), Th1 polarization and type II interferon production (10). Conversely, the inhibition of NET formation is able to attenuate the development of T1D (6).

In previous studies, increased circulating levels of neutrophil elastase (NE) and proteinase 3 (PR3), both serine proteinases produced by neutrophils and stored in their primary azurophilic granules, as well as increased levels of myeloperoxidase (MPO)-DNA complexes, were reported in patients with T1D (12), suggesting enhanced NET formation. However, another study refuted this observation and documented significantly decreased levels of NE and PR3 (9) in patients within 3 years of diagnosis, a finding possibly related to the previously shown gradual decrease of neutrophil counts after clinical onset of the disease (13).

In this study we aim to expand the spectrum of investigated NETosis related products to NE, PR3, MPO, peptidyl arginine deiminase 4 (PAD4) – an enzyme which facilitates chromatin decondensation vital for NET formation, LL37 – an antimicrobial cathelicidin, and DNA-histone complexes and assess their levels in the serum of a cohort of patients at the onset of T1D, with well-established disease and in their first-degree relatives, both autoantibody positive and negative.

METHODS

Cohort Description

Peripheral blood neutrophil counts were measured as part of complete blood count with differential, using routine in-house methods in 333 patients with long-term T1D at an average of 5.73 ± 3.82 years since clinical manifestation of T1D, 172 patients with newly diagnosed T1D sampled within 7 days of manifestation, 51 antibody positive [at least one of the following: anti-glutamic acid decarboxylase 65 (anti-GAD65), anti-tyrosine phosphatase-like insulinoma antigen 2 (anti-IA2), anti-indole-3-acetic acid (anti-IAA), anti-zinc transporter protein 8 (anti-ZNT8)] first-degree relatives of T1D patients, 122 antibody negative first-degree relatives of T1D patients and 17 healthy children.

Serum neutrophil products were measured in a subset of 31 patients with newly diagnosed T1D [8 who presented with diabetic ketoacidosis (DKA, pH < 7.3)], 32 patients at one year since clinical manifestation of T1D. Additionally, we investigated 32 antibody negative and 32 antibody positive healthy first-degree relatives. The control group with no personal history of autoimmune disease comprised 32 age and sex-matched healthy children.

Detailed cohort description data can be inspected in **Tables 1** and **2**.

Legal guardians of all study participants signed a written informed consent prior to entering the study. The study was approved by the institutional Ethics Committees of the University Hospital Motol and 2nd Faculty of Medicine, Charles University in Prague, Czech Republic and was conducted in accordance with the Declaration of Helsinki.

Neutrophil Counts and Autoantibody Determination

Anti-GAD65, -IA2, -IAA autoantibodies were measured using radioimmunoassay (RIA) based on 125I-labelled antigens (Medipan GmbH, Berlin, Germany). All three assays were evaluated using the Islet Autoantibody Standardization Program 2015. The following assay cut-offs were determined with receiver operating characteristic (ROC) plots using all the samples: 0.4 U/ml for anti-IAA, 1.0 U/ml for anti-GAD65 and 0.9 U/mL for anti-IA2. Anti-ZnT8 were examined by ELISA (RSR Limited, Wales, UK) as

TABLE 1 | Cohort description – neutrophil counts.

	Number (n)	Age (years, mean ± SD) (range)	Sex (n)	Time since diagnosis of T1D (years, mean ± SD) (range)	HbA1c (mmol/mol, mean ± SD) (range)
Healthy	17	14.29 ± 1.87 (11.81-17.37)	5 male, 12 female	NA	NA
Ab- relatives	122	9.11 ± 4.48 (0.3-20.7)	61 male, 61 female	NA	34.77 ± 2.76 (27–39)
Ab+ relatives	51	9.07 ± 4.37 (2.7-25.6)	29 male, 22 female	NA	32.83 ± 3.57 (25-40)
Recent onset	172	9.19 ± 4.44 (1.1-18.27)	85 male, 87 female	NA	97.63 ± 29.83 (33-172)
Long-term T1D	333	12.61 ± 4.21 (2.28-24.58)	168 male, 165 female	5.73 ± 3.82 (0.71-17.5)	64.48 ± 15.42 (35-143)

TABLE 2 | Cohort description – serum neutrophil products.

	Number (n)	Age (years, mean ± SD)	Sex (n)	HbA1c (mmol/mol, mean ± SD) (range)
Healthy	32	9.6 ± 4.2 (3.8-17.9)	16 male, 16 female	NA
Ab- relatives	32	9.5 ± 4.1 (2.0-17.4)	16 male, 16 female	33 ± 4.1 (28-43)
Ab+ relatives	32	9.9 ± 4.2 (3.2-17.6)	16 male, 16 female	33 ± 4.8 (27-40)
Recent onset	31	9.6 ± 4.2 (3.3-17.5)	15 male, 16 female	106.5 ± 25.5 (59-156)
Long-term T1D	32	10.1 ± 4.3 (3.2-17.5)	16 male, 16 female	50 ± 22.8 (32-125)

described previously (14). Complete blood count with differential was analysed on the Sysmex XN-3000 platform (Sysmex Europe, Norderstedt, Germany).

NET Components

Commercially available ELISA kits were used to quantify myeloperoxidase, neutrophil elastase, proteinase 3 (Abcam, Cambridge, USA), LL37 (Hycult Biotech, Wayne, USA), DNA-histone complexes (Sigma-Aldrich, St. Luis, USA) and PAD4 (LSBio, Seattle, USA) according to manufacturer's instructions.

Statistics

Statistical analyses were performed using Brown-Forsythe and Welch one-way analysis of variance (ANOVA), unpaired t-tests with Welch's correction and linear regression using GraphPad PRISM 8.0 (San Diego, CA, USA). Values of p=0.01-0.05 (*), p=0.001-0.01 (**), p<0.001 (***) and p<0.0001 (****) were considered statistically significant.

RESULTS

T1D Onset Is Accompanied by Transient Neutrophilia

In this study we observed substantial changes in absolute circulating neutrophil counts between healthy controls, first-degree relatives of T1D patients both negative and positive for T1D-specific autoantibodies, patients at clinical onset of the disease and patients with long-term disease (**Figure 1A**, Brown-Forsythe and Welch ANOVA $p < 0.0001$, summary data for other leukocyte populations shown in **Supplementary Table 1**).

In particular, we saw a modest but statistically insignificant decrease of neutrophils in relatives regardless of seropositivity and in patients with long-term disease, compared to healthy controls (**Figure 1A**), which was independent from age (**Figure 1B**). In contrast, patients at the onset of clinical disease had elevated absolute neutrophil counts compared to first-degree relatives ($p < 0.0001$ for antibody negative and $p = 0.001$ for antibody positive, unpaired t-test with Welch's

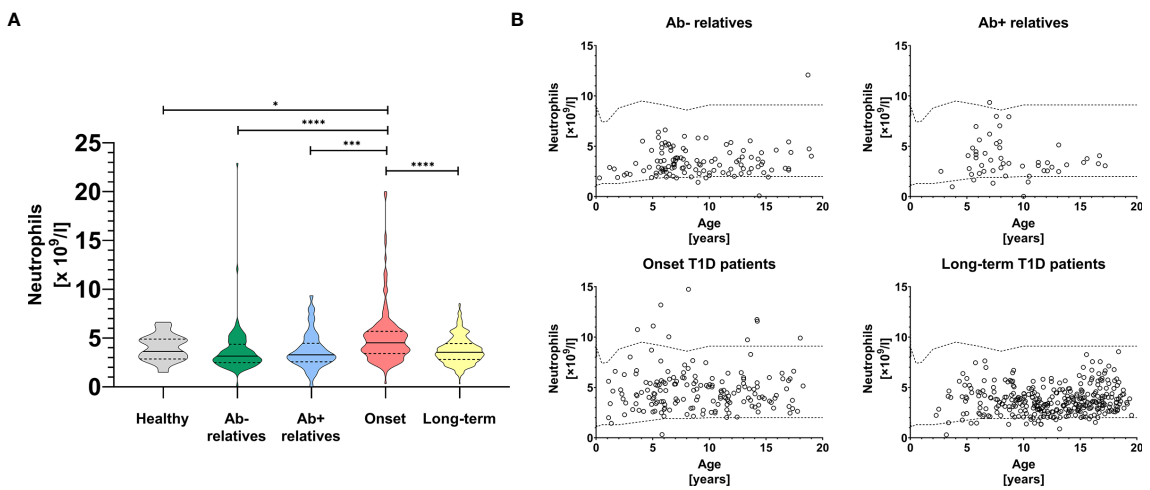

FIGURE 1 | Peripheral blood neutrophils. Absolute neutrophil counts in the peripheral blood of healthy controls, antibody negative and antibody positive first-degree relatives of T1D patients, T1D patients at the clinical onset of the disease and T1D patients with long-term, well-established disease **(A)**. Temporal development of absolute neutrophil counts in the peripheral blood of healthy controls, antibody negative and antibody positive first-degree relatives of T1D patients, T1D patients at the clinical onset of the disease and T1D patients with long-term, well-established disease **(B)**. Healthy in-house reference range visualized with dashed line. Unpaired t-test with Welch's correction p-values shown, p=0.01-0.05 (*), p=0.001-0.01 (**), p<0.001 (***), p<0.0001 (****).

correction), long-term T1D patients ($p < 0.0001$) and healthy controls ($p = 0.014$).

Increased Circulating Levels of NET-Associated Biomarkers Are a Hallmark of Recent Onset T1D Patients

Since the process of NETosis has previously been implicated in the pathogenesis of T1D and our patients displayed a discrete transient neutrophilia upon reaching clinical onset of T1D, we hypothesized that NET-associated biomarkers should be elevated in the serum of T1D patients, especially at the time of clinical manifestation of the disease.

Indeed, we were able to detect high levels of myeloperoxidase (MPO) (**Figure 2A**, Brown-Forsythe and Welch ANOVA $p = 0.0024$), neutrophils elastase (NE) (**Figure 2B**, $p = 0.0005$) and proteinase 3 (PR3) (**Figure 2C**, $p < 0.0001$), enzymes widely present in NET structures and released during the degranulation process, in the sera of T1D patients and their relatives, compared to healthy controls.

Antibody positive relatives (Ab+) also exhibited significantly elevated levels of LL37 (cathelicidin), an antimicrobial peptide commonly present in NET structures (**Figure 2D**, $p = 0.0058$). There was no relationship between a number of autoantibodies and NET-related products (**Supplementary Figure 1**).

Patients at the clinical onset of T1D displayed increased levels of cell-free DNA-histone complexes (**Figure 2E**, $p = 0.0031$) and peptidyl arginine deiminase 4 (PAD4), an enzyme which facilitates protein citrullination and has been intricately linked to NET formation (**Figure 2F**, $p < 0.0001$).

We did not observe significant difference in serum levels of any of the analytes and absolute circulating neutrophil counts in recent onset patients who presented with and without diabetic ketoacidosis (DKA), defined as pH < 7.3 (**Supplementary Figure 2**). MPO and DNA-histones levels significantly correlated with blood pH and MPO was slightly higher in patients with DKA but showed high variance (unpaired t-test with Welch's correction $p = 0.046$).

None of the NET-associated biomarkers was significantly associated with age (**Supplementary Figure 3**) or sex (**Supplementary Figure 4**). There was also no significant correlation between their serum levels and absolute counts of circulating neutrophils (**Supplementary Figure 5**).

Residual beta-cell activity was not directly quantified through the measurement of fasting or stimulated C-peptide, however there was no correlation between the metabolic control of T1D measured as glycated hemoglobin fraction HbA1c (**Supplementary Figure 6**) and NET-associated biomarkers in either recent onset or long-term patients.

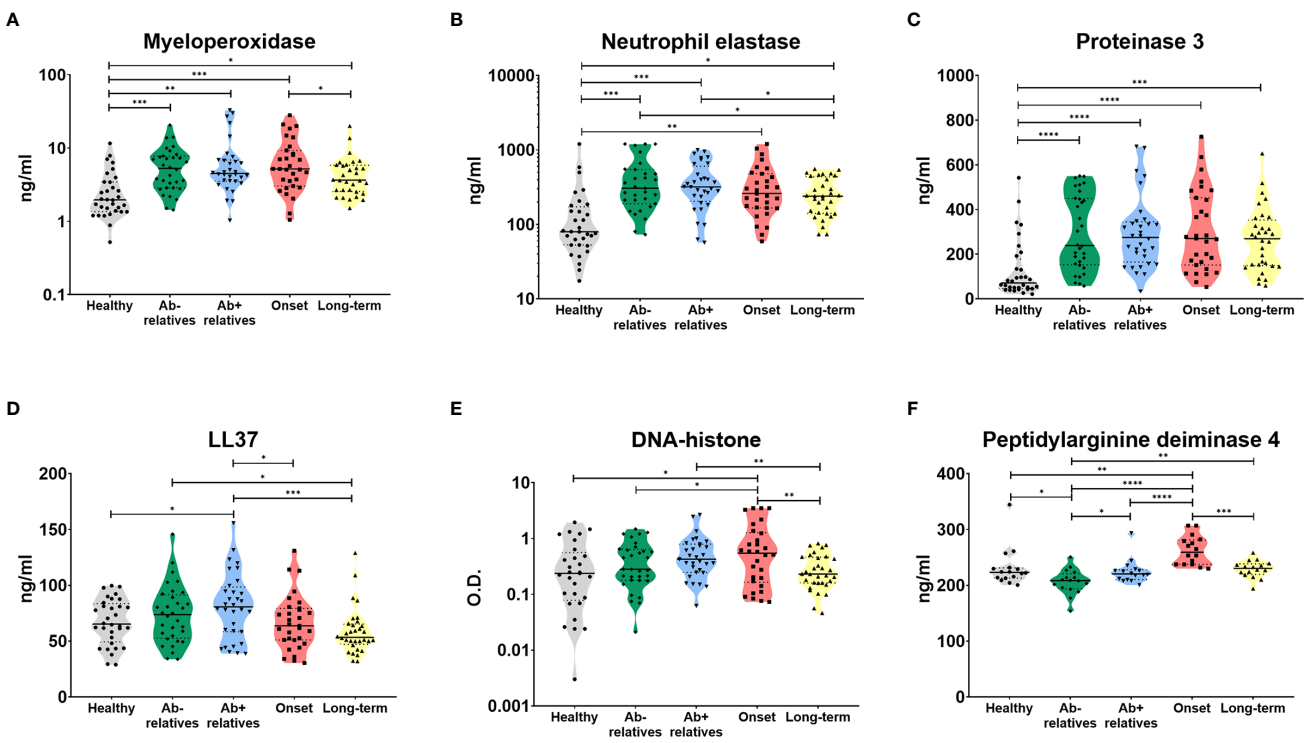

FIGURE 2 | Serum products of NETosis. Products of NETosis **(A)** myeloperoxidase, **(B)** neutrophil elastase, **(C)** proteinase 3, **(D)** LL37, **(E)** DNA-histone complexes and **(F)** peptidyl arginine deiminase 4) in the serum of healthy controls, antibody negative and antibody positive first-degree relatives of T1D patients, T1D patients at the clinical onset of the disease and T1D patients with long-term, well-established disease. Unpaired t-test with Welch's correction p-values shown, p=0.01-0.05 (*), p=0.001-0.01 (**), p<0.001 (***), p<0.0001 (****). O.D. = optical density.

DISCUSSION

In this brief report we demonstrate the elevation of neutrophils and indirect serum biomarkers of neutrophil NETosis – the enzymes myeloperoxidase (MPO), proteinase 3 (PR3), neutrophil elastase (NE) and protein arginine deiminase 4 (PAD4), the active form of the antimicrobial peptide cathelicidin, LL37, and cell-free DNA-histone complexes – in the blood of pediatric patients with recent onset type 1 diabetes.

Our study builds on and expands the previous works by Wang et al., who have shown similar results, but whose analysis was limited to PR3 and NE (12). We show for the first-time elevated serum levels of cell-free DNA and MPO. At the same time, the elevated concentration of PAD4 provides mechanistic insight into the process of NETosis in diabetic patients, as PAD4 citrullinates arginine residues on histones, reducing their positive charge and allowing chromatin decondensation vital for NET formation (15). LL37, which apart from its antimicrobial activity can suppress neutrophil apoptosis (16), was highest in antibody positive relatives, but quite normal in recent onset patients, suggesting concurrent activity of several pathways involving neutrophils.

The data concerning neutrophils and NETosis-biomarkers in T1D are not homogeneous. A study by Qin et al. (9) reported reduced NE and PR3 in T1D patients, which was associated with decreased neutrophils. While we too observed some correlation between MPO, NE, LL37 and neutrophil counts in T1D patients, this was not apparent in first-degree relatives and was driven by a single data point in each cohort. A possible explanation for these diverging results are the different inclusion criteria, as Qin's "recent-onset" cohort included patients up to 3 years after disease manifestation and featured mainly adults, whereas our recent onset cohort was sampled within 7 days of the clinical manifestation of the disease and comprised chiefly children under 10 years of age. A closer comparison may be drawn to the study by Valle et al., which reported decreased neutrophil counts in newly diagnosed pediatric T1D patients (1). The potential effect of recent metabolic stress on circulating neutrophils and NETosis cannot be discounted and presents a unique and currently unresolved challenge in determining which of these two is the initial driving factor, which will require further study. The slight neutropenia we reported in first-degree relatives is in agreement with previous observations by Vecchio et al. (2), suggesting that despite the absence of overt endocrinopathy, abnormalities in neutrophil biology are already present in at risk subjects. Further work on neutrophil phenotype in recent onset patients and at-risk relatives is warranted and could elucidate their activation status, maturity and more.

While we have already previously shown that T1D NETs activate dendritic cells and drive T cell polarization towards the IFN-γ Th1 response in a series of *in vitro* studies performed on material from patients with well-established disease (10), here for the first time we show NETosis biomarkers directly *ex vivo* and expand the studied cohorts to include recent onset patients and their relatives. As the current literature lacks any data analyzing the enzymatic activity of MPO, PAD4 and other enzymes, or the serum concentration of NET-derived citrullinated proteins in T1D patients, this report provides first indirect evidence of their role in T1D pathogenesis.

The double-edged role of NETosis in driving not only antimicrobial host defense, but also pathological inflammation, remains highly topical and has recently been shown in COVID-19, where elevated neutrophil counts predicted worse clinical outcome and serum MPO and cell-free DNA were elevated in patients requiring mechanical ventilation (17). The mass egress of proteolytic enzymes from neutrophils into circulation can trigger a proteolytic storm, drive activation of pro-enzymes and result in proinflammatory cytokine release and host damage (18).

We thus hypothesize that a yet unidentified trigger, perhaps a subclinical viral infection (19), contributes to the ongoing neutrophil activation and low-grade NETosis that we show already in antibody-positive relatives. The biological activity of NETs may then result in further accentuation of the inflammation, egress of neutrophils from the bone marrow – reflected in the transient neutrophilia we show – and neutrophil infiltration into the pancreas as documented by Diana et al. Ultimately, this vicious cycle leads to the destruction of insulin-producing beta-cells and clinical manifestation of diabetes mellitus.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional Ethics Committees of the University Hospital Motol and 2nd Faculty of Medicine, Charles University in Prague. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AK gathered and analyzed longitudinal data mapping peripheral neutrophil counts and co-wrote the article. PV and IZ performed experiments. JV, LP, ZS, and SP provided patient information and primary biomaterial and reviewed the manuscript. AS co-conceived the study, provided support and reviewed the manuscript. ZP conceived the article, designed experiments evaluating the serum concentration of NETosis products,

analyzed the data and co-wrote the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Exploiting Single-Cell Tools in Gene and Cell Therapy

Daniel Bode[1,2], *Alyssa H. Cull*[3], *Juan A. Rubio-Lara*[3] *and David G. Kent*[3*]

[1] Wellcome Medical Research Council (MRC) Cambridge Stem Cell Institute, University of Cambridge, Cambridge, United Kingdom, [2] Department of Haematology, University of Cambridge, Cambridge, United Kingdom, [3] York Biomedical Research Institute, Department of Biology, University of York, York, United Kingdom

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***Correspondence:**
David G. Kent
david.kent@york.ac.uk

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Single-cell molecular tools have been developed at an incredible pace over the last five years as sequencing costs continue to drop and numerous molecular assays have been coupled to sequencing readouts. This rapid period of technological development has facilitated the delineation of individual molecular characteristics including the genome, transcriptome, epigenome, and proteome of individual cells, leading to an unprecedented resolution of the molecular networks governing complex biological systems. The immense power of single-cell molecular screens has been particularly highlighted through work in systems where cellular heterogeneity is a key feature, such as stem cell biology, immunology, and tumor cell biology. Single-cell-omics technologies have already contributed to the identification of novel disease biomarkers, cellular subsets, therapeutic targets and diagnostics, many of which would have been undetectable by bulk sequencing approaches. More recently, efforts to integrate single-cell multi-omics with single cell functional output and/or physical location have been challenging but have led to substantial advances. Perhaps most excitingly, there are emerging opportunities to reach beyond the description of static cellular states with recent advances in modulation of cells through CRISPR technology, in particular with the development of base editors which greatly raises the prospect of cell and gene therapies. In this review, we provide a brief overview of emerging single-cell technologies and discuss current developments in integrating single-cell molecular screens and performing single-cell multi-omics for clinical applications. We also discuss how single-cell molecular assays can be usefully combined with functional data to unpick the mechanism of cellular decision-making. Finally, we reflect upon the introduction of spatial transcriptomics and proteomics, its complementary role with single-cell RNA sequencing (scRNA-seq) and potential application in cellular and gene therapy.

Keywords: cell therapy, gene therapy, single-cell sequencing, scRNA-seq, multimodal omics, multiomics, CAR T cell therapy, disease heterogeneity

INTRODUCTION

The crucial role that single-cell approaches play in understanding cell function has been recognised for decades. Early advances in immunology, and particularly hematopoiesis, have demonstrated the power of such approaches for ascribing functional properties to a single cell. Pioneering work by Till and McCulloch uncovered functional heterogeneity of hematopoietic stem cells (HSCs) by performing single cell-derived assays termed colony-forming unit spleen, or CFU-S, assays (1, 2). Similarly, early studies of single multipotent progenitors provided insights into the progenitor cell commitment and the development of mature immune cells, such as T and B lymphocytes (3, 4). Perhaps most transformative was the introduction of fluorescence activated cell sorting (FACS) which enabled the near-ubiquitous adaption of single-cell functional assays in immunology, hematopoiesis, and beyond (5–7).

Efforts to characterize the cellular function of single cells have fuelled an increased desire to understand detailed molecular mechanisms, but the technologies to do so in single cells have lagged substantially. The development of the polymerase chain reaction (PCR) for amplifying DNA ultimately paved the way for the first glimpse into the transcriptome of single cells (8, 9). The initial protocol for the amplification of cDNA using PCR from single macrophages was introduced by Brady et al. (10), where robust exponential amplification was achieved without disturbing the relative abundance of mRNA sequences, enabling the inspection of rare transcripts in a complex single cell-derived cDNA library. In parallel, Eberwine and colleagues developed a linear RNA amplification approach, based on the amplification of antisense RNA using a T7 RNA polymerase (11, 12). By inspecting mRNAs from single pyramidal neurons isolated from rat brains, they provided the first evidence for global molecular heterogeneity between morphologically similar cells (11).

While targeted single-cell PCR-based molecular screens revolutionized molecular biology, the low throughput and hypothesis-driven nature prevented unbiased exploratory screening. In 1991, Fodor and colleagues developed a novel photolithography-based approach for efficient synthesis of complex oligonucleotides on the microscale (13). This pioneering work would lead to the development of microarray technology where several years later, Schena et al. first applied this method for monitoring gene expression, examining the expression of 45 *Arabidopsis* genes from total mRNA (14). The following decade saw a rapid expansion of the technology, resulting in genome-wide genomic, transcriptomic and epigenetic screening using microarrays [reviewed elsewhere: (15–18)]. This ultimately enabled microarray analysis at single cell level (19), leading to insights into the molecular pathways governing cell fate (20, 21).

Microarrays, a hybridisation-based approach, assayed the known transcriptome and was therefore unsuitable for unbiased detection of novel transcripts. In 1977, Sanger and colleagues published the first genome to be sequenced (22) and soon after early generation sequencing methods began to rapidly develop (23). However, these approaches were extremely costly and time consuming (23). This opened up space for next generation sequencing (NGS) to lead to a revolution in molecular profiling, enabling low-cost, high-throughput and highly parallelised sequencing of nucleic acids. To date, a wide variety of NGS platforms have been developed [reviewed in (24, 25)] and in all cases, sheared DNA is bound to adapter sequences which are immobilised within flow cells, facilitating the synthesis of complementary DNA fragments for subsequent amplification (26). By using fluorophore-labelled nucleotides and simultaneous fluorescence readouts across the entire flow cell, the respective sequences can be determined and ultimately mapped against the reference genome (24, 27, 28). NGS for routine DNA and RNA sequencing provides multiple advantages over microarray technology, including reduced background noise, an increased dynamic range and the detection of novel transcripts (25, 29, 30).

For these reasons, NGS was rapidly adapted to a variety of model systems, including the inspection of rare cell types at single cell resolution (31–36). Tang et al. pioneered the first protocol for single-cell RNA sequencing (scRNA-seq) in single mouse blastomeres with improved performance compared to microarray-based single-cell protocols (36). Following this there has been an explosion of single-cell molecular technologies, enabling unbiased screening of the transcriptome (37, 38), genome (39, 40), DNA methylation (41), chromatin accessibility (42) and spatial resolution of gene expression (43). While these methods provide comprehensive snapshots of molecular states, their integration with cellular phenotype and function is less common and remains vital to the inspection of tissue complexity, disease progression, therapeutic intervention, and beyond. To achieve this goal, pioneering work to integrate omics protocols led to the development of several multimodal technologies. These include simultaneous screening of I) cell surface proteins and mRNA (44, 45), II) DNA methylation and mRNA (46), III) perturbations and mRNA (47), IV) DNA and mRNA (48), V) lineage tracing and mRNA, and VI) cellular function and mRNA (44, 49, 50).

Single-cell technologies have thus provided insight into a wide-range of disease mechanisms, especially in illnesses with significant heterogeneity (51), leading to a long list of potential new therapeutic options. In recent years, the fields of cellular and gene therapy have been steadily evolving for treatment of some monogenic diseases (gene therapy) and B cell leukemias (cell therapy) in particular (52, 53). However, to enable further improvements and applications to other more complex disease types such as autoimmune type 1 diabetes, key aspects such as characterizing target tissues, identifying novel targets in heterogeneous diseases and assessing efficacy of therapeutic interventions all require deeper interrogation. Recent advances in single-cell technologies are ideally positioned to address a number of these unmet needs (51).

In this review, we outline a wide range of recent technologies for screening the genome, epigenome, transcriptome and proteome of single cells and the multimodal integration of

these platforms. We focus on the integration of functional cellular phenotypes with molecular profiles and emphasise the use of single-cell technologies in gene and cell therapies.

A GOLDEN AGE FOR GENE THERAPY - RECENT SUCCESSES IN TREATING MONOGENIC DISORDERS

In its simplest form, gene therapy aims to cure a patient's disease by introducing a normal or corrected copy of a gene into target cells. In 1972, Friedmann and Roblin first proposed the concept of gene therapy as a treatment for inherited genetic defects that largely affected children, many of whom experienced severe, life-threatening symptoms (54). Initially, HSC transplantation represented the primary curative option for many of these disorders, but the availability of matched sibling donors and the risk of severe graft-versus-host disease were barriers for many patients (55). To circumvent these issues, the first gene therapy clinical trials used patient-derived differentiated (T lymphocytes) or immature (hematopoietic stem and progenitor cells, HSPCs) cells that were engineered *ex vivo* to express a disease-correcting transgene (56, 57). Pioneering studies in the late 1990s and early 2000s initially reported successful treatment of adenosine deaminase-deficient severe combined immunodeficiency (ADA-SCID) and other hematological disorders (56–59); however, these successes were soon overshadowed by reports of patients who experienced significant adverse events including the development of treatment-related leukemias and severe immune reactions (60–65). Many of these unanticipated biological effects were later directly linked to the viral vectors used for transgene delivery (66, 67). Consequently, research efforts became focused on improving the safety of viral vectors (68–70) and monitoring for pre-leukemic mutations became a standard feature of treatment follow-up (71–74).

Following these improvements, a number of clinical trials have demonstrated the long-term benefits achieved in individuals with various primary immunodeficiencies and monogenic blood disorders who have received gene therapy treatments (75–84). The follow-up data being reported for these patients mainly focus on disease-relevant parameters such as blood counts and overall clinical symptoms. As a result, numerous questions related to the gene therapy process still remain (**Figure 1**). For example, which HSPC populations are readily transduced during drug product creation and how does this impact outcomes? Do gene corrected terminally differentiated cells have any advantage over their non-transduced counterparts? These types of questions can best be answered using single-cell technologies. Another area of active research involves the development of *in vivo* non-viral delivery systems. These strategies include the use of nanoparticles, aptamers/oligonucleotides and extracellular vesicles to deliver transgenes or siRNAs/shRNAs (85–90). While *in vivo* treatments circumvent issues related to the isolation and manipulation of target cells, they have the potential to induce expression of transgenes or siRNAs/shRNAs in cell types that are not relevant to curing disease. High resolution single-cell transcriptomic and proteomic data will be vital in dissecting how these new treatments affect cell populations receiving the correcting vector. These types of information, especially at the level of preclinical studies, will greatly aid in the development of these technologies.

Moving beyond monogenic disorders, multi-target approaches may be useful in treating complex acquired diseases, such as cancers or autoimmune diseases like type 1

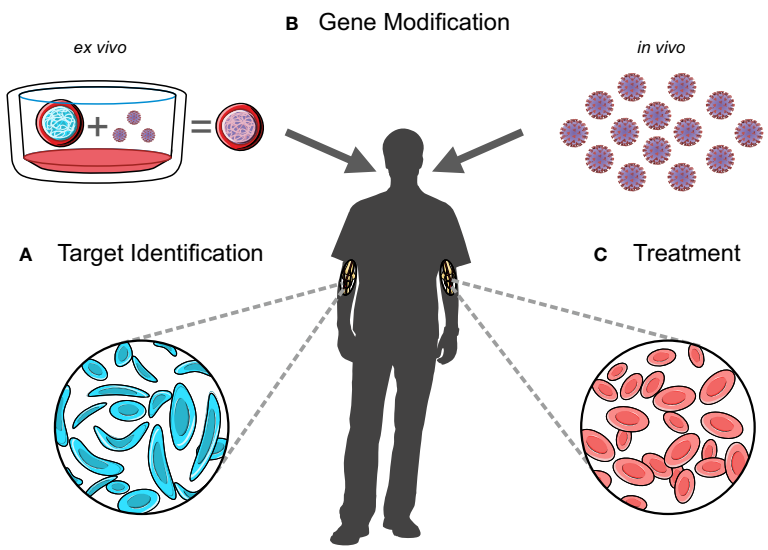

FIGURE 1 | A workflow for developing and administering gene therapy. Novel gene therapy approaches involve **(A)** the identification of therapeutic targets, **(B)** an *ex vivo* gene modification step to create a transduced drug product (left) or the production of an *in vivo* product (right), and **(C)** the infusion of these products into patients following myeloablative conditioning.

diabetes. Large-scale bulk pan-cancer genomics studies have suggested that tumors harbour an average of 4-5 driver mutations (91–94). While this represents an opportunity for the simultaneous manipulation of multiple drivers, the efficacy of this approach in individual patients depends on the specific combinations of these mutations within tumor cell subpopulations. As most genetic profiling of tumors is done using bulk sequencing, the resolution of major/minor clones and subclones becomes very difficult without the use of single-cell approaches. If individual cancers could be profiled to such high resolution, gene therapy strategies could be imagined to target genes essential to cancer cell survival (95–98) or disrupt processes such as angiogenesis that facilitate tumor growth (99–102). Combination therapies may also prove to be highly effective in some contexts (103, 104).

Type 1 diabetes is an autoimmune disease driven by loss of T cell tolerance resulting in islet autoimmunity. During disease development, insulin-producing β-cells in the pancreas are abnormally targeted by infiltrating immune cells (105). For monogenic disorders such as immune dysregulation polyendocrinopathy enteropathy X-linked syndrome where patients are at a much higher risk of developing secondary type 1 diabetes, gene therapy treatment could offer a potential cure (106). However, the genetic drivers of primary type 1 diabetes are complex and may act at the level of β-cells themselves and/or various T cell populations (105). Preclinical studies exploring the use of gene therapy to treat type 1 diabetes have clearly demonstrated the need for treatments that function on two levels - one to create or maintain functional insulin-secreting β-cells and another to protect these cells from autoimmune responses (107–110). Regardless of disease context, the overall diversity of cellular interactions driving human disease presents many challenges to the development of successful treatments. Single-cell studies can address questions pertaining to cell type interactions, disease-specific immunity, clonal dynamics of gene corrected cells and therapy-escape mechanisms, moving gene therapy forward to the next level.

CELL THERAPY AS A PROMISING TREATMENT FOR MORE COMPLEX DISEASES

While gene therapy has revolutionized the treatment of primary immunodeficiencies and monogenic disorders, other strategies may be required to treat more complex diseases. Currently, the primary standard of care for many cancers is chemotherapy, radiation therapy or, in the case of solid tumors, surgery. Immune-based treatments including cell therapy and immune checkpoint inhibitors are now being developed, already showing promise in treating refractory or relapsed patient cohorts. Cell therapy strategies involving chimeric antigen receptor (CAR) T cells have been particularly successful in the treatment of B-cell malignancies (111–113). In brief, these therapies use autologous lymphocytes with synthetically engineered antigen receptors to target tumor-specific antigens (114), thereby harnessing the immune system to trigger anti-tumor immunity (**Figure 2**). Pioneering work by several groups led to the first successful

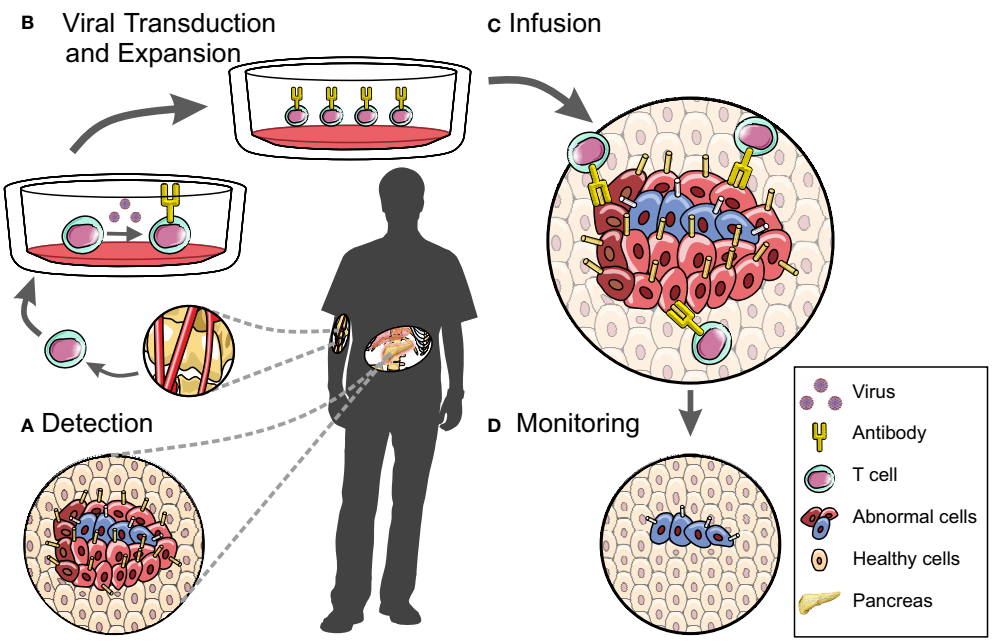

FIGURE 2 | A workflow for developing and administering cell therapy. CAR T cell-based therapies involve **(A)** the discovery of disease-associated antigens which can then be used to target the cytotoxic effects of engineered CAR T cells, **(B)** the isolation and manipulation of patient-derived T cell populations, **(C)** the infusion of these cells into patients, and **(D)** downstream monitoring of disease.

application of this technology in the treatment of B-cell malignancies (111–113), with the first therapy approved by the US-FDA in 2017 for use in B-cell acute lymphoblastic leukemia and diffuse large B-cell lymphoma (115).

Although stable remission is reportedly achieved in approximately 40-60% of patients with these B-cell malignancies (116), a number of significant barriers to increasing treatment efficacy have been identified. CAR T cell persistence and expansion has been shown to be variable between patients. Researchers have suggested that the use of less differentiated T cell subsets or T cells with an altered genetic background (for example, *TET2* disruption) during the manufacturing phase may improve outcomes (115, 117–123). However, a better understanding of the key molecular drivers of T cell expansion and persistence is required to inform future efforts to tailor the production of CAR T cells. Single-cell technologies can be used here to dissect these processes at the molecular level. In addition to increasing the overall performance of CAR T cells, another key aspect required to improve therapeutic outcomes is to control immune responses not directly mediated by CAR T cells (111–113, 124). In order to minimise these responses, a more thorough understanding of immune cell interactions must first be developed. In this context, single-cell approaches will provide the resolution required to dissect these complex systems. On a different level, selective pressures applied by anti-CD19 CAR T cells may also lead to antigen escape and lineage switching as 10-25% of patients go on to develop a $CD19^-$ cancer (125). While groups reported acquired *CD19* loss-of-function mutations (126) and abnormal splicing events leading to loss of CD19 expression (127, 128), the specific origin of $CD19^-$ cancer cells was not clear. A recent paper using single-cell techniques provides evidence that in at least some patients, treatment-resistant $CD19^-$ cancer cells exist prior to treatment (129), underscoring the vital role of single-cell approaches pinpointing the mechanisms by which cancer cells escape treatment and informing strategies targeting refractory disease.

On the other hand, there has been relatively limited success seen in CAR T cell treatments outside of B cell malignancies, despite the development of therapeutics targeting multiple antigens simultaneously or sequentially [reviewed in (130–132)]. In solid cancers, tumor-specific antigens (TSAs) first need to be comprehensively profiled to allow for selection of appropriate candidate TSAs (133) which is especially important when dealing with heterogeneous tumors. Understanding the consequences of on-target/off-tumor effects is also essential to creating safe and effective therapies as evidenced by recent reports of adverse events experienced by patients in two separate cell therapy clinical trials (134, 135). Even once promising TSAs have been selected and tested in both animal models and early phase clinical trials, a number of other tumor-specific factors will likely interfere with the effectiveness of this treatment strategy. For example, immunosuppressive mechanisms that dampen T cell anti-tumor responses may also impact CAR T cell function. Combination therapies or further disruptions to create CAR T cells that are resistant to these immune evasion pathways may therefore become essential (136, 137). Other CAR immune cell populations such as B cells, natural killer (NK) cells and macrophages may also be useful in treating certain diseases (138–140).

In the context of diabetes, both CAR T cell and regulatory T cell (Tregs)-based treatments are currently being developed (141–146). Under normal conditions, Tregs mediate immune tolerance by expressing anti-inflammatory cytokines and dampening the inflammatory or cytotoxic responses of other types of T lymphocytes (147). While patients with type 1 diabetes have similar frequencies of Tregs compared to control individuals, it has been shown that these Tregs have reduced immunosuppressive capacity (148–150). Adoptive Treg transfers from healthy donors into patients have shown promise in preclinical models for a number of different diseases driven by immune dysregulation including type 1 diabetes (145, 151–156). However, a thorough understanding of the heterogeneous cell types that facilitate disease initiation and progression will be crucial to optimizing these treatment regimens.

USING SINGLE-CELL APPROACHES TO REFINE TREATMENT AND INFORM THE DEVELOPMENT OF NOVEL THERAPEUTICS

Although great strides have been made in gene and cell therapy, applications to a wider range of diseases requires more information. Key aspects, such as characterizing target tissues, identifying novel targets in heterogeneous diseases and assessing efficacy of therapeutic interventions require deeper interrogation and single-cell approaches are well-positioned to provide this information.

While a number of groups have begun to use single-cell approaches to dissect various aspects of CAR T cell-based therapy (129, 157, 158), the gene therapy field has not explored this to the same extent. That said, a handful of studies have used bulk sequencing approaches to examine post-transplantation clonal dynamics in a small number of patients (159–161). Biasco and colleagues used this approach to estimate transduced HSPC population size and describe the contributions of HSPC subpopulations to various stages of hematopoietic reconstitution (159, 160). Most recently, Six and colleagues addressed questions pertaining to clonal selection following gene therapy in WAS, sickle cell disease (SCD) or beta-thalassemia patients and found no indications of clonal skewing caused by insertional mutagenesis (161). While all three of these studies provide important insights into human hematopoiesis, the reliance on bulk sequencing approaches to map viral integration sites means that several key questions remain unanswerable. For example, these methods do not allow unedited cells or low abundance clones to be tracked or the effects of multiple integration sites to be assessed. Furthermore, relationships between transduced and non-transduced cells cannot be assayed. These details can only be examined using strategies that analyse single cells and their clonal progeny (162).

In contrast, studies employing single-cell technologies have already begun to deconstruct the fundamental biology behind anti-CD19 CAR T cell therapeutic outcomes. Shieh et al. used single-cell transcriptomics to identify gene signatures associated with good treatment outcomes for patients with B cell malignancies, providing insights relevant to the optimisation of CAR T cell production (157). Deng et al. used a similar approach to discover transcriptional signatures connected to both complete and poor treatment responses (158). This study also identified a novel, transcriptionally distinct cell population found specifically in the infusion products of patients who went on to develop high-grade immune effector cell-associated neurotoxicity syndrome (158). This finding demonstrates the value of single-cell approaches in generating essential information that can then be fed back into clinical practice. Another recent publication applying single-cell technologies reported that the disease-driving clone observed in one patient's relapsed B cell acute lymphoblastic leukemia existed prior to anti-CD19 CAR T treatment (129). Taken together, these studies clearly illustrate how single cell-based datasets can provide clinically relevant insights into various aspects of the cell therapy process (**Figure 2**).

For every stage of the gene and cell therapy process, a number of important questions remain unanswered (**Table 1**). Ultimately, single-cell approaches will be instrumental both in informing our understanding of human disease and in developing the effective therapeutics required to treat them. Data generated using these methods has the potential to better inform our understanding of the numerous complex factors influencing treatment outcomes. The generation of novel targets and delivery methods for heterogeneous diseases relies on a high level of detail and the ability to map cell-cell interactions, especially for disorders with a strong immune component.

SINGLE-CELL MULTI-OMICS PLATFORMS AND THEIR PROSPECT IN GENE AND CELL THERAPY

A wide array of screening platforms have been developed to interrogate molecular states at the single cell level to give insight into tumor heterogeneity and clonal evolution of complex tissues. Here, we describe a selection of the most widely used omics tools and discuss their application in gene or cell therapy, including their potential role in addressing future clinical challenges.

Genome

The first protocol for DNA sequencing at the single cell level, termed single nucleus sequencing (SNS), was described by Navin and colleagues (40). Comparable and reproducible detection levels of copy number variations were observed in single cell and bulk (10^6) samples. By sequencing the genomes of 100 single monogenomic breast tumor cells and the associated liver metastatic tissue, the authors also observed substantial clonal

TABLE 1 | Unmet needs and addressable questions in gene and cell therapy.

Prior to therapy	
What is the underlying clonal diversity for complex diseases such as cancer or diabetes? Are there tumor-specific antigens/mutations or cell susceptibilities that can be used to target various disease subclones/abnormal cell populations? Can understanding the heterogeneity of diseases refined diagnosis?	
Isolation of cells to be edited/manipulated	
Gene therapy (*ex vivo* only)	**Cell therapy**
Which HSCs are mobilized and can gene therapy outcomes be improved if this is further optimized?	Are T cells obtained from different individuals inherently different? What contributes to CAR T cell product variability?
Manipulation of cells for therapeutic purposes	
Gene therapy (*ex vivo* only)	**Cell therapy**
Are some HSPCs easier to transduce than others? Can we adjust this to improve treatment efficacy? Do HSPCs acquire mutations or epigenetic changes during *ex vivo* expansion and transduction steps?	What makes a successful T cell product? Which T cell population should be used in the production of CAR T cells? How can CAR T cells be engineered to be more specific/minimise off-target immune cell activation?
Post-treatment follow-up	
Gene therapy (*ex vivo* and *in vivo*)	**Cell therapy**
What are the clonal dynamics of edited cells over time and how does that change in relation to unedited cells? When transgenes or shRNAs/siRNAs are expressed in HSPCs, what are the molecular consequences of these changes and how do the molecular signatures of these cells compare to HSPCs from age-matched healthy controls? Can low level leukemic clones be detected prior to overt leukemias for patients? When using *in vivo* approaches, what are the consequences of gene correction or transgene expression in cells that do not usually express the gene of interest? Can *in vivo* gene therapy approaches be designed to specifically target disease-causing cells?	Which factors contribute to the toxicities associated with CAR T cells [cytokine-release syndrome (CRS), hemophagocytic lymphohistiocytosis (HLH) and/or macrophage activation syndrome (MAS)]? How can on-target, off-tumor toxicities be minimized? Which CAR T cells survive over time and are some better at targeting tumor cells than others? Are there differences between CAR T cell populations in the blood versus those present in tumor tissue? How do cancer cells (especially in solid tumors) adapt to evade targeting by CAR T cells?

heterogeneity (40). After FACS of single nuclei and whole genome amplification (WGA), each nucleus is sequenced in an individual flow lane. The requirement of full sequencing lanes for single nuclei limited the throughput of such experiments and consequently, several groups introduced barcoding technologies to permit multiplexing of single cells in a single sequencing lane (163–167). To address this challenge, Amini et al. developed a combinatorial barcoding approach, first using Tn5 transposome-mediated labelling followed by PCR-based indexing to yield nearly 10,000 unique barcodes (165). In turn, Vitak et al. demonstrated the efficacy of a single-cell combinatorial indexed sequencing (SCI-seq) platform by acquiring >1500 single cell genomes from a primary pancreatic ductal adenocarcinoma sample (39). To date, a multitude of single-cell sequencing platforms rely on these barcoding principles (168, 169). However, only ~32% of sequenced cells had sufficient coverage for copy-number variation (CNV) detection (39). To address this issue and avoid amplification biases of exponential WGA, Chen and colleagues developed a linear amplification protocol, significantly reducing the required resolution for CNV calling and this was further complemented by experimental and computational approaches to improve the detection of single nucleotide variants (170, 171).

Despite experimental drawbacks related to coverage, single-cell whole genome sequencing (scWGS) has enabled an unprecedented insight into clonal dynamics during tumorigenesis and normal hematopoiesis (162, 172). One notable example includes a temporal study of single human B lymphocytes that explored the evolution of mutational signatures and age-related accumulation of oncogenic mutations (173), only achievable through scWGS.

While bulk WGS studies can infer which disease-causing mutations co-occur based on average variant allele frequencies, there is the potential to group populations of cells that in reality are part of distinct clonal entities. scWGS provides a more precise overview of clonal subpopulations while also capturing information that can be used to pinpoint mutation co-occurrence and order of acquisition (174–178). This approach has been used to profile mutant clones in diseases such as childhood acute lymphoblastic leukemia, childhood T cell acute lymphoblastic leukemia and adult acute myeloid leukemia (179–181). Rare cancer cell populations missed in bulk WGS may also be detected in scWGS assays, as demonstrated by Xu and colleagues (181). Capturing this heterogeneity is essential to understanding how clones with certain mutational profiles impact disease evolution and response to treatment.

Once gene corrected cells have been infused into a patient receiving gene therapy, it is important to track the clonal evolution of these corrected cells. scWGS could be used to track these dynamics as well as answer questions surrounding whether treatment-related mutations are acquired in cells during the gene therapy process. While this method is particularly effective at identifying copy number variants and aneuploidy, technical challenges exist such as low read coverage and sequencing depth. This may significantly hamper efforts to profile single nucleotide changes in gene corrected cells. For HSPCs, bulk WGS of single cell-derived clonal cultures or colonies has bypassed these obstacles (182); however, this approach is not feasible for cell types where *ex vivo* expansion is not possible. Provided that technical challenges are overcome, scWGS represents a promising avenue to explore clonal dynamics. However, the cost for sufficient whole genome coverage in bulk and scWGS currently remains a major barrier for routine adoption.

Following cell therapy treatments, scWGS can be used to assess mutation profiles at the single cell level for highly heterogeneous tumors during the follow-up stage. This information would be particularly helpful in determining why certain patients experience disease relapse, allowing for the identification of specific clones that are either highly susceptible or resistant to CAR T cell cytotoxicity. Additionally, building a more comprehensive understanding of tumor cell clonal dynamics will be key to dissecting out subpopulations that could then be profiled with the aim of identifying new TSAs. This type of approach can be applied to any group of diseases where complex mutation profiles are expected to impact the effectiveness of treatment.

Immune receptor repertoire analysis facilitates the interrogation of clonal dynamics of the adaptive immune response and thus provides a crucial tool for immunotherapy (183). In particular, the development of VDJ-sequencing and single-cell T cell receptor (TCR) sequencing enabled robust profiling of the output of VDJ recombination, using targeted PCR and NGS (184, 185). A multitude of studies outlined the efficacy of TCR sequencing for immune cell profiling in cancer patients to help stratify patient cohorts for immunotherapy, identify the T cell repertoire in the tumour microenvironment and determine the response to PD-1 therapy (186–188). Intriguingly, computational tools have also been developed to enable retrospective VDJ profiling from global single cell sequencing data, thus negating the need for separate immune receptor profiling (157). Nevertheless, limited availability of patient tissue samples and peripheral blood can prevent identification of rare clones and sequential PCR amplification increases risk of amplification biases (189).

Epigenome

The epigenome plays a crucial role in determining cell identity and function with chromatin organization playing a critical role in modulating gene expression and other regulatory functions (190). Chromatin accessibility is governed by the core epigenetic mechanisms of DNA methylation and post-translational modifications of histones (191). Thus, being able to screen DNA methylation, chromatin accessibility and histone modification at single cell resolution can provide crucial insight into tissue heterogeneity.

To identify open chromatin regions and characterize regulatory elements, Buenrostro and colleagues pioneered the assay for transposase-accessible chromatin using sequencing (ATAC-seq) protocol (192). In brief, this protocol leveraged the previously described hyperactive Tn5 transposase to

simultaneously fragment open chromatin regions and introduce sequencing adaptors for subsequent library synthesis (164, 192, 193). While the original ATAC-seq protocol required 500-50,000 cells, the adaptation to inspect single cells soon followed. Buenrostro et al. used the Fluidigm microfluidic platform, allowing single cell capture and downstream processing of hundreds or thousands of single cells (42). Since its inception, others have developed approaches to increase the throughput of scATAC-seq to tens, or even hundreds of thousands of cells (194, 195). Illustrating its power, Sapathy et al. generated scATAC-seq profiles for over 60,000 primary human bone marrow and peripheral blood mononuclear cells (PBMC) (194). Here, the authors identified cell-type specific *cis*-elements, key transcription factor (TF) activity across a broad range of hematopoietic populations and gene activity, using aggregate accessibility of multiple *cis*-elements for a single gene. Most intriguingly, such high density of single cell clusters permits the inference of complex differentiation trajectories. Using the well-characterized development of B cells, the authors were able to reconstruct the differentiation pathway, characterize *cis*-elements of each cell type, and identify active TF programs along the entire differentiation trajectory. Unsurprisingly, scATAC-seq enabled a previously unseen insight into tumor evolution, such as the role of naïve cell types in driving tumorigenesis (194, 196, 197).

DNA methylation of cytosine residues (5mC) plays a crucial role in epigenetic regulation, including the modulation of *cis*-regulatory elements (198). In particular, DNA methylation has been implicated in gene silencing to regulate transcriptional activity during development and altering transcription factor binding (199, 200). The development of bisulphite sequencing (BS-seq) enabled unbiased, genome-wide inspection of the DNA methylome (201). To enable BS-seq at single cell resolution (scBS-seq), pioneering work by Smallwood et al. adapted the existing post-bisulfite adapter tagging protocol to derive quantitative DNA methylation signatures at up to 50% of CpG islands (202–204). Smallwood et al. and others have extensively applied scBS-seq to interrogate mouse gastrulation, human implantation, embryonic stem cells and alternative splicing at single cell resolution (203, 205–207).

The clinically-relevant utility of scATAC-seq in building a comprehensive understanding of the tumor microenvironment has been clearly shown by Sapathy et al. (194) where chromatin accessibility was mapped for more than 37,000 cells from five sets of serial basal cell carcinoma tumor biopsies. Pre- and post-PD-1 inhibitor treated samples were profiled and cell types formed clearly defined clusters, with tumor cells and non-tumor populations clustering away from one another (194). One major strength of this method is the ability to assess chromatin accessibility at specific *cis*-elements in disease-associated loci across multiple cell types. This allows for the annotation of tumor-specific, immune cell population-specific or stromal-cell specific active *cis*-elements. Aside from describing active and inactive chromosomal regions for various cell populations, scATAC-seq can also be combined with individual lentiviral integration site mapping, enabling researchers to examine where these sites fall in relation to open chromosome regions (208). This type of information can be useful in assessing whether integration of viral components in or near specific genes can be connected to robust expansion or *in vivo* persistence of CAR T cells (208). The same approaches could be used to assess how viral integration in certain chromosomal regions affects outcomes in gene therapy. These studies clearly demonstrate how this approach permits comparison of diverse cell populations that directly impact both the disease microenvironment and response to treatment.

In some diseases, therapeutic benefits may be attained through the de-repression of epigenetically silenced genes. One such example involves triggering the expression of fetal gamma-globin (HbF) to correct the pathophysiological defects associated with SCD (80, 209). One preclinical study aiming to identify a novel treatment for Fragile X syndrome used a directed DNA demethylation tool to remove methylation marks in the *FMR1* promoter region, leading to increased *FMR1* expression (210). Newly developed CRISPR/Cas9-mediated demethylation and methylation tools allow for the manipulation of the methylome (211–214). In order for these strategies to be developed into viable treatments, techniques such as scBS-seq will be required to ensure that targeting is specific and that it does not lead to outgrowth of modified cells.

Recent evidence suggests that changes in CAR T cell global methylation status may have some bearing on treatment efficacy. One study found enhanced proliferation and persistence of a dominant CAR T clone with biallelic disruption of the *TET2* gene, which encodes a demethylating enzyme (121). Another study provided evidence that decitabine treatment-mediated epigenetic reprogramming of CAR T cells led to enhanced cytotoxicity and persistence (215). scBS-seq profiling of CAR T cells in a variety of patient samples has the potential to identify novel mechanisms that play a role in determining overall treatment response.

Single-cell epigenomic screening, such as scATAC-seq and scBS-seq, can provide crucial insights into the disease microenvironment, tumor-infiltrating lymphocytes or epigenetic disruption in disease. However, the rapid technological advances in single cell epigenomics posed a new challenge – the computational analysis of large data volumes. In addition, high background noise levels, low sequencing depth and limited capture rates of single-cell epigenetic screens restricts the analytical scope of pipelines developed for bulk sequencing protocols (216). Hence, current analytical strategies leverage a pseudo-bulk approach. First, single cells are aggregated for peak calling, then individuals cells are inspected for identified pseudo-bulk peaks (217). More recently, comprehensive tools have been developed to integrate dimensionality reduction, peak calling, identification of variable peaks, motif analysis, prediction of gene association and differentiation trajectories into single pipelines (218, 219).

Transcriptome

Single-cell RNA sequencing (scRNA-seq) is arguably the most widely applied and established single-cell molecular screening platform. Consequently, a multitude of novel scRNA-seq protocols and adaptations have been developed [extensively

reviewed elsewhere: (220, 221)]. Amongst these, two major groups have emerged, primarily differing in sequence coverage to either profile full-length transcripts or sequence the 3' or 5' ends of captured transcripts. Picelli and colleagues pioneered Smart-seq2 for full-length transcriptomic profiling of hundreds of cells (38). Alternatively, platforms for 3' mRNA profiling, such as Drop-seq (37) and more recently Chromium (10X Genomics) (222), utilise droplet-based microfluidic devices and unique molecular identifiers for massively high-throughput single-cell screens. This technological advance allowed profiling of tens or hundreds of thousands of cells at significantly reduced sequencing costs per cell compared to full-length profiling protocols. These high throughput techniques enable deep molecular profiling of complex tissues and are particularly beneficial for the identification of rare cell types. In contrast, full-length profiling protocols are not compatible with droplet-based approaches, thus reducing the throughput by 10- to 1000-fold at increased sequencing cost per cell (221). However, Smart-seq2 provides deeper sequencing coverage, resulting in the detection of a larger number of genes with fewer sequencing dropouts (223, 224), allowing much more robust conclusions about transcript co-expression in single cells. Increased sequencing depth also provides increased detection of low-abundance transcripts. Perhaps most useful, full-length transcript profiling also permits the detection of alternative splicing and novel transcripts (221). Taken together, both sequencing platforms provide a diverse toolbox to cover a broad range of biological questions, but it is imperative to choose the right tool for the biological question being addressed.

Multiple studies have demonstrated the utility of scRNA-seq in describing cell-cell interactions, discovering unique disease-associated cell populations, identifying minimal residual disease following treatment and even distinguishing host- versus donor-derived cells following transplantation (222, 225–228). These types of applications can easily be used to address a number of currently unanswered questions relating to all phases of the gene therapy process (**Table 1**). As a lower-cost alternative to WGS, scRNA-seq can be used to identify single nucleotide variants (SNVs) and splice variants in gene corrected cells (221, 229). Given that scRNA-seq is also particularly powerful in separating heterogeneous groups of cells (225), these datasets can be very useful in identifying genes and pathways relevant to the function of abnormal cell types that participate in the establishment of diseases such as diabetes (230, 231). In turn, this information can be employed to develop new therapeutic avenues.

Similar to its applications in gene therapy, scRNA-seq can also be used to dissect basic biological processes such as T cell development (232), aspects of which may inform the optimization of CAR T cell therapies. As discussed above, a number of studies profiling anti-CD19 CAR T cell populations before and after infusion into patients have been able to draw clinically relevant conclusions about transcriptional profiles that mark CAR T cells associated with both good and poor clinical outcomes (158, 232). scRNA-seq studies can also be used to examine interactions occurring within the tumor microenvironment between various endogenous immune cell types and CAR T cells (233).

Proteome

The eukaryotic proteome provides the greatest molecular complexity within the genotype-phenotype paradigm. With the addition of post-translational modification, the number of functionally distinct proteins considerably exceeds the ~20,000 identified protein-coding genes (234). In addition to the complexity of the proteome, the absence of protein amplification tools has limited our ability to perform unbiased proteomic screens. Traditional hypothesis-driven approaches, such as high-resolution microscopy, flow cytometry and immunohistochemistry, have enabled protein quantification at single cell resolution (235); however, these techniques are limited by the number of screened proteins, cell throughput, and the need to know the target *a priori*. These limitations are partly addressed by mass cytometry, a high-throughput quantitative screen for up to 60 proteins using currently available protocols and a theoretical capacity of up to 120 proteins (236). The principle of mass cytometry, or cytometry by time-of-flight (CyTOF), was based on the core concept of covalent conjugation of multiple individual antibodies with unique heavy metal reporter isotopes with district ion masses (237). In brief, single cells, labelled with a complex set of reporter-conjugated antibodies, are vaporised by inductively coupled plasma to release reporter ions for analysis by time-of-flight mass spectrometry (238–240). Unique ion mass sizes permit deconvolution and ultimately the quantitative comparison of labelled proteins on individual cells.

Pioneering work by Palii and colleagues utilised CyTOF to determine the role of lineage-specific transcription factors (LS-TF) in hematopoietic lineage specification (241). By performing a temporal screen during erythropoiesis, the authors demonstrated that multipotent progenitor populations undergo gradual LS-TF changes to commit to single lineages at the single cell level. Furthermore, CyTOF has been widely applied in immune cell profiling, biomarker discovery and treatment response studies (236, 242, 243). Such findings demonstrate the power of single-cell approaches to decipher complex molecular interactions, which would otherwise be masked in bulk studies.

As previously mentioned, one of the potential risks of virus-based gene therapy is the development of an immune response targeting the delivery vehicle. A major strength of CyTOF is its ability to profile multiple cell types simultaneously, allowing researchers to create snapshots of proteins being expressed both on the cell surface and intracellularly (244, 245). With the aim of determining whether healthy donor PBMCs were reactive to viral vector components used in many gene therapy clinical trials, Kuranda et al. simultaneously profiled cytokine secretion, immune cell activation, and T cell exhaustion using CyTOF (246). Different immune cell responses were observed, some of which correlated with whether or not the donor had previously been exposed to the virus originally used to develop clinical viral vectors. These findings indicate that it may be possible to predict

which patients will go on to develop vector immunogenicity (246). This type of approach can also be applied to the monitoring of immune cell interactions following CAR T cell infusion.

While CyTOF was originally developed for the screening of suspension cells, Giesen et al. pioneered imaging mass cytometry (IMC) to introduce spatially resolved mass cytometry of ~30 proteins (247). Giesen and colleagues elegantly combined traditional immunohistochemistry with laser ablation and mass cytometry, thus enabling mass cytometric screening across tissue sections with subcellular resolution. Two concurrent studies utilised IMC for screening islets and the immune cell compartment of type 1 diabetes patients at single-cell resolution (248, 249). The authors demonstrated the alterations in islet topology during disease progression and the role of T lymphocytes in β-cell destruction.

As outlined above, high-throughput single-cell phenotyping plays a crucial role in gene and cell therapy. CyTOF and other flow cytometry-based technologies, such as full spectrum flow cytometry (FSFC) and Chipcytometry, enable phenotyping of dozens of distinct cell types (250, 251). In brief, Chipcytometry utilises microfluidics to enable iterative inspection of cell surface markers, while FSFC relies on full spectral acquisition to enable parallel screening of dozens of cell surface markers (250, 251). Near limitless throughput and high capture efficiency paired with the ability to distinguish rare cell populations provides a powerful tool for immunophenotyping. Indeed, FSFC has been successfully applied to identify therapy-mediated alterations in peripheral blood mononucleocyte profiles of head and neck squamous cell carcinoma patients (252).

Despite these advances, the high cell throughput and complexity of acquired CyTOF data provides a significant computational challenge and remains a key focus area for technical development [comprehensively reviewed elsewhere: (253)]. Recent technological advances in mass spectrometry and upstream sample processing have also raised the prospect of unbiased proteomic screens. Separate work by the Slavov and Mann groups have shown a capacity to capture ~3000 and ~800 proteins per cell, respectively (254–256). At present, however, the technology is prohibitive for routine application and will require substantial development to become a powerful tool in the near future.

MULTIMODAL SEQUENCING OF COMPLEX TISSUES

The development of single-cell uni-modal sequencing platforms to independently interrogate the genome, epigenome, transcriptome or proteome has raised the prospect of screening multiple components simultaneously (multimodal profiling).

Numerous approaches for separating genomic DNA and mRNA from the same single cell have been proposed [various approaches extensively reviewed elsewhere: (163)]. Amongst these, the elegant G&T-seq protocol, pioneered by Macaulay et al., separates mRNA from genomic DNA by using magnetic beads and biotinylated oligo(dT) primers against poly-A tails of mRNA molecules (**Figure 3** and **Table 2**) (48). The full-length transcript profiling in G&T-seq assays provides a powerful tool for identifying alternatively spliced transcripts, fusion transcripts and expression of single nucleotide variants (SNVs) (269). The ability to associate such information with DNA copy number and structural variants at the single cell level allows unprecedented insight into the relationship of the genotype and its gene expression profiles. Nevertheless, manual separation of DNA and mRNA during the G&T-seq protocol increases sample handling, thereby limiting the throughput to hundreds of cells (269) which is further compounded by the high sequencing costs to ensure sufficient genome coverage.

Whole genome sequencing (WGS) approaches provide a crucial tool for characterizing genomic abnormalities in primary tumors (270). Zhu et al. recently applied G&T-seq to a subset of lymphovascular invasive cells, isolated from a breast cancer patient (271), describing the relationship between RNA and CNV clones and outlining multiple functionally distinct clones and their role in metastatic dynamics. This illustrates the power of G&T-seq to uniquely integrate genomic abnormalities with transcriptional consequences, potentially of substantial utility in deciphering tumor heterogeneity and intra-tumoral clonal dynamics post CAR T therapy.

Existing epigenetic single-cell assays have also been adapted to enable multimodal approaches (**Figure 3** and **Table 2**). For example, Angermueller et al. adapted the existing principles of G&T-seq by introducing a bisulfite treatment step which allowed DNA methylation profiles and gene expression to be obtained from the same cell (scM&T-seq) (46). A more recent adaptation to the scM&T-seq protocol introduced chromatin accessibility as the third dimension for simultaneous single-cell nucleosome, methylation and transcription sequencing (scNMT-seq) (257). Here, a methyltransferase is used to label accessible DNA prior to scBS-seq. Such labelling permits downstream computational deconvolution of DNA methylation and chromatin accessibility profiles (272). To date, scM&T-seq and scNMT-seq have provided intriguing insight into stem cell biology and mouse gastrulation. For instance, pioneering work by Argelaguet and colleagues described the role of epigenetic priming at lineage-specific enhancers during lineage commitment (205). A second pioneering study revealed that changes in DNA methylation drive increasing transcriptional heterogeneity during stem cell ageing (273). These studies demonstrate the impact of a multi-modal scNMT-seq for characterising the role of the epigenome in complex tissues and biological processes, including the underlying cellular heterogeneity.

Taking into account the role of DNA methylation in driving autoimmune defects, age-related diseases and tumorigenesis (274, 275), scNMT-seq can provide a powerful and versatile tool for uncovering novel therapeutic avenues. These principles can also be applied for assessing the extent to which normal tissue function can be restored following corrective gene therapies. Similarly, multimodal epigenetic and gene expression profiling can provide a valuable tool for characterizing the tumor microenvironment and its interaction with CAR T cells to

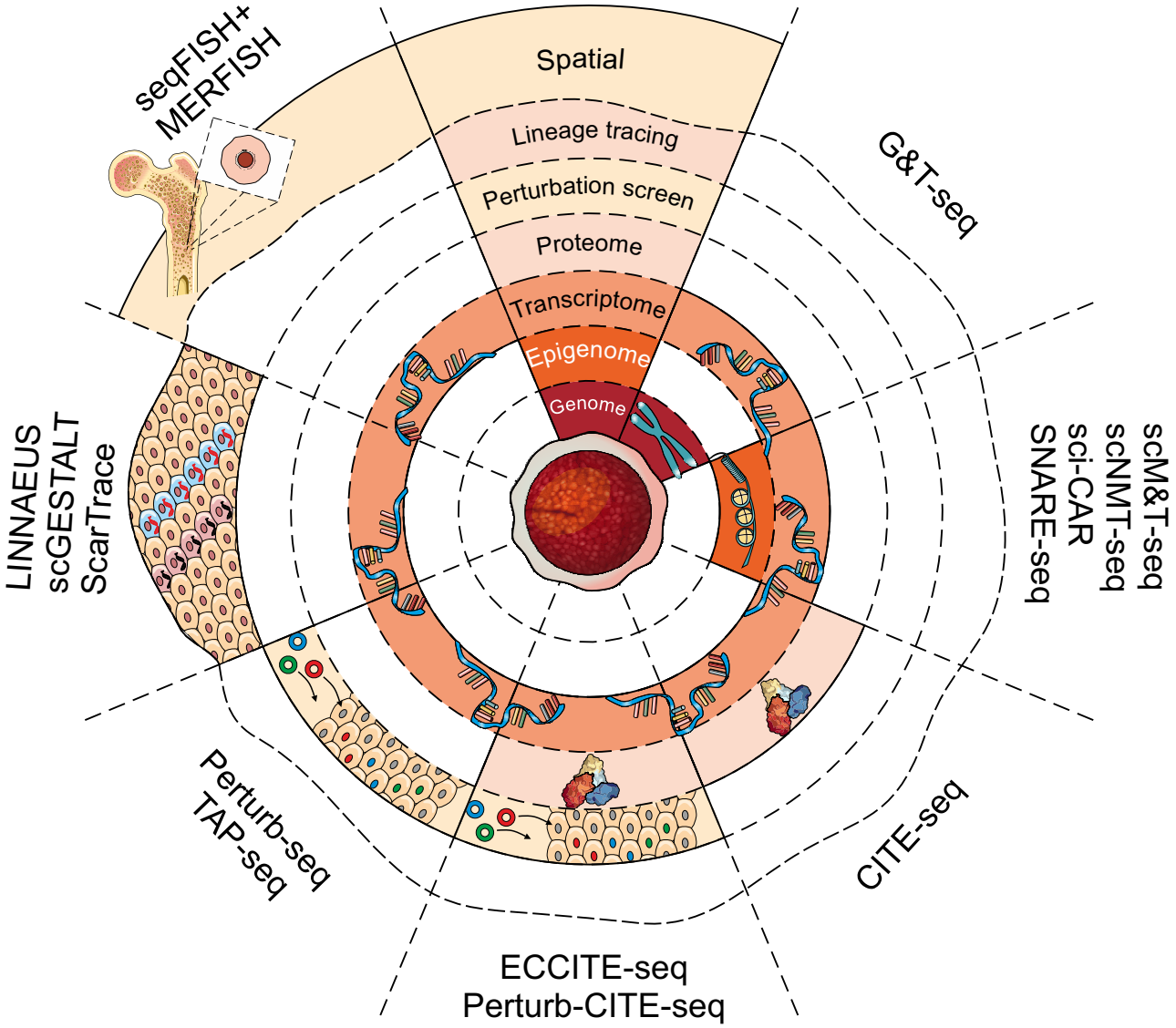

FIGURE 3 | Single-cell multimodal platforms and their uses. A number of recently developed technologies can be used to assess the genomic, transcriptomic, epigenomic and proteomic landscape of a single cell. Each layer of the concentric circle represents a different molecular dimension that can be assessed using each method (from inside to outside: genome, epigenome, transcriptome, proteome, genetic perturbation, lineage tracing, spatial transcriptome). Method names are indicated along the periphery.

increase therapeutic efficacy. However, the relatively low-throughput of scNMT-seq can limit the coverage of large, complex tissues.

To determine the impact of *cis*- and *trans*-regulatory elements on gene expression profiles, collecting chromatin accessibility and gene expression profiles from the same cell are of paramount importance. Cao et al. pioneered sci-CAR to simultaneously perform nuclear scRNA-seq and scATAC-seq (258) by adapting previously established principles of single-cell combinatorial indexing to barcode mRNA and open chromatin regions from single nuclei extracts. Shortly thereafter, Chen and colleagues developed SNARE-seq for performing simultaneous gene expression and chromatin accessibility profiling (259). In contrast to sci-CAR, SNARE-seq utilized the high-throughput Drop-seq platform to incorporate single nuclei and adapter-coated beads. Upon nuclei lysis within each droplet, released nuclear RNA and chromatin fragments bind to the uniquely barcoded beads allowing connectivity of ATAC-seq and RNA-seq profiles of individual cells. Furthermore, SNARE-seq enabled significantly improved capture of chromatin fragments and improved the transcript sequencing depth (259). That said, the potential of SNARE-seq is partially restricted by the complexity of

TABLE 2 | Multimodal single-cell tools.

Name	Modalities	Feature coverage	Throughput	Cost	References
G&T-seq	Genome + Transcriptome	Whole Genome + Whole Transcriptome	100-1000	$$$	(48)
scM&T-seq	Epigenome + Transcriptome	Whole Genome + DNA methylation	100-1000	$$$	(46)
scNMT-seq	Epigenome + Transcriptome	Whole Genome + DNA methylation + chromatin accessibility	100-1000	$$$	(257)
sci-CAR	Epigenome + Transcriptome	Chromatin accessibility + Whole transcriptome	1,000-20,000	$$	(258)
SNARE-seq	Epigenome + Transcriptome	Chromatin accessibility + Whole transcriptome	5,000-20,000	$$	(259)
CITE-seq	Transcriptome + Proteome	Whole transcriptome + 200 proteins	5,000-30,000	$$	(45)
ECCITE-seq	Transcriptome + Proteome + Perturbation	Whole transcriptome + 200 proteins + sgRNAs + VDJ recombination	5,000-30,000	$$	(260)
Perturb-CITE-seq	Transcriptome + Proteome + Perturbation	Whole transcriptome + 200 proteins + sgRNAs	5,000-30,000	$$	(261)
Perturb-seq	Transcriptome + Perturbation	Whole transcriptome + sgRNAs	5,000-100,000	$$	(47)
TAP-seq	Transcriptome + Perturbation	Hundreds of genes + Thousands of gRNAs	5,000-250,000	$	(262)
LINNAEUS	Transcriptome + Lineage Tracing	Whole transcriptome + Lineage	1,000-10,000	$$	(263)
scGESTALT	Transcriptome + Lineage Tracing	Whole transcriptome + Lineage	1,000-10,000	$$	(264)
scarTrace	Transcriptome + Lineage Tracing	Whole transcriptome + Lineage	1,000-10,000	$$	(265)
seqFISH+	Transcriptome + Spatial	Up to 10,000 genes + Subcellular location	Thousands (limited by field of view and imaging time)	$$$	(266)
MERFISH	Transcriptome + Spatial	Up to 10,000 genes + Subcellular location	Thousands (limited by field of view and imaging time)	$$$	(267, 268)

downstream data analysis and this prompted the development of integrated analysis pipelines, such as Signac (218) and the Chromium Single-Cell Multiome ATAC + Gene Expression platform. The simplification of the sample preparation process and analysis pipelines will be required to facilitate the wider adoption of multi-modal epigenetic and gene expression screening.

A vast array of computational tools has been developed for the analysis of unimodal single cell data. For instance, advances in dimensionality reduction, clustering and algorithms for identifying marker genes, constructing lineage trajectories and batch correction contributed greatly to current widespread access to scRNA-seq analysis tools (163). The assembly and curation of key tools into unified analysis pipelines, such as Seurat, SCRAN or SCANPY, has enabled bench-trained scientists to independently analyse scRNA-seq data (276–279). Datasets from multimodal analysis with distinct cellular dimensions inherently do not share common features (280), making data integration across distinct modalities from the same cell a profound and novel computational challenge. To integrate multiple modalities collected from the same cells into a single reference describing cell identities, Hao et al. developed a Weighted Nearest Neighbour (WNN) framework (281). In brief, WNN utilises nearest neighbour analysis and computes modality weights to derive a single landscape, reflecting the similarities of all modalities. The increased adoption of single-cell multimodal screens provides another computational challenge - the integration of multimodal data across distinct experiments, platforms and batches. While multiple strategies to integrate and batch-correct unimodal scRNA-seq datasets have been proposed (278, 282), their applicability to multimodal datasets is limited. To overcome this limitation, Stuart and colleagues adapted canonical correlation analysis and L2 normalisation to derive anchors for data integration (283). To enable integration in a variety of experimental settings, several anchoring methods have been proposed [reviewed in (284)]. Nevertheless, the rapidly expanding landscape of novel multimodal screening technologies continues to require bespoke analytical approaches and recent developments in multimodal data analysis are expansively reviewed elsewhere (163, 280).

Overall, technological advances have resulted in an unprecedented proliferation of novel single-cell molecular assays. Intriguingly, the capability of incorporating such approaches to acquire multiple elements from single cells has allowed the interrogation of the direct relationship of multiple molecular dimensions. Such extensive single-cell profiling is particularly beneficial for application in future cell therapies where the interrogation of tumor infiltrating lymphocytes and tumor microenvironments will provide a crucial component for target discovery and monitoring of therapeutic efficacy. Due to the heterogeneous nature and shifting clonal dynamics of malignant tissues, single-cell approaches are of paramount importance for the development of effective cell therapies.

MULTIMODAL SINGLE-CELL APPROACHES INTEGRATING FUNCTIONAL AND MOLECULAR DATA

Simultaneously acquiring functional and molecular readouts from the same cells have historically represented an experimental challenge, as omics profiling tools typically result in destruction of the target cell. This is particularly challenging when the functional state of a cell is determined by a retrospective assay, thereby making its prospective isolation and molecular characterization impossible. Hence, most technical developments that combine functional and molecular multimodal approaches have focused on capturing cellular function prior to a destructive single-cell assay.

Transcriptome and the Cell Surface Proteome

One of the first applications of multimodal omics technologies arose from the desire to connect cell surface phenotypes with gene expression profiles. Several well-characterized biological systems, particularly immune cell subtypes and hematopoiesis, have benefited from in-depth characterization of cell surface markers for a variety of functionally distinct cellular populations (285). As a result, quantitative phenotypic information of selected cell surface markers can permit inference of cellular function. Fluorescence-activated cell sorting (FACS) in combination with index sorting allows simultaneous recording of cell surface protein levels prior to deposition in lysis buffer for downstream destructive molecular assay, such as the Smart-seq2 protocol for gene expression profiling (38). The application of such approaches has allowed the linkage of stem cell function with global molecular profile for the first time and provided numerous insights into our understanding of transcriptional heterogeneity throughout hematopoiesis (44, 285–287).

Strategies involving index sorting and downstream scRNA-seq are particularly powerful when combined with functional outcome analyses. Wilson et al. and others have shown how these methods can be applied to understanding the heterogeneity inherent to many normal tissues and identifying features that differentiate normal and disease-causing cell types (44, 287–292). These methods would be particularly useful in linking T cell function to distinct gene expression profiles, allowing for the identification of subpopulations of cells that are associated with specific clinical outcomes.

Nevertheless, isolation strategies of functional cell types frequently do not achieve homogeneity and contaminating cells cannot be fully excluded from destructive molecular assays. This is in contrast to selective single-cell functional assays that can distinguish truly functional cells from contaminants, meaning that cellular heterogeneity is often the first to be identified (i.e., they drop out of the assay and do not generate a confusing data point) (293). Furthermore, cell isolation by FACS requires prior knowledge of distinct cell types, thereby precluding the discovery of novel cell types. In addition, index-sorting FACS-based approaches are not compatible with droplet-based high-throughput sequencing platforms. To overcome these limitations, Stoeckius et al. pioneered CITE-seq (cellular indexing of transcriptomes and epitopes by sequencing, **Figure 3** and **Table 2**) (45). Here, antibodies against cell surface proteins of interest are labelled using unique oligonucleotide barcodes. Antibody-labelled cells are subjected to the Drop-seq protocol, encapsulating single cells in droplets containing beads to introduce unique cellular barcodes to mRNA and the antibody-derived tags (ADTs). Subsequently, ADT counts are used to quantify antibody-bound cell surface proteins and provide a link to the corresponding single-cell gene expression profiles. Consistent surface proteome quantification and resolution were achieved compared to traditional flow cytometry approaches, while providing a theoretically unlimited scope for antibody multiplexing (45).

The application of CITE-seq in tumor microenvironment biology has been noted previously (294, 295). Praktiknjo et al. screened healthy and tumor-bearing mouse salivary glands, including the immune compartment of the tissue (295). By performing CITE-seq, the authors were able to construct a comprehensive gene expression atlas and simultaneously recorded a comprehensive set of 63 immune-specific cell surface proteins. Most notably, they derived a comprehensive cell atlas of the tumor microenvironment, using gene expression profiles and quantification of cell surface proteins, underscoring the utility of CITE-seq in the discovery of novel tumor-specific cell surface antigens for cell therapy. By linking surface protein quantification with gene expression profiling at single cell resolution, CITE-seq can identify novel antigens associated with specific clones within heterogeneous cancer tissues, ultimately raising the prospect of a broader spectrum of effective cell therapies. The efficacy of multimodal single-cell screens, such as CITE-seq has been particularly evident throughout the scientific response to the COVID-19 outbreak. Combined efforts to screen >780,000 single PBMCs from COVID-19 patients and healthy donors using CITE-seq revealed the immune response to COVID-19 infections and its role in disease pathology (296). Such studies provide a prominent example how single-cell multiomics can provide rapid insight into previously unknown diseases and help inform the development of effective therapeutics.

Perturbation Screens

Large-scale perturbation screens have previously provided unprecedented insights into gene functions and their role in complex biological mechanisms (297). The advent of CRISPR/Cas9 has revolutionized our ability to conduct high-throughput perturbation screening and multiple groups have now developed multimodal single-cell perturbation screens, combining CRISPR technology with scRNA-seq (47, 298–301). In Perturb-seq (**Figure 3** and **Table 2**), a pool of barcoded single-guide RNAs (sgRNAs) is constructed against a set of 24 transcription factors and transduced cells are subjected to high-throughput droplet-based sequencing, whereby unique cell barcodes are also introduced. The dual barcoding approach allows connection of single-cell gene expression profiles with a respective perturbation. Such single-cell CRISPR screens and their ability to interrogate transcriptional consequences of perturbations provided a novel method to assess the functional effectors of complex biological mechanism and tissues (301, 302). Of note, Jin et al. demonstrated the application of Perturb-seq in an *in vivo* setting (303). To interrogate the underlying molecular mechanisms driving autism, the authors introduced a guide RNA pool against risk genes to the forebrain of a developing embryo *in utero*. The progeny of perturbed cells was then collected at P7 for downstream scRNA-seq analysis, providing key insights into the molecular mechanisms of neocortical cell types.

Perturb-seq can be very useful in trying to understand larger pathways that integrate multiple signals. For example, Adamson et al. used Perturb-seq to understand how activation of the

unfolded protein response (UPR) differed between individual cells (301). This type of data has the potential to disentangle larger signaling networks, all of which is important for understanding complex processes such as immune responses.

Despite the demonstrated efficacy, application of Perturb-seq is limited by the sequencing depth of high-throughput approaches. Acquired data is subject to significant background noise and low-abundant transcripts are frequently missed (47, 298). Furthermore, the multiplicity problem of combining multiplexed perturbations with single-cell gene expression profiles poses a computational challenge. Schraivogel proposed an intriguing adaptation, termed targeted Perturb-seq (TAP-seq) (262). By performing targeted amplification of a selected set of genes prior to sequencing, the cost and analytical complexity could be significantly reduced. This approach provides a powerful tool for screening cellular pathways with defined genetic biomarkers. In the context of cell therapy, TAP-seq could thus provide a cost-effective tool for identifying underlying molecular mechanisms of immune cell evasion of CAR T therapy.

There have been a wide variety of additional approaches to integrate single-cell perturbation screens with the surface proteome of the same cell. Most notably, Mimitou et al. proposed ECCITE-seq (260) and Frangieh et al. described Perturb-CITE-seq (261). In brief, Mimitou et al., adapted the existing CITE-seq protocol by introducing addition oligonucleotides against unique sgRNA identifiers to cellular barcoding beads. Thus, sgRNA, transcripts, antibody-oligonucleotides and up to 2 other parameters can be recorded for individual cells (260). More recently, Frangieh et al. proposed Perturb-CITE-seq to provide a scalable solution for Perturb-seq with simultaneous screening of cell surface proteins (261). Here, the authors demonstrated the benefits of Perturb-CITE-seq by identifying molecular pathways driving immune evasion of a melanoma cell line against primary tumor infiltrating lymphocytes (261). Overall, the ability to connect gene expression profiles and the cell surface proteome from single cells under perturbation provides a comprehensive characterisation of complex molecular systems. As demonstrated by Frangieh et al., such technologies can help identify and characterize immune evasion drivers and ultimately reveal novel targets that might lead to enhanced therapeutic potency of immunotherapies.

Clonal Tracking and Lineage Tracing

Recent work by Lee-Six et al. outlined the application of whole genome sequencing (WGS) approaches to establish the clonal dynamics of human HSPCs (182). The authors isolated single HSPCs from a healthy donor and were able to retrospectively reconstruct the phylogenetic tree of single cell-derived colonies, based on a broad set of shared or unique acquired somatic mutations. By simultaneously screening mature cells isolated from peripheral blood samples of the same individual, Lee-Six et al. were able to infer the progeny and extended relatedness of stem cell clones. Using this approach in a 59 year old human, the authors could map all the way back to the most recent common ancestor for blood and buccal epithelium, observed an early expansion of the stem cell compartment and confirmed hematopoietic activity of a large number of diverse HSC clones estimated to be between 50,000 and 200,000 actively contributing HSCs (162, 182).

This technique could be powerfully applied to gain insight into the clonal dynamics of HSCs used in gene therapy. Careful patient monitoring must be undertaken to ensure therapeutic efficacy and restoration of normal tissue function. As multipotent cells provide the most common target for gene therapies, gene corrections can significantly impact the clonal dynamics of the target tissue. Intriguingly, previous efforts to track therapeutic efficacy of corrective therapies large depended on monitoring progeny cells, their homeostatic function and particularly the proportion of target cells expressing the desired gene edit (159, 304). However, such approaches do not provide sufficient resolution to fully characterize clonal dynamics of corrected cell types and their impact on homeostatic tissue function. WGS of single cell-derived colonies allows to monitor naturally occurring somatic mutations in multipotent cells and their progeny to establish their relationship and infer clonal dynamics of single cells (162). When applied to a pool of edited cell and mature cell progeny post-gene therapy, such approaches can provide a direct insight into therapeutic efficacy and long-term tissue health.

In contrast, upfront labelling of target cells followed by temporal tracking of their progeny can reveal patterns of clonal evolution. Here, the advent of routine and cost-effective sequencing also revolutionised lineage tracing, providing a compelling alternative to traditional imaging-based approaches. In the context of diabetes, lineage tracing has been used to track the various cell types which originate from pancreatic progenitor cell populations (305–307) and identify cell types that are able to transdifferentiate into insulin-secreting cells (110, 308, 309). High-throughput screening at single cell resolution and integration into multimodal approaches greatly expand the scope of lineage tracing (310). While fluorescent tags limit the capacity of parallel barcoding, DNA sequence complexity provides a scalable barcoding approach. In principle, unique DNA barcodes are first introduced into a large population of target cells. Subsequently, amplification of the unique set of DNA barcodes in cell progeny can be used to compute lineage phylogenies (311, 312). A prominent barcoding approach relies on CRISPR/Cas9-mediated dynamic lineage tracing. Here, CRISPR/Cas9-mediated double-stranded breaks are introduced at defined genomic loci (313). The resulting insertions and deletions (indels) create unique cellular barcodes, which evolve over time. By sequencing such regions, the mutational patterns can be used to establish phylogeny and clonal evolution. Multiple groups have independently pioneered such CRISPR/Cas9-based lineage tracing approaches, which predominantly differentiate in the number of loci used to store lineage barcodes (263, 265, 314–318). Of note, using genome editing of synthetic target arrays for lineage tracing (GESTALT), McKenna et al. were able to trace and reconstruct early developmental pathways in a whole organism.

Dynamic lineage tracing protocols outlined above have been integrated in multimodal screens to link cellular progeny to their

respective gene expression profiles, including single-cell GESTALT (scGESTALT), linear tracing by nuclease-activated editing of ubiquitous sequences (LINNAEUS) and ScarTrace (**Figure 3** and **Table 2**) (263–265). Raj et al. integrated the underlying principles of GESTALT with scRNA-seq to simultaneously acquire lineage information and gene expression profiles of the same cell (264). Instead of targeted sequencing of genomic DNA, scGESTALT relies on sequencing of expressed transgenes, which encode the unique cellular barcode. The use of droplet-based high-throughput gene expression thus provides cell type information, otherwise lost in previous lineage tracing protocols. Intriguingly, the LINNAEUS and ScarTrace protocols introduce barcodes in fluorescent transgenes to allow monitoring of successful integration of cellular barcodes. Thus, providing a crucial quality control mechanism prior to performing computational- and capital-intense sequencing (263, 265).

While prospective lineage tracing is not possible in humans, the use of these techniques in preclinical studies has the potential to unlock cellular relationships that are relevant to understanding cell origins in normal and diseased tissues. Furthermore, lineage tracing may also be used to link immature immune cell types to their immunologically active terminally differentiated counterparts. This could feed into refinements of CAR T cell production protocols for example, allowing for the selection of specific populations with maximal effector function (117).

Nevertheless, these multimodal lineage tracing technologies are currently in their infancy and a variety of experimental and computational limitations require attention. Shallow sequencing depth of high-throughput approaches can prevent barcode detection and CRISPR/Cas9-induced cell toxicity has recently been described, thus potentially disrupt the effective construction of phylogeny or distort separation of cell types (310, 319, 320). Furthermore, Spanjaard et al. noted the probability of double scarring, whereby a subset of non-homologous end joining-mediated errors have a higher probability of occurring (263). Thus, if not excluded, high-frequency scars can result in false inference of lineage relationship. To address the issue of barcode duplications and noise, Zafar et al. recently proposed a novel analytical pipeline for improved lineage tree reconstruction and integration of separate single-cell lineage tracing experiments (49). While these advances are promising, further computational innovation will be of paramount importance for the adoption of single-cell lineage tracing in gene and cell therapy developments.

INTRODUCING SPATIAL RESOLUTION IN GENE AND CELL THERAPY

Single-cell sequencing technologies and their multimodal integration continue to push the boundaries of understanding the mechanisms governing complex tissue organization. However, such single-cell screening protocols are largely based on removing the cells and destroying them, typically discarding any spatial information of the underlying tissue from which they were extracted. The crucial role of cellular location and spatial gene expression throughout early embryogenesis has been widely recognized (321). Similarly, cellular location in heterogeneous tumors and the surrounding tumor microenvironment are vital to cell function (322). Therefore, resolving spatial dimensions and linking these with gene expression profiles to infer gene function and cell identity can help us understand disease pathology and complex tissue function. Here, we discuss selected technological developments in spatial transcriptomics and their prospect in the development of novel cell and gene therapies [spatial omics protocols are comprehensively described elsewhere: (321, 323)].

The development of fluorescence *in situ* hybridisation (FISH) techniques first enabled the detection of DNA and RNA molecules in structurally preserved, fixed tissue sections (43, 324, 325). Oligonucleotides, complementary to a target nucleotide sequence, are labelled with single or multiple fluorophores. In turn, fluorescently labelled oligos bound to a target region can be observed using optical microscopy. Ultimately, the principles of FISH facilitated quantitative detection of mRNA at subcellular resolution (43, 324, 326). Here, the authors constructed a library, consisting of short single fluorophore-labelled oligos, against a single mRNA target to estimate the number of mRNA molecules in a single cell, screening up to 3 mRNA sequences in parallel.

To enable high-throughput spatial transcriptomic screening, Lubeck et al. first established the principles of sequential FISH (seqFISH), providing a strategy with theoretically whole transcriptome coverage (327, 328). In brief, multiple single fluorophore-labelled probes are used for mRNA labelling during a single hybridization round. By stripping probes and performing multiple rounds of hybridisation, the number of unique barcoding increases exponentially. Shah et al. demonstrated the efficacy of seqFISH for screening hundreds of genes at sub-cellular resolution, providing a novel insight into the spatial organisation and transcriptional heterogeneity of the mouse brain (329). The recent introduction of an additional fluorophore to sequential hybridisation allowed further scaling of seqFISH (seqFISH+) (**Figure 3** and **Table 2**) (266). This strategy avoids optical crowding by effectively diluting mRNA molecules into separate images. The result was a robust protocol for screening 10,000 genes in spatially resolved tissues, spanning thousands of cells (266). Here, the use of confocal microscopy for the seqFISH+ protocol provides a key advantage to facilitate wider adaption. A recent study by Lohoff et al. applied seqFISH to construct spatially resolved gene expression profiles for mouse organogenesis using a computational framework for the integration of spatially-resolved gene expression maps with scRNA-seq profiles of cell types in early mouse development (330, 331). In parallel, Chen et al. pioneered a multiplexed error-robust FISH (MERFISH) approach which combined error-corrected barcoded probes and sequential imaging to perform a multiplexed screen of hundreds of genes (**Figure 3** and **Table 2**) (267). Further MERFISH developments, such as the use of expansion microscopy, enabled quantification of thousands of genes in hundreds spatially resolved cells at a

detection efficiency of ~80% (268). This high capture efficiency is a major advantage of MERFISH.

While the methods outlined above drove innovation in spatial transcriptomics, their relative infancy is accompanied by experimental and computational complexity, which currently provides a barrier to wide-spread adoption. Several commercially available platforms have been established to provide a standardised experimental framework. The Visium platform utilised NGS for deriving spatially resolved gene expression profiles (323, 332). Here, a tissue section of interest is deposited onto a slide, coated with uniquely barcoded arrays (barcode spacing permits 55um resolution). Following barcoding of captured mRNA molecules, cDNA libraries are subjected to high-throughput NGS and spatial deconvolution based on the unique barcoding. However, the current barcode spacing prevents interrogation of neighbouring cells. Here, *in situ* analysis can provide a complementary approach, allowing interrogation of a defined set of mRNA targets at spatial singe cell resolution (333–335).

Collectively, spatial transcriptomics technologies are currently in the developmental and early adaption phase. As a result, several key limitations persist. For instance, the tissue-dependent optimisation and sequential hybridisation rounds require significant experimental time, while the use of customised equipment also impacts implementation. However, increasing throughput and the desire to reach whole-transcriptome coverage will greatly increase imaging time and data complexity, making the most prominent limiting factor the development of robust analytical tools. To overcome the computational barrier, recent advances aim to address key unmet needs in data analysis and its scalability (336, 337).

Despite these challenges, several major advances have already been made using spatial transcriptomics, including studies in tumor heterogeneity and transcriptional changes in the microenvironment. In one study, Berglund et al. constructed a comprehensive spatial map of tumor and healthy prostate tissue biopsies from a prostate adenocarcinoma patient (322). The authors uncovered significant transcriptional differences between the tumor core and its periphery. Intriguingly, thorough interrogation of stromal and immune cell types, surrounding the primary tumor, facilitated the identification of heterogeneous gene expression networks in the tumor microenvironment (322). Spatial transcriptomics has also been applied for mapping the localisation of Cxcl12-abundant reticular cells in the bone marrow niche and for the characterisation of stromal cell heterogeneity in tumor microenvironments (338, 339). These and other studies demonstrate that the potential of spatial transcriptomics in deciphering tumor architecture, heterogeneity and microenvironments has been widely recognised. Beside its role in therapeutic discovery and disease pathology, spatially resolved gene expression profiles can become of paramount importance for monitoring therapeutic outcomes of cell therapies and identify evasion mechanisms in response to cell therapies. In addition, spatial characterisation post CAR T cell therapy could provide an insight into the impact of off-target effects on the function of proximal tissues. Similarly, spatial transcriptomics could aid in long-term monitoring of patients undergoing corrective gene therapies.

CONCLUDING REMARKS

The past decade has produced an abundance of novel single-cell molecular tools, facilitating the unbiased screening of a wide array of molecular dimensions at unprecedented resolution. Unimodal sequencing technologies have proved particularly impactful in the first wave of wide-scale adoption, but more approaches have been focused on combining such techniques into multimodal screens to allow simultaneous capture of multiple molecular dimensions from the same cell. These technologies have allowed researchers to unpick the molecular mechanisms driving disease pathology at a scale not previously considered possible. Tissue and disease heterogeneity, previously masked in bulk sequencing approaches, are now routinely being explored at single cell resolution.

Techniques such as scRNA-seq have been widely adopted due to the production of robust experimental protocols and increasing consensus surrounding the computational approaches for quality control and data analysis. On the other hand, multimodal screens have not yet enjoyed similar uptake due to their reliance on high sequencing costs, advanced integrative computational tools and technical expertise. However, just as moving to single cells was a technical hurdle of 10 years ago, the research benefits derived from novel multimodal screening platforms will push the limits of discovery and accelerate technical development and method standardization. The next few years should see these technical and computational approaches streamlined to create reproducible protocols and standardised analytical pipelines to facilitate rapid adoption rates, as has occurred for scRNA-seq historically.

Concomitant with the technical challenges and need for standardization, the increased accessibility of single-cell technologies has exponentially increased the amount of data generated during these studies. This provides a unique opportunity to leverage the power of these studies by integrating datasets but also makes for substantial computing and processing challenges. Batch correction and data integration across experiments and different sequencing platforms are areas that will require particular attention and novel computational approaches for handling and analysing increasing amounts of data will be of paramount importance. Ultimately, the continuous technical improvements and aggregation of data could provide the foundation for a fully characterized reference atlas of the human body at single cell resolution. The drive towards such a resource is evident in the recently announced efforts to establish a common coordinate framework (CCF) for data collection and integration (340). In line with that, initiatives such as the Human Biomolecular Atlas Program and the CCF aim to provide a publicly available tool to help researchers map data from diseased states onto healthy single-cell datasets and

provide a reference for the entire scientific community (340, 341).

A number of recent studies have clearly demonstrated the utility of these approaches in (1) understanding complex biological processes such as cell fate determination and immune response, (2) dissecting tissue and disease heterogeneity, and (3) stimulating innovative research aimed at developing novel therapeutics (342–344). Within the next decade, it is anticipated that an increasing number of patients across many disease types will be treated with gene and cell therapy. Using samples obtained from these growing patient cohorts, single-cell technologies will undoubtedly be used to answer essential questions related to the relationships between disease-causing cells, normal or corrected cell types, tumor-targeting lymphocytes such as CAR T cells, and endogenous immune populations. For autoimmune diseases such as type 1 diabetes where the risk of relapse is relatively high due to immunogenicity, this level of detail will be essential to finding new ways to increase treatment efficacy. Additionally, due to the relatively recent wider application of these therapeutics, only a limited number of gene or cell therapy clinical trial patients have been monitored for more than 10 years following treatment initiation (65, 84, 345–347). Depending on the stability of edited cells and the influence of other comorbidities, detailed studies using single-cell approaches may also become relevant during long-term follow up. As patients enter the later decades of life, the intersection of age-related and treatment-related abnormalities may present unique clinical challenges. Further refinements and innovations to single-cell profiling technologies have the potential to unlock and disentangle relationships between key drivers of disease phenotypes, leading to wider delivery of authentic personalised medicine.

AUTHOR CONTRIBUTIONS

DB and AC wrote and compiled the review. JR-L designed and created the figures. DK supervised the work and edited the manuscript. All authors contributed to the article and approved the submitted version.

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IL-10 Deficiency Accelerates Type 1 Diabetes Development *via* Modulation of Innate and Adaptive Immune Cells and Gut Microbiota in *BDC2.5* NOD Mice

Juan Huang[1,2], *Qiyuan Tan*[2,3], *Ningwen Tai*[2†], *James Alexander Pearson*[2,4†], *Yangyang Li*[2,5], *Chen Chao*[1], *Lucy Zhang*[2], *Jian Peng*[2], *Yanpeng Xing*[2,6], *Luyao Zhang*[2,6], *Youjia Hu*[2], *Zhiguang Zhou*[1], *F. Susan Wong*[4] *and Li Wen*[2*]

[1] National Clinical Research Center for Metabolic Diseases, Key Laboratory of Diabetes Immunology (Central South University), Ministry of Education, and Department of Metabolism and Endocrinology, The Second Xiangya Hospital, Central South University, Changsha, China, [2] Section of Endocrinology, Department of Internal Medicine, School of Medicine, Yale University, New Haven, CT, United States, [3] Department of Endocrinology and Metabolism, Shanghai Jiaotong University Affiliated Sixth People's Hospital, Shanghai, China, [4] Division of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, United Kingdom, [5] Department of Endocrinology, The Second Hospital of Jilin University, Changchun, China, [6] Department of Gastrointestinal Surgery, The First Hospital of Jilin University, Changchun, China

Type 1 diabetes is an autoimmune disease caused by T cell-mediated destruction of insulin-producing β cells. *BDC2.5* T cells in *BDC2.5* CD4$^+$ T cell receptor transgenic Non-Obese Diabetic (NOD) mice (*BDC2.5*$^+$ NOD mice) can abruptly invade the pancreatic islets resulting in severe insulitis that progresses rapidly but rarely leads to spontaneous diabetes. This prevention of diabetes is mediated by T regulatory (Treg) cells in these mice. In this study, we investigated the role of interleukin 10 (IL-10) in the inhibition of diabetes in *BDC2.5*$^+$ NOD mice by generating *Il-10*-deficient *BDC2.5*$^+$ NOD mice (*BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice). Our results showed that *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice displayed robust and accelerated diabetes development. *Il-10* deficiency in *BDC2.5*$^+$ NOD mice promoted the generation of neutrophils in the bone marrow and increased the proportions of neutrophils in the periphery (blood, spleen, and islets), accompanied by altered intestinal immunity and gut microbiota composition. *In vitro* studies showed that the gut microbiota from *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice can expand neutrophil populations. Moreover, *in vivo* studies demonstrated that the depletion of endogenous gut microbiota by antibiotic treatment decreased the proportion of neutrophils. Although *Il-10* deficiency in *BDC2.5*$^+$ NOD mice had no obvious effects on the proportion and function of Treg cells, it affected the immune response and activation of CD4$^+$ T cells. Moreover, the pathogenicity of CD4$^+$ T cells was much increased, and this significantly accelerated

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***Correspondence:**
Li Wen
li.wen@yale.edu

†These authors have contributed equally to this work

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the development of diabetes when these CD4$^+$ T cells were transferred into immune-deficient NOD mice. Our study provides novel insights into the role of IL-10 in the modulation of neutrophils and CD4$^+$ T cells in *BDC2.5*$^+$ NOD mice, and suggests important crosstalk between gut microbiota and neutrophils in type 1 diabetes development.

Keywords: type 1 diabetes, interleukin-10, neutrophils, gut microbiota, CD4$^+$ T cells

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease that results from the destruction of insulin-producing β cells. T cells are the predominant component of the infiltrates in these pancreatic islet lesions (1, 2). Reports from bone marrow transplantation studies in identical twins in humans (3) and adoptive transfer of T cells from diabetic Non-Obese Diabetic (NOD) mouse donors to non-diabetic NOD mouse recipients (4) demonstrated that type 1 diabetes is an immune cell-mediated disease. T cell receptor (TCR) transgenic NOD mice provide critical tools for investigation of the important roles that T cells play in the immunopathogenesis of T1D development.

The *BDC2.5* TCR transgenic NOD (*BDC2.5*$^+$ NOD) mouse was generated from a diabetogenic CD4$^+$ T cell clone, designated as *BDC2.5*, from a new-onset diabetic NOD mouse (5). In these mice, ~90% CD4$^+$ T cells express the transgenic TCR (6). Interestingly, despite the potent ability of the parental *BDC2.5* T clone in inducing type 1 diabetes development, *BDC2.5*$^+$ NOD mice showed severe insulitis as young as 2-3 weeks of age but rarely developed diabetes (7, 8). However, *BDC2.5*$^+$ CD4$^+$ T cells from the *BDC2.5*$^+$ NOD mouse could rapidly transfer diabetes into severe combined immune-deficient NOD (NOD.*scid*) recipients after activation *in vitro (*9). Moreover, if *BDC2.5*$^+$ NOD mice are on an immunodeficient background, such as a recombination-activating gene (*Rag*) deficiency (10) or NOD.*scid* (9), the mice develop rapid diabetes at a very early age, suggesting intrinsic immune regulatory factors suppress the development of diabetes in *BDC2.5*$^+$ NOD mice in immuno-sufficient hosts. Studies have shown that Foxp3$^+$CD4$^+$ regulatory T (Treg) cells play a protective role by regulating autoimmune responses in *BDC2.5*$^+$ NOD mice (11–13). Our previous studies found that B cell depletion in *BDC2.5*$^+$ NOD mice induced transient aggressive behavior in diabetogenic *BDC2.5*$^+$ CD4$^+$ T cells with reduction in Treg cell number and Treg cell suppressive functions (14). However, after B cell reconstitution, *BDC2.5*$^+$ CD4$^+$ T cells were less aggressively pathogenic due to the increased number of Treg cells and enhanced suppressive function of Tregs cells to CD4$^+$CD25$^-$ T effector cells, as well as increasing IL-10 producing Bregs (14).

Although the destruction of insulin-producing pancreatic beta cells seen in type 1 diabetes is primarily characterized by autoreactive T cells, recent studies suggest that neutrophils also play an essential role in the development of type 1 diabetes. Diana et al. found that neutrophils infiltrate the islets of NOD mice at an early age, which was required for the initiation of the diabetogenic T cell response (15). The authors also showed that neutrophils interacted with B-1a cells and plasmacytoid dendritic cells in the development of type 1 diabetes (15). Their further study showed that macrophages and β-cells in the pancreas were responsible for neutrophil recruitment to the pancreas (16). In patients with type 1 diabetes, the level of circulating neutrophil elastase released from activated neutrophils was positively associated with the numbers and titers of the autoantibodies against β-cell-specific antigens, suggesting that neutrophil activation leading to the elevated proteases might be involved in the process of β-cell autoimmunity (17). Moreover, the changes in circulating neutrophil numbers were found to be associated with β-cell specific autoimmunity and the HLA-DR3-DQ2/DR4-DQ8 high risk genotype (18–22). Studies have shown that gut microbiota also regulated neutrophil aging and homeostasis (23, 24) and increasing evidence suggests that gut microbiota play an important role in modulating type 1 diabetes development in NOD mice (25–29) and in patients with type 1 diabetes (30, 31). It is known that IL-10 is an important immuno-regulatory cytokine in homeostasis of gut mucosal immunity (32–34) and IL-10 also mediates immune supression by Treg cells (35–37). However, it is not clear how IL-10 mediates the immune regulation seen in *BDC2.5*$^+$ NOD mice and if gut microbiota also modulate islet β cell autoimmunity through regulation of neutrophil homeostasis in *BDC2.5*$^+$ NOD mice. To fill those knowledge gaps, we generated *BDC2.5*$^+$ NOD mice with *Il-10* deficiency (*BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice) and investigated the action of IL-10 in modulating diabetogenic CD4$^+$ T cells, neutrophils and the gut microbiota in type 1 diabetes development.

METHODS AND MATERIALS

Mice

Mice used in this study were housed in specific pathogen-free (SPF) facilities with a 12-hour-dark/light cycle at Yale University. NOD mice, *BDC2.5*$^+$ NOD mice, *Il-10*$^{-/-}$ NOD mice and NOD.*scid* mice were originally obtained from the Jackson Laboratory and maintained at Yale University. *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice were generated by

Abbreviations: FITC, fluorescein isothiocyanate; G-CSF, granulocyte colony-stimulating factor; IL-10, interleukin 10; MyD88, myeloid differentiation factor 88; NOD, non-obese diabetic; OTU, operational taxonomic unit; PBS, Phosphate Buffered Saline; PCoA, Principal Coordinate Analysis; PLN, pancreatic lymph node; Rag, recombination-activating gene; Scid, severe combined immune deficiency; SPF, specific pathogen-free; TLR4, Toll-like receptor 4.

breeding *BDC2.5*$^+$ NOD mice with *Il-10*$^{-/-}$ NOD mice, followed by intercrossing the *BDC2.5*$^+$*Il-10*$^{+/-}$ NOD mice. The use of animals in this study was approved by the Institutional Animal Care and Use Committee of Yale University (approval number 2016-07911). Except for the experiments to observe diabetes incidence and adoptive transfer experiments using splenocytes from diabetic mice, all the other experiments were performed using 4-5 week-old non-diabetic mice. The detail of the number of animals used and the number of replicates are included in the figure legends for each experiment and also see **Table S1**.

Natural History of Diabetes Development

BDC2.5$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice (both sexes) were observed for spontaneous diabetes development by screening for glycosuria (Bayer Diastix) weekly and diabetes onset was confirmed by blood glucose ≥ 250 mg/dl (13.9 mmol/l).

Islet and Islet-Infiltrating Immune Cell Isolation

Pancreata removed from 4-week-old *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice were agitated in a 37°C shaking water bath after addition of 1.5 mg/ml collagenase (Sigma) and 62.5 units/ml DNase-I (Sigma). Collagenase activity was stopped by adding complete RPMI-1640 media after digestion. Islets were hand-picked under a light microscope, and subsequently incubated in a 37°C water bath for 6 minutes in the presence of 500 µl Cell Dissociation Buffer (Gibco), for immune cell isolation. After washing, isolated immune cells were filtered and re-suspended in complete RPMI-1640 media before staining.

Intraperitoneal Glucose Tolerance Test (IPGTT)

BDC2.5$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice were fasted overnight prior to intraperitoneal injection with 20% glucose (2 g/kg). Blood glucose was measured before glucose injection and at different time points after glucose injection.

Cell Purification

CD4$^+$ T cells, antigen-presenting cells, Treg cells and neutrophils were each purified from the splenocytes of 4-week-old *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice, *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice, or wild type NOD mice. Splenic CD4$^+$ T cells were purified by depletion, of CD8$^+$ T cells (clone T1B105), MHC class II$^+$ cells (clone 10.2.16), and B cells (anti-mouse IgM and IgG), incubating the cells with monoclonal antibody (mAb) hybridoma supernatants, followed by magnetic bead separation. For splenic antigen-presenting cell (APC) isolation, anti-Thy1 (Y19) mAb hybridoma supernatant and complement was used to remove Thy1$^+$ T cells. The supernatants of different mAb hybridomas were kindly provided by the late Charles Janeway (Yale University). Magnetic beads conjugated with goat anti-mouse IgG, goat anti-mouse IgM, or goat anti-rat IgG were purchased from QIAGEN. Treg cells were isolated using MojoSort™ Mouse CD4$^+$ T cell Isolation Kit (BioLegend), followed by CD25 positive isolation using a PE-anti-mouse CD25 antibody (Clone, PC61, BioLegend) and MojoSort™ Mouse anti-PE Nanobeads (BioLegend). The remaining CD4$^+$CD25$^-$ T cells were used as effector CD4$^+$ T cells. Neutrophils were isolated according to the manufacturer's instructions using MojoSort™ Mouse Neutrophil Isolation kit (BioLegend).

In Vitro Culture

Splenic CD4$^+$ T cells (1×10^5/well) purified from 4-week-old *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice were stimulated with different concentrations of mimotope peptide in the presence of mitomycin-c-treated APCs for 3 days at 37°C. CD4$^+$CD25$^+$ Tregs (5×10^4/well) purified from 4-week-old *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD were co-cultured with effector CD4$^+$ T cells (CD4$^+$CD25$^-$ T cells, 1×10^5/well) from either *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice or *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice, in the presence of 5 ng/ml mimotope peptide (amino acid sequence RTRPLWVRME) and mitomycin-c-treated APCs (5×10^4/well), which were purified from wild-type NOD mice. Cells were incubated for 3 days at 37°C. Purified splenic neutrophils (5×10^4/well) from *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice were co-cultured with purified splenic CD4$^+$ T cells (1×10^5/well) isolated from either *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice or *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice in the presence of mitomycin-treated APCs (5×10^4/well) from wild type NOD mice and mimotope peptide (5 ng/ml). Cell proliferation was determined by ^{3}H-thymidine incorporation over 16 hours, with supernatants collected prior to ^{3}H-thymidine addition.

Adoptive Transfer Experiments

Total splenocytes (10×10^6) from diabetic *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice were injected (*i.v.*) into irradiated 4-week-old wild-type NOD mice. Total splenocytes (10×10^6), or purified splenic CD4$^+$ T cells (7×10^6) from non-diabetic *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice were transferred (*i.v.*) into 4-week-old NOD.*scid* mice. Recipients were monitored for glycosuria (Bayer Diastix) weekly and diabetes was confirmed by blood glucose ≥ 250 mg/dl (13.9 mmol/l).

Extraction of Gut Bacterial DNA

Fecal pellets collected from 4-week-old *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice were resuspended in 300 µl Tris-EDTA buffer (10 mM Tris and 1 mM EDTA, pH8) containing 7.5 µl 0.5% SDS and 3 µl Proteinase K (200 µg/ml). The samples were then incubated at 37°C for 1 h and homogenized in solution, containing one volume of phenol/chloroform/isoamyl alcohol (25:24:1), 200 µl 20% SDS and 0.3 g zirconium silica beads, with a mini-bead-beater (BioSpec) for 2 minutes. Phenol/chloroform/isoamyl alcohol was then added to the samples prior to centrifugation (4°C, 12000 g, 15 mins), with the upper aqueous layer, containing DNA, transferred to a new tube. Bacterial DNA was subsequently precipitated with isopropanol, washed with 70% ethanol, air-dried, and resuspended in 100 µl of sterile water.

16S rRNA Sequencing and Data Analysis

The V4 region of the bacterial 16S rRNA gene was amplified from each DNA sample by PCR using barcoded broadly-conserved primer pairs (5'-GTGCCAGCMGCCGCGGTAA-3') and (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR products were purified using gel extraction kits (QIAGEN) with DNA concentration quantified on a Nanodrop spectrophotometer. Equimolar amounts of each sample were pooled for pyrosequencing using the Ion Torrent Personal Genome Machine (PGM) sequencing system (Life Technologies). The sequencing results were analyzed with the Quantitative Insights Into Microbial Ecology (QIIME) software package (version 1.8) and UPARSE pipeline (version 7.0). β-diversity was analyzed to compare differences between microbial communities, and the data are shown as a Principal Coordinate Analysis (PCoA). Taxonomy assignment was performed at various levels using representative sequences of each operational taxonomic unit (OTU).

Pre- and Neo-Natal Antibiotic Treatment *In Vivo*

For depletion of endogenous commensal microbiota, *BDC2.5^{+}Il-10$^{+/-}$* NOD breeders were treated with an antibiotic cocktail containing 0.5 g/l vancomycin, 1 g/l ampicillin, 1 g/l metronidazole, and 1 g/l neomycin added in drinking water, from one week before delivery to 4 weeks after birth.

Cytokine ELISA

Murine IL-17A, IL-10 and IFN-γ from the serum and gut flush from different sections of the intestine were measured using the Mouse ELISA kits (BioLegend), following the manufacturer's instructions. Serum samples were diluted 1:100 in PBS before measurement. Gut flush was obtained by infusing 10 ml PBS to the gut lumen, after termination of the mice, and removal of the intestine. The collected fluid used for ELISA, after removing the solid material by centrifugation (12,000 g, 5 min, RT).

Gut Permeability Assay

Four-week-old *BDC2.5^{+}Il-10$^{+/+}$* NOD mice and *BDC2.5^{+}Il-10$^{-/-}$* NOD mice were fasted overnight for 13 hours. Food was resupplied to the mice two hours post-gavage with 0.6 mg/g FITC-dextran (MW 3,000-5,000, Sigma). Blood samples were collected from the mice two hours after food restoration and were centrifuged (2300 g, 5 min, RT) for serum separation. Serum samples were diluted 1:1 in PBS with the FITC-dextran concentration determined using a fluorescence spectrophotometer (Perkin Elmer). Standard curves were generated using known concentrations of FITC-dextran, diluted in serum from untreated NOD mice. The concentrations in serum from FITC-dextran gavaged *BDC2.5^{+}Il-10$^{+/+}$* NOD mice and *BDC2.5^{+}Il-10$^{-/-}$* NOD mice were determined using linear regression.

In Vitro Bacterial Stimulation

Fresh stool samples, collected from 4-week-old *BDC2.5^{+}Il-10$^{+/+}$* NOD mice and *BDC2.5^{+}Il-10$^{-/-}$* NOD mice, were resuspended at 1 g/mL in sterile PBS, and homogenized by vortexing vigorously for 30 secs. The samples were then centrifuged (300 g, 1 min, RT) to remove large debris and subsequently further centrifuged at 12,000 g for 5 minutes to pellet the bacteria. Bacteria from *BDC2.5^{+}Il-10$^{+/+}$* NOD mice and *BDC2.5^{+}Il-10$^{-/-}$* NOD mice were re-suspended in sterile PBS, heat-inactivated at 95°C for 30 min, and co-cultured overnight (10^{8} CFU) with 2 million total splenocytes or purified splenic neutrophils from *BDC2.5^{+}Il-10$^{+/+}$* and *BDC2.5^{+}Il-10$^{-/-}$* NOD mice. Stimulated splenocytes or neutrophils were further analyzed by flow cytometry and real-time qPCR.

Real Time Quantitative PCR (qPCR)

RNA from purified neutrophils (un-stimulated or stimulated with gut microbiota) or small intestinal tissue was extracted using Trizol reagent and an RNeasy mini plus kit (QIAGEN). After quantification, RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Invitrogen). Samples were analyzed on an iCycler qPCR machine (Bio-rad). Gene expression level was determined using the $2^{-\Delta\Delta Ct}$ method and normalized with the housekeeping gene, *Gapdh*. Primers sequences are listed in **Table 1**. Each sample was assayed in duplicate and the experiments were repeated at least twice.

Flow Cytometry

0.5 - 1 × 10^{6} single-cell suspensions from different lymphoid tissues were incubated with Fc block at room temperature for 20 min, before cell surface staining. For intracellular cytokine staining, cells were incubated at 37°C for 4 h in the presence of 10 ng/ml phorbol myristate acetate (PMA, Sigma), 500 ng/ml of ionomycin (Sigma) and 1 µl of Golgi plug™ (BD Bioscience), followed by cell surface staining, washing, fixation and permeabilization and intracellular cytokine staining. For intranuclear staining, cells were fixed and permeabilized using the Transcription Factor Staining Buffer Set (Tonbo Biosciences).

TABLE 1 | Primer information.

Genes	Primers	Sequence
Tnf-α	Forward	CAAATGGCCTCCCTCTCAT
	Reverse	TGGGCTACAGGCTTGTCACT
Il-1β	Forward	TGGAGAACACCACTTGTTGCTCCA
	Reverse	AAACAGATGAAGTGCTCCTTCGAGG
Inos	Forward	AGATTGGAGTTCGAGACTTCTG
	Reverse	TGGCTAGTGCTTCAGACTTC
Nos2	Forward	CCAAGCCCTCACCTACTTCC
	Reverse	CTCTGAGGGCTGACACAAGG
Arg1	Forward	CTCCAAGCCAAAGTCCTTAGAG
	Reverse	AGGAGCTGTCATTAGGGACATC
Caspase9	Forward	TCCTGGTACATCGAGACCTTG
	Reverse	AAGTCCCTTTCGCAGAAACAG
Zonulin1	Forward	CACCGGAGTGATGGTTTTCT
	Reverse	CCACCTCTGTCCAGCTCTTC
Reg3β	Forward	CTGCCTTAGACCGTGCTTTC
	Reverse	CCCTTGTCCATGATGCTCTT
Reg3γ	Forward	TTCCTGTCCTCCATGATCAAAA
	Reverse	CATCCACCTCTGTTGGGTTCA
Defcr6	Forward	CAGGCTGTGTCTGTCTCTTTTG
	Reverse	TAAATGACCCTTTCTGCAGGTC
Gapdh	Forward	GGGGTCGTTGATGGCAACA
	Reverse	TGTAGACCATGTAGTTGAGGTCA

After incubation, the cells were stained with an anti-Foxp3 antibody (clone: FJK-16s, eBioscience) and T-bet (clone:4B10, BioLegend). Cells were stained with mAbs to the following surface markers: CD45 (clone: 30-F11), TCR-β (clone: H57-597), CD4 (clone: GK1.5), CD8 (clone: 53-6.7), CD11b (clone: M1/70), CD62L (clone: MEL-14), ICOS (clone: 15F9), CD69 (clone: H1.2F3), CD25 (clone: 3C7), CD19 (clone: 6D5), all from BioLegend, and Ly6G (clone: 1A8) from BD Biosciences. mAbs to intracellular cytokines include TNF-α (clone: MP6-XT22), IFN-γ (clone: XMG1.2) and IL-17A (clone: TC11-18H10.1) were purchased from Biolegend. Samples were analyzed on a BD LSRII flow cytometer and subsequently analyzed by FlowJo 8.8.6 software (Tree star). Immune cells were gated based on their FSC-A/SSC-A properties. Single cells and subsequent live cells were gated on their FSC-A/FSC-H properties and live/dead staining, respectively.

Statistics

Diabetes incidence was compared using a log-rank test for survival. Insulitis scores were analyzed using a *Chi*-square test. Statistical analysis of microbial β-diversity was conducted using an analysis of similarities (ANOSIM). Differences between microbial species were determined following analysis using multiple *t*-tests with Bonferroni correction. Data from experiments *in vitro* were assessed for normality and subsequently analyzed using a two-tailed Student's *t* test (if data were normally distributed), a two-tailed Mann-Whitney test (if data were not normally distributed), or a two-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

In the Absence of IL-10, BDC2.5$^+$ NOD Mice Develop Accelerated Diabetes at a Very Young Age

To assess the role of IL-10 in diabetes protection observed in *BDC2.5*$^+$ NOD mice, we first monitored the natural history of type 1 diabetes development in *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice. Interestingly, both female and male *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice developed accelerated diabetes at a very young age (**Figures 1A, B**). In line with islet β cell destruction, *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice displayed impaired glucose tolerance compared with *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice (**Figure 1C**). We found no significant difference in severity of insulitis between *BDC2.5*$^+$*Il-10*$^{+/+}$ and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD

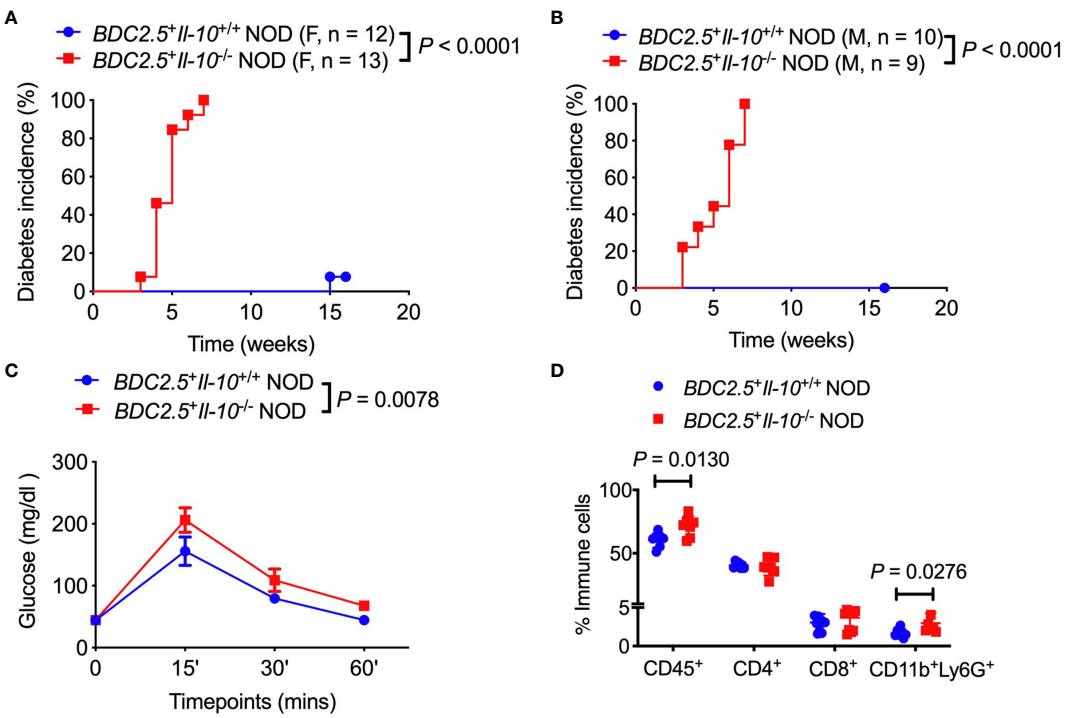

FIGURE 1 | *Il-10* deficiency in *BDC2.5*$^+$ NOD mice induced rapid development of type 1 diabetes. **(A, B)** Natural history of type 1 diabetes development in *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD littermates from females [**(A)**, n = 12-13/group] and males [**(B)**, n = 9-10/group]. **(C)** Intraperitoneal glucose tolerance test (IPGTT) in *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice (n = 5/group). **(D)** Infiltrated immune cells in the islets of mice (*n* = 7-11/group). In flow cytometric analysis, CD45$^+$ immune cells were gated from live cells. CD4$^+$ T cells, CD8$^+$ T cells, CD11b$^+$Ly6G$^+$ neutrophils were gated from CD45$^+$ immune cells. Data in **(A, B, D)** were pooled from two or more independent experiments. The experiment in **(C)** was performed twice, and consistent results were obtained. Data were analyzed using a log-rank test for survival **(A, B)**, a two-way ANOVA **(C)**, or a two-tailed Student's *t*-test [**(D)**, Data are presented as mean ± SD]. $P < 0.05$ was considered statistically significant.

mice (**Figures S1A, B**); however, *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice had more CD45$^+$ immune cells, especially CD11b$^+$Ly6G$^+$ neutrophils, infiltrating the islets than *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice, but no significant differences in the proportion of CD4$^+$ T cells, CD8$^+$ T cells, B cells, macrophages, and dendritic cells (**Figure 1D** and data not shown). Our data showed that in the absence of IL-10, *BDC2.5*$^+$ NOD mice develop accelerated diabetes at a very young age.

IL-10 Deficiency Promotes the Expansion of Bone Marrow and Peripheral Neutrophils in BDC2.5$^+$ NOD Mice

We observed an increased frequency of neutrophils infiltrating the islets of *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice (**Figure 1D**). To investigate the crosstalk between IL-10 and neutrophils, we assessed the neutrophil population in both the bone marrow and peripheral tissues of *BDC2.5*$^+$*Il-10*$^{+/+}$ and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice. Consistent with the results from the islets, we also found a higher percentage of neutrophils in the spleen, bone marrow and blood of *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice, compared to *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice (**Figures 2A–E** and **S2A, B**). Moreover, there was a higher proportion of IFN-γ-producing splenic neutrophils in *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice compared with *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice (**Figures 2F, G**). The expression levels of cytokines in the neutrophils from the blood and islets in *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice were the same as those in *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice (**Figures S3A–G**). Additionally, we found that neutrophils from the islets of the *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice had lower levels of CD62L (**Figure 2H**). We then purified neutrophils and found no significant differences in the gene expression of *Inos*, *Nos2*, *Arg1*, and *Caspase9* in the spleen (**Figures S4A–D**); however, we found increased *Nos2* and *Il-1β* in purified neutrophils from the bone marrow of *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice (**Figures 2I, J**), but no significant differences in the expressions of *Inos*, *Arg1*, *Caspase 9*, and *Mmp9* (**Figures S4E–H**). Next, we assessed the phagocytotic ability of neutrophils

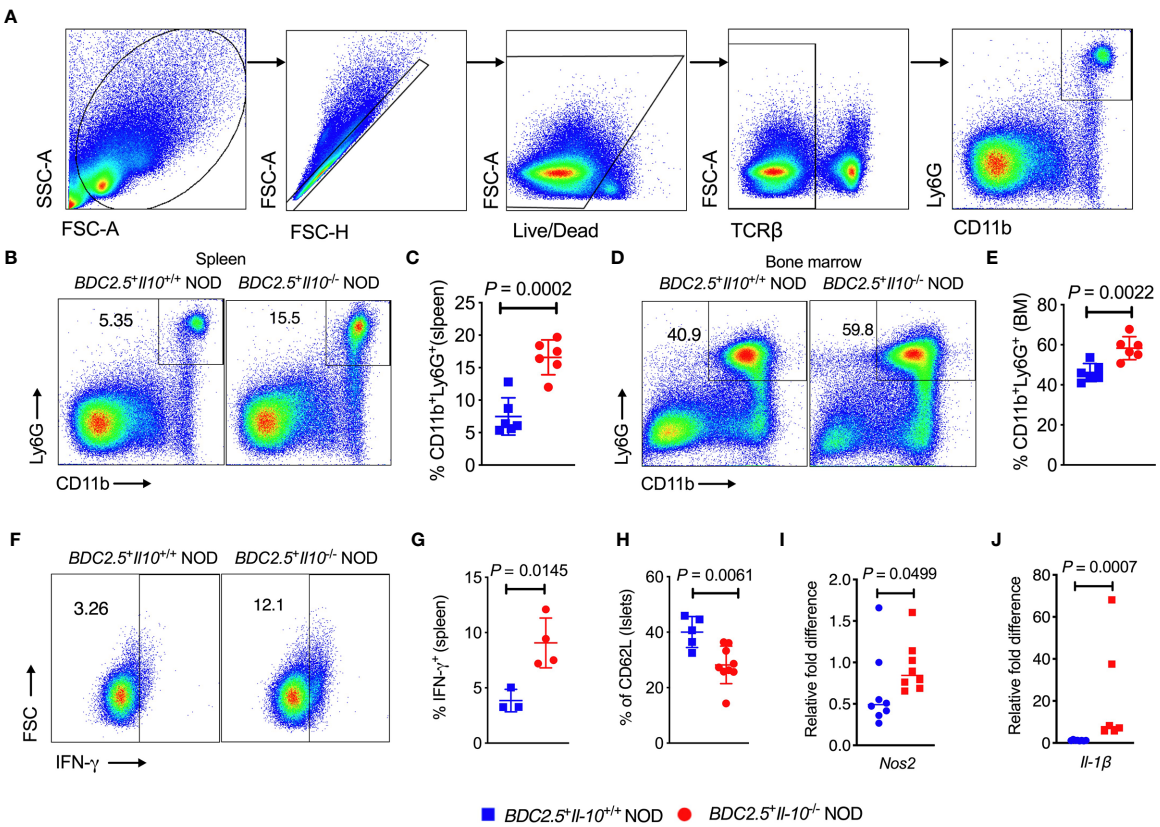

FIGURE 2 | Altered neutrophil homeostasis in *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice compared with *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice. **(A)** Gating strategies of CD11b$^+$Ly6G$^+$ neutrophils from the spleen. **(B, C)** Proportion of CD11b$^+$Ly6G$^+$ neutrophils in the spleen (n = 6/group). Representative flow cytometric profiles **(B)**, and summary of CD11b$^+$Ly6G$^+$ neutrophils gated from TCR-β$^-$ cells **(C)**. **(D, E)** Proportion of CD11b$^+$Ly6G$^+$ neutrophils in the bone marrow (n = 6/group). Representative flow cytometric profiles **(D)**, and summary of CD11b$^+$Ly6G$^+$ neutrophils gated from TCRβ$^-$ cells **(E)**. **(F, G)** IFN-γ expression in splenic CD11b$^+$Ly6G$^+$ neutrophils (n = 3-4/group). Representative flow cytometric profiles **(F)**, and summary of IFN-γ expression **(G)**. **(H)** CD62L expression in pancreatic CD11b$^+$Ly6G$^+$ neutrophils (n = 5-9/group). **(I, J)** Gene expression of *Nos2* **(I)** *and Il-1β* **(J)** in splenic neutrophils from *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice (n = 6-8/group). Data in **(C, E, H–J)** were combined from two or more independent experiments. The experiment in **(G)** was performed twice and consistent results were obtained. Data in **(C, E, G, H)** are shown as mean ± SD and were analyzed using a two-tailed Student's t-test. Data in **(I, J)** are presented as median and were analyzed using a two-tailed Mann-Whitney test. $P < 0.05$ was considered statistically significant.

and found that neutrophils from *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice were not different from those from *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice (**Figures S5A, B**). Taken together, our data suggested that *Il-10* deficiency altered neutrophil homeostasis in *BDC2.5*$^{+}$ NOD mice.

In the Absence of IL-10, CD4^{+} T Cells Are More Activated and Pathogenic in BDC2.5^{+} NOD Mice

Next, we assessed the phenotype and function of different T cell populations in *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice. Interestingly, unlike the composition of the immune cells seen in the islet infiltrate (**Figure 1D**), *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice had a higher proportion of splenic CD4^{+} T cells when compared with *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice (**Figure 3A**), while no significant difference was found in the percentage of CD8^{+} T cells (**Figure 3A**). The proportions of both CD4^{+} and CD8^{+} T cells were increased in the pancreatic lymph node (PLN) of *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice (**Figure 3B**). We found that splenic CD4^{+} T cells from *BDC2.5*$^{+}$*Il-10*$^{-/-}$ mice expressed a higher proportion of ICOS, T-bet and CD69 (**Figure 3C**) while splenic CD8^{+} T cells expressed higher level of T-bet only (**Figure 3D**). Moreover, splenic CD4^{+} T cells from *BDC2.5*$^{+}$*Il-10*$^{-/-}$ mice showed increased expressions of inflammatory cytokines including IFN-γ and IL-17A in the spleen (**Figure 3E**) and more TNF-α in CD4^{+} T cells from PLN (**Figure 3F**). Additionally, splenic CD8^{+} T cells in *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice expressed more TNF-α (**Figure 3G**). Interestingly, splenic CD4^{+} T cells from *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice showed stronger proliferative responses to their antigenic peptide

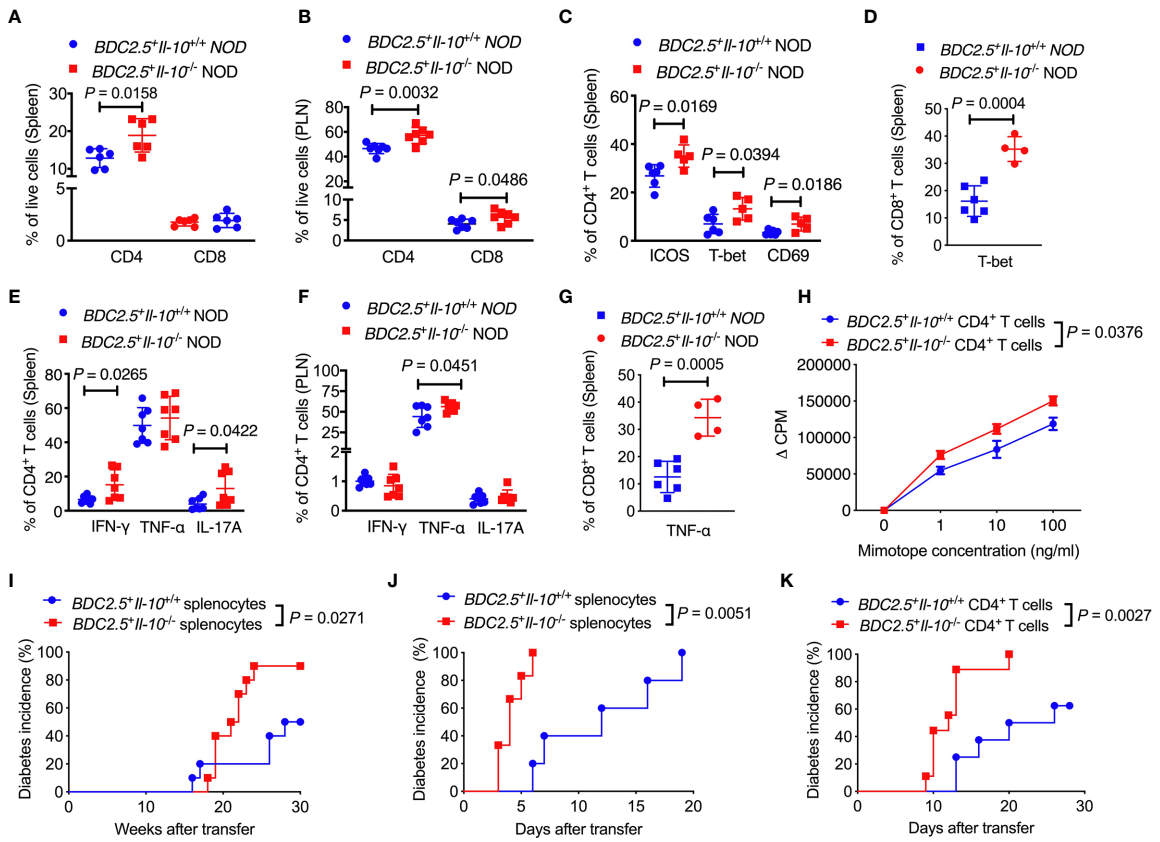

FIGURE 3 | *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice have more activated and pathogenic CD4^{+} T cells. **(A)** The percentage of CD4^{+} T cells and CD8^{+} T cells in the spleen of *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice (n = 6/group). **(B)** The percentage of CD4^{+} T cells and CD8^{+} T cells in the pancreatic lymph node (PLN) of *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice (n = 7/group). **(C)** The proportion of ICOS^{+}, T-bet^{+} and CD69^{+} splenic CD4^{+} T cells in *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice (n = 5-6/group). **(D)** The proportion of T-bet^{+} splenic CD8^{+} T cells in *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice (n = 4-6/group). **(E)** The proportion of IFN-γ^{+}, TNF-α^{+}, and IL-17A^{+} splenic CD4^{+} T cells in *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice (n = 7/group). **(F)** The proportion of IFN-γ^{+}, TNF-α^{+}, and IL-17A^{+} CD4^{+} T cells in the PLN from *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice (n = 7/group). **(G)** The proportion of TNF-α^{+} splenic CD8^{+} T cells in *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice (n = 4-6/group). **(H)** Proliferation of purified splenic CD4^{+} T cells from *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice in the presence of different concentrations of mimotope. **(I)** Adoptive transfer of diabetic splenocytes from *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice into irradiated wild-type NOD mice (n = 10/group). **(J)** Adoptive transfer of non-diabetic splenocytes from *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice into NOD-*scid* mice (n = 7-9/group). **(K)** Adoptive transfer of purified splenic CD4^{+} into NOD-*scid* mice (n = 8-9/group). Data in **(A–C, E, F, I–K)** were combined from two or more independent experiments. The experiments in **(D, G, H)** were performed two or more times, with similar results obtained. Data in **(A–G)** are shown as mean ± SD. Data were analyzed using a two-tailed Student's *t*-test **(A–G)**, a two-way ANOVA **(H)**, or log-rank test for survival **(I–K)**. $P < 0.05$ was considered statistically significant.

(mimotope) compared to $CD4^+$ T cells from *BDC2.5*$^+$*Il-10*$^{+/+}$ splenocytes (**Figure 3H**). No significant differences were found in the proportion of $CD8^+$ T cells expressing IFN-γ, TNF-α, or IL-17A in the PLN between *BDC2.5*$^+$*Il-10*$^{+/+}$ and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice (**Figures S6A–C**). To determine the effect of IL-10 on diabetogenicity of the T cells from *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice *in vivo*, we adoptively transferred total splenocytes from diabetic *BDC2.5*$^+$*Il-10*$^{-/-}$ or *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice into 4-week-old irradiated wild-type female NOD mice. Supporting the *in vitro* data, splenocytes from diabetic *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice showed more potent diabetogenicity by inducing a higher incidence of diabetes in the recipients, compared with the splenocytes from *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice (**Figure 3I**). Moreover, when we transferred total splenocytes from non-diabetic *BDC2.5*$^+$*Il-10*$^{-/-}$ or *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice into NOD.*scid* mice, splenocytes from *BDC2.5*$^+$*Il-10*$^{-/-}$ mice induced rapid diabetes in the NOD.*scid* recipients as early as 3-days after transfer (**Figure 3J**). We further confirmed the enhanced diabetogenicity by BDC2.5 $CD4^+$ T cells by adoptive transfer with purified splenic $CD4^+$ T cells from non-diabetic *BDC2.5*$^+$*Il-10*$^{-/-}$ or *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice into NOD.*scid* recipients. Similar to the adoptive transfer with total splenocytes, $CD4^+$ T cells from *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice induced rapid onset of diabetes compared to their counterparts from *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice (**Figure 3K**). To determine the effects of neutrophils on $CD4^+$ T cells, we performed criss-cross co-culture in which purified splenic neutrophils from *BDC2.5*$^+$*Il-10*$^{+/+}$ *or* *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice were cultured with purified splenic $CD4^+$ T cells from *BDC2.5*$^+$*Il-10*$^{-/-}$ or *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice. Interestingly, neutrophils from both *BDC2.5*$^+$*Il-10*$^{+/+}$ and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice effectively inhibited the proliferation of $CD4^+$ T cells from *BDC2.5*$^+$*Il-10*$^{+/+}$ and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice when stimulated by antigenic peptide (**Figures S7A, B**). However, there was no significant difference in the inhibitory effect of neutrophils from *BDC2.5*$^+$*Il-10*$^{+/+}$ and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice (**Figures S7A, B**). When we transferred purified splenic $CD4^+$ T cells alone or purified splenic neutrophils together with $CD4^+$ T cells from *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice into *Rag*$^{-/-}$ NOD mice, no significant difference in the diabetes incidence was found, indicating that the altered neutrophils in *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice may contribute to the disease development, but not by direct effects on $CD4^+$ T cells (**Figure S7C**). We examined the Treg cells in *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice. Surprisingly, there were no significant differences in both the proportion and the suppressive function of Treg cells between *BDC2.5*$^+$*Il-10*$^{+/+}$ and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice (**Figures S8A–C**). Taken together, our data showed that in the absence of *Il-10*, $CD4^+$ T cells in *BDC2.5*$^+$ NOD mice were more activated and highly pathogenic. However, Tregs did not appear to be directly affected by the absence of *Il-10*.

IL-10 Deficiency Alters the Composition of Gut Bacteria and Intestinal Immunity in BDC2.5$^+$ NOD Mice

To study whether *Il-10* deficiency also alters the gut bacteria of TCR transgenic *BDC2.5*$^+$ NOD hosts, we investigated the gut bacterial composition of *BDC2.5*$^+$*Il-10*$^{+/+}$ and *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice. *Il-10*-deficient *BDC2.5*$^+$ NOD mice had an altered composition of gut bacteria, indicated by β-diversity of the gut microbiota shown in the Principal Coordinate Analysis (PCOA; **Figure 4A**), compared to *Il-10*-sufficient *BDC2.5*$^+$ NOD mice. Further analysis also revealed differences at the phylum, class, and species levels. At the phylum level, *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice had increased levels of *Proteobacteria* compared with *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice (**Figures 4B, C**), with increased abundance of *Epsilonproteobacteria* and *Gammaproteobacteria* (**Figure 4D**). Moreover, the relative abundance of *Escherichia* from *Proteobacteria* was significantly different between the two groups (**Figure 4E**). We also assessed gut permeability *in vivo* to determine whether the altered gut bacteria affected the gut barrier in *Il-10*-deficient *BDC2.5*$^+$ NOD mice. Indeed, *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice exhibited increased gut permeability compared to *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice as demonstrated by the increased concentration of serum FITC-dextran (**Figure 5A**). We found decreased expression of the intestinal tight junction protein *Zonulin1* in *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice (**Figure 5B**), but not *Claudin2* or *Occludin* (data not shown). Moreover, when we assessed the inflammatory cytokines in the gut lumen, we found an increased level of IL-17A (**Figure 5C**) in *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice. Additionally, the intestinal expression of *Myd88*, which plays an essential role in the activation of innate immunity, was also increased in *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice when compared with that in *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice (**Figure 5D**). Interestingly, *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice showed increased expression of intestinal antimicrobial peptide genes, including *Crp*, *Defcr6*, *Reg3γ*, and *Reg3β* (**Figures 5E–H**), which could be induced to defend against the altered bacteria in the gut. Additionally, we found that the percentages of TCR-γδ$^+$ cells and NKP46$^+$ (one of the ILC markers) cells decreased in *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice, but no significant differences in $CD11c^+$ cells, IFN-γ$^+$CD4$^+$ (Th1) cells, IL-17$^+$CD4$^+$ (Th17) cells, and CD117$^+$ (one of the ILC markers) cells between the two groups (**Figures S9A–F**). Taken together, *Il-10* deficiency in *BDC2.5*$^+$ NOD mice not only altered the gut bacterial composition, but also changed the intestinal immune responses of the host.

Altered Gut Bacteria Contribute to the Altered Neutrophil Homeostasis in *BDC2.5*$^+$*Il-10*$^{-/-}$s NOD Mice

To determine whether the altered gut bacteria in *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice contribute to the increased frequency of neutrophils, we first depleted the endogenous commensal bacteria of *BDC2.5*$^+$*Il-10*$^{+/-}$ NOD breeders by treating the mice with a cocktail of antibiotics (0.5 g/L vancomycin, 1 g/L ampicillin, 1 g/L metronidazole, and 1 g/L neomycin) in drinking water and investigated the changes in neutrophils. Our results revealed that the antibiotic treatment significantly decreased the proportions of neutrophils in the islet, bone marrow, spleen and peripheral blood (**Figures 6A–H**). We also had the wildtype mice (*BDC2.5*$^+$*Il-10*$^{+/+}$) as controls, treated with or without antibiotic, and found that neutrophils were only decreased in the spleen in *BDC2.5*$^+$*Il-10*$^{+/+}$ NOD mice (**Figure S10**). To further confirm the role of gut microbiota from *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD mice in modulating neutrophil homeostasis, we co-cultured *BDC2.5*$^+$*Il-10*$^{-/-}$ NOD

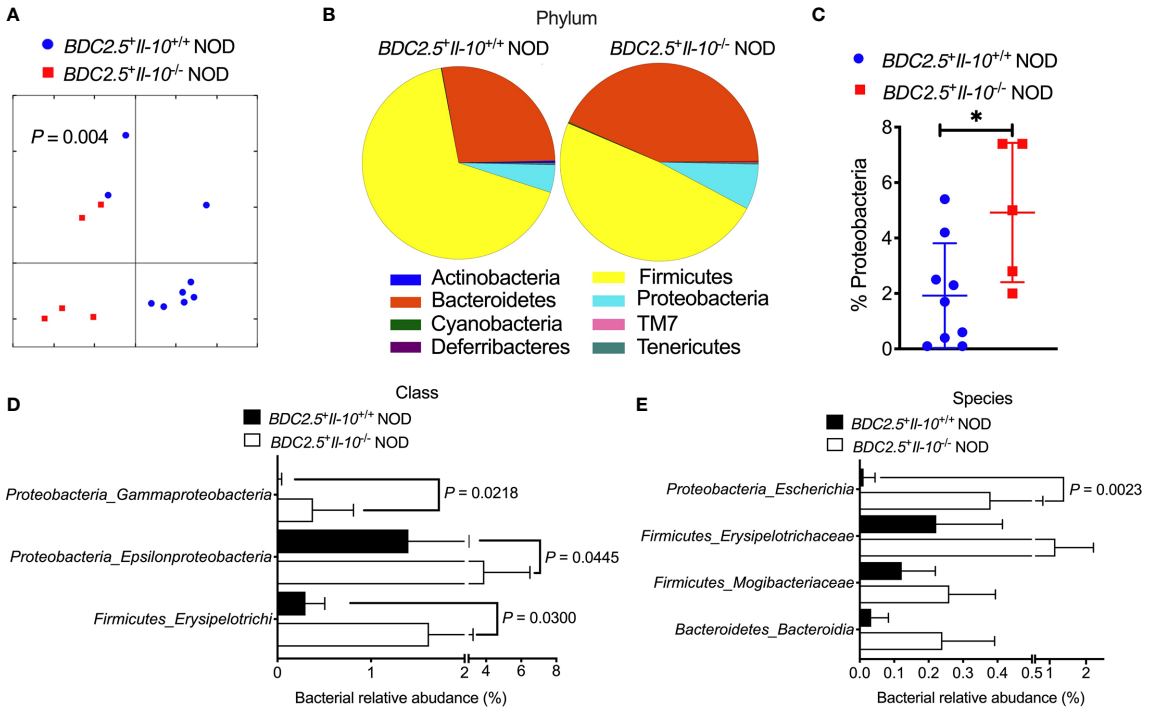

FIGURE 4 | Gut bacterial composition of *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice showed distinct differences compared with *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice. Gut bacteria in fecal pellets were analyzed by 16S rRNA sequencing (n = 5-9/group). **(A)** β-diversity of the gut bacteria indicated by unweighted PCoA. **(B, C)** Gut bacterial composition at the phylum level including representative composition **(B)** and summarized percentage of *Proteobacteria* as an example **(C)**. **(D)** Gut bacterial composition at the class level. **(E)** Gut bacterial composition at the species level. Statistical analysis was performed by an analysis of similarities (ANOSIM) **(A)**, a two-tailed Student's *t*-test **(C)**, or multiple *t*-tests with Bonferroni correction **(D, E)**. $P < 0.05$ was considered statistically significant. *$P = 0.0261$.

splenocytes *in vitro* with heat-inactivated gut bacteria from either *BDC2.5*$^{+}$*Il-10*$^{+/+}$ or *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice. Interestingly, we found that gut microbiota from *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD further expanded the neutrophil population significantly (**Figures 6I, J**). Next, we purified splenic neutrophils from *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice and stimulated them with heat-inactivated gut bacteria from either *BDC2.5*$^{+}$*Il-10*$^{+/+}$ or *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice, followed by assessment of gene expression. Our qPCR results showed that *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD gut microbiota promoted *Ifn-γ* gene expression (**Figure 6K**), which was consistent with the intracellular cytokine staining results seen in *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice (**Figures 2F, G**). Similar to the results using fresh *ex-vivo* splenic neutrophils (**Figures S4A, B**), there were no significant differences in the gene expressions of *Inos* and *Nos2* in the neutrophils after *in vitro* stimulation (**Figures 6L, M**). Taken together, our data suggested that altered gut bacteria from *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice modulated neutrophil homeostasis, which play a role in the development of type 1 diabetes.

DISCUSSION

In this study, we developed a markedly accelerated model of type 1 diabetes by introducing an *Il-10* deficiency to *BDC2.5*$^{+}$ NOD mice, whereas *Il-10* sufficient *BDC2.5*$^{+}$ NOD mice develop a very low incidence and delayed spontaneous diabetes. Although no significant difference was found in the severity of insulitis between *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice and *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice, *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice had more immune cells, especially neutrophils, infiltrating into the islets. In this mouse model, *BDC2.5*$^{+}$ NOD CD4^{+} T cells are more activated and pathogenic than CD4^{+} T cells from *Il-10* sufficient *BDC2.5*$^{+}$ NOD mice. Furthermore, *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice displayed increased proportions of neutrophils in the bone marrow, peripheral blood and spleen, which was closely associated with altered gut microbiota. We postulate that the altered microbiota may be central in increasing the neutrophils, which also play a role in increasing pathogenic CD4^{+} T cells.

IL-10 is a multifunctional cytokine that plays crucial roles in limiting the immune response and regulating the growth and/or differentiation a variety of immune cells (38, 39). The reports of the role of IL-10 in the development of type 1 diabetes are conflicting. It has been documented that early exposure to IL-10 accelerates the disease development (40), while IL-10 exposure during the later prediabetic phase inhibits disease (41). Additionally, the effects of IL-10 on autoimmune diabetes of NOD mice are associated with the location of IL-10 expression. Pancreatic expression of IL-10 can up-regulate the expression of intercellular adhesion molecule 1 (ICAM-1) on vascular endothelium (42) and promotes diabetes development (43),

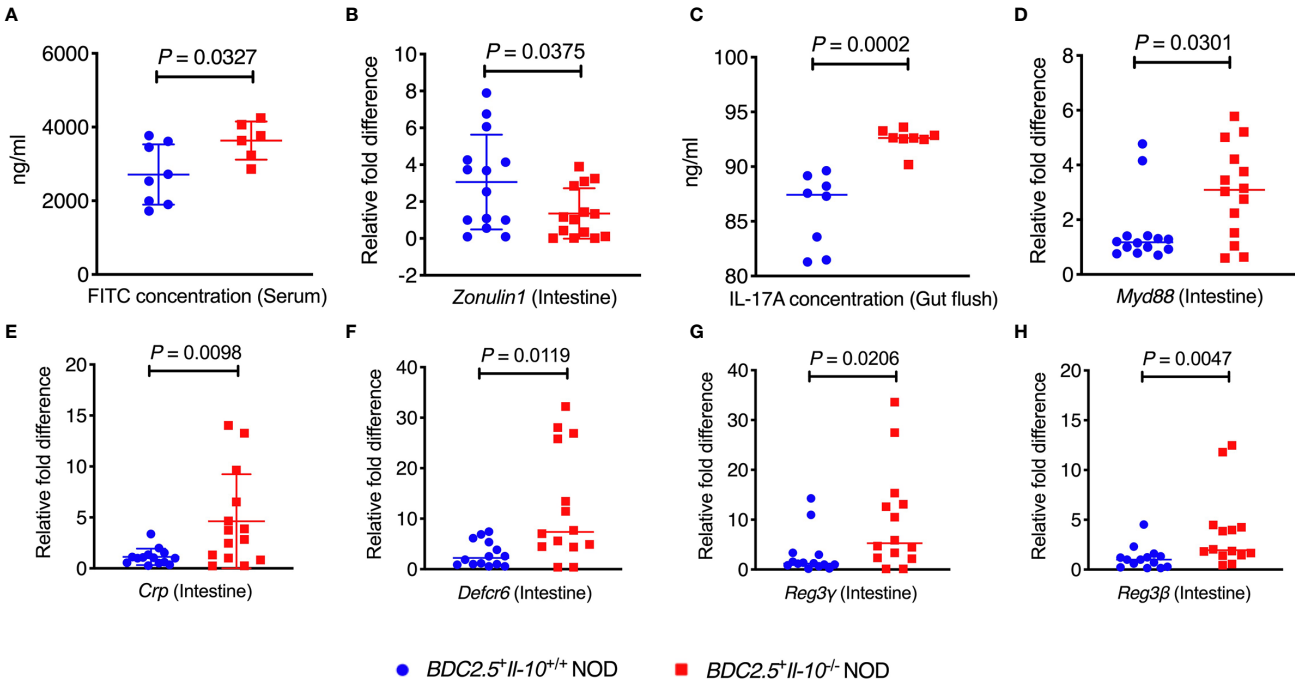

FIGURE 5 | *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice showed altered intestinal responses compared with *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice. **(A)** Gut permeability assessed following FITC-dextran uptake into the blood from the gut (n = 6-8/group). **(B)** Gene expression of *Zonulin1* (n = 14/group). **(C)** IL-17A concentration in the gut flush of the large intestine (n = 8/group). **(D)** Gene expression of *Myd88* (n = 14/group). **(E–H)** Gene expression of intestinal antimicrobial peptide genes, including *Crp* **(E)**, *Defcr6* **(F)**, *Reg3γ* **(G)**, and *Reg3β* **(H)** (n = 14/group). Data in **(A–H)** were combined from two or more independent experiments are presented as mean ± SD **(A, B, E)** or Median **(C, D, F–H)**. Statistical analysis was performed by a two-tailed Student's *t*-test **(A, B, E)** or a two-tailed Mann-Whitney test **(C, D, F–H)**. $P < 0.05$ was considered statistically significant.

but systemic IL-10 is dispensable for autoimmune diabetes (44). IL-10 has the ability to drive the generation and differentiation of Treg cells that can inhibit the antigen-specific immune responses (45, 46). Moreover, IL-10 also affects the function of Treg cells (35). In NOD mice, overexpression of IL-10 can dramatically induce Treg cells and therefore ameliorates the development of type 1 diabetes (47). However, we found that *Il-10* deficiency in *BDC2.5*$^{+}$ NOD mice did not affect the frequency and function of Treg cells. Consistent with previous findings that IL-10 strongly inhibited the cytokine production (48) and the proliferation of CD4^{+} T cells (49), we found that *Il-10* deficiency significantly affected the cytokine expression in CD4^{+} T cells and enhanced the immune response of *BDC2.5*$^{+}$ CD4^{+} T cells to specific antigen stimulation. Moreover, *Il-10* deficiency modulated the activation of *BDC2.5*$^{+}$ NOD CD4^{+} T cells, with increased expression of T-bet, CD69, IFN-γ, TNF-α and IL-17A. Most importantly, *Il-10* deficiency changed the function of CD4^{+} T cells as *BDC2.5*$^{+}$*Il-10*$^{-/-}$ CD4^{+} T cells were more pathogenic and induced rapid diabetes onset in NOD.*scid* mice compared with those CD4^{+} T cells from *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice. Therefore, *Il-10* deficiency in *BDC2.5*$^{+}$ NOD mice significantly affects the activation, proliferation, and function of CD4^{+} T cells, and thus contributes to the development of type 1 diabetes.

Although T cells have been well documented to play predominant roles in type 1 diabetes development, there is increasing evidence from both animal models and human beings that neutrophils from the innate immune system also contribute the initiation and progression of type 1 diabetes (15, 17, 21, 22). In addition to any effects on CD4^{+} T cells, *Il-10* deficiency had a significant impact on homeostasis of neutrophils in different tissues and expanded neutrophils in the bone marrow, peripheral blood, spleen, and islets in *BDC2.5*$^{+}$ NOD mice. Interestingly, we found that the change in the frequency of neutrophils was mediated by the altered gut microbiota in *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice, as this expansion was ameliorated by the depletion of the endogenous commensal microbiota *via* antibiotic treatment. Our work furthers the current understanding from other studies where gut microbiota was shown to regulate the host immunity by influencing neutrophil production and activation (50). Khosravi et al. also demonstrated that germ-free (GF) animals displayed reduced proportions of neutrophils in bone marrow and spleen (51). Microbially-derived components can regulate neutrophil homeostasis as the neutrophil reduction was rescued by treatment with microbe-associated molecular patterns from heat-killed *E coli* or autoclaved cecal content (51). Gut microbiota have been documented to regulate granulocytosis and neutrophil homeostasis by influencing the intestinal IL-17-producing cells and the release of granulocyte colony-stimulating factor (G-CSF) in a Toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88)-dependent manner (23). Additionally, Zhang and colleagues found that gut

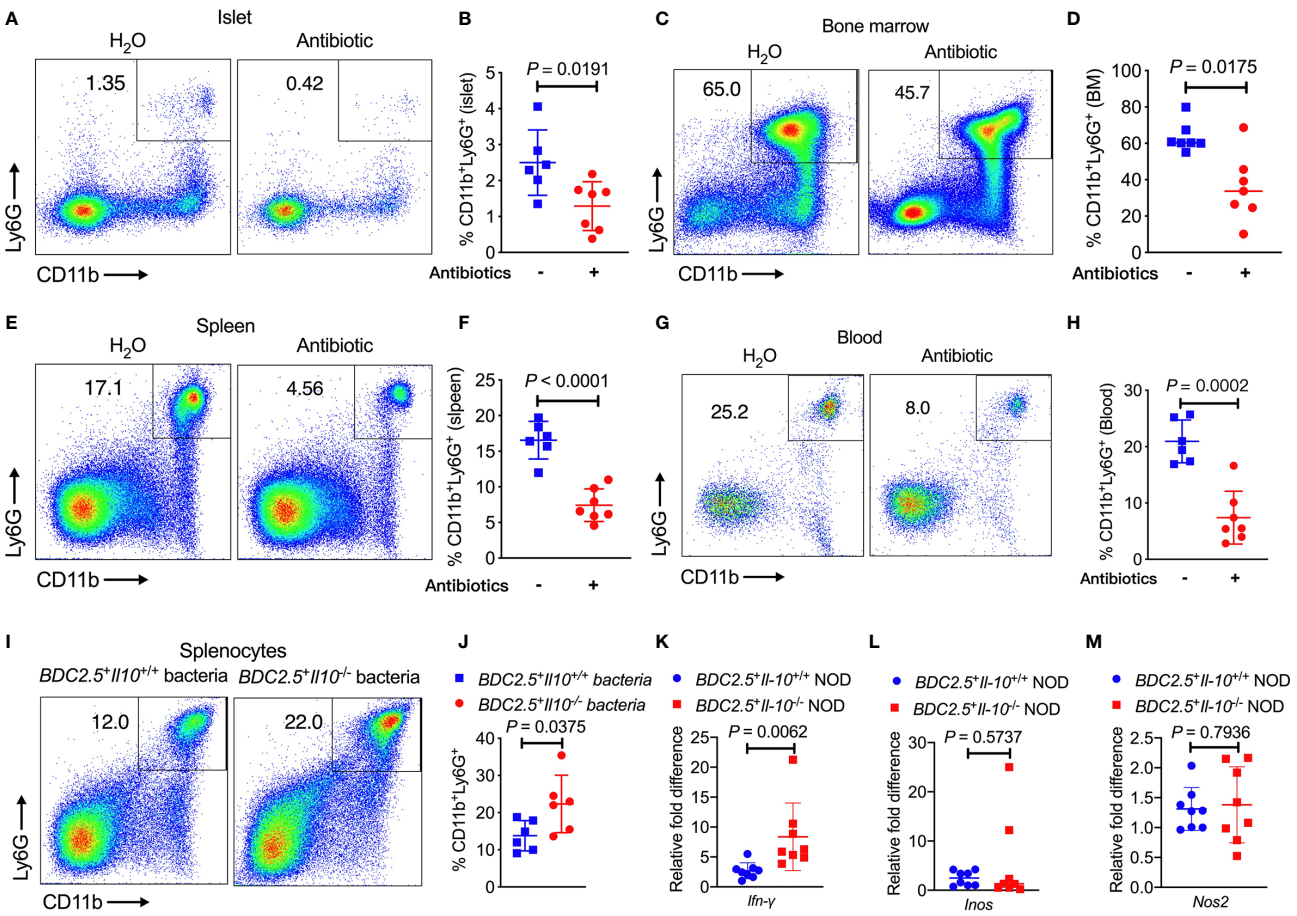

FIGURE 6 | *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD gut bacteria modulate neutrophil homeostasis. **(A–H)** *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice showed altered percentages of neutrophils after antibiotic treatment (n = 6-7/group). Representative flow cytometric profiles, and summary of CD11b^{+}Ly6G^{+} neutrophils in the islets gated from CD45^{+} immune cells **(A, B)**, in the bone marrow gated from TCRβ^{-} cells **(C, D)**, in the spleen gated from TCRβ^{-} cells **(E, F)**, and in the blood gated from live cells **(G, H)**. **(I, J)** Proportion of CD11b^{+}Ly6G^{+} neutrophils in the splenocytes stimulated with heat-inactivated gut bacteria from either *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice or *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice (n = 6/group). Representative flow cytometric profiles **(I)**, and summary of CD11b^{+}Ly6G^{+} neutrophils gated from TCR-β^{-} cells **(J)**. **(K–M)** Gene expression of *Ifn-γ* **(K)**, *Inos* **(L)**, and *Nos2* **(M)** from purified splenic neutrophils after stimulation with heat-inactivated gut bacteria from either *BDC2.5*$^{+}$*Il-10*$^{+/+}$ NOD mice or *BDC2.5*$^{+}$*Il-10*$^{-/-}$ NOD mice (n = 8/group). Data in **(B, D, F, H, J–M)** were combined from two or more independent experiments. Data in **(B, F, H, J, K, M)** are presented as mean ± SD and statistical analysis was performed by a two-tailed Student's t-test. Data in **(D, L)** are presented as Median and were analyzed using a two-tailed Mann-Whitney test. $P < 0.05$ was considered statistically significant.

microbiota modulate the neutrophil ageing and the depletion of the microbiota can significantly reduce the number of circulating aged neutrophils (24). Our studies suggest that gut microbiota might affect type 1 diabetes development through modulating neutrophil hemostasis, especially by increasing neutrophil infiltration in the islets. Further investigation is needed to elucidate how gut microbiota modulate neutrophils and their role in the development of type 1 diabetes. To better understand the role of IL-10 in regulation of gut microbiota and gut microbiota-associated neutrophil hemostasis, the ideal approach would be to generate germ free *Il-10* deficient mice. This would be our future direction.

Taken together, by depleting *Il-10* in *BDC2.5*$^{+}$ NOD mice, we generated a markedly accelerated model of type 1 diabetes. Our studies showed that *Il-10* deficiency in *BDC2.5*$^{+}$ NOD mice significantly altered the immune response and function of CD4^{+} T cells. Importantly, we showed that the effect of IL-10 on the homeostasis of neutrophils was mediated by the altered gut microbiota. Thus, our study suggests that gut microbiota might contribute to the development of type 1 diabetes by regulation of neutrophil homeostasis. These findings may provide novel insights into the role of IL-10 in the modulation of both innate immune cells and adaptive immune cells and the development of autoimmunity, such as type 1 diabetes.

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 animal study was reviewed and approved by The Institutional Animal Care and Use Committee of Yale University.

AUTHOR CONTRIBUTIONS

LW conceived the project. JH, QT, NT, JAP, YL, CC, LZ, JP, YX, LYZ, and YH researched the data. ZZ contributed to the discussion. JH wrote the manuscript. LW, FW, and JAP revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Visceral Adipose Tissue: A New Target Organ in Virus-Induced Type 1 Diabetes

Danny Zipris *

Innate Biotechnologies LLC, Denver, CO, United States

Type 1 diabetes (T1D) is a proinflammatory pathology that leads to the specific destruction of insulin producing β-cells and hyperglycaemia. Much of the knowledge about type 1 diabetes (T1D) has focused on mechanisms of disease progression such as adaptive immune cells and the cytokines that control their function, whereas mechanisms linked with the initiation of the disease remain unknown. It has been hypothesized that in addition to genetics, environmental factors play a pivotal role in triggering β-cell autoimmunity. The BioBreeding Diabetes Resistant (BBDR) and LEW1.WR1 rats have been used to decipher the mechanisms that lead to virus-induced T1D. Both animals develop β-cell inflammation and hyperglycemia upon infection with the parvovirus Kilham Rat Virus (KRV). Our earlier *in vitro* and *in vivo* studies indicated that KRV-induced innate immune upregulation early in the disease course plays a causal role in triggering β-cell inflammation and destruction. Furthermore, we recently found for the first time that infection with KRV induces inflammation in visceral adipose tissue (VAT) detectable as early as day 1 post-infection prior to insulitis and hyperglycemia. The proinflammatory response in VAT is associated with macrophage recruitment, proinflammatory cytokine and chemokine upregulation, endoplasmic reticulum (ER) and oxidative stress responses, apoptosis, and downregulation of adipokines and molecules that mediate insulin signaling. Downregulation of inflammation suppresses VAT inflammation and T1D development. These observations are strikingly reminiscent of data from obesity and type 2 diabetes (T2D) in which VAT inflammation is believed to play a causal role in disease mechanisms. We propose that VAT inflammation and dysfunction may be linked with the mechanism of T1D progression.

Keywords: type 1 diabetes, Kilham rat virus, Inflammation, visceral adipose tissue (VAT), beta cells

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Li Wen,
Yale University, United States

Reviewed by:
Aditi Arun Narsale,
San Diego Biomedical Research Institute, United States
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The University of Iowa, United States

***Correspondence:**
Danny Zipris
zdanny@innatebiotechnologies.com

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INTRODUCTION

Type 1 diabetes (T1D) is a multi-step proinflammatory pathology that culminates in the specific destruction of islet β-cells and lack of insulin secretion (1–3). The Centers for Disease Control and Prevention have estimated that ~1.25 million Americans are currently living with T1D and 40,000 new cases of T1D are being diagnosed in the U.S each year and it is estimated that five million Americans will live with T1D by mid-century (4).

It is thought that both genetic and environmental factors are key players in the mechanism that triggers diabetes (5–7). The risk for T1D development is substantially increased in relatives of T1D patients, since ~6% of children of a diabetic parent, 5% of siblings and 50% of monozygotic twins develop T1D compared to only 0.4% in the general population (8, 9). More than 50 T1D genetic risk loci have been identified to be associated with disease progression (10).

There is ample evidence from humans and animals supporting the notion that the environment plays a key role in mechanisms that trigger β-cell autoimmunity (11–18), and viruses have been postulated to play a pivotal role in these mechanisms (16, 17, 19–27). Due to ethical reasons, it is almost impossible to establish a causal role for microbial infections in triggering T1D, or address virus-induced disease mechanisms in humans. Furthermore, identifying microbes involved in triggering T1D may be hindered since by the time T1D is detected, the individual might have been infected with multiple viruses and the virus triggering the disease might have been cleared (28, 29). We have therefore used the BBDR and LEW1.WR1 rat models that develop T1D following infection with Kilham Rat Virus (KRV) (30) to identify how infections lead to β-cell inflammation and destruction.

Emerging evidence suggests that inflammation plays a key role in triggering numerous inflammatory disorders (31–35). We recently hypothesized that innate immune upregulation is associated with promoting virus-induced T1D (30, 36–43). Our recent data provided for the first time evidence linking inflammation in VAT with mechanisms of T1D (44). Inflammation in VAT is detectable soon after infection prior to insulitis and hyperglycemia and is characterized by infiltration of macrophage to the site of inflammation and proinflammatory cytokine and chemokine upregulation and tissue dysfunction (44). On the basis of these observations, we hypothesize that VAT inflammation and dysfunction may be associated with T1D mechanisms.

KILHAM RAT VIRUS

KRV is a rat-specific virus environmentally ubiquitous and a member of the *Parvoviridea*, a virus group of small single-stranded DNA viruses with an average genome size of 5 Kbp encapsidated by protein in an icosahedral non-enveloped particle (45). This virus group infects various animal species, including humans (46) and rodents (47). KRV encodes three overlapping structural proteins, VP1, VP2, and VP3, and two overlapping nonstructural proteins, NS1 and NS2 (47). There human parvovirus B19 has been linked with pro-inflammatory autoimmune disorders like acute myocarditis (48, 49), rheumatoid arthritis (50), systemic lupus erythematosus (51), and Sjögren's syndrome, as well as other autoimmune conditions (50). Infection with B19 has been associated with the appearance of elevated levels of autoantibodies against nuclear antigens and double-stranded DNA (50). KRV infection can occur in natural environment leading to T1D without the need for virus injection (52). Known routes by which KRV transmission may occur are direct contact, aerosol, and oral (52).

RAT MODELS OF VIRUS-INDUCED T1D

There are two inbred rat strains that have been most used to address virus-induced T1D mechanisms, the BBDR and LEW1.WR1 rats. These animals are the only genetically un-manipulated animal models in which infection with a virus triggers anti-ß-cell autoimmunity (41). BBDR rats have normal levels and function of peripheral T cells (53, 54), and spontaneous diabetes does not develop in viral antibody–free BBDR rats (55). However, insulitis, hyperglycemia, and severe ketosis occur in animals after inducing innate immunity with Poly(I:C) plus elimination of regulatory ART2+ T cells (55), or following virus infection (52). T1D in the BBDR rat is mediated by the immune system since the transfer of lymph nodes from animals with diabetes to RT1u MHC compatible T cell deficient WAG nu/nu rats results in diabetes progression (56).

The LEW1.WR1 rat has also normal levels and function of T lymphocytes (57). The LEW1.WR1 rat has a higher degree of disease penetrance compared with that of BBDR rats as evidenced by the observation that elimination of ART2.1+ cells by itself can result in diabetes (57). As seen in the BBDR rat, KRV infection leads to hyperglycemia by specific loss of islet ß-cells, glycosuria, ketonuria, and polyuria (55, 57).

Infecting LEW1.WR1 and BBDR rats with KRV leads to specific β-cell inflammation, islet cell death and permanent T1D occurring following insulitis, 2-4 weeks following virus inoculation with disease rate of ~20 and 60%, respectively (30, 34, 35, 52). It is noteworthy that the ability of virus infection to trigger T1D or inflammation in the rat is not limited to KRV, since β-cell autoimmunity in the rat can be triggered by two other viruses, rat CMV (58). Furthermore, Poly I:C, a synthetic analogue of double stranded RNA which mimics viral infection, synergizes with low KRV titers, that by themselves do not induce T1D, on disease progression (41). Because double stranded RNA molecules can be expressed by different viruses, this may suggest that microbes other than KRV could also be associated with initiating T1D development (44). Indeed, multiple viruses have been hypothesized to be involved in triggering human T1D (5, 6, 16, 17, 19–26, 59).

A key factor linked with the mechanism leading to T1D in both animals and humans is likely to be linked with proinflammatory pathways that can potentially be upregulated by different virus groups (60). It is therefore plausible to hypothesize that while a human KRV homologue may not necessarily be involved in triggering T1D in humans, viruses that induce proinflammatory pathways similar to those induced by KRV may be linked with promoting β–cell autoimmunity in genetically susceptible individuals. Identifying mechanisms of KRV-induced T1D in rat models of virus-induced T1D could therefore provide valuable data on mechanisms mediating the human disease.

The relevance of the BBDR and LEW1.WR1 rat models to the human disease is supported by data from our laboratory and others. T1D in the rat better resembles the human disorder than the mouse model with respect to histopathology (61). Similar to the rat, there is no significant infiltration of immune cells around the islet ("peri-insulitis") prior to disease onset and insulitis is morphologically mild and more similar to that detected in human T1D (62–64). As seen in humans, disease in the rat is not influenced by gender (65) and is MHC-dependent (61, 66).

The mechanism of T1D in the LEW1.WR1 rat is believed to be fundamentally different than that leading to T1D in the NOD mouse. In contrast to the rat, T1D development in the mouse is not dependent on microbial infections as germ-free mice retain the ability to develop disease (67). While β-cell autoimmunity in the mouse appears to be independent of the MyD88 signaling pathway (68), our studies demonstrated that the disease in the rat is mediated *via* the MyD88-TLR9 signaling axis (40). Finally, innate immune activation with exogenous activators of TLR2, TLR3/MDA-5, TLR4, TLR7/8, and TLR9, and exacerbates T1D in the rat (41, 69), but protects NOD mice from β–cell autoimmunity (70–73).

Innate Immunity and Inflammation

Inflammation is a physiological reaction of the innate immune system to microbial infection or tissue injury leading to the secretion of numerous inflammatory mediators, such as cytokines and chemokines, which orchestrate cellular defense mechanisms and injured tissue repair (74, 75). In contrast to adaptive immunity that identifies antigenic molecules using highly specific receptors expressed on T and B lymphocytes, inflammation is the less specific arm of the immune system (76).

Innate immune sentinel cells such as dendritic cells (DCs), macrophages, and neutrophils and recognize invading microbes *via* pattern recognition receptors (PRRs) activating downstream innate immune pathways aiming to eliminate infections (77, 78). A key PRR group is the Toll-like receptors (TLRs) family each member of which recognizes a different type of conserved pathogen-associated molecular patterns (PAMPs), such as TLR2 that senses cell wall molecules of gram-positive bacteria lipoteichoic acid and TLR3 and TLR9 that sense double stranded RNA and microbial DNA, respectively reviewed in refs. (79–88). Recognition of PAMPs by PRRs induces proinflammatory responses and activation of host defense mechanisms (79–88). The interaction of TLRs with their agonists induces in addition to proinflammatory cytokine and chemokine responses, the expression of MHC Class II and costimulatory molecules on antigen presenting cells (APCs), thus enabling these cells to effectively activate antigen-specific T cells to specifically attack invading pathogenic microbes (79–88). In addition to sentinel cells, innate immunity also has a humoral arm comprised of pattern recognition molecules (PRMs), such as lectin, ficolins, pentraxins, and the complement component C1q (89, 90).

Role of Inflammation in KRV-Induced T1D

Infection with KRV induces a global innate immune upregulation detected in various lymphoid organs, such as the spleen, pancreatic lymph nodes, Peyer's patches and thymus involving the induction of numerous proinflammatory cytokines, including IL-1ß, IFN-γ, and IL-12 3-5 days after infection, prior to insulitis and diabetes (30, 34, 35, 66). The rats develop humoral and cellular anti-KRV responses and clear the virus (91). We proposed that KRV-induced inflammation is associated with mechanisms of disease development (30, 36–43). We were the first to implicate TLR signaling in T1D progression (40, 41). We demonstrated that innate immune activation with ligands of TLRs synergizes with KRV infection on T1D development (41). Furthermore, we observed that the highly homologous H-1 parvovirus does not activate the innate immune system and fails to induce diabetes development in the BBDR rat (41). Our *in vivo* studies have shown that blocking IL-1 signaling with IL-1RA (39), or suppressing inflammation with a number of immunomodulatory agents, such as steroids (69), histone deacetylase inhibitor (38), antibiotics (30), or short chain fatty acids (92) prevents diabetes. Our hypothesis on the role of innate immunity in T1D is further supported by earlier data implicating TLR9 pathways in KRV-induced T1D mechanisms (40). We demonstrated that *in vitro* KRV-induced innate immunity is blocked by inhibitors of TLR9 and blockers of PKR and NF-κB (40). Finally, pharmacological suppression of TLR9 *in vivo* prevents T1D (40).

COVID-19 and T1D

Given that COVID-19 induces robust inflammation in infected individuals, it has recently been hypothesized that this virus could potentially drive T1D *via* mechanisms associated in part with immune upregulation (93, 94). The data on the ability of COVID-19 to induce autoimmunity are mixed and clear evidence that COVID-19 activates anti-β–cell autoimmunity is not yet available (94–103). Moreover, the observations implicating COVID-19 in T1D development are based primarily on anecdotal data (95–101). Because hyperglycemia is only the end stage of the anti-islet autoimmune process that may start many years prior to disease onset (104–106), long-term follow up epidemiological studies will be required to determine whether COVID-19 infection increases the risk for T1D development in genetically-susceptible individuals.

KRV-INDUCED INFLAMMATION IN VISCERAL ADIPOSE TISSUE (VAT)

In the course of our studies on the role of inflammation in virus-induced T1D, we observed that infection of LEW1.WR1 rats with KRV leads to inflammation in VAT detectable as early as day 1 post-infection, long before β-cell inflammation and hyperglycemia. This inflammation is characterized by an influx of $CD68^+$ macrophages into VAT seen in the interstitial space surrounding adipocytes in KRV-infected animals but not control rats injected with PBS. In sharp contrast to VAT, subcutaneous adipose tissue (SAT) was observed to be free of cell infiltration (44). Activation of innate immunity with Poly (I:C) in the absence of virus also induces VAT inflammation. Because i.p. injection of KRV induces inflammation in proximal and distal organs, and since Poly (I:C) itself, in the absence of virus, can

induce VAT inflammation, it is unlikely that the route of virus inoculation or site of infection play a critical role in triggering VAT inflammation. Unlike VAT, the exocrine tissue and islets from day 5-infected rats are insulitis-free, whereas ß-cells from day-14-infected animals are inflamed or show signs of tissue destruction (44). KRV induces the expression of virus transcripts and proinflammatory cytokines such as IL-1ß, IL-6, and IL-12p40 and chemokines in VAT *in vivo* and in purified adipocytes *in vitro* (44). Furthermore, KRV induces ER and oxidative stress response and activation of apoptotic pathways in infected VAT *in vivo* (44). KRV also downregulated the expression of adipokines and genes associated with mediating insulin signaling in VAT (44). Brief therapy with dexamethasone early in the disease course (days 1-5) prevents VAT inflammation and T1D. Based on these data, we hypothesized that VAT inflammation and dysfunction may be linked with early mechanisms of virus-induced disease development.

ROLE OF ADIPOSE TISSUE IN GLUCOSE METABOLISM AND IMMUNITY

There are several types of adipose tissue, i.e. white adipose tissue (WAT), brown adipose tissue (BAT) and beige adipose tissue (107). WAT is the most abundant fat accounting for 5% to 50% of human body weight (107). It plays a key role in metabolic homeostasis by storing fat for long-term survival and by functioning as an endocrine organ (107–109). WAT is a main source of many adipokines, peptides or proteins with hormone-like properties that regulate metabolic homeostasis through local paracrine effects and endocrine effects (107–109). The metabolic characteristics of WAT is determined by its location in the body, commonly classified into subcutaneous fat and visceral fat depots (107–109). Adipose tissue can release and respond to cytokines and may therefore exert immune modulatory functions on non-adipose tissues (107). The discovery of leptin and adiponectin was the first indication that adipose tissue is an endocrine organ with the ability to regulate systemic energy homeostasis and glucose metabolism as well as mediate immunity. The metabolic effects of leptin and adiponectin on target tissues were observed to be robust (110).

Why KRV induces inflammation in VAT and not SAT is unclear. It may be that this is the result of differences in the function of VAT *versus* SAT. SAT is less active metabolically than VAT (111). It has been shown that adipocytes of VAT undergo more lipolysis than SAT and therefore contribute larger amounts of fatty acids to the circulation (111–113). On the other hand, SAT is considered to have a better capability of storing fatty acids, implying that it could store energy in periods of excess nutrition and supply fatty acids in periods of starvation (111).

Leptin has been suggested to play a key role in T2D development (reviewed in ref. 104). The long form of the leptin receptor (ObRb) capable of intracellular signaling is expressed in ß-cells, and exogenous leptin inhibits insulin production and secretion from human islets implying a direct action of leptin on β-cell function (105, 106, 114–118). Furthermore, mice deficient of leptin have increased appetite, weight gain, insulin resistance and diabetes, conditions that can be improved with leptin therapy (104, 119–124). In addition to its role in controlling energy balance, leptin can also influence immune functions reviewed in ref. (119). Indeed, macrophages express the leptin receptor (119) and leptin can increase the proliferation of monocytes and induce the expression of inflammatory cytokines such as TNF-α and IL-6 and other surface activation molecules (125).

Adiponectin has been shown to have beneficial effects on insulin sensitivity (110, 126) and β-cell regeneration in mice with STZ-induced diabetes (127). Adiponectin has also been demonstrated to protect ß-cells from the detrimental effects of free fatty acids (128) *via* as yet unidentified mechanisms (118). Adiponectin is an endogenous insulin sensitizer in the skeletal muscle and liver, and administering mice with adiponectin results in lower blood glucose levels and the reversal of insulin resistance in mouse models of obesity (119). The receptor for adiponectin is expressed in macrophages, and adiponectin can suppress the production of TNF-α and IL-6 and induce the production of the anti-inflammatory mediators IL-10 and IL-1 receptor antagonist (119). Mice deficient in adiponectin have increased numbers of activated M1 macrophages in their adipose tissue with increased production of TNF-α, IL-6, and MCP-1 (119).

CROSSTALK BETWEEN INNATE IMMUNITY AND GLUCOSE METABOLISM

The hypothesis that there is interplay between the innate immune system and glucose metabolism emerged after it was observed that administering low doses of lipopolysaccharide (LPS) leads to hyperglycemia mediated primarily by IL-1 pathways (129). Innate immune mediators such as IL-1 may play a beneficial role in maintain a normal glucose homeostasis by inducing insulin secretion and biosynthesis and β-cell proliferation reviewed in ref. (130).

In obesity, increased fat mass can result in adipocyte hypertrophy, hypoxia, death and ER stress response reviewed in refs. (119, 130). The adipose tissue death and dysfunction lead to the induction of chronic inflammation associated with the expression of proinflammatory cytokines such as IL-1, IL-6 and TNF-α and chemokines such as MCP-1 in adipocytes. MCP-1 and other chemokines released by adipocytes and immune cells in fat tissue further promote infiltration of monocytes and other immune cells into adipose tissue (130–132). Macrophages are the most abundant innate immune cells infiltrating and accumulating into adipose tissue of obese individuals (133).

Chronic inflammation in adipose tissue is believed to play a key role in the development of insulin resistance that is a hallmark of T2D in obese individuals reviewed in ref. (133). Insulin resistance may culminate in aberrant glucose uptake and glycogen synthesis (134). Consequently, ß-cells attempt to

compensate for insulin resistance by increasing insulin secretion to restore normal glucose homeostasis (134). A further decline in insulin sensitivity makes the ß-cells exhausted, leading to hyperglycemia and T2D (135).

VAT INFLAMMATION AND DYSFUNCTION IN KRV-INDUCED T1D *VERSUS* T2D

The underlying mechanisms and pathways critically involved in KRV-induced inflammation and T1D remain to be identified. The data from our laboratory implicating VAT inflammation and dysfunction in T1D development are highly reminiscent of observations from obesity and T2D in which VAT inflammation and dysfunction have been hypothesized to play a causal role in mechanisms that result in islet damage and diabetes progression (136–143). Although the level of the proinflammatory response detected in VAT from infected LEW1.WR1 rats is substantially greater than that typically seen in adipose tissue from T2D (44, 136, 137, 141, 142, 144–155), one cannot ignore the remarkable commonalities between inflammation observed in KRV-induced T1D *versus* T2D. Most notably, in both conditions, VAT is targeted by the innate immune system. Moreover, VAT inflammation in both disorders is linked with 1) macrophage infiltration into VAT, 2) expression of proinflammatory cytokines such as IL-1, IL-6 and TNF-α and as well as chemokines such as CCL2, CCL5, and CXCL-10, 3) oxidative stress response, 4) apoptosis, 5) adipocyte death, and 6) tissue dysfunction (136, 137, 141, 142, 144–155).

ROLE OF VAT INFLAMMATION AND DYSFUNCTION IN VIRUS-INDUCED T1D

Whether and how VAT inflammation and dysfunction play a role in KRV-induced T1D mechanisms remain to be further elucidated. We propose a model that may explain how VAT inflammation and dysfunction lead to T1D (see model in **Figure 1**). We hypothesize that infection with KRV results in VAT infection and TLR-induced macrophage activation and infiltration into VAT. Inflammation in VAT associated with a robust proinflammatory cytokine response may lead to adipose tissue hypoxia, ER and oxidative stress responses and apoptosis and consequently aberrant adipokine expression (118, 119, 133, 156, 157). In Obesity, free fatty acids, and lipid intermediates synergistically induce adverse effects on both β-cell mass and function contributing to the progressive loss of functional β-cell mass reviewed in ref. (118). Likewise, circulating factors such as cytokines released from inflamed tissues such as adipose tissue and activated innate immune cells can adversely affect ß-cells by impairing their functions and limiting cell mass (118). In a similar manner, KRV-induced excessive lipolysis resulting from adipocyte death can result in excess of free fatty acids in the circulation, which can induce lipotoxicity. KRV-induced proinflammatory cytokines such as IL-1ß can enter the circulation and from there to the pancreas where it may exert toxic effects on islets, potentially leading to metabolic and cellular stress in ß-cells (136, 158). Furthermore, a rise in glucose levels in the microenvironment of ß-cells can activate the inflammasome in pancreatic ß-cells, further increasing the

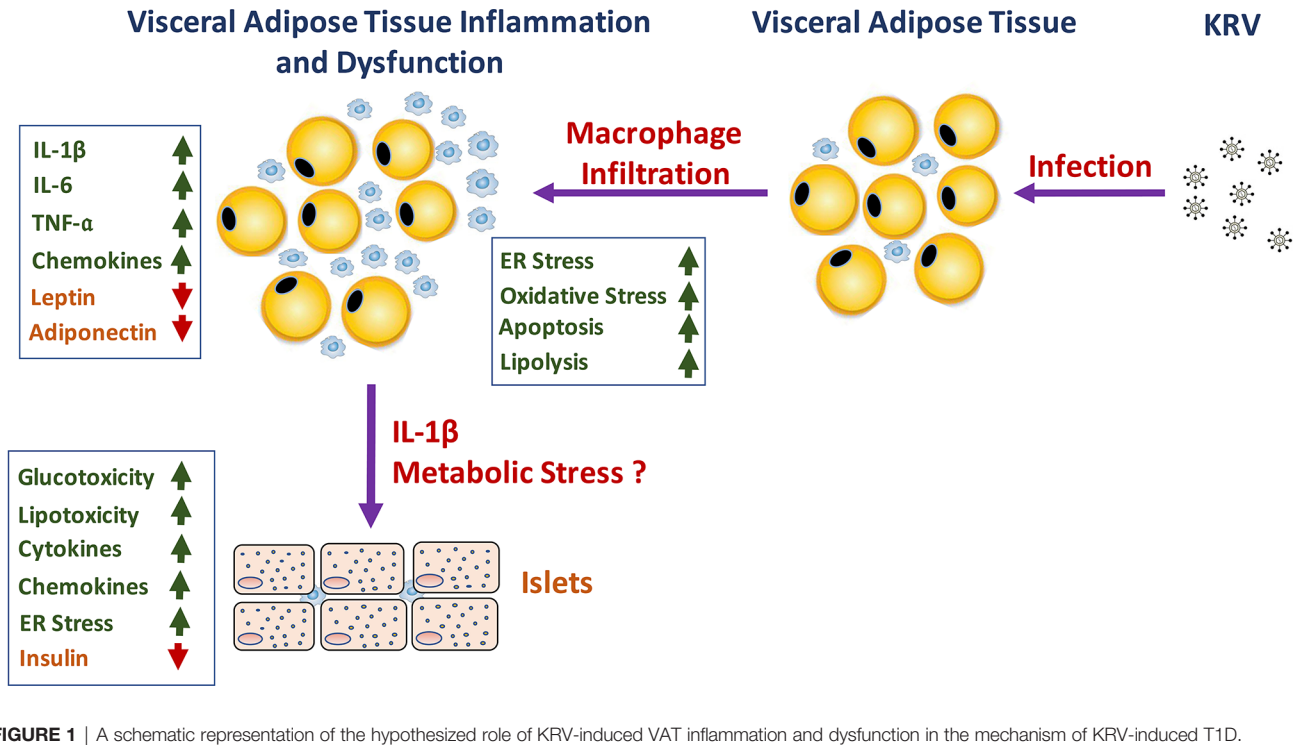

FIGURE 1 | A schematic representation of the hypothesized role of KRV-induced VAT inflammation and dysfunction in the mechanism of KRV-induced T1D.

expression of IL-1ß (136, 158). Consequently, IL-1ß released from ß-cells may trigger the recruitment and activation of innate immune cells, which may then release more IL-1ß. IL-1ß in the islet microenvironment can exacerbate ß-cell dysfunction, and trigger apoptosis in ß-cells (30, 36, 40, 136, 158). Finally, islet impairment and damage may ultimately signal innate and adaptive immunity to attack and destroy ß–cells leading to permanent hyperglycemia (159–162).

CONCLUSIONS AND FUTURE PERSPECTIVES

Earlier data demonstrated that the mechanism of KRV-induced T1D is associated with innate immune activation early in the disease course. We recently reported that infection with KRV results in VAT inflammation and dysfunction detected soon after infection. There are marked similarities between inflammation detected in VAT from infected LEW1.WR1 rats and inflammation detected in VAT from T2D patients. Whether as found in T2D, a cause-and-effect relationship exists between VAT inflammation and islet autoimmunity remains to be determined. As discussed in this Review, there is crosstalk between the innate immune system and glucose metabolism. We propose a paradigm by which virus-induced global innate immunity resulting in proinflammatory cytokine and chemokine upregulation and aberrant adipokine profile and lipolysis in VAT lead to metabolic stress and β–cells inflammation and destruction. Future studies will identify the interplay between the innate immune system and metabolic pathways and its role in triggering virus-induced disease. Identification of critical metabolic and immune pathways linked with β–cell autoimmunity will open new avenues for the development of targeted therapies for disease amelioration.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Transient Depletion of Foxp3$^+$ Regulatory T Cells Selectively Promotes Aggressive β Cell Autoimmunity in Genetically Susceptible DEREG Mice

*Deepika Watts[1,2,3], Marthe Janßen[1,2,3], Mangesh Jaykar[1], Francesco Palmucci[1,2,3], Marc Weigelt[4], Cathleen Petzold[1], Angela Hommel[4], Tim Sparwasser[5], Ezio Bonifacio[2,3,4] and Karsten Kretschmer[1,2,3]**

[1] Molecular and Cellular Immunology/Immune Regulation, Center for Regenerative Therapies Dresden (CRTD), Center for Molecular and Cellular Bioengineering (CMCB), Technische Universität Dresden, Dresden, Germany, [2] Paul Langerhans Institute Dresden (PLID) of the Helmholtz Zentrum München at the University Hospital and Medical Faculty Carl Gustav Carus of TU Dresden, Dresden, Germany, [3] German Center for Diabetes Research (DZD e.V.), Neuherberg, Germany, [4] Regenerative Therapies for Diabetes, Center for Regenerative Therapies Dresden (CRTD), Center for Molecular and Cellular Bioengineering (CMCB), Technische Universität Dresden, Dresden, Germany, [5] Institute of Infection Immunology, TWINCORE/Centre for Experimental and Clinical Infection Research, Hanover, Germany

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Anne Cooke,
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Helen Thomas,
University of Melbourne, Australia
Colin C. Anderson,
University of Alberta, Canada

***Correspondence:**
Karsten Kretschmer
karsten.kretschmer@tu-dresden.de

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Type 1 diabetes (T1D) represents a hallmark of the fatal multiorgan autoimmune syndrome affecting humans with abrogated Foxp3$^+$ regulatory T (Treg) cell function due to *Foxp3* gene mutations, but whether the loss of Foxp3$^+$ Treg cell activity is indeed sufficient to promote β cell autoimmunity requires further scrutiny. As opposed to human Treg cell deficiency, β cell autoimmunity has not been observed in non-autoimmune-prone mice with constitutive *Foxp3* deficiency or after diphtheria toxin receptor (DTR)-mediated ablation of Foxp3$^+$ Treg cells. In the spontaneous nonobese diabetic (NOD) mouse model of T1D, constitutive Foxp3 deficiency did not result in invasive insulitis and hyperglycemia, and previous studies on Foxp3$^+$ Treg cell ablation focused on Foxp3^{DTR} NOD mice, in which expression of a transgenic BDC2.5 T cell receptor (TCR) restricted the CD4$^+$ TCR repertoire to a single diabetogenic specificity. Here we revisited the effect of acute Foxp3$^+$ Treg cell ablation on β cell autoimmunity in NOD mice in the context of a polyclonal TCR repertoire. For this, we took advantage of the well-established DTR/GFP transgene of DEREG mice, which allows for specific ablation of Foxp3$^+$ Treg cells without promoting catastrophic autoimmune diseases. We show that the transient loss of Foxp3$^+$ Treg cells in prediabetic NOD.DEREG mice is sufficient to precipitate severe insulitis and persistent hyperglycemia within 5 days after DT administration. Importantly, DT-treated NOD.DEREG mice preserved many clinical features of spontaneous diabetes progression in the NOD model, including a prominent role of diabetogenic CD8$^+$ T cells in terminal β cell destruction. Despite the severity of destructive β cell autoimmunity, anti-CD3 mAb therapy of DT-treated mice interfered with the progression to overt diabetes, indicating that the novel NOD.DEREG model can be exploited for preclinical studies on T1D under

experimental conditions of synchronized, advanced β cell autoimmunity. Overall, our studies highlight the continuous requirement of Foxp3$^+$ Treg cell activity for the control of genetically pre-installed autoimmune diabetes.

Keywords: type 1 diabetes, immune regulation, Treg cells, Foxp3, cell ablation

INTRODUCTION

Type 1 diabetes (T1D) is a chronic disease under complex environmental, immunological and genetic control, which is manifested by the autoimmune destruction of functional insulin-producing β cells of pancreatic islets caused by islet-infiltrating diabetogenic CD4$^+$ and CD8$^+$ T cells (1, 2). The concept of Foxp3$^+$ regulatory T (Treg) cell-based therapies to interfere with β cell autoimmunity in T1D has been fueled in large part by evidence indicating that diminished Treg cell activity may contribute to disease pathogenesis. In fact, many of the T1D genetic susceptibility loci have been implicated in Foxp3$^+$ Treg cell function, either through indirect (e.g., *IL2*) or direct (e.g., *IL2R, CTLA4, PTPN22, IL10*) mechanisms (2). Likewise, T1D is considered a hallmark (3) of the fatal multiorgan autoimmune syndrome (IPEX; immune dysfunction, polyendocrinopathy, enteropathy, X-linked) affecting humans with abrogated Treg cell function due to genetic *FOXP3* gene mutations (4, 5), demonstrating that a severe Treg cell defect is sufficient to promote destructive β cell autoimmunity independently of other genetic and environmental factors. However, loss-of-function studies in non-autoimmune and diabetes-prone mice have not been able to demonstrate an unequivocal link between Foxp3$^+$ Treg cell deficiency and catastrophic β cell autoimmunity. In mice on a non-autoimmune genetic background, constitutive genetic *Foxp3* deficiency (6, 7) or acute Foxp3$^+$ Treg cell ablation based on Foxp3-driven expression of a human diphtheria toxin receptor (DTR) (8–10) recapitulates many clinical features of the human IPEX syndrome, but the manifestation of β cell autoimmunity has not been reported. Moreover, Foxp3-deficient *scurfy* mice on the diabetes-prone NOD background (NOD.Foxp3^{sf}) develop exocrine pancreatitis and peri-insulitis, but do not develop insulitis and overt diabetes, unless the CD4$^+$ T cell receptor (TCR) repertoire of NOD.Foxp3^{sf} mice is artificially restricted to a single highly diabetogenic specificity by transgenic expression of the BDC2.5 TCR (11). These observations could be interpreted as evidence that Foxp3$^+$ Treg cells are dispensable for the autoimmune β cell protection in the NOD model, but the interpretation of data in the constitutive absence of Treg cells is hampered by potentially confounding effects of immune adaptations and severe systemic autoimmunity, including premature death and alterations in thymic T cell development (12). The administration of mAbs directed against CD25 as 'surrogate' Treg cell marker largely preserves systemic immune homeostasis and has been employed to examine the role of Foxp3$^+$ Treg cells in the control of β cell autoimmunity. However, CD25 is not uniquely expressed on Foxp3$^+$ Treg cells, and anti-CD25 mAb treatment has been proposed to act on Treg cells by functional inactivation, rather than physical depletion (13–15), while sparing Foxp3$^+$ Treg cells with a CD25$^-$ phenotype. These limitations in specificity and efficiency of CD25-targeted interference with Foxp3$^+$ Treg cell activity may account for the largely contradictory range of data in the NOD model, including precipitation of overt diabetes (16–19), accelerated diabetes progression in young but not adult mice (20), as well as maintenance of β cell tolerance (21–23), or even delayed onset of diabetes (22).

Foxp3-driven DTR expression has been successfully employed for the specific and temporally controlled ablation of Foxp3$^+$ Treg cells (8–10, 24), while the outcome can considerably differ between independent Foxp3^{DTR} mouse lines, depending on the transgenic strategy of Foxp3-driven DTR/GFP expression. In 'knock-in' mice expressing a DTR/GFP fusion protein from an IRES down-stream of the endogenous *Foxp3* gene (Foxp3$^{IRES\text{-}DTR/GFP}$) on a non-autoimmune genetic background, DT-mediated Treg cell-ablation in young and adult Foxp3$^{IRES\text{-}DTR/GFP}$ mice resulted in an autoimmune disease similar to that observed in Foxp3-deficient mice (8). In the DEREG ('depletion of regulatory T cell') model, in which DTR/GFP is expressed from a transgenic Foxp3 bacterial artificial chromosome (Foxp3$^{BAC\text{-}DTR/GFP}$), administration of DT into newborns resulted in *scurfy*-like symptoms, while adults were found to be protected from autoimmune diseases, despite efficient GFP$^+$ Treg cell depletion (9). In fact, and as opposed to Foxp3$^{IRES\text{-}DTR/GFP}$ mice, the Foxp3$^{BAC\text{-}DTR/GFP}$ transgene of DEREG mice is not physically linked to the endogenous *Foxp3* gene, allowing for the accumulation of DT-resistant Foxp3$^+$GFP$^-$ Treg cells (25–28). These unique features prompted us to hypothesize that the DEREG model is particularly suited to study the role of Foxp3$^+$ Treg cells in organ-specific autoimmunity in mice on a genetically susceptible genetic background, while keeping collateral autoimmune damage to a minimum. In previous studies employing a NOD model, in which the spontaneous diabetes development was constrained by transgenic BDC2.5 TCR expression on all CD4$^+$ T cells (29), acute Foxp3$^+$ Treg cell ablation was shown to unleash a highly aggressive form of autoimmune diabetes (30), which fully abrogated the sex bias usually observed in spontaneous diabetes progression of NOD mice (31). Here, we report on the specific effects of acute Foxp3$^+$ Treg cell ablation on the physiologic disease course in autoimmune diabetes-prone DEREG mice in the context of a polyclonal TCR repertoire.

MATERIALS AND METHODS

Mice

NOD/ShiLtJ, NOD.Rag1$^{-/-}$, NOD.BDC2.5 mice (all Jackson Laboratories, Bar Harbor, USA), and DEREG mice (9) on different genetic backgrounds (C57BL/6, BALB/c, or NOD)

were housed and bred at the Animal Facility of the CRTD under specific pathogen-free conditions. NOD.DEREG mice were obtained by backcrossing C57BL/6.DEREG mice onto the NOD/ShiLtJ background for ≥13 generations. NOD.DEREG mice were intercrossed with NOD.BDC2.5 mice to obtain NOD.DEREG × BDC2.5 mice. All NOD mouse lines were fed with NIH #31M rodent diet (Altromin, Germany). Blood glucose levels were measured using whole blood from the tail vein and Accu-Chek® Aviva (Roche). If not stated otherwise, blood glucose levels were routinely determined once a week. Mice were considered diabetic at blood glucose levels above 200 mg/dl on at least two consecutive measurements or with blood glucose levels once above 400 mg/dl. All animal experiments were performed as approved by the Landesdirektion Dresden (24-9168.24-1/2014-1, DD 24-5131/338/38 (TVV37/2015).

DT-mediated Foxp3$^+$ Treg Cell Ablation and Adoptive Cell Transfers

Mice were *i.p.* injected with 0.5 µg DT (Merck Millipore - Calbiochem, Darmstadt, Germany) in 200 µl of sterile PBS on two consecutive days, if not stated otherwise. Where indicated, DT-treated NOD.DEREG mice were additionally *i.v.* injected with 5 µg anti-CD3e mAb (145-2C11). For total splenocyte transfers, single-cell suspensions were prepared from pooled spleens of 4-5-week-old (n = 4) and 16-18-week-old (n = 6) NOD.DEREG females, and 5 x 10^6 total cells were *i.v.* injected into NOD.Rag$^{-/-}$ recipient mice, followed by two consecutive injections of DT at day 7 and 8. For CD4$^+$BDC2.5$^+$ T cell transfers, conventional T (Tcon) cells (CD4$^+$CD62L^{hi}Vβ-4$^+$CD25$^-$GFP$^-$) and Foxp3$^+$ Treg cells (CD4$^+$Vβ-4$^+$CD25^{high}GFP$^+$) were FACS-isolated (99.3 – 99.8% purity) from pooled spleen and LN of NOD.DEREG × BDC2.5 mice. FACS-purified DTR$^-$ Treg cells (CD4$^+$Vβ-4$^+$CD25^{high}) from DEREG$^-$ NOD.BDC2.5 mice were included to control for DT toxicity. Diabetogenic Tcon cells (5×10^5) were *i.v.* injected into NOD.Rag$^{-/-}$ recipient mice, either alone or co-injected with DTR$^+$ or DTR$^-$ Treg cells (1×10^5), followed by DT injection on three consecutive days in week 6 after adoptive transfer.

Immunohistochemistry

Pancreatic cryosections (5µm) were fixed in 4% formalin and stained for C-peptide using polyclonal rabbit anti-C-peptide Ab (Cell Signaling, Germany), followed by Alexa Fluor 488-labeled polyclonal goat anti-rabbit IgG Ab (Invitrogen). Subsequently, detection of CD3 was carried out using rat anti-CD3 mAb (CD3-12) (AbD Serotec), followed by staining with Alexa Fluor 568-labeled polyclonal goat anti-rat secondary Ab (Invitrogen). Nuclei were visualized using 4′-6-Diamidino-2-phenylindole (DAPI). Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA), using standard protocols. All images were acquired with a Leica SP5 upright Laser Scanning confocal microscope. For evaluation of lymphocyte infiltration (insulitis), at least three sections were collected at 50 µm intervals and 6-12 pictures per pancreas were taken, using the following scale (32): 0, no infiltration; 1, minimal focal infiltration; 2, peri-islet infiltration (<50%); 3, intra-islet infiltration (>50%); 4, extensive infiltration (100%).

Flow Cytometry and Cell Sorting

Pancreatic islets were isolated by collagenase digestion (0.7 mg/ml) (Sigma-Aldrich Chemie GmbH) and discontinuous Ficoll density gradient. Single-cell suspensions of pancreatic islets and lymphoid tissues were prepared using 70 µm cell strainers (Becton Dickinson, San Diego, CA, USA) and Hank's buffer [1 x HBSS, 5% (v/v) FCS, 10mM HEPES; all Invitrogen]. Single cell suspensions from spleen were additionally subjected to red blood cell lysis (erythrocyte lysis buffer EL, Qiagen). Peripheral blood mononuclear cells (PBMCs) were obtained by retro-orbital sinus puncture [PBS supplemented with 10% (v/v) Heparin (Biochrom AG, Berlin, Germany)] and Ficoll (VWR, Darmstadt, Germany) gradient centrifugation. mAbs to CD3 (145-2C11), CD4 (RM4-5, GK1.5), CD8 (53–6.7), CD25 (PC61, 7D4), CD44 (IM7), CD62L (MEL-14), Vβ-4 (KT4), and CD49b (R1-2) were purchased from eBioscience (Frankfurt, Germany) or BD (Heidelberg). The samples were analyzed using a LSRII or sorted on a FACS Aria (all BD). Data were analyzed with the FlowJo software (Tree Star).

Immunophenotyping

For flow cytometry-based immunophenotyping, male and female cohorts of adult, age-matched NOD.DEREG mice were either left untreated or injected with DT on two consecutive days. Pancreatic islets, pancreatic lymph nodes (pLN), and a collection of other lymphoid tissues [subcutaneous LN (scLN), spleen, and thymus] were harvested before (day 0) or at different days after (day 1-7, day 10) administration of the first dose of DT (3-6 mice per timepoint). Single-cell suspensions were subjected to multicolor flow cytometry for the quantification of immune subsets (αβ T cells, NKT cells, and NK cells: mAbs directed against CD3, CD4, CD8, CD25, and CD49b; B cells, granulocytes, macrophages, and dendritic cells: mAbs directed against CD19, Gr1, CD11b, and CD11c).

Gene Expression Analysis

Freshly isolated pancreata were subjected to rapid freezing and grinding in liquid nitrogen, followed by total RNA extraction using Trizol® (Life Technologies), the RNeasy Mini Kit, and DNase I digestion (Qiagen, Hilden, Germany). Total RNA was extracted from pLN using the RNeasy Mini Kit, DNase I digestion. For real-time RT-PCR, cDNA was synthesized using Oligo-d(T) primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. The QuantiFast SYBR Green PCR kit (Qiagen) and a Mastercycler ep realplex thermal cycler (Eppendorf) was used to analyze cDNA in replicates. The following primers were used: β-Actin, 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3' and 5'- TAA AAC GCA GCT CAG TAA CAG TCC G-3'; GzmA, 5'-TTT CAT CCT GTA ATT GGA CTA A-3' and 5'-GCG ATC TCC ACA CTT CTC-3'; IFN-γ, 5'-GGC TGT TAC TGC CAC GGC ACA-3' and 5'-CAC CAT CCT TTT GCC AGT TCC TCC-3'; GITR, 5'-GAC GGT CAC TGC AGA CTT TG-3' and 5'-GCC ATG ACC AGG

AAG ATG AC-3'; NKG2D, 5'-ACG TTT CAG CCA GTA TTG TGC-3' and 5'-GGA AGC TTG GCT CTG GTT C-3'.

RESULTS

Generation of Autoimmune Diabetes-Prone DEREG Mice

The DEREG mouse line was originally developed on the non-autoimmune prone C57BL/6 background (9). Here, we introduced the Foxp3$^{BAC\text{-}DTR/GFP}$ transgene of DEREG mice into the autoimmune diabetes-susceptible NOD background by extensive backcrossing (see *Materials and Methods*). We preferred this strategy, rather than generating a novel Foxp3-DTR transgenic line directly on the NOD genetic background, because adult DEREG mice on a non-autoimmune prone genetic background have been shown to be resistant to autoimmune diseases or *scurfy*-like symptoms after DT treatment (9, 25, 26). Confirming the validity of our NOD.DEREG model, DT-untreated female mice spontaneously developed overt diabetes, which was accompanied by an early onset of insulitis at ≤ 4 weeks of age and progressive loss of insulin-producing β cells (see below), resulting in the manifestation of hyperglycemia from 12 weeks of age (**Supplementary Figure S1**). In line with previous reports on efficient Treg cell depletion in C57BL/6.DEREG mice (9), administration of two consecutive daily doses of DT into NOD.DEREG mice resulted in an > 98% depletion of CD4$^+$GFP$^+$ Treg cells in peripheral blood (day 3, **Figure 1A**), while total CD4$^+$ T cell proportions (mean ± SD: day 0: 58.3 ± 5.0, day 3: 53.3 ± 4.3, day 7: 52.6 ± 6.0) did not significantly change over time (Unpaired t-test, n = 8). We observed a similar efficiency of

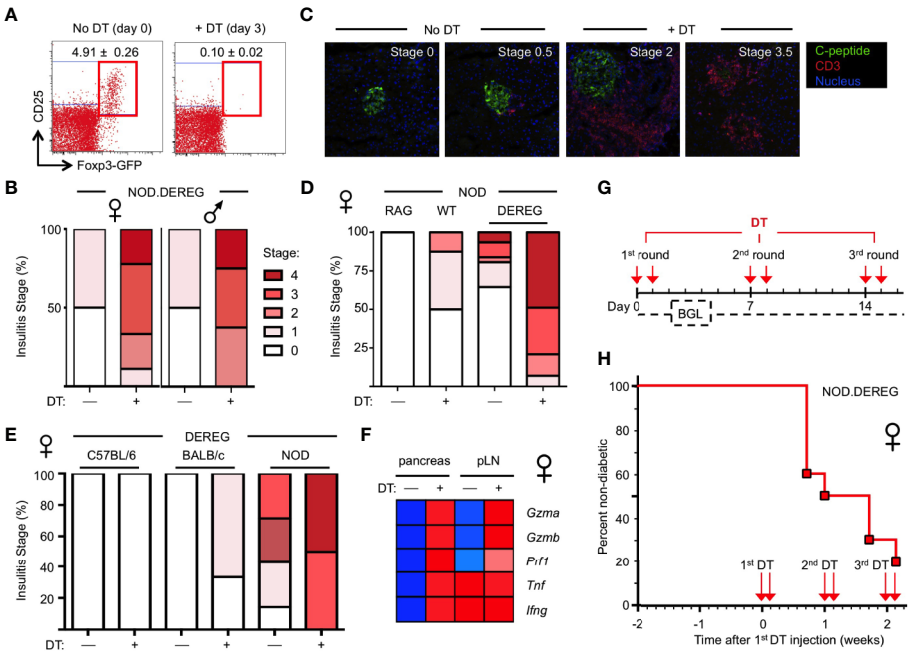

FIGURE 1 | Rapid progression to overt diabetes after acute Treg cell ablation in adult NOD.DEREG females. Mice were either left untreated or injected with DT on two consecutive days, and subjected to further analysis on day 14, unless otherwise stated. **(A)** Efficiency of Treg cell ablation. Representative dot plots of CD4-gated cells from peripheral blood of 16-week-old NOD.DEREG females before (day 0) and after (day 3) injection with DT. Numbers in dots plots indicate mean percentage ± SD of 8 mice (unpaired t-test, $p < 0.001$) from a single experiment representative of 6 experiments performed (5-9 mice per experiment). **(B–E)** Pancreatic pathohistological changes. Histological sections were scored as described in Materials and Methods. **(B)** Insulitis scores of 4-5-week-old female (left, n = 10) and male (right, n = 9) NOD.DEREG mice. As all mice remained normoglycemic by day 7 after the first DT injection, two additional doses of DT were administered (day 7 and 8) prior to histology on day 14. **(C)** Representative histology and **(D)** insulitis score of 8-9-week-old NOD.DEREG females (8-10 mice per group). Untreated NOD.RAG (n = 4) and DT-injected DEREG$^-$ NOD (n = 5) females were included as controls. **(E)** Insulitis scores of 16-week-old DEREG females on different genetic backgrounds (4-7 mice per group). **(F)** Expression of mRNA encoding immune effector molecules with known function in autoimmune β cell destruction. Freshly isolated mRNA from pancreas and pLN (pooled from 4-5 mice per group) of 10-12-week-old NOD.DEREG females was subjected to real-time RT-PCR using β-Actin for normalization. Heat map (row normalized) shows mean values of triplicate samples from a single experiment representative of at least two independent experiments performed. Blue and red represent lowest and highest gene expression values, respectively. Pancreas, Unpaired t-test: $p < 0.001$: *Gzma, Gzmb, Prf1, Tnf*; $p \leq 0.01$: *Ifng*. pLN, Unpaired t-test: $p < 0.001$: *Gzma, Gzmb*; $p \leq 0.01$: *Prf1*; not significant: *Tnf, Ifng*. **(G, H)** Manifestation of overt diabetes. **(G)** Cohorts of 16-week-old, initially non-diabetic NOD.DEREG mice were repeatedly injected with 2 doses of DT at 5-day-intervals, as indicated. Blood glucose levels (BGL) were determined at least three times per week. **(H)** Diabetes incidence of female NOD.DEREG mice. Mice were considered diabetic at blood glucose levels above 200 mg/dl on at least two consecutive measurements or with blood glucose levels once above 400 mg/dl. Data are from a single experiment (n = 10) representative of > 8 experiments performed. Note that the diabetes incidence of male NOD.DEREG mice is depicted in **Supplementary Figure S3**.

DT-mediated Treg cell depletion in both sexes of NOD.DEREG mice, and over a wide age range (4 weeks – 12 months).

Massive β Cell Loss and Hyperglycemia Within Days after Acute Treg Cell Ablation

In previous studies on a non-autoimmune-prone background, DT-mediated Treg cell depletion resulted in autoimmune diseases only when injected into newborn DEREG mice (9). Consistently, and despite multiple repeated injections of DT, Treg cell depletion in cohorts of young, 4–5-week-old NOD.DEREG mice caused worsening of initially mild insulitis in both sexes (**Figure 1B**), but the majority of mice (≥ 90%) remained normoglycemic and showed no *scurfy*-like symptoms (scaliness and crusting of eyelids/ears/tail, hepatomegaly, splenomegaly, enlarged lymph nodes, early death) (data not shown). In contrast, the pancreas of adult NOD.DEREG females showed strong pathohistological changes, when only two doses of DT were administered on consecutive days, and histological analysis of the pancreas was performed on day 12 (**Figures 1C–E**). This included massive $CD3^+$ T cell infiltrates throughout the islet space and complete islet disaggregation with only a few, if any, residual insulin-producing β cells in DT-treated NOD.DEREG mice (**Figure 1C**). In the control cohorts, DT-treated DTR^- littermates or sham-injected DTR^+ NOD mice exhibited no or only minimal peri-islet infiltration (**Figures 1C, D**). With regard to the susceptibility to multiple autoimmune diseases in the NOD background, histological analysis of adult, 9-12-week-old females of our NOD.DEREG colony revealed marked thyroid immune infiltrates, as expected (33), but the severity of autoimmune thyroiditis appeared similar between sham- and DT-injected mice, irrespective of whether they remained nondiabetic or progressed to hyperglycemia (**Supplementary Figure S2A**). We observed only very rare cases (<5%) of autoimmune neuropathy (34) among diabetic Treg cell-depleted mice, as indicated by the manifestation of hind limp paralysis with histological evidence for immune infiltration in the peripheral nerves (**Supplementary Figure S2B**).

Overall, the manifestation of destructive β cell autoimmunity was strictly dependent on the NOD genetic predisposition, as the pancreata of DT-treated DEREG females on the C57BL/6 or BALB/c background were devoid of immune infiltration and evidence for β cell death (**Figure 1E).** Notably, DT treatment was found to preserve the age-dependent differences in the severity of spontaneous insulitis that is found in DT-untreated NOD.DEREG females, when comparing different age groups (**Figure 1B**, 4-5 weeks; **Figure 1D**, 8-9 weeks; **Figure 1E**, 16 weeks). Thus, it appears that acute Treg cell ablation in NOD.DEREG mice exacerbates pre-established β cell autoimmunity, while preserving key features of spontaneous diabetes development in the NOD model (35, 36). Consistently, DT treatment of prediabetic NOD.DEREG adults increased mRNA expression of the Th1 cytokines TNF-α and IFN-γ selectively in the pancreas, while up-regulation of other autoimmune effector molecules with known functions in β cell destruction could be observed in both pLN and pancreas, such as Granzyme A, Granzyme B or Perforin (**Figure 1F**). The expression of mRNA encoding pro-inflammatory cytokines, such as innate-derived IL-1β or Th2 and Th17 signature cytokines (*e.g.*, IL-4, IL-13, IL-21, IL-22), remained below the detection limit in DT-treated NOD.DEREG mice, but were readily detectable in adolescent $Foxp3^{sf}$ mice (data not shown). Finally, in cohorts of adult NOD.DEREG mice, ≥ 50% of female (**Figures 1G, H**, *see also* **Figure 3E**) but only ≤ 10% of male (**Supplementary Figure S3**) mice progressed to overt diabetes within 5 days after the administration of two doses of DT. In these experiments, the administration of DT on two consecutive days was required to reproducibly promote overt diabetes at a high incidence. A single dose of DT or the repeated injection of three single doses of DT at 7-day-intervals into cohorts of adult (8-16-week-old) or aged (6-12-months-old) females only sporadically resulted in the induction of diabetes, with an incidence ranging from 0-10% (data not shown).

Mechanisms That Constrain Autoimmunity in DT-Treated NOD.DEREG Mice

In DT-treated NOD.DEREG mice, the manifestation of overt diabetes in the absence of other autoimmune symptoms suggests an intricate balance between diabetogenic and tolerogenic mechanisms, which constrain aggressive autoimmunity primarily to pancreatic β cells. Overall, the efficiency of DT-mediated Treg cell depletion was comparable between different lymphoid tissues and pancreas, reaching its maximum on day 3 after the first of two doses of DT at all anatomical sites (**Figure 2A**). However, the kinetics of Treg cell recovery differed between anatomical sites. Consistent with the thymus as a primary site of Treg cell *de novo* generation, $CD4^+GFP^+$ cells became first detectable in the thymus (day 4, **Figure 2A**), followed by the continuous replenishment of the $CD4^+GFP^+$ Treg cell compartments at peripheral sites, resulting in a recovery rate of 60-70% in spleen and scLN by day 10 (**Figure 2A**). In comparison, the kinetics of GFP^+ Treg cell recovery in pancreas and pLN was somewhat delayed, reaching ≤ 35% at day 10 (**Figure 2A**), which is likely to facilitate local autoimmune responses, while the rapid GFP^+ Treg cell recovery in secondary lymphoid tissues is providing a rather narrow time window for the manifestation of systemic autoimmunity.

Given that the endogenous *Foxp3* gene and the $Foxp3^{BAC\text{-}DTR/GFP}$ transgene are not physically linked, the activity of $Foxp3^+$ Treg cells with a DTR/GFP^- phenotype may represent another mechanism that limits catastrophic autoimmunity in DT-treated NOD.DEREG mice. While the expression of GFP and Foxp3 closely correlates in steady-state DEREG mice, DT administration can result in the proliferative expansion of an initially minute population of DT-resistant DTR/GFP^-Foxp3^+ Treg cells (37). In fact, 43.1 ± 3.5% (mean ± SD, n = 3) of pancreatic $CD4^+CD25^+GFP^-$ cells expressed Foxp3 protein at day 5 after DT administration, as compared to 9.1 ± 1.5% (mean and range of duplicate samples) in DT-untreated NOD.DEREG mice, as judged by the analysis of Foxp3 protein expression using anti-Foxp3 mAb (data not shown). Furthermore, the restoration of a pancreatic GFP^+ Treg cell compartment was preceded by the accumulation of such DTR/GFP^-CD25^+ T cells within 1-2 days after DT administration (**Figure 2B**). DT-resistant DTR/GFP^-CD25^+ T cells

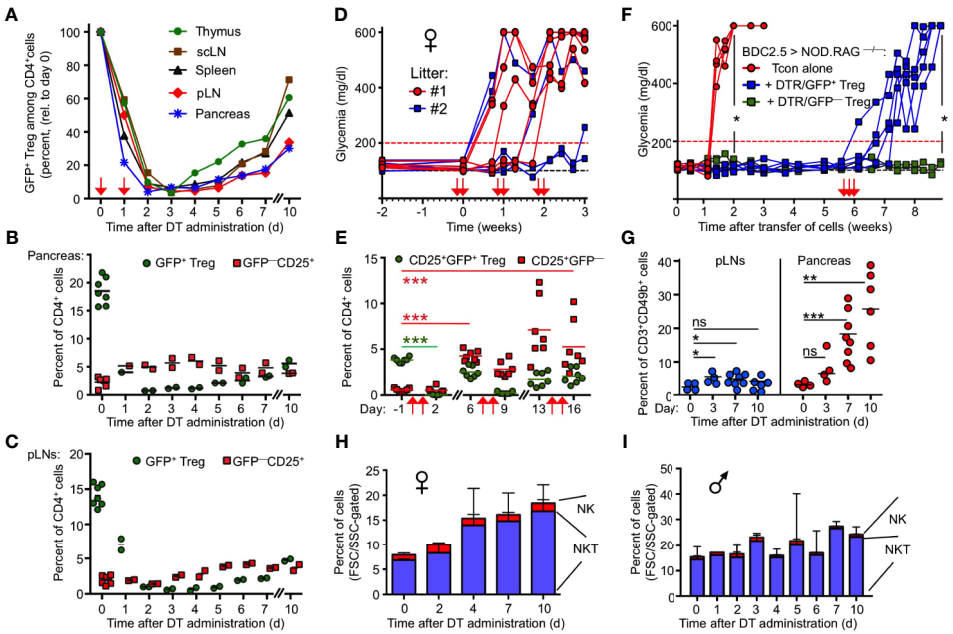

FIGURE 2 | Transient nature of Treg cell ablation in NOD.DEREG mice. **(A)** Kinetics of DT-mediated ablation and subsequent recovery of the CD4$^+$GFP$^+$ Treg cell compartment in pancreas and indicated lymphoid organs of 10-12-week-old NOD.DEREG mice. Data are from a single experiment (2 mice/timepoint) representative of 3 experiments performed. Arrows indicate days of DT injection. **(B, C)** GFP$^+$ Treg cell recovery in **(B)** pancreas and **(C)** pLN is preceded by the rapid accumulation of CD4$^+$CD25$^+$ cells lacking DTR/GFP expression (GFP$^-$). Each symbol corresponds to an individual mouse (10-12-week-old). **(D, E)** Impact of repeated DT administration on glycemic state and Treg cell depletion. **(D)** Blood glucose concentrations of individual NOD.DEREG mice presented in **Figure 1 (H)**. Arrows indicate days of DT injection. **(E)** Kinetics of CD25$^+$GFP$^+$ and CD25$^+$GFP$^-$ cells among CD4-gated cells, as revealed by flow cytometry among peripheral blood-derived CD4$^+$ T cells at different timepoints (Two-way ANOVA in combination with Bonferroni's Multiple Comparison post-test: ***p < 0.001). **(F)** Acute Treg cell ablation in the absence of Treg cell rebound. NOD.RAG1$^{-/-}$ mice were repopulated with diabetogenic BDC2.5 T conventional cells, either alone (red circles) or with GFP$^+$ (blue squares) or DTR/GFP$^-$ (green squares) Treg cells, and blood glucose levels of recipients were routinely assessed at least twice a week for up to 9 weeks. DT was injected at three consecutive days of week 6 (arrows). (Mann-Whitney-U test; two weeks: p = 0.0159, nine weeks: p = 0.0476). **(G–I)** Pancreatic NKT cell accumulation after Treg cell ablation. **(G)** Percentages of CD3$^+$CD49b$^+$ NKT cells among FSC/SSC-gated cells in pLNs (left) and pancreas (right) of 12-16-week-old NOD.DEREG females before (day 0) and at indicated days after DT administration. Symbols and horizontal lines indicate individual mice (4-8 mice per timepoint) and mean values, respectively. Unpaired t-test: ns, not significant; *p ≤ 0.05, **p ≤ 0.01, ***p < 0.001). **(H, I)** Kinetics of pancreas-infiltrating CD3$^+$CD49b$^+$ NKT and CD3$^-$CD49b$^+$ NK cells (FSC/SSC-gated) in **(H)** female and **(I)** male NOD.DEREG mice (10-12-week-old). Shown are mean percentages ± SD (day 0: n = 7) or mean percentages and range of replicate mice (days 1-7, day 10: n = 2).

also accumulated at other anatomical sites of DT-treated NOD.DEREG mice, although with delayed kinetics (**Figure 2C**). The rapid dynamics of Treg cell rebound was further illustrated by longitudinal studies concerned with the impact of prolonged DT administration (**Figures 1H**) on the progression to hyperglycemia (**Figures 2D**) and the GFP$^-$/GFP$^+$ Treg cell compartment size in peripheral blood (**Figure 2E**) of individual mice. Consistent with our data on pancreas (**Figure 2B**) and lymphoid tissues (**Figures 2A, C**), the population size of both GFP$^-$ and GFP$^+$ cells in blood markedly increased by day 6 after the first round of two DT doses (**Figure 2E**). The repeated injection of two doses of DT at 5-day intervals further enhanced the accumulation of DT-resistant CD25$^+$DTR/GFP$^-$ Treg cells, while the depletion efficiency of the CD25$^+$DTR/GFP$^+$ compartment appeared to decrease (**Figure 2E**).

Next, we aimed to assess the impact of DT-mediated Treg cell depletion on β cell autoimmunity mediated by defined numbers of diabetogenic T cells, in the absence of potentially confounding effects of 'rebounding' Treg cells. For this, immunodeficient NOD.Rag1$^{-/-}$ mice (no B and T/NKT cells) were reconstituted with 5 x 10^5 diabetogenic CD4$^+$BDC2.5$^+$ T cells, either alone or with small numbers of DTR/GFP$^+$ Treg cells (5 x 10^4 cells), followed by the injection of DT in week 6 (**Figures 2F**). In this experimental setting, the *de novo* generation of Foxp3$^+$ Treg cells is precluded by the complete block of thymic T cell development in NOD.Rag1$^{-/-}$ recipients, and DT-resistant DTR/GFP$^-$ Treg cells were excluded by FACS-based isolation of GFP$^+$ Treg cells prior to adoptive transfer. While co-transferred Treg cells efficiently interfered with the manifestation of overt diabetes, all recipients of DTR/GFP$^+$ Treg cells nearly synchronously developed severe hyperglycemia within 1 week after DT-mediated depletion (**Figure 2F**). DT-treated NOD.Rag1$^{-/-}$ recipients of diabetogenic CD4$^+$BDC2.5$^+$ T cells and DTR$^-$ Treg cells remained normoglycemic, excluding a major role of DT toxicity on β cell death (**Figure 2F**).

Overall, these data highlight the continuous requirement for Treg cell-mediated suppression in the control of destructive β cell autoimmunity, and suggest Treg cell rebound as plausible mechanism underlying the incomplete diabetes penetrance in

DT-treated NOD.DEREG mice (**Figure 1H**). Additionally, kinetics studies employing flow cytometric immunophenotyping (see Materials and Methods) indicated that $CD3^+CD49b^+$ NKT cells were initially less abundant in the pancreas of female NOD.DEREG mice, but their population size gradually increased after DT administration (**Figures 2G, H**), whereas the pancreatic NKT cell compartment in males remained largely constant (**Figure 2I**). Although their exact role in T1D has been controversially discussed (38–41), these data suggest that NKT cells may exert a tolerogenic function in Treg cell-depleted NOD.DEREG females.

Anti-CD3 mAb Therapy Following Treg Cell Depletion Interferes With Diabetes

With the exception of NKT cells (*see* **Figure 2G, H**), our flow cytometric immunophenotyping revealed no other quantitative changes of major immune cell subsets in the pancreas of NOD.DEREG mice (data not shown). This also holds true for $CD3^-CD49b^+$ NK cells (**Figure 2H**), which have previously been shown to undergo massive proliferative expansion (up to 5-fold) within 48 hours after DT-mediated Treg cell depletion in a $Foxp3^{BAC\text{-}DTR/GFP}$ NOD mouse line carrying the diabetogenic BDC2.5 TCR as additional transgene (30). Additionally, acute Treg cell ablation in adult NOD.DEREG mice had no impact on the population size of pancreatic $CD4^+$ and $CD8^+$ T effector cells (**Supplementary Figure S4**), which are thought to play a major role as physiologic mediators of β cell destruction in the NOD model (2, 35). However, DT administration increased the frequency of $CD8^+$ T cells with an effector/memory phenotype, in pancreas (**Figure 3A**) and pLN (**Figure 3B**), and to a lesser extend in scLN (**Figure 3C**), which could be attributed to an increase in the compartment size of $CD62L^{low}CD44^{high}$ effector/memory T cells at the expense of $CD62L^{high}CD44^{low}$ naïve T cells. We made similar observations for conventional $CD4^+$ T effector cells (**Supplementary Figure S5**), but anti-CD4 mAb (GK1.5) administration into cohorts of Treg cell-depleted NOD.DEREG mice (n = 10) did not appreciably interfere with the manifestation of overt diabetes, whereas anti-CD8α mAb (53.6.72) administration reduced the diabetes incidence to 10%, as compared to 50% in untreated and 60% in anti-CD4 mAb-treated mice (data not shown). One interpretation of these data is that $CD4^+$ T cells are dispensable at this stage of the disease,

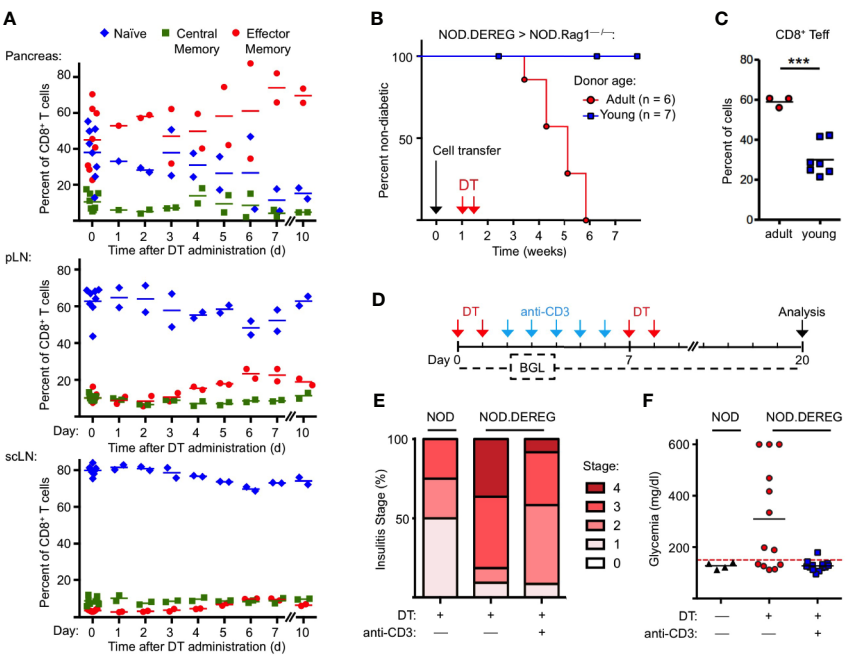

FIGURE 3 | Anti-CD3 mAb therapy after Treg cell ablation ameliorates insulitis and prevents progression to hyperglycemia. **(A)** Kinetics of $CD8^+$ T cells with a naïve ($CD62L^{high}CD44^{low}$), central memory ($CD62L^{int}CD44^{int}$), and effector/memory ($CD62L^{low}CD44^{high}$) phenotype in pancreas (top), pLN (middle), and scLN (bottom) of 10-12-week-old NOD.DEREG mice before (day 0) and at indicated days after DT administration. **(B, C)** Adoptive splenocyte transfer model. **(B)** Splenocytes from adult (16-18-week-old) but not young (4-5-week-old) DTR/GFP^+ NOD.DEREG donors promote overt diabetes in NOD.$RAG1^{-/-}$ recipient mice after DT treatment. Black arrow indicates the day of splenocyte transfer (2 x 10^6 total cells), and red arrows indicate the days of DT administration. (Log-Rank (Mantel-Cox) test, p = 0.0097). **(C)** Proportions of $CD8^+$ T cells with a $CD62L^{low}CD44^{high}$ effector/memory phenotype in the pLN of NOD.$RAG1^{-/-}$ recipients of splenocytes from young (red circles) or adult (blue squares) NOD.DEREG donors. Unpaired t-test: ***p < 0.001. **(D–F)** anti-CD3 mAb treatment of Treg cell-depleted NOD.DEREG mice. **(D)** Scheme of experimental design. Cohorts of adult NOD.DEREG females were injected with DT only (n = 13), or were additionally treated with anti-CD3 mAb (n = 13). Untreated DTR/GFP^- NOD.DEREG mice were included for comparison (n = 4). Arrows: red, DT injection; blue, anti-CD3 injection; black, analysis by histology and flow cytometry. Blood glucose concentrations were assessed before (day 0) and every second day after the first injection with DT until the end of the observation period on day 14. **(E)** Histological insulitis score of indicated experimental groups, and **(F)** blood glucose levels of individual mice (one-way ANOVA: p ≤ 0.01). Each symbol in **(A, C, F)** corresponds to an individual mouse.

while $CD8^+$ T cells represent the final effector cells. As the CD8α chain is not exclusive to αβ T cells, but also expressed on other immune cells (such as NKT or DCs), we next assessed the diabetogenic potential of NOD.DEREG splenocytes after injection into lymphopenic NOD.$Rag1^{-/-}$ mice (**Figure 3D**). In this adoptive transfer model, the manifestation of overt diabetes has been shown to be strictly $CD8^+$ T cell-depend, with numbers of diabetogenic $CD8^+$ T cells closely correlating with the kinetics of β cell destruction (42). Our results show that DT administration into NOD.$Rag1^{-/-}$ recipients of splenocytes from cohorts of adult NOD.DEREG females promotes overt diabetes in all recipients (**Figure 3B**), which correlated with an enrichment of effector/memory-type $CD8^+$ T cells in the pLN (**Figure 3C**) but not scLN (data not shown). In contrast, all NOD.$Rag1^{-/-}$ recipient mice of splenocytes from young NOD.DEREG donors maintained normoglycemia during the entire observation period of 8 weeks (**Figure 3B**). These data further indicate that $CD8^+$ T cells are key mediators of destructive β cell autoimmunity in the NOD.DEREG model, and provide a mechanistic basis for our observation that young, DT-treated NOD.DEREG mice are largely refractory to the manifestation of diabetes (**Figure 1B**).

We also assessed the impact of anti-CD3 mAb therapy on β cell autoimmunity in Treg cell-depleted NOD.DEREG mice (**Figure 3D**). In the NOD model, treatment with anti-CD3 mAb at recent diabetes onset has been shown to restore normoglycemia and long-term immune tolerance, but was ineffective in preventing destructive β cell autoimmunity, when injected at earlier stages of diabetes development (43, 44). Our results show that anti-CD3 mAb administration following DT administration ameliorated the strong pathohistological changes observed in the pancreas of Treg cell-depleted cohorts of adult NOD.DEREG females (**Figure 3E**) and interfered with the manifestation of hyperglycemia in all anti-CD3-treated mice (**Figure 3F**). Normoglycemia was also maintained when DT was repeatedly injected after discontinuation of anti-CD3 mAb treatment (**Figures 3D, F**), indicating that anti-CD3 mAb-mediated β cell protection is independent of repopulating GFP^+ Treg cells.

DISCUSSION

Here, we show that the specific ablation of $Foxp3^+$ Treg cells in DEREG mice can reproducibly precipitate severe insulitis and stable hyperglycemia in the context of a polyclonal TCR repertoire, provided that the autoimmune susceptibility is pre-installed by the NOD genetic background. One of the strengths of the NOD.DEREG model is that it preserves key aspects of the physiologic disease course in Treg cell-proficient NOD mice (*e.g.*, Th1 bias, role of $CD8^+$ T cells, female sex bias), while the transient nature of DT-mediated Treg cell depletion minimizes potentially confounding effects of systemic autoimmunity. The reappearance of $Foxp3^+$ Treg cells shortly after withdrawal of DT has also been observed in other $Foxp3^{DTR}$ lines (8, 45), including $Foxp3^{BAC\text{-}DTR/GFP}$ mice (9), but the apparent differences in the depletion efficiency and recovery kinetics between mouse lines indicate that the DEREG model is particularly suitable for studies on the Treg cell-mediated control of organ-specific autoimmune responses (26).

Overall, our findings indicate that the diabetes incidence in Treg cell-depleted NOD.DEREG mice is largely determined by the extent of preformed pancreatic lesions and numbers of diabetogenic $CD8^+$ T cells at the time of DT administration. This is in line with the observation that $Foxp3^{sf}$ mice (11) and 4-5-week-old, DT-treated NOD.DEREG mice (**Figure 1B**) are refractory to the manifestation of severe insulitis, despite a comparable efficiency in the DT-mediated depletion of the pancreatic Treg cell compartment in young and adult NOD.DEREG mice (data not shown). However, the severity of destructive β cell autoimmunity in DT-treated adult NOD.DEREG females appears rather unexpected, in particular in light of previous studies on $Foxp3^+$ Treg cell targeting using anti-CD25 mAb and the moderate spontaneous diabetes incidence that we observed in the present study in DT-untreated NOD.DEREG mice (**Supplementary Figure S1A**). Importantly, and consistent with strong pancreatic lesions (**Figures 1C–E**), many Treg cell-depleted NOD.DEREG mice nearly synchronously progressed to overt diabetes within 3 days after the administration of only 2 doses of DT (**Figure 2D**). In fact, this rapid kinetics was comparable to previous observations made in BDC2.5 TCR-transgenic DEREG (31)and $NOD^{BAC\text{-}DTR/GFP}$ mice (30). We also addressed the possibility that alleviating the metabolic stress of residual β cells by insulin replacement therapy for the duration of Treg cell recovery may help restoring metabolic homeostasis (*e.g.*, by promoting the regeneration of functional β cells) (46, 47). However, the administration of exogenous insulin into DT-treated NOD.DEREG mice shortly after diagnosis of overt diabetes restored normoglycemia for several weeks, but all mice returned to high blood glucose levels, once insulin therapy was discontinued (data not shown), further illustrating the destructiveness of β cell autoimmunity.

Our data suggest a scenario, in which pancreatic $Foxp3^+$ Treg cells in prediabetic NOD.DEREG mice interfere rather late in the cascade of events ultimately leading to the spontaneous progression of overt diabetes, highlighting the important role of continuous Treg cell activity in constraining terminal β cell destruction by pancreas-infiltrating $CD8^+$ T cells. This interpretation is further supported by our observation that, in contrast to anti-CD4 mAb, anti-CD8 and anti-CD3 mAb (44) administration into DT-treated NOD.DEREG mice interfered with the progression to overt diabetes (**Figure 3F**), despite substantial histopathological changes (**Figure 3E**). In this context, anti-CD3 mAb is of particular interest, as targeting CD3 is a promising approach currently being pursued for the therapy of human T1D (48, 49). Several non-mutually exclusive mechanisms underlying the action of anti-CD3 mAb therapy have been proposed, including the induction of recessive tolerance in pathogenic T effector cells (50), and of dominant tolerance by promoting $Foxp3^+$ Treg cell activity (51, 52). Notably, in the BDC2.5-transgenic NOD^{Rag} model of autoimmune diabetes, anti-CD3 treatment has been shown to induce massive proliferation of

an initially constrained population of BDC2.5$^+$Foxp3$^+$ Treg cells and long-term protection from diabetes development, which could be abrogated by subsequent DT-mediated Treg cell depletion (51). Here, we show that anti-CD3 mAb therapy in Treg cell-depleted NOD.DEREG mice potently interfered with diabetes development (**Figures 3E, F**), probably by mechanisms independent of Foxp3$^+$ Treg cells. One plausible explanation of these data is that anti-CD3 mAb can also exert its protective effect by acting on diabetogenic CD8$^+$ T cells (49, 53, 54). Clearly, further studies are warranted to more precisely determine the relative contribution of recessive and dominant tolerance mechanisms to the anti-CD3 mAb-mediated effects on β cell autoimmunity.

In summary, the NOD.DEREG line represents a novel tool to analyze the specific role of Foxp3$^+$ Treg cells in the control of β cell autoimmunity, resolving some of the previous limitations of NOD mice with constitutive Foxp3 deficiency or transgenic expression of a diabetogenic TCR. This includes mechanistic studies on novel Treg cell-based therapies under experimental conditions of synchronized, advanced β cell autoimmunity.

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 animal study was reviewed and approved by Landesdirektion Dresden, Germany.

AUTHOR CONTRIBUTIONS

DW designed, performed, and analyzed the experiments, and contributed to the data interpretation and assisted in manuscript preparation. MJan, MJay, FP, MW, CP, AH contributed to the acquisition, analysis, and interpretation of data. TS, EB, and KK conceived the research. KK guided its design, analysis and interpretation, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.720133/full#supplementary-material

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Natural Killer Cells as Key Mediators in Type I Diabetes Immunopathology

*Graeme Gardner and Christopher A. Fraker**

Tissue and Biomedical Engineering Laboratory, Leonard M. Miller School of Medicine, Diabetes Research Institute, University of Miami, Miami, FL, United States

The immunopathology of type I diabetes (T1D) presents a complicated case in part because of the multifactorial origin of this disease. Typically, T1D is thought to occur as a result of autoimmunity toward islets of Langerhans, resulting in the destruction of insulin-producing cells (β cells) and thus lifelong reliance on exogenous insulin. However, that explanation obscures much of the underlying mechanism, and the actual precipitating events along with the associated actors (latent viral infection, diverse immune cell types and their roles) are not completely understood. Notably, there is a malfunctioning in the regulation of cytotoxic CD8+ T cells that target endocrine cells through antigen-mediated attack. Further examination has revealed the likelihood of an imbalance in distinct subpopulations of tolerogenic and cytotoxic natural killer (NK) cells that may be the catalyst of adaptive immune system malfunction. The contributions of components outside the immune system, including environmental factors such as chronic viral infection also need more consideration, and much of the recent literature investigating the origins of this disease have focused on these factors. In this review, the details of the immunopathology of T1D regarding NK cell disfunction is discussed, along with how those mechanisms stand within the context of general autoimmune disorders. Finally, the rarer cases of latent autoimmune, COVID-19 (viral), and immune checkpoint inhibitor (ICI) induced diabetes are discussed as their exceptional pathology offers insight into the evolution of the disease as a whole.

Keywords: natural killer cells, type 1 diabetes, beta cell, immunopathology, autoimmune

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***Correspondence:**
Christopher A. Fraker
cfraker@med.miami.edu

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INTRODUCTION

Type 1 Diabetes (T1D) is a debilitating autoimmune disease that affects at least 1.6 million people in the US, accounting for ~5% of all diagnosed cases of diabetes, with an estimated 5 million people to be diagnosed by 2050 (1). Worldwide, of the ~463 million people living with diabetes, up to 10% have type 1 (2), representing an increasing incidence within an otherwise serious and increasing epidemic (3). It is well-established at this point that T1D results from autoimmunity, potentially involving both the innate and adaptive arms of the immune system. Indeed, most of the genes associated with greater risk in developing T1D point toward such an origin (4). However, the exact mechanism and the interplay between autoimmunity as well as the influence of environmental factors are still debated and investigated. The primary conundrum is that our understanding of these two components of the immune system is ever-evolving, and the conclusions made by

studying *in vitro* or *in vivo* models like the non-obese diabetic mouse (NOD) or streptozotocin (STZ)-induced animals do not necessarily correlate one-to-one to the pathophysiology in humans. In addition, certain hypotheses involving multifactorial origins of T1D are difficult to test experimentally and frequently rely on correlative or epidemiological data rather than a discrete causality. It is likely that there are multiple etiologies and therefore, perhaps no two cases of T1D are the same necessarily.

At its core, T1D is a chronic autoimmune disease caused by destruction of the insulin-producing islet β cells, therefore rendering patients with the requirement of lifetime exogenous insulin supplementation (5). Oftentimes, diagnosis occurs at an early age, with clinical features indicative of hyperglycemia, such as increased thirst and frequent urination. Decreased circulating c-peptide levels and presence of autoantibodies, even prior to clinical manifestation portends the underlying immune-mediated attack. Early studies on autoimmunity focused on identifying autoantibodies and characterizing the pathogenesis, whereby autoreactive CD8+ T cells are the primary active immune cell in β cell death (6, 7). Islet autoantibodies for glutamic acid decarboxylase (GAD65), islet antigen 2A and insulin suggests a role for B cells, but to a lesser extent than the CD4+ T helper cells (e.g. Th1 and 2) thought to provide the pro-inflammatory cytokine profile necessary for activation of cytotoxic CD8+ T cells. More recent work suggests a more complete picture with innate immunity involvement – either in a destructive or regulatory role. Natural Killer cells (NK's) are a bridge between the adaptive and innate arms of the immune system. They are capable of fighting pathogens or cancerous cells directly, and yet also generate memory cells and respond *via* antigen-mediated attack. They have long been associated with autoimmune diseases, and studies of their concentration, phenotype (frequency and function) and *in vitro* functionality in peripheral blood and tissue are numerous.

In this review, the immunoregulatory role of natural killer cells in the development of T1D will be presented, along with discussion of viral etiology, genetic risk, environmental factors, and even rare cases of T1D induced by cancer immunotherapy. The primary points of discussion will be the phenotypic character of pro-inflammatory and regulatory NK's, their interplay with viral mechanisms of T1D induction in human and animal studies, and some alternative hypotheses involving late onset autoimmune diabetes and gut microbiome health that interweave nicely with the immunoregulatory role for NK's. The central takeaway being the breakdown of self-tolerance that leads to T1D development is due ultimately to dysfunctional peripheral tolerance mechanisms associated with natural killer cells.

NATURAL KILLER CELLS AND DIABETES

Prior to the discussion of their role in the development of T1D, it would be prudent to briefly introduce the nature and function of natural killer cells (NKs), and to distinguish them from other immune cell subtypes that also play a critical role in the development of this disease, such as macrophages, T and B cells. Natural killer cells can be characterized as somewhat a hybrid between the innate or adaptive arms of the immune system. They mature from common lymphoid progenitor cells (CLP), recognize MHC Class I molecules, and exhibit targeted killing of virus-infected or transformed tumorigenic cells without prior sensitization *via* "missing self"-directed pathways (8, 9). They have a large cell body filled with cytolytic granules (perforin, granzyme B) similar to CD8+ effector T cells, but their activity is coordinated by a multitudinous array of both inhibitory and activating receptor-ligand interactions that can alter the NK cell status depending on levels of expression (9). It is conceivable that the evolution of NKs is a response to viral evasion of the adaptive immunity, thus giving rise to their innate phenotype with adaptive genotypic signature (10).

Distinct populations have been described for both mouse and human lineages, where they perform a Janus-type role of pleiotropic pro-inflammatory and regulatory functions (11, 12), somewhat analogous to the macrophage subtypes M1 and M2s (2a,b,c,d). They are the bone marrow-derived, thymus-independent third arm of the lymphocyte lineage that comprise 5-15% of peripheral blood mononuclear cells (PBMC's) and take up residency canonically in the spleen and liver with small tissue-resident populations elsewhere (e.g. skin, liver, uterus). There are also subpopulations of NK cells that are capable of secreting anti-inflammatory cytokines (e.g. IL-10, IL-13, IL-27, TGF-β, IL-23) (13). Conversely, the more conventional populations can perform antigen-mediated cell lysis and apoptosis in addition to rapidly producing large quantities of inflammatory or directly cytotoxic molecules [principally IFN-γ (14), also TNF, GM-CSF, IL-5, IL-13, IL-22, and macrophage inflammatory proteins (MIP)] (15–17). Therefore, their role in immune system homeostasis is critical. Phenotypically, they carry quite a large array of distinguishing biomarkers, but in a simplified form, they are CD45+/CD3-, CD56+ (dim or bright), and CD16+/- depending on maturity. Some important receptors they carry involved in innate activation include the killer cell lectin-like receptors NKG2D and KLRG1, and natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46, while on the other hand receptors like the CD94/NKG2A dimer and the killer cell immunoglobulin receptors (KIRs in humans, Ly49 in mice) are usually inhibitory (18, 19). What is particularly interesting in the context of adaptive immunity, is how NK cells interplay with the activity of CD8+ cytotoxic T cells and CD4+ helper T cells (20, 21). It has been asserted that NKs act as initiators, mediators, and a hybrid of both, for which other reviews are available (22). Needless to say, they have a well-documented ability to prevent and control the CD8+ effector cytotoxic T cell response implicated in autoimmunity (21). As a results, they are frequently hypothesized as having an outsized role in the development of several autoimmune conditions (13, 23, 24), as they are the first in line in terms of developing an inappropriate response to a "self"-antigen or lack of sufficient presentation.

Tissue-Resident Natural Killer Cells

The populations of NK cells resident within tissue (trNKs) (16) may possess more than just superficial phenotypic differences, perhaps even forming distinct lineages from NKs circulating

within peripheral blood (cNKs) (16, 25, 26). The NKs that reside in the liver and skin are distinct from those in the blood and thymus, and from those that are intimately involved in preventing maternal rejection of the fetus within the uterus during pregnancy [uterine or decidual NK cells (16, 27–29)]. The exact function of these tissue-resident immune cells is unclear, but from an observational point of view, it is more nuanced than just cytotoxic mediators in the early stages of viral infection or tumor development and they are recruited for reasons outside of localized inflammation. It seems that they play a role in tissue homeostasis, and dysfunction or imbalances here could lead to several disease states, including autoimmunity. Resident NKs have been found in the pancreas of both diabetes-prone and normal mice (30), and possess an activated phenotype distinct from cNKs. It was also observed that they accumulate in the pancreas long before T cells and illustrate an exhausted and hyporesponsive state during later stages of disease (30). A similar effect was confirmed in a model for the autoimmune disease myasthenia gravis (EAMG) where the NK cells degenerated during the progression of disease and were mediated through an IL-21-dependent pathway by autoreactive CD4+ T cells (31). These observations are important to keep in mind during the interpretation of results from human studies where NK populations are decreased or non-functional.

Natural Killer Cell Receptors and Their Ligands

The activity of natural killer cells is dictated by a balance between activating and inhibitory receptor-ligand interactions, some of which are immunoregulatory and therefore critical in the development of autoimmune disease. The NKG2D receptor is expressed by NKs among other immune cell subtypes in both human and mouse, binding to induced-self antigens of the MHC Class I polypeptide-related sequence (MIC) A/B which are overexpressed in infected (32) or otherwise transformed cells (e.g. tumorigenic) (33, 34). However, they have been reported to be constitutively expressed at low levels in many tissues including the pancreas (35). It is part of the greater NKG2 family of C-type lectin-like receptors. Unlike the CD94/NKG2A receptor dimer which also binds to MHC-I ligands (i.e. HLA-E), NKG2D is involved in activation/stimulation rather than inhibition and is costimulatory with CD8+ T cells (36). Effector status of NKs depends critically on the frequency and expression levels of this receptor (37, 38) and is therefore involved in regulating the activity of CD8+ T cells (36). The expression of NKG2D and, by extension, the activity of NK cells can be controlled by regulatory T cells (Treg) through a TGF-β mediated pathway (39), where Tregs are thought to down regulate its expression – leading to deleterious effects in the context of tumor surveillance but a pathway to understanding autoimmunity (40, 41). Although its ligands MIC A/B are normally expressed at sub-activating levels, NKG2D can accept a diverse array of ligands (42), one of which is retinoic acid early inducible 1 (RAE1, or ULBP in human), which is also constitutively expressed by pancreatic β cells and whose transcription is upregulated during viral infection in mice (25, 43, 44). This results in a precarious balance in the context of pancreatic trNKs, with both activating and inhibitory ligands being expressed constitutively. The activating NCR receptor NKp46 (NCR1 in mice) is considered especially important in the context of NKs and T1D since it is almost exclusively expressed by nearly all NKs (43, 45). Its function is also critical in terms of effective immunity toward viral infection, as noted by lethal influenza infection in NCR1 knockout mice (46). However, its ligands are yet to be fully characterized (47) and cross-reactivity toward molecular mimics is possible (45). Pancreatic β cells are thought to express from early development a yet unknown ligand for this receptor (48). This exposes these cells to potential NK attack if immunoregulatory/inhibitory receptors or ligands are insufficiently expressed. Regarding inhibitory receptors, the dimeric CD94/NKG2A serves an important role since it recognizes "self" antigens in the MHC-I family, including the non-classical HLA-E molecule. The expression of HLA-E is regulated by a complex set of processes but can be reduced or masked by some viral infections, which will be discussed more below. A related molecule in the non-classical MHC-I family is HLA-G, a ligand involved with immune protection/tolerance from NKs in the fetal trophoblast and anterior eye cell layers. It happens to be expressed by pancreatic β cells, which is hypothesized to be tied in with their insulin secretory activity as exocytosis exposes the extracellular space to myriad potential autoantigens (49). Its associated gene locus has naturally low-level polymorphism, suggesting small mutations could easily lead to a breakdown of immune tolerance, and there is some evidence from genetic studies correlating that region of the genome toward T1D susceptibility (50). Another set of inhibitory receptors in the killer cell lectin-like receptor subfamily (KLRs) include KLRG1 and KLRB1 (aka CD161) which are considered markers for activation (51) and senescent (52) phenotypes, respectively, but may play a role in regulating both cytolytic NK and T cell activity, potentiated by expression levels of its ligand lectin-like transcript 1 (LLT1) (53). The relationship between these receptors and T1D will be discussed at various points throughout the review. The importance of their role in disease etiology is a frequent point of contention, but regardless, they are ubiquitous throughout the literature.

NK Observations in Humans

Early reports on NK cells from the peripheral blood mononuclear cells (PBMCs) of T1D patients showed a significant decrease in their proportion when compared to healthy individuals (54), which in one case was proposed as a possible explanation for higher occurrence of neoplastic tumors (54). Also among these early studies were reports from Negishi et al. that showed significantly decreased direct cytotoxicity *versus* relevant control samples against the K562 cell line with simultaneous increase in directed islet toxicity (55, 56). Some authors hypothesized aberrant NKG2D signaling in addition to decreased NK cell number as the primary driver for T1D development (57). However, the NKs present in peripheral blood only tell part of the story, as sequestered cNKs or potentially trNKs that are localized to the pancreas – where the important events unfold – may account for that deficit. Also, the functionality of these NKs from primarily long-standing patients may not be relevant to

recent onset patients early in disease progression, as they are likely entering a 'hyporesponsive' phenotype (25, 31). The only consistent trend between NK populations and T1D in human patients is that the population in the peripheral blood is typically lower when compared to age/sex matched controls. In an analysis of the immune cell infiltrates of post-mortem pancreas samples of T1D patients, the most abundant cell type was CD8+ T cells, with very little NK detection (7). However, when an analysis of the tissue-resident immune cells of the pancreas of non-diabetic donors was performed (58), the majority of cells were also CD8+ T cells expressing markers for resident memory cells (CD69 and CD103). Here, NK's represented only ~3% of the lymphocyte infiltrates. Given the similarity in distribution during healthy and diseased patients, it seems that what is being captured during this post-mortem examination may not be representative of critical phases in disease progression. That is where longitudinal studies such as those being carried out by the JDRF network of Pancreatic Organ Donors (nPOD) will be more revealing in terms of the evolution of immunophenotype at various time points along disease progression (59, 60). Other studies have stressed the importance of the natural cytotoxicity receptor (NCR) NKp46 expression on NK cells of diabetic patients (43, 61) which will be discussed from a mechanistic standpoint in animal models more below. In a study of isolated primary human islets, the presence of a ligand specific for the activating receptor was implicated in the NK cell mediated destruction of β cells, *in vitro*. It was found that the binding site on the receptor specific for its β cell ligand also binds viral and tumor associated proteins (48, 62). A takeaway lesson from these human studies is that the timing and nature of the sampling process is important when interpreting the results, as the peripheral blood cells of long-standing T1D patients may not provide the most accurate snapshot of the initial immune system alterations and dysfunction.

Animal Model and Mechanistic Studies

Early studies on animal models yielded mostly conflicting results, albeit with some support of observed NK depletion (4, 63). For instance, a paper published in 1991 reported lower incidence of diabetes in a streptozotocin (STZ) mouse model when an NK specific antibody was administered before the first does of STZ, *versus* saline and non-specific Ig controls (64). However, just as early from Ellerman et al., it was demonstrated that in the BB/Wor rat model of diabetes, knocking down the population of peripheral NK cells with a 3.2.3 monoclonal antibody (mAb) did not prevent or delay diabetes onset, even though their critical role was hypothesized (63). Recent animal model work has demonstrated that after infection of rat insulin promoter RIP-GP mice with LCMV, induction of diabetes resulting from T cell activation (LCMV-gp) was regulated by NK cell levels and expression (20). Counterintuitively, the pancreatic tissue destruction was much worse in mice that were injected with low dosage virus (103 plaque-forming units) when compared to high dosage virus (65). The observed effect correlated with much greater NK cell activation and lower levels of tissue antigen-specific CD8+ T cells when high dosage of virus was used. When taken from high dose blood samples, those NK cells were directly cytotoxic toward autoreactive CD8+ T cells *in vitro*. The exact mechanism appears to be dependent on the expression of the NCR1 (NKp46) receptor in these NK cells, which was upregulated only in the case of high dosage, whereas expression of the receptor NKG2D was upregulated comparably in both viral dosages. A study in NCR1 knockout mice infected with LCMV confirms the observed mechanism of CD8+ T cell regulation (21). Strangely, this is in contradiction to previous observations in which NCR1 (NKp46) deficient mice were observed to have reduced T1D development (43), and where treatment of NOD mice with anti-NKG2D antibody prior to disease onset halted progression altogether (66, 67). Simultaneously, NKG2D ligands seem to be upregulated on target cells of diabetic model organisms (57). What role the NKp46 and NKG2D receptors play in T1D animal models is therefore a matter of contention, but may be resolved by considering the expression levels, the location of their respective ligands, the strength of the inhibitory signaling, and finally the longitudinal time of analysis, since NK effector status is always dictated by this balance. In the mice given high viral doses, the upregulation of NKp46 may have reflected its role in NK attack of CD8+ T cells with concomitant halting of disease progression. When β cell destruction is mediated through an antigen-specific process (aka after viral infection, discussed below), it follows that NKs targeting those T cells would inhibit that process. If there is an alternative pathway for the development of T1D, potentially *via* innate autoimmunity, the converse might be true, as in a case of NK activation *via* NKG2D/NKp46 ligand expression on β cells. The NKp46 receptor itself can be probed directly for its role in T1D development. Two separate studies from Mandelboim et al. showed that NCR1/NKp46 recognizes ligands expressed on mouse and human pancreatic β cells that specifically induce NK degranulation and subsequent cytotoxicity (43). Treatment of NOD mice *via* direct injection of a monoclonal antibody raised against the murine NCR1 receptor down-regulated its surface expression (68). This in turn led to a lower overall incidence in T1D development compared to appropriate controls, also observed in NCR1 knockout mice treated with STZ to induce diabetes (43). These animal models – while important for studying potential mechanisms – may lead to specious conclusions if the results from human studies are disregarded. Nonetheless, they still demonstrate the important role for NK activating receptors as well as their respective ligand interactions in both inflammatory and regulatory processes.

Natural Killer T Cells

Not to be confused with natural killer cells, invariant natural killer T cells (iNKT) may also play a role in the autoimmune regulation and development of T1D (69). iNKT's are tissue-resident innate-like immune cells whose defining quality is the expression of an invariant T cell receptor α-chain, and their recognition of CD1b. CD1b is an antigen-presenting molecule (MHC-I class-like) associated with dendritic cells (and some other APC's) that displays lipid and glycolipid antigens of invading microbial pathogens. Although they do express cell surface markers of NK, such as CD161 (aka KLRB1) in humans, the expression of T cell receptors puts them distinctly into the latter class of immune cells descendent from the common lymphoid progenitor. They are

primarily involved in defense against invading pathogens, tumor growth, and metastasis, but also play a regulatory role and can quickly release large amounts of cytokines like IL-4 and IFN-γ. Several studies using NOD mice have confirmed their effect on reducing the likelihood of diabetes development, which has been reviewed elsewhere (69). Suffice it to say, a similar effect as described above in the LCMV treated mice was also attributed to activation of NKT cells where they indirectly mediate CD8+ cytotoxic T cells *via* induction of TGF-β-producing Tregs (70). However, contradictory results in the number and type of NKT's in human studies, in part due to very low number (~0.1%) in the peripheral blood and variable frequency in the general population makes it difficult to form definitive conclusions about their role in disease. This redundancy in the immune system reflects the hypothetical ease by which an autoimmune reaction could become problematic.

GENETIC RISK FACTORS AND AUTOIMMUNITY

Although an auto-immune disorder of multifactorial origin, T1D does have associated genetic risk markers, suggesting a possible inherited risk. The observations of imbalance in population and aberrant behavior of NK cells in T1D patients certainly suggests a possible causal relationship in terms of islet cell destruction but this does not elucidate the related immune system malfunction, or, as in the case of viral infection, β cell susceptibility. Therefore, the associated genetic polymorphisms may be useful in identifying a link. Out of the >60 genes or loci that have been linked to a greater risk of developing T1D, the strongest correlations have been found with the HLA genes, specifically the class II alleles (71–74). This family of alleles is intimately involved in antigen presentation and recognition, a pathway involving APCs, B cells, and CD4+ T helper cells. Although adaptive immune response is important to the ultimate progression of disease, and abnormalities in the presentation of antigenic peptides by HLA molecules clearly may affect outcome, these correlations are not very useful in identifying the genetic role in the early precipitating events. This further supports the potentially larger role of environmental factors like viral infection relative to genetic predisposition towards a breakdown of central tolerance. It is likely that both are necessary for disease development with an environmental trigger that is amplified by genetic predispositions that manifest in defective immune response phenotypes. A fact supported by the tepid genetic linkage between T1D and other autoimmune disorders that are non-endocrine in origin (75). It has also been hypothesized that there exists a correlation between another allele, MHC Class I chain-related A (MICA), and risk for T1D, which similarly is involved in cell-cell communication and is a ligand for the activating receptor NKG2D. However, when a meta-analysis of ~5,000 patients with and without T1D was performed, variants of the MHC Class I chain-related A (MICA) were not found to be significantly correlated to T1D occurrence (76). Finally, the insulin molecule itself has been implicated in genetic predisposition (77), with some evidence to suggest CD8+ reactivity toward a pre-proinsulin epitope (78), which would fit in well with a disease progression that culminates with a primed adaptive immune system but still not explaining instigating events. In many of these studies, it is difficult to provide associative risk with absolute certainty due to the complexity in both the techniques used, and their accompanying analysis. However, emerging evidence in studies that look deeper than simple genetic mutation have revealed that even single nucleotide polymorphisms (SNPs) can alter how immunoregulatory genes are expressed (79), meaning the underlying genetic associations and/or susceptibilities have a complicated role in defining risk. Finding a concrete genetic link may be obscured underlying epigenetic factors that influence disease development. The role of microRNAs (or miRNA) in autoimmune disease in general has seen a tremendous surge in research effort (80, 81), and there is reason to suspect involvement in the development of type 1 diabetes (82). MicroRNA's are involved in post-transcriptional regulation, in most cases silencing translation of target mRNAs, which in the context of autoimmune disease and T1D could mean a multitude of potential regulatory checkpoints. In addition, the discovery of circulating miRNAs associated with T1D could lead to their use as biomarkers for early detection or to identify at-risk individuals (83). Among the profile of miRNAs identified in exosomes isolated from human blood samples in one study, seven were differentially expressed in patients with diabetes (84).

VIRAL-MEDIATED TYPE 1 DIABETES

Viral infections are hypothesized to be involved in myriad autoimmune diseases (85, 86). As alluded to above, viruses play a critical role in the onset or potential for acquiring T1D reflected by their prevalence in animal model studies (87, 88). Their use in eliciting the understanding of disease progression with regard to NK cells is invaluable, as the two are inexorably linked (28, 89). The etiology suggests that enteroviruses (90, 91) (e.g. CV-B4) or those belonging to the Herpes family (92, 93) are the most likely contributors in humans, as many recent-onset patients show enteroviral nucleic acid or other viral biomarkers like viral capsid protein and IgM indicative of recent infection. Conversely, the prevalence of viral biomarkers in control populations of healthy individuals is significantly lower (88, 90, 94, 95). Due to improved detection and sampling methods, only recently has a definitive link been established (96, 97). However, it is not completely clear whether the presence of virus indicates causality or is a result of diminished ability to fight off viral infection due to reasons like suppressed/altered NK levels or dysfunctional adaptive immunity. The role for virally-mediated development of T1D has been reviewed in great detail elsewhere (89, 98–101), and therefore only the relevant material will be discussed here.

Viral-Mediated β Cell Destruction

The general mechanism by which viral infection can lead to autoimmune disease is assumed to be the following: (1) infection

localized to some target organ first activates an innate immune response (e.g. macrophages, NKs) (2) those cells become cytotoxic toward the autologous infected cells causing tissue damage beyond what is sufficient to clear infection (3) subsequent antigen release/processing recruits an already primed adaptive immunity in a runaway inflammatory cascade leading to lasting or permanent damage of the tissue/organ. Given the right mix of genetic risk and environmental factors, the process can easily lead to an autoimmune disease state. Viruses have evolved countless ways to outsmart adaptive immunity designed to seek them out *via* modulation of the expression of MHC class I peptide complexes (102, 103), which underlines how important NK cell function is. NKs are the principal defenders against viral invaders, secreting copious IFN-γ and inducing cytotoxicity in infected cells without the need for a priming phase *via* the "missing-self" mechanism. This leads to one hypothesis being that defective NKs result in viral-induced T1D development, and that process can go one of two ways. In the "pro-inflammatory" defective state, NKs are far too aggressive in viral clearance and T cell recruitment. In an "immunosuppressive" defective state, NKs do not respond appropriately to viral infection, allowing for chronic or persistent infection and/or β cell destruction by uncontrolled cytotoxic adaptive T cells. As evidence for the pro-inflammatory defect, one study showed that type I interferon (IFN-1) transcriptional signatures are associated with an increased activated innate immune response in patients pre-disposed to developing T1D, and confirmed after a longitudinal study that those with the strongest signature went on to develop the disease (104). One of the genes identified with increased T1D risk, IFIH1, encodes the MDA5 receptor that recognizes viral RNA and induces IFN-1 signaling. Reduction of that receptor by >50% (using a IFIH1 knockout) on an NOD mouse model protected them from T1D development without diminished ability to clear virus (105). Arguments for the overly immunosuppressive side have been put forth as well. In their normal regulatory capacity, NKs secrete IL-10, which has been observed to play a role in immunosuppression during systemic infection but less so local infection (106). An infection localized to the pancreas would be unlikely to induce such expression, but it has been hypothesized that infected islet cells can secrete IL-10 to avoid extensive T cell recruitment (90). Perhaps in the context of β cell infection and subsequent insulitis, NK cells are not appropriately activated and do not secrete sufficient IFN-γ to recruit effector CD8+ T cells to efficiently clear virus, which has been demonstrated in a recent study of RIP-GP mice at low levels of viral infection (20). It would seem then that counterintuitively, NK cells in T1D development are defective on two fronts – simultaneously attacking β cells and producing pro-inflammatory cytokines that lead to T cell recruitment, while unable to clear infection which allows for persistent and destructive insulitis. Another hypothesis which has been proffered could better explain this etiology, centered on the reasoning that viral modulation of the immune response causes defective NK-signaling. For example, β cells could be particularly susceptible to specific viruses leading to pervasive infection and improper clearance (65), or chronic infection and immune-evasive tactics of some viruses may ultimately lead to destruction, as might be expected for viruses undergoing lysogenic-lytic cycles. One interesting hypothesis that has emerged is the role of reactivated human endogenous retrovirus (HERV), whereby environmental or inflammatory stimulus (e.g. other viral infection) allows for activation of HERV transcription and gene expression that could once again either cause direct damage to islet cells, or induces an autoreactive immune response by affecting activating or inhibitory receptor-ligand interactions (107). A hypothesis that might aid in the understanding of this viral-mediated process is the following (**Figure 1**). In a normal response, sentinel pancreatic NK cells take on a regulatory phenotype after the initial phases of innate activation leading to effector status toward CD8+ T cells, thereby preventing β cell destruction and T1D. However, in a dysfunctional response, one of two things (or combined effect) occurs. Either (1) NKs become exhausted/hyporesponsive, diminish in activity and number, and allow for what is typically understood as the major mechanism for β cell destruction, aka CD8+ T cell autoreactivity, (2) Viral pathogens hijack mechanisms for immune modulation (like over-expression of HLA-G and modulation of HLA-E) thereby turning NK cells into a suppressive force that allows the adaptive response to go unchecked. The regulatory or immunosuppressive capacity of NKs has been demonstrated in both systemic (106) and local (20, 21) infection, and it stands to reason that dysregulation at this junction could be a deciding factor in T1D development, perhaps reconciling the observations of impaired T regulatory ability as well (108).

Sars-CoV-2 and Diabetes

Considering the recent pandemic, it would be appropriate to examine the recent cases of T1D following COVID-19 infection. Diabetes, especially type II, has been established as an associated increased risk factor for developing severe disease, but does the SARS-CoV-2 virus itself present as a possible cause of diabetes? There have now been more than merely isolated cases of hyperglycemia, lasting β cell damage and other severe metabolic complications in COVID-19 patients, in some cases remitting after a few weeks, but in others developing into lasting disease (109, 110). The virus enters the cell *via* the angiotensin-converting enzyme 2 (ACE2), a receptor expressed on multiple cell types, including endocrine cells of the pancreas, making SARS-CoV-2 a plausible case for COVID-19 induced diabetes. Indeed, it is documented that coronaviruses can cause multi-organ damage by entering through these receptors (111), and *in vitro* pancreatic-like organoids derived from induced pluripotent stem cells are susceptible to viral entry *via* a spike-protein mediated attack (112). Curiously, in comparison to the other cell types generated, the pancreatic organoids were much more permissive to viral entry. In an analysis of post-mortem COVID-19 patient samples and ex vivo islets, the presence of SARS-CoV-2 protein colocalized with the NKX6.1 β cell marker was confirmed. Interestingly, infection elicited an interferon transcriptional signature reminiscent of that which proceeds T1D (104, 113). Studies on the links between the latest

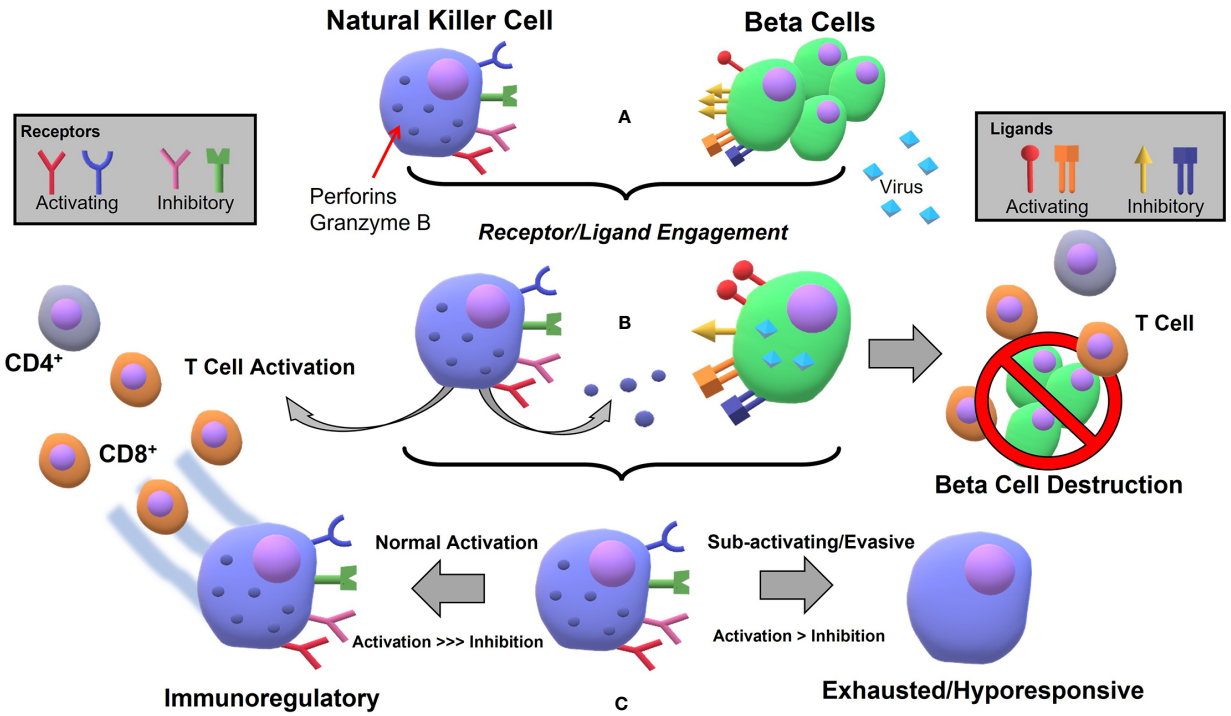

FIGURE 1 | Hypothetical process for NK mediated β cell destruction and subsequent autoimmune T cell reaction following viral infection in pancreatic tissue: **(A)** Natural Killer cells express several activating (NKp46, NKG2D, some KIRs) and several inhibitory (CD94/NKG2A, KLRG1, KLRB1, some KIRs) receptors for ligands which can be expressed at varying levels on β cells during normal stasis. Ligands include the inhibitory set of MHC-I molecules (e.g. HLA-E) and the activating inducible MIC A/B molecules, constitutively expressed RAE-1/ULBP, and the unknown ligand for NKp46 **(B)** Viral infection leads to NK activation, cytokine release, whereby adaptive immunity is recruited and NKs degranulate, killing infected cells **(C)** If the viral infection is at low-level, persistent, or the virus is able to use evasive or immunosuppressive tactics, the NKs will not react appropriately and the immunoregulatory feedback does not occur, leading to exhausted or hyporesponsive state and β cell destruction by autoimmunity.

coronavirus and new onset diabetes are nascent and ongoing, and it remains to be seen if and how it fits in with the analysis presented here.

UNIQUE CASES

LADA

Latent autoimmune diabetes in adults (LADA) is characterized by a pathological state that is clinically defined as having characteristics of both Type 1, with the presence of autoantibodies (GAD), and Type 2 diabetes with typically – though not always – later onset (114, 115). In simple terms, age, and insulin dependence at the time of diagnosis are considered critical factors. However, LADA can be viewed as a milder or slower moving case of T1D since autoantibodies and β cell reactive T cells are still present and exogenous insulin supplementation is usually required. Therefore, the disease allows for careful longitudinal study of the progression of autoimmune diabetes. As with recent onset T1D patients, individuals with recently diagnosed LADA exhibit a decrease of NKs in the peripheral blood when compared to healthy individuals (116). In one case of recently diagnosed LADA patients, however, it was reported that NK frequency increased, especially of activated NKp46+ cells (61). The expression of the activating receptor NKG2D and inhibitory receptor KIR3DL1 was increased and decreased in these patients, respectively, and a reduced frequency of CD4+CD25+ T regulatory cells was observed (116, 117). Notably, a lower proportion of APC's and higher number of regulatory B cells (IL-35+) was observed in LADA patients when compared to healthy control and T1D patients (118). These combined observations lead to another important inference about the phenotype of those with this form of the disease. The immune cells and their receptors that are ultimately responsible for activating/regulating the β cell destructive CD8+ cytotoxic T cells are decreased/increased, respectively in LADA compared to T1D. However, they are still increased/decreased compared to healthy controls, representing an intermediate immunophenotype. Whether this observation is a result of disease pathology or is a causal agent has not been elucidated. Still the correlation supports the notion that the cytotoxic CD8+ T cell "finishes the job" after recruitment to the target organ *via* NK-mediated pathways. It has been hypothesized that the CD4+CD25+ regulatory T cells regulate NK cells' NKG2D expression *via* a TGF-β-dependent pathway. A disruption of said pathway may lead to the upregulation of this activating receptor (40, 41). If this inhibitory signaling is outpaced, a clear imbalance

results. In developing cases of latent diabetes, therefore, a treatment to prevent total islet destruction may be possible. For instance, the monoclonal antibody drug Monalizumab, which targets the inhibitory natural killer cell receptor NKG2A, is currently under clinical investigation for use in the treatment of some cancers and autoimmune conditions like RA (119). An analog targeting the NKG2D receptor may be useful in terms of preventing the full transition to insulin dependent type 1 diabetes when administered early in disease progression. In a clinical trial evaluating hematopoietic stem cell transplantation to treat T1D, patients that required lower exogenous insulin saw increased TGF-β and IL-10 immunoregulatory and decreased IFN-γ, IL-2 inflammatory cytokines (120).

Immune Checkpoint Inhibitor Diabetes

ICI chemotherapy (immunotherapy) is a recently approved cancer treatment (121), but there are non-phenomenological case reports and clinical reviews that definitively demonstrate immune-related adverse events (IRAEs) leading to the development of diabetes or at the very least diabetic ketoacidosis (122–125). Although the cause of diabetes or other autoimmune side effects in these cases does not coincide with the paradigm of normal pathogenesis of the disease, it is worthwhile to briefly examine how the two are related, especially within the context of participating immune cells. In the case of programmed death-1 (PD-1) inhibitors, the prevailing therapy associated with these outcomes (124), their mechanism of is to bind to the transmembrane protein located on the surface of activated T cells in order to block the "hand-shake" interaction with its associated ligands, PDL-1/2. This interaction limits autoimmunity during inflammatory responses. As a result, activated T cells can directly target the proliferating tumor cells, and to the detriment of a very small number of patients (~1%) act upon the β cells of the pancreas leading to diabetes development. It is possible the susceptibility is genetic and related to altered or lowered PD-1 expression that is also observed in T1D patients (126–128). However, the fact that these patients who developed diabetes only after immunotherapy treatment were of relatively advanced age, and in many cases had disease reversal upon cessation of treatment suggests that the PD-1 related susceptibility is not sufficient in and of itself for developing disease. Once again, a role for regulatory immune cells, like the NKs alluded to above, may prevent this effect from being more common among ICI patients and treatments utilizing transformed NKs are becoming more acceptable (129).

Gut Microbiome

Although the implications of the health of gut microbiota sometimes stretch further than what is empirically proven, it is obvious that there is a potential role for the microbiome in autoimmunity and even the development of T1D. Several reviews poring over the mechanistic and genomic details that underpin the relationship between the two are available (130–132). For the sake of brevity, we highlight a few important studies that complement the pathology described above. Some early evidence exemplifying the role of environment and microbiome in animal model studies was that the incidence of NOD mice developing diabetes is drastically increased when raised in completely "germ-free" environmental conditions (133, 134). In a separate study, NOD mice given an intraperitoneal administration of a bacterial extract containing a cocktail of bacteria that cause respiratory tract infections either prevented or delayed the onset of disease (135). The effect was neutralized by administration of anti-TGF-β antibody, suggesting a role for and potential increase in concentration of this regulatory cytokine after extract administration. It was suggested that the pathway would involve NKT cells, but Cd1d-/NOD mice did not show much difference in their response. As mentioned above and in ref (40), it is thought that TGF-β mediates the expression of NKG2D on natural killer cells, naturally modulating their innate immune activity toward potentially infected or transformed cells. This suggests the extract may supplement natural TGF-β production needed to suppress NKG2D receptor activation, attenuating NKs that may otherwise target β cells. Further study should target NK deficient animal models instead to pin down the culprit immune cell(s).

DISCUSSION

The development of autoimmune diabetes is generally thought to progress as follows. A susceptible person has at most minor abnormalities in the number and phenotype of immune cells such as NKs as a result of genetic and/or environmental factors (e.g. microbiome activity, endogenous virus, epigenetic regulation). An external stimulus – most likely viral infection – is key in precipitating a peripheral immune reaction that leads to formation of autoreactive T cells and antibodies that ultimately leads to the destruction of the functional pancreatic islet cells and necessitation of insulin dependence. NKs are involved at an early stage, where external stimulus takes place and peripheral tolerance breaks down. The receptors as well as their ligands that are involved in NK activation are both aberrantly activated, and β cell attack becomes inevitable. It should be emphasized that this represents the collapse of a very fragile balance, where the combination of several small factors exponentially precipitates into a catastrophic event. The existence and rarity of late-onset autoimmune diabetes exemplifies this fact, as avoiding the confluence of these small events late into adulthood is highly unlikely.

IMPLICATIONS FOR TREATMENT AND CONCLUDING REMARKS

In many of the studies discussed above, the natural assumption can be made that only preemptive surveillance and hypervigilance would make it possible to prevent the development of T1D. After the initial signs of β cell loss, it seems that there is little that can be done to reverse its course outside of auto/allo-transplantation of functional tissue under the blanket of systemic immunosuppression. Unfortunately, that limits the clinical reach of T1D treatment to patients with severe hypoglycemic unawareness. Refinements in donor islet and stem-cell derived tissue implantation have come a long way and increased the available tissue source. Additionally, there is a concerted effort to eliminate systemic immunosuppression with

efforts targeting localized delivery of immune modulatory agents coupled with immune evasion through encapsulation and/or genetic manipulation. While currently in their infancy, immune cell therapies could one day play a role as well, still requiring further study before clinical applications could be explored. Through a detailed study and understanding of the progression to T1D onset, it may be possible to develop prevention strategies without undue burden of painstaking surveillance. Routine genetic screens are now commonplace for many hereditary diseases and adding another T1D-specific panel would not be prohibitively costly. Also, with emerging scientific consensus on the importance of a healthy gut microbiome as an environmental factor, strategies to improve gut health would be easy to implement.

Although it is still a subject of ongoing investigation, the defining picture of T1D autoimmunity is becoming clearer, albeit perhaps more complex than originally thought. Conflicting results that arise from a limited pool of samples, sample selection, stage of disease, etc, and inappropriate *in vitro* or *in vivo* models have confounded progress. However, recent research efforts to expand sample availability and collaborative efforts, such as the JDRF-nPOD, have accelerated discovery on many fronts. As it stands today, it is becomingly increasingly obvious that the progression of T1D occurs because of improper activation and dysregulation of the immune system starting with natural killer cells and viral infection. In this review, we focused on the topic from the standpoint that the primary breakdown occurs at peripheral immune tolerance, as brought out by a dysfunctional set of primarily pro-inflammatory natural killer cells that precipitates the adaptive response and autoimmunity characterized by the disease. The reason for this breakdown is hypothesized to be a combination of the overexpression of activating receptors/ligands ascertained from genetic risk factors, lack of immunosuppressive support from the microenvironment, a likely viral triggering event. For the next steps, a method by which to recognize the early signs of this action and slow or halt its progression will be an ideal treatment to put an end to this pandemic.

AUTHOR CONTRIBUTIONS

CF and GG contributed to the conception of the manuscript. GG wrote the original draft. GG, and CF contributed to the manuscript revision and editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Regulatory B Cells: Role in Type 1 Diabetes

Joanne Boldison[1]* *and F. Susan Wong*[2]

[1] Institute of Biomedical & Clinical Science, University of Exeter, Exeter, United Kingdom, [2] Division of Infection & Immunity, School of Medicine, Cardiff University, Cardiff, United Kingdom

Regulatory B cells (Bregs) have an anti-inflammatory role and can suppress autoimmunity, by employing both cytokine secretion and cell-contact mediated mechanisms. Numerous Breg subsets have been described and have overlapping phenotypes in terms of their immune expression markers or cytokine production. A hallmark feature of Bregs is the secretion of IL-10, although IL-35 and TGFβ–producing B cells have also been identified. To date, few reports have identified an impaired frequency or function of Bregs in individuals with type 1 diabetes; thus our understanding of the role played by these Breg subsets in the pathogenesis of this condition is limited. In this review we will focus on how regulatory B cells are altered in the development of type 1 diabetes, highlighting both frequency and function and discuss both human and animal studies.

Keywords: IL-10, B cell, type 1 diabetes, frequency, function

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***Correspondence:**
Joanne Boldison
J.Boldison@Exeter.ac.uk

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INTRODUCTION

It is now well-established that regulatory B cells (Bregs) can dampen immune responses and play a role in maintaining immune tolerance. These immunosuppressive Bregs are generally named for the anti-inflammatory cytokines that they produce to exert their regulatory effects, and so a variety of Bregs have been identified. The cytokine most widely associated with Bregs is Interleukin-(IL-)10 (1) and thus has been the major focus of many studies into the failure of Bregs to suppress inflammation in autoimmune conditions. IL-10 independent mechanisms have been identified, including suppression mediated by contact of cell surface molecules (2, 3) or other soluble mediators such as the production of TGFβ (4) and IL-35 (5). However, currently there are no reports of alterations in these IL-10 independent regulatory B cell populations, either in number or function, in human type 1 diabetes; thus their contribution to type 1 diabetes remains an outstanding question.

In type 1 diabetes B cells are typically understood to play a pathogenic role in disease, likely through the production of inflammatory cytokines and presentation of autoantigens to T cells (6). This has been emphasized by the use of Rituximab in clinical trials and the observed temporary delay in the loss of C-peptide (7). However, studies of other autoimmune diseases have highlighted the essential role for regulatory B cells (8) and this has now been reflected in type 1 diabetes, although comparatively with fewer studies. Regulatory B cells in other autoimmune diseases, including diabetes, has recently been reviewed (9). It is imperative that we further understand the balance between effector and regulatory B cells in order to improve immunotherapeutic treatments targeting these lymphocytes, including utilizing Bregs as a therapeutic option. This review will focus on the emerging literature on Bregs and discuss their role in type 1 diabetes.

REGULATORY B CELL PHENOTYPES

Studies in both human and mouse have contributed to identifying numerous IL-10-producing Breg subsets using a variety of immune markers, some of which overlap, to indicate a regulatory population. In humans, several Breg subsets enriched at different stages of B cell maturation, including immature B cells ($CD24^{hi}CD38^{hi}$) (10), memory B cells ($CD24^{hi}CD27^{+}$ [B10]) (11) and plasmablasts ($CD27^{int}CD38^{+}$) (12) have been identified. Similarly, in mice, various subsets have been identified in the transitional (13) and marginal zone (14) B cell compartments, including specific mouse subsets that parallel human B10 cells (11, 15) and human plasmablasts (12).

Other human regulatory B cell subsets have also been described including $CD19^{+}Tim\text{-}1^{+}$ B cells (16) and $CD39^{+}CD73^{+}$ Bregs (17), with equivalent subsets described in mice (18, 19). In addition, human $CD25^{hi}CD71^{hi}$ B cells produce IgG4 and are designated as regulatory Br1 cells (20). However, these subsets have not yet been described in human type 1 diabetes. The diversity and identification of Breg phenotypes has been reviewed extensively (9, 21, 22). The range and variability in methods which induce IL-10-producing B cells, along with a lack of a key definitive marker, makes it difficult to define a Breg cell without assessing IL-10 production, as a key function. Therefore, the evaluation of IL-10-production during the differentiation and developmental stages of B cells is important, as demonstrated by Iwata et al. reporting the distinction between B10 cells and B10-progenitor cells ($B10_{PRO}$) (11). The different subsets of Bregs that have been assessed, specifically in studies of type 1 diabetes, is discussed (in *Impaired Regulatory B Cell Mechanisms in type 1 diabetes*) and **Table 1**.

BREG INDUCTION AND TYPE 1 DIABETES

The heterogeneity of Bregs, both in phenotype and response to stimuli, and the absence of a definitive single marker (so far) has led to the hypothesis that any B cell can differentiate into a Breg depending on their prevailing environment, rather than a subset derived from a distinct lineage (21). Indeed, signals required for the induction or the promotion of regulatory B cells are the result

TABLE 1 | Evidence for numerical defects in Bregs in type 1 diabetes.

Study	Phenotype of B cell	Change in cell frequency (*vs.* healthy donors)	Stimulus for IL-10 induction	Diabetes duration (years)	Age of donors with diabetes (years)	Age of healthy donors (years)
De Filippo., et al. (23)	$CD5^{+}CD19^{+}$	Increase (median 250 *vs.* 95 [cells mm^3])*	NM	<30days diagnosis	Mean ±SD: 6.7±2.5	Age-matched
Deng., et al. (24)	$CD19^{+}CD5^{+}CD1d^{hi}$ (B10 cells)	Decrease (Median, values not described, [B10% of $CD19^{+}$]***	NM	Mean ±SD: 3.1 ± 3.5	Mean ±SD: 28.53 ±16.21	Mean ±SD: 41.37 ± 13.52
Habib., et al. (25)	$CD19^{+}CD27^{-}$ $CD10^{+}CD24^{hi}CD38^{hi}$	Increase (Mean, values not described, [%transitional/$CD19^{+}$]*	NM	Not reported	Range: 19-36	Range: 19-46
Hanley., et al. (26)	$CD24^{hi}CD38^{hi}$	Decrease (Mean ±SD: 1.54± 0.85 *vs.* 2.67 ±1.15 [% of $CD19^{+}$]**	NM	Mean ±SD: 19.25 ± 10.99	Mean ±SD: 34.75 ± 13.13	Mean ±SD: 31.75 ±8.17
Thompson., et al. (27)	$CD19^{+}CD27^{-}$ $CD24^{hi}CD38^{hi}$ (transitional)	No difference (P=0.50)	NM	Range: 0.2-31. Median: 1.8	Range: 9-42. Median: 20	Range:18-37. Median: 27
	$IL\text{-}10^{+}$ B cells	No difference (P=0.74)	Anti-CD40 + IL-21 (3 days) + CpG + LPS (last 5hrs)			
Kleffel., et al. (28)	$CD19^{+}IL\text{-}10^{+}$ B cells	Decreased (Mean ±SEM, values not described, [IL-10%]**	CD40L + LPS (4 days)	Mean ±SEM: 35 ±2.4	Mean ±SEM: 53.2 ± 2.3	Mean ±SEM: 32.1 ± 2.2
Saxena., et al. (29)	$CD5^{+}IL\text{-}10^{+}$ B cells	No difference (P=0.31)	PMA/Ionomycin	Range: 1.5-31.5	Range: 18-49.2	Range: 19.2-46
Wang., et al. (30)	$CD24^{hi}CD38^{hi}$	Decreased (Mean ±SEM, 5.6 ± 3.5 *vs.* 6.9 ± 3.3 [%])*	NM	Mean ±SEM 5.38± 0.72	23.76± 5.89§ (Range 7-29)	24.91± 2.92§ (Range 20-30)
	$CD24^{hi}CD38^{hi}IL\text{-}10^{+}$	Decreased (Mean ±SEM, values not described, [IL-10%])***	CD40L + CpG (3 days)			
El-Mokhtar., et al. (31)	$CD24^{hi}CD38^{hi}IL\text{-}10^{+}$ $CD24^{+}CD27^{+}IL\text{-}10^{+}$	Decreased (% $CD24^{hi}CD38^{hi}IL\text{-}10^{+}$, Mean ±SEM, 0.48 ± 0.54 *vs.* 1.3 ± 0.57)*** (% $CD24^{+}CD27^{+}IL\text{-}10^{+}$, Mean ±SEM, 0.49 ± 0.57 *vs.* 1.3 ± 0.53)***	PMA/Ionomycin	Range 0.1-4.85, Median 1.6	Range 3.4-11, Median 7	Range 2.6-8.5, Median 7

All studies measured IL-10 production by intracytoplasmic staining. NM (not measured). Versus and compared to healthy donors. All studies performed in human peripheral blood. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. §*Average age, SEM or SD not stated.*

of an activated inflammatory environment, including pro-inflammatory cytokines, engagement of Toll-like receptors (TLRs) and costimulatory signals (32, 33). This has been reviewed extensively (34). Certainly, evidence from mouse studies show that Bregs are induced in response to inflammation or autoimmunity (13, 35). Moreover, a number of cytokines are involved in promoting Breg responses, many of which have been associated with autoimmune disorders. In autoimmune diabetes a number of cytokines including IL-1β, IL-6 and Interferon (IFN)α, play a role in the development of disease and can contribute to pancreatic β cell death (36). The same cytokines, as well as IL-21, have been shown to activate or expand Breg function (33, 37). IFNα secreted from plasmacytoid DCs (pDCs), in combination with CD40 ligation, can induce IL-10-producing Bregs (37). B cells stimulated with cytosine-phosphate-guanine (CpG) dinucleotides in combination with IL-2, IL-6 and IFNα induced an enhanced IL-10 response (12). Furthermore, IL-1β and IL-6 can drive B cell IL-10-production and Breg differentiation (33). Interestingly, this raises the question of why then in some studies Bregs are numerically or functionally defective in autoimmunity that includes type 1 diabetes (see *Impaired Regulatory B Cell Mechanisms in type 1 diabetes*). One possible reason for this paradox could be explained by other mechanisms required for Breg induction, which are altered in autoimmunity (**Figure 1**).

In a human study of SLE, the failed Breg expansion is attributed to elevated levels of IFNα produced from pDCs during disease, which drives plasmablast differentiation rather than Breg expansion (37). Therefore, it is suggested the concentration levels of cytokine are an important factor in Breg induction, and chronic exposure during inflammation can impair Breg frequency and function (37, 38). Type 1 diabetes, like SLE, is associated with an IFN signature. IFNα expression detected in the pancreatic islets (39) and IFN-associated genes are overexpressed in islets of individuals with type 1 diabetes (40). Additionally, an IFN transcriptional signature has been shown to be increased, even before the onset of human islet autoimmunity (41).

Both IL-21 and CD40 receptor engagement are required for the maturation and function of IL-10-producing B cells, a key

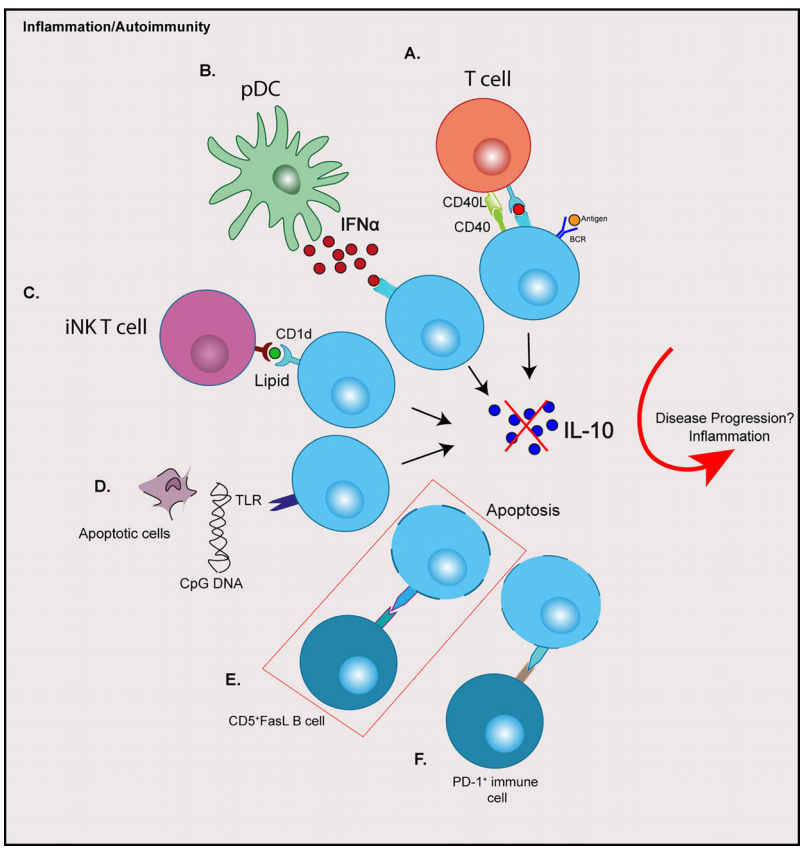

FIGURE 1 | Possible contributions of immune cell crosstalk resulting in dysregulation of regulatory B cells in type 1 diabetes. **(A)** Aberrant CD40:CD40L signalling through T cells **(B)** Elevated IFNα production from pDCs **(C)** Altered iNK T cells and CD1d expression on B cells **(D)** TLR signalling from apoptotic cell debris or the presence of viruses or microbes **(E)** Increased expression of Fas on IL-10^{+} B cells are targeted by CD5^{+}FasL B cells **(F)** PD-L1: PD-1 engagement resulting in increased Breg apoptosis. Red box depicts a possible mechanism reported in type 1 diabetes. CPG, cytosine-phosphate-guanine; BCR, B cell receptor; IFN, Interferon; iNK, invariant natural killer; pDCs, plasmacytoid dendritic cells; TLR, toll-like receptor; FasL, Fas-ligand; PD-L1, programmed death-ligand 1; PD-1, programmed cell death protein 1.

study demonstrated in mice (42). Interestingly, naïve B cell responses to IL-21 are diminished in established human type 1 diabetes; however this response is enhanced in pre-diabetic individuals with multiple islet autoantibodies (43). Furthermore, CD4 T follicular helper (Tfh) cells in patients with type 1 diabetes have increased IL-21 production, compared to healthy donors (44, 45).

The importance of CD40: CD40L signaling has been noted in autoimmunity. For example, in autoimmune diabetes, the influence of CD40L blockade on the development of diabetes has been demonstrated in the NOD mouse model (46). This fundamental signaling pathway is important in both T and B cells. In people with type 1 diabetes, $CD4^{lo}CD40^{+}$ T cells (T_{CD40}) are expanded in peripheral blood (47). In another autoimmune disease, SLE, aberrant expression of CD40L in circulating B cells, in addition to T cells has been noted (48). Furthermore, reduced numbers of $CD40^{+}$ B cells is observed in individuals with type 1 diabetes, compared to healthy donors; however, the levels of CD40 expression on B cells were not measured in this study (28).

Other mechanisms are necessary for the generation or expansion of Bregs and these include both adaptive and innate immune pathways. B cell receptor (BCR) signaling (32, 49), is diminished in B cells from individuals with established type 1 diabetes (25, 43). Signaling through TLR9 changes the frequency and function of IL-10 producing B cells in NOD mice; TLR9 deficiency specifically in B cells increased IL-10 producing cells and protected against diabetes (50). No direct study has demonstrated a mechanism that drives a Breg defect in type 1 diabetes in humans however, and this remains an outstanding question (see *Discussion and Outstanding Questions*).

IMPAIRED REGULATORY B CELL MECHANISMS IN TYPE 1 DIABETES

Studies on the numerical and functional defects of Bregs have been described in various autoimmune diseases, including SLE, RA and MS and overall an inverse correlation between the frequency of Bregs and disease activity has been observed (10, 51). It should be noted, however, that studies have also reported either no differences or an increased frequency in these cells between autoimmune individuals and healthy donors (11, 25). Others have demonstrated different levels of $CD24^{hi}CD38^{hi}$ Bregs in various autoimmune conditions, compared with healthy controls (52). This theme of contrary results is echoed in studies of type 1 diabetes, which are summarized in **Table 1**.

It still remains unclear whether a defect or an impaired function of Bregs contributes to the development of diabetes or if the observed aberrant frequency and function is a result of chronic inflammation. Studies in the Experimental autoimmune encephalomyelitis (EAE) mouse model of MS has implicated Bregs in disease initiation rather than late-phase progression (53, 54). Moreover, in NOD mice, early treatment (5-6 weeks old) with BCR-activated B cells both delayed and reduced diabetes onset; however later treatment at 9 weeks of age only delayed onset of disease (55). Determining how Bregs contribute to the onset of type 1 diabetes will be of significance when considering immunotherapies targeted at B cells. Future real-time studies of regulatory B cells in islet autoantibody-positive individuals, who have not yet developed overt type 1 diabetes, would improve understanding of this.

Evidence for Numerical Defects in Regulatory B Cells in Type 1 Diabetes

Specific cell subsets that are associated with regulation, and B cells actively producing IL-10 after *ex vivo* stimulation, have been evaluated to ascertain if Breg frequencies are altered in type 1 diabetes. Whether the frequency of Breg-associated populations are altered, which include $CD5^{+}CD1d^{hi}$ and transitional $CD24^{hi}CD38^{hi}$ B cells, has been inconclusive when comparing patients to healthy donors (**Table 1**). A likely contributor to the disparity in these studies is the different sets of immune markers used to distinguish discrete populations or analysis of different Breg subsets. Use of an increased number of immune markers and high-dimensional profiling will help to determine more discrete B cell subsets and may resolve these dichotomies. For example, detailed characterisation has shown that human B cells which readily produce IL-10 are enriched in both T2 ($CD27^{-}IgM^{+}IgD^{+}$) and $CD27^{+}$ B cells in the transitional $CD24^{hi}CD38^{hi}$ compartment (52). Furthermore, stimulation *via* TLR9 resulted in enhanced IL-10 expression in the transitional T3 subset (52).

Direct assessment and evaluation of IL-10 production from B cells requires exogenous stimuli. Targets include either the innate TLRs or other receptors such CD40 or the BCR, either separately or by co-engagement, and if IL-10 is measured by intracytoplasmic staining, the addition of PMA/Ionomycin is also required (56). So far in type 1 diabetes, the studies employing CD40L and TLR stimulants - either LPS [TLR4] or CpG [TLR9] in culture before assessment, or with PMA/ionomycin alone - have shown a decrease in numerical frequency of IL-10-producing B cells from peripheral blood samples (28–30). However, when a combination of LPS and CPG was used, with the addition of IL-21, which can drive IL-10 production from B cells (42), the investigators found no difference in $IL\text{-}10^{+}$ B cells, in either naïve or memory compartments (27). A detailed summary of these studies is described in **Table 1**. It is clear that both the stimulation conditions and the appropriate markers to identify distinct populations are necessary for a more accurate overview on how B cell subsets are altered in type 1 diabetes.

In addition, a key disparity between studies is how accurately healthy donors were age-matched (**Table 1**). It is clear that subsets, such as transitional $CD24^{hi}CD38^{hi}$ B cells, enriched with $IL\text{-}10^{+}$ B cells, decline with age (27, 57), which is an important note for future studies. Recently, in children with type 1 diabetes, a decrease in both the $CD24^{hi}CD27^{+}$ (B10) and transitional $CD24^{hi}CD38^{hi}$ $IL\text{-}10^{+}$ B cells but not in $CD38^{hi}CD27^{+}IL\text{-}10^{+}$ plasmablasts was found (31). This numerical decrease was also negatively correlated with HbA1c levels (31), as was the frequency of $CD24^{hi}CD38^{hi}$ B cells in a study by Wang et al. (30). In view of the recent observation that the frequency of pancreatic $CD20^{+}$ B cells correlates with earlier diagnosis of a rapidly progressing and more aggressive disease (58), considering both age and clinical parameters in studies assessing regulatory B cells will be particularly important.

Currently, very few studies have assessed Breg populations in individuals with multiple islet autoantibodies who are classed as 'at risk' or in 'stage 1' or 'stage 2' (59) of developing diabetes. Kleffel et al. reported that individuals with multiple islet autoantibodies (like individuals with diabetes) had significantly fewer IL-10$^+$ B cells, compared to healthy controls (28). However, Saxena et al. observed that antibody positive individuals had increased CD5$^+$IL-10$^+$ B cells, compared to both healthy and diabetic controls (29). Overall, whether numerical differences exist in IL-10-producing B cells in individuals with islet autoantibodies remains a key outstanding question, which needs to be addressed in order to refine and improve immunotherapy targeted at B cells.

Although evidence has been provided in mouse models that IL-10$^+$ B cells can control autoimmune diabetes (55), few studies have addressed the number of IL-10-producing B cells in mice that have developed overt disease. Recent work from our group has demonstrated that NOD mice that developed diabetes showed a reduced splenic IL-10$^+$ B cell population, measured by intracytoplasmic staining, compared to mice that were long-term normoglycemic or 'naturally-protected' from diabetes (>35 weeks old) (60). Also, the frequency of IL-10$^+$ B cells was dependent on the B cell stimulation used, with anti-CD40 ligation highlighting the greatest loss in frequency of IL-10$^+$ B cells in diabetic NOD mice (60). This again focuses our attention on the need for better understanding and a more comprehensive use of different, combined stimuli. Additionally, we observed either no difference or increased IL-10 secretion in the mice that had developed diabetes, dependent on the stimulus used for study of the B cells (60). To date, type 1 diabetes studies reporting differences in IL-10$^+$ B cells have not evaluated IL-10 secretion. Increased IL-10$^+$ B cell frequency has been demonstrated in long-term normoglycemic or 'naturally protected' NOD mice in pancreatic islets (28, 61), suggesting a Breg-mediated protection against β cell destruction. For further discussion of Bregs related to pancreatic islets see *Regulatory B cells in Pancreatic Islets*.

Impaired Regulatory B Cell Function in Type 1 Diabetes

Functional studies in Bregs have described numerous immunosuppressive mechanisms of IL-10-producing B cells, including inhibiting pro-inflammatory cytokines from immune cells and promoting regulatory T cell differentiation (10, 51, 62), together with dampening of antigen presenting cell (APC) responses (11, 12). In autoimmune conditions, failed mechanisms of Breg immunosuppression are observed. In SLE patients, B cells fail to produce IL-10 in response to CD40 ligation and are unsuccessful in suppressing Th1 responses (10). CD24^{hi}CD38^{hi} Bregs from individuals with active RA are unable to convert CD4$^+$CD25$^-$ into Tregs or suppress Th17 responses (51). Moreover, CD19$^+$CD27$^+$IL-10$^+$ B cells from donors with RA fail to suppress IFNγ from CD4$^+$ T cells, compared to healthy individuals (62).

Evidence for diminished Breg function in human type 1 diabetes studies is limited. A recent study demonstrated that a numerical deficiency of Bregs was coupled with a functional defect in patients (30). Here, IL-10-producing B cells in healthy volunteers were enriched in the CD24^{hi}CD38^{hi} transitional subset, after CD40L and CPG stimulation, as shown previously (10). Furthermore, CD24^{hi}CD38^{hi} B cells inhibited effector cytokines from CD4$^+$ T cells and promoted CD4$^+$FoxP3$^+$ Tregs, in an IL-10-dependent manner (10). However, in patients with type 1 diabetes, CD24^{hi}CD38^{hi} B cells failed to reduce IFNγ, TNFα and IL-17 production from CD4$^+$ T cells (30). Conversely, Kleffel et al. showed that expanded IL-10-producing B cells from individuals with type 1 diabetes could suppress IFNγ production in PBMC cultures, in the presence of IA-2 peptide (28). However, the generation of IL-10$^+$ B cells from both individuals with type 1 diabetes and those with multiple islet autoantibodies was significantly impaired compared to healthy donors (28).

Murine studies have illustrated how regulatory B cells can control autoimmunity (8). Research has focused on how B cells can suppress autoimmune diabetes, demonstrating a role for IL-10-independent (63) and IL-10-dependent (55) mechanisms of B cell-mediated immunosuppression. However, data describing impaired regulatory B cell responses in mice, NOD or otherwise, are limited. TLR4-activated B cells from NOD mice that have developed diabetes suppress insulin-specific CD8 T cells, and in a B cell: DC : CD8 T cell co-culture produced significant amounts of IL-10 (60). This required the presence of the pathogenic CD8 T cells, because without pathogenic CD8 T cells in the cultures, the TLR4-induced B cells produced significantly less IL-10 and were less efficient in reducing DC activation. We also showed, in NOD mice with established diabetes, that CD40-ligation on B cells, followed by co-culture with DCs, the ability to reduce DC activation was decreased and resulted in a contact-dependent increase in IFNγ secretion, compared to NOD mice naturally-protected from autoimmune diabetes (60). In line with these observations, B cells from hyperglycemic NOD mice adoptively transferred into B cell-depleted long-term normoglycemic NOD animals promoted diabetes onset (28).

Other mechanisms of Breg suppression, independent of IL-10 expression and dependent on cell-contact have been noted. For example, PD-L1 and FasL exert suppression *via* apoptosis of target cells upon engagement with their receptors (2, 64). B cells that express FasL can induce apoptosis and suppress proliferation of CD4$^+$ T cells (3, 65). In mice, FasL can be induced by TLR4 activation in CD5$^+$CD1d$^+$ Bregs (65) and in the NOD mouse model can be activated with LPS (TLR4), resulting in TGFβ production, which inhibits Th1 responses and diabetes progression (63). In humans FasL^{hi}CD5$^+$ B cells are increased in frequency in individuals with type 1 diabetes, compared to both islet autoantibody positive and healthy donors (29), although here the levels of TGFβ production with stimulation was not assessed. Interestingly, in this study the frequency of CD5$^+$IL-10$^+$ B cells did not differ between healthy and diabetes donors (described in **Table 1**), but the percentage of Fas-expressing CD5$^+$IL-10$^+$ B cells was elevated in donors with type 1 diabetes (29). This is indicative of Fas-FasL B cell interplay, with elevated CD5$^+$FasL B cells targeting more

apoptosis-sensitive $CD5^+IL\text{-}10^+$ B cells, which results in fewer $IL\text{-}10^+$ B cells in individuals with autoimmune diabetes (29) (**Figure 1**, red box).

It remains inconclusive if there is an intrinsic developmental Breg defect that contributes to disease progression in individuals that develop type 1 diabetes, and is complicated by the lack of a definitive Breg marker and their heterogeneity. It is possible the differences in Bregs observed in some studies (described in **Table 1**) results from the inflammatory environment that occurs with the progression of disease, which indirectly impacts the size or function of the Breg compartment (see **Figure 1**). Indeed, IL-10-producing B cells are expanded in mice predisposed to autoimmunity, compared to non-susceptible mice (32). Furthermore, $IL\text{-}10^+$ splenic B cells are expanded in 4-week-old NOD mice and $IL\text{-}10^+$ B cells from normoglycemic NOD mice are still capable of suppressing T cell-mediated diabetes (28).

Overall, these studies described above in type 1 diabetes suggest that further interrogation is warranted on the defective or dysfunctional Bregs observed, including the autocrine B cell mechanisms and crosstalk with other immune cells (see *Discussion and Outstanding Questions*). Further studies, using both human peripheral blood and tissue sites in different cohorts, taking into account that $IL\text{-}10^+$ B cell immune-phenotypes are variable with age (66), will provide insight into Breg defects.

REGULATORY B CELLS IN PANCREATIC ISLETS

B cells residing in pancreatic islets during inflammation contribute to the destruction of β cells, and consequently a loss in the secretion of insulin. Evidence for this direct pathogenic role has been shown by B cell depletion studies in the NOD mouse model, highlighting a reduction in effector T cell function inhibiting tissue-specific inflammation in treated mice (67, 68). In NOD mice, B-1a cells located in the pancreas, early in diabetes, play a role in initiation of disease (69). Furthermore, the observation of different profiles of insulitis in human pancreatic islets, with increased frequency of CD20 B cells correlate with a more progressive earlier diagnosis (58).

Previously, we have alluded to proposed interactions between regulatory B cells and the inflammatory pancreatic islet environment, and how Bregs can control inflammation (70). Islet-specific B cells in naturally-protected normoglycemic NOD mice have increased IL-10 and CD40 expression (28). More recently, we have corroborated this work and demonstrated B cells from naturally-protected NOD mice have an increased frequency of B cells expressing IL-10, CD80 and CD40 (61). In this study we also described an enrichment of $CD19^{int}CD138^{hi}CD44^{hi}Ki67^+$ dividing plasmablasts in naturally-protected NOD mice (61) a phenotype attributed to IL-10 production (12). Alongside this increase in regulatory B cells, a significant increase of $CTLA4^+FoxP3^+$ Tregs was also observed (61), possibly indicating some Breg-Treg crosstalk, which suppresses local pancreatic inflammation. However, it is unknown if this crosstalk is dependent on the expression of IL-10. It is possible that other IL-10-independent Treg induction by B cells may occur, as shown by the requirement for Breg expression of GITR ligand (71). Moreover, it is currently unclear if the altered pancreatic milieu in naturally-protected NOD mice is responsible for the induction of these regulatory immune cells, or a result of expanded $IL\text{-}10^+$B cells in the periphery (60). $IL\text{-}10^+$ B cells can be detected in the pancreatic islets of younger NOD mice after CD40 ligation along with a PMA/Ionomycin stimulation, albeit the frequency of $IL\text{-}10^+$ B cells was very low (28, 61), and it is unknown if they have any role in controlling local β cell damage *in vivo*.

DISCUSSION AND OUTSTANDING QUESTIONS

As discussed above, studies of regulatory B cells in type 1 diabetes are limited in comparison to other autoimmune diseases that include SLE, RA and MS, and thus lessons can be learned in order to extrapolate the findings to direct key research in type 1 diabetes. Finally, we discuss future and outstanding research questions that will advance the treatments of type 1 diabetes.

1. A deeper understanding of the different Breg repertoires that are IL-10-producing or IL-10-competent, together with the altered frequency and function in different stages of autoimmune diabetes development.

The complex picture described, so far, in diabetes and other autoimmune diseases may reflect the divergent role of Breg subsets in various disease settings. Different subsets of Bregs, based on their maturity, may be more influenced by the level of inflammation and disease stage of the individual. As previously shown, different immune profiles for B cell and T cell responses are dependent on disease stage or progression (43). It should also be noted that IL-10-producing B cells can also secrete TNF and IL-6 and so there is heterogeneity in Breg cytokine production (72).

2. Breg interplay and crosstalk with both other B cell populations and different immune cells to dissect the relationships that impact frequency and function (**Figure 1**).

Interrogating Breg: immune cell crosstalk will uncover aberrant regulatory feedback loops. Cell subsets like pDCs (37) or other DC subsets will reveal how Bregs dampen, or fail to dampen APCs. Other Breg studies highlight immunosuppressive mechanisms *via* invariant NKT cells dependent on the surface molecule CD1d (73). Other studies describe a feedback loop between T cells and B cells, *via* CD40:CD40L interactions, to develop regulatory function, which differentially regulate T cell proliferation and Th1 responses (74).

3. Determine the impact of defective Breg frequency and function. Do impaired Bregs contribute to diabetes initiation or progression or both?

Determining if the defect in Breg frequency and/or function is a consequence of chronic inflammation or a contributor to the development of diabetes will have an impact on how B cell depletion therapy is exploited in individuals during various stages of disease progression. Furthermore, understanding if impaired Bregs contribute to disease due to the lack of immunosuppressive action or if Breg plasticity results in a further progression of disease under certain chronic conditions should be addressed.

4. The use of immunotherapies to either selectively expand Bregs or target pathogenic B cells but spare regulatory B cells.

So far, only a pan-B cell depletion approach has been trialed in type 1 diabetes (Rituximab) (7), and therefore we can only discuss preclinical studies that approach expanding Bregs *in vivo* or targeting a specific B cell population. Expansion of $CD73^+$ regulatory B cells after treatment with a small molecule inhibitor that disrupts the *Aicda*-encoded activation-induced cytidine deaminase protein (AID) results in the inhibition of diabetes development in the NOD mouse (75). Conversely, AID deficiency in the NOD mouse model can accelerate type 1 diabetes development (76) and therefore the role of AID in diabetes progression requires further investigation. An additional B cell-targeted therapeutic approach is to selectively deplete effector B cells preserving regulatory B cells; however this is complicated by the lack of a definitive Breg marker. Interestingly, targeting of B cells *via* the blockade of the B cell activating factor (BAFF) induced an increase of IL-10^+ B cells and diabetes protection (77). Furthermore, in this study, anti-CD20 treatment depleted this IL-10-producing B cell population, suggesting that Bregs are more sensitive to deletion during anti-CD20 treatment (77). This Breg sensitivity may have contributed to the limited success of the Rituximab clinical trial (7). However, as discussed above, a deeper understanding of Bregs during the development of type 1 diabetes is needed to harness and develop successful B cell targeted immunotherapies.

Overall, the pathogenesis of type 1 diabetes is complex and multi-stage, and requires a number of pathogenic cell types that give rise to the development of disease. Equally, it is clear that balanced against these pathogenic cells are regulatory cells, that include both T and B cell subsets. Defining the roles of these less-understood Breg subsets will provide important information to be further studied in humans with the aim of increasing therapeutic opportunities.

AUTHOR CONTRIBUTIONS

JB wrote and edited the manuscript. FSW edited the manuscript. All authors contributed to the article and approved the submitted version.

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Alpk1 Sensitizes Pancreatic Beta Cells to Cytokine-Induced Apoptosis *via* Upregulating TNF-α Signaling Pathway

Fei Ding[1†], *Xi Luo*[1†], *Yiting Tu*[2†], *Xianlan Duan*[1], *Jia Liu*[3], *Lijing Jia*[1*] *and Peilin Zheng*[1*]

[1] Department of Endocrinology, Shenzhen People's Hospital, The Second Clinical Medical College of Jinan University, The First Affiliated Hospital of Southern University of Science and Technology, Shenzhen, China, [2] Department of Neurology, Shenzhen Samii International Medical Center (The Fourth People's Hospital of Shenzhen), Shenzhen, China, [3] School of Medicine, Southern University of Science and Technology, Shenzhen, China

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***Correspondence:**
Peilin Zheng
plzheng83@163.com
Lijing Jia
jialijing2012@126.com

[†]*These authors have contributed equally to this work*

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Pancreatic beta cell failure is the hallmark of type 1 diabetes (T1D). Recent studies have suggested that pathogen recognizing receptors (PRRs) are involved in the survival, proliferation and function of pancreatic beta cells. So far, little is known about the role of alpha-protein kinase 1 (ALPK1), a newly identified cytosolic PRR specific for ADP-β-D-manno-heptose (ADP-heptose), in beta cell survival. In current study we aimed to fill the knowledge gap by investigating the role of Alpk1 in the apoptosis of MIN6 cells, a murine pancreatic beta cell line. We found that the expression of Alpk1 was significantly elevated in MIN6 cells exposed to pro-inflammatory cytokines, but not to streptozotocin, low-dose or high-dose glucose. Activation of Alpk1 by ADP heptose alone was insufficient to induce beta cell apoptosis. However, it significantly exacerbated cytokine-induced apoptosis in MIN6 cells. Mechanistic investigations showed that Alpk1 activation was potent to further induce the expression of tumor necrosis factor (TNF)-α and Fas after cytokine stimulation, possibly due to enhanced activation of the TIFA/TAK1/NF-κB signaling axis. Treatment of GLP-1 receptor agonist decreased the expression of TNF-α and Fas and improved the survival of beta cells exposed to pro-inflammatory cytokines and ADP heptose. In summary, our data suggest that Alpk1 sensitizes beta cells to cytokine-induced apoptosis by potentiating TNF-α signaling pathway, which may provide novel insight into beta cell failure and T1D development.

Keywords: Alpk1, pancreatic beta cells, apoptosis, pro-inflammatory cytokines, TNF-α signaling

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by the infiltration of inflammatory immune cells into pancreatic islets and progressive destruction of insulin-producing beta cells (1, 2). Both innate and adaptive immunity are involved in the injury of beta cells. To date, a variety of pathogen recognizing receptors (PRRs) have been identified, including C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), Toll-like receptors (TLRs), and retinoid acid inducible gene I (RIG-I)-like receptors (RLRs) (3).

Previous studies have shown that several PRRs are expressed in pancreatic beta cells in both humans and animal models (4, 5). Furthermore, activation of TLR3 or TLR4 induces the apoptosis of beta cells, while TLR9 suppresses the differentiation and function of beta cells (6–8). These findings collectively demonstrate that PRRs are involved in the survival and function of pancreatic beta cells.

Alpha-kinase 1 (ALPK1, also known lymphocyte α-kinase) has been recently identified as a new cytosolic PRR specific for ADP-β-D-manno-heptose (ADP-heptose), a metabolite in the biosynthesis of Lipopolysaccharide (LPS) (9). A series of studies have demonstrated that ALPK1 is an upstream kinase to induce the phosphorylation and oligomerization of RAF-interacting protein with forkhead-associated domain (TIFA), and subsequently TGF-β-activated kinase 1 (TAK1) phosphorylation, nuclear factor kappa B (NF-κB) activation and pro-inflammatory cytokine production (10–13), suggesting that ALPK1 is an essential player in inflammation and innate immune responses.

So far, the role of ALPK1 in beta cell survival and function is little known. A recent paper has shown that *Alpk1*-overexpressed C57BL/6 mice exhibited a decreased insulin level and severe hyperglycaemia than wild type mice after streptozotocin (STZ) treatment (13), suggesting that Alpk1 might participate in the destruction of pancreatic beta cells. Therefore, in this study we investigated the role of Alpk1 in the injury of MIN6 cells, a murine pancreatic beta cell line.

MATERIALS AND METHODS

Cell Culture and Treatment

MIN6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 15% heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/ml penicillin,100 U/ml streptomycin (Gibco), and 50 μM β-mercaptoethanol (Gibco). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. MIN6 cells were exposed to a mix of pro-inflammatory cytokines including 10 ng/ml interleukin (IL)-1β, 10 ng/ml tumor necrosis factor (TNF)-α, and 10 ng/ml γ-interferon (IFN-γ) (hereafter referred to as cyto mix), or 10 mM STZ, or 32 μM ADP heptose, or various concentrations of glucose (5.5, 11.1, 25, 33.3 mM) for indicated durations. In some experiments, cells were simultaneously treated with cyto mix and ADP heptose at aforementioned concentrations.

Chemicals

Recombinant murine IL-1β, IFN-γ, and TNF-α were purchased from Peprotech. ADP heptose (>95% purity) was obtained from J&K Scientific. STZ was purchased from Macklin. GLP-1-receptor-agonist liraglutide (Victoza) was from Novo Nordisk.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA from the cells was extracted using Direct-zol RNA MiniPrep Plus kit (Zymo Research), and cDNA was synthesized from 1 μg of total RNA by PrimeScript reverse reaction kit, according to the manufacturer's protocols (Takara). qRT-PCR analysis of Alpk1, TNF-α, IL-1β, and IFN-γ was performed in a StepOne Plus PCR system (Applied Biosystems). The gene expression levels were quantified as a fold change against β-actin by using the $2^{-\Delta\Delta CT}$ method (14). The following primers were used: mouse *Alpk1* (forward: 5' -CAGGTTCACGGA TGTGACCA-3', reverse: 5'-GCCCTGTGCATATTTCAGCG-3'); mouse *IFN-γ* (forward: 5'- TCAAGTGGCATAGATGTGG AAGAA-3', reverse: 5'- TGGCTCTGCAGGATTTTCATG-3'); mouse *Fas* (forward: 5'- AGCCCGTTGGAGTGATTCAA-3', reverse: 5'- CCCCCTGCAATTTCCGTTTG-3'); mouse TNF-α (forward: 5'- AATGGCCTCCCTCTCATCAGT-3', reverse: 5'-GCTACAGGCTTGTCACTCGAATT-3'); mouse IL-1β (forward: 5'-TGCCACCTTTTGACAGTGATG-3', reverse: 5'-TGTGCTGCTGCGAGATTTG-3'); mouse β-actin (forward: 5'-CCCAGCACAATGAAGATCAAGATCAT -3', reverse: 5'- ATCTGCTGGAAGGTGGACA -3') (15).

Western Blotting

Cells were lysed using SDS lysis buffer containing protease and phosphatase inhibitors. The anti-mouse Alpk1 antibody (1:1000) was from Proteintech. The anti-mouse TNF-α, Fas and specificity protein 1 (SP1) antibodies (all 1:500) were purchased from Beyotime. The anti-cleaved Caspase 3 (1:1000), anti-NF-κB P65 (1:1000), anti-tubulin (1:2000) antibodies were from Cell Signaling Technology. The anti-TNF receptor associated factor (TRAF) 2 (1:1000), anti-TRAF6 (1:1000), anti-p-NF-κB P65 (S536, 1:1000), anti-p-TAK1 (S412, 1:1000), anti-TAK1 (1:1000) antibodies were from ABclonal. The anti-p-TIFA (T9, 1:1000), anti-TIFA (1:1000) antibodies were from Abcam.

Apoptosis Assay

For apoptosis assessment, $1x10^5$ MIN6 cells were plated and treated with cyto mix, or ADP heptose, or cyto mix together with ADP heptose for 24 hours. The dead cells were examined by addition of CellTox Green Cytotoxicity dye. The plate was read on a SPARK 10M reader (TECAN). In some experiments, cells were treated and then stained with Annexin V/PI solution (RiboBio) and measured on a DxFLEX flow cytometer (Beckman Coulter). Data were analyzed with Flowjo version 10.0.7 (Treestar). For TUNEL staining, $1x10^5$ MIN6 cells were plated onto coverslips in 24-well culture plates and treated. A riboAPO One-Step TUNEL Apoptosis Kit (RiboBio) was used to detect DNA fragmentation in cells according to the manufacturer's instructions. The nuclei were stained with DAPI. TUNEL staining was evaluated by a fluorescence microscopy (Leica TCS SP8). Cells double labeled with DAPI and TUNEL in the nuclei were considered as dead cells.

Cell Viability Assay

Cell viability was assessed by a CCK-8 kit (Med Chem Express). Briefly, $1x10^5$ MIN6 cells were incubated with ADP heptose, or cyto mix, or cyto mix plus ADP heptose for 24 hours. CCK-8 solution was added and OD (450 nm) was measured on a SPARK 10M reader (TECAN).

EdU Cell Proliferation Assay

The proliferation of MIN6 cells was assessed by a Cell-Light EdU Kit (RiboBio) according to manufacture instructions. The nuclei were stained with DAPI. EdU incorporation was evaluated by a fluorescence microscopy (Leica TCS SP8). Cells double labeled with DAPI and EdU in the nuclei were considered as dividing cells.

RNA-Seq Analysis

RNA-Seq analysis was performed by BGI-Shenzhen. Briefly, total RNA was extracted from MIN6 cells treated with cyto mix alone or together with ADP heptose for 24 hours using Trizol (Invitrogen) according to manual instruction. RNA was qualified and quantified using a Nano Drop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific). Oligo (dT)-attached magnetic beads were used to enrich mRNA, which subsequently fragmented into small pieces. The First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by a second-strand cDNA synthesis and addition of A-Tailing Mix and RNA Index Adapters. The cDNA fragments were validated on the Agilent Technologies 2100 bioanalyzer, and then denatured and circularized into single strand circle DNA (ssCir DNA). The final library was amplified with phi29 to make DNA nanoball (DNB) which had more than 300 copies of one molecular, DNBs were loaded into the patterned nanoarray and single end 50 bases reads were generated on BGIseq500 platform. The original sequencing data were deposited to Sequence Read Archive (SRA) with the accession number PRJNA726429 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA726429).

Isolation of Nuclear Proteins

Nuclear proteins of MIN6 cells were isolated according to manufacturer's instructions (Thermo Scientific, subcellular protein fractionation kit for cultured cells). Briefly, MIN6 cells were treated and washed twice with ice-cold PBS. 5×10^6 cells were then to a 1.5mL microcentrifuge tube for nuclear protein isolation.

Statistical Analysis

Statistical analyses were performed with Prism version 8.3.0 (GraphPad). Data were analyzed with unpaired Student's t-test (two-tailed) or one-way analysis of variance followed by Tukey's *post hoc* test. $p<0.05$ was considered statistical significant.

RESULTS

Pro-Inflammatory Cytokines Induced the Expression of Alpk1 in MIN6 Cells

Pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-1β, STZ and glucose toxicity play essential roles in the failure of pancreatic beta cells (1, 16, 17). To investigate the role of Alpk1 in beta cell injury, we first used cyto mix (10 ng/ml IL-1β, 10 ng/ml TNF-α, and 10 ng/ml IFN-γ), or various concentrations of glucose, or STZ to treat MIN6 cells, and examined the expression of Alpk1 by qRT-PCR. The mRNA level of *Alpk1* was significantly induced when cells were exposed to cyto mix for 24 hours (**Figure 1A**). Time-course profiling showed that following cyto mix treatment, *Alpk1* mRNA level was significantly increased even at 1 hour, maintained at a nearly 10-fold increase from 3 to 24 hours, and then decreased to baseline at 48 and 72 hours (**Figure 1B**). Correspondingly, Alpk1 protein was significantly elevated in response to cyto mix at 24 hours (**Figure 1C**). No alterations of *Alpk1* expression were observed in cells treated with low or high concentrations of glucose, or STZ (**Figures 1D, E**). Activation of Alpk1 by ADP heptose also did not affect *Alpk1* expression (**Figure 1F**). Collectively, these data indicated that Alpk1 might be involved in cytokine-induced beta cell injury.

Alpk1 Activation by ADP Heptose Impaired the Viability of Cytokine-Treated MIN6 Cells

ALPK1 is a PRR that can be activated by its agonist, ADP heptose. We next investigated whether ADP-heptose-induced Alpk1 activation impaired beta cell survival. A little surprisingly, the viability of MIN6 cells was not affected when exposed to a series concentration of ADP heptose (**Figure 2A**). Considering Alpk1 expression was induced by cyto mix and both pro-inflammatory cytokines and PRR agonists were elevated in the sera of T1D patients (18, 19), we investigated the combinational effects of cyto mix and Alpk1 activation on beta cell survival. Cyto mix impaired beta cell viability. Of note, Alpk1 activation exacerbated cytokine-induced beta cell death in a dose dependent manner (**Figure 2B**). We further determined which component of cyto mix were synergized with Alpk1. It was found that two cytokines, TNF-α and IFN-γ, could synergize with Alpk1 to reduce beta cell viability (**Figure 2C**).

Alpk1 Activation by ADP Heptose Exacerbated Cytokine-Induced Beta Cell Death

We proceeded to ask whether the decreased viable cell number was due to suppressed proliferation or enhanced apoptosis after cyto mix and ADP heptose treatment. We found that activation of Alpk1 by ADP heptose did not alter cell proliferation (**Supplementary Figures 1A, B**). In contrast, Alpk1 activation significantly exacerbated apoptosis in cyto mix-treated beta cells, as revealed by the staining of a dead cell dye (**Figure 3A**), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, **Figures 3B, C**) and Annexin V/Propidium Iodide (**Figures 3D, E**). We also evaluated the effects of ALPK1 activation on the apoptosis of cells treated with individual cytokine. Consistent with the cell viability data, Alpk1 activation could synergize with TNF-α or IFN-γ to induce more cell death (**Figure 3F**). Although STZ exerted little effect on the expression level of *Alpk1* in MIN6 cells, we further investigated whether these two could be functional synergistic. Intriguingly, the addition of ADP heptose did not alter the apoptosis of STZ-treated cells (**Figure 3G**).

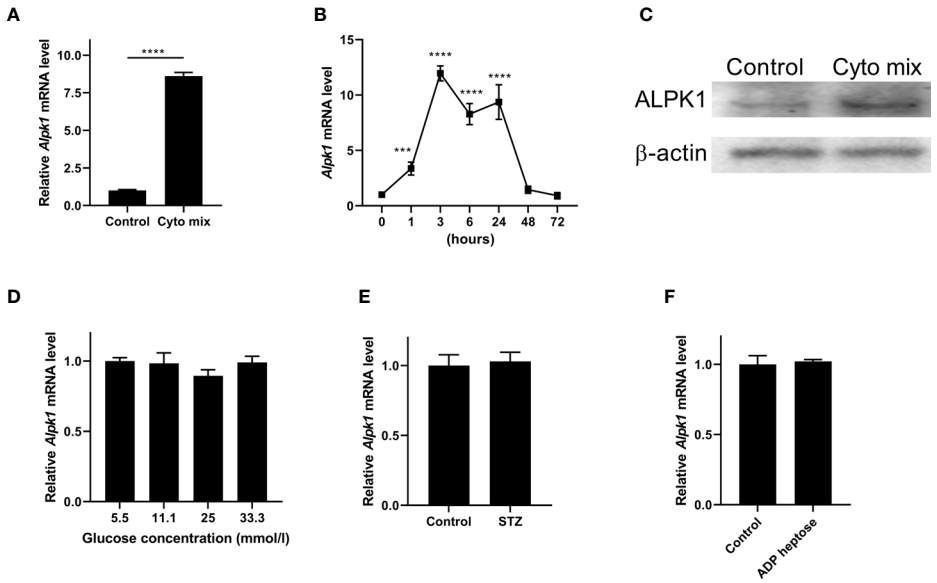

FIGURE 1 | Alpk1 expression was induced by pro-inflammatory cytokines in MIN6 cells. **(A)** MIN6 cells were untreated or treated with a mix of pro-inflammatory cytokines (cyto mix; 10 ng/ml IL-1β, 10 ng/ml TNF-α, and 10 ng/ml IFN-γ) for 24 hours. *Alpk1* mRNA level was measured by qRT-PCR. **(B)** Time-course expression of *Alpk1* exposed to cyto mix. **(C)** MIN6 cells were untreated or treated with cyto mix for 24 hours. Alpk1 protein level was measured by western blotting. **(D)** MIN6 cells were treated with various concentrations of glucose for 24 hours. **(E)** MIN6 cells were treated with vehicle or 10 mM STZ for 24 hours. **(F)** MIN6 cells were treated with vehicle or 32 μM ADP heptose for 24 hours. *Alpk1* mRNA level was measured by qRT-PCR. Data show mean ± SD and are representative of 3 independent experiments. The data shown in **(A, E, F)** are were analyzed with unpaired Student's t-test, and data in **(B, D)** were analyzed with ANOVA followed by Tukey's *post hoc* test. ***P < 0.001; ****P < 0.0001. Cyto mix, a mix of pro-inflammatory cytokines; STZ, streptozotocin.

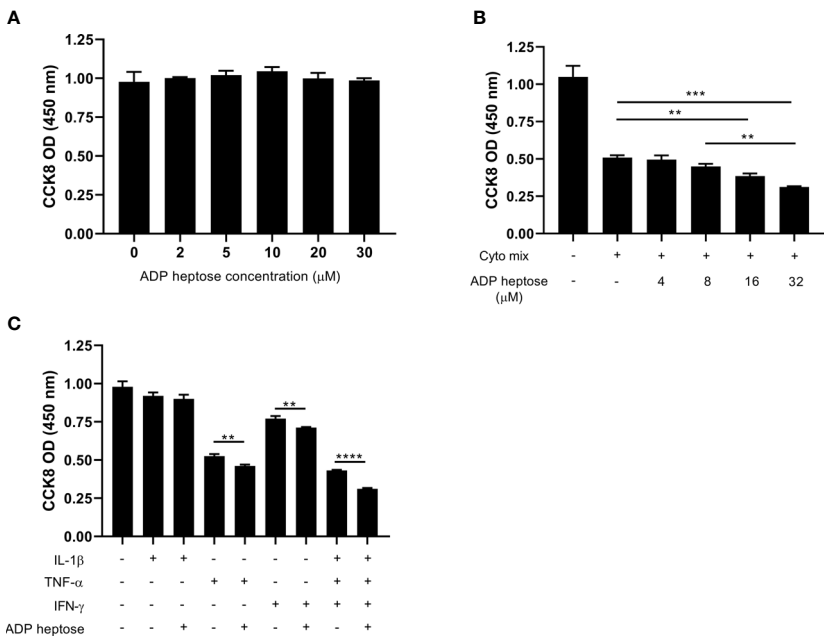

FIGURE 2 | Alpk1 activation synergized with cyto mix to reduce cell viability. The number of viable cells were assayed by CCK-8 solution. **(A)** $1x10^5$ MIN6 cells were treated with vehicle (sterile distilled water), or a series concentration of ADP heptose for 24 hours. **(B)** MIN6 cells were treated with vehicle (sterile distilled water), or cyto mix, or cyto mix together with elevated concentrations of ADP heptose for 24 hours. **(C)** MIN6 cells were treated with the indicated cytokine with or without ADP heptose (32 μM) for 24 hours. Data show mean ± SD and are representative of 3 independent experiments. The data shown in **(A, B)** are were analyzed with ANOVA followed by Tukey's *post hoc* test, and data in **(C)** were analyzed with unpaired Student's t-test. **P < 0.01; ***P < 0.001; ****P < 0.0001.

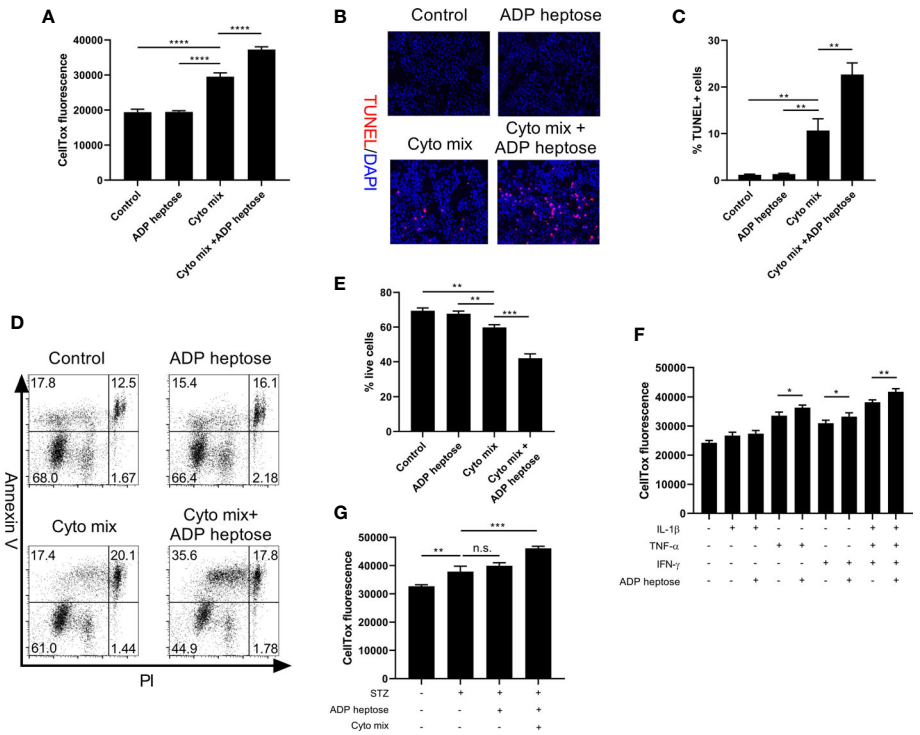

FIGURE 3 | Alpk1 activation exacerbated cytokine-induced beta cell death. 1x10^5 MIN6 cells were treated with vehicle, or ADP heptose (32 μM), or cyto mix, or cyto mix plus ADP heptose (32 μM) for 24 hours. **(A)** The death of MIN6 cells as assayed by the staining of CellTox Dye. **(B)** Representative images with at 100X magnification showing apoptotic MIN6 cells (TUNEL$^+$DAPI$^+$) with summary **(C)**. Red, TUNEL; Blue, DAPI. **(D)** Representative flow plots showing Annexin V/PI staining of MIN6 cells with summary **(E)**. **(F)** The death of MIN6 cells exposed to individual cytokine with or without ADP heptose, as assayed by the staining of CellTox Dye. **(G)** The death of MIN6 cells exposed to vehicle, or STZ, or STZ and ADP heptose, or STZ, ADP heptose and cyto mix, as assayed by the staining of CellTox Dye. Data show mean ± SD and are representative of 3 independent experiments. Data were analyzed with unpaired Student's t-test. **P < 0.01; ***P < 0.001; ****P < 0.0001. TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI, 4',6-diamidino-2-phenylindole; PI, Propidium Iodide; n.s., not significant.

ADP-Heptose-Induced Alpk1 Activation Enhanced TNF-α Signaling Pathway in Cytokine-Treated Beta Cells

Since we demonstrated that Alpk1 activation sensitized pancreatic beta cells to cytokine-induced apoptosis by various approaches, we next investigated the potential mechanism by RNA-Seq analysis. RNA from MIN6 cells exposed to cyto mix alone or cyto mix together with ADP heptose were extracted and sequenced. Notably, we found that TNF-α signaling pathway was the most enriched pathway for differentially expressed genes (DEGs) in cells simultaneously treated with cyto mix and ADP heptose (**Figure 4A**). Two upstream factors, TNF-α and Fas, were significantly upregulated among 20 DEGs in enriched TNF-α signaling pathway (**Figure 4B**). The elevated expression of TNF-α and Fas were further confirmed in separate experiments by qRT-PCR and western blotting (**Figures 4C–E**). We also investigated whether Alpk1 activation induced the expression of IL-1β and IFN-γ, which were the other two components in cyto mix. Interestingly, *IL-1β* and *IFN-γ* mRNA levels were comparable in cytokine-treated cells with or without ADP heptose (data not shown). Caspase-3 is one of essential downstream effectors in TNF-α signaling pathway. Western blotting analysis showed that the amount of cleaved caspase-3 was elevated in cells treated with cyto mix, and was further increased in the presence of cyto mix and ADP heptose (**Figure 4F**). Consistently, the abundance of nuclear NF-κB, a pivotal transcription factor in TNF-α signaling pathway, was most increased when cells were exposed to cyto mix and ADP heptose (**Figure 4G**). To further investigate the possible underlying molecular mechanism for the synergistic effect of ALPK1 and TNF-α, we evaluated the activation of key molecules in the ALPK1 and TNF-α signaling pathways. Intriguingly, TNF-α exposure in MIN6 cells was sufficient to phosphorylate and activate TIFA, a direct downstream effector of ALPK1. The addition of ADP heptose further enhanced TIFA activation, upregulated the expression of TIFAsome components such as TRAF2 and TRAF6, and subsequently induced an increased phosphorylation of TAK1 and NF-κB P65 (**Figure 4H**).

Glucagon-Like Peptide-1 Receptor (GLP-1R) Agonist Ameliorated Apoptosis in Beta Cells Exposed to ADP Heptose and Cyto Mix

A previous study have shown that GLP-1R agonist could improve pancreatic beta cell survival when exposed to

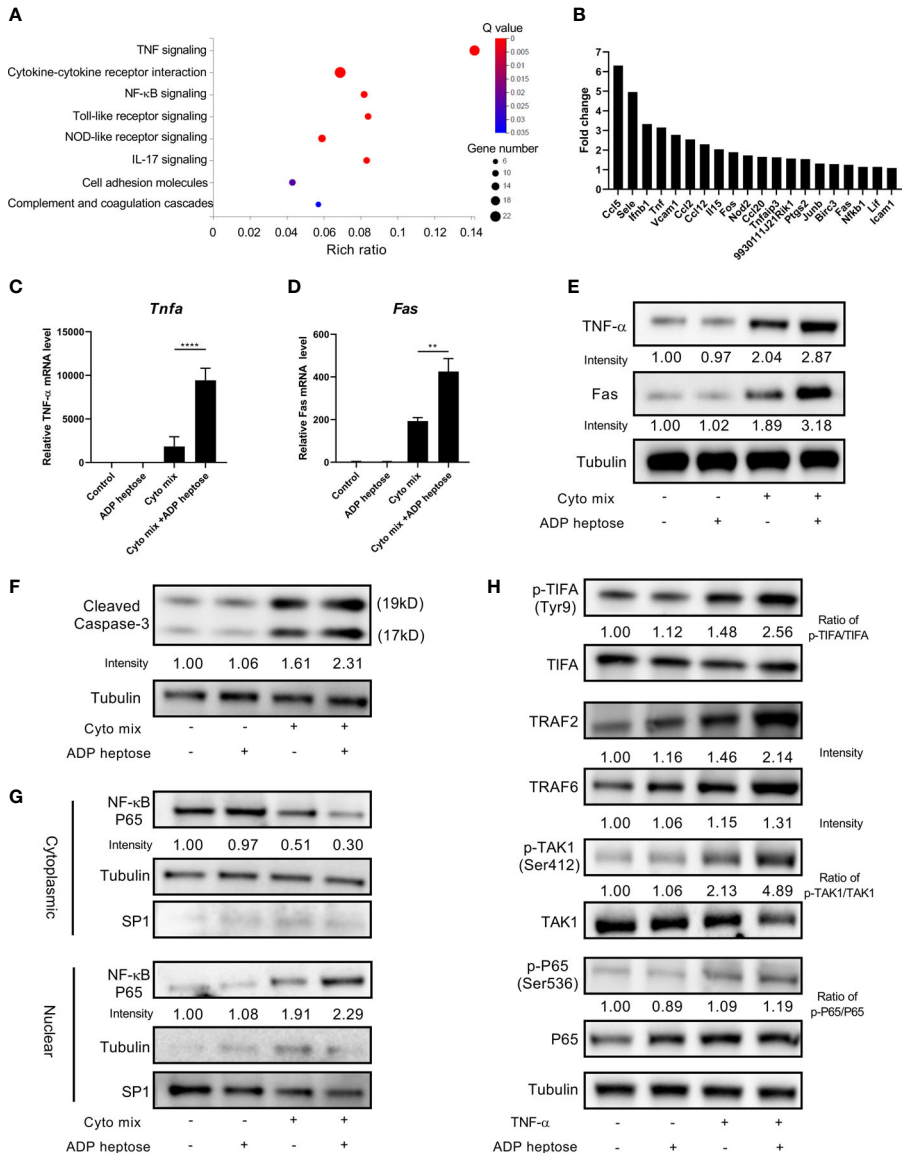

FIGURE 4 | ADP-heptose-induced ALPK1 activation enhanced TNF-α signaling pathways in cytokine-treated beta cells. MIN6 cells were treated with cyto mix or cyto mix plus ADP heptose for 24 hours. RNA from treated cells were extracted and sequenced. **(A)** Bubble chart showing the significantly enriched KEGG pathways for differentially expressed genes in MIN6 cells simultaneously treated with cyto mix and ADP heptose (Q value < 0.05). The size of the bubble represents the number of genes annotated to KEGG Pathway. The color represents the enriched significance. The redder the color, the smaller the significance value. **(B)** The expression fold change of twenty differentially expressed genes in enriched TNF-α signaling pathway. MIN6 cells were treated with vehicle, or ADP heptose (32 μM), or cyto mix, or cyto mix plus ADP heptose (32 μM) for 24 hours. *TNF-α* **(C)** and *Fas* **(D)** mRNA level was measured by qRT-PCR. **(E)** TNF-α and Fas protein levels were examined by western blotting. **(F)** The level of cleaved Caspase-3 was examined by western blotting. **(G)** The nuclear proteins of MIN6 cells were fractioned by subcellular protein fractionation kit for cultured cells (Thermo Scientific, #78840). The levels of cytoplasmic and nuclear NF-κB P65 were detected by western blotting. The cytoplasmic protein Tubulin and nuclear protein SP1 were served as the internal control. **(H)** MIN6 cells were treated with 10 ng/ml TNF-α, and or 32 μM ADP heptose for 4 hours. Cells were then lysed in lysis buffer containing phosphatase and proteinase inhibitors. Data show mean ± SD and are pooled from 3 independent experiments. The data shown in **(C, D)** were analyzed with unpaired Student's t-test. **P < 0.01; ****P < 0.0001.

endoplasmic reticulum stress (20). Therefore, we investigated whether GLP-1R agonist could improve beta cell survival in Alpk1 and cytokine-induced inflammation. We found that GLP-1R activation significantly diminished the apoptosis of cells treated with ADP heptose and cyto mix (**Figure 5A**). The mRNA and protein levels of TNF-α and Fas were significantly reduced (**Figures 5B–D**). The activation of Caspase-3 (**Figure 5E**) and NF-κB (**Figure 5F**) was also dramatically decreased after GLP-1R agonist treatment.

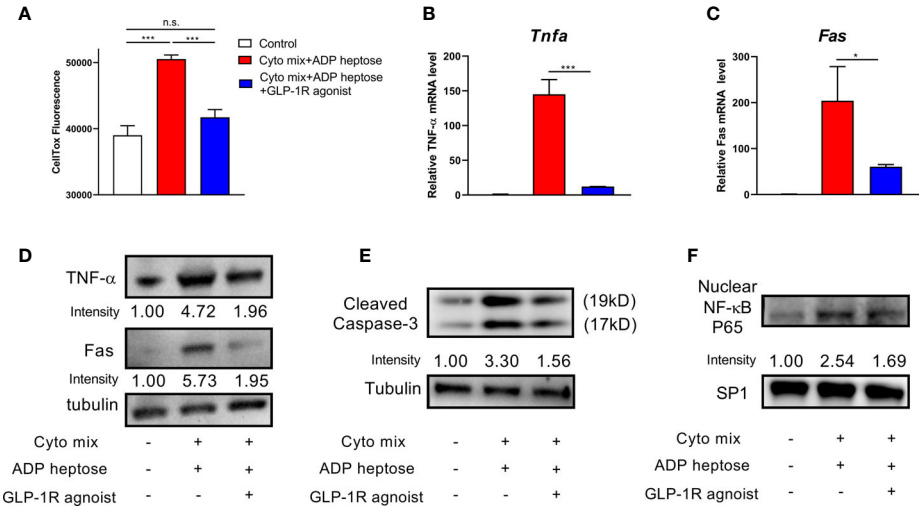

FIGURE 5 | GLP-1R agonist ameliorated apoptosis in beta cells exposed to ADP heptose and cyto mix. **(A)** The death of MIN6 cells as assayed by the staining of CellTox Dye. The changes of mRNA **(B, C)** and protein **(D)** levels of TNF-α and Fas when cyto mix and ADP heptose treated MIN6 cells exposed GLP-1R agonist (100 nM) for 24 hours. **(E)** The level of cleaved Caspase-3 was examined by western blotting. **(F)** The nuclear proteins of MIN6 cells were fractioned, and the level of NF-κB P65 was detected by western blotting. The nuclear protein SP1 was served as the internal control. Data show mean ± SD and are representative of 3 independent experiments. The data shown in **(A–C)** were analyzed with unpaired Student's t-test *P < 0.05; ***P < 0.001; n.s., not significant.

DISCUSSION

In this study, we provide the first evidence that Alpk1, a newly identified PRR, is involved in the survival of pancreatic beta cells. We showed that pro-inflammatory cytokines, but not STZ, glucose toxicity and ADP heptose, induced Alpk1 expression in pancreatic beta cells. In turn, activation of Alpk1 further sensitized beta cells to cytokine-induced apoptosis, possibly by selectively up-regulating the expression of TNF-α and Fas to elicit a more profound Caspase-3 activation and NF-κB nuclear translocation. Finally, GLP-1R agonist could inhibit TNF-α signaling pathway, and ameliorated the apoptosis of cells exposed to cyto mix and ADP heptose. Our results thus demonstrated a novel link between Alpk1 and TNF-α signaling pathway in beta cell injury.

Previous studies mainly focused on the function of PRRs on immune cells in T1D studies. TLRs constitute a major class of PRRs. The role of TLRs in immune regulation and T1D development has been uncovered by several studies. Deletion of *Tlr7* protected NOD mice from T1D, which might be due to the altered differentiation and reduced antigen-presenting functions of B cells (21). *Tlr9* deficient NOD mice exhibited a decreased incidence of T1D. The disease protection could be imparted by enhanced CD73 expression in T cells and impaired IFNα expression in dendritic cells (22, 23). Of note, specific knockout of *Tlr9* in B cells of NOD mice replicated disease protection, possibly mediated by B cell hyporesponsiveness and elevated IL-10-producing B cells (24). Recent studies have demonstrated that in addition to the dysfunction of immunity, the injury of β cells also plays an essential role in the pathogenesis of T1D (15, 25). So far, the studies of PRRs in pancreatic beta cells are limited. *Tlr9* deficiency in NOD mice did not alter beta cell death, but enhanced CD140a expression and subsequently increased beta cell proliferation and mass (9). Activation of TLR4 by LPS led to enhanced expression of pro-inflammatory cytokines and chemokines, as well as elevated apoptosis in both human and murine pancreatic beta cells (7, 19, 26). Quite differently, in current study we found that activation of Alpk1 alone was insufficient to induce the apoptosis of MIN6 cells. However, Alpk1 activation could exacerbate beta cell death in the presence of pro-inflammatory cytokines. So far we don't know the underlying mechanism for this distinction. A possible explanation may be that TLR4 is located on the plasma cell surface to form the first line to sense pathogen and counteract infection. On the contrary, Alpk1 is a cytosolic PRR, which might form the second line to amplify danger signals and trigger a more severe inflammation in pancreatic beta cells. Another possible explanation can be that in the resting condition Alpk1 expression is pretty low in MIN6 cells (cycle threshold value >32).Therefore, ADP-heptose-induced Alpk1 activation may be insufficient to trigger inflammatory responses. Cyto mix significantly induced Alpk1 expression, which could promote Alpk1 activation and the downstream inflammatory pathways.

Intriguingly, Alpk1 activation could synergize with TNF-α or IFN-γ, but not IL-1β, to induce more cell death. It has been well documented that NF-κB predominantly mediates IL-1β as well as ALPK1 signaling pathways (10, 27, 28). In contrast, transcription factors such as NF-κB, activating transcription factor 2 (ATF2), C/EBP Homologous Protein (CHOP), cAMP Response Element-Binding Protein (CREB) and ETS Like-1 (ELK1) are involved in TNF-α signaling pathway, while interferon regulatory factor 1 (IRF-1) and signal transducer

and activator of transcription 1 (STAT1) participates in IFN-γ mediated signaling transduction (29, 30). Therefore, a possible explanation can be that the excess usage of NF-κB in cells treated with both IL-1β and ADP heptose might hinder their potential synergic effects. Of note, we found that TNF-α treatment was capable of activating TIFA in MIN6 cells, which was consistent with a previous observation that TNF-α stimulation induced TIFA phosphorylation in 293T cells (31). In contrast, ADP heptose stimulation slightly increased TIFA phosphorylation, which might explain why ADP heptose alone had a minimal effect on the apoptosis of MIN6 cells.

Multiple injections of low-dose STZ (MLDS) can cause a low-grade injury of pancreatic beta cells accompanied by elevated inflammation and consequently apoptosis of beta cells (32). A previous study have showed that C57BL/6 mice with *Alpk1* overexpression exhibited normal blood glucose level. After MLDS treatment, those mice showed a lower level of insulin and severe hyperglycemia (13). In line with this observation, we found that Alpk1 activation alone did not alter the proliferation or apoptosis of MIN6 cells. In the presence of inflammation, activation of Alpk1 enhanced the expression of TNF-α and Fas, and might subsequently exacerbate beta cell death. A previous study have showed that treatment of high-dose glucose (200 mg/dL) for 48 hours significantly elevated the expression of Alpk1 in THP1 and HK2 cells (33). However, in this study we found that Alpk1 expression was selectively induced by pro-inflammatory cytokines. It seems that Alpk1 expression can be indeed induced under stress conditions, but may depend on specific cell types or stress.

ADP heptose is an intermediate for the biogenesis of LPS in gram negative bacteria (34). In this study we used a synthesized ADP heptose to investigate the effects of Alpk1 activation on beta cell death *in vitro*. Several studies have illustrated the importance of gut microbiome in the pathogenesis of T1D. The increased intestinal permeability, which precedes the onset of disease, is observed in both T1D patients and animal models (35, 36). The altered permeability of intestinal barriers allows gut bacteria translocating into pancreatic lymph nodes and trigger T1D onset (37–39). Therefore, we speculate that *in vivo* ADP heptose might be derived from microbiome in the leaky gut and contribute to T1D development, which requires further studies.

In current study we used a mix of pro-inflammatory cytokines to induce the injury of pancreatic beta cells, which is more relevant to the pathology of T1D (17). A series of studies have demonstrated that a chronic and low-grade inflammation, characterized by the elevated pro-inflammatory cytokines such as TNF-α and IL-6, exists in obese and type 2 diabetic patients (40, 41). Intriguingly, *ALPK1* variant was reported to be associated with type 2 diabetes (42, 43), suggesting that ALPK1 might also be involved in the injury of pancreatic beta cells in the scenario of type 2 diabetes.

The limitation of the present study is the lack of *in vivo* validation for the role of Alpk1 on beta cell survival. Considering that Alpk1 is pleiotropic and impacts multiple cell types, transgenic mice with beta cell-specific deletion of *Alpk1* may contribute to solve this issue. In conclusion, our data suggest that Alpk1 can sensitize pancreatic beta cells to cytokine-induced apoptosis by upregulating TNF-α signaling pathway. Inhibition of Alpk1 might delay beta cell failure and be an important therapeutic approach for T1D.

DATA AVAILABILITY STATEMENT

The raw sequencing data have been uploaded and released on BioProject - accession number PRJNA726429 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA726429).

AUTHOR CONTRIBUTIONS

FD, XL, YT, and PZ designed the experiments. XL, FD, XD, and JL conducted the experiments. FD, XL, FD, YT, XD, LJ, and PZ analyzed data. PZ and LJ supervised the study. YT and PZ wrote the manuscript. This project was conceived by PZ who assumes responsibility for the work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.705751/full#supplementary-material

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REVIEW
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Insights From Single Cell RNA Sequencing Into the Immunology of Type 1 Diabetes- Cell Phenotypes and Antigen Specificity

Stephanie J. Hanna[1]*, *Danijela Tatovic*[1], *Terri C. Thayer*[1,2] and *Colin M. Dayan*[1,3]

[1] Diabetes Research Group, Division of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, United Kingdom, [2] Department of Biological and Chemical Sciences, School of Natural and Social Sciences, Roberts Wesleyan College, Rochester, NY, United States, [3] Wellcome Centre for Human Genetics, University of Oxford, Oxford, United Kingdom

In the past few years, huge advances have been made in techniques to analyse cells at an individual level using RNA sequencing, and many of these have precipitated exciting discoveries in the immunology of type 1 diabetes (T1D). This review will cover the first papers to use scRNAseq to characterise human lymphocyte phenotypes in T1D in the peripheral blood, pancreatic lymph nodes and islets. These have revealed specific genes such as IL-32 that are differentially expressed in islet –specific T cells in T1D. scRNAseq has also revealed wider gene expression patterns that are involved in T1D and can predict its development even predating autoantibody production. Single cell sequencing of TCRs has revealed V genes and CDR3 motifs that are commonly used to target islet autoantigens, although truly public TCRs remain elusive. Little is known about BCR repertoires in T1D, but scRNAseq approaches have revealed that insulin binding BCRs commonly use specific J genes, share motifs between donors and frequently demonstrate poly-reactivity. This review will also summarise new developments in scRNAseq technology, the insights they have given into other diseases and how they could be leveraged to advance research in the type 1 diabetes field to identify novel biomarkers and targets for immunotherapy.

Keywords: type 1 diabetes, scRNAseq, immunology, lymphocytes, TCR - T cell receptor, BCR - B cell receptor

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Edited by:
Anne Cooke,
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***Correspondence:**
Stephanie J. Hanna
HannaSJ@cf.ac.uk

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INTRODUCTION

It is widely accepted that in T1D "there remains a paucity of robust and accepted biomarkers that can effectively inform on the activity of T cells during the natural history of the disease or in response to treatment" (1). Furthermore, the phenotype and roles of autoreactive B cells in T1D have received less attention than T cells (2–4). Whilst flow and mass cytometry approaches have

Abbreviations: AAB+, Autoantibody positive; DE cell, dual expressor cell reported to express both a TCR and a BCR'; DEG, differentially expressed genes; GEX, gene expression; scRNAseq, single cell RNA sequencing.

enabled many insights into cell phenotypes and antigen specificity in type 1 diabetes [reviewed (5)], they allow detection of a relatively small number of markers, limiting the potential to discover truly novel biomarkers. In turn this limits the ability to monitor the natural history of diabetes development and patient responses to immunotherapy. In addition, although a number of immunomodulatory agents are in clinical trials for type 1 diabetes, these are generally non-specific in their actions (for example targeting CD3 or CD20) (6), and there remains a need to identify and target pathways that are perturbed specifically in islet-antigen specific lymphocytes.

Traditional RNA sequencing involves taking all cells of interest, and combining their RNA in a single sample before sequencing. In contrast, single cell RNA sequencing isolates individual cells, either through sorting into wells, or using droplet based technology (7). Transcripts from each cell are barcoded (a unique molecular identifier is also added to each transcript to circumvent any amplification bias), before being combined for sequencing. This allows quantification of the expression of every gene in every individual cell, so that cell phenotypes and heterogeneity can be fully elucidated. Of particular interest to immunologists are scRNAseq methods that allow sequencing across the V(D)J region of TCRs and BCRs. This allows capture of the paired TCRα and β chains (or paired heavy and light chains of BCRs) which is key to determining antigen specificity (8, 9) and being able to reconstitute the receptor in a cell line or to express it as a secreted antibody. A single cell sequencing approach also avoids much of the bias of bulk RNAseq of receptors (10).

There are a variety of methods used for scRNAseq [reviewed (7)] although the 10x Genomics platform has come to dominate the field, due to the relatively large number of cells that can be sampled and options of combining, for example, protein expression and V(D)J sequencing with standard gene expression (GEX) data (11, 12). In parallel, there has been an explosion in techniques to deal with the vast quantity of data generated, perform quality control and extract meaningful findings (13).

However, scRNAseq also comes with a number of caveats. Firstly it is technically challenging and poor sample preparation can lead to doublet formation in a similar manner to that seen in flow cytometry, but in addition scRNAseq samples are susceptible to contamination with RNA from dying cells and the downstream clustering algorithms can also produce seemingly novel cell populations which are in fact artefacts (14). Secondly, the high cost can make it somewhat inaccessible and limit sample numbers and sizes. Lastly, it requires stringent statistical analysis to avoid type 1 errors, preferably backed up by follow up experiments to verify findings (13).

Nevertheless, scRNAseq offers an exciting opportunity to identify novel biomarkers that could be indicative of diabetes progression in at risk individuals, and allow real-time monitoring of clinical trials through tracking expression of specific immuno-receptor sequences and cell phenotypes. Furthermore, it has the potential to discover novel targets for immunotherapy of type 1 diabetes, through the identification of genes that are differentially expressed in islet-antigen specific lymphocytes.

USING scRNAseq TO IDENTIFY BIOMARKERS FOR PROGRESSION TO TYPE 1 DIABETES AND PHENOTYPES IN T1D

scRNAseq's potential is demonstrated in a paper by Kallionpää et al. They revealed that high IL-32 expression in PBMCs was strongly associated with seroconversion and progression to T1D, contributed mainly by activated, highly differentiated, T cells and NK cells. Interestingly insulin (INS), glucagon (GCG), and REG1A were found to be upregulated in T1D and AAB+ individuals in the bulk RNAseq of PBMC but not in scRNAseq (15). These genes are normally associated with the pancreas, but expressed at the mRNA level in whole blood and lymph nodes at much lower levels (www.genecards.org). For insulin in particular this wider expression is thought to be involved in peripheral maintenance of tolerance (16).

We can also glean insight into the immunology of T1D from scRNAseq studies of the pancreas, as in T1D these will include infiltrating immune cells. For example, the Vahedi group performed scRNAseq of human pancreatic islet cells and found particular enrichment of antigen-presenting cells and macrophages in T1D (17). In a strong replication of Kallionpää et al.'s findings, an analysis of differentially expressed genes (DEG) in immune cells between healthy and type 1 diabetes pancreas samples identified REG1B, REG1A, INS and REG3A and IL-32 as highly differentially expressed (17). As with INS, GCG and REG1A, REG1B and REG3A are highly expressed in the pancreas but at lower levels in the blood and lymph nodes. Furthermore REG genes are reported to be upregulated in the pancreas not only in people that have T1D, but also those who are autoantibody positive (18). They are upregulated in inflammatory conditions and are thought to be important in the survival of beta cells in T1D (18). An alternative explanation for the association of these RNA transcripts with immune cells is that RNA transcripts from dying beta cells are contaminating other cell types during the scRNAseq process (19). A similar scRNAseq analysis of the NOD mouse pancreas has also been conducted (20) and scRNAseq has been used to characterise hESCs differentiating into beta cells (21). Studies using scRNAseq to investigate the human pancreas and T1D are summarised in **Table 1**.

Closely related to scRNAseq is scATACseq, whereby the DNA from individual cell nuclei is analysed to identify open or accessible chromatin regions and hence predict which genes are being expressed in each cell. Recently, Chiou et al. combined scATACseq with bulk ATACseq and scRNAseq approaches to link cis-regulatory elements (CREs e.g. gene promoters and enhancers) in peripheral blood cells and pancreatic cells with GWAS of diabetes risk (23). As would be expected this identified

TABLE 1 | human scRNAseq gene expression studies relevant to type 1 diabetes.

Paper	Tissues	Antigen receptors	T1D status	Citation	Notes
Fasolino et al.	Pancreas	no	Healthy donors, AAB+, T1D	(17)	
Kallionpää et al.	PBMC	no	Healthy donors, AAB+	(15)	Children <3 2/4 AAB+ rapidly developed T1D
Xin et al.	Pancreas	no	Healthy donors, T1D	(22)	All islet cells sequenced, but analysis of beta cells only
Chiou et al.	PBMC pancreas	no	Healthy donors, T1D	(23)	scATACseq of PBMCs and pancreas of healthy donors. Reanalysis of healthy donor and T1D islet scRNAseq (22)
Cerosaletti et al.	PBMC	TCRs	Healthy donors, T1D	(24)	Islet-reactive T cells
Fuchs YF et al.	PBMC	TCRs	Healthy donors, T1D	(25)	Only one T1D sample
Culina S et al.	PBMC	TCRs	Healthy donors, T1D	(26)	Sorted ZnT8 186-194 MMr+CD8+ T cells.
Heninger et al.	PBMC	TCRs	Healthy donors, children who later progressed to T1D	(27)	GAD65- and proinsulin-responsive CD4+ T cells, limited genes sequenced in scRNAseq
Ahmed R, et al.	PBMC	TCRs, BCRs	T1D	(28)	Single donor
Hao Y et al.	Pancreas	no	Healthy children-older adults (29) Unspecified (30) Unspecified (31) Healthy controls and T2D (32) Healthy controls and T2D (33) Unspecified (34) Healthy controls (35)	(12)	Combines multiple previous scRNAseq datasets to make a reference dataset and app, Azimuth

many CREs used in T cells and beta cells that had genetic variants associated with T1D susceptibility. For example CREs that controlled CTLA4 and CCR7 expression in T cells had variants associated with T1D. Importantly, this paper also identified CREs used in pancreatic cells that had polymorphisms associated with T1D risk, particularly those used in acinar and ductal cells. They were further able to map the T1D risk allele of rs7795896 to a CRE used in ductal cells. The risk variant was associated with decreased CFTR expression in ductal cells. Mutations in CFTR itself cause cystic fibrosis, frequently associated with pancreatic exocrine and endocrine abnormalities, but this is this first demonstration of a role for it, and may other genes expressed in the exocrine pancreas, in T1D pathogenesis. This paper also produced a reference map of single-cell chromatin accessibility from T1D-relevant cells from healthy donors (i.e. lymphoid, myeloid and pancreatic endocrine and non-endocrine cells). Interestingly scRNAseq of the human pancreas also identified multiple changes in gene expression in ductal cells in T1D (17). In particular expression of MHC Class II pathway and interferon alpha and beta pathway genes were increased. Other developments in the field of epigenetics of T1D and the interplay with environmental triggers [reviewed (36–38)] have also started to yield evidence of pathogenic roles for molecules such as BACH2, IL23A, IL6R and IL6ST in T cell function in T1D (39). It will be of great interest to see how our understanding of epigenetics in T1D develops at the single cell level.

scRNAseq OF TCRS

Methods to Identify Antigen Specific T Cells

As discussed above, there are many advantages of single cell sequencing TCRs over bulk TCR repertoire sequencing. Before the advent of large scale scRNAseq, many people in the type 1 diabetes field appreciated the importance of sequencing immunoreceptors on a single cell basis and linking this to antigen specificity and affinity (40–42). As of 2017 there were 1655 clonotypes of known specificity for T1D autoantigens (41), a number which has increased substantially with the advent of higher throughput scRNAseq.

These have been identified through a numbers of methods. HLA class I or class II multimers may be used to select antigen specific T cells. This has the advantage of being able to select cells from the peripheral blood but is limited by HLA restriction and to known epitopes or mimotopes (43). In addition non-specific binding may yield false-positive TCRs. Alternatively peripheral blood T cells can be stimulated *in vitro* with islet peptide pools and selected on the basis of upregulation of activation markers, allowing wider specificities and HLA compatibilities, but with the risk of bystander activation again resulting in false negatives. A third approach is to sample T cells directly from the pLN or pancreas, where islet-specific T cells will be massively enriched. These cells can then either be stimulated *in vitro* with peptide pools the TCRs re-expressed *ex vivo* to determine specificity. Alternatively, TCR sequences can be compared to those in the literature known to be islet antigen specific.

Diabetes Autoantigen- Specific Paired TCRs in the Peripheral Blood

Eugster and colleagues performed an heroic effort to sequence paired TCRs from 1650 T cells that either bound a GAD tetramer or responded to GAD *in vitro*, by sorting single cells from the peripheral blood and performing plate based scRNAseq (44). GAD specific TCRs were highly heterogenous both within and between donors, with no shared TCRs between donors, although individual TCRα or TCRβ chains were often shared. Moreover, there was limited overlap between the TCRs identified by tetramer binding and T cell activation methods, indicating that epitope recognition and MHC usage by GAD specific TCRs was likely to be broad (44).

Cerosaletti et al. performed scRNAseq of islet-reactive TCRs from the peripheral blood (identified by *ex vivo* response to stimulation with islet-peptide pools). They found that T cells from T1D had higher numbers of identical CDR3, which had arisen by clonal expansion (i.e. T cells with identical TCRα and TCRβ chains, that have arisen by division of a parent cell), rather than convergent recombination (24). By re-expressing the TCRs in cell lines it was found that many of these TCRs in people with T1D were IGRP specific (24). It was further shown that donors with T1D had large clonal expansions of IGRP-reactive T cells in the peripheral blood and frequently used a specific shared TCRα chain, which was paired with different beta chains in each donor (45). Preferential usage of TRAJ53 and TRAV29 and TCRα chains bearing the motif SGGSNYKLTF were identified in single cell TCR sequencing of people with T1D. When a bulk sequencing approach was taken, a particular TCRα chain bearing this motif was highly enriched in the memory CD8+ T cells of autoantibody positive people and those with T1D compared to controls. Clones bearing the motif were also shown to directly kill IGRP- peptide bearing cells (45). T cell clones bearing both IGRP (24, 45) and hybrid insulin peptide-responsive TCRs are persistent over time (46). However, others have examined TCR repertoires in children progressing to diabetes and shared TCRs were not seen either between children or within the same child over time, indicating high diversity in the peripheral blood at this age (27).

Diabetes Autoantigen- Specific Paired TCRs in the Pancreas and Pancreatic Lymph Nodes

Early work examined T cells from the pancreatic lymph nodes (pLN) of people with T1D and found high clonal expansions (47). Additionally there were many T cells that shared a TCRβ but had divergent TCRα. Many clonally expanded CD4+ T cells recognised insulin A1-15 in the context of DR4 (47). Pathiraja et al. grew out CD4+T cells from the pancreatic islets of a donor with T1D using anti CD3 and cytokine stimulation. Over 25% of these clones had TCRs that responded to proinsulin peptides restricted by HLA-DQ8 or the HLA-DQ8 transdimer and 30% of clones used TRBV5–1*01 (48). Whilst it is difficult to make direct comparisons to frequencies of islet-reactive T cells in the peripheral blood (26), it is clear that in the peripheral blood frequencies are much lower [around 0.01-0.05% of T cells in people with T1D (3, 44, 49)]. Most T cells isolated from the pancreas had unique clonotypes, whilst the majority of *in vivo* clonally expanded T cells were specific for proinsulin (48). It has also been found that ZnT8- reactive T cells were present at similar frequencies in the blood of healthy controls and people with T1D, but were enriched in the pancreas of the latter (26). Single cell sequencing of TCRs found a public ZnT8 specific CDR3B in the peripheral blood, and enriched in the pancreas of people with T1D, although the full TCRβ had divergent sequences due to different gene usages. ZnT8 reactive T cells also showed a bias towards TRBV19 and TRAV12-2 usage (26).

Seay et al. also found sharing of CDR3s between donors in the pancreas, with a TCRβ with homology to a known GAD reactive TCR found in 7/18 T1D donors (50). Furthermore a shared CDR3β chain was found in all people with T1D in the conventional T cell compartment, whilst in healthy controls it was predominantly in the Treg compartment (50). Interestingly TCR sequencing of GAD-responsive CD4+IL-13+ T cells from patients who had received injected GAD Alum found that they often used a highly public TCRβ (TCRα sequencing was not available) (51).

Direct capture of pancreatic T cells by the Nakayama group enabled single cell TCR sequencing and confirmed infiltration of proinsulin specific cells into the pancreas in T1D. Of the hundreds of TCRs sequenced, most were present only on a single cell, indicating a diversity of response even years after diagnosis. Clonal expansions were more likely in CD8+ T cells and these clones were found in multiple islets from the same donor, indicating *in vivo* migration. Furthermore, across three donors it was noted that whilst there were no identical TCRs, there were identical TCRα sequences and TCR subunits (52). When the TCRs were re-expressed, the B9-23 reactive TCRs isolated from the pancreas induced much higher IL-2 secretion compared to control B9-23 TCRs isolated from peripheral blood (52) which may indicate the former have a higher affinity for B9-23. Moreover, only the pancreas-derived TCRs were capable of a response to whole proinsulin presented by APCs (52).

Recently the Nakayama group has reconstructed individual TCRs from the pancreas of people with T1D. TCRs were selected for re-expression on the basis of clonal expansion or V gene usage previously associated with proinsulin C19-A3 specificity, and were found to recognise epitopes across preproinsulin and presented by a variety of MHC class II (53). Many TCRs recognised peptides in the region of B9-23, but others, (many from clonally expanded cells) recognised peptide right across from the signal peptide to the A chain. Furthermore, these TCRs recognised peptides in the context of diverse MHCII, although a preference was shown for DQ (53). Even with these constraints of the selection criteria in this study, this single cell approach showed a diversity of peptide and MHCII specificity that would have been missed using tetramers.

New Avenues for scRNAseq of TCRs

Taken together, the evidence suggests that T cells with TCRs with higher affinity for diabetes autoantigens are more likely to be found in the pancreas than in the peripheral circulation. This represents a major challenge in T1D research as in other autoimmune diseases it is relatively straightforward to obtain samples from the site of autoimmune attack (54). For example in psoriatic arthritis, extraction of viable T cells directly from the affected joints enabled sequencing of paired TCR receptors and scRNAseq profiling of cells phenotypes (55). Even in the pancreas, clonal expansion is modest and whilst CDR3 sequences specific for many diabetes autoantigens are shared between donors, there is not yet evidence of truly public TCRs with identical TCRα and β chains. However, more widespread use of the VDJdb repository (56), IEDB (57) and the JDRF/nPOD CloneSearch might allow enriched motifs to become apparent across different experiments, although this would still

be limited by HLA restriction. To further complicate the picture, scRNAseq has demonstrated that islet antigen –reactive T cells (24) and HIP reactive T cells in particular (58) sometimes express two TCRα chains, which are known to contribute to autoimmunity (59, 60).

Phenotypes of Antigen-Specific T Cells: Combining TCR Sequencing With Gene Expression

Combining TCR sequencing (or selection based on autoantigen reactivity), with scRNAseq has the potential to give further insights into T cell function. This has not always been straightforward to demonstrate, for example analysis of IGRP- specific T cells from the peripheral blood did not show a distinctive gene expression (GEX) pattern in response to stimulation (25). Similarly scRNAseq of ZnT8 reactive cells from the peripheral blood of people with T1D showed similar GEX profiles to healthy controls, indicating that these peripheral T cells may not be playing a driving role in T1D, although T1D patients had higher expression of aryl hydrocarbon receptor (AHR) and aurora kinase A (AURKA) and lower expression of RORA (26).

The approach was more successful for Heninger et al, who demonstrated that proinflammatory responses to diabetes autoantigens were dominant in children who progressed to autoantibody positivity, whilst regulatory T cell responses were seen in those who didn't (27). An algorithm based on gene expression in response to autoantigens enabled identification of which children would later progress to autoantibody positivity. As this group developed autoantibodies the GEX profiles of their CD4+ T cells changed towards increased expression of Th1 genes (27). These findings suggest that biomarkers of T1D susceptibility may allow identification of at risk children prior to seroconversion (61).

In addition, Cerosaletti et al. used islet peptide pools to stimulate T cells from the peripheral blood *in vitro* and characterised those that activated by scRNAseq. They did not observe a significant level of differentially expressed genes between healthy controls to those from people with T1D. However, when they focussed on cells from people with T1D that were highly clonally expanded (termed T1D-E cells), they found that these cells did have a unique transcriptional profile compared to islet reactive T cells from healthy controls or those from people with T1D that were not clonally expanded. T1D-E cells preferentially expressed genes associated with T cell activation and leukocyte differentiation (24). These experiments demonstrate how focussing on antigen specificity can enhance findings from scRNAseq.

scRNAseq OF BCRS

Early Work to Determine Antibody Sequences

Early interest in autoantibodies in T1D, before the advent of scRNAseq, focussed on isolating GAD-specific B cells from people with T1D (62) and sequencing the BCRs from clones, which provided evidence that GAD autoantibodies have frequently undergone somatic maturation and are therefore from antigen-experienced B cells (63, 64). Similarly IA-2 specific antibodies sequenced from B cells from people with T1D also show evidence of somatic mutation (65–67). Anti-insulin antibodies have been sequenced from people with T1D, but may have arisen in response to injected insulin rather than endogenous insulin (68, 69) [reviewed (70)]. BCR sequencing combined with phenotyping of B cells has given great insight into B cell response in other autoimmune diseases (71) and in response to vaccinations (72) and in B cell lymphoma (73). Yet without the equivalent of a tetramer approach to identifying autoreactive B cells, phenotyping and characterisation of the BCR has lagged behind T cell research in T1D.

Identifying Islet-Reactive BCRs in the Periphery, Pancreatic Lymph Nodes and Pancreas

Smith et al. developed an approach to isolate insulin reactive B cells from the peripheral blood of people with T1D, by flow cytometric sorting B cells that bound insulin conjugated to fluorescent tags. The authors were then able to sequence BCRs from individual cells. They demonstrated that insulin binding BCRs preferentially used JH6 gene segments which have previously been associated with autoreactivity and were biased towards use of positively charged amino acids in the CDR3 region (74). When re-expressed as antibodies, BCRs from anergic naive IgD^+, IgM^- B cells demonstrated binding at levels thought to induce anergic B cell responses, whilst those from naïve B cells bound weakly and would likely be ignorant of insulin under physiological conditions (74).

scRNAseq has been used to characterise a novel lymphocyte population that express both TCRs and BCRs (28). It is suggested that these "dual expressors" (DE) are increased in frequency in type 1 diabetes and that in people with type 1 diabetes there is a public BCR which can stimulate insulin-reactive CD4+ T cells. However, this work remains controversial as others have been unable to replicate the enrichment of DE in T1D nor the specific public BCR sequence (75). This highlights the importance of good quality control at every step of scRNAseq experiments.

Isolation of CD19+IgG+ B cells from pancreatic lymph nodes from autoantibody positive donors and single cell sequencing of their BCRs demonstrated that no clonally expanded B cells were identified in the pLN. Antibodies were reconstructed from BCR sequencing, although very few of these were found to be specific for IA2 (none were specific for GAD and insulin was not tested) (76). Seay et al. also sorted and single cell sequenced the BCRs from pancreatic LNs. They found an enrichment of insulin binding motifs in pLN from people with T1D compared to controls (50). They also observed sequence overlap with autoreactive BCRs cloned from precursor (early immature) B cells from healthy donors previously published by Wardemann et al. (77). Wardemann et al. observed that not only are many BCRs from healthy donor precursor B cells insulin reactive, they are often also polyreactive to other autoantigens for example dsDNA, ssDNA or nuclear proteins (77). This polyreactivity has also been noted for both IgM and IgG insulin antibodies (68).

Similarly Smith et al. demonstrated that all of their high affinity insulin binding BCRs were also reactive to LPS and chromatin (74). Polyreactive antibodies have been postulated to play a key role in the healthy immune system but are also implicated in a variety of autoimmune diseases (78, 79). It therefore appears that autoreactive B cells in T1D may span a wide range of phenotypes and the antibodies produced may often be polyreactive, however the limited number of studies make it difficult to draw firm conclusions.

FUTURE PERSPECTIVES ON scRNAseq IN TYPE 1 DIABETES

New Single Cell Methods and Analysis Tools

scRNAseq is beginning to give fascinating insights into type 1 diabetes and new approaches may yield further discoveries. The first of these is spatial transcriptomics (80). In this technique, indexed oligos capture RNA from either fresh-frozen or formalin-fixed, paraffin-embedded tissue sections. This allows determination of gene expression on a level that is fast approaching single cell resolution. It has already been used to give insights into cell interactions in other diseases such as rheumatoid arthritis, where infiltrating leukocytes interact with target cells (81, 82). Spatial transcriptomics therefore has great potential to unravel lymphocyte interactions with beta cells in the pancreas and to give insight into different patterns of immune cell infiltration (2). In both type 1 (23) and type 2 diabetes (83) scATACseq has recently been used to link GWAS to epigenetic regulation of gene expression. New methodologies enabling combination of ATACseq, and CITEseq with scRNAseq in the same experiment will also contribute to the field (84). New analysis tools such as CellPhoneDB give the ability to map interactions between subsets of cells, based on DEG in scRNAseq datasets, which would allow identification of novel interactions between immune cells and beta cells in the pancreas (85, 86). This may become increasingly important as we begin to understand the role of beta cell stress and signalling in type 1 diabetes (6) as well as the involvement of other pancreatic cells in diabetes development (23). CellPhoneDB has been used to identify crosstalk between T cells and epithelial cells in ulcerative colitis (87) whilst in rheumatoid arthritis scRNAseq has revealed interaction pathways between B cells, fibroblasts and monocytes (88). Additionally, recent work from the Satija lab has brought together previously published scRNAseq datasets of pancreatic cells, including immune cells from healthy pancreatic samples (12), which will facilitate this type of analysis. This would be further enhanced were there a unified repository for T1D scRNAseq datasets, similar to those for COVID-19 (89).

Technological and Analytical Approaches to Enhance Immunoreceptor Sequencing

We have seen how combining GEX with V(D)J sequencing has increased insights into T1D. The recent development of DNA barcoded multimers will allow now the determination of T cell antigen specificity in scRNAseq experiments (90, 91), whilst conjugation of whole proteins or large folded protein fragments to DNA barcodes will facilitate identification of antigen specific B cells (92).

Computational approaches to determine the likely interaction of an immunoreceptor with target antigen also have the potential to revolutionise the search for antigen specific TCRs and BCRs. Approaches such as tcrdist (93), GLIPH (94) and immune receptor network generation for BCRs (95) enable BCR and TCR sequences to be mapped and visualised, and those that differ by only one or two amino acids are assumed to target the same antigens. NetTCR (96) and TCRex (97) use neural networks and machine learning algorithms to cluster TCRs predicited to bind the same epitope. Recent advances such as ICON and TCRAI leverage scRNAseq technology along with oligo labelled dextramers. They utilise the paired TCRα and TCRβ transcripts to build libraries of antigen specific receptors, with a neural network to predict antigen specificity of TCRs. However, many of these approaches have been validated using viral or tumour antigens with well-defined epitopes. As we have seen in the sections above, whilst there definitely are peptide sequences from diabetes autoantigens that are widely recognised, the immune response also targets diverse sequences in different individuals. Furthermore, auto-antigenic TCRs tend to bind pMHC with lower affinity than TCRs targeting pathogens (98, 99) as high affinity self-reactive TCRs are generally deleted in the thymus. It is not clear how this lower affinity and lack of public TCRs may impact upon the usefulness of computational approaches for T1D.

Biomarkers in Clinical Trials

In 2019, it was demonstrated that teplizumab could delay progression to T1D in high risk individuals (100). Further work confirmed a correlation between fold change in C-peptide and change in frequency of CD8+KLRG1+TIGIT+ T cells (101). scRNAseq of T cells from the clinical trials of teplizumab and other immunotherapies in T1D could offer an amazing opportunity to identify all biomarkers predictive of successful treatment. For example, scRNAseq studies have shown a variety of phenotypic markers induced *in vitro* with anti-CD3 antibodies in human PBMC, including a variety of interleukin receptors and markers of regulation and exhaustion including FOXP3, CTLA4, TNFRSF18, LAG3 and PDCD1 (102). In contrast, anti-CD3/CD28 stimulation of PBMC analysed with scRNAseq and CITEseq, showed phenotypes strongly associated with activation (although memory subsets also upregulated senescence) (103).

In the future, a deeper understanding of TCRs and BCRs has the potential to better quantify the risk of progression in autoantibody positive people. Monitoring the abundance and phenotypes of lymphocytes bearing specific CDR3 sequences or using specific V genes may also prove useful in monitoring immunotherapies, particularly antigen specific immunotherapies, where phenotypic changes in whole lymphocyte populations may not be so obvious (1, 104–106). In addition, BCRs also have the potential to be used in CAR-Treg cell immunotherapy as has been demonstrated in the NOD mouse (107).

A Computational Approach to Move Beyond scRNAseq

scRNAseq has demonstrated its great potential to identify novel biomarkers both in T1D and other autoimmune diseases. However, it is both technically challenging and expensive. Therefore it is crucial that researchers should be able to translate findings from scRNAseq into more accessible diagnostic and monitoring tests, for example using standardised flow cytometry or qPCR panels as is starting to happen in cancer research (108, 109). Similarly in IBD, a machine learning approach allowed identification of a CD8+ T cell signature that could predict prognosis. These biomarkers were then developed into a commercially available whole blood qPCR test to facilitate personalised therapy (110).

In T1D, recent advances in computational analysis are beginning to allow discrimination of changes in cell subsets from bulk RNAseq. Mehdi et al. identified a peripheral blood transcriptomic signature that predicted autoantibody development (111). Of the DEG identified, many were associated with the ubiquitin-proteasome pathway, DC and T cell function and were potentially targets of drugs approved for other conditions (111). Xhonneux et al. (112), demonstrated from transcriptomics of whole blood that they could undertake "digital cytometry", by mapping groups of genes back to cell types. Children who developed autoantibodies against insulin first, had a signature of increased NK cells and CD4+ memory T cells. In contrast, those who first developed autoantibodies to GAD had a reduced percentage of CD4+ memory T cells and NK cells, but increased activated NK cells. Harmonizome (113) was used to identify a G protein–coupled receptor, GPR171, predicted to control the immune signature found in IAA+ children (112). Adding gene expression information to predictive models, increased their accuracy in predicting later T1D development in children under 18 months (112).

DISCUSSION

The first papers to analyse lymphocytes from type 1 diabetes using scRNAseq have provided fascinating insights into phenotypes involved in driving the disease and identified new potential targets for immunotherapy, such as IL-32 (15, 17). scRNAseq of TCRs involved in T1D has revealed that autoantigen specific TCRs have a wide range of targets and that whilst single chains or CDR3s are often shared between donors, it is rare to see TCRs with both chains identical in multiple donors; hence public TCRs remain elusive. In the peripheral blood, diabetes autoantigen reactive cells do not always have distinct phenotypes in healthy donors compared to those with T1D (25, 26), and enrichment of islet reactive cells is much more pronounced in the pancreas and pancreatic lymph nodes. Combining TCR sequencing with T cell phenotyping has led to a deeper understanding of islet antigen-specific cells in the peripheral blood (24, 27). A key challenge, for which scRNAseq is ideally suited, will be to develop methods to identify which T cells in the periphery are truly involved in beta cell destruction, and which are simply able to bind islet antigen multimers but are not capable of either trafficking to the islets or contributing to beta cell killing. Looking to the future, it is clear that combining antigen specificity with scRNA phenotyping and new computational approaches, such as those that can give insight into interactions between islet cells and infiltrating lymphocytes, have the potential to revolutionise the field.

Relatively few papers have tackled single cell sequencing (or indeed bulk sequencing) of BCRs repertoires in T1D, but those available suggest that these BCRs have unique properties and are often polyreactive (50, 74, 77). New approaches to identify islet-antigen specific B cells with scRNAseq (92) will therefore have much to contribute to our knowledge of how islet autoantibodies develop and are involved in disease progression.

scRNAseq is ideally suited to identifying subtle phenotypic differences between cohorts and has demonstrated promise in identifying differentially expressed genes in people that will later progress to autoantibody positivity and T1D (27). Developing this approach will be key to identifying at-risk individuals and matching them to a novel immunotherapy that is appropriate for their stage and phenotype of disease (100, 101, 114, 115). Furthermore, new analytical approaches will enable scRNAseq findings to be translated into new immunotherapies and biomarkers to monitor effectiveness of those already in clinical trials.

AUTHOR CONTRIBUTIONS

SH wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Environmental Determinants of Type 1 Diabetes: From Association to Proving Causality

Lauren M. Quinn[1,2]*, *F. Susan Wong*[3] *and Parth Narendran*[1,2]

[1] Institute of Immunology and Immunotherapy, Research College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom, [2] Diabetes Research Group, Division of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, United Kingdom, [3] Department of Diabetes, University Hospitals of Birmingham NHS Foundation Trust, Birmingham, United Kingdom

The rising incidence of type 1 diabetes (T1D) cannot be ascribed to genetics alone, and causative environmental triggers and drivers must also be contributing. The prospective TEDDY study has provided the greatest contributions in modern time, by addressing misconceptions and refining the search strategy for the future. This review outlines the evidence to date to support the pathways from association to causality, across all stages of T1D (seroconversion to beta cell failure). We focus on infections and vaccinations; infant growth and childhood obesity; the gut microbiome and the lifestyle factors which cultivate it. Of these, the environmental determinants which have the most supporting evidence are enterovirus infection, rapid weight gain in early life, and the microbiome. We provide an infographic illustrating the key environmental determinants in T1D and their likelihood of effect. The next steps are to investigate these environmental triggers, ideally though gold-standard randomised controlled trials and further prospective studies, to help explore public health prevention strategies.

Keywords: type 1 diabetes (T1D), seroconversion, auto-antibodies, autoimmunity, environmental factors, gut micro biome, obesity, infection - immunology

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University of Baghdad, Iraq

***Correspondence:**
Lauren M. Quinn
l.quinn.1@bham.ac.uk

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INTRODUCTION

An estimated 1.1 million people under 20 years of age are affected by type 1 diabetes (T1D) worldwide (1, 2). T1D represents 5-10% of the global diabetes burden (3) and is not a disease of childhood alone, with almost half diagnosed in adulthood (4–6). Overall annual increase in T1D is estimated at 3% (2-5%) (1, 7), with rising trends observed across all age groups over the last three decades (1, 3). Some of the greatest increases are observed in historically low prevalence countries (4, 8).

T1D is a chronic autoimmune condition, characterised by hyperglycaemia and long-term insulin dependency (9). T1D pathophysiology is defined by three stages of disease progression (10). Stage one is seroconversion to one or more autoantibodies (10), including glutamic acid decarboxylase (GAD), anti-insulin, insulinoma-associated antigen 2 (IA2) and zinc-transporter 8 (Zn-T8). Presence of two or more antibodies will see 70% of children develop T1D in the next 10 years, whilst four autoantibodies invariably confer 100% risk (11). Stage 2 is damage to the beta-cells causing pre-symptomatic dysglycaemia and stage 3 is overt T1D due to beta-cell failure with requirement for

exogenous insulin (10). This provides different targets for prevention at the stages of primary prevention (preventing seroconversion, in those genetically at risk), and secondary prevention (preventing loss of and damage to the beta-cells in individuals with autoimmunity/autoantibodies) (12).

The primary risk factor for T1D is genetic. It is strongly associated with HLA-DR3-DQ2 and/or HLA-DR4-DQ8 haplotypes (13). The significance of genetics is further evidenced by the increased risk observed where a sibling (8%), father (5%) or mother (3%) has T1D. The major histocompatibility complex (MHC) encoding the HLA region confers 50% of the genetic risk for T1D (13). Genome-wide association studies have identified an additional 50 loci that confer susceptibility (13).

However, genetics alone does not equate to causality and an array of environmental factors are implicated to trigger T1D seroconversion and disease progression (14). The threshold hypothesis describes a model in which genetic and environmental factors represent intersecting and reciprocal trend lines, defined by odds ratios in rank order, which cumulatively confer risk of progression to T1D until a critical threshold is met (15).

Evidence to support the nurture side of the argument for T1D continues to expand. Firstly, the rising incidence of T1D in recent decades is too rapid to be explained by genetic changes alone (8). Secondly, T1D concordance rates between monozygotic twins is <50% (16). Thirdly, T1D incidence also demonstrates significant geographic and latitudinal differences, with higher incidence in Nordic countries (17–20), and migration studies show that the incident risk of the new location is assumed (21). Fourthly, incidence is increasing across all age groups, and the incidence in younger children is rising (1), despite highest risk genotypes declining over the last 20-40 years (22, 23). The highest rates are observed in previously low incidence countries and countries with the highest economic growth (8).

It may be more than coincidence that T1D is therefore a heterogenous condition determined by a combination of genetic, immunological, and metabolic factors, and this complexity reflects the array of environmental triggers implicated in the pathogenesis.

Numerous other reviews have explored environmental contributions to T1D (8, 12, 14, 24, 25). These have largely outlined association studies between different environmental determinants and the development of T1D. Some of these reviews also predate the results of the seminal, 'The Environmental Determinants of Diabetes in the Young' (TEDDY) study, from which different aspects have been published over the last 6 years (26).

In this narrative review, we explore the putative environmental risk factors for T1D with an emphasis on testing causality. Since causality is best tested in the setting of a double-blind randomised controlled trial (RCT), we outline the different RCTs in this area. As part of the review, we outline potential underlying mechanisms to the different environmental determinants and the stage of pre-T1D at which they could exert an influence.

We undertook this review though searching PubMed and Medline. We used the following search terms 'environment AND type 1 diabetes'; type 1 diabetes (T1D) OR islet autoimmunity (IA) combined with the following - enterovirus; rotavirus; influenza; COVID-19; vaccine OR vaccination; birth weight; weight gain; BMI; childhood obesity; gut microbiome OR gut microbiota; diet; breast milk OR breastfeeding; cow's milk; formula milk; gluten; antibiotic; probiotic; vitamin D; and nicotinamide; omega-3. We included systematic reviews, meta-analyses, RCT, cohort studies (prospective and retrospective) and case-control studies. We have included the largest retrospective and prospective European cohort and case control studies, including 'Type 1 Diabetes Prediction and Prevention' (DIPP) (27), 'Early Childhood Diabetes in Finland' (DiMe) (28), 'Danish National Birth Cohort' (DNBC), 'Norwegian Mother and Child cohort' (MOBA) (29), 'Diabetes Autoimmunity Study in the Young' (DAISY) (30), DIABIMMUNE, Environmental Triggers of T1D (MIDIA - Norwegian acronym) (31), Finnish Dietary Intervention Trial for the Prevention of T1D (FINDIA) (32), TEDDY study (26), and trials including 'Trial to Reduce IDDM in the Genetically at Risk' (TRIGR) (33), the 'European Nicotinamide Diabetes Intervention Trial' (ENDIT) (34), and the Deutsche Nicotinamide Intervention Study (DENIS) (35). Reference lists were also screened for relevant articles.

INFECTIONS AND VACCINATIONS

Viruses are important contenders for environmental triggers of T1D (36, 37).

Acute fulminant diabetes, termed type 1b diabetes, is reported following infection with Mumps, Coxsackie B3 and B4, Rubella, and Influenza B infection (38). The hyperglycaemic ketosis is sudden, symptoms occur for one week, islet antibodies are negative, and C-peptide is extremely low. In this instance, beta-cell damage occurs secondary to direct lytic effects from viral invasion, causing widespread beta-cell destruction and absolute insulin deficiency, without autoimmunity (37, 38).

An alternative association, expanded below, appears to be a more chronic, repeated viral exposure. Implicated mechanisms here include molecular mimicry, where viral epitope sequences bear resemblance to beta-cell antigens and potentially trigger a cross-reactive autoimmune response (36). Viral infection of beta-cells will also result in over-expression of MHC class I, resulting in presentation of self-antigens and perpetuation of further autoimmunity (36, 37, 39). Viruses implicated in this more chronic-repeated infection model are outlined below.

Enterovirus

The most robust evidence for a viral trigger exists for enteroviruses (EV) (40–42). T1D incidence correlates with enteroviral infection rates and the seasonal variation in T1D is preceded by enteroviral epidemics (41, 43). However, EV is a common childhood infection, hence HLA susceptibility to T1D, combined with genetically determined susceptibility and

inflammatory response to EV are critical determinants of risk (41, 44–46). EV infection potentially initiates and accelerates all three stages of T1D pathophysiology (42, 43).

EV spreads and replicates *via* the upper respiratory and gastrointestinal tracts, and invades the beta islet cells *via* the coronavirus adeno receptor (CAR) (36). Inefficient viral clearage of EV (47) and induction of a chemokine response from the beta-cells triggers islet autoimmunity (IA) through molecular mimicry, inflammation, bystander effects, and T cell suppression (48). EV chronicity appears critical to sustain beta-cell autoimmunity; repeated infection with EV strains further increases risk (36).

Systematic reviews (41, 49) and cohort studies (27, 28, 50, 51) demonstrate positive associations between persistent EV infection, autoimmunity, and progression to stage 3. The DiMe study (28) and the DIPP (27) study showed that EV infection in pregnancy or early childhood respectively increased risk of T1D. Interestingly, studies show evidence of seroconversion to islet autoantibodies in both mother postnatally and child, following enteroviral infection during pregnancy (52, 53). A meta-analysis showed that maternal infections were also significantly associated with T1D progression, and most notably for maternal enterovirus infections, odds ratio (OR) 1.54 (confidence interval (CI) 1.05-2.27) (54). The postulated mechanism is transfer of epitopes/molecular mimicry that triggers autoimmunity in the offspring, but the supporting evidence is limited. Cross-reactivity between EV with GAD and IA2 epitopes could trigger seroconversion, but equally, secondary to chronic and cumulative viral infections, primed auto-reactive T cells could promote progression to stage 2, in auto-antibody positive individuals (36).

Most recently, the TEDDY study showed from a genetically predisposed cohort of children (n=8676) followed-up over 15 years, that chronicity of Coxsachie B (EVB), from persistent shedding in the stool, predicted development of IA, particularly anti-insulin antibodies (51, 55, 56). Conversely, acute EVB infection, without prolonged stool shedding, did not associate with autoimmunity or T1D in this cohort (51). A systematic review and meta-analysis by Yeung et al. (41) further showed that persistent EVB increases risk of IA and T1D with an OR of 3.7 (CI: 2.1-6.8) and 9.8 (5.5-17.4) respectively. Problems remain in detecting the specific strain(s) of EV that confer the highest risk.

Based on this strong association between chronic EV infection and T1D (51, 56), a RCT with a polyvalent vaccine to EV is currently planned. An EV vaccine would target primary and secondary prevention by reducing recurrent EV infection, limiting exposure to potential reactive epitopes and chronic beta-cell inflammation that could contribute to IA (48, 57).

Rotavirus

Introduction of the childhood rotavirus (RV) vaccination in Australia, and the fall in T1D incidence that followed, was postulated to implicate a causal environmental trigger (58). The outer capsid protein of the human RV (VP7) shares 56% identity and 100% similarity with a dominant epitope in IA2, and both bind to (HLA-DR4) (*0401) (59), suggesting molecular mimicry and cross-reactive T cells as mediators of IA (59, 60). However, the DIPP longitudinal cohort study exploring RV in T1D pathophysiology failed to show an association between RV and auto-antibodies (61). A study demonstrated that RV infection prior to 6 months of age was significantly associated with human and bovine-insulin binding antibodies, but this was strongest in children receiving cow's milk prior to 3 months (62), representing an important confounding factor.

An Australian interrupted time series analysis found a 15% T1D risk reduction in children aged 0-4 years receiving the RV vaccine compared to those not vaccinated (58). However, the Finnish population-based study with 11–14 year follow-up did not replicate these findings (63, 64). Although Rogers et al. (65) found a 33% reduced risk of T1D in vaccinated compared to unvaccinated children, Burke et al. found no association between RV vaccination and T1D incidence in a US cohort of children with commercial insurance (66). Similarly, Glanz et al. (67) found no association. Hence the evidence supporting RV vaccination as a protective environmental factor is inconclusive.

Influenza

Studies investigating the impact of the influenza infection on stage 1 and 2 T1D risk, have also been inconclusive. Valdes et al. (68) and Nenna et al. (69) showed increased risk of T1D following influenza infection whereas Kondrashova et al. (70) showed no increased risk in children genetically susceptible to T1D.

With regard to vaccination, Ruiz et al. (71) and Bardage et al. (72) showed no association between T1D risk and influenza vaccination. The Pandemrix vaccine, which caused narcolepsy in genetically susceptible individuals, raised concerns for T1D cross-reactivity and was investigated in the TEDDY study in a Finnish and Swedish cohort (73). Here, the Pandemrix vaccine did not increase risk of seroconversion for one or two islet autoantibodies [HR 0.75 (0.55-1.03) and HR 0.85 (0.57-1.26)] respectively, after adjusting for confounders (73). Interestingly, 73% received a second dose in Sweden compared to 0.6% in Finland, and the Finnish cohort had a lower risk of IA; one antibody [HR (0.47 (0.29-0.75)], or more than one antibody [HR 0.50 (0.28-0.90)], and risk of T1D [HR 0.38 (0.20-0.72)] (73). The TEDDY study is ongoing to explore links between influenza vaccination and progression to stage 3 T1D, but no RCTs are planned to test the associates listed above.

COVID-19

Individuals with T1D are at greater risk of the Sars-CoV2 coronavirus (COVID-19) and more susceptible to severe infection (74). Global studies have suggested an increased incidence of T1D during the COVID-19 pandemic and higher frequency of presentation in severe diabetic ketoacidosis (DKA) (37, 75–78). However, important confounders include delay in seeking medical assistance and the resulting later presentations in DKA (37, 75, 78), as well as the high numbers of patients with type 2 diabetes presenting in DKA. The latter is evidenced by cases of COVID-19 associated DKA who were eventually weaned off insulin onto oral therapies (79, 80).

The coronavirus gains access to lung and gut epithelium *via* the angiotensin converting enzyme-2 (ACE2) functional receptor, which is also highly expressed in islet cells (81–83). The previous SARS-CoV1 2003 epidemic was associated with elevated fasting plasma glucose, with hyperglycaemia as an independent predictor of morbidity and mortality, even in mild pneumonitis cases with no steroid requirement (84–86). Similarly, in SARS-CoV2, hyperglycaemia in non-diabetic patients is attributed to the inflammatory response and cytokine activation, in addition to viral infection of beta-cells, which decreases insulin production (37, 86). It remains unclear if COVID-19 is simply triggering a fulminant diabetogenic state or presents the final trigger for T1D progression. However, in the former scenario, the beta-cell damage has not always persisted, evidenced by cases that were weaned off insulin and onto oral therapies (79, 80). Moving forward, the CoviDiab study (87) and roll-out of COVID-19 vaccination programmes (88) may help delineate causal relationships between COVID-19 and T1D risk.

Vaccinations

The steady rise in autoimmune and allergic diseases in industrialised countries has been linked to a reduction in infectious diseases. Contributing factors include geography and climate (North-South gradient), childhood mixing and subsequent exposure to childhood infection (20), and vaccination programmes, and this relationship underlines the hygiene hypothesis. The incidence of T1D is lower in countries without nationwide vaccination programmes and contrasts starkly to countries with established (or newly implemented) vaccination programmes, where there is a rising T1D incidence (8). *In vivo* studies have shown in nonobese diabetic mice for T1D models, T1D incidence is higher in the mice bred in specific pathogen free (SPF) conditions compared to those bred in conventional facilities (20, 89, 90).

In terms of vaccinations being directly linked to T1D, cohort studies and meta-analysis performed to explore a causative association have failed to identify a link to date (91, 92). Childhood vaccinations for measles, rubella, mumps, pertussis, Bacillus Calmette-Guerin (BCG), Haemophilus influenza B (HiB), Tetanus Diptheria poliomyelitis, Measles/Mumps/Rubella and Diptheria/Tetanus/Pertussis showed no association with T1D, harmful or protective (91). Kühtreiber et al. performed a randomised 8 year study in T1D and showed that three years after receiving two doses of the BCG vaccine, HbA1c was lowered to near normal levels for five years (93). The mechanism was demonstrated *in vitro* and *in vivo*, and can be explained by a metabolism shift from oxidative phosphorylation to aerobic glycolysis, the latter of which is a high glucose usage state. The BCG vaccine also had a role in re-programming tolerance, through epigenetic demethylation of regulatory T cell signature genes, resulting in upregulation of mRNA expression and subsequent induction of T regulatory cells (93).

Infections and Vaccinations – A Summary

The evidence of viral infections as a risk factor for T1D is strong. The strongest evidence appears to be through a direct lytic effect on beta-cells (37), for example following infection with mumps. Alternatively, the more insidious classical autoimmune T1D appears to have the strongest association with EV infection (36) and RCT involving vaccination programmes to test a causative link are in development. Associations between T1D and RV or COVID-19 need further evidence - either way there are other major population benefits to vaccinating against these two diseases. There is currently no evidence that any of the childhood vaccination programmes associate with T1D risk.

BIRTH WEIGHT, INFANT GROWTH, AND CHILDHOOD OBESITY

Worldwide prevalence of obesity has risen to 5.6% in girls and 7.8% in boys (94), which is a 10-fold rise in four decades (8, 95–98). The roots lie in genetic, epigenetic, and environmental factors (99, 100). We have observed a rising incidence of childhood obesity along with that of T1D, depicting a double diabetes state which combines features of autoimmunity with insulin resistance (8, 99, 101). The obesity induced insulin resistance in children, increases the burden on the islets cells and potentially initiates, and accelerates the autoimmune processes in genetically predisposed individuals (102, 103). This has been termed the accelerator hypothesis (102, 103). Adiposity also contributes to systemic chronic inflammation, which together contributes to beta-cell damage and apoptosis, and progression to stage 2 and 3 of T1D (104).

It has been suggested that the stress on these beta-cells, either through inflammation or metabolic demand risks derailing stringently controlled events relating to protein transcription, translation or folding (26, 105–107). The aberrant proteins and peptides resulting from this "beta-cell stress", in conjunction with local inflammation, are then capable of stimulating an autoimmune response (108–110).

Birth Weight

Higher birth weight and infant growth rate contribute to T1D pathogenesis. A systematic review and meta-analysis by Harder et al., comprising 2.4 million children, found high birth weight (>4kg) increased risk of T1D by 17% (1.09-1.26) (111). The Danish National Birth Cohort (DNBC) and Norwegian Mother and Child cohort (MoBa) comprised 99,832 children and found an increased birth weight up to 12 months of age was significantly associated with T1D risk [HR 1.24 (1.09-1.41)] (29). Similarly, the Goldacre UK population study found that children with higher birth weight (3.5-<4kg and 4-5.49kg) compared to medium birth weight (3-3.49kg) had higher incidence of T1D, HR 1.13 (1.03-1.23) and HR 1.16 (1.02-1.31) respectively (112).

Infant Growth and Body Mass Index (BMI)

The TEDDY study showed that higher infantile weight gain was associated with increased risk of IA [HR 1.09 per 1 kg/year (1.02-1.17)] (113). In children with first autoantibody GAD, there was

also an increased risk of progression from IA to overt T1D [HR 2.57 per 1 kg/year (1.34-4.91)] (113). Increased progression from IA to T1D was also observed when height-growth pattern was lower in infancy, but higher in early childhood (113). Interestingly, Yassouridis et al. (114) used pooled analyses to show that IA was only linked with rising BMI up to three years of age, in non-diabetic mothers (adjusted OR 2.02 (1.03-3.73) (114). Finally, in the TRIGR study, although annual growth did not associate with autoantibody status, being overweight at 2-10 years of age increased risk (HR 2.39 (1.46-3.92) of progression to T1D (stage 2-3) but not risk of seroconversion (stage 1) (33).

A meta-analysis showed a positive dose response relationship between childhood BMI and T1D risk, OR 1.25 (1.04–1.51) (115). A Mendelian randomisation study found an OR of 2.76 (1.40-5.44) for T1D risk (116). Further, a Danish study showed that higher BMIz score at 7 years [OR 1.23 (1.09-1.37)] and 13 years [OR 1.20 (1.04-1.40)] of age was associated with an increased risk of T1D (117). The TrialNet pathway to prevention study found no link between BMI, BMI percentile, insulin resistance of progression to T1D (118), although Ferrara et al. showed that cumulative excess BMI was associated (119).

Given birth weight (111), body weight (115) and weight gain (113) correlate with T1D risk at an early stage, and this excess weight is most amenable to intervention in early childhood (120), this justifies early recognition and treatment. However, to date there are limited studies exploring whether reducing obesity decreases T1D risk. A number of studies have explored whether exercise programmes that reduce the insulin resistance and weight associated with obesity also reduce T1D. Exercise in both the NOD mouse model of T1D (121–124) and in people newly diagnosed with T1D appear to preserve beta-cell function (125), with evidence of reduced immune cell inflammation and insulitis in the former model (126, 127).

Birth Weight, Infant Growth, and Obesity – A Summary

There is now reasonable evidence that increased birth weight (111), early weight gain (113) and obesity in children (115) matters, and associates with risk of IA as well as overt T1D, i.e. that this environmental factor may act to progress people into stage 1, 2, and stage 3 pre-T1D (51, 112, 113, 115). Unfortunately, RCT evidence to test causality are lacking. Surrogate studies demonstrating that exercise interventions can preserve beta-cells at stage 3 T1D do however show promise (125).

THE GUT - MICROBIOME AND DIET

The Gut Microbiome

The gut microbiota, established in early life, is influenced by perinatal factors and nutrition, and modulates the innate and adaptive immune systems (128, 129). Signature profiles of gut flora are observed in those genetically predisposed to, and incident cases of, T1D (130). The hallmark characteristics are decreased bacterial diversity, reduced microbiota stability, increased frequency of *Bacterioides* species and decreased frequency of P*revotella*, *Bifidobacteria*, and *Lactobacillus*, the latter of which confer immunomodulatory properties through production of short chain fatty acids (SCFA) (130). The gut microbiome potentially contributes to beta-cell autoimmunity through enhanced intestinal inflammation, increased permeability, loss of barrier function, and subsequent exposure to dietary antigens (129).

The TEDDY study used 16S ribosomal ribonucleic acid (rRNA) and metagenomic sequencing to reveal gut taxonomy from stool samples of healthy controls compared to genetically at-risk children, aged 3-46 months (131). Weak associations were identified between the gut microbial taxonomies and progression from stages 1-3 of T1D (seroconversion or progression to overt T1D) (131). Conversely, Vatanen et al. (132) showed the gut microbiome in healthy controls expressed genes which stimulated fermentation and synthesis of SCFA, although taxonomy did not significantly differ from the case subjects. This reflects functionally protective properties among healthy gut flora which are lost in predisposed and seroconverted individuals (132). The DIABIMMUNE study, which included 1000 genetically predisposed newborns, showed reduction in gut microbial diversity when progressing from autoantibody positivity to T1D. In autoantibody positive subjects, gene expression was shifted to enhance sugar transport and reduce amino acid biosynthesis, compared to non-seroconverters (31). The ABIS study, included 17,000 babies from Sweden born between 1997 and 1999 followed-up for 12 years, and showed that HLA haplotype determines gut microbial composition (133). Zhao et al. compared serial faecal samples in 11 seroconverted cases (5 of whom developed T1D) compared to controls, and found a higher bacteriophage Shannon diversity index in the controls, and these differences increased with age (134). Random Forests analysis revealed T1D-associated viral bacteriophage contigs, separate from the age-associated bacteriophage contigs, which were linked to gut microbial composition. The best predictive contig had nucletoid sequence homolog consistent with B.dorei (134). Overall, the gut microbiome is a key window to IA.

In the TEDDY study, mode of birth delivery was an important determinant of gut taxonomy in the first year of life (131). Mode of delivery cultivates the neonatal gut microbiome, which determines microbial composition, succession and function, and contributes to risk of autoimmune disease, allergy and obesity in later life (130). Vaginal delivery leads to neonatal gut colonisation that reflects the vaginal flora, comprising *Lactobacillus* and *Bifidobacterium* (130). The TEDDY study suggests that vaginal delivery leads to *Bacteroide* colonisation, which supports gut maturation and enhances microbial diversity (130, 131). Alternatively, delivery by caesarean section (CS) is associated with gut microbiota seeded from the mothers skin commensals, namely *Clostridium* and *Staphylococcal* species (130). The lack of colonisation by *Lactobacillus* and *Bifidobacterium species* confers dysfunctional immunomodulation with resultant implications for autoimmunity (130, 131). Risk of T1D following CS vs vaginal delivery was evaluated in a meta-analysis, including 20 studies.

After adjustment for confounders, CS increased risk of T1D by 23% (1.15-1.32) compared to vaginal delivery (135). Another systematic review, comprising 9 observational studies and including 5 million births found that elective CS increased childhood T1D risk by 12% (1.05-1.20) compared to vaginal delivery. However, following adjustment, risk differences did not remain due to large study heterogeneity (136). Separate analyses focussed on cohort studies, which reduced the heterogeneity and showed T1D risk was significantly higher in elective CS [OR 1.12 (1.01-1.24)]. In contrast, the DIPP (137) and DIABIMMUNE (132) studies both demonstrated higher levels of *Bacteroide* colonisation in genetically predisposed children who seroconverted and progressed to T1D, contrary to evidence that early colonisation with *Bacteroidetes* species comprised healthy gut flora (130). The gut microbiome is therefore complex, and we need large metagenomic studies to taxonomise the gut microbiota and identify the species which confer protective vs damaging effects, and how they relate to each other.

Indeed, multiple environmental factors shape the gut microbiome in the first years of life, including geography and household exposures such as pets and siblings (131, 138). The ABIS study showed that exposure during pregnancy to cats and dogs conferred no increased risk of T1D, but hamsters did (139).

Obesity also negatively influences the gut microbiome. The obese adult individuals' gut microbiome lacks diversity and the resultant dysbiosis, triggers immune dysregulation, inflammation and promotes diet-sustained obesity (140). The lack of diversity and composition in the gut of an obese individual is therefore similar to the T1D gut milieu. Consequently, there is research interest in interventions which negate these effects and help restore healthy gut microbiota. In mouse studies, faecal transplant from obese humans to germ free mice triggered greater weight gain, and the opposite also remains true. Allogenic healthy donor faecal transplant to individuals with metabolic syndrome improved insulin sensitivity and restored healthy gut flora (140). Trials of donor faecal transplant for T1D prevention have yet to be attempted, but de Groot et al. performed an RCT in new-onset T1D (<6 weeks) and found preservation of C-peptide following faecal transplantation (141).

In all cases, further studies need to address the range of factors which cultivate the gut microbiome, but dysbiosis appears to be an important hallmark for T1D pathogensis (142, 143).

Breast Milk

TEDDY showed that the most significant determinant of gut taxonomy in the first year of life is breastfeeding (131). The World Health Organisation (WHO) recommend exclusive breastfeeding until ≥6 months of age, to support growth, development, immunity and the developing gut microbiome (144). However, practices differ across societies and cultures regarding duration of breast feeding, and the type and timing of solid foods (145). Unique benefits of breast feeding include transference of biologically active substances, such as antibodies, cytokines and hormones that modulate the developing immune system (130, 146). It is postulated that breast milk also confers protection from T1D through reduced frequency of infantile respiratory and gastrointestinal infections (147), delayed exposure to dietary antigens (gluten and bovine insulin) (12), and promotion of a healthy gut flora, seeding *Bifidobacterium* species (131). The DNBC and MOBA (29) population-based cohort studies, included 155,392 children and showed a two-fold increased risk [HR 2.29 (1.14-4.61)] of T1D in children not breastfed at 6-12 months compared to any breastfeeding for ≥12 months (30). There was no difference in T1D incidence between those fully or partially breastfed, and no association with age of introduction of solid foods (30). The MIDIA study explored breast feeding and age at introduction of solid foods with T1D risk in genetically susceptible children (148). Similarly, they found breastfeeding for ≥12 months predicted decreased risk of progression to T1D (HR 0.35 (0.13-0.94), with no effect on IA (148). Duration of full breastfeeding, age at introduction of solid foods and combination with breastfeeding, did not associate with risk of IA or T1D (148). Importantly, the prospective TEDDY study (149, 150) and the TRIGR RCT (151) showed no effect with duration of exclusive breastfeeding on seroconversion or progression to T1D. Despite the mixed results, ability to extrapolate further insights is limited, as the general health benefits of breastfeeding outweigh risk.

Cow's Milk and Formula Feeds

Cow's milk, which contains bovine insulin, could potentially induce autoimmune responses through molecular mimicry to human insulin, leading to T1D seroconversion in children (152). A Finnish cohort study found that children exposed to cow's milk formula before 3 months of age, had higher rates of IgG binding to bovine insulin antigen and these antibodies cross-reacted with human insulin (153); however none of these children went onto develop IA. The bovine insulin binding antibodies also inversely correlated with age at introduction of formula feed. Bovine insulin autoantibodies declined at 12 and 18 months, except in the anti-insulin antibody seropositive children, where levels significantly increased (153). The FINDIA study investigated bovine-insulin free formula feed with randomisation to three treatment arms (cow's milk, whey-based hydrolysed formula and bovine-insulin free formula) and showed a reduced incidence of seroconversion in the bovine-insulin free formula feed group (32).

In light of concerns around introduction of cow's milk (standard/conventional formulas), protein hydrolysed formula alternatives were trialled to determine risk reduction in T1D. In the Finnish TRIGR study, genetically susceptible children were randomised to either cow's milk (CM) or casein-hydrosylate formula (CHF) feed, during the first 6-8 months of life where breast feeding was not possible, and found a reduced incidence of IA in the CHF group compared to CM group, with one [HR 0.51 (0.28-0.91)] or ≥two autoantibody positivity [HR 0.47 (0.19-1.07)] (154). The TRIGR study was a double-blind RCT including 2159 genetically at-risk children from 15 countries,

followed-up for at least 10 years (33). TRIGR showed that weaning to hydrolysed formula compared with conventional formula (casein hydrosylate or adapted cow's milk formula) did not decrease the cumulative incident risk of T1D after 11.5 years follow-up (33). Similarly, the TEDDY study generally showed no significant association between IA and hydrolysed or conventional formula feed (155). However, extensively hydrolysed formula feed was associated with an increased risk of IA when introduced in the first 7 days of life [HR 1.57 (1.04-2.38)] (155).

Gluten

Coeliac disease is triggered by an autoimmune reaction to gluten, leading to villous atrophy in the small intestine and subsequent malabsorption (156). Coeliac disease affects 2.5% to 16.4% (5.7% overall) of individuals with T1D (157). Gluten is thought to trigger progression to beta-cell autoimmunity through molecular mimicry (158). The Finnish DIPP study, in 5545 genetical predisposed children, showed that higher intake of oats and gluten-containing foods increased risk of IA (159). The DAISY study showed cumulative gluten intake in the first 12 months did not associate with IA or T1D; however, introduction of gluten prior to 4 months of age significantly increased risk of T1D (160). On the contrary, the prospective TEDDY study showed that delaying introduction of gluten increased the risk of IA. Risk of developing islet antibodies was lower with introduction of gluten at <4 months of age compared to 4-9 months [HR 0.68 (0.47-0.99)], but higher compared to >9 months [HR 1.57 (1.07-2.31)] (161). However, TEDDY also showed that higher gluten intake in the first 5 years of life was associated with an increased risk for celiac disease (156).

Risks of other solid foods included in a weaning regimen have been explored but evidence is limited. The DIPP study linked early introduction of fruits, berries, and root vegetables, between 3-4 months of age, with increased risk of IA in genetically predisposed infants (162). Moreover, the TEDDY study showed protection against IA with introduction of egg, but the association was weak and did not remain when examined in a dose-response relationship (161). Virtanen et al. showed that early introduction of egg, at <8 months of age increased risk of IA in the first three years of life, but the relationship did not remain beyond 3 years follow-up (163).

Overall, we can deduce that introduction of solid foods presents a critical window to the gut microbiota, which may be protected by continuation of breastfeeding during this period (164, 165).

Antibiotic Use

Antibiotics carry potential to chronically disrupt the gut microbiome, particularly in immunosuppressed individuals (130). The same concern applies to antibiotic treatment in early life, where new environmental exposures can shift microbial colonisation, conferring risk to T1D. Mikelson et al. (166) showed in a population case-control study that broad-spectrum antibiotic use in the first two years of life increased risk of T1D. A Finnish case-control study found T1D risk was associated with maternal pre-natal phenoxymethyl penicillin [OR 1.70 (1.08–2.68)] or quinolone use [OR 2.43 (1.16–5.10)] (167). Importantly though, antenatal antibiotic use did not affect risk. The UK Health Improvement Network (THIN) database revealed increased antibiotic exposure was associated with T1D risk, observed when taking 2-5 courses of cephalosporins [OR 1.41 (1.11–1.78)] or >5 courses of penicillins [OR 1.63 (1.26–2.11)] (168). However, the TEDDY study showed cumulative antibiotic use within the first four years of life did not associate with seroconversion [HR 0.98 (0.95-1.10)] or autoantibody progression [HR 0.99 (0.95-1.02)] (169). Similarly, Tapia et al. (170) showed no link between acetaminophen use in the first 6-9 months of life and risk of T1D in a Norwegian cohort.

Probiotic Use

Agents which alter gut bacterial flora provide opportunities to restore a healthy microbiome for primary and secondary preventative purposes (171). However, evidence in support of their beneficial impact in reducing T1D risk is lacking. Probiotics consist of live micro-organisms and are engineered to restore healthy gut microbiota; protect gut membrane integrity; increase SCFA/butyrate production; reduce proinflammatory cytokines; and promote anti-inflammatory cytokines (171). In the TEDDY study, probiotic use in the first 27 days of life reduced risk of IA, compared to probiotic use after 27 days of life or no probiotic use, HR 0.66 (0.46-0.94), but this was only observed in genetically predisposed individuals (150). A double-blind placebo RCT compared maternal and infant probiotic supplementation in 1223 babies at risk of allergy and found no association with IA by 5 years, or overt T1D by 13 years, but this was a small sample size in a population not at risk of T1D (172). Prebiotics similarly aim to restore healthy gut flora and confer immunomodulatory benefits. Prebiotics comprise fructo-oligosaccharides, galacto-oligosaccharides, lactulose, or indigestible carbohydrates, are selectively up taken by gut microbiota and are associated with SCFA production, but have not been tested as a protective agent in T1D. Overall, evidence to support probiotics or prebiotics in the primary or secondary prevention of T1D is limited, but represent novel targets for therapeutic trials in genetically predisposed and seroconverted individuals (171).

Vitamin D

Vitamin D is a candidate for protection against T1D due to its anti-inflammatory effects, role in regulation of the immune system and induction of T regulatory cells, which modulate autoimmune risk (8). Cathelicidin was recently proposed to link vitamin D with the gut microbiota and protective effects on beta-cell function (173). Further evidence stems from the higher incident cases of T1D observed at northern latitudes and in winter months compared to summer, where sunlight exposure inversely correlates with T1D cases on a monthly basis (174). However, studies exploring the relationship between vitamin D

concentration and supplementation with T1D risk demonstrate mixed results (175–178).

A higher serum vitamin D reduces risk of IA, as demonstrated by a dose-response meta-analysis which found a U-shaped relationship with an OR 0.91 (0.90-0.93) for T1D per 10nmol/L increase in vitamin D (179). In contrast, the prospective DAISY study found no association between vitamin D concentration and seroconversion or T1D disease progression in IA positive individuals (175). This finding was corroborated by the prospective DIABIMMUNE study (180). Importantly however, the TEDDY study confirmed that higher plasma 25-hydroxyvitamin D correlated with lower risk for IA in genetically predisposed children (181). More copies of the Vitamin D Receptor allele (VDR) due to a Single Nucleotide Polymorphism (SNP-86), conferred greater protection. Interestingly, dairy product vitamin D supplementation in Finland has since been associated with the stabilising incidence of T1D in this region (181).

Regarding supplementation, the Finnish birth cohort study found that in cases where the recommended dose was supplemented in the first year of life, >2000 units per day compared to <2000 units per day, relative risk (RR) for T1D was much reduced at 0.22 (0.05-0.89) (182). A Norwegian study showed that vitamin D and cod liver oil supplementation from 7-12 months of age reduced risk of T1D compared to supplementation from birth to 6 months of age (183). Further, the EURODIAB study showed that vitamin D supplementation in infancy was associated with reduced risk of T1D (184). A meta-analysis also showed a 29% (0.60-0.84) risk reduction for T1D with vitamin D supplementation (178). However, the DAISY prospective cohort did not identify an association between vitamin D and IA or risk of progression to T1D (175). In the ABIS study, infantile, intermediate vitamin D supplementation also did not associate with IA (176). Analysis of the TEDDY cohort for infantile vitamin D supplementation and T1D risk is awaited. However, the TEDDY study and a meta-analysis showed no association between maternal vitamin D supplementation and offspring's T1D risk (177). The jury is out but further trials are warranted to further explore the value of vitamin D in the T1D risk story.

Nicotinamide

Nicotinamide delays beta-cell failure enhances resistance to beta-cell toxins and increased regenerative capacities observed in NOD mice (185, 186). The ENDIT RCT investigated islet cell antibody (ICA) positive, first degree relatives of people with T1D but found no significant association with T1D (34). The DENIS study similarly showed no benefit with high dose nicotinamide at 3 years follow-up in genetically predisposed first-degree relatives (35).

Omega-3 Poly-Unsaturated Fatty Acids

Omega-3 poly-unsaturated fatty acids (PUFA) reduce pro-inflammatory cytokines and may protect against T1D (187). Studies exploring benefit with omega-3 supplementation however have shown mixed results (188, 189). The TRIALNET Pathway to Prevention study compared omega-3 supplementation in the third trimester of pregnancy compared to infants aged 5 months and found no difference in pro-inflammatory cytokine profiles (188). In the DAISY study, Norris et al. identified a risk reduction in IA in infants supplemented with omega-3 PUFA from 12 months of age (189). This association was strongest in participants who were positive for more than 2 autoantibodies. The DAISY study further showed this increased risk was associated with reduced omega-3 PUFA in the red blood cell membranes. Reduced membrane concentration of docosapentaenoic acid predicted increased risk of IA and an individual's genotype determined protective effects of α-linolenic acid supplementation (165, 190).

The Gut – A Summary

The role of the gut microbiome and diet has been an area of active interest and research. There is strong evidence for an association between the microbiome (and factors that affect it) and T1D, and this is worth further exploration (129–132). However, the association of T1D with the many dietary agents that have been postulated remain to be confirmed and tested in an RCT setting.

DISCUSSION

Despite over 40 years of investigation, with multiple, international case-control, cohort, and prospective studies, we are still in search of those critical environmental triggers for T1D. The TEDDY study has provided the largest evaluation of environmental triggers in genetically predisposed children to date (26). Lessons learned are that T1D is a highly heterogenous condition, influenced by both genetic (13) and environmental factors (14), which interact through the threshold hypothesis (15, 26), to initiate and promote T1D over time.

We would suggest that a way forward for this field is first to explore and establish those environmental factors that probably associate with risk for IA and/or T1D. Once identified, they can then be tested, ideally through a RCT.

This proposal comes with challenges. The challenges of recruiting, defining and measuring exposure to the environmental agent, and allowing a sufficient period of follow up for IA and T1D to develop should not be under-estimated and has been outlined by others (191). Bearing these issues in mind, our review suggests probable associations with enterovirus infections; birth weight; early growth; childhood obesity; and with changes in the gut microbiome (**Table 1**). Several other possible associations exist but these need further evaluation. **Figure 1** summarises the likelihood of effect influenced by the environmental discussed in this review.

The subsequent testing of 'probable association' also brings challenges. Some agents do not lend themselves easily to testing with a gold-standard RCT (birth weight and rate of childhood growth), and others cannot be tested because programmes to

TABLE 1 | List of the key environmental determinants outlined in this review and the evidence supporting a causal framework.

Class of agent	Agent	Current strength of association with IA or T1D	Proving contribution to causality	Supporting References
Infections and vaccinations	Enterovirus	Probable	Vaccination trials in planning	(48, 57)
	Rotavirus	Possible	Rotavirus vaccinations being incorporated into childhood vaccination programmes in some countries	(58, 65)
	Influenza	Unlikely	Studies show inconsistent results	(70–73)
	COVID-19	Possible	Vaccination programmes being set up	(37, 75–78, 87)
	Childhood vaccinations	Unlikely	Studies show inconsistent results	(91, 92)
Weight	Birthweight	Probable	RCT and intervention studies needed	(29, 111, 112)
	Infant growth	Probable	RCT and intervention studies needed	(113, 114) (192)
	Childhood obesity	Probable	RCT and intervention studies needed	(115–119)
The Gut	Microbiome	Probable	RCT needed	(31, 129, 131–133)
	Breastfeeding	Possible	RCT evidence supports no role	(131, 151)
	Cow's milk/formula feeds	Unlikely	RCT evidence supports no role	(32, 33, 153, 155)
	Gluten	Possible	Studies show inconsistent results	(156, 159–161)
	Antibiotic use	Possible	Studies show inconsistent results	(166, 168–170)
	Probiotic use	Possible	RCT evidence supports no role but small study	(150, 172)
	Vitamin D	Possible	Conflicting RCT results of vitamin D supplementation	(176–178, 182–184)
	Nicotinamide	Unlikely	RCT evidence supports no role	(34, 35)
	Omega-3 (PUFA)	Possible	Conflicting RCT results of PUFA supplementation	(188, 189)

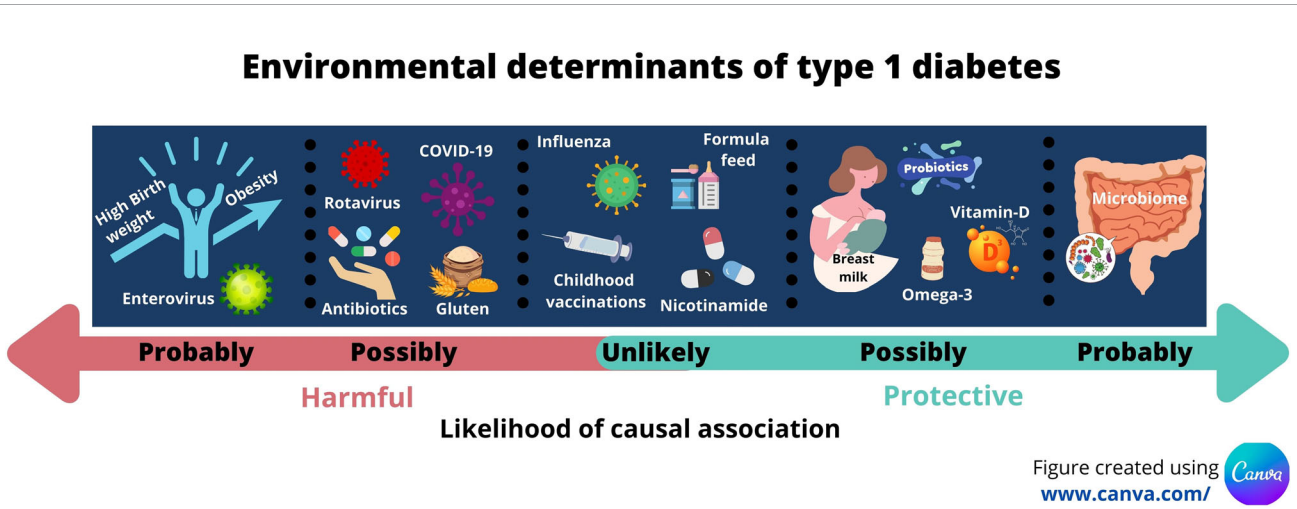

FIGURE 1 | Infographic illustrating the key environmental determinants of type 1 diabetes and their likelihood of contributing to causality.

control the putative agent have been, or are being, implemented for other public health reasons (rotavirus, COVID-19) (88, 193). Yet other environmental agents such as childhood obesity may be considered unethical to test because there are good arguments for establishing a national programme to address this major global health burden (8). Proving causality for these agents will require means of assessment other than RCTs. However, well conducted RCTs, as was undertaken for the TRIGR study comparing hydrolyzed infant formula compared to cow's milk-based formula (33), can be effective at addressing long-standing concerns about the T1D risk of particular environmental agents.

In conclusion, we present a summary of the environmental determinants according to the leading hypotheses; infection and vaccinations, the accelerator hypothesis, and the gut microbiome, and we outline the necessary routes to transition from association to causality.

AUTHOR CONTRIBUTIONS

LQ, FW, and PN made substantial contributions to the following: conception or design of the work; drafting the work or revising it critically for important intellectual content; providing approval for publication of the content; and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

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REVIEW
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Partners in Crime: Beta-Cells and Autoimmune Responses Complicit in Type 1 Diabetes Pathogenesis

Eliana Toren[1,2†], KaLia S. Burnette[2,3†], Ronadip R. Banerjee[4], Chad S. Hunter[1,2*] and Hubert M. Tse[2,3*]*

[1] Department of Medicine, Division of Endocrinology Diabetes and Metabolism, University of Alabama at Birmingham, Birmingham, AL, United States, [2] Comprehensive Diabetes Center, University of Alabama at Birmingham, Birmingham, AL, United States, [3] Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, United States, [4] Division of Endocrinology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, United States

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Edited by:
F. Susan Wong,
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Stephan Kissler,
Joslin Diabetes Center and Harvard Medical School, United States
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Institut National de la Santé et de la Recherche Médicale (INSERM), France

***Correspondence:**
Hubert M. Tse
htse@uab.edu
Chad S. Hunter
huntercs@uab.edu
Ronadip R. Banerjee
rrbanerjee@jhmi.edu

[†]These authors share first authorship

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Type 1 diabetes (T1D) is an autoimmune disease characterized by autoreactive T cell-mediated destruction of insulin-producing pancreatic beta-cells. Loss of beta-cells leads to insulin insufficiency and hyperglycemia, with patients eventually requiring lifelong insulin therapy to maintain normal glycemic control. Since T1D has been historically defined as a disease of immune system dysregulation, there has been little focus on the state and response of beta-cells and how they may also contribute to their own demise. Major hurdles to identifying a cure for T1D include a limited understanding of disease etiology and how functional and transcriptional beta-cell heterogeneity may be involved in disease progression. Recent studies indicate that the beta-cell response is not simply a passive aspect of T1D pathogenesis, but rather an interplay between the beta-cell and the immune system actively contributing to disease. Here, we comprehensively review the current literature describing beta-cell vulnerability, heterogeneity, and contributions to pathophysiology of T1D, how these responses are influenced by autoimmunity, and describe pathways that can potentially be exploited to delay T1D.

Keywords: beta-cell, beta-cell heterogeneity, pancreatic islet, autoimmunity, ER stress, oxidative stress, Type 1 Diabetes

INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease in which loss of beta-cell mass and subsequent insulin-insufficiency leads to hyperglycemia. T1D is linked to secondary complications including cardiovascular disease, kidney disease, and neuropathy (1). T1D is the most common form of diabetes in children, comprising approximately 75% of new diabetes diagnoses in patients under 19 years of age (2). Nonetheless, T1D is not a disease only of the young. Epidemiological studies now show that the incidence of autoimmune diabetes in adults (age 30 – 49 years) is at least as high as young adults (age 15 – 19 years) (3). Incidence of T1D is between 4 and 41 per 100,000 in the United States, but interestingly there is significant geographic variation in incidence rates worldwide. Asian countries have relatively lower rates of T1D, while Switzerland, Finland, Norway, the UK, and Sardinia have

among the highest rates, with values greater than 20 per 100,000 people (4). Even considering the large geographic variability, overall new diagnoses are on the rise in both childhood and adult populations.

T1D is a multifactorial disease; both genetic and environmental factors contribute to risk. While incompletely understood, putative environmental triggers include microbial infections, neonatal nutrition status/weight, and exposure to certain toxins, such as nitrates (5). Following a triggering event in genetically-susceptible individuals, immune effector cells infiltrate the pancreas and activate inflammatory pathways to mediate targeted destruction of insulin-producing beta-cells. Since the early 1970's, when the genetic connection between human leukocyte antigen (HLA) and T1D was first discovered, pathogenesis of T1D was largely defined by autoimmunity and the selective presentation of islet autoantigens. Strictly defining T1D by an immunological mechanism, however, does not acknowledge any potential role for the beta-cell itself in promoting disease pathology. Mounting evidence indicates the beta-cell is more than just a passive target in the development of T1D: the lack of long-term success with immune intervention therapies, the existence of islet autoimmunity without T1D development, and the persistence of beta-cells after diagnosis and T1D progression, all provide evidence that the beta-cell is an active participant along with the immune system in T1D pathogenesis (6, 7).

In this review, we will focus on the beta-cell in both healthy and T1D environments. We will explore inherent beta-cell heterogeneity and vulnerabilities, contributions to the local inflammatory environment, and how the beta-cell response to metabolic stress can perpetuate disease. Shifting focus from the beta-cell as a passive target to an active participant in disease progression will help identify novel therapeutic approaches, potentially leveraging these unique beta-cell responses and susceptibilities for both treatment and prevention of T1D.

THE BETA-CELL: CHARACTERISTICS THAT IMPART VULNERABILITY

In 1985, Dr. Gian Franco Bottazzo's lecture titled "Death of a Beta Cell: Homicide or Suicide?" posed the idea of beta-cell fragility (8). Dr. Bottazzo questioned whether beta-cells were innocent bystanders of immune attack or contributors to their own destruction (9). Beta-cells must rapidly respond to glucose fluctuations by secreting the appropriate amount of insulin to maintain euglycemia, a taxing process that, even in healthy cells, makes them vulnerable to stressors such as inflammation and nutrition excess. The metabolic demand associated with tightly regulated insulin secretion, paired with a highly vascularized environment, reduced antioxidant defense mechanisms, and sensitivity to proinflammatory cytokines, makes beta-cells uniquely susceptible to autoimmune-mediated destruction (**Figure 1**).

Secretory Demand

The beta-cell is responsible for producing and secreting multiple secretory granule proteins including insulin, chromogranin-A (ChgA), and islet amyloid polypeptide (IAPP). The endoplasmic reticulum (ER) is the site of protein production and relies heavily on Ca^{2+} concentrations to maintain the environment needed for proper protein synthesis and folding (**Figure 2A**) (10). Insulin secretory demand makes the beta-cell particularly vulnerable to exceeding ER protein folding capacity, which leads to the accumulation of misfolded proteins and a disruption of ER homeostasis (**Figure 2F**) (11). This physiological state is termed ER stress (12). Prolonged efforts by the cell to correct misfolded proteins can lead to unregulated changes in enzyme activity, reduced beta-cell function, and induction of apoptosis (13–16). To meet the metabolic demands of glucose-stimulated insulin secretion (GSIS), the beta-cell requires a tightly-coupled process with cellular metabolism to properly maintain euglycemia (17). In brief, glucose is transported into the beta-cell *via* the glucose transporter 2 (Glut2) in rodents (GLUT1 and 3 in humans), converted to pyruvate, and shuttled into the mitochondria where it is used for ATP production (18). Changes in the ATP to ADP ratio lead to beta-cell depolarization, Ca^{2+} influx, and insulin release (19–21). *Insulin* mRNA is translated at the ER following nutrient stimulation, which in rodents can signal up to a 10-fold increase in insulin synthesis at a rate of 1 million molecules per minute (22, 23).

To meet these high demands, beta-cells have an extensive ER with multiple chaperones to aid in protein folding, packaging, and secretion. However, high protein synthesis puts a significant amount of stress on the ER. The unfolded protein response (UPR) is triggered when an excessive amount of misfolded proteins accumulate in the ER, which can be caused by overnutrition, increased reactive oxygen species (ROS), or proinflammatory cytokines (24, 25). Three major sensors of the UPR are protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 alpha (IRE1α) and activating transcription factor 6 (ATF6) (13, 26). In an unstressed state, these sensors are bound to the ER chaperone binding immunoglobulin protein (BiP) (**Figure 2A**). Accumulation of misfolded proteins leads to the dissociation of BiP from the three UPR sensors (**Figure 2F**) (27, 28). Together, the UPR sensors alleviate ER stress by attenuating global protein synthesis to reduce the load of unfolded proteins, increasing chaperone synthesis to guide protein degradation or refolding misfolded proteins, and synthesizing lipids to increase ER volume (29).

In addition to protein synthesis, the ER is also responsible for storage of intracellular Ca^{2+} and therefore, regulates calcium-dependent signaling within the cell, such as protein folding and enzymatic function (13). ER stress disrupts intracellular Ca^{2+} balance influencing multiple processes, including activation of cytosolic post-translational modification (PTM) enzymes by facilitating their translocation into subcellular compartments. This imparts downstream changes in gene expression, protein conformation, and enzyme activity (30–33). Dysregulation of PTM enzymes has been linked to the development of rheumatoid arthritis, celiac disease, and T1D (34–38). In T1D, this includes citrullinating peptidyl arginine deiminase (PAD) enzymes and tissue transglutaminase 2 (tTG2) deaminating enzyme (13, 39). PAD, tTG2, and similar enzymes can alter the binding affinity of

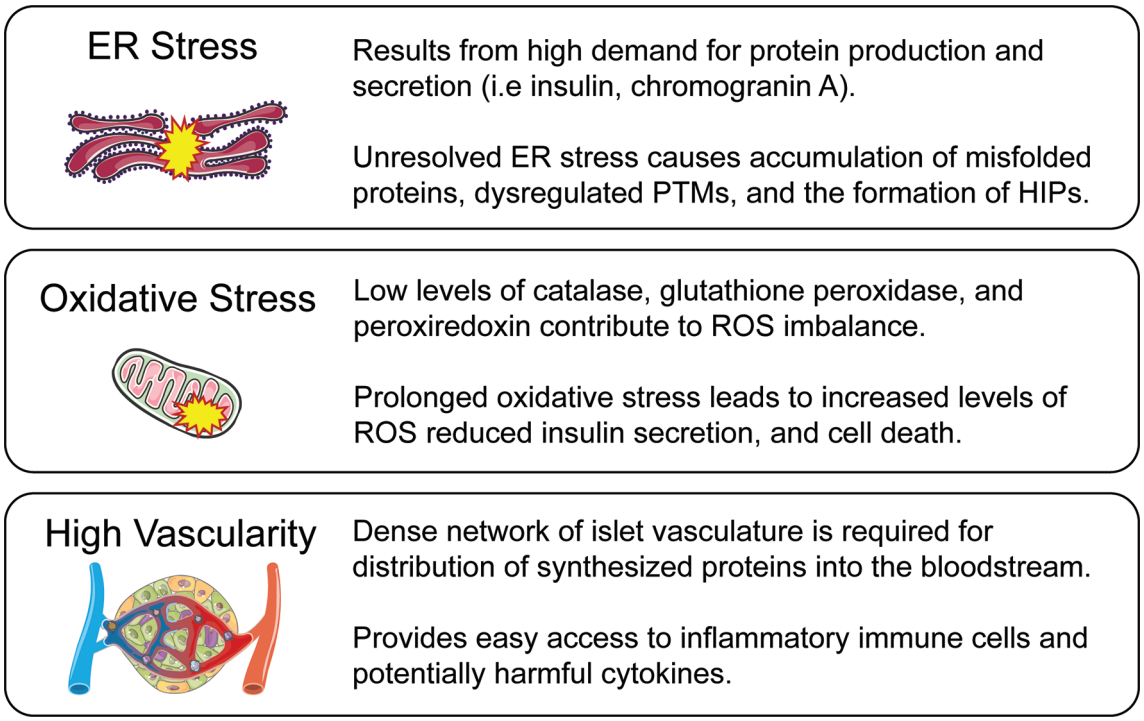

FIGURE 1 | Beta-cell vulnerabilities. While autoimmunity is a major driver of T1D pathogenesis, innate features of beta-cell biology make it a complicit partner in disease progression. These beta-cell characteristics are a result of normal beta-cell function while also active contributors to disease amplification. ER stress is caused by the high protein production and secretory demand of the beta-cell, but in excess leads to misfolded protein response and the generation of HIPs through PTMs. Oxidative stress is caused by an imbalance between the generation of ROS and their detoxification by antioxidants. The reduced antioxidant capabilities of the beta-cell can lead to impaired function and cell death. A densely vascularized environment is required for secretion of insulin and other peptides directly into the bloodstream, but creates a direct dialogue between the beta-cell and potentially harmful immune cells and inflammatory cytokines which may further lead the cell toward stress and apoptosis.

peptide epitopes, such as insulin, to major histocompatibility complex (MHC) class II, resulting in increased CD4 T cell activation (36, 40). Inhibition of systemic PAD enzymes in NOD mice can protect against diabetes progression, suggesting a role in T1D initiation (41).

Stress-induced PTMs can also result in the creation of neo-antigens in peripheral tissues for which the thymus has not established tolerance. Many T1D neo-antigens generated from PTMs have been identified (42). Some PTMs can lead to non-functional protein products resulting from alternately spliced RNA called defective ribosomal products (DRiPs) (43). Increased expression of DRiPs from insulin have been measured in beta-cells in response to ER stress and can be recognized by T cells from patients with T1D (44, 45). Hybrid insulin peptides (HIPs) are another group of neo-antigens generated from transpeptidation, a PTM where insulin peptides are covalently linked to other beta-cell granule peptides including insulin C-peptide, IAPP, and ChgA (**Figure 2G**) (46, 47). HIPs are not only recognized by autoreactive CD4 T cells in mouse models of T1D, but $CD4^+$ T cells from patients with T1D recognized HIPs as well, signifying their potential role in disease initiation and progression (48–50). Our understanding of how neo-antigens are generated and contribute to the development of autoreactivity in T1D is currently unknown. Future studies are warranted to further define how ER stress and subsequent downstream disruptions induced by the secretory demands of the beta-cell can influence autoreactive T cell responses and beta-cell vulnerability in T1D (**Figure 1**).

Oxidative Stress

Oxidative stress occurs when there is an imbalance between ROS generation and antioxidant activity (51). Superoxide is primarily a byproduct of normal cellular metabolism that is generated in the mitochondria and cytoplasm (**Figure 2B**) and is an initiating free radical that can result in the formation of other reactive species such as hydrogen peroxide (H_2O_2), hydroxyl radical, and peroxynitrite (52). Free radicals are highly reactive and can induce cellular damage, but antioxidants including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), peroxiredoxins, thioredoxin, and glutathione protect the cell by detoxifying these reactive species (53, 54). SOD dismutates superoxide to molecular oxygen and H_2O_2, a less destructive oxidant and signaling molecule. H_2O_2 regulates insulin secretion by activating the second messenger c-Jun N-terminal Kinase (JNK) (55). This leads to decreased insulin production through the translocation of the transcription factor pancreatic and duodenal homeobox 1 (Pdx1) from the nucleus to the cytoplasm, resulting in decreased *Insulin* transcription (56)

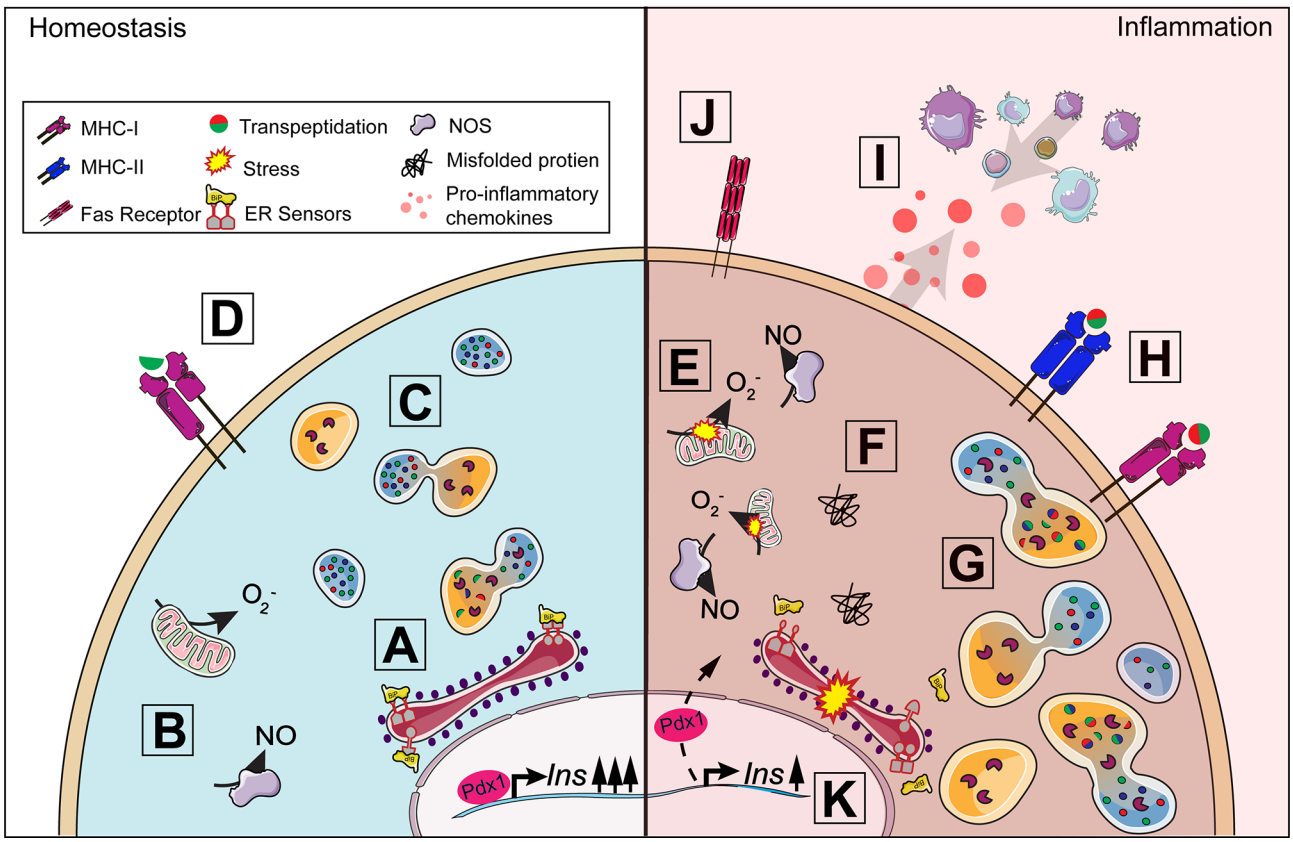

FIGURE 2 | Beta-cell response to inflammation. Under homeostasis conditions, insulin production is tightly coupled with cellular metabolism including protein synthesis in the endoplasmic reticulum (ER) **(A)** and mitochondrial function **(B)**. When insulin secretory granule proteins are in excess, they can be broken down and recycled by crinophagy, a process by which granules fuse with lysosomes **(C)**. Some peptides from this degradation process are presented on MHC-I **(D)** and, in healthy cells, should not lead to activation. A proinflammatory environment around the islet exacerbates ER and oxidative stress **(E)** contributing to the dysregulation of multiple processes in the beta-cell. The accumulation of misfolded proteins can result in the activation of the unfolded protein response **(F)** and increase lysosomal degradation of insulin secretory granule proteins **(G)**. Protein degradation under stress can lead to the production of neo-antigens, such as hybrid insulin peptides, through transpeptidation. ER and oxidative stress results in the upregulation of MHC-I and the unique expression of MHC-II **(H)** by the beta-cell allowing for increased presentation of potential neo-antigens to T cells. Fas receptor expression **(J)** makes the beta-cell vulnerable to Fas-mediated apoptosis. The release of chemokines **(I)** from the beta-cell further contributes to immune cell recruitment and the development of insulitis. Insulin production can be affected as disturbances in cellular homeostasis can lead to the translocation of Pdx1 from the nucleus to the cytoplasm, decreasing insulin production **(K)**.

(**Figure 2K**). H_2O_2 is further converted to oxygen and water by catalase, GPx, and peroxiredoxin. Increased levels of H_2O_2 can form extremely reactive hydroxyl radicals through Fenton reactions with free iron present in the cytoplasm, which has downstream negative effects on intracellular calcium levels, protein synthesis, glycosylation, and redox status (53). Beta-cells, however, have decreased antioxidant levels and therefore, are highly susceptible to free radical-mediated damage (**Figure 2E**). Rodent and human beta-cells have reduced transcriptional and protein levels of cytosolic copper/zinc (Cu/Zn) SOD1, mitochondrial manganese (Mn) SOD2, catalase, and GPx, which can result in exacerbated levels of superoxide, H_2O_2, hydroxyl radical, and peroxynitrite that are implicated in beta-cell death in T1D (57–61). The inability to properly restore cellular homeostasis due to the negative effects of oxidative and ER stress can induce apoptosis in insulin-secreting beta-cells. Increased beta-cell apoptosis has been measured in patients with T1D and NOD mice when compared to healthy controls (14, 62–66). In addition to oxidative stress, the beta-cell is also impacted by the islet microenvironment in which it is closely associated.

Islet Vascularization and Exposure to Cytokines

Importantly for T1D pathology, islets are highly vascularized. This provides an interface by which immune cells, even from distant sites, can gain local access to pancreatic islets (67, 68). Islets contain a glomerular-like network of fenestrated capillaries that comprise about 8-10% of islet volume. Islet capillary density is estimated to be 10 times higher than that of the exocrine pancreas and is driven by high local production of VEGF-A (69, 70). This rich vascularization and high islet blood flow is autonomously regulated through complex interactions between hormones, metabolites, and the nervous system. While islet blood flow is innately required for and coupled to insulin

sensing and release, extensive vasculature also makes the beta-cell uniquely poised for interactions with the immune system.

The dense islet vasculature network facilitates activated immune cell trafficking across the vascular endothelium into the islet (**Figure 1**). This causes a local inflammatory microenvironment which in turn, further increases permeability, facilitating access even for naïve T cells (71). Interestingly, this "open" environment remains, even after reversal of diabetes with anti-CD3 treatment. In addition to naïve T cell infiltration, activated immune cells that are primed locally in the pancreatic lymph nodes (pLNs) can also cross the vascular endothelium. pLNs may contribute to T1D pathogenesis as drainage from the pancreas and local gut regions provides a crossroad for the immune cells traveling between these compartments (67, 72). The interplay between the microbiome, the immune system, and a "leaky gut" has been implicated as a key factor in T1D pathogenesis (73, 74). Toll-like receptors (TLRs) are a family of innate pattern recognition receptors important for microbial clearance by the immune system. Many TLRs signal through the MyD88 adapter protein. NOD mice deficient in MyD88 exhibit microbiota-dependent protection from autoimmunity development (75). Specific manipulation of TLR expression and microbiota composition can further regulate disease progression or prevention (76). Sex-specific autoimmune risk can also be influenced by microbiome manipulation (77–79). With new data unveiling the importance of the microbiome for the gut immune environment and shaping peripheral tolerance, the relationship between pLNs, islet vasculature, and immune cell trafficking is increasingly relevant to beta-cell vulnerability and T1D pathogenesis (80, 81). In addition to facilitating interactions between islet cells and immune cells, the islet vasculature also sensitizes the beta-cell to the damaging effects of circulating proinflammatory cytokines.

Beta-cells are sensitive to cytokine-mediated damage. Cytokines can alter crucial beta-cell characteristics including insulin secretion, mitochondrial function, and intracellular calcium stores (82, 83). Inflammatory cytokines including tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and interleukin-1 beta (IL-1β), cause beta-cell dysfunction by impairing ATP production, inducing DNA damage, and promoting apoptosis (**Figure 3**) (84, 85). Proinflammatory cytokine exposure inhibits GSIS due to the limited availability of ATP in both rodent and human islets, as well as in beta-cell lines (86–89). Islet exposure to cytokines triggers nuclear factor κB (NFκB) induction of inducible nitric oxide synthase (iNOS), which increases NO formation in the beta-cell (87). NO has temporal effects on beta-cell responses, as early and transient levels of NO (less than 24 hours) facilitate the repair of cytokine-induced DNA damage by inhibiting the activation of the DNA damage response and preventing the induction of apoptosis (90). However, prolonged exposure to NO can induce beta-cell death due to DNA damage, UPR activation, and decreased mitochondrial oxidation (i.e., ATP production) (87, 91). In cultured islets, pre-exposure treatment with NO inhibitors, such as aminoguanidine, attenuates cytokine-mediated beta-cell death (92). Cytokine-mediated beta-cell death becomes exacerbated in an inflammatory microenvironment in the pancreas, creating a positive feedback loop resulting in more inflammation, stress, vulnerability, and eventually cell death (**Figure 3**). Unfortunately, clinical trials with anti-cytokine therapies such as Anakinra, an IL-1 receptor antagonist, were not efficacious in delaying T1D, suggesting a more complex interaction between cytokines and beta-cells *in vivo* (92). Nonetheless, not every beta-cell is lost in T1D, nor do they all respond negatively to ER and/or oxidative stress, indicating an intrinsic beta-cell heterogeneity in response to disease promoting factors.

BETA-CELL HETEROGENEITY

It is challenging to fully understand the response and contribution of the beta-cell to the T1D disease state without understanding beta-cell heterogeneity. Many groups have focused on determining whether different subtypes of beta-cells exist, and if so, how they might differ in functional ways such as proliferative and secretory capacities (93–95). Identifying subpopulations, and then understanding their inter- and intra-islet communication, has uncovered a level of complexity and diversity not previously appreciated. We will briefly explore the recent findings regarding functional and transcriptional heterogeneity of the beta-cell and discuss potential impacts on susceptibility to T1D.

Functional Diversity

Evidence for functional beta-cell heterogeneity in calcium flux, metabolism, ion channel conductance, and insulin secretion has been appreciated for almost 30 years (96, 97). More recently, this functional diversity has been specifically defined by many research groups into beta-cell subpopulations (**Table 1**). Using novel cell surface markers (ST8SIA1 and CD9) identified by immunizing mice with human islets, four human beta-cell subtypes with unique basal and GSIS responses were defined as β1-4 (93). All subtypes contain insulin granules but exhibit variable functionality and abundance; β1 is the most abundant and glucose responsive, while β4 is the rarest and least responsive, with highest basal insulin secretion. Interestingly there are no correlations found between subtype ratios and sex, age, or obesity, but subtype abundance is altered and much more variable in Type 2 diabetes (T2D). For example, β3 and β4 subsets are unusually overrepresented in T2D islets, but abundance of these two subsets varied much more than in control non-diabetic tissue. It is unknown if β1-4 cells exist in unique patterns before or after T1D diagnosis and disease progression.

In 2016, Johnston et al. discovered specialized beta-cells they termed "hub" cells that exert disproportionate control over blood glucose (94). Hub cells, comprising 1-10% of islet beta-cell mass, are metabolically active, exhibit evidence of transcriptional immaturity (low or absent Pdx1 and Nkx6.1 transcription factor levels), and are hypothesized to act as a pacemaker within the islet. Supporting this model, calcium tracing showed that surrounding cells, termed "followers", respond to glucose stimulation slightly after the hub cell. Using optogenetic and pharmacological techniques, silencing hubs caused desynchrony

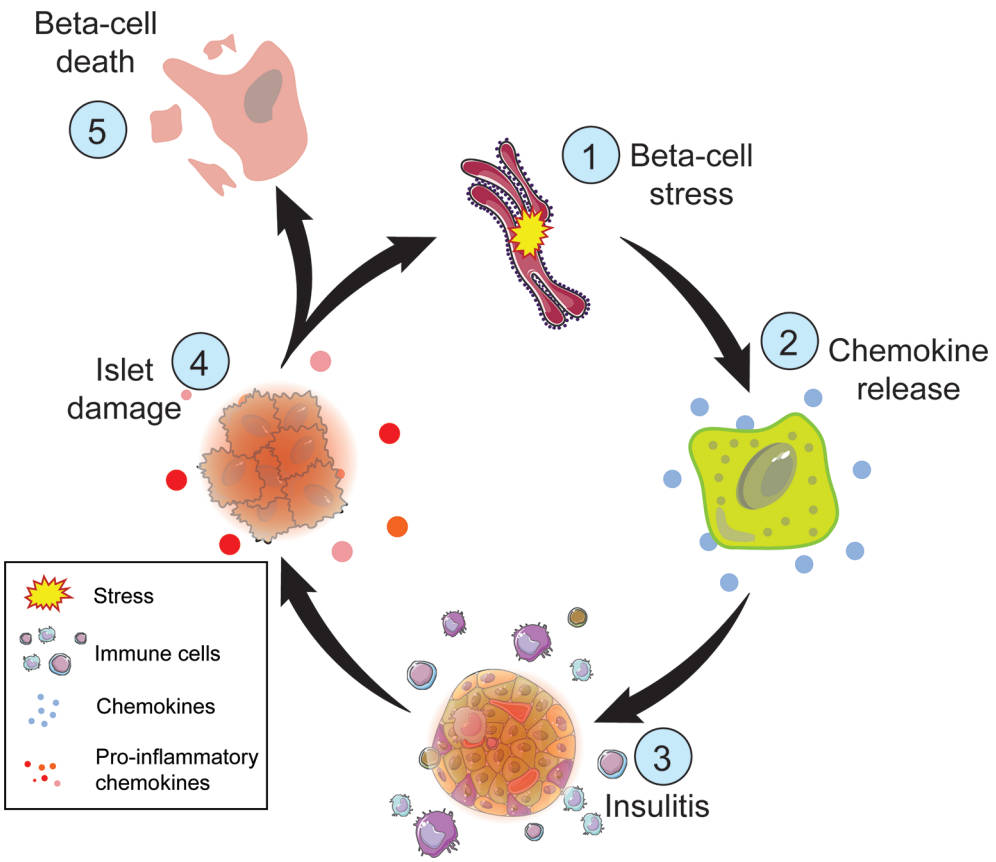

FIGURE 3 | Steps of beta-cell death in T1D. Metabolic demand of nutrient challenge results in ER and oxidative stress (1) followed by chemokine release by the beta-cell (2). Chemokines attract immune cells (3), such as macrophages and T cells, which can damage the islet (4) directly though T cell interactions and indirectly through the release of inflammatory cytokines and reactive oxygen species. Cellular damage exacerbates ER and oxidative stress perpetuating this cycle. The inability to restore cellular homeostasis will result in beta-cell death (5).

in the calcium-induced response of beta-cells. The Huising group reported on a beta-cell subtype located in the islet periphery that is both transcriptionally and functionally immature, called the "virgin" beta-cell (95). Virgin cells lack the maturity marker urocortin 3 (UCN3) and are incapable of sensing glucose or proper calcium influx. Using lineage tracing, they found that these cells are transdifferentiation intermediates between alpha- and beta-cells. This work defined a "neogenic niche" of new beta-cells originating from an alpha-cell lineage, establishing a plasticity between these cell types that had also been suggested by models of extreme beta-cell loss (100). The hub versus virgin subpopulations can be compared to flattop^{+} and flattop^{-} populations, where this novel marker differentiates between a mature, functional beta-cell population (flattop^{+}) and an immature and highly proliferative one (flattop^{-}) (101).

In 2020, the Benninger lab defined yet another subpopulation that is functionally distinct from those previously described (102). These cells are termed "first-responder" beta-cells, as defined by calcium dynamics. They found that first-phase response time of beta-cells is spatially organized and dependent on the physical distance from the first-responder cell. How these sub-populations of cells as defined by these specific markers may be unique or overlapping remains to be determined.

Transcriptional Diversity

The implementation of single-cell transcriptomics has allowed exploration of beta-cell heterogeneity on a scale that was not previously possible. The Kubicek lab published the first application of single-cell transcriptomics to human islet cells in 2016 (103); while the number of beta-cells identified was extremely low, they showed transcriptome heterogeneity for genes such as *DLK1*, a T1D associated gene by GWAS (Genome-Wide Association Study) which will be discussed later in more detail. That same year the Kaestner lab published single-cell mass cytometric analysis of human islets and found heterogeneity in markers including Ki67, identifying four distinct beta-cell subpopulations (104). Several groups have since published large-scale single-cell transcriptome analyses of human and mouse beta-cells and identified unique subpopulations. These populations display differential levels of various beta-cell characteristics including maturity (UCN3),

TABLE 1 | Selected pancreatic beta-cell subtypes. Heterogeneity in beta-cell response has led to the identification of beta-cell subtypes. These subtypes may vary in spatial location within the islet, speed of response to a stimulus, and secretory capacity. The names of beta-cell subtypes, description of characteristics, and whether they were identified in mouse or human pancreata are defined below.

Name of Subtype	Description of Characteristics	Mouse or Human	Reference
β1	Highest GSIS, least abundant in T2D tissue	Human	Dorrell et al., 2016 (63)
β2	$CD9^+$, $ST8SIA1^-$, stimulation index second highest after β1	Human	
β3	$CD9^-$, $ST8SIA1^+$, increased in T2D	Human	
β4	Lowest GSIS, high basal secretion, increased in T2D	Human	
Hub	Pacemaker, responds quickly to calcium influx, makes up 1-10% of beta-cell mass	Both	Johnston et al., 2016 (98)
Virgin	Transcriptionally and functionally immature (UCN^-), located at islet periphery, incapable of glucosensing	Both	Van der Meulen et al., 2017 (65)
Flattop+	Mature, functional secretory granules, increased with high fat diet	Mouse	Bader et al., 2016 (69)
Flattop-	Immature, highly proliferative, Wnt^+ (Become $flattop^+$)	Mouse	
Top	Present in non-T1D setting, glucose responsive, express maturity markers	Mouse	Rui et al., 2017 (99)
Bottom	Population appears in a T1D environment, resistant to immune killing, unresponsive to glucose, express stemness markers	Mouse	
First-Responder	First to respond to calcium influx, other beta-cell response based on distance from these	Mouse	Kravets et al., 2020 (70)
Extreme	High levels of proinsulin and ribosomes, low insulin protein content, increased in *db/db* mice	Mouse	Farack et al., 2019 (78)

aging (IGF1R), and ER stress response (SRXN1, SQSTM1) (105, 106). Genes involved in ER/oxidative stress have been some of the strongest distinguishers of subpopulation clusters in various studies, with ER stress markers also correlating to proliferation and reduced beta-cell function (107–109). As discussed earlier, beta-cell ER stress is extremely relevant to an understanding of T1D contribution and response. Perhaps certain populations of proliferative-ER stressed beta-cells are the first to be lost during T1D pathogenesis.

For years, technical hurdles to interrogating mRNA in islets due to the digestive enzymes present in the surrounding exocrine pancreas limited our understanding of transcriptional dynamics and heterogeneity in the beta-cell. Dr. Shalev Itzkovitz at the Weitzmann Institute developed methods to visualize the dynamics of beta-cell mRNAs. His group designed an optimized single-molecule FISH (fluorescence *in situ* hybridization) protocol that allowed for assessment of transcriptional heterogeneity within the beta-cell population (110). This method revealed a subpopulation of "extreme" beta-cells that contain high levels of *insulin* and secretory factor mRNAs (*IAPP*, *ChgA*), but interestingly, low levels of insulin protein. The investigators suggested this may impart a specialization for basal insulin secretion. Additionally, beta-cell mRNAs displayed a uniquely polarized pattern, with elevated *Insulin* mRNA concentration in apical ER enriched compartments of the cell. The ratio of extreme beta-cells was increased in *db/db* diabetic mice, potentially facilitating increased requirements for basal insulin. This work gives rise to another unappreciated aspect of beta-cell heterogeneity: transcriptional heterogeneity. Future studies are needed to explore the proportion of extreme beta-cells in T1D and how this type of transcriptional variability may affect vulnerability to autoimmune recognition and attack.

The relationship between these different beta-cell subpopulations is still being defined, as the markers discussed above may represent unique or overlapping populations. For example, each of these functional and transcriptional subpopulations represents a unique niche that may have specific susceptibilities or contributions to T1D. Is a hub cell the same as a β1 or $flattop^+$ cell? What are the differences and are they physiologically relevant? What is the relationship between these cell types and how do those relationships change with age and nutrition state? Understanding the relationship between functionality, heterogeneity, and vulnerability will provide a deeper understanding of T1D etiology, potentially setting the stage for more effective therapeutic strategies.

BETA-CELL CONTRIBUTIONS TO INFLAMMATION

Therapeutic strategies for preventing or treating T1D have historically focused on modulating the immune response to the beta-cell. Emerging strategies that instead focus on beta-cell dysfunction through manipulation of ER, oxidative, or cytokine-induced cell stressors may prove to be beneficial, as the beta-cell itself actively contributes to inflammatory responses in T1D. The following section will discuss beta-cell contributions to the T1D inflammatory environment, which may represent optimal targets for future combinatorial therapies.

Chemokine Production

Chemokines are a family of small molecules involved in lymphoid physiology, pathology, and hemopoietic cell migration (111, 112). Chemokines can be broadly separated into two categories: constitutive and inducible (113). Constitutive chemokines perform homeostatic functions involving non-inflammatory leukocyte trafficking, while inducible chemokines are produced in response to inflammation to recruit activated leukocytes to the sites of damage or stress (114). Multiple chemokines, detailed below, are secreted by the beta-cell and contribute to immune infiltration into the islet (**Figure 2I**). These secreted factors make the beta-cell a target for immune invasion and destruction.

CCL2

The chemokine C-C ligand 2 (CCL2) also known as monotype chemoattractant protein (MCP)-1 is an inducible chemokine

involved in monocyte, NK cell, and T cell recruitment during inflammation (115–117). Human and NOD islets cultured with proinflammatory cytokines IL-1β and IFNγ can induce CCL2 production (118–120). Beta-cells express *Ccl2* in an NF-κB –dependent manner and can be induced *in vivo* by environmental triggers such as viral infections leading to inflammation and macrophage recruitment (121, 122). Macrophages are the first and most abundant immune cell to infiltrate the islet during the progression of T1D in NOD mice and have also been identified in islets from patients with recent-onset T1D (115, 123, 124). CCL2 may be responsible for this influx in macrophages, as transgenic overexpression of *Ccl2* in murine beta-cells results in increased monocyte recruitment, insulitis, and islet destruction (115). Binding of CCL2 to its receptor C-C chemokine receptor-2 (CCR2) in macrophages leads to the production of proinflammatory cytokines and chemokines such as TNFα, IL-1β, IL-12, and CXCL10 to exacerbate the inflammatory environment of the islet (125). Prolonged exposure to proinflammatory cytokines leads to ER stress, oxidative stress, and cell death (115) (**Figure 3**).

CCL5

CC ligand 5 (CCL5) also called RANTES (regulated on activation, normal T cell expressed and secreted) is a chemoattractant for T cells, eosinophils, and monocytes involved in inflammatory responses. CCL5 has been measured in rodent islets and from cell sorted beta-cells in response to inflammatory cytokines TNFα, IL-1β, and IFNγ (126, 127). Increased expression of CCR5, one of the cognate receptors for CCL5, was detected on T cells from patients with T1D and NOD mice (128, 129). Blocking CCR5 using neutralizing antibodies in 2-month-old NOD mice (after islet infiltration, but before overt diabetes) inhibits future immune infiltration and prevents development of diabetes (126).

CXCL10

C-X-C motif chemokine ligand 10 (CXCL10) also called IP-10 (IFNγ-induced protein 10) is a chemokine secreted by many cell types including monocytes, neutrophils, and endothelial cells (130, 131). CXCL10 is increased in the serum and tissues of patients with various autoimmune diseases including T1D (99, 132). Human islets, murine islets, and NIT-1 NOD beta-cells secrete CXCL10 when cultured with pro-inflammatory cytokines IL-1β and IFNγ (127). CXCL10 binds the seven transmembrane G protein coupled receptor CXC receptor 3 (CXCR3), expressed on both immune and non-immune cells (133, 134). In lymphocytes, CXCR3 mediates chemotaxis, while in non-lymphocytes CXCR3 regulates tissue repair, proliferation, and angiogenesis (135, 136). Mice lacking CXCR3 and infected with lymphocytic choriomeningitis virus-WE strain (LCMV-WE), an established model to study T1D, exhibited a delay in insulitis, while overexpressing CXCL10 in mouse islets accelerated LCMV-induced diabetes (127, 137, 138). As predicted, using neutralizing antibodies to block CXCL10 also decreased T cell trafficking to the islet and abrogated diabetes development (139–141). In culture, the NIT-1 NOD beta-cell line was found to secrete CXCL10 in response to inflammatory cytokines IL-1β, TNFα, and IFNγ (127). These data suggest that elevated CXCL10 secretion by the beta-cell may occur early in T1D progression. CXCL10 not only contributes to immune cell recruitment but is also directly toxic to beta-cells (142). In addition to CXCR3, CXCL10 also binds Toll-like receptor-4 (TLR4), a pattern recognition receptor involved in the immune response to microbial pathogens (143). The CXCL10:TLR4 signaling pathway in beta-cells leads to cleavage and translocation of activated protein activated kinase 2 (PAK-2) into the nucleus, contributing to apoptotic signaling within the cell (144, 145). Islets from C57BL/6 *Tlr4*$^{-/-}$ knockout mice are protected against CXCL10-induced damage. Therefore, CXCL10 released by the beta-cell contributes to cell death by attracting activated immune cells and inducing apoptosis within the beta-cell.

Beta-cells produce proinflammatory CCL2, CCL5, and CXCL10 chemokines when exposed to inflammatory conditions or environmental triggers and can perpetuate the recruitment of immune cells to initiate insulitis. Once present, these immune cells can damage the beta-cell by synthesizing ROS, proinflammatory cytokines, and expressing receptors that can directly mediate beta-cell death (**Figure 2**).

Beta-Cell Promotion of Cellular Death

Of the infiltrating cells causing insulitis in T1D, T cells are the major destroyer of beta-cells, with both CD4 and CD8 T cells being required to effectively transfer disease (146–148). CD4 and CD8 T cells have different roles in disease development (149). When activated, CD4 T cells or T "helper" cells influence the activation of surrounding immune cells through the production of pro- or anti-inflammatory cytokines (149). Human CD4 T cells conventionally recognize peptides presented on HLA-II molecules expressed by antigen-presenting cells (APCs), but the expression of HLA-II has also been detected on beta-cells from patients with T1D (150). CD8 T cells recognize peptides presented on MHC-I on mouse cells and HLA-I on human cells (**Figure 2D**). Islets biopsied from patients with T1D displayed HLA-I hyperexpression, which warrants their susceptibility to CD8 T cell-mediated destruction (151). The activation of CD8 T cells leads to the differentiation of CD8 T cells to become cytolytic T lymphocytes (CTLs) resulting in the directed release of cytotoxic cytokines, cytolytic granules, and Fas ligand (FasL)-mediated death of the target cell. Beta-cells from diabetic patients not only express HLA-I/II molecules, but also the Fas receptor (CD95/Apo-1) (152–154) (**Figure 2J**). Fas/FasL signaling is suggested to play a role in T1D pathology as NOD mice deficient in Fas do not develop inflammation or diabetes (155). Fas-deficient mice are also protected against adoptive transfer of splenocytes from diabetic NOD mice. In rodent and human islets, the expression of Fas receptor in beta-cells is induced by proinflammatory cytokines IL-1α, IL-1β, IFNγ, and the upregulation of iNOS, as sequestering NO in the beta-cell decreases Fas expression (156, 157). Fas/FasL signaling in the beta-cell leads to apoptosis *via* the activation of caspase 8 and the mitochondrial pathway of apoptosis (158). Beta-cell-derived proinflammatory chemokines, HLA-I/II (or MHC-I/II) molecules, and Fas/FasL receptors can perpetuate T1D disease progression by promoting immune cell recruitment, T cell activation, and subsequent beta-cell destruction. Since

autoantibodies can be detected in circulation for years prior to disease onset (159) and patients from the Medalist study (discussed below) retain a portion of insulin-secreting beta-cells, these observations provide evidence that at least some beta-cell populations may possess mechanisms to evade the immune response.

THE T1D BETA-CELL

In addition to the intrinsic, "baseline" heterogeneity of beta-cells, heterogeneity of disease progression within islets from individual patients, and heterogeneity of disease progression amongst patients with T1D are becoming apparent through longitudinal clinical studies and new analytical techniques examining T1D animal models. The Joslin Medalist Study of T1D patients with disease duration of 50 years or longer revealed that some insulin producing beta-cells persist, ostensibly even after years in a chronic inflammatory environment (160). This highlights that some level of heterogeneity is present in the T1D islet, supporting that certain beta-cell populations may be protected from autoimmune destruction. The expansion of single-cell transcriptomics has contributed to our understanding of cell populations dynamics, but whether it be mouse or human, almost all published studies have used either healthy or T2D islets.

Exciting work in the past few years has given rise to the idea that disease-specific beta-cell heterogeneity may arise during T1D progression, with certain populations that are more vulnerable than others to autoimmune-mediated death. The Herold lab was one of the first to identify distinct cell populations in T1D with their discovery of a low granularity beta-cell population termed "bottom" cells in the NOD mouse model (161) (**Table 1**). They found that this non-glucose-responsive population emerges prior to hyperglycemia and immune infiltration and expands over time, comprising over 50% of the beta-cell population by 12 weeks of age. The bottom cells express "stemness" markers and were found to be less sensitive to treatment with cytokines and immune infiltrates compared to their "top" counterparts, suggesting they may evade immune attack (161). As we continue to understand disease etiology more deeply, these resistant populations may provide a novel target for treatment.

The discovery of distinct T1D endotypes associated with age of diagnosis has recently contributed to our knowledge of the T1D beta-cell (162). Using immunohistochemical analysis of pancreas samples from patients diagnosed under the age of 30, Leete et al. found a distinct pattern of insulin/proinsulin localization in the beta-cell that is not present in non-T1D controls. Specifically, they found high insulin/proinsulin colocalization in patients who were diagnosed under 13, and even more consistently in patients diagnosed before 7 years of age. Similar subtypes had been described regarding insulitis, with two discrete histological profiles associating strongly with age of diagnosis (163, 164). The authors postulate that discovery of these histologically distinct phenotypes points to disease endotypes that could even be described as T1DE1 and T1DE2 and may require different immunotherapeutic options based on age of diagnosis. While this work is not necessarily beta-cell specific, the heterogenous nature of disease that the field continues to uncover further points to the importance of understanding beta-cell heterogeneity and response to autoimmunity. We propose that an understanding of beta-cell dynamics prior to, during, and after immune-cell infiltration in T1D will be vital to development of therapies that can not only combat T1D development, but perhaps even precede and bypass it.

T1D-ASSOCIATED BETA-CELL SNPs

T1D pathogenesis involves both genetic and environmental triggers and susceptibilities. Thus, examining genetic associations that link specific loci to T1D vulnerability has been a major area of research focus. Surprisingly, the Wellcome Trust Case Control Consortium (WTCCC) established GWAS found relatively few novel risk loci for T1D. It was not until the T1D Genetics Consortium (T1DGC) conducted a meta-analysis that approximately 41 distinct susceptibility loci were identified (165). Fine mapping of these loci using ImmunoChIP established credible sets of single nucleotide polymorphisms (SNPs), most of which are found in non-coding DNA regulatory regions, including tissue-specific enhancers (166). Most of our understanding of the identified SNPs has been centered around the *HLA* loci that are strongly associated with the disease and T cell autoreactivity. While these studies of the immune arm of pathogenesis are invaluable, not only do SNPs in the *INSULIN* (*INS*) gene remain one of the highest risks (167), but approximately 60% of all T1D susceptibility genes are expressed in the islet (168). These data further support the concept that the beta-cell has a larger role in its own destruction than previously appreciated, and that genetic susceptibility is not solely based on the status of the immune system. Below we describe beta-cell-associated SNPs and what is known thus far about their T1D implications.

Insulin

As mentioned above, after the *HLA* locus, the 5' upstream region of the *INS* locus is the genomic region with the strongest association with T1D risk (167). Specifically, it is the INS-VNTR (variable number tandem repeat) locus that confers susceptibility differences. The VNTR alleles are defined by two classes: class I (26-63 repeats) and class III (140-200 repeats). The shorter class I VNTRs confer a 2-5-fold increase in T1D risk while the longer class III allele is protective against T1D, this is thought to be due to effects on proinsulin expression in the thymus (169). Class III VNTRs are associated with increased *INSULIN* transcription in the thymus during induction of central immune tolerance. The authors proposed that these increased thymic insulin levels may promote negative selection of insulin-specific T cells, ultimately leading to a protective effect on T1D susceptibility. Class I VNTRs result in decreased *INSULIN*

transcription in the thymus and potentially allow insulin-specific autoreactive T cells to escape from the thymus due to defects in central tolerance and negative selection. This class I versus III allele-specific mechanism illustrates the complexity of T1D risk, and while this susceptibility locus can absolutely be seen as a beta-cell associated SNP, the proposed mechanisms that have been defined thus far are still largely immune system focused. So, despite the 100 years since the discovery of insulin, large gaps in our understanding of how it is involved in the response of the beta-cell itself in T1D remain.

GLIS3

Due to the scarcity of studies of beta-cell contributions to T1D, many associations have been made between beta-cell death and failure in T1D and T2D. Remarkably, GWAS indicates there are very few susceptibility loci associated with both maladies. Variations of the Kruppel-like zinc finger transcription factor *GLIS3* are one of the few that have been strongly associated with both T1 and T2D (170). Also a known MODY (Maturity Onset Diabetes of the Young) gene, *GLIS3* is expressed predominantly in the pancreas, thyroid, and kidney. While there have been some discrepancies in the literature regarding the exact timing of expression, in GLIS3-EGFP knock-in mice, GLIS3 mainly co-expresses with Sox9 in bipotent pancreatic islet progenitor cells and is absent from the acinar progenitors at embryonic day (E) 13.5. This pattern correlates with its importance in development of the pancreatic endocrine lineage and seeming negligibility for the exocrine portion of the pancreas. The association between GLIS3 and T1D was first identified in European populations but has more recently been recapitulated in a Pakistani cohort (171, 172). Interestingly, a GLIS3 variant (A908V) is associated with T1D resistance in Japanese patients (173). Considering the thymic expression of GLIS3, the authors propose that perhaps this variant induces central or peripheral immune tolerance more efficiently than the wild-type variant, but more studies are needed to understand this mechanism. Regarding the molecular mechanisms that underlie the associations of GLIS3 and diabetes, little was known until multiple groups independently generated both global and beta-cell-specific *GLIS3* knockout models (174–176). Not only do *GLIS3*$^{-/-}$ mice die within the first few days of life, but their islet area is approximately 15% that of littermate controls (177). Insulin production was reduced by 80%, making it difficult to assess GSIS in these mice. These groups also found that the endocrine progenitor gene, *Ngn3*, is a GLIS3 target, and that GLIS3 physically and functionally interacts with the beta-cell transcription factor Pdx1 to regulate *insulin* transcription. Interestingly, GLIS3 overexpression leads to an upregulation of *Ngn3* mRNA in ductal cells, further supporting the role of GLIS3 in pancreatic islet progenitor specification. Pancreatic progenitors, as well as the adult acinar compartment, seem to be unperturbed in GLIS3 knockouts showing an islet progenitor specificity to its role during development. These mechanisms do not tie directly to T1D association but understanding the role of GLIS3 in beta-cell identity may help unveil the mechanisms involved in both T1 and T2D disease susceptibility.

CLEC16A

C-type lectin domain family 16, member A (*CLEC16a*) is a gene locus associated with T1D, multiple sclerosis, and adrenal dysfunction (98, 178, 179). Though genetic associations have been long established, until the work of Soleimanpour et al., a molecular basis for how CLEC16A might increase T1D was unknown. Interestingly, these investigators found that mouse Clec16a interacts with Nrdp1 (an E3 ubiquitin ligase) and has roles in normal GSIS in the beta-cell (180). Pancreatic *Clec16a* deletion causes reduced ATP levels and mitochondrial oxygen consumption, establishing the factor as a novel regulator of beta-cell mitophagy. Additionally, patients with the T1D-associated SNP in the *CLEC16A* gene exhibit reductions in CLEC16A expression and perturbed insulin secretion.

These observations of impaired insulin and glucose homeostasis, along with ER-stress in their mouse model, are some of the few providing insight into the non-immune related mechanisms of T1D (180). ER-stress and perturbations in first-phase insulin release are among the earliest signs of T1D, predating immune infiltration and insulitis (181, 182). The role of Clec16a in these processes not only highlights its crucial role in beta-cell function, but also establishes it as a potential player in the first steps of beta-cell vulnerability in T1D.

DLK1

Delta-like 1 (DLK1), also known as DLL1 or Pref-1 (preadipocyte factor 1), is a transmembrane protein that belongs to the Delta-Notch signaling family. Both mouse and human *Dlk1* are known to be subject to genomic imprinting, and Dlk1 is paternally inherited, with the maternal gene being silenced during development. This becomes potentially interesting considering the sexual discordance in inheritance risk in T1DM, as risk of transmission to offspring is 1.7 fold higher from diabetic fathers than mothers (183). However, Wurst et al. found that, in the case of gestational diabetes mellitus (GDM), serum Dlk1 levels were not significantly different between diabetic and control patients (184). Mouse Dlk1 is expressed highly and ubiquitously during development in the embryo and placenta, starting around E11.5, but becomes downregulated in most adult tissues. Adult Dlk1 expression becomes restricted to the beta-cell, bone marrow, pituitary, and adrenal glands. Some evidence suggests that Dlk1 may help undifferentiated cells maintain their pluripotent state, working as a growth factor to maintain proliferation. In preadipocytes, Dlk1 must be downregulated for differentiation to occur (185). Rodent models have remained somewhat controversial, as *Dlk1* null mice have partially penetrant neonatal lethality and complex adult and developmental phenotypes, yet conditional loss of function models in various tissues using floxed mice failed to recapitulate null phenotypes (186, 187). Dlk1 beta-cell knockout mice were found to be fully viable with normal islet architecture up to six weeks of age, though glycemic control was not assessed. More thorough analyses of Dlk1 in the beta-cell, including insulin secretion and glucose tolerance, are required to fully understand if and how it may contribute to both function and potentially pathogenesis of T1D.

The genetic basis of T1D pathogenesis is complicated and still poorly understood. A majority of these studies have been conducted using data exclusively from Caucasian patients, and inclusion of multi-ethnic populations is required for a more complete and accurate understanding of genetic variants. The few studies using African-ancestry participants have already yielded unique haplotypes and signatures (188, 189). Additionally, as mentioned above, non-coding DNA regulatory regions make up a majority of T1D associated SNPs, which suggests that genetic variation may be impacting regulatory functions rather than gene-coding abilities (165, 166). Gene expression can be controlled *via* long-range interactions, with regulatory elements impacting genes that are hundreds of kilobases away. Understanding these potential interactions requires employment of techniques such as chromatin conformation capture, building a more complete picture of how these SNPs may regulate distant genes through physical contact with non-adjacent promoters. Recently, the use of chromatin-accessibility quantitative trait loci (caQTL) and fine mapping analysis expanded the genetic variants and loci associated with T1D and provided novel molecular targets to investigate (190). Whether it be immune or beta-cell related, understanding these "true" gene targets is a vital steppingstone in leveraging this genetic information to develop diagnostic and therapeutic solutions to T1D.

CLINICAL APPLICATIONS: BETA-CELL DIRECTED THERAPEUTICS

Therapeutic strategies for preventing T1D in high-risk patients have often focused on modulating the immune response to the beta-cell. Newer strategies include methods that focus on the beta cell: reduction of beta-cell dysfunction through the manipulation of ER, oxidative, or cytokine induced cell death. Additionally, functional beta-cell mass replacement strategies through alternative sources, such as stem cells, are being exploited in the field. These strategies, however, will likely have limited clinical utility until autoimmune destruction of the beta-cell replacement can be avoided. Therefore, combinatorial therapeutic programs will likely be required to truly prevent or reverse T1D. Here we describe the state of a few current beta-cell focused therapeutics.

Modulation of ER-Induced Beta-Cell Death

Development of compounds targeting ER-stress pathways are being explored to prevent beta-cell death in early onset T1D. The three UPR sensors PERK, ATF6, and IRE1α regulate apoptosis and thus make a promising target for reducing ER stress and subsequent death in the beta-cell (191). Tauroursdoxycholic acid (TUDCA), a naturally occurring bile acid, can reduce ER-stress by inhibiting the dissociation of BiP from PERK, preventing cell death (192). In a multiple low-dose STZ C57BL/6 mouse model of beta-cell death, TUDCA improved glucose tolerance, increased beta-cell mass, and improved glycemia compared to control diabetic mice (193). The benefits of TUDCA and other UPR chaperones continue to be investigated for their ability to prevent ER-stress induced apoptosis in T1D (194).

An ongoing clinical trial using imatinib mesylate (brand name Gleevec), a tyrosine kinase inhibitor, shows promising results in targeting beta-cell ER stress (195). The efficacy of imatinib for the treatment of various immune-mediated diseases is currently being tested. Initially found to abrogate type 2 diabetes in *db/db* mice, imatinib treatment in the NOD mouse was able to reverse autoimmune diabetes (196, 197). By blunting IRE1α RNase hyperactivity, imatinib reduces beta-cell apoptosis and preserves physiological function. In humans, a clinical trial found imatinib preserved beta-cell function at 12 months in adults with recent-onset T1D (195). Ongoing studies will investigate dose and duration of therapy as well as safety and efficacy for use in children.

Targeting Oxidative Stress

Considering the major role of oxidative stress in T1D pathogenesis, therapies designed to improve antioxidant defenses in beta-cells are another promising avenue for clinical use. Thioredoxin interacting protein (TXNIP), a thioredoxin (TRX) inhibitor of the peroxiredoxin/thioredoxin detoxification pathway has demonstrated clinical potential in both animal models and initial clinical trials (198). The binding of TXNIP to TRX promotes oxidative stress by preventing peroxide clearance. TXNIP is elevated in patients with T1D and T2D (199). *In vivo* overexpression of TXNIP in mouse beta-cells induces apoptosis, while inhibition is protective against STZ-induced diabetes (200). Anti-diabetic agents including insulin and metformin, were found to augment TXNIP degradation through activation of adenosine monophosphate activated protein kinase (AMPK), supporting the idea that TXNIP may be a viable clinical target (201). Verapamil, which blocks voltage-gated calcium channels, decreased TXNIP and enhanced beta-cell survival in both human and rodent islets (202). A clinical trial in which Verapamil was administered (along with insulin therapy) promoted patient beta-cell function and lowered exogenous insulin requirements (203). TXNIP is expressed in multiple cell types throughout the body and additional clinical trials (NCT04545151, NCT04233034) are ongoing to explore the efficacy of TXNIP inhibition in protecting beta-cells and affecting autoimmune responses in patients with T1D.

For both imatinib, an FDA approved anti-leukemia drug, and verapamil, a widely used anti-hypertensive, the ability to repurpose drugs already on the market and with established safety profiles for T1D is an attractive way to expedite the often lengthy and costly process of bringing drugs to market.

Stem Cell Derived Beta-Cells and Emerging Technologies

The development of human stem cells for clinical use may provide a long-term solution to T1D without the challenge of organ shortage and HLA mismatch. While for years now, stem cell-derived insulin-producing cells can be generated and studied in the lab, improvements in cell viability, identity, and reproducibility may be needed before they can be applied as a safe and affordable therapy (204). Beta-cell differentiation from multiple cell sources

have been attempted, the most promising of which seem to be induced pluripotent stem cell (iPSC)-derived beta-cells (205). Clinical trials are in progress investigating the efficacy of treating T1D with one version of iPSC derived cells designed by Viacyte, Inc. and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) Therapeutics (NCT03163511).

Combining iPSC-derived cells with CRISPR gene editing technology allows the potential to correct monoallelic mutations in genes, such as those that cause MODY diabetes. Optimal for T1D treatment, some therapies are also focused on creating "stealthy" beta-cells and islets, that can be specifically engineered to evade immune recognition. One gene of interest is *renalase* (*Rnls)*, which encodes for an FAD-dependent amine oxidase enzyme that was identified in NIT-1 cells. *Rnls* deletion elicits beta-cell protection against autoimmune attack. NIT-1 cells carrying the *Rnls* mutation improved graft survival when transplanted into diabetic NOD mice (206). Using CRISPR, human iPSCs were generated lacking *RNLS*. These *RNLS*$^{-/-}$ iPSCs could be successfully differentiated and exhibited normal insulin secretion *in vitro*. Thus far, the *in vivo* function of these cells has not been reported. Similarly, some groups have recently generated iPSC lines that are "hypoimmune" by inactivating MHC class I and II genes and overexpressing protective marker CD47 (207, 208). These stem cells evade immune rejection in fully competent recipients, while maintaining their pluripotency. Hypoimmune stem cells have been used to treat pulmonary and cardiovascular disease and have major implications for universal transplantation (209). The widespread availability of techniques such as single-cell RNA sequencing can bolster the design of iPSC derived beta-cells by identifying the gene expression repertoire needed to obtain the appropriate distribution of cell types within the islet (210).

An exciting new technology that may aid in our understanding of the T1D beta-cell is Patch-Seq, a powerful method that can link single-cell transcriptomes with electrophysiology measurements (211). Groups using this technique in the beta-cell may be able to uncover various levels of beta-cell heterogeneity and link it to functionality in both healthy and T1D contexts. The Yoshihara group is also working on combining concepts with their work reproducing disease with 3D organoids engineered to model immune invasion (212). Overexpression of PD-L1 in human islet organoids was able to protect xenografts from immune invasion and restore glucose homeostasis for 50 days in immune competent mice. Similarly, the Melero-Martin group has used a combinatorial approach to incorporate the importance of islet vasculature in their studies. Their "vascular organoids" include microvessels that become perfused during transplantation and even reduce the islet requirement for transplantation, highlighting the importance of vascular cell types for ideal glucose regulation (213). More combinatorial therapies should be explored as we continue to uncover the nuances of the beta-cell and immunological interface of T1D pathology.

CONCLUSIONS

We have highlighted the crosstalk between pancreatic beta-cells and the immune system in T1D and potential mechanisms by which innate beta-cell characteristics contribute to T1D initiation and progression. Beta-cells display an increased vulnerability to destruction and can also perpetuate inflammatory and autoimmune responses in a destructive positive feedback loop. Despite recent advances in technologies such as single-cell sequencing and the optimization of differentiation protocols for stem cell-derived beta-cells, we still have an incomplete understanding regarding the dynamics of beta-cell biology in T1D. In particular, the relationship between the transcription factors involved in beta-cell heterogeneity that can influence immune evasion versus immune susceptibility need to be further defined. Beta-cell vulnerability to oxidative stress needs to be further explored, as redox-dependent signaling pathways influence numerous facets of beta-cell biology including the differentiation of beta-cell subtypes in T1D. Many of the aforementioned emerging technologies have been examined in T2D in the islet but have not been studied in T1D. Despite the challenges, more studies of human islets before, during, and after autoimmunity in T1D should be performed to improve our understanding of beta-cells that can resist immune destruction, and therefore our ability to design more effective treatments. Could an exploitation of beta-cell populations that are less vulnerable prevent or delay T1D onset? Perhaps as we understand these "resistant" populations more fully, therapies can target and pharmacologically expand them.

AUTHOR CONTRIBUTIONS

ET, KB, HT, CH, and RB outlined, wrote, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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GLOSSARY

ATF6	activating transcription factor 6
AMPK	adenosine monophosphate activated protein kinase
APC	antigen-presenting cell
BiP	binding immunoglobulin protein
CCR2	C-C chemokine receptor-2
CCL2	C-C ligand 2
JNK	c-Jun N-terminal Kinase
Clec16A	C-type lectin domain family 16, member A
CXCL10	*C-X-C* motif chemokine ligand 10
Ca^{2+}	calcium
GPx	glutathione peroxidase
CCL5	CC ligand 5
ChgA	chromogranin-A
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
CXCR3	CXC receptor 3
DLK1	Delta-like 1
LCMV-WE	diabetogenic lymphocytic choriomeningitis virus-WE strain
E	embryonic day
ER	endoplasmic reticulum
FasL	Fas ligand
FISH	fluorescence *in situ* hybridization
GDM	gestational diabetes mellitus
Glut2	glucose transporter 2
GSIS	glucose-stimulated insulin secretion
Gad65	glutamic acid decarboxylase 65
GWAS	Genome-Wide Association Study
HLA	human leukocyte antigen
HIPs	hybrid insulin peptides
H_2O_2	hydrogen peroxide
iPSC	induced pluripotent stem cell
iNOS	inducible nitric oxide
IRE1a	inositol-requiring enzyme 1 alpha
INS	*INSULIN*
IFNγ	interferon gamma
IL-1β	interleukin-1 beta
IP-10	IFNγ inducible protein 10
IAPP	islet amyloid polypeptide
LADA	latent autoimmune diabetes in adults
MHC	major histocompatibility chain
MODY	maturity onset diabetes of the young
MCP)-1	monotype chemoattractant protein
NOD	non-obese diabetic
NFκB	nuclear factor κB
Pdx1	pancreatic and duodenal homeobox 1
pLNs	pancreatic lymph nodes
PAD	peptidyl arginine deiminase
PTM	post-translational modification
Pref-1	preadipocyte factor 1
PAK-2	protein activated kinase 2
PERK	protein kinase RNA-like endoplasmic reticulum kinase
RANTES	regulated on activation, normal T cell expressed and secreted
ROS	reactive oxygen species
Rnls	renalase
SNPs	single nucleotide polymorphisms
SOD	superoxide dismutase
T1DGC	T1D Genetics Consortium
TUDCA	tauroursdoxycholic acid
TRX	thioredoxin
TXNIP	thioredoxin interacting protein
tTG2	tissue transglutaminase 2
TLR4	Toll like receptor-4
TNFα	tumor necrosis factor alpha
T1D	Type 1 diabetes
T2D	Type 2 diabetes
UPR	unfolded protein response
UCN-3	urocortin-3
VNTR	variable number tandem repeat
WTCCC	Wellcome Trust Case Control Consortium

frontiers in Immunology

REVIEW
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Virus Infection Is an Instigator of Intestinal Dysbiosis Leading to Type 1 Diabetes

Zachary J. Morse and Marc S. Horwitz *

Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada

In addition to genetic predisposition, environmental determinants contribute to a complex etiology leading to onset of type 1 diabetes (T1D). Multiple studies have established the gut as an important site for immune modulation that can directly impact development of autoreactive cell populations against pancreatic self-antigens. Significant efforts have been made to unravel how changes in the microbiome function as a contributor to autoimmune responses and can serve as a biomarker for diabetes development. Large-scale longitudinal studies reveal that common environmental exposures precede diabetes pathology. Virus infections, particularly those associated with the gut, have been prominently identified as risk factors for T1D development. Evidence suggests recent-onset T1D patients experience pre-existing subclinical enteropathy and dysbiosis leading up to development of diabetes. The start of these dysbiotic events coincide with detection of virus infections. Thus viral infection may be a contributing driver for microbiome dysbiosis and disruption of intestinal homeostasis prior to T1D onset. Ultimately, understanding the cross-talk between viral infection, the microbiome, and the immune system is key for the development of preventative measures against T1D.

Keywords: type 1 diabetes, microbiome, autoimmunity, coxsackievirus, dysbiosis, gut

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Matthias G. Von Herrath,
La Jolla Institute for Immunology (LJI), United States
Mikael Knip,
University of Helsinki, Finland
Emma Estelle Hamilton-Williams,
The University of Queensland, Australia

***Correspondence:**
Marc S. Horwitz
mhorwitz@mail.ubc.ca

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INTRODUCTION

Type 1 diabetes (T1D) is a persistent autoimmune disorder where immune cells attack and destroy the insulin-producing beta cells of the pancreas. Eventually, once enough beta cell mass is lost, individuals will begin to experience loss of natural blood glucose regulation and become reliant on exogenous administration of insulin. Numerous studies have characterized genetic variance and single nucleotide polymorphisms associated with T1D, which can explain why some individuals are more predisposed than others (1–6). Genome-wide association studies have found that Human leukocyte antigen (HLA) and insulin genes are responsible for a significant portion of the genetic risk for T1D. Additionally, many polymorphisms have been identified within immune-related genes including *PTPN22*, *IFIH1*, *CTLA4*, and *IL2RA* (5, 6). However, genetic make-up only accounts for part of the equation. After all, the immune system is shaped to an incredible extent by non-heritable forces and instead moulded largely by environmental exposures (7).

An array of exogenous stressors have been associated with precipitating autoimmunity (8). However, understanding exactly how environmental factors contribute to disease pathogenesis is a messy ordeal. Dysbiosis, infection, exposure to dietary antigen, and vitamin D deficiency have all

been significantly implicated in altering susceptibility to T1D (9, 10). With such complicated etiology, incorporation of multi-faceted approaches, which take into account the extensive amount of cross-talk that occurs between each of these influences on the host, should be strongly considered in future studies.

Virus infections may be an instigating factor for the gut pathology and dysbiosis that is observed in patients leading up to islet autoimmunity and/or T1D onset (**Figure 1**). Clinical evidence suggests that diabetic patients experience prolonged enterovirus infections associated with the gut mucosa, resulting in persistent inflammation. Furthermore, patients with islet autoimmunity have increased intestinal permeability, low-grade enteropathy, and a dysbiotic microbiome. Seasonal patterns observed in T1D and other autoimmune disease diagnosis could, at least partially, be explained by seasonal variations in infection (11, 12). In this review, we will examine the known effects of virus infection on the microbiome and gastrointestinal (GI) physiology, and how this modulation may relate to T1D pathogenesis.

VIRUS INFECTIONS ARE ASSOCIATED WITH T1D

Numerous viruses, particularly those associated with the gut, have been connected with T1D pathogenesis including enterovirus, rotavirus, cytomegalovirus, and norovirus (13–17). The enterovirus, coxsackievirus B (CVB), has been the virus most frequently associated with T1D. So much so, that recently there has been movement and discussion towards the necessity to develop a vaccine specific for coxsackievirus to help mitigate the globally increasing rates of T1D (18–21). CVB binds to the coxsackie and adenovirus receptor (CAR), which is highly expressed on the insulin-secreting beta cells in the pancreatic islets (22). Variance in CAR expression has been correlated with increased predisposition for T1D (23). In both human populations and experimental mouse models, infection with enteroviruses has been identified to precede onset of islet autoimmunity (24–26). A recent large-scale study looking at virus shedding in the stool of children found that while those with islet-autoantibodies had no difference in total incidence of infection, they did experience a higher rate of sustained enterovirus B (particularly of CVB serotype) infections, which may be contributing to islet autoimmunity (23). Interestingly, this study also found association of other mammalian viruses including human masadenovirus-C (HAdV-C), which actually correlates with reduced incidence of auto-reactivity. The authors suggest that this may be due to HAdV-C competitively inhibiting CAR engagement or through sustained activation of innate immunity resulting in protection from other strains of virus including enterovirus. Children who developed T1D and islet-specific autoantibodies also have a history of increased incidence of respiratory infections in early adolescence (27). It is unclear, however, if there may be underlying immune differences that cause these populations to have increased susceptibility to both these types of infections and T1D autoimmunity. But, children who experience early loss of B cell tolerance to insulin exhibit weak humoral protection against CVB, whereas those with autoantibodies to the T1D biomarker, glutamic acid decarboxylase (GAD), have competent CVB responses - signifying viral clearance may be altered in individuals with T1D-related autoimmunity (28).

Rotavirus infection in children with a genetic predisposition to T1D is associated with increased islet autoimmunity, signifying that infection may exacerbate autoimmunity and diabetes (14). In non-obese diabetic (NOD) mice, rotavirus infection has also been shown to accelerate onset of T1D (29). However, pre-existing autoimmunity is necessary to accelerate disease onset (29). Thus, rotavirus may likely promote pathogenic events rather than serving as a trigger of diabetes.

Antiviral responses to viruses including CVB can likely have direct effects within the pancreas in precipitating T1D (30–32). While CVB has been shown to impair beta cell function *in vitro*, evidence suggests that the virus itself does not destroy beta cells through cytopathy (30, 33). Antiviral responses are largely mediated through expression of the three classes of interferon (IFN): type I (IFN-α and IFN-β), type II (IFN-γ) and type III (IFN-λ). Innate viral receptor engagement and ensuing immune pathway activation can have a significant role in T1D initiation and pathogenesis (34, 35). A transient type I IFN signature has been observed preceding islet autoantibody development in genetically-susceptible children, but is lost by the time of diabetes diagnosis (36–38). This IFN signalling may be a significant contributor to the hyperexpression of major histocompatibility complex (MHC) class I, endoplasmic reticulum stress, epigenetic and transcriptional/translational modifications observed in the islet microenvironment prior to T1D development. Recently, researchers were able to detect viral signatures (enteroviral protein and dsRNA) in the islets of autoantibody-positive and recent-onset T1D donors along with increased interferon and microbial stress markers (39). There has also been some suggestion that terminally-deleted viral genomes are able to persist in the islet microenvironment causing inflammation and increased immune cell recruitment (40).

Abbreviations: AMP, antimicrobial peptide; APC, antigen-presenting cell; CAR, coxsackie and adenovirus receptor; CARD, caspase activation and recruitment domains; CTLA4, cytotoxic T-lymphocyte associated protein 4; CVB, coxsackievirus B; dsRNA, double stranded RNA; DSS, dextran-sulfate sodium; EBV, Epstein-Barr virus; FMT, fecal microbiome transfer; GAD, glutamic acid decarboxylase; GI, gastrointestinal; HAdV-C, human masadenovirus-C; HERV, human endogenous retroviruses; HHV, human herpes viruses; HLA, Human leukocyte antigen; IEB, intestinal epithelial barrier; IFIH1, interferon induced with helicase C domain 1; IFN, interferon; IGF, insulin-like growth factors; IL2RA, interleukin-2 Receptor alpha; LPS, lipopolysaccharide; MAIT, Mucosa-associated invariant T cells; MHC, major histocompatibility complex; mLN, mesenteric lymph node; MNV, murine norovirus; MR1, MHC class 1-related protein; MyD88, Myeloid differentiation primary response 88; NOD, non-obese diabetic; NOD2, nucleotide-binding oligomerization domain-containing protein 2; NOR, non-obese diabetes resistant; PAMP, pathogen-associated molecular patterns; pDC, plasmacytoid dendritic cells; PRR, pattern recognition receptor; PTPN22, protein tyrosine phosphatase 22; SCFA, short chain fatty acid; SLE, systemic lupus erythematosus; T1D, type 1 diabetes; Th, T helper cell; TLR, toll-like receptor; Treg, regulatory T cell; VILP, viral insulin/insulin-like growth-1-like peptides.

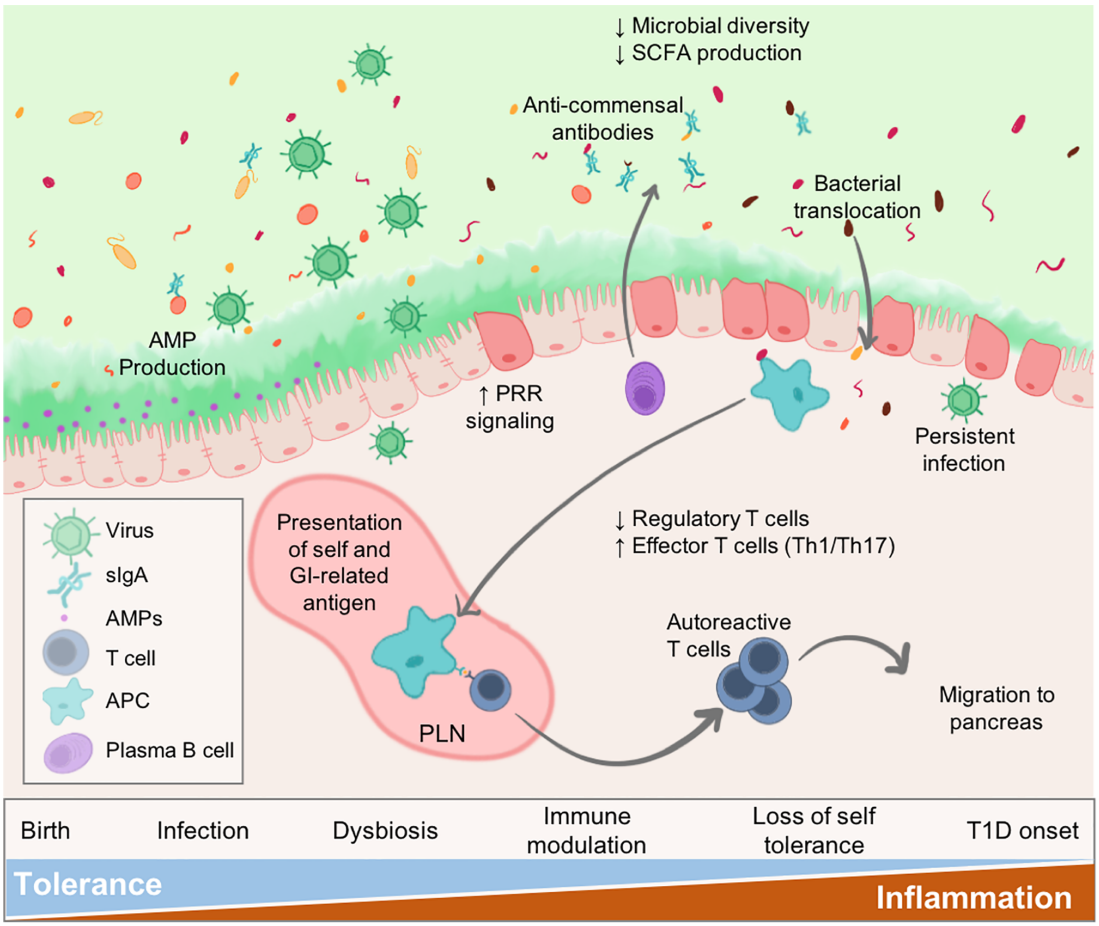

FIGURE 1 | Virus infections alter intestinal homeostasis to contribute to T1D. The GI environment is tightly regulated by numerous mechanisms. Perturbations such as virus infection results in dysbiosis and disruption to the enteric environment. Microbial dysbiosis is characterized by loss of species diversity and production of SCFAs including butyrate and acetate. As a result of dysbiosis and inflammation, the epithelial barrier becomes more permeable due to loss of tight junctions between epithelial cells, alteration of secreted IgA (sIgA) antibodies, and diminished mucus production. Some persistent infections may be maintained contributing to sustained inflammatory signalling within the gut. Both pancreatic self-antigens and commensal microbial antigens are taken up by APCs and presented to T cells in the pLN causing loss of self-tolerance. These autoreactive T cells migrate to the pancreas to contribute to anti-islet responses and destruction of insulin-secreting beta cells. Individuals would progress to T1D once sufficient beta cell mass is lost resulting in loss of blood glucose regulation.

While there are existing direct links to virus-causing inflammation and modulation of the immune system within the islet microenvironment, there are likely secondary effects of infection, which are also long-term contributors to disease pathogenesis including microbial dysbiosis.

THE MICROBIOME AND T1D

Comprising of a rich diversity of bacteria, archaea, viruses, fungi, and helminths – the microbiome is a dynamic environment that is constantly shifting. This review primarily focuses on the impact of commensal bacterial communities and later the collective virome. The microbiome has a substantial role in shaping peripheral immune tolerance, activation, migration, and differentiation, as well as local inflammatory responses (41). In response, the immune system is in constant communication to respond to these fluctuations in stimuli (42). Alterations in the microbiome have been heavily implicated in the pathogenesis of T1D (43–45) and genetic risk for T1D autoimmunity even confers differences in the bacterial microbiome (46–48). The intestinal microbiota can exert potent influence on immune homeostasis through the production of various metabolites and particularly short chain fatty acids (SCFAs). Both clinical studies and mouse models have established SCFAs including butyrate, propionate, and acetate as significant factors affecting immune regulation in T1D pathogenesis (43, 49). Metabolite-related dietary patterns have been shown to influence T1D susceptibility and metabolomic alterations precede the development of islet autoantibodies in children (50, 51).

While the human microbiome can be quite heterogenous and studies examining the relationship between the microbiome and diabetes have produced highly variable results, there are some

notable microbial hallmarks which have been often identified in individuals with T1D and islet-autoimmunity including: a marked decrease in the diversity of bacteria colonizing the gut, increased abundance of bacteria within the *Bacteroides* phylum, the loss of *Firmicutes*, and decreased production of SCFAs among other variances (52–55). However, understanding the effects of perturbations in humans while also controlling for extraneous factors is incredibly difficult. The use of NOD mice as a model for spontaneously developing diabetes has given significant insight into understanding the disease pathogenesis of T1D. While the autoimmunity experienced by NOD mice is not the same as that experienced by humans, it allows the use of environmental and genetic interventions in order to understand how they may impact diabetes development (56). Dysbiosis occurs in both humans and NOD mice prior to disease onset and diabetes incidence can even be predicted in these mice based on sampling from various mucosal microbiomes (43, 57, 58). A "diabetogenic" microbiome from a diabetes-prone NOD mouse is sufficient to promote insulitis when transferred to a non-obese diabetes-resistant (NOR) mouse (59). Typically, female NOD mice are more susceptible to developing autoimmune diabetes than male mice; however, this difference is not observed in germ-free mice (60). This discrepancy in sex bias can at least partially be explained by microbial stimulation of testosterone (60).

Pattern recognition receptors (PRRs) including toll-like receptors (TLRs), RIG-I-like receptors, and caspase activation and recruitment domains (CARDs) are innate sensors that can detect viral and microbial pathogen-associated molecular patterns (PAMPs). Signalling through these receptors can be detrimental for modifying susceptibility to T1D development (34). There are 13 total types of TLRs, each of which is specific for various bacterial (LPS, flagellin, peptidoglycan, etc.) and/or viral (dsRNA, CpG DNA, viral protein, etc.) antigens. Microbiota can regulate T1D through TLR signaling differences (61, 62). For instance, imbalance between TLR2 *vs*. TLR4 stimulation can determine T1D susceptibility where TLR2 provides a pro-diabetic signal whereas TLR4 provides microbiota-induced tolerization (61). This overlap in bacterial and virus infection immune signaling may signify a role between commensal microbes and virus infection in host immune regulation.

VIRUS INFECTIONS AS A SOURCE OF DYSBIOTIC PERTURBATION

In the first few years of life, colonization of the GI tract plays an indispensable role in shaping host immune development, regulation, and maintenance (63, 64). With age, the microbiome experiences decreasing plasticity and tolerance for new antigen exposure and environmental disruptions (63, 65). Following infancy, the microbiome seems to stabilize with relatively established communities that continue to shape mucosal and systemic immune homeostasis into adulthood (66). Thus, timing of environmental perturbations is likely an important factor for producing dysbiosis, which impacts disease susceptibility. The "Hygiene Hypothesis" proposes that exposure to antigens in early life during immune development can have profound effects for the development of autoimmune and allergic disease later on. Evidence suggests that instigating factors leading to T1D occur early in life – especially since a majority of early-onset individuals who progress to overt T1D before adolescence develop autoantibodies by 3 years of age (1, 67). However, most individuals are diagnosed with T1D in adulthood, hinting that tolerance for environmental stressors may not necessarily be limited to a defined age or that triggering events can occur long before disease onset (68).

Infections that are relatively mild later in life, may have the ability to be quite detrimental early in life at promoting T1D, as the immune system is not yet fully developed and may lack the ability to properly defend the host (69). Viruses cause dysbiosis (70–72), potentially signifying lasting consequences whereby individuals may develop a more autoimmune-skewed microbiome that might be characterized by decreased diversity and less beneficial bacteria (e.g., less butyrate producers). Studies in NOD mice have shown early life exposure to a "diabetogenic microbiome" through fecal microbiome transfers (FMT) can regulate B cell activation and promote T1D onset later on (69). However, when mice are given this same microbiome composition post-adolescence they do not experience the same modulation of the immune system and increased incidence of diabetes autoimmunity. Thus, there may be a unique window, particularly early in life, whereby disruptions in the microbiome from exogenous stressors like infection can have much larger implications on future health.

T1D: A CONSEQUENCE OF INTESTINAL DYSBIOSIS AND RESIDENT IMMUNE POPULATION CONTROL

Studies have found that detection of enteric infection precedes islet autoimmunity by 6 months or more (24, 73, 74). The existing confluence between intestinal inflammation and T1D maintain the gut as an important site for immune modulation that has implications for islet autoreactivity. While some viruses may have deleterious effects on the microbiome, others may actually promote tolerance. For example, norovirus infection was shown to protect from T1D through modulation of the microbiome (75). In this study, Pearson et al. found that NOD mice infected with murine norovirus (MNV) had significantly lower diabetes incidence, less immune infiltration into the islets, increased bacterial diversity, and an increased regulatory rather than inflammatory T cell profile.

Islet-autoreactive $CD8^+$ T cells circulate in the blood in approximately the same quantities between healthy and diabetic patients – suggesting that these cells are a normal part of the T cell repertoire (76). However these cells are more abundant in the pancreata of T1D patients, indicating that they must home to the pancreas due to altered immunoregulatory signalling, proinflammatory islet environment, and/or peripheral activation (76). The GI tract plays a fundamental part in communicating between the host and microbiota. Even at healthy steady-state

conditions, there is significant T cell trafficking between the gut and pancreatic tissues (77). Existing within this gut-pancreas axis, the pancreatic lymph nodes (pLN), which drain from the duodenum and pancreas, are sampling antigen heavily from both organs. The pLN resides at a critical and significant confluence whereby intestinal stress can alter the presentation of pancreatic self-antigens (77). It has even been suggested that this may be the portal connecting celiac disease with T1D, where GI inflammation due to gluten-sensitivity potentially stimulates anti-islet immune activation in the pancreas (77, 78). Diabetic patients experience prolonged enterovirus infections associated with the gut mucosa, resulting in persistent inflammation (79). This sustained inflammation may be sufficient to result in loss of self-tolerance and T1D development.

Adaptive Cells

Resident T and B cells hold specificity for commensal microbes even under healthy homeostatic conditions (80). T cell polarization into T helper 1 (Th1), Th2, Th17, or regulatory (Treg) cell phenotypes can be driven in the gut by presence and abundance of specific microbes in autoimmunity (42, 81, 82). For instance, *Bifidobacteria* species can drive Th17 cell responses (83) – while *Akkermansia*, *Bacteroides*, and most notably *Clostridium* species, have been shown to promote Treg populations (82, 84). Produced in large quantities, particularly by *Clostridium* bacteria, the SCFA butyrate is a potent inducer of Treg delineation through histone modification promoting *Foxp3* expression and, by eliciting high levels of transforming growth factor β (TGF-β), expression in gut-related $CD103^+$ dendritic cells (85, 86).

Regulatory T Cells

Insight into the pathogenesis of T1D has revealed that Treg cells can be potent mediators for the suppression of autoreactive T cells and promotion of tolerance to islet antigen (87). Inflammasome-deficient mice have a microbiome that is protective for T1D (88). When NOD mice are co-housed with these protected mice they experience a corresponding reduction in diabetes incidence (89). This is attributed to an expansion of type 1 regulatory T cells in the gut, which home to the pancreas and secrete IL-10 to reduce inflammation in the pancreatic microenvironment. This microbiome-driven alteration in Treg populations is likely due to production of bacterial SCFA metabolites since administration of butyrate to NOD mice also causes initial expansion of Tregs in the colon, mesenteric lymph nodes (mLN), and Peyer's patches with a subsequent migration to the pancreas and pLN to reduce T1D onset (90). Expansion of *Ruminococcus* species of bacteria can also promote $CD8^+$ Treg cells to prevent diabetes in NOD mice and a streptozotocin-induced model. Furthermore, healthy human donors have increased $CD8^+$ Tregs along with increased *Ruminococcus* when compared to T1D patients (91). These gut-primed Tregs may have a profound impact on maintaining pancreatic tolerance and may be limited in infection since enterovirus detection in young children is associated with ensuing depression of Treg responses and increased inflammatory Th1/Th17 responses (92).

Mucosa-Associated Invariant T Cells

Mucosa-associated invariant T cells (MAIT) are innate-like T cells expressing MHC class 1-related protein (MR1) that specifically binds microbial metabolites originating from riboflavin metabolite biosynthesis in bacteria. These cells are present in several tissues, and like their name suggests, they are important at mucosal sites (93). MAIT cells exist at an interesting interface and may be a key mediator between microbes, virus infection, and T1D. Germ-free mice lack MAIT cells, thus indicating that they likely rely on commensal bacteria for their development and maintenance (93). In fact, differences in bacterial metabolism can regulate MAIT cell activation (94, 95). Typically, MAIT cells are thought to have a protective phenotype whereby they promote intestinal homeostasis and have a role in supporting the gut epithelial barrier *via* secretion of IL-22, IL-12, and IL-17a (96). However, MAIT cells can also take on a more pathogenic nature in certain circumstances.

Rouxel et al. found that both recent-onset and established T1D patients have altered MAIT cell populations circulating in their blood whereby they are less abundant, express more activation/exhaustion markers, Th1-skewed, and are more cytotoxic (97). In NOD mice, MAIT cells seem to show a dimorphic phenotype depending on tissue specificity where MAIT cells in the lamina propria express IL-22 and IL-17a in non-diabetic mice; however, cells that infiltrate the pancreatic islets express IFN-γ and granzyme B to participate in beta cell destruction. Furthermore, the authors showed that MAIT cell-deficient (MR1-restricted) NOD mice have increased rates of diabetes and have a modified gut mucosal environment – suggesting that they can be protective (97). Beyond their role in sensing bacterial products, MAIT cells have also been identified to hold potent inflammatory responses in both acute and chronic virus infections (98). This is due to activation, which is independent of MR1 stimulation and instead due to cytokine signaling largely through type-1 interferon, IL-12, and IL-18 (98, 99). Ultimately, collective signalling from bacterial metabolites and cytokine profiles in infection may be detrimental in skewing MAIT populations toward either a protective or pathogenic nature in T1D pathogenesis.

B Cells

Mariño et al. found that providing diets to NOD mice that yield increased production of acetate and/or butyrate are largely protected from autoimmune diabetes (49). These two SCFAs accomplish this through their own distinct mechanisms. While butyrate primarily boosted Tregs, acetate decreased frequency of islet-specific autoreactive T cells by modulating antigen presentation in B cell populations residing in the spleen and intestinal Peyer's patches. Cross presentation of islet antigen by B cells in the pLN has been previously been shown to activate self-reactive $CD8^+$ T cells (100).

Antigen-Presenting Cells

Plasmacytoid dendritic cells (pDC) play an important part in mediating antiviral intestinal immunity. These cells extend their dendrites across the epithelial cell barrier to sample microbial antigen in the GI tract to present to resident adaptive immune

cells and can produce a significant amount of IFN in infection. pDCs infected with rotavirus can induce bystander activation of islet-reactive T cells *via* type I interferon signalling (17). Mucosa-associated pDCs likely detect virus infection and travel to the mesenteric and/or pLN to promote B cell expression of MHC-I and proinflammatory T cell cytokine secretion to aid in inflammation (101, 102). Phagocytosis of *Lactococcus lactis* bacteria by pDCs can stimulate robust IFN-α secretion *via* TLR9 and MyD88 signalling (103). Oral administration of the *L. lactis* colonization factor antigen I fimbriae can also prevent T1D in NOD mice by promoting expansion of IL-10 and IFNγ while decreasing Th1 T cells (104).

MNV infection alters recruitment of macrophages in the pLN where they are deficient in CD86, signifying a decreased capacity to activate T cells leading to protection from T1D (75). Furthermore the offspring of antibiotic-treated pregnant NOD mice also experience reduced T1D incidence by instigating tolerized APCs (105). These APCs have a diminished ability to activate cytotoxic $CD8^+$ T cells and thus represent the importance for microbiome-specific education of developing immune self-tolerance. Macrophages which lack previous exposure to bacteria in antibiotic-treated mice have reduced responses to LPS antigens (106). Decreased inflammatory responses by these APC populations due to microbiome differences may be sufficient to prevent autoreactivity – especially since islet resident macrophages are detrimental for the instigation of T1D autoimmunity in NOD mice (107).

INFECTION AND A LEAKY GUT

Containment of commensal bacteria and dietary antigens within the intestinal lumen relies on several physiological and molecular barriers. The first line of defense is a layer of mucus created by O-linked glycoproteins (mucins) secreted from intestinal goblet cells combined with luminal saccharides. In the colon, a double layer of mucus serves as a physical barrier. The apical layer is typically colonized with various mucus-degrading microbes including those within the *Akkermansia* family. The innermost mucus layer, however, is predominately uncolonized and creates a largely impenetrable barrier for bacteria. A single cell layer of epithelial cells (IEB) is joined through tight junctions to create a continuous cellular barrier throughout the GI tract. This IEB can be maintained by cytokines including IL-22 produced by group 3 innate lymphoid cells and IL-17A from Th17 lamina propria T cells. Epithelial cells and resident lamina propria immune cells constantly sample the mucosal environment and respond to changes in microbial and viral stimuli. Commensal bacteria populations are regulated through production of antimicrobial peptides (AMPs) and by secreted IgA antibodies. AMPs are bactericidal for specific bugs, particularly within the small intestine where the mucus barrier can be more discontinuous. Colonization of bacteria within the GI tract is also highly regulated by IgA antibodies, which can coat bacteria for neutralization and opsonization. Most secreted IgA is polyreactive and holds an innate specificity to multiple strains of bacteria, but can also undergo somatic hypermutation to produce highly specific IgA against particular bacteria (91, 108).

Autoimmune disorders including T1D, rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus (SLE) have all been associated with increases in intestinal permeability - or a so-called "leaky gut" (109–111). Clinical studies have found that individuals with islet autoimmunity experience increased intestinal permeability and low-grade enteropathy (112–116). Loss of integrity occurs prior to T1D development in both human and mouse models, indicating that it may be a significant trigger – rather than a result – of autoimmunity (112, 117). In fact, Sorini et al. found that breaking the intestinal barrier using low-dose dextran-sulfate sodium (DSS) treatments in NOD mice was sufficient to increase onset of autoimmune diabetes (109). This subsequent loss of intestinal integrity can induce activation of islet-specific immune cells in the gut to travel to the pancreas and promote onset of diabetes in T cell receptor-transgenic BDC2.5 crossed NOD mice. Activation of these T cells also appeared to be dependent on the presence of the gut microbiome; however, microbial dysbiosis caused by the DSS-treatment alone was not sufficient to promote autoimmunity.

Bacterial Translocation

Breakage of the tight junctions, which glue together the intestinal epithelial barrier, may be a contributing factor in allowing permeability and contribute to T1D pathogenesis (118). As a result of reduced intestinal integrity, bacteria can cross mucosal barriers and leak into systemic circulation and various tissues. When disseminated systemically, commensal bacteria antigens can rapidly promote diabetes autoimmunity in NOD mice (119). Translocation of bacteria can contribute to autoreactivity in the following ways: 1) by directly damaging the beta cells (120) 2) through presentation of bacterial antigen to autoreactive T cells (109) 3) in promoting inflammation through innate receptor stimulation (121) 4) through bacterial molecular mimicry of self-antigens (122). In fact, translocation to the pLN has been observed in NOD mice prior to diabetes onset (123). It can also trigger activation of the innate bacterial peptidoglycan receptor, NOD2, to contribute to T1D development in a streptozotocin-induced mouse model (121). Islets exposed to translocated bacteria can directly mount anti-bacterial responses and promote inflammation (124). These responses may ultimately aid in recruitment and activation of autoreactive cells within the pancreatic environment.

Intestinal Homeostasis in the NOD Mouse

Miranda et al. performed an extensive analysis looking at alterations at the mucosal immune environment in NOD mice prior to diabetes development (123). This study found that the mice developed impaired mucin production, dysbiosis, modified secretion of bacteria-specific IgA, and alterations in lamina propria dendritic and T cell populations – which skew toward an inflammatory rather than regulatory profile. Some of these changes were shown to be microbiome-driven since cross-fostering NOD pups with NOR mothers can restore mucus production. The intestinal mucus layers represent the first line

of defense against intestinal microbes and can be modulated by the presence of specific bacteria. Specifically, butyrate-producing and mucin-degrading bacteria can improve intestinal integrity through regulation of epithelial tight junctions and stimulate production of mucin synthesis, respectively (49, 125, 126). Acetate- and butyrate-yielding diets correspond to a reduced concentration of bacterial lipopolysaccharide (LPS) antigens detected in the serum of mice - indicating reduced bacterial dissemination (49). The mucin-degrading bacteria, *Akkermansia muciniphilia*, can reduce intestinal permeability through fortification of epithelial tight junctions (126). Administration of *A. muciniphilia* can reduce diabetes incidence in NOD mice by modulating mucus production and expression of antimicrobial peptides (127). Furthermore, the colonization of *A. muciniphilia* decreased islet expression of TLRs and promoted regulatory T cells (127). This potentially signifies a change in the host's ability to respond to subsequent infections and susceptibility to infection-induced diabetogenic responses.

Commensal-Specific Antibody Responses

Alterations in the abundance of certain bacterial antigens have been previously observed to elicit specific IgG antibody responses to commensal bacteria suggesting that B cell receptor and TLR stimulation can alter GI-related B cell profiles (128). Furthermore, SCFA metabolite concentration can drive production of bacteria strain-specific IgA in a T cell-dependent mechanism involving TLR recognition – resulting in altered bacterial colonization of mucosal environments (108). The presence of T1D and/or autoimmune risk alleles confers alterations in IgG and IgA anti-commensal microbial responses in HLA haplotype-dependent and -independent mechanisms (129). For example, Huang et al. observed that newly-diagnosed T1D patients have increased secretory IgA responses along with dysbiosis and decreased SCFA production (130). Performing FMTs to transfer the microbiota from these T1D patients to germ-free NOD mice results in similar alteration in IgA-mediated immunity in these mice. However, administration of the SCFA acetate is able to recover this modulation and restore IgA responses. It has yet to be determined if dysregulation of IgA-mediated control of commensal bacteria communities and intestinal homeostasis has role in contributing to T1D autoimmunity or if it is a by-product of dysbiosis and/or metabolic pathogenesis. Some evidence has indicated that changes in the anti-commensal antibody milieu occurs after seroconversion, but prior to T1D onset (48).

Infection as an Instigator of Intestinal Permeability

Collectively, research included in this review suggests that a "leaky gut" is a natural part of T1D pathophysiology that likely triggers and/or progresses disease (**Table 1**). Virus infections may be a causative agent to aid in microbiome-related promotion of autoreactivity. Increased gut inflammation invariably leads to loss of epithelial integrity and a breakdown of the barriers – thereby allowing dissemination of bacteria from the gut and increasing immune accessibility to antigens within the GI tract. Chronic viral infection is sufficient to drive sustained intestinal permeability (133). This infection-induced epithelial damage can be mitigated through blockade of type I IFN or depletion of $CD8^+$ T cells (133). Infection with *Citrobacter rodentium* is able to produce barrier disruption along with increased insulitis in NOD mice (134). Respiratory infections are known to cause gastrointestinal distress, dysbiosis, and increased intestinal permeability despite an absence of virus in the GI environment (135). SARS-CoV2 patients experience noted dysbiosis and loss of intestinal integrity corresponding with more severe systemic inflammation, bacteremia, and higher mortality rate – potentially signifying a leaky gut as a contributor to worsening disease outcomes (136, 137). Additionally, human immunodeficiency virus (HIV) infection has been shown to cause systemic immune activation and AIDs-related morbidity due to translocation of bacteria from the intestinal lumen (138). In fact, HIV positive individuals can experience systemically disseminated bacteria resulting in stimulation of anti-$CD4^+$ T cell autoantibody production (139).

VIROME AS A CONTRIBUTOR TO HOST IMMUNITY AND MICROBIAL REGULATION

The intestinal virome is made up of rich and diverse prokaryotic and eukaryotic viral communities, which are shaped by numerous factors including diet, genetics, disease, and geography (140). While a vast majority of the viruses in the body are bacteria-infecting phages, the human virome is also made up of: genomically-integrated human endogenous retroviruses (HERVs); latently-infecting viruses, such as human herpes viruses (HHVs); and potentially persistent/chronic infections – including common enteric viruses previously discussed in this review (CVB, norovirus, rotavirus, etc.) (23, 141). With the GI tract being the most abundant site of viral colonization, the intestinal virome is crucial for maintaining homeostasis and regulating disease pathogenesis through interaction with both commensal bacteria as well as the host (142). Typically germ-free and antibiotic-treated mice face immune dysfunction and altered intestinal morphology. However, infecting these mice with MNV mitigates these aberrations in the intestinal environment (143). Norovirus is therefore sufficient to preserve gut homeostasis and intestinal immunity in a manner that is typically served by microbiota. With potential for such an influential impact, it should be no surprise that alterations and dysbiosis in the viral composition have been associated with several diseases and can alter host immune homeostasis, particularly within mucosal environments (142, 144).

Virus-Mediated Regulation of Bacterial Communities

Using metagenomic analyses, researchers have observed the intestinal virome dramatically shifting prior to onset of T1D

TABLE 1 | Highlighted recent studies depicting intestinal changes associated with T1D.

Organism	Virus	Result	Microbiome Dysbiosis	Intestinal Pathology	Intestinal Immune Changes	Ref
NOD mice	None	Butyrate and acetate SCFA administration protects from T1D	Increased *Bacteroides*	SCFA treatment reduced systemic bacterial translocation and increased expression of tight junction proteins	SCFA treatment promotes increased Treg populations, altered B cell differentiation and function, increased serum IL-22, and decreased serum IL-21.	Marino et al. (49)
NOD Mice	None	NOD mice receiving FMT from T1D patients had modified IgA immunity to GI bacteria. Acetate treatment reverses IgA dysfunction.	Decreased diversity, decreased *Firmicutes* in mice receiving FMT from T1D patients	NOD mice receiving FMT from T1D donors experience heightened intestinal permeability, increased IgA immunity, and decreased AMP expression	Acetate treatment increases gut-associated Tregs and decreases IgA+ B cells.	Huang et al. (130)
NOD mice	None	Low-grade DSS administration is able to induce T1D.	DSS treatment alters microbiome, however FMT of dysbiotic DSS-induced microbiome to naïve mice is insufficient to promote T1D alone	Increased permeability triggers T1D (NOD mice have decreased tight junction protein expression, and reduced mucosal barrier	Increased intestinal permeability activates islet-reactive T cells and increased gut related T cell infiltration into the pancreatic islets.	Sorini et al. (109)
NOD mice	None	Intestinal homeostasis is altered in NOD prior to T1D onset.	Increased *Firmicutes* and reduced *Actinobacteria* prior to T1D development	Prediabetic NOD mice have increased intestinal permeability, diminished mucus production, bacterial translocation, and reduced IgA.	Prior to T1D onset, mice have elevated Th1 and Th17 responses as well as decreased Th2 cells, ILC2s, and Tregs in the small intestine.	Miranda et al. (123)
NOD mice	None	TLR4-defiecient NOD mice have accelerated T1D onset.	T1D was associated with increased *Bacteroides*, lower *Firmicutes*, and decreased peripheral SCFA levels.	Increased bacterial translocation (Serum LPS levels)	ND	Simon et al. (62)
NOD mice	None	Offspring of NOD mice treated with Vancomycin had increased autoimmunity and those treated with Neomycin experienced protection.	Both case group mice had less segmented filamentous bacteria. Offspring of neomycin-treated mice had less gram-positive bacteria overall, and more *Actinobacteria*.	ND	Neomycin-treated mice had significantly less co-stimulatory molecule expression on APCs, and decreased Th1 and Th17 T cells.	Hu et al. (105)
NOD mice	MNV	MNV infection protects from T1D development.	Increased alpha-diversity, increased *Firmucutes*/*Bacteroides* ratio, and reduced *Akkermansia* in infected mice	MNV infection causes altered Tuft cell gene expression. No changes in permeability, tight junction, or AMP expression in infected mice.	Infected mice had increased systemic Tregs, reduced inflammatory T cells and cytokine secretion, altered mucosa-associated B cell populations, and increased macrophage recruitment in pLN	Pearson et al. (75)
Humans	Unknown	Human T1D patients have decreased acetate levels and increased IgA production.	T1D patients had increased bacterial diversity, with decreased *Firmicutes* species prevalence, and decreased stool acetate and butyrate levels	T1D patients had increased IgA-coated bacteria in their stool.	ND	Huang et al. (130)
Humans	Enterovirus	Small bowel mucosa from T1D patients have increased prevalence of enterovirus. Children who progress to T1D experience sustained enterovirus infections prior to autoimmunity.	ND	Virus positive and T1D patients had increased mucosal IgA deposits.	Virus positive patients had increased CD3 intra-epithelial leukocytes. T1D patients (without celiac disease) had increased HLA-DR expression.	Oikarinen et al. (79) Honkanen et al. (24)
Humans	Unknown	Children with islet autoantibodies and who progress to T1D experience intestinal dysbiosis.	Case subjects had decreased anti-inflammatory Prevotella and Butyricimonas bacteria as well as overall decreased microbial diversity.	Individuals with islet autoantibodies and those who progressed to T1D had increased intestinal permeability and decreased mucus production	Seropositive subjects had decreased IgA (decreased stool IGHA1)	Harbison et al. (115) Gavin et al. (131)

(Continued)

TABLE 1 | Continued

Organism	Virus	Result	Microbiome Dysbiosis	Intestinal Pathology	Intestinal Immune Changes	Ref
Humans	Enterovirus B and intestinal virome	Children with islet autoantibodies experience sustained enterovirus B shedding. Changes in the virome precede T1D-related autoantibody detection.	Genetic risk for T1D confers altered virome. Increased prevalence of *Bacteroides dorei* bacteria and *Bacteroides*-associated phages prior to seroconversion.	ND	ND	Vehik et al. (23) Zhao et al. (132)

ND, no data.

(23, 132, 145). Zhao et al., for instance, found that healthy donors had significantly higher viral diversity and increased abundance of *Circoviridae*-related sequences when compared to children who developed autoantibodies and T1D (132). These differences were observed prior to seroconversion and were also reflected in coinciding dysbiosis in bacterial communities. This suggests that there is a viral-bacterial relationship in precipitating autoimmunity. While modulation of commensal bacteria through phage bactericidal predation is not well understood, the ability of certain phages to affect bacterial abundance and modify bacterial fitness is particularly exemplified by the success of phage therapies in treating antibiotic-resistant bacterial infections (146). A study by Hsu et al. showed how phage-mediated killing has cascading effects within the microbiome, resulting in expansion or attrition of non-target bacterial populations and causing altered gut metabolomic profiles (147). These results suggest that lytic bacteriophages and the induction of prophages can be potent modulators of the bacterial microbiome and their effects can be amplified between molecular and cellular signals in the GI environment.

Immune Regulation by Commensal Viruses

An exhaustive study by Dallari et al. characterized host immune responses to several asymptomatic virus infections (acute and persistent strains of MNV, mastadenoviruses, astrovirus, parvoviruses, and reoviruses) in conventional and germ-free mice (144). The authors identified both distinct and common immune modulation contributed by viral and bacterial microbes. Viruses were generally responsible for eliciting Th1- and IL-22-mediated immunity as well as B cell and bacterial response pathway activation. While each virus exposure promoted profound immunomodulation, there was little consistency in immune pathways activated by each virus examined. Viral genome type, virus persistence, and viral load were only modestly attributed to the observed immune variance suggesting there is a largely individualistic and strain-specific contribution to intestinal immunity. While bacterial members of the microbiome have been the major focus of research in respect to their ability to shape mucosal immunity, this highlights importance and impact virus exposure also has within both GI-related and systemic immune homeostasis.

Despite eukaryotic cells not being a natural target for bacteriophages, their presence can alter host immune profiles. This is most often accomplished by bacteriophage stimulation of viral PRRs, including TLRs or RIG-I-like receptors. One study showed how phage taken up in antigen-presenting cells activates TLR3 signalling and subsequently type I IFN expression (148). Another study demonstrated *Lactobacillus*, *Bacteroides*, and *Escherichia* phages can promote IFN-γ-producing T cells along with IL-6, IL-10, and IL-12 secretion *via* TLR9 activation in germ-free mice (149). These changes can alter susceptibility to ensuing bacterial and viral infection. For instance, the presence of murine astrovirus has been shown to protect against MNV and rotavirus infection *via* stimulation of type III interferon signalling in the gut epithelium (150). Type III IFN expression in epithelial cells may also be detrimental in determining persistence of CVB in enteric environments (151). Phage-mediated cell lysis of bacteria would also result in increased release of antigenic bacterial PAMPS that go on to initiate inflammation through PRR activation. Bacteriophage induced amyloid production in *E. coli* has been associated with subsequent seroconversion and development of T1D (152). This effect is hypothesized to be caused by the release of *E. coli* amyloid-DNA PAMPs, which are known inducers of TLR2 and TLR9 and have been previously shown to trigger SLE autoimmunity in mice (153). However, more evidence is needed to determine if this mechanism can be directly contributing to T1D.

Human Endogenous Retroviruses

Ancestral viruses have integrated into the mammalian genome over millions of years of evolution, resulting in human endogenous retroviruses (HERVs). Some estimates attribute approximately 8% of the human genome to a viral origin (154). These genomic viral remnants largely go unexpressed. However, they can be induced by exogenous stressors including CVB and other viral infections (155–157). Expression of HERV antigen, particularly from the HERV-W family, has been associated with both T1D and multiple sclerosis autoimmunity in humans and mouse models (158–161). Mycobacterial infection can stimulate expression of the HERV-W envelope antigen, resulting in increased cross-reactive autoantibody expression in children at higher risk of T1D (162). Murine ERV antigens can be detected in the islets of NOD mice as disease progresses and anti-ERV immunity correlates with anti-islet reactivity (158). Furthermore, inducing expression of HERV-W-Env protein in mice causes hyperglycemia, reduced insulin production, and increased immune infiltration into the pancreas (159). This indicates a potential role in promoting

inflammatory events within the islet microenvironment. While the exact role is to be determined, HERV-W-Env involvement in autoimmunity has been at least partially attributed to its signalling *via* CD14 and TLR4 PRR stimulation in APCs resulting in activation of Th1 and antimicrobial immune pathways (163).

Molecular Mimicry in the Virome

Antigenic similarity between viral and host proteins can also potentially contribute to autoimmune responses. Antibodies against CVB4 viral protein can positively recognize beta cell antigen and induce cell apoptosis (164). Commensal viruses including Poxviruses, HHVs and other dsDNA viruses have been shown to exhibit sequence homology with multiple human peptide hormones such as insulin, insulin-like growth factors (IGFs), adiponectin, and resistin (165). Viruses in the *Iridoviridae* family express viral insulin/insulin-like growth-1-like peptides (VILPs), which share a significant homology with human insulin/IGF-1. These VILPS are able to adequately bind to, and cause activation of, their respective hormone receptors in both humans and mice (165). Whether this similarity can contribute to antigenic cross-reactivity against endogenous insulin in T1D has yet to be seen.

INTESTINAL COMMENSAL BACTERIA CAN INFLUENCE VIRUS OUTCOMES

There is a significant degree of bidirectional influence between the microbiome and antiviral response. Not only does infection alter the microbial homeostasis, but the microbiome can also have a significant impact on the outcome of virus infection and the ensuing immunological responses (144, 166–168). The microbiome has been shown to determine severity of viral infection and promote resistance to enteric infection (169–171). Certain species of commensal bacteria can colonize intestinal lymphoid tissues including the Peyer's patches and mLNs to modify antigen-presenting cell cytokine expression even under healthy homeostatic conditions (172). There is some evidence that microbial antigens may even share sufficient homology to induce cross-reactive T cells against pancreatic targets (76, 122). For instance, an integrase protein expressed by many bacteria within the *Bacteroides* genus is capable of serving as a low-avidity mimotope of pancreatic autoantigen (173).

Commensal bacteria can aid or limit virus infection through enhanced viral genetic recombination, stabilization of virus particles, promotion of virion dissemination to permissive cells, and modification to immune homeostasis (174). Surface bacterial polysaccharides, such as peptidoglycan and LPS, have been shown to promote virion stability and receptor engagement to increase poliovirus and reovirus infectivity in mice (170). Certain *Bifidobacteria* and *Lactobacillus* species have even exhibited an inhibitory potential of CVB4 *in vitro* (175, 176). Additionally, depleting microbiota through use of antibiotics is able to reduce rotavirus infection by promoting virus-specific humoral responses (177). Infection with H3N2 and H1N1 influenza strains in mice causes intestinal dysbiosis and results in reduced SCFA production and diminished immune responses to secondary infections (135). Conversely, commensal bacteria LPS and extracellular matrix-binding proteins have also been shown to destabilize influenza virions and block infection at mucosal sites, respectively (178, 179).

Microbial Activation of Antiviral Immunity

Intestinal bacteria can elicit prolonged steady-state activation of the innate and adaptive immune system to modify susceptibility to subsequent infection (180–182). For instance, commensal microbes can limit persistence of MNV infection in mice through stimulation of interferon signalling (183). Ultimately, bacterial stimulation of immune pathways may play an important role in setting the thermostat for ensuing pathogenic infections particularly in the intestinal environment (184). Antibiotic-treated mice have compromised innate and adaptive antiviral immune responses resulting in impaired ability to clear virus infection (181). This is likely because the sustained immunological stimulation from commensal microbiota lowers the activation threshold in order to establish a robust immune response against an invading pathogen. In fact, intestinal bacteria can send signals to lung stromal cells to maintain a primed baseline IFN signature to prepare against subsequent influenza infection and limit early viral replication (185). The antiviral thermostat may be altered in some individuals due to genetic variance and/or environmental stimulation. This may allow the establishment of persistent infections which have been observed prior to disease onset in individuals with islet-autoimmunity and T1D (23).

CONCLUSION

Understanding how enteric viruses contribute to homeostatic regulation of immunity and may contribute to autoimmune disorders is of great importance. Consequences of virus exposure within the intestinal environment are difficult to determine due to a lack of established animal models and confounding variables including commensal microbes commonly found in murine colonies (e.g., SFB, astrovirus), which may limit viral infection and skew results (150, 186). Ultimately, mice also exhibit differences in viral susceptibility, tropism, and pathogenesis when compared to humans.

Changes in the microbiota have been observed to occur prior to autoimmunity development, which suggests that dysbiosis has a causative role in T1D rather than a result of autoreactive or metabolic pathophysiological responses (58). Intervention studies in humans modulating the microbiome through dietary means or FMT have shown some success in improving T1D outcomes and prevention (187). However, conclusive results in these studies may be limited and require further work.

While there remains much controversy with regards to the precise role and importance of virus infection and the microbiome in determining whether a genetically susceptible individual will lose self-tolerance, significant efforts are being made to understand the patterns and commonalities that are able

break through the heterogeneity of human data, background noise, and experimental limitations currently impeding understanding of these issues. Mouse models certainly provide a great deal of potential mechanistic insight into T1D; however, longitudinal human studies integrating clinical data for microbiome differences, infection history, and susceptibility to T1D-related autoimmunity are absolutely necessary to dissect the complicated etiology leading to diabetes development. Blood and stool samples from these large cohort studies can shed light on changes in the microbial, viral, and immunological landscape prior to disease onset. Furthermore, intestinal inflammation and potential increases in gut permeability can be identified by determining abundance of blood markers, clinical tests, and presence of translocated bacterial antigen (188).

Communication between the intestinal microbiota and resident immune populations likely have a profound role in dictating susceptibility and immune system response to virus infection. The intimate inter-relatedness of genetic susceptibility, viral responses, dysbiosis, and host immune state produces an incredibly complex web whereby perturbation can cause a myriad of effects. Understanding the experimental complexity in host-virus-microbe interactions is a monumental challenge. It is difficult to determine which factors and pathways are active contributors to, rather than incidental by-products of, disease. Though challenging, exploring this relationship further is necessary to inform the ultimate prevention, detection, and treatment of autoimmunity.

AUTHOR CONTRIBUTIONS

ZM and MH conceptualized, wrote, and edited the manuscript. ZM created the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Thymic B Cells as a New Player in the Type 1 Diabetes Response

Richard B. Greaves, Dawei Chen and E. Allison Green *

Centre for Experimental Medicine and Biomedicine, Hull York Medical School, University of York, York, United Kingdom

Type 1 diabetes (T1d) results from a sustained autoreactive T and B cell response towards insulin-producing β cells in the islets of Langerhans. The autoreactive nature of the condition has led to many investigations addressing the genetic or cellular changes in primary lymphoid tissues that impairs central tolerance- a key process in the deletion of autoreactive T and B cells during their development. For T cells, these studies have largely focused on medullary thymic epithelial cells (mTECs) critical for the effective negative selection of autoreactive T cells in the thymus. Recently, a new cellular player that impacts positively or negatively on the deletion of autoreactive T cells during their development has come to light, thymic B cells. Normally a small population within the thymus of mouse and man, thymic B cells expand in T1d as well as other autoimmune conditions, reside in thymic ectopic germinal centres and secrete autoantibodies that bind selective mTECs precipitating mTEC death. In this review we will discuss the ontogeny, characteristics and functionality of thymic B cells in healthy and autoimmune settings. Furthermore, we explore how *in silico* approaches may help decipher the complex cellular interplay of thymic B cells with other cells within the thymic microenvironment leading to new avenues for therapeutic intervention.

Keywords: type 1 diabetes, thymic B cells, autoimmunity, computational modelling, negative selection

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***Correspondence:**
E. Allison Green
allison.green@york.ac.uk

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INTRODUCTION

Type 1 diabetes (T1d) is an autoimmune condition characterised by the destruction of the insulin producing beta (β) cells in the islets of Langerhans by co-operative interaction between the innate and adaptive immune systems; the final assault being perpetuated by $CD8^+$ cytotoxic T cells (1–3). The non-obese diabetic (NOD) mouse that spontaneously develops T1d in a manner thought to be similar to man, has been an important resource in analysing the complexity of the T1d immunopathology (4). In line with the important role that the thymus plays in purging thymocytes with autoreactive T cell receptors (TcRs) (5), T1d progression is linked to defects in central tolerance increasing the output of islet-reactive T cells (6, 7), although definitive understanding of why negative selection of islet-reactive T cells occurs remains elusive.

Studies in NOD mice deficient in B cells have revealed an initially unappreciated role for B cells not only in helping the $CD4^+$ T cell response to islet antigens (8–10), but also promoting the survival of islet-reactive $CD8^+$ CTL *in situ* (11). This important role for B cells in the T1d process has been translated to man, where T1d progression risk is determined by the number of serum antibodies to islet antigens (12) and evidence that T1d severity is characterised by increased infiltration of islets letion of B cells in people newly diagnosed with T1d results in transient remission of the condition (14).

Recently, we reported a new role for B cells in the T1d process- mediators of breakdown in thymic central tolerance (15). We showed that thymic B cells, normally a minor constituent of a healthy thymus in both man and mouse (16, 17), rapidly increase following the initiation of islet infiltration with immune cells (termed insulitis) but prior to overt clinical manifestation of T1d. Further, we showed that thymic B cells reside in putative thymic germinal centres, undergo *in situ* class switching and differentiation into plasma cells. Autoantibodies from these plasma cells targeted specific medullary thymic epithelial cells (mTECs) for antibody-mediated apoptosis, leading to increased output of T cells that bypassed negative selection thereby enhancing T1d progression. Our finding was reminiscent of the immunopathology of the autoimmune conditions of myasthenia gravis (18) and systemic lupus erythematosus (19, 20), suggesting that thymic B cell abnormalities may be a common link between certain autoimmune conditions.

Analysis of thymic B cells is challenging; thymic involution as we age necessitates human thymic studies to be largely restricted to foetal or paediatric tissue which, quite rightly, is ethically sensitive to procure. Although it could be argued the availability of murine thymi negates issues of thymic cellularity during the involution process, researchers have an ethical responsibility to minimise the size of murine cohorts undergoing experimental procedures. Systems biology, which incorporates the development of computational models that can recapitulate the complexity of the immune response in defined tissues- offers an attractive approach to evaluate the molecules and signal pathways that contribute to the thymic B cell-mediated progression to T1d.

Examples of existing simulation studies in biology are numerous: granuloma formation (21–23), breast cancer metastasis (24) and lymphoid tissue formation (25, 26). There has also been a considerable body of work on modelling epithelial tissues (27, 28). Following this precedent it will be instructive to create a computer simulation of the early thymic events in T1d development.

This review is, therefore, structured in two parts; first we will discuss our current understanding of the ontogeny, phenotype and function of thymic B cells in health and disease, and secondly, we review the development and challenges of the systems biology approach of generating a computational model of thymic autoimmunity.

ONTOGENY OF THYMIC B CELLS

Thymic B cells were initially discovered during immunohistochemical studies of human thymic tissue (17) and subsequently in mice (29, 30).Thymic B cells, a minor population of cells within the thymic cellular pool, are present from embryonic age through to adulthood, the number of cells remaining stable throughout life, although reports of the ageing murine thymus suggest increasing numbers of thymic B cells are a characteristic of the thymic involution (6, 7, 31). Since the discovery of thymic B cells, the origin of the cells- *in situ* development versus recirculation from the periphery- has been debated. For example, Sato et al., demonstrated that peripheral B cells preferentially migrate to the thymus in response to increasing levels of intrathymic B lymphocyte chemoattractant CXCL13, although it was noted this occurred in aged mice (32). In contrast, parabiosis models argue against peripheral B cell migration to the thymus (33). Our adoptive transfer studies in NOD mice, revealed splenic B cells had minimum capacity to migrate to the thymus even when transferred at the post-insulitic, pre-diabetic phase when thymic B cell numbers rapidly increase (Davis and Green, unpublished observations). Interestingly, B cells isolated from the thymus almost exclusively migrated back to this organ following adoptive transfer into recipient mice. These findings related to peripheral B cell thymic migration, align with others, and suggest that thymic B cells originate from *in situ* development (34, 35). Further support for this hypothesis was provided by studies in NOD-RAG2p-GFP reporter mice (15), that enable monitoring of recombination activating gene (RAG) activity in developing B and T cells (36). Comparison of thymic B cell RAG activity in NOD mice revealed a significant increase in rearrangement of the B cell receptor (BcR) in the post-insulitic, pre-diabetic phase compared to pre-insulitic phase (15). Interestingly, the level of thymic B cell RAG activity was similar between NOD mice in the pre-insulitic phase to the levels seen for control, age-matched mice, and in this latter strain thymic B cell RAG activity remained at a constant level as mice aged. Although it could be speculated that increased RAG activity in thymic B cells may represent aberrant re-ignition of RAG genes in peripheral B cells that migrate to the thymus and potentially undergo receptor editing of the BcR, studies by Gay et al., demonstrating that peripheral RAG^- B cells were unable to reactivate RAG following a series of mitogenic and antigenic B cell stimulations, argue against this possibility (37). Similar findings looking at the ability of immunisation to re-ignite RAG activity supports the hypothesis that RAG expression in B cells is restricted to their developmental stages (38, 39). Thus, the thymus of mice *per se* can support B cell development, but in the context of T1d, there is an acceleration in B cell development specifically during the post-insulitis, prediabetic phase, and this coincides with a rapid increase in thymic B cell numbers (15).

If thymic B cells develop *in situ*, what are the progenitor cells and signal pathways involved? Although it is well established that the thymus is seeded with common lymphoid progenitor cells (CLP) that have T, B and NK cell potential (40), it has been challenging to map the thymic B cell development pathway assuming it originates from the CLP population. Several studies have identified B lineage-committing transcription factors and cells within the thymus that have a phenotype akin to B cell committed precursors in their natural developmental habitat, the bone marrow (34). In addition, McKenna et al., demonstrated that addition of bone marrow isolated pro-B cells into thymic organ cultures stimulated with Fms-related receptor tyrosine kinase 3 (FLT3) and IL-7 induced immature B cell development (41). Interestingly, similar culture of pro-B cells with cell-lines derived from bone marrow or thymic stroma cultured did not induce complete B cell development in the

presence of FLT3 and IL-7. However, these studies are based on B cell committed progenitors, that is pro-B cells, and it has been far more challenging to identify within the thymus the cell(s) that lie upstream of the pro-B cell that have been identified in the bone marrow (42, 43).

As well as speculation on the definitive progenitor from which intrathymic B cells develop, there also been controversy in the signal pathways that lead to intrathymic B cell development. For example, it has been proposed that intrathymic B cell development may be a default pathway resulting from perturbation of the T cell developmental pathway due to impaired CD3 or T cell receptor β (TcRβ) signalling (44) or inappropriate Notch signalling (45). However, Feyerabend et al., using the cre-lox system to delete Notch in the DN1 population, a population that has both B and T cell potential, did not divert development down the B cell developmental pathway (46). More understanding is required as to both the progenitor and signal pathways that enable the development of thymic B cells. Such studies will be invaluable in determining whether increased thymic B cell development characteristic of T1d progression in NOD mice relates to perturbation of these signal pathways.

PHENOTYPE OF THYMIC B CELLS

B cells can be divided into several classifications: follicular (FO), marginal zone (MZ), peritoneal (B1a) and regulatory (Bregs). Each subgroup of B cells can be identified by their surface markers, and each has particular functions to play in the immune response. Although Bregs have been identified in the thymus (47) Bregs will be discussed elsewhere in this Special Edition and will not be considered here. MZ B cells have received particular attention in the autoimmune setting, due to an aberrant increase in both their numbers and location in murine autoimmune models (48–50) as well as in humans living with certain autoimmune conditions (51, 52). Innate-like MZ B cells express high levels of IgM and low levels of IgD alongside coexpression of CD21 and CD35 (53). They can function to remove apoptotic cell debris impeding autoimmunity (54), or conversely, they may promote autoimmunity *via* their polyreactive receptors (48). Furthermore, weak signalling through the BcR (55, 56) or perturbation of the negative regulators of BcR signalling- including FcγRIIB- promotes expansion of MZ B cells that are more efficient at presenting antigen to T cells (57), including potentially autoreactive T cells. In light of the strong link between mutations in FcγRIIB and T1d in both mouse and man, we assessed whether thymic B cells in NOD mice had a MZ or FO phenotype (15). Our data showed that although MZ-like cells are detectable in the thymus of both NOD and control mice, there are significantly fewer in the NOD mouse thymus compared to control animals. It is intriguing that MZ-like cells are present in the thymus of mice, and it will be interesting to see if they function here as surveillance cells against infection or participate in the removal of apoptotic thymocytes.

Nevertheless, our data overwhelmingly ascribes thymic B cells to have a FO phenotype in the NOD mouse, a finding that is in line with several studies in mice and man (33). In NOD mice, CD5, which has been described as a marker for B1a cells in the peritoneal cavity, is also expressed on the thymic FO B cells, although not as extensively as seen in non-NOD strains (29). Thymic FO B cells, in comparison to splenic FO B cells, express much higher levels of MHC class I and II, as well as costimulatory molecule CD40, signalling through which may regulate expression of CD5 (58). Although the majority of thymic B cells in the NOD mice expressed an IgM^+IgD^+ BcR, class-switched B cells expressing $IgM^-IgD^-IgG^+$ BcRs were readily detectable, although notably control mice also had these class-switched cells too. In contrast to this shared phenotype of NOD thymic B cells with non-NOD strains of mice, the NOD thymus harbours class-switched unusual $IgM^-IgD^+IgA^+$ and $IgM^-IgD^+IgG^+$ FO B cells where such cells were largely absent in control mice (15). Interestingly, human peripheral B cells with this unusual expression of IgD^+ in the absence of IgM has recently been described in people living with T1d (59). In people with T1d, these IgD^+ B cells express polyreactive receptors and can interact with insulin. Although we established that insulin-reactive B cells reside in the thymus of NOD mice, similar numbers of insulin-reactive B cells were present in control, non-autoimmune mice (15). Further, our studies focused on the thymic B cell population in its entirety, not this specific subgroup of B cells. Thus, the antigen specificity of these unique thymic IgD^+IgG^+ B cells in NOD mice is yet to be resolved, but potentially harbours an autoreactive BcR repertoire.

ACTIVATION OF THYMIC B CELLS – A ROLE FOR THYMIC GERMINAL CENTRES

B cell activation takes place in specialised germinal centres (GCs) within B cell follicles of secondary lymphoid organs. GCs tend to form in response to infection, although small transient GCs have been seen in non-inflammatory conditions. GC formation requires cross-talk between stromal cells and immune cells, and are integral for the somatic hypermutation, class switching and differentiation of activated B cells into plasma cells and memory B cells reviewed by (60). Aside from conventional GCs, B cell activation can also take place in extrafollicular structures and ectopic GCs. These ectopic GCs occur in non-lymphoid tissue, and result from the remodelling of the tissue stromal cell network in response to inflammation (61). Ectopic GCs are of particular importance in autoimmunity, and several conditions have reported the presence of these structures in the target tissue (62–64), including in the islets of T1d murine models (65), and the presence of ectopic GCs correlates with disease severity. Like conventional GCs, ectopic GCs are compartmentalised into B and T cell areas (64), and several cytokines have been attributed to their localised formation including IL-22 (66) and IL-23 (67). Interferon gamma (IFNγ) seems to be one of the most critical players in ectopic GCs formation (68–70). Despite the remarkable knowledge we now have on conventional and ectopic GCs, little is known about GCs that can form in thymic tissue despite being a characteristic of autoimmune conditions like myasthenia gravis (18, 71), systemic lupus erythematosus (19) and T1d (15). Consistent among the autoimmune conditions where thymic GC occur, the GCs form at the cortical-medullary junction, and in NOD mice, such structures only materialise at the post-insulitic, pre-diabetic phase. Furthermore, the thymus becomes enriched with IL-21 as thymic GCs form, a cytokine that is critical for regulating GC maintenance

and promotion of B cell differentiation and proliferation (72). Expression of activation-induced cytidine deaminase (AID) increases, suggesting active somatic hypermutation/class-switching is ongoing. IL-21 has proven a particularly interesting cytokine in promoting T1d both in man (73) and NOD mice (74). At the heart of the link between IL-21 and T1d are the T follicular helper (Tfh) cells (73) and in children at risk of T1d progression, circulating Tfh cell numbers peak around onset of clinical symptoms (75). In man it has been described that Tfh cells within conventional GCs may be identifiable from their recirculating counterpart on the basis of expression of the master transcription factor for Tfh cells- Bcl-6; whereas GC residing Tfh cells express Bcl-6, recirculating Tfh cells may not (76, 77). Nevertheless, circulating Tfh cells are uniquely gifted at entering inflamed tissue, and participating in ectopic GC formation (78) and *in vitro*, can promote B cell class switching retaining characteristics of GC Tfh cells (79, 80). As expected, considering their essential role in GC/ectopic GCs, Tfh cells are enhanced in the thymus of NOD mice at an age when thymic GC formation occurs. These Tfh cells express Bcl-6 and IL-21, as well as other known markers of Tfh cells (15). This suggests that intrathymic Tfh cells may derive from *in situ* $CD4_+$ T cells, unless circulating Tfh cells that migrate to the thymus take on the phenotype of GC Tfh cells with respect to Bcl-6 expression.

Certain questions remain about the nature of the thymic GCs in T1d; do they contain follicular dendritic cell structures and what is the source of the inflammation to push thymic GC formation? GCs are populated with specialised follicular dendritic cells (FDC) that act as a depot for antigen presentation within the GC (81). However, we have yet to confirm such cells exist in our thymic GCs (Pinto and Green, unpublished observations). Interestingly, it has been postulated that the type of FDC present in a GC/ectopic GC may be unique to the tissue and type of inflammation (67, 82) and markers of conventional GCs may not be present on thymic FDCs. In terms of inflammation, it could be speculated that viral infections linked to T1d development in man may also infect the thymus (83) or endogenous retroviral infection (84) could induce thymic GC formation. Alternatively thymic GC formation may simply be a reflection of an accelerated ageing of the thymus (85). Indeed, others, have documented accelerated thymic involution in NOD mice (6). It will be important to determine if people who develop T1d also have thymic GCs, and potentially accelerated ageing of this tissue.

THYMIC B CELL FUNCTION

The immunohistochemical evidence that thymic B cells form follicles at the cortico-medullary junction, and can form rosette-like structures around T cells in the medulla (86, 87) led to early speculation that thymic B cells were involved in negative selection of autoreactive T cells. Subsequent studies from different groups have substantiated that hypothesis (discussed below), although whether thymic B cells positively or negatively contribute to negative selection seems dependent on the autoimmune nature of the mammal studied. Before we discuss the evidence for the role of thymic B cells in negative selection, let's first consider their antigen specificity.

We earlier touched on the finding that thymic B cells in NOD mice or control animals expressed receptors for insulin i.e. they were autoreactive. In normal B cell development and maturation, efficiency in removing B cells with autoreactive BcRs is high, with approximately 20% of circulating B cells bearing autoreactive BcRs (88). Interestingly, the removal of B cell with autoreactive BcRs occurs in two stages; initially at the immature B cell stage in the bone marrow and subsequently during the transition of immature B cells to mature B cells following their recent egress from the bone marrow, with the early immature to immature B cell development stage exhibiting the largest removal of self-reactive B cells from the repertoire (88). Thus the earliest stages of B cell development in the bone marrow the repertoire of B cells has a high level of self-reactivity. To determine if the thymic B cell repertoire, similar to bone marrow-derived B cells, had self-reactivity, Rother et al. performed comparative sequencing studies of single cell sorted paediatric thymic B cells versus foetal bone marrow B cells (89). Such studies demonstrated that thymic B cells had a greater specificity for self-peptide autoantigens than similar sequenced foetal bone marrow B cells, these latter cells being more specific for dsDNA. Furthermore, thymic B cells had polyreactivity, recognising multiple autoantigens, including insulin. This prevalence of thymic B cells to harbour an autoreactive BcR has been shown by others (90). It can be envisaged that autoreactive thymic B cells may participate in negative selection by presenting 'free' autoreactive antigens- either trafficked to the thymus or captured from dying medullary thymic epithelial cells. Indeed, thymic B cells have been shown to efficiently present antigens their BcR is specific for to developing T cells (91) promoting efficient deletion of autoreactive T cells during T cell development (92–95). However, capture of autoantigens by the thymic B cell autoreactive BcR is not the sole way thymic B cells contribute to negative selection. Yamano et al., using a transgenic autoimmune regulator (AIRE) gene locus encoding a chimeric influenza haemagglutinin protein and human CD2 promoter demonstrated that 50% of thymic B cells express AIRE. In contrast, splenic or bone marrow B cells in the transgenic animal did not (94). Interestingly these $AIRE^+$ thymic B cells resided in the medulla, and comparative studies of tissue restricted antigen (TRA) expression between $AIRE^+$ thymic B cells and $AIRE^+$ medullary thymic epithelial cells found there was no overlap between the two cell types in the TRAs presented. This suggests that thymic B cells may work in concert with medullary thymic epithelial cells to negatively select a greater range of autoreactive T cells. The work of Yamano builds on early reports that thymic B cells deleted superantigen specific T cells and that B cell specific expression of myelin oligodendrocyte glycoprotein enhanced negative selection of MOG-reactive transgenic T cells (16).

This ability of thymic B cells to participate in negative selection is suggested to be linked to their intrathymic class-switching activity (96), autoreactive thymocytes enabling selection and expansion of their cognate autoreactive thymic B cell counterpart. Perera et al., used an AID reporter mouse in a parabiotic model to show that self-antigen can drive class-switching of thymic B cells *in situ*, and the

class-switched cells predominantly expressed IgG2b and IgA BcRs (96), and on an autoimmune background, class-switching of autoreactive thymic B cells numbers was enhanced. Further, they showed that impeding class switching of thymic B cells impaired their ability to negatively selective autoreactive T cells.

Others have documented a role for thymic B cells in the development of thymic T regulatory cells (Treg), and animals with expanded thymic B cell compartments have a correlating expanded thymic Treg compartment too (97, 98).

It would appear that there is strong evidence that thymic B cells are important in central tolerance. However, this positive role for thymic B cells in central tolerance is contrasted by the evidence that thymic B cells are key players in mediating tissue damage in myasthenia gravis (18, 63, 71, 99) and more recently systemic lupus erythematosus (19, 100) and T1d (15). Myasthenia gravis is the most documented condition where thymic B cells participate negatively in the autoimmune outcome. In myasthenia gravis, the thymus is the key source for pathogenic acetylcholine receptor antibodies that target this receptor on muscles leading to chronic muscle weakness. The thymus in myasthenia gravis patients have medullary thymic epithelial hyperplasia (18) with autoreactive thymic B cells secreting antibodies to acetylcholine receptors expressed on the medullary thymic epithelial cells triggering their demise *via* Complement-mediated attack (101).

In animal models of systemic lupus erythematosus, thymic B cells have a distinct transcriptome compared to thymic B cells from non-autoimmune prone, with increased prevalence of genes related to B cell survival (19). These increased thymic B cells were shown to promote expansion of the Tfh cells which in turn could enhance the systemic autoantibody response.

Our own studies in NOD mice, suggest that in T1d, thymic B cells may act in a manner similar to that seen in myasthenia gravis. Enhancement of thymic B cell numbers, the formation of thymic germinal centres results in a significant increase in intrathymic antibody levels in contrast to non-autoimmune prone mice. These antibodies are predominantly of the IgG1 and IgA subclass and were unique to the thymic compartment (15). Furthermore, *in situ* binding of these IgG antibodies to thymic medullary epithelial cells correlated with enhanced apoptosis of these antibody-selected cells. We have yet to establish that the antigen recognised by the autoantibodies is insulin, however, we showed that loss of certain medullary thymic epithelial cells resulted in decreased negative selection of autoreactive T cells, and enhanced survival of insulin-reactive thymocytes (15).

These contrasting roles for thymic B cells in negative selection are intriguing. Whether thymic B cells harbour pro-negative selection and anti-negative selection subpopulations, with the latter population having an advantage over the former in the autoimmune setting remains to be established. Alternatively, it may be that as an autoimmune process is ongoing, pro-negative selection thymic B cells switch to an anti-negative selection functionality. The potential mechanisms that promote these divergent properties of thymic B cells is shown in **Figure 1**. Studies that address these hypotheses will be important in understanding the relationship between thymic B cells and central thymic tolerance.

COMPUTATIONAL APPROACHES TO DEVELOP A THYMIC *IN SILICO* MODEL

Many challenges face immunologists studying the thymus; early involution of thymic tissue meaning most human studies are based on procured tissue from foetuses, neonates or young children, and acquiring such tissue is not readily accessible due to, among other issues, ethical reasons. Studies of the thymus, particularly the ageing thymus and its many intricacies in cellular cross-talk, requires large cohorts of mice, again rearing questions of ethics. Recent years have seen a drive towards using computational algorithms and *in silico* models to recapitulate the dynamic environments of immune tissues, and assess the role of candidate molecules in a particular pathway. In this last part of the review, we will discuss the potential of developing *in silico* computational models to identify therapeutic avenues for manipulating thymic B cells in autoimmune disorders like T1d.

IN SILICO APPROACHES TO MODELLING BIOLOGICAL SYSTEMS

Systems biology is the integration of wet-lab experimentation and computational research in order to understand complex biological systems. Computational biology provides tools for the theoretical exploration of biology, permitting scientists to address critical questions directly (102). Simulation is one facet of computational biology that is finding increasing usage (103–106).

The use of simulation would bypass the necessary ethical, financial and practical issues surrounding acquisition of thymic tissue. In addition, the very nature of the system lends itself very precisely to Agent Based Modelling and Simulation (ABMS); consisting as it does of very many heterogeneous individuals of different types e.g. B cells, T cells, thymic medullary epithelial cells. These cell populations can interact with each other in specific ways under specific conditions. The cells will also possess some concept of location (the medulla or the cortico-medullary junction) and state (e.g. some stage of the cellular life cycle). Use of agent-based modelling will permit us to investigate how manipulating cell behaviours will give rise to altered system-wide ('emergent') behaviour. The results will be easily interpretable and so simple to put into words accessible to a wider audience.

MATHEMATICAL VERSUS COMPUTATIONAL MODELS

A simulation is typically either mathematical or computational. Mathematical models are generally based on series of differential equations (107) and cells are modelled as populations rather than as individuals (108). Such models are often seen as opaque to non-mathematicians (109) and the model is typically difficult to extend should further cell types need to be considered. A further perceived disadvantage of mathematical models is that the resulting differential equations tend be complicated to solve

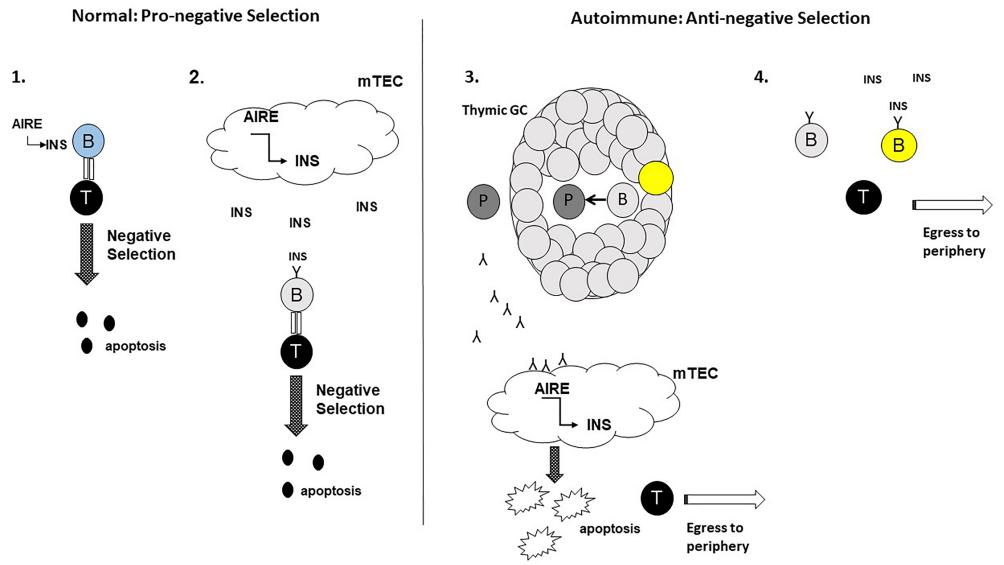

FIGURE 1 | Potential roles for thymic B cells in the thymic negative selection process: a hypothesis. In the normal setting, thymic B cells enhance the negative selection of autoreactive T cells. 1. Aire$^+$ Thymic B cells (blue) express and present self-antigens e.g. insulin (INS) to autoreactive T cells with high affinity TcRs for the self-antigens leading to T cell apoptosis. 2. Self-antigens e.g. INS secreted by Aire$^+$ mTECs are acquired by thymic B cells expressing self-reactive BcRs (light grey). Internalisation, processing and presentation of the self-antigen to autoreactive T cells leads to T cell apoptosis. In the autoimmune setting, thymic B cells impede the negative selection of autoreactive T cells. 3. The emergence of thymic GCs results in thymic B cells receiving signals to develop into plasma cells (dark grey) secreting autoantibodies for self-antigens expressed by mTECs. Binding of the autoantibodies to mTECs leads to mTEC apoptosis, leading to decreased negative selection of autoreactive T cells and increased egress of the T cells to the peripheral tissues. 4. Somatic hypermutated (yellow) thymic B cells egressing from thymic GCs, may outcompete 'normal' thymic B cells for the binding of self-antigen in the thymic milieu impeding negative selection. For this scenario, the sm thymic B cells would either fail to adequately present the self-antigens to autoreactive T cells to support negative selection.

exactly and often require solving *via* numerical methods which entails potentially unacceptable approximations to the model.

On the other hand, computational simulation methodologies such as Agent Based Modelling (ABM) are typically conceptually much simpler (110, 111). Cellular populations are modelled as sets of individuals (112) that share similar behaviours e.g. a population of Single Positive T cells. In this way more descriptive explanations of experimental observations may be proposed from simulation outcomes. Additionally agent-based models are better suited than other modelling techniques to capturing the system-wide, or emergent, behaviour of the system (113). Here, emergent behaviour is understood to be the system behaviour arising from the combined behaviours of the component entities e.g. cells.

An ABM will represent some abstraction of the system created jointly by a scientist, expert in the system of interest, and a software developer. The model should aim to include all factors e.g. cells and signalling molecules, generally held to be essential to system function. It is also important to include any potential roles for the biological environment in the model.

CREATING AN AGENT-BASED MODEL

A number of short tutorials on the creation of agent based models can be found in the literature e.g. Bandini et al., (114). Generally, the principal steps in developing an agent-based simulation are:

i. Identify the agents that are important to what you want to model:

For example in a model of negative selection events in the thymic medulla, we might wish to model the behaviours of Single Positive T cells, B cells and medullary thymic epithelial cells (mTECs).

ii. Identify the environment in which your agents exist:

We might for instance break the thymus into three distinct environments: the cortex, the cortico-medullary junction (CMJ) and the medulla; placing relevant cells in each.

iii. Identify mechanisms whereby the agents interact with each other and with their environment:

We might consider that cells behave differently in different environments. For example, SP T cells will not tend to remain in the cortex, but will rather migrate across the CMJ to the medulla where they will interact with mTECs, *via* recognition of the insulin fragments presented by the mTECs, to facilitate negative selection. Aggressive B cells will also be able to interact directly with mTECs, also *via* recognition of insulin presented by the mTECs.

iv. Consider how best to implement the model as computer code and any assumptions and simplifications necessary in achieving this:

It will be very difficult to exactly replicate precise biological behaviour as computer code. For example, cells are unlikely to be of regular shapes e.g. circular, so the geometry of the system will not be exactly reproducible *in silico* and appropriate approximations will be necessary. In the case of thymic B cells it will be necessary to decide how we will differentiate between those B cells which serve to enhance the negative selective of autoreactive T cells and those that prevent the negative selection of autoaggressive T cells.

CHALLENGES AND LIMITATIONS OF THE APPROACH

Despite the benefits of employing simulation as an investigative tool, it is wise to be mindful of the potential pitfalls in the use of the technique.

As an experiment, a simulation must be reproducible. It is therefore of paramount importance that simulation design be comprehensively documented. The documentation must incorporate all assumptions and design decisions to make them available to the scientific community to assess. The CoSMoS simulation design protocol (115) provides guidance in documentation and development of simulations, in which the user can have confidence.

The first challenge in the design of agent-based models is to correctly capture the relevant entities (cells, signalling molecules etc.) of the system and their behaviours in the model. If a key component of the system (or its behaviour) is omitted from the model then the model will be unfit for the purpose for which it was designed. As an example, two different types of behaviour in the B cell population have been noted. Some cells appear to be essentially quiescent, though may play an active role in sustaining negative selection, whereas others adopt a more aggressive role, apoptosing mTECs and thus triggering the breakdown of negative selection and hence central tolerance. Effective communication between the software developer and the expert biologist will help to mitigate this problem.

Also, simulation outcomes will be determined by the choice of values for the required simulation parameters. Model parameters may represent quantities such as the duration of a particular cell cycle stage or the concentration of cytokine that brings about progression to the next differentiation state. Such values will, in all likelihood, not be available from experiment and must be estimated. Chosen parameter values will ultimately impact on overall system behaviour and simulation output. Calibration is the process of adjusting parameter values so as to align *in silico* simulation results with observed *in vivo* behaviours. The issues surrounding simulation parameterization are discussed briefly below, but are addressed more fully in the relevant literature:

Optimization of parameter values is the subject of numerous widely used techniques such as the Latin Hypercube (116). This technique is based on the random sampling of the entire parameter space and is time and resource intensive to perform for a typical biological system.

The stability of simulation performance with parameter perturbation can be assessed using two different types of analysis. Robustness analysis is used to gauge the impact of simulation parameters on the simulation's ability to execute stably i.e. which parameter values might cause the simulation to crash (117). Sensitivity Analysis can be used to investigate the effects of individual parameters or combinations of parameters on simulation outputs *via* the systematic variation of parameter values and observation of the effect on simulation performance (117). Sensitivity analyses are further discussed in (118, 119).

Recent research has aimed to refine techniques that facilitate calibration of simulations involving large numbers of parameters in a less resource intensive manner than the Latin Hypercube method described above. These novel techniques include the use of Machine Learning and multi-objective calibration (120, 121).

Although the scope and particularly the calibration of computational simulations is potentially limited by the scale of the computational resources available, there is a trend towards easier access to powerful high performance computer clusters which makes these concerns less relevant.

Despite the challenges entailed in employing an ABMS approach to biological simulation, ABMS remains a highly descriptive and powerful methodology for elucidation of cellular and molecular mechanisms of disease and many of the challenges posed by the use of the technique, particularly those relating to parameterization, are becoming more easily addressed using recent developments.

FINAL COMMENTS

Commonalities in autoimmune conditions offer insights into novel therapies that may target multiple autoimmune conditions. Evidence that abnormality in the thymic B cell compartment is a shared characteristic between several autoimmune conditions highlights the need for further studies into this enigmatic cell. Nevertheless, increasing ethical considerations and availability of thymic tissue for analysis makes studies of thymic B cells somewhat challenging, particularly in man. Computational models of the dynamic thymic environment may offer a new approach to address the role of thymic B cells in health and disease, leading to candidate molecules or signal pathways as novel therapeutic targets in autoimmune conditions, including T1d.

AUTHOR CONTRIBUTIONS

DC and EAG wrote the thymic B cell section. RG wrote the computational modelling section. All authors contributed to the article and approved the submitted version.

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Changes in MDA5 and TLR3 Sensing of the Same Diabetogenic Virus Result in Different Autoimmune Disease Outcomes

Pamela J. Lincez[1], *Iryna Shanina*[2] *and Marc S. Horwitz*[2*]

[1] Michael Smith Laboratories, The University of British Columbia, Vancouver, BC, Canada, [2] Department of Microbiology & Immunology, Life Sciences Institute, The University of British Columbia, Vancouver, BC, Canada

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F. Susan Wong,
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***Correspondence:**
Marc S. Horwitz
mhorwitz@mail.ubc.com

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Seemingly redundant in function, melanoma differentiation-associated protein 5 (MDA5) and toll-like receptor- 3 (TLR3) both sense RNA viruses and induce type I interferon (IFN-I). Herein, we demonstrate that changes in sensing of the same virus by MDA5 and TLR3 can lead to distinct signatures of IFN-α and IFN-ß resulting in different disease outcomes. Specifically, infection with a diabetogenic islet β cell-tropic strain of coxsackievirus (CB4) results in diabetes protection under reduced MDA5 signaling conditions while reduced TLR3 function retains diabetes susceptibility. Regulating the induction of IFN-I at the site of virus infection creates a local site of interferonopathy leading to loss of T cell regulation and induction of autoimmune diabetes. We have not demonstrated another way to prevent T1D in the NOD mouse, rather we believe this work has provided compounding evidence for a specific control of IFN-I to drive a myriad of responses ranging from virus clearance to onset of autoimmune diabetes.

Keywords: autoimmunity, diabetes, interferon, MDA5, TLR3, coxsackievirus, interferonopathy

INTRODUCTION

As is commonly described for most autoimmune diseases, type 1 diabetes (T1D) is a disease that results from changes in specific genes, disruption in the balance of immune responses, and exposure to environmental agents like viruses (1–3). With studies and data that continue to emerge from major T1D pancreatic tissue biobanks (4, 5), it is increasingly clear that genetics alone cannot account for the beta cell destruction and insulitis that ensues in the pancreas of people (mostly children) suffering with T1D.

Although not included as type 1 interferonopathies, organ-specific autoimmune diseases such as T1D have been strongly associated with upregulation of the type 1 interferon (IFN-I) response. In children at risk for T1D, an IFN-I transcriptional signature precedes islet autoimmunity (6). In recent onset studies, patients with insulitis-affected islets have an overexpression of interferon-stimulated genes (ISGs), comparable to responses seen in islets infected with virus or treated with inflammatory agents like IFN-α or IFN-γ (7, 8). Included in the overexpressed ISGs identified in

T1D patients, are the genes that express the enterovirus sensors melanoma differentiation-associated protein 5 (MDA5) and toll-like receptor- 3 (TLR3).

And, in a recent study using a reporter cell line infected with enterovirus strains isolated from T1D patients, immune transcriptome data supports the hypothesis of enterovirus-induced immune changes leading to the development of autoimmunity (9).

Infection by enteroviruses, such as coxsackieviruses, has been strongly associated with the autoimmune disease process T1D (2, 10). MDA5 and TLR3 are double-stranded RNA virus sensing proteins that specifically detect and protect from coxsackievirus infection and upon viral RNA detection, MDA5 and TLR3 activate and stimulate a cascade of anti-viral responses leading to the production of IFN-I (11, 12). Depending on their levels of expression and the timing and location of signaling, MDA5 and TLR3 can also protect from the onset of autoimmune diabetes following infection with diabetogenic viruses like coxsackieviruses and the pancreatropic RNA virus encephalomyocarditis virus strain D (EMCV-D) (1, 3, 12–15). In MDA5 heterozygous NOD mice, reduced expression of MDA5 induces a unique IFN-I signature with greater IFN-β and this protects mice from T1D after CB4 infection (13). Further, blocking IFN-α, but not IFN-β prevented T1D in the RIP-LCMV Tg model post LCMV infection (16).

Herein, we will show in greater detail, how changes in sensing of the same virus by MDA5 and TLR3 can lead to distinct signatures of interferon (INF)-α and IFN-ß resulting in different autoimmune diabetes disease outcomes. These findings add to the growing knowledge of interferonopathy as a contributor in enterovirus-driven autoimmunity (2, 3, 17) and highlight further, the importance of the local immune environment at the site of autoimmunity as an indicator of disease susceptibility.

MATERIALS AND METHODS

Mice

NOD/ShiLtJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). $MDA5^{+/-}$ mice were backcrossed from C57BL/6 $MDA5^{-/-}$ mice onto the NOD background as previously described. We confirmed by SNP analysis (in house and by DartMouse, Lebanon, NH) that they carry the full complement of NOD *idd* alleles. More importantly, we confirmed that littermates to the backcrosses that were either heterozygous or wild-type for the $MDA5^{-/-}$ alleles mice had the ability to develop spontaneous diabetes which is strongly indicative that the required susceptibility loci had crossed over. $TLR3^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, USA) and were backcrossed and maintained on the NOD/ShiLtJ mouse background. NOD $TLR3^{+/-}$ and $TLR3^{+/+}$ progeny were bred for use in experiments. Mice were maintained in the Modified Barrier Facility (Pharmaceutical Sciences Building, Vancouver, British Columbia) and kept in a pathogen-free environment. Diabetes incidence was monitored by non-fasting blood glucose measurements. Disease onset was determined by two consecutive blood glucose levels exceeding 300 mg/dL. Only pre-diabetic mice were used for experiments. All animal work was performed under strict accordance with the recommendations of the Canadian Council for Animal Care. The protocol was approved by the Animal Care Committee (ACC) of the University of British Columbia.

Western Blotting

Mice were stimulated by intraperitoneal injection with 100μg of polyinosinic:polycytidylic acid (P1530, Sigma, St. Louis, MO). After 24 hours stimulation, spleens were isolated and homogenized by sonication and tissue homogenates were lysed with CellLytic MT Mammalian Tissue Lysis Reagent (Sigma, St. Louis, MO). Samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gels, transferred to polyvinylidene fluoride membranes, blocked with Odyssey Blocking Buffer (LI-COR, Lincoln, NE) probed with monoclonal mouse anti-TLR3 (Novus Biologicals, Littleton CO) and polyclonal goat anti-tubulin Santa Cruz Biotech, Santa Cruz, CA) primary antibodies and IRDye 800CW and IRDye 680 RD secondary antibodies (LI-COR, Lincoln, NE). Membranes were scanned with the LI-COR Odyssey Scanner (LI-COR, Lincoln, NE). Protein was quantified using LI-COR Odyssey 3.0 Software.

Virus

Ten-to 12-week old mice were infected intraperitoneally with sublethal doses of 400 plaque-forming units (PFUs) of CB4 Edwards strain 2 or coxsackievirus group B type 3 (CB3, Nancy Strain) diluted in DMEM. As there is no gender bias in CB4-mediated T1D, equal numbers of male and female mice were infected with CB4. Both male and female mice were infected with CB3. Virus stocks were prepared and free virus particles were detected from tissue homogenates by plaque assay as described previously (13).

Immunohistochemical and Immunofluorescent Staining

Single cell-suspensions from pancreatic lymph nodes and spleens were restimulated for 4 hours at 37°C in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum with 500ng/ml PMA, 10 ng/ml ionomycin and Golgi Plug (BD Biosciences). Cells were stained for surface and intracellular markers (**Table S2**), fixed, permeabilized, stained for inflammatory cytokines like γ-interferon (IFN-γ) and analyzed by flow cytometry. Cytokines IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, and IFN-γ were measured from serum days 0, 3 and 7 post-CB4 infection in a multiplexed format using a Cytometric Bead Array (mouse Th1/Th2/Th17 cytokine kit; BD Biosciences, Mississauga, ON). IFN-Is, IFN-α and-β were measured from serum by ELISA using VeriKine Mouse Interferon-α and β ELISA kits (PBL Interferon Source, Piscataway, NJ). All mAbs were purchased from eBiosciences (San Diego, CA) with the exception of Helios from BioLegend (San Diego, CA). Stained cells were analyzed by flow cytometry with the BD Biosciences LSR II (San Jose, CA) and Flow Jo vX.0.6 software (TreeStar, Ashland, OR).

Reverse Transcription and Quantitative Real-Time PCR

Organs were removed and immediately snap frozen in TRIzol reagent (Life Technologies Inc, Burlington, ON). Tissues were weighed and organs were homogenized using QIAGEN stainless steel beads and TissueLyser II benchtop homogenizer at 19/s for 10 min. Total RNA was prepared with TRIzol reagent according to the manufacturer's protocol (TRIzol, Life Technologies). RNA was quantified using a NanoDrop-ND-1000 (VERIFY) (Thermo Scientific, Wilmington, DE).

cDNA was prepared for 1μg of RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Reverse transcription-PCR was performed with the BioRad T-100 Thermal Cycler. cDNA was diluted with UltraPure™ DNase/RNase-Free Distilled Water (Life Technologies) and a final RNA concentration equivalent to 10μg/μl was used for real time (RT)-PCR. Gene expression for MDA5, IFN-α, IFN-β, TLR3 and GAPDH was quantified using the iQ™ SYBR® Green Supermix (BioRad, Mississauga, ON) PCR amplification was performed in 384-well plates with the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). All samples from three independent experiments were evaluated in duplicate amplification reactions. mRNA expression was normalized to GAPDH. The comparative C_t method was used as previously described and data is shown as -Δ C_t and fold change of -Δ C_t relative to Wt (NOD) samples (18). Primers used in this study are listed in **Table S2**.

Poly I:C Treatment

At days 3 and 5 post-infection with CB4, mice were stimulated by intraperitoneal injection with 100μg of polyinosinic:polycytidylic acid (P1530, Sigma, St. Louis, MO).

Statistical Analysis

GraphPad Prism 6.0 software (GraphPad, San Diego, CA) using the Student t test (two-tailed distribution) and a P value <0.05 determined statistical significance. Serum cytokine concentrations were determined with FCAP Array Software (BD Biosciences, Mississauga, ON). Data are presented as means ± SEM.

RESULTS

To better understand how immune pathologies like IFN-I responses that result from MDA5 and TLR3 signaling influence T1D development, we challenged heterozygous NOD mice that retained MDA5 and TLR3 function with known diabetes and IFN-I inducers like coxsackievirus B4 (CB4).

Western blots confirmed a 50% reduction in MDA5 (13) and a 30% reduction in TLR3 expression (**Figure 1A**) in the spleens of our MDA5 and TLR3 heterozygous mice who were stimulated with an RNA mimetic polyinosinic:polycytidylic acid (poly I:C). Expression of MDA and TLR3 is below the level of detection in unstimulated mice (not shown). Complete deficiency in TLR3 expression ($TLR3^{-/-}$) inhibits survival after CB4 infection (12), whereas a slight loss in TLR3 expression ($TLR3^{+/-}$) retains sufficient signaling function for protection against CB4 (**Figure 1B**) and another coxsackievirus associated with T1D, coxsackievirus B1 (CB1, not shown). TLR3 expression, at reduced levels in $TLR3^{+/-}$ mice, is not successful in protecting from CB4-induced T1D (**Figure 1B**) as up to 16 days post-infection, $TLR3^{+/-}$ mice show a higher incidence of T1D compared to infected control wild type $TLR3^{+/+}$ mice. Disease incidence in $TLR3^{+/-}$ mice continued to rise to 50% by day 20 post-infection, just under the 60% disease incidence observed in $TLR3^{+/+}$ mice. Though critical for survival, TLR3 expression does not contribute to protection from CB4-induced T1D, contrary to previous observations in $MDA5^{+/-}$ mice, where a reduction in MDA5 increased survival and protected against the development of T1D after CB4 infection [23].

To examine antiviral responses in CB4 infected mice deficient in TLR3, inflammatory cytokines including IFN-I were measured in $TLR3^{+/-}$ and $TLR3^{+/+}$ mice post-infection at multiple time points using ELISA, cytometric bead array and real-time PCR (RT-PCR). Changes in TLR3 signaling induction of IFN-β compared to MDA5 signaling after infection with the same virus are further reflected in the ratio of sera IFN-β *versus* IFN-α levels. $TLR3^{+/-}$ mice have three times the amount of IFN-β than IFN-α in the sera at baseline, prior to CB4 infection, compared to wt mice and by day 3 post infection, $TLR3^{+/-}$ mice dramatically lose IFN-β production *versus* IFN-α in the sera (ratio of 0.14) compared to wt mice (ratio 0.71, **Figure 1C**).

This suggests that changes in TLR3 expression support the systemic production of IFN-α rather than IFN-β after CB4 infection, contrary to what we have previously observed for $MDA5^{+/-}$ mice, where IFN-β responses are more significantly favored (13). Alterations in TLR3 signaling also induces the production of other inflammatory cytokines after CB4 infection (**Figure 1D** unlike negligible responses observed with changes in MDA5 signaling (13). Serum levels of IL-17a at day 2 post-infection and IL-6, and IL-10 at day 3 post-infection are increased in $TLR3^{+/-}$ compared to CB4-infected wt mice (**Figure 1D**). In $TLR3^{+/-}$ mice, IFN-β is significantly reduced and IFN-α is increased in both the pancreas and spleen (**Figure 1E**). The expression of IFN-I stimulators MDA5 and TLR3 is also tissue-specific. In CB4-infected NOD mice heterozygous for MDA5 (13) expression of both MDA5 and TLR3 is increased compared to wt NOD mice at day 3 post-infection, whereas in CB4-infected $TLR3^{+/-}$ mice only TLR3 expression is increased compared to wt mice in the pancreas (**Figure 1E**). CB4 infection in $TLR3^{+/-}$ mice also induces a significant reduction in TLR3 and increased MDA5 expression in the spleen (**Figure 1E**), opposite to infected $MDA5^{+/-}$ mice that have decreased MDA5 and increased TLR3 expression in the spleen (13). Overall $TLR3^{+/-}$ and $MDA5^{+/-}$ mice have distinctive tissue specific expression of IFN-I inducers TLR3 and MDA5 and unique tissue-specific IFN-I responses after infection with the same virus.

Since we know from previous studies (2, 10, 13, 19) that an imbalance in cellular immune responses, especially locally, can

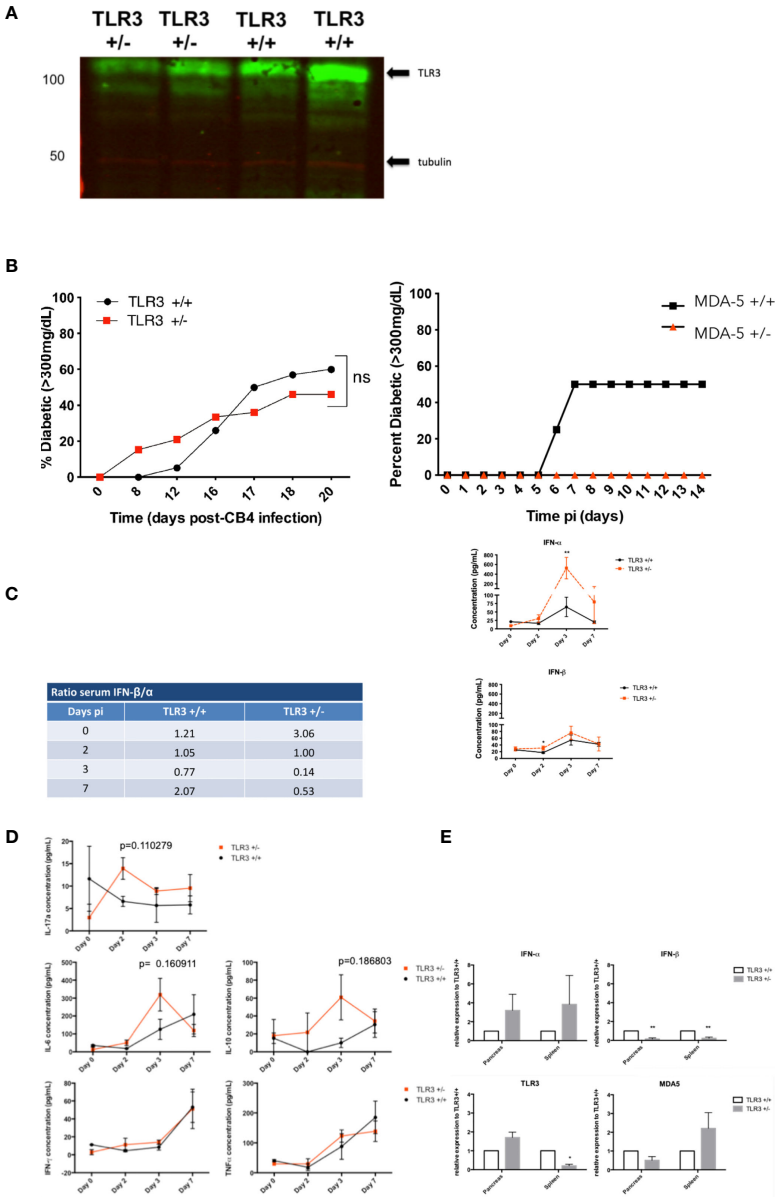

FIGURE 1 | Diabetes incidence in TLR3$^{+/-}$ and TLR3$^{+/+}$ mice post- CB4 infection. **(A)** TLR3 expression in the spleens of TLR3 +/+, TLR3+/-, and TLR3-/- (not shown) mice that were stimulated with poly I:C as described in Materials and Methods. Twenty-four hours after stimulation, a reduction in TLR3 protein from TLR3 +/- spleens compared to TLR3 +/+ was confirmed by Western Blot. **(B)** TLR3 +/- (n = 13) and TLR3 +/+ (n = 20) (left) and MDA5+/- (n = 15) and MDA5 +/+ (n = 20)(right) were infected ip with 400 pfu CB4. Diabetes incidence was monitored up to 20 days post-infection. Two consecutive blood glucose levels greater than 300mg/dL determined diabetes incidence. Systemic and local inflammatory cytokines in TLR3$^{+/-}$ and TLR3$^{+/+}$ mice post-CB4 infection. **(C, D)** TLR3 +/- (n = 4-8) and TLR3 +/+ (n = 4-8) were infected by an intraperitoneal i.p. injection with 400 pfu CB4. IFN-α/β Inflammatory cytokine concentrations (pg/mL) were measured from sera by ELISA and IL-6, IL-10, IL-17a, IFN-γ, and TNF-α by FACS bead array at days 0, 2, 3 and 7 post-infection (pi). Ratios of the average IFN-β concentrations *versus* IFN-α (left) and the individual concentrations (right) **(C)** and averages of inflammatory cytokines **(D)** measured from sera at each time point are shown. **(E)** Relative mRNA expression levels of TLR3, MDA5, IFN-α and β from the spleen and pancreas of TLR3+/+ (n = 4-8) and TLR3+/- mice (n = 4-8) at day 3 post-CB4 infection were quantified by quantitative real time PCR and normalized to GAPDH. The comparative Ct method was used to calculate mean relative expression ± SEM against TLR3+/+ mice as described in *Materials and Methods* section. Data shown are from duplicate samples from two independent experiments. *p < 0.05, **p < 0.01 and ns, not significant.

lead to the development of T1D following virus challenge, we investigated the regulatory and effector cell responses in the pancreatic lymph nodes and spleen of CB4-challenged mice. Similar to MDA5$^{+/-}$ mice (13), at day 7 following CB4 infection in comparison to wt mice, TLR3$^{+/-}$ mice have significantly increased regulatory Foxp3^{+} T cells in the pancreatic lymph nodes (PLNs) and in the spleen, while statistical significance was not achieved, there was a propensity for increased Tregs

(**Figure 2A**). Though unlike CB4-infected MDA5$^{+/-}$ mice that have a T cell response skewed towards protection from T1D, infected TLR3$^{+/-}$ mice have significantly increased effector T cells in the PLN (IFN-γ- CD4^{+} T cells) and are not protected from T1D.

While statistical signficance was not achieved, in comparison to wt mice at 7 days PI, infected TLR3$^{+/-}$ mice have a tendency for increased CD44^{hi}CD62L^{lo} CD4^{+} effector T cells in the PLNs and spleen(**Figure 2B**). Statistically significant increased levels of IFN-γ-producing CD4^{+} T cells are observed in the PLNs compared to infected wt mice (**Figure 2C**). The increase in IFN-γ and adaptive responses at the site of autoimmunity is thus greatly apparent with changes in TLR3 expression and begs the question whether it is the receptor expression and signaling or the location of viral infection and subsequent interferon and T cell responses rather that influences susceptibility to disease. To address this potential spatial relevance in the function of interferon and T cell responses against viral infection, we turned to our MDA5 model and compared the inflammatory responses from two pancreatropic infections.

To test if MDA5 signaling within the pancreatic islets protects from T1D, we infected MDA5$^{+/-}$ mice with the beta cell tropic-B4 strain and in another set of mice, the non-beta cell tropic virus B3 strain of coxsackievirus. In NOD mice, both the B4 and B3 strains of coxsackievirus infect and cause significant inflammatory pathology in the acinar tissue in the pancreas (19) though CB4 and not CB3 infects the pancreatic beta cells and induces T1D.

Though CB3-infected MDA5$^{+/-}$ mice have similar levels of inflammatory cytokines TNF-α, IFN-γ, IL-6, IL-17a, and IL-10 (data not shown) by day 7 following infection, CB3-infect MDA5$^{+/-}$ mice have greater systemic levels of both IFN-α and IFN-β compared to CB3-infected wt mice (**Figure 3A**). CB4-infected MDA5$^{+/-}$ mice also have increased systemic IFN-α at day 7 post-infection compared to infected wt mice, though they have a significant decrease in IFN-β (**Figure 3A**).

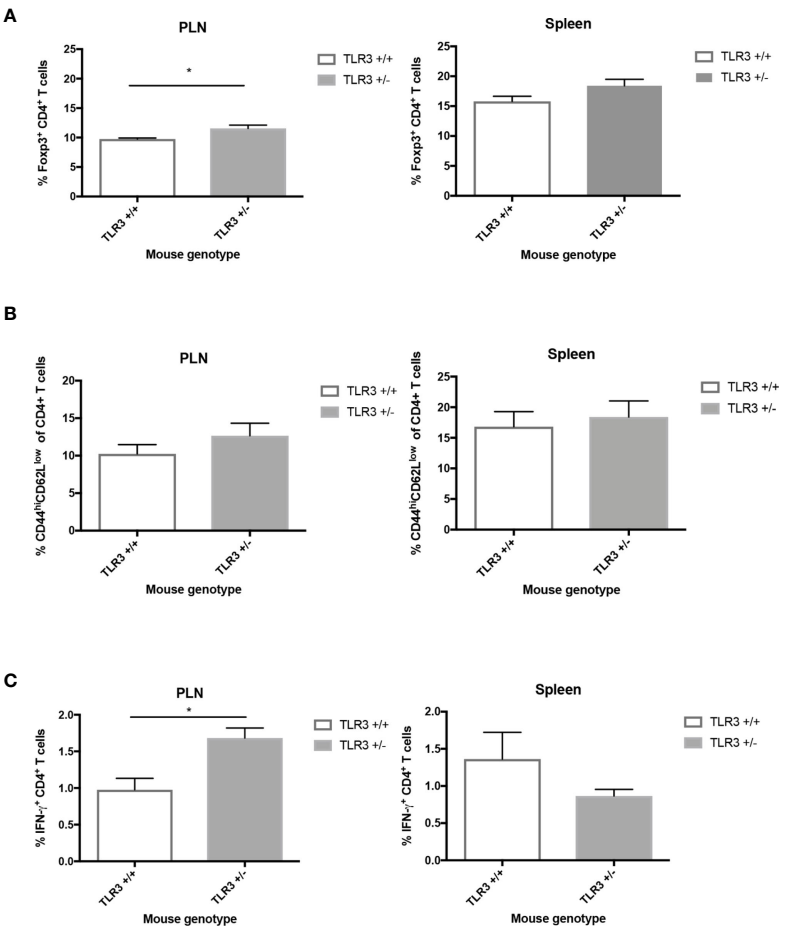

FIGURE 2 | Regulatory and effector CD4+ T cells from the PLNs and spleen of TLR3$^{+/-}$ and TLR3$^{+/+}$ mice post-CB4 infection. **(A)** Regulatory T cells (Foxp3^{+} CD4^{+}) and **(B, C)** effector T cells (CD44^{hi}CD62^{low}CD4^{+} and IFNγ-producing CD4^{+}) were isolated from the pancreatic lymph nodes (PLNs) and spleens of TLR3+/+ (n = 5) and TLR3+/- mice (n = 5) at day 7 post CB4-infection and were stained with classical activation and maturation marker antibodies for FACS analysis. Results are shown as mean ± SEM of a representative from three independent experiments. *$p < 0.05$.

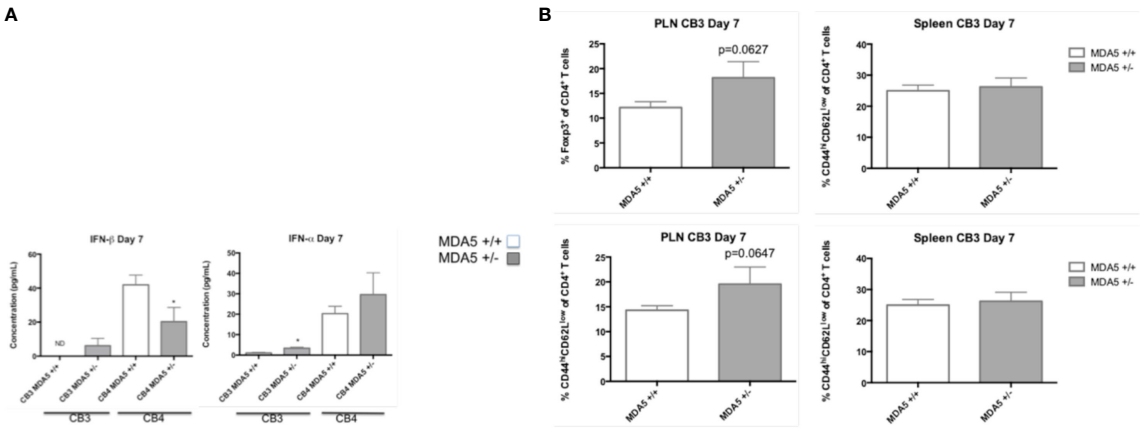

FIGURE 3 | Serum levels of IFN-I and $CD4^+$ T cell responses in $MDA5^{+/-}$ and $MDA5^{+/+}$ mice after CB3 and CB4 infection. **(A)** IFN-α/β Inflammatory cytokine concentrations (pg/mL) were measured from CB4 and CB3 infected $MDA5^{+/-}$ (n = 7) and $MDA5^{+/+}$ (n = 7) mice sera by ELISA. **(B)** Regulatory T cells ($Foxp3^+$ $CD4^+$) and effector T cells ($CD44^{hi}CD62^{low}CD4^+$ and IFNγ-producing $CD4^+$) were isolated from the pancreatic lymph nodes (PLNs) and spleens of CB3 infected $MDA5^{+/-}$ (n = 5) and $MDA5^{+/+}$ (n = 5) mice at day 7 post CB4-infection and were stained with classical activation and maturation marker antibodies for FACS analysis. Results are shown as mean ± SEM of a representative from three independent experiments. *$p < 0.05$.

In $MDA5^{+/-}$ mice, CB4 infection also results in increased regulatory $Foxp3^+$ $CD4^+$ T cells at the site of autoimmunity [23]. Infection with the non-diabetogenic non-beta cell tropic virus CB3 in $MDA5^{+/-}$ mice, however, induces a more inflammatory response with the increased presence of both regulatory $Foxp3^+$ $CD4^+$ T cells and effector $CD44^{hi}CD62L^{lo}$ $CD4^+$ T cells at the site of autoimmunity, in the pancreatic lymph nodes (PLNs) (**Figure 3B**) compared to infected wt mice. And also unlike CB4-infected $MDA5^{+/-}$ mice (13), CB3-infected $MDA5^{+/-}$ mice have similar regulatory and effector $CD4^+$ T cell responses in the spleen compared to infected wt mice (**Figure 3B**). With CB4 infection, effector T cells ($CD4^+$ and $CD8^+$) are significantly reduced in $MDA5^{+/-}$ mice PLNs and spleen (13). These observations demonstrate that infection outside the islets with CB3, leads to systemic, and localized inflammatory responses. Infection within the islets, with CB4, induces regulatory, suppressive responses.

As humans encounter various pathogenic insults from their environment, it is likely that these pathogenic insults induce frequent bursts of IFN-I signaling as a result of innate immune responses. Consequently, innate responses and pathogen clearance may also frequently alter the IFN-I signature in the host and as such, alter immune homeostasis. To simulate alterations in immune homeostasis with frequent IFN-I production and to test whether the IFN-I signature and protective adaptive responses we observe in CB4-infected $MDA5^{+/-}$ mice can be maintained despite additional bursts of IFN-I, we stimulated CB4-infected $MDA5^{+/-}$ and $MDA5^{+/+}$ mice 3 and 5 days post-CB4 infection with the dsRNA mimetic poly I:C.

Contrary to what was expected, a reduction in MDA5 impressively held a unique IFN-I signature despite additional IFN-I stimuli following CB4 infection. At day 7 post-infection, $MDA5^{+/-}$ mice treated with poly I:C at days 3 and 5, maintain lower IFN-β levels similarly to unstimulated $MDA5^{+/-}$ mice. Poly I:C stimulated $MDA5^{+/-}$ mice do, however, show an increase in IFN-α production by day 7 post-infection compared to unstimulated CB4-infected $MDA5^{+/-}$ mice (**Figure 4A**). Poly I:C treatment holds a greater effect on $MDA5^{+/+}$ mice, where both IFN-α and β serum levels are increased at day 7 post-infection compared to untreated CB4-infected wt mice (**Figure 4A**).

To further study the immune consequences of additional IFN-I stimulation in CB4-infected $MDA5^{+/-}$ mice, we examined the expression of significant APC activation markers CD40, CD80 (data not shown) and CD86 in poly I:C treated, CB4-infected $MDA5^{+/-}$ mice. After poly I:C treatment and seven days of CB4 infection, APCs ($CD11b^+CD11c^+$, $CD11b^+CD11c^-$) isolated from PLNs and spleens of $MDA5^{+/-}$ mice express similar levels of CD40 and CD86 compared to untreated CB4-infected $MDA5^{+/-}$ mice (**Figure S2**). With insignificant APC activation, it is also no surprise that pro-inflammatory cytokine production is also insignificant at day 7 infection (data not shown). Further, T cell responses in poly I:C treated and CB4-infected $MDA5^{+/-}$ remain similar with the response in untreated CB4-infected $MDA5^{+/-}$ mice while the number of $Foxp3^+$ $CD4^+$ regulatory T cells remained elevated in the PLNs relative to the number of effector $CD44^{hi}$ $CD62L^{lo}$ $CD4^+$ T cells (**Figure 4B**). This suggests that APC activation and subsequent T cell polarization in $MDA5^{+/-}$ is unaffected by additional IFN-I stimulus.

Injections of poly I:C have a more dramatic effect on CB4-infected $MDA5^{+/+}$ mice. Although CD86 expression remained unchanged in CB4-infected $MDA5^{+/+}$ mice following poly I:C treatment, CD40 expression on $CD11b^+CD11c^+$ cells was decreased (**Figure S2**), regulatory T cells were slightly increased in the spleens (**Figure 4B**) and notably, regulatory T cells were significantly decreased in the PLNs (**Figure 4B**) compared to untreated, infected $MDA5^{+/+}$ mice. These results

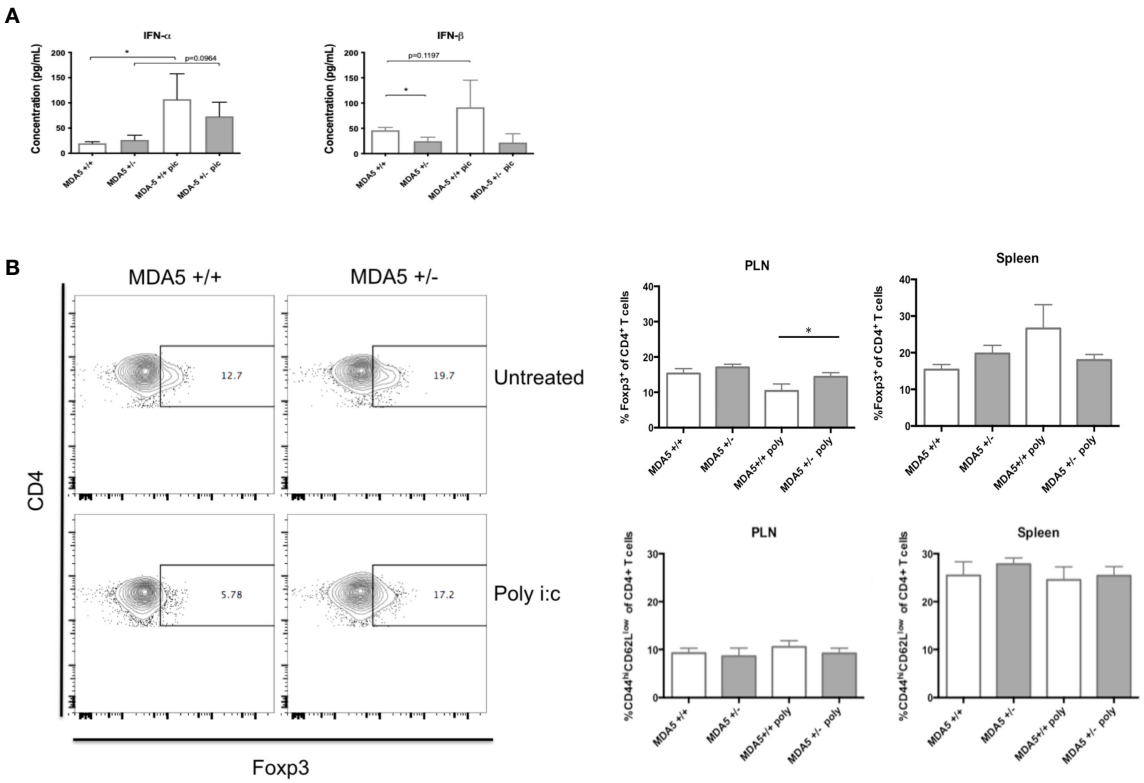

FIGURE 4 | Systemic IFN-I levels, regulatory and effector $CD4^+$ T cell responses in $MDA5^{+/-}$ and $MDA5^{+/+}$ mice after poly I:C stimulation and CB4 infection. **(A)** IFN-α/β Inflammatory cytokine concentrations (pg/mL) were measured from $MDA5^{+/-}$ (n = 7) and $MDA5^{+/+}$ (n = 7) mice sera at day 7 post-infection by ELISA. **(B)** Regulatory T cells ($Foxp3^+$ $CD4^+$) and effector T cells ($CD44^{hi}CD62^{low}CD4^+$ and IFNγ-producing $CD4^+$) were isolated from the pancreatic lymph nodes (PLNs) and spleens of poly I:C treated and untreated $MDA5^{+/-}$ (n = 5) and $MDA5^{+/+}$ (n = 5) mice at day 7 post CB4-infection and were stained with classical activation and maturation marker antibodies for FACS analysis. Results are shown as mean ± SEM of a representative from three independent experiments. GraphPad Prism 6.0 software (GraphPad, San Diego, CA) using the Student t test (two-tailed distribution) and a P value < 0.05 determined statistical significance *p < 0.05.

emphasize the strength of the regulatory protective phenotype observed in $MDA5^{+/-}$ mice to remain at the site of autoimmunity, which is not observed in $MDA5^{+/+}$ mice. Despite the additional poly I:C treatment and potential for greater IFN-I, by day 7 post-infection reduced MDA5 signaling still results in a lower IFN-I level with greater regulatory T cells in the PLNs, at the site of autoimmunity.

DISCUSSION

Establishing a balance between immune activation and immune protection is essential in an already fragile autoimmune-susceptible state. Double stranded RNA sensors like MDA5 and TLR3 are critical in recognizing specific RNA viruses and inducing an inflammatory IFN-I antiviral response to help clear the invading RNA virus. Here, we have provided evidence of a unique and protective IFN-I and regulatory T cell signature induced with a reduction in MDA5 that is specific to changes in MDA5 signaling and not with a reduction in TLR3 signaling. Rather, the two viral sensors signal co-operatively, similar to a thermostat or counter-balance to achieve a range of immune responses.

Although TLR3 signaling is critical for survival against CB4 in NOD mice (12), we observed that IFN-I and adaptive responses produced as a result from TLR3 signaling to clear the virus are not similarly compatible with protection from autoimmunity. CB4-infected $TLR3^{+/-}$ mice have skewed IFN-I responses that favor increases in IFN-α and increases in effector T cells at the site of autoimmunity that ultimately do not protect from T1D.

In diabetes resistant C57BL/6 mice, MDA5 and TLR3 signaling are both required to prevent diabetes following infection with a pancreatropic virus encephalomyocarditis virus strain D (EMCV-D). EMCV-D, however, induces diabetes through the direct destruction of β cells rather than T cell–mediated autoimmunity that we observe with CB4 infection. Interestingly, MDA5 and TLR3 exert different IFN-I response kinetics following EMCV-D infection in C57BL/6 mice, with IFN-I responses detected in $MDA5^{-/-}$ at 15 hours post-infection and at a later time in $TLR3^{-/-}$ mice (15). The potential cooperative role in IFN-I signaling seems to be specific to the

EMCV-D infection model in diabetes resistant C57BL/6 mice. With our CB4-infection model in diabetes susceptible NOD mice we observe distinct IFN-I responses from MDA5 and TLR3 signaling and we do not see a cooperative mechanism from either receptor attempting to compensate for the lack in expression of the other in our heterozygous or knockout (not shown) mice. From what we have demonstrated with our model, TLR3 signaling is more important for anti-viral responses and rather the IFN-I signature and adaptive responses from MDA5 signaling, though still critical for the anti-viral response, are significant in protecting from autoreactive responses.

The location of viral infection and thus the location of MDA5 signaling in response to viral infection is also important in considering anti-viral immune responses such as IFN-I that could exacerbate and activate pre-existing autoreactive responses. IFN-I are primarily produced by the β cells in the pancreas with coxsackievirus infection and intraislet IFN-I production has shown to prevent CB4 replication (20, 21). In T1D patients, a unique IFN-I signature precedes islet autoimmunity and expression of interferon-stimulated genes is exacerbated in insulitic islets (6). The nature of the IFN-I response within the islets is therefore a critical component in the pathology that leads to autoimmunity in T1D.

We previously demonstrated that CB4-infected MDA5$^{+/-}$ mice have tissue-specific and unique systemic IFN-I and adaptive responses that ultimately lead to protection from T1D (13). Here we sought to determine whether MDA5 signaling and anti-viral IFN-I responses within the islets would contribute to the protective phenotype. After challenging MDA5$^{+/-}$ mice with the B3 strain of coxsackievirus that infects and causes significant inflammatory acinar tissue pathology outside of the islets, in comparison to mice challenged with the β- cell tropic virus CB4 we determined whether viral islet tropism could change anti-viral and autoreactive responses. We observed that it is likely that the location of virus infection in MDA5$^{+/-}$ mice that alters IFN-I and adaptive responses. With CB3 infection, we observed in MDA5$^{+/-}$ mice, an increase in systemic IFN-I levels and localized numbers of effector T cells at the site of autoimmunity, similarly to CB3-infected MDA5$^{+/+}$ mice and in contrast to the phenotype observed with CB4 infection.

MDA5 signaling of CB4 and likely other β-cell tropic viruses followed by the presentation of β-cell antigens is therefore necessary to maintain a unique IFN-I signature and regulatory T cell responses at the site of autoimmunity that can ultimately protect from T1D. By regulating the induction of IFN-I at the site of infection, virus infection creates a local site of interferonopathy leading to altered T cell responses, loss of T cell regulation and induction of autoimmune diabetes. As CB4-MDA5$^{+/-}$ mice have a burst in IFN-β shortly after infection that subsides by day 7 post-infection, it is likely that controlled, local, IFN-β signaling at the site of the islet β cell, leads to protection from exacerbated autoreactive responses and ensuing autoimmunity. Studies investigating the role of localized regulation of IFN-I within the islets could establish a new therapeutic avenue for preventing the autoimmune process and susceptibility to T1D.

In addition to considering the localized islet environment, it is also important to consider the environment of the host. As individuals are exposed to multiple environmental factors including viruses, they are exposed to frequent sources of IFN-I stimuli. Frequent bursts of IFN-I, depending on the location and susceptibility of the host, could lead to abrogated immune responses and lead to autoimmunity. To reproduce an environment where the host is exposed to frequent IFN-I stimulation and determine whether additional IFN-I responses are capable in offsetting an existing protective IFN-I signature, we infected MDA5$^{+/-}$ and MDA5$^{+/+}$ controls with CB4 and at days 3 and 5 post-infection, injected CB4-infected mice with the artificial dsRNA mimetic poly I:C.

In NOD mice, at a dose of 5μg/g body weight, poly I:C protects from T1D (22) and in NOD BDC2.5 transgenic mice that do not develop spontaneous diabetes, a similar poly I:C dose is not capable of inducing resting autoreactive memory T cells and diabetes (19). Though poly I:C signals MDA5 and activates IFN-I responses, the dsRNA mimetic does not induce β cell damage directly like β -cell tropic agents such as CB4. In asking whether recurrent IFN-I responses induced by subsequent treatments of poly I:C following CB4 infection could offset an existing IFN-I and immunoregulatory phenotype in MDA5$^{+/-}$ mice, we did not expect and were not surprised to observe that poly I:C treatment did not further accelerate disease onset in our mice following CB4 infection. Instead, it might be expected that in addition to an already existing breakdown in tolerance, a second insult inducing IFN-I, such as poly I:C treatment, could regress tolerogenic mechanisms and progress disease pathogenesis to autoimmunity. As such, we expected that supplemental IFN-I stimulation with poly I:C post-CB4 infection would abrogate the IFN-I signature with higher systemic IFN-I levels and polarized T cell responses typically observed in CB4-infected, untreated MDA5$^{+/-}$ mice would shift to effector rather than regulatory CD4+ T cells dominating in the PLNs. To our delight, we observed that despite the additional IFN-I stimulation with CB4 infection and two doses of poly I:C, MDA5$^{+/-}$ mice maintain IFN-I and immunoregulatory responses similar to the protective phenotype observed in untreated CB4-infected MDA5$^{+/-}$ mice. Though poly I:C is known to stimulate APC maturation and polarize T cell responses in certain susceptible models, we did not observe changes in the expression of classic APC markers CD40, CD80 or CD86 or changes in T cell responses in poly I:C treated CB4-infected MDA5$^{+/-}$ mice from the untreated phenotype.

The reduction and changes in MDA5 signaling at the site of autoimmunity in MDA5$^{+/-}$ mice likely allows for a balanced anti-viral and immunoregulatory adaptive response in the events of exposure to frequent IFN-I stimulation. With low-dose injections (0.05μg/g body weight) in the BioBreeding (BB), diabetes-resistant rat model, poly I:C protects rather than accelerates disease, mostly attributing to the induction of suppressor T cell activity (23). With the B7.1 C57BL/6 model, where the B7.1 costimulatory molecule is expressed in islets, the level of poly I:C–induced IFN-α determines the frequency and timing of diabetes onset, where higher levels of IFN-α coincide

with accelerated, earlier onset of T1D (24). These models along with our MDA5$^{+/-}$ model suggest that low level IFN-I signaling, as a result of a reduction in MDA5, can help avoid and regulate rather than activate autoreactive responses in a genetically susceptible host. Local anti-viral IFN-I responses in the pancreas likely create a site of interferonopathy that shifts the balance from immunoprotective to fully regressed immunosusceptible and prone to autoimmunity.

CONCLUSIONS

With our MDA5$^{+/-}$ and TLR3$^{+/-}$ CB4-infection models, we have identified, using a virus clinically linked to T1D, how changes in MDA5 and not TLR3 signaling are critical in producing an IFN-I response and subsequent adaptive responses that protect from T1D. By challenging MDA5$^{+/-}$ mice with different strains of coxsackieviruses we have teased out the location specific importance of IFN-I signaling and the interferonopathy within the pancreas that changes with a reduction in MDA5 and allows for IFN-I and T cell responses in favor of protection from T1D. Conversely, challenging TLR3$^{+/-}$ mice resulted in the opposite effect by retaining an IFN-I response that resolves to T1D. This further dissects the IFNα/β responses to separate sensors demonstrating a need, and not a redundancy, for both TLR3 and MDA5 to sense and respond differentially to infection. Further, in treating MDA5$^{+/-}$ mice with poly I:C, an additional IFN-I inducer, following CB4 infection, we simulated the environmental context in which humans are exposed to multiple IFN-I stimuli, and demonstrated the strength of the protective phenotype that results with a reduction in MDA5 signaling in withstanding additional IFN-I chaos. MDA5 serves as a cellular protector from viral invasion and as such, with inter-individual variability, MDA5 activity will change with variations at the genetic and immunological expression level. Establishing low level IFN-I signaling from MDA5 may be host specific as we see with IFIH1 heterozygous individuals that carry the protective allele. Regulating IFN-I levels to evade interferonopathies whether systemic or organ-specific still remains an effective strategy in evading autoimmunity as we have demonstrated using an autoimmune diabetes model. We have not demonstrated another way to prevent T1D in the NOD mouse, rather we believe this work has provided compounding evidence for a specific control of IFN-I to drive a myriad of responses ranging from virus clearance to onset of autoimmune diabetes.

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 animal study was reviewed and approved by UBC Animal Care Committee.

AUTHOR CONTRIBUTIONS

Conceptualization, PL and MH. Methodology, PL, IS, and MH. Software, X.X. Validation, PL, IS, and MH. Formal analysis, PL and MH. Investigation, PL and MH. Resources, MH. Data curation, PL. Writing—original draft preparation, PL. Writing—review and editing, PL and MH. Supervision, MH. Funding acquisition, MH. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.751341/full#supplementary-material

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REVIEW
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Using the T Cell Receptor as a Biomarker in Type 1 Diabetes

Maki Nakayama[1,2,3*] *and Aaron W. Michels*[1,2,3,4]

[1] Barbara Davis Center for Childhood Diabetes, University of Colorado School of Medicine, Aurora, CO, United States, [2] Department of Pediatrics, University of Colorado School of Medicine, Aurora, CO, United States, [3] Department of Immunology and Microbiology, University of Colorado School of Medicine, Aurora, CO, United States, [4] Department of Medicine, University of Colorado School of Medicine, Aurora, CO, United States

T cell receptors (TCRs) are unique markers that define antigen specificity for a given T cell. With the evolution of sequencing and computational analysis technologies, TCRs are now prime candidates for the development of next-generation non-cell based T cell biomarkers, which provide a surrogate measure to assess the presence of antigen-specific T cells. Type 1 diabetes (T1D), the immune-mediated form of diabetes, is a prototypical organ specific autoimmune disease in which T cells play a pivotal role in targeting pancreatic insulin-producing beta cells. While the disease is now predictable by measuring autoantibodies in the peripheral blood directed to beta cell proteins, there is an urgent need to develop T cell markers that recapitulate T cell activity in the pancreas and can be a measure of disease activity. This review focuses on the potential and challenges of developing TCR biomarkers for T1D. We summarize current knowledge about TCR repertoires and clonotypes specific for T1D and discuss challenges that are unique for autoimmune diabetes. Ultimately, the integration of large TCR datasets produced from individuals with and without T1D along with computational 'big data' analysis will facilitate the development of TCRs as potentially powerful biomarkers in the development of T1D.

Keywords: T cells, TCR sequencing, autoimmunity, type 1 diabetes, HLA, MHC

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***Correspondence:**
Maki Nakayama
maki.nakayama@cuanschutz.edu

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INTRODUCTION

A T cell receptor (TCR) determines antigen specificity of T cells by interacting with a peptide-major histocompatibility complex (peptide-MHC), and signals received through the TCR along with the CD3 complex are the primary components that regulate function and fate of T cells. Individual T cells express unique TCRs, and therefore TCR sequences can be used as an identifier of T cells that are specific to particular antigens and involved in immune responses. In this review, we will focus on the potential use of TCR sequences as non-cell based T cell biomarkers for type 1 diabetes (T1D), a tissue-specific autoimmune disease targeting insulin-secreting pancreatic beta cells (1–3).

Several features of self-reactive T cells make it challenging to develop T cell biomarkers in diabetes (4). First, the frequency of autoreactive T cells is extremely low in the peripheral blood, estimated to be $1/10^5$ – $1/10^6$. Second, response to peptide-MHC by autoreactive T cells tends to be minimal compared to anti-cancer or anti-pathogen T cell responses (5, 6). Third, healthy individuals with T1D-risk MHC molecules can have autoreactive T cells that are quantitatively and functionally similar to those in T1D patients (7). TCR sequencing allows for the analysis of TCR

clonotypes from tens of millions of T cells using nucleotide samples rather than living cell biospecimens and may overcome many of these challenges when appropriately utilized. Advantages provided by TCR biomarkers include (1) living T cells are not required for assays; (2) intra- and inter-assay variations due to cell conditions and operator performance are minimized; and (3) extremely infrequent and low-responding T cells are detectable by recently emerging high-throughput sequencing technologies. Here, we will review current knowledge about TCR repertoires and clonotypes specific for T1D and address the knowledge gaps to develop TCR biomarkers that can stratify individuals throughout the stages of T1D development.

TRI-MOLECULAR COMPLEX CONSISTING OF TCR, PEPTIDE, AND MHC MOLECULES

TCRs expressed by classical T cells are composed of alpha and beta chains, both of which are formed by somatic recombination of the variable (V) and joining (J) segment genes (and diversity [D] for beta chains). In humans, 45 TRAV and 52 TRAJ genes have been identified as functional V and J segment genes for alpha chains (8, 9). Likewise, there are 49 TRBV, 2 TRBD, and 13 TRBJ functional V, D, and J segment genes in the beta chain locus (10, 11). During maturation in the thymus, individual T cells undergo rearrangement of segment genes, resulting in one V, one D (for beta), and one J segment genes assembled adjacent to each other. Since additional nucleotides are often inserted or deleted between the segments, billions of junction sequences with hundreds of different V, D, J combinations are possibly assembled (12–14). Experimentally, each adult person is expected to have over 100 million TCR clonotypes uniquely expressed by hundreds of billions of individual T cells in the body (15–19). There are three regions, called complementarity determining regions (CDR), that directly interact with peptide-MHC complexes, thereby crucial to determine antigen specificity (20–22). Two CDR regions, CDR1 and CDR2, are included in the V segment, and the CDR3 region is formed at the junction between V, D (for beta), and J segments. Amino acid residues in the CDR3 regions closely interact with peptide, and thus are considered to be important to determine antigen specificity and are often used as a property of each TCR clonotype for TCR repertoire analysis.

MHC MOLECULES IN T1D

The major genetic determinant in susceptibility to most autoimmune diseases reside in the human MHC that contains the human leukocyte antigen (HLA) region. MHC molecules are heterodimers formed between alpha and beta chains that function to present peptides to TCRs on T cells. Class I molecules are on all nucleated cells and present antigens to CD8 T cells, while class II molecules are expressed by antigen presenting cells (e.g. B cells, dendritic cells, and macrophages) and present peptides to CD4 T cells. In T1D, specific HLA class I and II alleles are associated with increased risk (23, 24). Several HLA class I and II alleles confer risk for T1D and are associated with other autoimmune disorders (**Table 1**) (25, 26). DR is in close linkage disequilibrium with DQ such that the DR4-DQ8 and DR3-DQ2 haplotypes confer the greatest risk for T1D development. Both the alpha and beta chains of DQ molecules are polymorphic, and have the ability to form mixed molecules in cis and trans. As an example, the alpha chain of DQ2 can pair with the beta chain of DQ8 to form DQ8-trans (*DQA1*05:01-DQB1*03:02*) when both DQ2 and DQ8 are in the genotype. DQ8-trans has an odds ratio of disease development for T1D at 35 (35 times more likely to develop diabetes compared to those without these alleles), compared to odds ratios of ~11 and ~4 for DQ8 and DQ2, respectively (27, 28). Interestingly, HLA-DQ6 (*DQA1*01:02-DQB1*06:02*) provides dominant protection for T1D development with an odds ratio of only 0.03 (29, 30). The stark dichotomy of risk between DQ molecules highlights the important role of antigen presentation to TCRs in T1D.

TABLE 1 | Common HLA alleles associated with type 1 diabetes risk.

Name	Allele	Associated Autoimmune Diseases
HLA Class II		
DQ8	*DQA1*03:01-DQB1*03:02*	Celiac disease, Addison's disease
DQ2	*DQA1*05:01-DQB1*02:01*	Celiac disease, Addison's disease
	*DQA1*02:01-DQB1*02:02*	Celiac disease, Addison's disease
DQ8-trans	*DQA1*05:01-DQB1*03:02*	Celiac disease
DR4	*DRB1*04:01*	Rheumatoid Arthritis, Thyroid disease, Addison's disease, Alopecia Areata
	*DRB1*04:02*	Rheumatoid Arthritis (protective), Thyroid disease, Addison's disease
	*DRB1*04:04*	Rheumatoid Arthritis, Thyroid disease, Addison's disease
	*DRB1*04:05*	Rheumatoid Arthritis, Thyroid disease, Addison's disease
DR3	*DRB1*03:01*	Systemic Lupus Erythematous (SLE), Neuromyelitis Optica (NMO), Myasthenia Gravis, Thyroid disease, Addison's disease
HLA Class I		
A2	*A*02:01*	Vitiligo
A24	*A*24:02*	unknown
B39	*B*39:06*	unknown
B18	*B*18:01*	unknown
B7	*B*07:05*	unknown

DIVERSITY OF TCR REPERTOIRES

Adults have approximately 10^8-10^{10} unique TCR clonotypes (15, 17, 18, 31). With an assumption that the TCR repertoire size may represent a capacity for responding to diverse antigens, the TCR repertoire diversity in the blood has been examined to determine whether it is associated with immune conditions. For example, having diverse TCR repertoires is associated with desirable responses to immune therapies in cancer (32–34). In T1D, it has been reported that TCR repertoires in peripheral blood of T1D patients are less diverse compared with those without T1D (35). Thus, there may be trends of TCR repertoire sizes that are preferred by a certain immune condition. However, it should be noted that the diversity of TCR repertoires cannot specify a certain disease.

USE OF TCR CLONOTYPES AS SURROGATES TO QUANTIFY ANTIGEN-SPECIFIC T CELLS

TCR clonotypes determine antigen specificity, and therefore they can be utilized as a surrogate marker to evaluate the presence and prevalence of antigen-specific T cells in the blood. Frequencies of these antigen-specific TCR clonotypes can be quantified by high-throughput sequencing, which is expected to be more specific to individual diseases compared to surveying the broad TCR repertoire. Furthermore, once a panel of antigen-specific TCR clonotypes are determined, a single TCR sequencing assay allows for evaluating specificity to many antigens rather than needing to test specificity to each individual antigen. TCR sequencing has been done from different tissues in many disease states (36), including autoimmune disorders (37) and cancer (38–40). Remarkably, TCR sequencing has been shown to differentiate early-stage cancer patients from healthy individuals (41, 42). This strategy requires a list of TCR clonotypes beforehand that can be searched in blood samples, and such TCR clonotypes used as surrogate biomarkers need to satisfy three factors: (1) publicity (i.e. commonality and shared between individuals), (2) abundancy, and (3) disease specificity. Namely, T cells expressing the same or similar TCR clonotypes need to be commonly present in a number of people; frequency of such T cells in the blood of each person needs to be high enough for quantification; and presence or absence of such T cells needs to be associated with a disease state. In addition, with larger numbers of TCR clonotypes in a given panel, the more specific and sensitive an assay will become. Thus, identifying disease-specific TCR candidates is essential to establish a robust TCR sequencing assay that can discriminate a subset of individuals having a specific stage or feature of T1D such as those who have potential to respond to an interventional therapy.

There are several strategies to identify disease-specific TCR clonotypes. Since a significant portion of disease-specific TCRs are likely to recognize islet antigens, TCR clonotypes expressed by islet antigen-specific T cells are reasonable candidates for TCR biomarkers. Such T cell sources include peripheral blood T cells responding to islet antigen stimulation or enriched by staining with fluorescence-conjugated multimers consisting of an islet-derived peptide and a particular HLA molecule (43–45). Alternatively, TCR clonotypes identified in the target organ (i.e. pancreas or pancreatic islets) or draining lymph nodes may be also disease-specific. In any of these T cell sources, specificity (i.e. potential contamination of non-disease associated T cells) as well as sensitivity (i.e. missing a portion of antigen-specific T cells) needs to be carefully considered. For example, T cell samples enriched by antigen stimulation may contain only a few clonotypes that readily proliferate in response to the stimulation or could be non-specific T cells that proliferate due to "bystander effect." Likewise, T cells in the pancreas and pancreatic lymph nodes may not necessarily be islet-reactive or disease-specific (46). On the other hand, T cell populations enriched by multimer staining may contain only those having high affinity to bind peptide-MHC complexes, and TCRs weakly binding to peptide-MHC may be missed. This possibility is likely important for autoreactive TCRs since T cell responsiveness to self-antigens tends to be low compared to pathogen T cell responses. Nevertheless, identifying TCR clonotypes from samples enriched with antigen-specific T cells is indispensable to identify disease-specific TCR candidates. These TCR clonotypes should then be assessed for frequency in peripheral blood of individuals with different stages of T1D to determine the ultimate association with disease status. The next subsections will summarize features of TCR clonotypes specific to islet-specific autoantigens as well as those potentially associated with T1D pathogenesis.

Lessons From Islet-Specific TCRs in T1D Animal Models

Non-diabetic (NOD) mice spontaneously develop autoimmune diabetes and represent many features of human T1D including a T1D-susceptible MHC allele (I-A^{g7}), homologous to HLA-DQ8, the development of insulin autoantibodies prior to diabetes onset, and insulitis. A number of T cell clones reacting with islet tissues have been isolated from pancreatic islets and spleens of NOD mice in the past few decades and further characterized for antigen specificity as well as TCR clonotypes (47). In the 1990's, Santamaria and colleagues discovered that a large portion of CD8 T cells infiltrating NOD islets share an identical Valpha segment (i.e. TRAV16) along with a specific junction motif (i.e. MRD or MRE) (48), and subsequently identified a peptide derived from islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) as an epitope targeted by these CD8 T cells (49). Likewise, CD4 T cell clones as well as T-hybridoma cells that are reactive to insulin B-chain peptides have been established from NOD islets by a number of investigators using different methods (50–56). The majority of these T cells expresses TCRs containing specific Valpha and Jalpha segment motifs, TRAV5D-4 or TRAV10 along with TRAJ53 or TRAJ42. When mice are forced to have only T cells expressing TCRs containing TRAV5D-4, approximately one percent of CD4 T cells becomes specific to an insulin B chain 9-23 peptide (57), and the mice are susceptible to develop anti-insulin autoimmunity (58, 59).

Alanine scanning and crystal structure analyses identified several amino acid residues in the TRAV5D-4 and TRAV10 CDR1 and CDR2 regions that are crucial to interact with the insulin peptide-MHC complex (59, 60). Also, among insulin B chain-specific CD4 T cells, those particularly recognize an insulin B chain 12-20 peptide prefer to express TCR beta chains containing a negatively charged amino acid (i.e. aspartic acid [D] or glutamic acid [E]) in the junction region (56). This observation is consistent with a notion that the I-A^{g7} T1D-susceptible MHC class II molecule, which has a positively charged patch in the surface area near the p9 pocket due to the lack of a negatively charged amino acid residue at the beta 57 position, engage TCRs having a negatively charged residue when p9 of peptides is not negatively charged (the position 20 of insulin B chain is glycine). Thus, these studies provide a molecular elucidation of how TCR motif selection occurs by interaction with a particular peptide-MHC complex.

T1D-specific TCR repertoires in rat models have been extensively studied by the group of Mordes and Blankenhorn (61). Of note, diabetes-susceptible rat strains have a T1D risk MHC haplotype (RT1B/D^u), which lacks a negatively charged amino acid residue at the beta chain 57 position and is homologous to HLA-DQ8 (61). In addition to the HLA gene locus, *Iddm14*, which contains the TCR beta chain genes, is a T1D-susceptible locus (62). The group identified a TCR Vbeta allele, *Tcrb-V13S1A1*, that is shared among T1D-susceptible rat strains but not with T1D-resistant ones (63), and demonstrated that genetic elimination of this allele or depletion of T cells expressing TCRs containing Vbeta13a (product of the *Tcrb-V13S1A1* gene) abrogates diabetes development in T1D-susceptible rats (64–66). A series of these studies elegantly linked the genetic risk with a functional mechanism in which a particular TCR motif facilitates T1D development with a specific MHC molecule.

In sum, these animal studies demonstrate the presence of preferred TCR motifs in both germline-encoded and rearranged regions to recognize particular epitope sequences, which can be reasonably explained by molecular interaction between the TCR – peptide – MHC molecule. From a view of TCR biomarker development, TCR motifs shared by antigen-specific or disease-susceptible T cells can be utilized to enrich and classify TCR clonotypes that are distinctive of T1D.

TCR Repertoires in the Pancreas of Humans

Emerging sequencing technologies and increasing availability of human samples, in particular pancreas and peripheral immune tissues isolated from organ donors having T1D, facilitate identification of islet antigen-specific or T1D-associated T cells and TCR clonotypes (7, 67–74). In the 1990's, two groups in Spain and Japan separately analyzed TCR repertoires in the pancreas and demonstrated clonal expansion of T cells with particular Vgene segment usage in individual patients (75, 76). Importantly, the same group in Spain demonstrated that a clonally expanding TCR in islet and pancreas samples was detected in the blood of the same individual, indicating that islet-residing TCR clonotypes are detectable in peripheral blood samples (77). More recently, Brusko and colleagues further corroborated this concept by studying a larger number of individuals using a next generation sequencing technology that allows to analyze much higher numbers of T cells (72, 78). This high resolution analysis discovered that CD8 TCR clonotypes in the pancreas and draining lymph nodes are detected in peripheral blood more frequently than those expressed by CD4 T cells and provided important insights about the depth of TCR sequencing to achieve quantitative measurement.

Another important concept is to consider commonality of TCR repertoires in the pancreas across patients. We recently determined thousands of TCR clonotypes expressed by T cells in the islets of organ donors with and without T1D (73, 74). Our analysis indicated clonal expansion in the pancreas of individual donors regardless of the disease, but also found that the frequency of TCR clonotypes shared between donors is limited. This low frequency of shared TCR clonotypes may be due to diverse HLA restrictions present in different individuals. Another reason could be the fact that T cells in the islets may not be necessarily islet-specific. Indeed, multiple studies analyzing islet T cell specificity found that over half of T cell clones and lines derived from the islets did not respond to preproinsulin and other known islet epitopes (46, 70, 71, 73, 74). However, it should be noted that collecting TCR clonotypes from a larger number of donors significantly increases the number of shared clonotypes and such large TCR repertoire information allows for identifying common motifs even when not sharing entire TCR sequences, which will be essential to precisely cluster TCRs recognizing the same epitope (see below regarding TCR clustering). Thus, continuing efforts to accumulate TCR sequence information from the target organ along with epitope identification is crucial to establish a sufficient list of TCR clonotypes that can be used for disease-associated TCR biomarkers.

Islet Antigen-Specific TCR Clonotypes in Humans

TCRs expressed by islet-reactive T cells may be another optimal source that can be used as clonotypes for T1D biomarkers, especially if they circulate in the peripheral blood. Such clonotypes could come from T cell clones, T cell lines, hybridomas, and transductant cells that have been confirmed to respond to islet antigens, cell subsets enriched by multimer staining, and those activated or proliferated by antigen stimulation. TCR clonotypes for which reactivity to epitopes has been confirmed at a single cell level would be the most reliable source. Here we summarize islet antigen-specific TCR clonotypes that were isolated from individuals having T1D (**Table 2**). To date, over a hundred TCR alpha and beta paired sequences specific to common islet epitopes have been reported by a number of investigators, and it is notable that the majority of these TCRs were identified in the past several years (7, 45, 70, 73, 74, 79–99). However, hundreds of disease-associated TCR clonotypes are far too small to cover T1D patients having heterogeneous antigen specificity. With rapidly evolving sequencing technologies, future efforts to identify islet epitope-

TABLE 2 | T cell receptors specific to islet epitopes.

Clone/ Sequence ID	Epitope	Epitope sequence	HLA[#]	TRAV	TRAJ	CDR3a	TRBV	TRBJ	CDR3b	Source of T cells	Method to confirm reactivity	Reference
BRI4.13	GAD65:555-567	NFFRMVISNPAAT	DR4	TRAV19	TRAJ44	CALSENRGGTASKLTF	TRBV5-1	TRBJ1-1	CASSLVGGPSSEAFF	PBMC CD4	Clone/ Transgenic cells	(79–81)
BRI164	GAD65:555-567	NFFRMVISNPAAT	DR4	TRAV19	TRAJ56	CALSEEGGGANSKLTF	TRBV5-1	TRBJ1-6	CASSLAGGANSPLHF	PBMC CD4	Clone/ Transgenic cells	(80, 82)
T1D2-1&2	IGRP:305-324	QLYHFLQIPTHEEHLFYVLS	DR4	TRAV29	TRAJ40	CAATRTSGTYKYIF	TRBV6-6	TRBJ2-3	CASSPWGAGGTDTQYF	PBMC CD4	Clone/TCR transductant	(81)
T1D4-3&4	IGRP:305-324	KWCANPDWIHIDTTPFAGLV	DR4	TRAV2	TRAJ15	CAVEDLNQAGTALIF	TRBV5-1	TRBJ2-1	CASSLALGQGNQQFF	PBMC CD4	Clone/TCR transductant	(81)
23.G8	PPI:36-50	VEALYLVCGERGFFY	DR4	TRAV39	TRAJ56	CAWRTGANSKLTF	TRBV24-1	TRBJ2-2	CATGLAANTGELFF	Islet CD4	TCR transductant	(83)
SD52.c1	PPI:72-90	PGAGSLQPLALEGSLQKRG	DR4	TRAV4	TRAJ27	CLVGDSLNTNAGKSTF	TRBV27	TRBJ1-5	CASSWSSIGNQPQHF	PBMC CD4	Clone	(82, 84)
95.A9-1	PPI:87-101	QKRGIVEQCCTSICS	DR4.4	TRAV9-2	TRAJ18	CALRTDRGSTLGRLYF	TRBV11-2	TRBJ1-6	CASSLQSSYNSPLHF	Islet CD4	TCR transductant	(83)
Mi.1	Insulin A:1-15 (PPI: 90-104)	GIVEQCCTSICSLYQ	DR4	TRAV8-3	TRAJ44	CAVGALAGTASKLTF	TRBV29-1	TRBJ2-3	CSVEATRADTQYF	PLN CD4	Clone	(85)
Ba.14	Insulin A:1-15 (PPI: 90-104)	GIVEQCCTSICSLYQ	DR4	TRAV39	TRAJ33	CAVVNMDSNYQLIW	TRBV5-1	TRBJ2-3	CASSLATSGGGSDTQYF	PLN CD4	Clone	(85)
Ba.11	Insulin A:1-15 (PPI: 90-104)	GIVEQCCTSICSLYQ	DR4	TRAV22 TRAV26-2 [##]	TRAJ52 TRAJ47	CADAGGTSYKLF CIPGSEEYGNKLVF	TRBV5-1	TRBJ2-3	CASSLATSGGGSDTQYF	PLN CD4	Clone	(85)
6.H11	PPI:94-108	QCCTSICSLYQLENY	DR4.2	TRAV26-1	TRAJ13	CIVRVYSGGYQKVTF	TRBV30	TRBJ2-3	CAWSARLAGGPRTQYF	Islet CD4	TCR transductant	(83)
SD32.5	PPI:94-110	QCCTSICSLYQLENYCN	DR4	TRAV26-1	TRAJ23	CIVRVSSAYYNQGGKLIF	TRBV27	TRBJ2-3	CASSPRANTDTQYF	PBMC CD4	Clone	(82, 84)
B3.3	Proinsulin:52-62 (PPI:76-86)	SLQPLALEGSL	DR4	TRAV17	TRAJ54	CATGPIQGAQKLVF	TRBV6-5	TRBJ1-1	CASSYAWGRATEAFF	PBMC CD4	Clone	(86)
K4.4/K6.4	Proinsulin:54-63 (PPI:78-87)	QPLALEGSLQ	DR4	TRAV10	TRAJ17	CVVSAKAAGNKLTF	TRBV7-8	TRBJ2-7	CASSLAGTDHYEQYF	PBMC CD4	Clone	(86)
23.F7	PPI:24-38	AFVNQHLCGSHLVEA	DR1	TRAV8-2	TRAJ29	CAVIASGNTPLVF	TRBV19	TRBJ2-3	CASKGPGTVIRADTQYF	Islet CD4	TCR transductant	(83)
55.B3	PPI:37-51	EALYLVCGERGFFYT	DR9	TRAV21	TRAJ29	CAVLPPTPLVF	TRBV18	TRBJ1-1	CASSYPGTGGARTEAFF	Islet CD4	TCR transductant	(83)
55.C10	PPI:58-72	AEDLQVGQVELGGGP	DR53	TRAV26-1	TRAJ26	CIVRSHGQNFVF	TRBV20-1	TRBJ2-7	CSARPGTRNYEQYF	Islet CD4	TCR transductant	(83)
Clone 5	Insulin B:9-23 (PPI: 33-47)	SHLVEALYLVCGERG	DQ8	TRAV21	TRAJ6	CAVKRTGGSYIPTF	TRBV11-2	TRBJ2-2	CASSSFWGSDTGELFF	PBMC CD4	Clone/TCR transductant	(82, 87, 88)
GSE.6H9	Insulin B:9-23 (PPI: 33-47)	SHLVEALYLVCGERG	DQ8, DQ8-trans	TRAV26-1	TRAJ40	CIVRVDSGTYKYIF	TRBV7-2	TRBJ2-1	CASSLTAGLASTYNEQFF	Islet CD4	TCR transductant	(73, 83)
GSE.20D11	Insulin B:9-23 (PPI: 33-47)	SHLVEALYLVCGERG	DQ8	TRAV12-3	TRAJ4	CAILSGGYNKLIF	TRBV2	TRBJ2-5	CASSAETQYF	Islet CD4	TCR transductant	(73, 83)
T1D#3 C8	Insulin B:11-23 (PPI: 35-47)^{R22E}	LVEALYLVCGEEG	DQ8	TRAV17	TRAJ23	CATDAGYNQGGKLIF	TRBV5-1	TBBJ1-3	CASSAGNTIYF	PBMC CD4	Clone	(82, 89)

(Continued)

TABLE 2 | Continued

Clone/ Sequence ID	Epitope	Epitope sequence	HLA#	TRAV	TRAJ	CDR3a	TRBV	TRBJ	CDR3b	Source of T cells	Method to confirm reactivity	Reference
T1D#3 C10	Insulin B:11-23 (PPI: 35-47)^{R22E}	LVEALYLVCGEEG	DQ8	TRAV12-3	TRAJ26	CATAYGQNFVF	TRBV4-1	TRBJ2-2	CASSRGGGNTGELFF	PBMC CD4	Clone	(82, 89)
19.A4	PPI:55-69	RREAEDLQVGQVELG	DQ8	TRAV8-6	TRAJ32	CAVRETGATNKLIF	TRBV20-1	TRBJ2-7	CSARPQGFSSYEQYF	Islet CD4	TCR transductant	(83)
GSE.8E3	PPI:72-87 hEL:C-peptide (HIP11)	PGAGSLQPLALEGSLQ SLQPLALEAEDLQV	DQ8, DQ8-trans	TRAV2	TRAJ37	CAVDGSGNTGKLIF	TRBV4-1	TRBJ2-7	CASSQDLAGVREQYF	Islet CD4	TCR transductant	(73, 83)
6.G4	PPI:86-100	LQKRGIVEQCCTSIC	DQ8, DQ8-trans	TRAV26-1	TRAJ8	CIVRVRNTGFQKLVF	TRBV27	TRBJ1-1	CASSPGPGNTEAFF	Islet CD4	TCR transductant	(83)
56.B1	PPI:40-54	YLVCGERGFFYTPKT	DQ2	TRAV13-1	TRAJ40	CAVLSPSGTYKYIF	TRBV7-9	TRBJ1-4	CASSLMGNPHEKLFF	Islet CD4	TCR transductant	(83)
53.A4-1	PPI:72-87	PGAGSLQPLALEGSLQ	DQ2	TRAV39	TRAJ33	CAVDPMDSNYQLIW	TRBV29-1	TRBJ2-6	CSVGTDPSGANVLTF	Islet CD4	TCR transductant	(83)
23.G6	PPI:29-43	HLCGSHLVEALYLVC	DP4	TRAV9-2	TRAJ6	CALSISGGSYIPTF	TRBV5-1	TRBJ2-5	CASSFRQGEQETQYF	Islet CD4	TCR transductant	(83, 90)
A4.13	Proinsulin:41-51 (PPI:65-75)	QVELGGGPGAG	DQ8	TRAV6	TRAJ36	CALKYGANNLFF	TRBV18	TRBJ1-1	CASSPTTGGDEAFF	Islet CD4	Clone	(70)
A1.1	Proinsulin:50-59 (PPI:74-83)	AGSLQPLALE	DQ8	TRAV25	TRAJ16	CAGGFSDGQKLLF	TRBV20-1	TRBJ2-7	CSARTEAYEQYF	Islet CD4	Clone	(70)
A1.2	Proinsulin:50-58 (PPI:74-82)	AGSLQPLAL	DQ8	TRAV20	TRAJ58	CAVIETSGSRLTF	TRBV20-1	TRBJ2-3	CSARDQQRVDTQYF	Islet CD4	Clone	(70)
A2.4	Proinsulin:52-62 (PPI:76-86)	SLQPLALEGSL	DQ8-trans	TRAV19	TRAJ49	CALSRAGTGNQFYF	TRBV5-1	TRBJ2-4	CASSLGLRGENIQYF	Islet CD4	Clone	(70)
B3.1	Proinsulin:48-59 (PPI:72-83)	PGAGSLQPLALE	DQ8	TRAV12-1	TRAJ9	CVVKSTGGFKTIF	TRBV20-1	TRBJ2-5	CSAGGLAGASQETQYF	PBMC CD4	Clone	(86)
K3.2/K9.5	Proinsulin:54-62 (PPI:78-86)	QPLALEGSL	DQ2	TRAV3	TRAJ31	CAVRGDNNARLMF	TRBV7-2	TRBJ2-2	CASSPIIWGTGELFF	PBMC CD4	Clone	(86)
K6.2	Proinsulin:49-58 (PPI:73-82)	GAGSLQPLAL	DQ8-trans	TRAV8-2/8-4	TRAJ11	CAVTPKSGYSTLTF	TRBV20-1	TRBJ2-3	CSARDLAIPDTQYF	PBMC CD4	Clone	(86)
K9.6	Proinsulin:41-51 (PPI:65-75)	QVELGGGPGAG	DQ8	TRAV26-1	TRAJ54	CIVRVEIQGAQKLVF	TRBV3-2	TRBJ2-1	CASSSPGTEYNEQFF	PBMC CD4	Clone	(86)
D1.1/D1.4	Proinsulin:34-43 (PPI:58-67)	AEDLQVGQVE	DQ8	TRAV13-1	TRAJ38	CAARNAGNNRKLIW	TRBV4-2	TRBJ2-3	CASSFRGLGGGTDTQYF	PBMC CD4	Clone	(86)
T6.1	Proinsulin:52-63 (PPI:76-87)	SLQPLALEGSLQ	DQ2, DQ2-trans	Functional alpha not detected			TRBV9	TRBJ2-1	CASSVDPGVYNEQFF	PBMC CD4	Clone	(86)
T6.6	Proinsulin:56-62 (PPI:80-86)	LALEGSL	DQ2	TRAV35	TRAJ28	CAAALSGAGSYQLTF	TRBV19	TRBJ2-3	CASRLDPSTDTQYF	PBMC CD4	Clone	(86)
T17.1	Proinsulin:56-62 (PPI:80-86)	LALEGSL	DQ2, DQ2-trans	TRAV35	TRAJ28	CAAALSGAGSYQLTF	TRBV19	TRBJ2-3	CASRLDPSTDTQYF	PBMC CD4	Clone	(86)
H3.3/H6.4	Proinsulin:52-61 (PPI:76-85)	SLQPLALEGS	DQ8-trans	TRAV19	TRAJ57	CALSGRGSEKLVF	TRBV5-1	TRBJ2-7	CASSTRTGQGGNEQYF	PBMC CD4	Clone	(86)
H3.7/H7.4/ H8.5	Proinsulin:50-58 (PPI:74-82)	AGSLQPLAL	DQ8	TRAV12-1	TRAJ20	CVVNPTDDYKLSF	TRBV20-1	TRBJ2-3	CSARSLASGGPDTQYF	PBMC CD4	Clone	(86)

(Continued)

TABLE 2 | Continued

Clone/ Sequence ID	Epitope	Epitope sequence	HLA[#]	TRAV	TRAJ	CDR3a	TRBV	TRBJ	CDR3b	Source of T cells	Method to confirm reactivity	Reference
H11.5	Proinsulin:42-51 (PPI:66-75)	VELGGGPGAG	DQ8	TRAV26-1	TRAJ36	CIVRVVTGANNLFF	TRBV5-1	TRBJ2-5	CASSLERETQYF	PBMC CD4	Clone	(86)
E2.3	Proinsulin:54-62 (PPI:78-86)	QPLALEGSL	DQ2	TRAV30	TRAJ37	CGTEKPGSGNTGKLIF	TRBV20-1	TRBJ1-4	CSARDGARGEKLFF	PBMC CD4	Clone	(86)
E2.5	Proinsulin:35-46 (PPI:59-70)	EDLQVGQVELGG	DQ8	TRAV12-3	TRAJ5	CVISPPGRRALTF	TRBV5-4	TRBJ2-2	CASSSGTSAGTGELFF	PBMC CD4	Clone	(86)
A3.10	hEGGG : IAPP2 (HIP6)	GQVELGGGNAVEVLK	DQ8	TRAV38-1	TRAJ54	CAFFGQGAGKLVF	TRBV5-1	TRBJ2-3	CASSLSASGGATDTQYF	Islet CD4	Clone	(70, 91, 92)
A1.9	Proinsulin:42-50 (PPI:66-74) hEGGG : IAPP2 (HIP6)	VELGGGPGA GQVELGGGNAVEVLK	DQ8	TRAV20	TRAJ7	CAVQAGGNNRLAF	TRBV5-1	TRBJ1-2	CASSLERDGYTF	Islet CD4	Clone	(70, 92)
A6.15/A5.8	Proinsulin:42-50 (PPI:66-74) hEGGG : IAPP2 (HIP6)	VELGGGPGA GQVELGGGNAVEVLK	DQ8	TRAV26-1	TRAJ21	CIAIYNFNKFYF	TRBV5-1	TRBJ1-6	CASSLEASSYNSPLHF	Islet CD4	Clone	(70, 92)
A2.13	Proinsulin:42-50 (PPI:66-74) hEGGG : IAPP2 (HIP6)	VELGGGPGA GQVELGGGNAVEVLK	DQ8	TRAV26-1	TRAJ39	CIVSHNAGNMLTF	TRBV5-1	TRBJ2-5	CASSLERETQYF	Islet CD4	Clone	(70, 92)
A5.5	Proinsulin:42-50 (PPI:66-74) hEGGG : IAPP2 (HIP6)	VELGGGPGA GQVELGGGNAVEVLK	DQ8	TRAV26-1	TRAJ54	CIVRVEIQGAQKLVF	TRBV5-1	TRBJ2-5	CASSLGPGQRETQYF	Islet CD4	Clone	(70, 92)
A2.11	hEGGG : IAPP2 (HIP6)	GQVELGGGNAVEVLK	Not reported	TRAV38-1	TRAJ54	CAFMGAGAQKLVF	TRBV4-3	TRBJ2-3	CASSQILRGGPPDTQYF	Islet CD4	Clone	(70, 91)
HIP14-G10/D3	hEL : IAPP2 (HIP14)	SLQPLALNAVEVLK	DR	TRAV16 TRAV5 ##	TRAJ37 TRAJ40	CARSHGSGNTGKLIF CAESIASGTYKYIF	TRBV27	TRBJ2-5	CASSSGYGGETQYF	PBMC CD4	Clone	(93)
E2b	hEL:C-peptide (HIP11)	SLQPLALEAEDLQV	DQ2	TRAV8-4	TRAJ43	CAVGATNNNDMRF	TRBV5-4	TRBJ2-1	CASSPIGASGGNEQFF	PBMC CD4	Clone	(94)
ET650-2	Proinsulin:42-50 (PPI:66-74) hEGGG : IAPP2 (HIP6) HIPL11C	VELGGGPGA GQVELGGGNAVEVLK GQVELGGGNAVEVCK	DQ8	TRAV26-1	TRAJ39	CIVRVGYNAGNMLTF	TRBV20-1	TRBJ1-5	CSAIAGPNQPQHF	PBMC CD4	Clone	(92)
ET650-4	Proinsulin:42-50 (PPI:66-74) hEGGG : IAPP2 (HIP6) HIPL11C	VELGGGPGA GQVELGGGNAVEVLK GQVELGGGNAVEVCK	DQ8	TRAV26-1	TRAJ42	CIVRVAIEGSQGNLIF	TRBV5-1	TRBJ1-3	CASSLRRGDTIYF	PBMC CD4	Clone	(92)
ET650-5	hEGGG : IAPP2 (HIP6) HIPL11C	GQVELGGGNAVEVLK GQVELGGGNAVEVCK	DQ8	TRAV26-1	TRAJ9	CIVRLQSGGFKTIF	TRBV20-1	TRBJ1-2	CSAYSPGDRDFSNYGYTF	PBMC CD4	Clone	(92)
ET672-1	Proinsulin:42-50 (PPI:66-74) hEGGG : IAPP2 (HIP6) HIPL11C	VELGGGPGA GQVELGGGNAVEVLK GQVELGGGNAVEVCK	DQ8	TRAV12-2	TRAJ48	CAVNHGNEKLTF	TRBV18	TRBJ1-1	CASSPWEGRMDTEAFF	PBMC CD4	Clone	(92)
1E6	PPI:15-24	ALWGPDPAAA	A*02:01	TRAV12-3	TRAJ12	CAMRGDSSYKLIF	TRBV12-4	TRBJ2-4	CASSLWEKLAKNIQYF	PBMC CD8	Clone	(82, 95)
1D5/1D10/ 2B3/4C6/ 3E7	PPI:3-11	LWMRLLPLL	A*24:02	TRAV5	TRAJ37	CAEPSGNTGKLIF	TRBV7–9	TRBJ2-7	CASSLHHEQYF	PBMC CD8	Clone	(96)

(Continued)

TABLE 2 | Continued

Clone/ Sequence ID	Epitope	Epitope sequence	HLA#	TRAV	TRAJ	CDR3a	TRBV	TRBJ	CDR3b	Source of T cells	Method to confirm reactivity	Reference
Clone 7	IGRP:265-273	VLFGLGFAI	A*02:01	TRAV41	TRAJ48	CAVTSNFGNEKLTF	TRBV6-2/6-3	TRBJ2-7	CASSSRFVGEGLFRYGYEQYF	PBMC CD8	Clone/ Transgenic cells	(97, 98)
Clone 32	IGRP:265-273	VLFGLGFAI	A*02:01	TRAV12-1	TRAJ48	CVVNILSNFGNEKLTF	TRBV20-1	TRBJ2-1	CSASRQGWVNEQFF	PBMC CD8	Clone/ Transgenic cells	(97, 98)
Clone 16/ 17	IGRP:265-273	VLFGLGFAI	A*02:01	TRAV25	TRAJ53	CAGLGDSGGSNYKLTF	TRBV3-1	TRBJ2-4	CASSQDRWDVMSKNIQYF	PBMC CD8	Clone	(45)
Clone 22/ 27	IGRP:265-273	VLFGLGFAI	A*02:01	TRAV29/ DV5	TRAJ53	CAASGGSNYKLTF	TRBV10-3	TRBJ1-2	CAISDRFMREGMTYGYTF	PBMC CD8	Clone	(45)
Clone#1	INS-DRiP:1-9	MLYQHLLPL	A*02:01	TRAV12-2	TRAJ34	CAVNKTDKLIF	TRBV6-1	TRBJ1-2	CASSVTGNGYTF	PBMC CD8	Clone	(99)
Clone#2	INS-DRiP:1-9	MLYQHLLPL	A*02:01	TRAV10	TRAJ8	CVVNMNTGFQKLVF	TRBV12-3/12-4	TRBJ2-2	CASSPPQGGNTGELFF	PBMC CD8	Clone	(99)
1.C1	INS-DRiP:1-9	MLYQHLLPL	A*02:01	TRAV12-1	TRAJ39	CGENNAGNMLTF	TRBV27	TRBJ2-5	CASSLQPPGTSTETQYF	Islet CD8	TCR transductant	(74)
96.A9	INS-DRiP:1-9	MLYQHLLPL	B*08:01	TRAV12-2	TRAJ39	CAVNVYNAGNMLTF	TRBV30	TRBJ1-1	CAWSVRGGSYMNTEAFF	Islet CD8	TCR transductant	(74)
D222D Clones 2	ZNT8:186-194	VAANIVLTV	A*02:01	TRAV17	TRAJ36	CAVTGANNLFF	TRBV19	TRBJ2-2	CASSIEGPTGELF	PBMC CD8	Clone	(7)
D010R clone 1E2	ZNT8:186-194	VAANIVLTV	A*02:01	TRAV35	TRAJ36	CAGTRNNLFF	TRBV19	TRBJ2-7	CASGGSSYEQYF	PBMC CD8	Clone	(7)
D010R clone 1D3	ZNT8:186-194	VAANIVLTV	A*02:01	TRAV25	TRAJ20	CAGGSNDYKLSF	TRBV6-1	TRBJ2-3	CASSSVGVDTQYF	PBMC CD8	Clone	(7)
D267T 33B8	ZNT8:186-194	VAANIVLTV	A*02:01	TRAV19	TRAJ23	CALSEATYNQGGKLIF	TRBV19	TRBJ1-3	CASSIFPNPGNTIYF	PBMC CD8	Clone	(7)
D349D 178B9	ZNT8:186-194	VAANIVLTV	A*02:01	TRAV14/ DV4	TRAJ9	CAMREGLTGGFKTIF	TRBV11-2	TRBJ1-1	CASSPFLTGSNTEAFF	PBMC CD8	Clone	(7)
D351D 188D3	ZNT8:186-194	VAANIVLTV	A*02:01	TRAV19	TRAJ20	CALSPAETSDYKLSF	TRBV19	TRBJ1-1	CASTLTGFAEAFF	PBMC CD8	Clone	(7)
23.F9	PPI:1-11	MALWMRLLPLL	C*03:04	TRAV12-3	TRAJ48	CAMSALGNFGNEKLTF	TRBV19	TRBJ2-1	CASSIAGGNEQFF	Islet CD8	TCR transductant	(74)
19.A1	PPI:1-11	MALWMRLLPLL	C*03:04	TRAV8-4	TRAJ11	CAVSDQGSGYSTLTF	TRBV28	TRBJ1-5	CASSWTANQPQHF	Islet CD8	TCR transductant	(74)
20.E5	PPI:1-11	MALWMRLLPLL	C*03:04	TRAV14/ DV4	TRAJ52	CAMSNAGGTSYGKLTF	TRBV28	TRBJ1-4	CASSLARYNEKLFF	Islet CD8	TCR transductant	(74)
20.F1	PPI:1-11	MALWMRLLPLL	C*03:04	TRAV14/ DV4	TRAJ43	CAMRLHNNNDMRF	TRBV28	TRBJ1-5	CASIASRYNQPQHF	Islet CD8	TCR transductant	(74)
22.A10	PPI:1-11	MALWMRLLPLL	C*03:04	TRAV8-1	TRAJ13	CAVNAAGGYQKVTF	TRBV28	TRBJ2-1	CASIPDRYNEQFF	Islet CD8	TCR transductant	(74)
1.C8	PPI:1-11/2-12/2-10	MALWMRLLPLL ALWMRLLPLLA ALWMRLLPL	A*02:01	TRAV24	TRAJ58	CAFKRETSGSRLTF	TRBV13	TRBJ2-1	CASSTRLAGDEQFF	Islet CD8	TCR transductant	(74)
1.F3	PPI:2-12	ALWMRLLPLLA	A*02:01	TRAV39	TRAJ39	CAVENAGNMLTF	TRBV10-2	TRBJ2-1	CASWTVSYNEQFF	Islet CD8	TCR transductant	(74)

(Continued)

TABLE 2 | Continued

Clone/ Sequence ID	Epitope	Epitope sequence	HLA[#]	TRAV	TRAJ	CDR3a	TRBV	TRBJ	CDR3b	Source of T cells	Method to confirm reactivity	Reference
96.F5	PPI:3-11	LWMRLLPLL	A*02:01	TRAV8-6	TRAJ48	CAVSDISNFGNEKLTF	TRBV9	TRBJ2-3	CASSVVGLGTDTQYF	Islet CD8	TCR transductant	(74)
23.H5	PPI:3-13	LWMRLLPLLAL	A*02:01	TRAV38-2/DV8	TRAJ22	CAYRSPARQLTF	TRBV6-1	TRBJ2-7	CASSEGWGVPSYEQYF	Islet CD8	TCR transductant	(74)
1.B10-1	PPI:15-23	ALWGPDPAA	A*02:01	TRAV8-3	TRAJ33	CAVVADSNYQLIW	TRBV4-2/4-3	TRBJ2-2	CASSQTKGTGELFF	Islet CD8	TCR transductant	(74)
1.F1	PPI:15-24	ALWGPDPAAA	A*02:01	TRAV39	TRAJ41	CAVSNSGYALNF	TRBV29-1	TRBJ2-5	CSVFHRGETQYF	Islet CD8	TCR transductant	(74)
23.C12	PPI:15-24/15-25	ALWGPDPAAA ALWGPDPAAAF	A*02:01	TRAV41	TRAJ42	CAVSGGSQGNLIF	TRBV28	TRBJ1-2	CASSPPTGWGGYTF	Islet CD8	TCR transductant	(74)
93.D1	PPI:15-25	ALWGPDPAAAF	A*02:01	TRAV5	TRAJ8	CAVTKDTGFQKLVF	TRBV20-1	TRBJ2-1	CSARDHFGGSGYEQFF	Islet CD8	TCR transductant	(74)
10.C6-1	PPI:23-32	AAFVNQHLCG	C*12:03	TRAV19	TRAJ39	CALSGALNNAGNMLTF	TRBV27	TRBJ2-5	CASSLFGYRQETQYF	Islet CD8	TCR transductant	(74)
28.D3	PPI:31-41/34-41	CGSHLVEALYL HLVEALYL	A*02:01	TRAV26-2	TRAJ26	CILTDNYGQNFVF	TRBV27	TRBJ1-1	CASSLIGLNTEAFF	Islet CD8	TCR transductant	(74)
28.E6	PPI:46-54/47-54	RGFFYTPKT GFFYTPKT	A*29:02	TRAV19	TRAJ28	CALSEAGAGSYQLTF	TRBV2	TRBJ2-5	CASSPSGTSSQETQYF	Islet CD8	TCR transductant	(74)
20.G1	PPI:69-77/69-79	GGGPGAGSL GGGPGAGSLQP	C*03:04	TRAV1-2	TRAJ8	CAVRMNTGFQKLVF	TRBV9	TRBJ2-1	CASSVGMDPGLGYNEQFF	Islet CD8	TCR transductant	(74)
96.B4	PPI:91-99	IVEQCCTSI	C*05:01	TRAV12-2	TRAJ31	CAVNNARLMF	TRBV6-5	TRBJ2-1	CASRPTSGGYNEQFF	Islet CD8	TCR transductant	(74)
86.C1	PPI:91-100/92-100/92-102	IVEQCCTSIC VEQCCTSIC VEQCCTSICSL	B*41:02	TRAV19	TRAJ16	CALSEAGFSDGQKLLF	TRBV19	TRBJ2-1	CASSIQFSYNEQFF	Islet CD8	TCR transductant	(74)
84.D9	PPI:91-100/92-100/92-102	IVEQCCTSIC VEQCCTSIC VEQCCTSICSL	B*41:02	TRAV29/DV5	TRAJ43	CAASNSNDMRF	TRBV7-9	TRBJ2-1	CASSLAQREQFF	Islet CD8	TCR transductant	(74)
28.E11	PPI:91-100	IVEQCCTSIC	B*18:01	TRAV12-2	TRAJ49	CAVSMNTGNQFYF	TRBV29-1	TRBJ2-1	CSVQVYNEQFF	Islet CD8	TCR transductant	(74)
1.E9-1	PPI:92-99	VEQCCTSI	B*50:01	TRAV12-2	TRAJ34	CAVNIRYNTDKLIF	TRBV6-2/6-3	TRBJ1-5	CASSSIQGSGSGQPQHF	Islet CD8	TCR transductant	(74)
86.G3-2	PPI:94-102	QCCTSICSL	B*35:01	TRAV8-6	TRAJ33	CAVSDGYQLIW	TRBV6-1	TRBJ2-7	CASSGREAPYEQYF	Islet CD8	TCR transductant	(74)
54.F1	PPI:96-103	CTSICSLY	A*01:01	TRAV3	TRAJ26	CAVPDNYGQNFVF	TRBV7-2	TRBJ2-2	CASSLVVELFF	Islet CD8	TCR transductant	(74)

[#]*HLA class II alleles: DR4 (DRB1*04:01); DR4.4 (DRB1*04:04); DR4.2 (DRB1*04:02); DR1 (DRB1*01:01); DR9 (DRB1*09:01); DR53 (DRB4*01:01); DQ8 (DQA1*03:01-DQB1*03:02); DQ8-trans (DQA1*05:01-DQB1*03:02); DQ2 (DQA1*05:01-DQB1*02:01/02:02), DQ2-trans (DQA1*03:01-DQB1*02:01); DP4 (DPA1*01:03-DPB1*04:01).*

[##]*Two in-frame alpha chains detected. Functional alpha not determined.*

specific TCR clonotypes is essential to develop TCR biomarkers for T1D. In addition to TCR clonotypes listed in **Table 2**, Bonifacio and colleagues reported hundreds of TCR sequences expressed by T cells that were stained with multimer composed of islet epitopes or those proliferated in response to islet antigens (44, 45). While it is necessary to carefully validate true reactivity to antigens, this type of analysis is an excellent resource to gain T1D-associated TCR clonotypes. Computational tools to decrease the "noise" (i.e. eliminating non-specific binding TCR clonotypes) may help to enrich truly antigen-specific clonotypes (100, 101). Further, these candidate TCR clonotypes could be validated for disease specificity using larger cohorts analyzed with whole blood TCR sequencing, and then clonotypes that were detected only in individuals having various stages of T1D could be assessed for functional reactivity (**Figures 1A–C**). Retro/lentiviral transduction systems, especially in a moderate to high throughput multiplex assay, will facilitate verifying reactivity to antigens (82, 98, 102, 103).

Identification of Disease-Specific TCR Clonotypes Using Big Data

Big data analysis, which seeks to classify TCR repertoires in a specific condition using a large number of TCR samples, is an emerging strategy to identify disease-associated TCR clonotypes. A major advantage of this approach is the capability to identify disease-associated TCR clonotypes without knowing antigen specificity, thereby allowing one to include TCRs that are potentially disease-associated but not islet-specific and also those having low affinity to antigens. Indeed, specificities of large proportions of T cells in the islets are unknown (46, 70–74). Virus infections such as enterovirus and coxsackie B virus (CVB) are suggested to be involved in T1D development (104–106), and TCRs specific to these viruses could be identified by big data analysis by comparing TCR repertoires of individuals having or not having different stages of T1D. Although it has been demonstrated in infectious diseases that big data analyses can identify pathogen-specific TCR clonotypes, it has not yet been successful at identifying T1D-associated TCR clonotypes using PBMC samples from individuals with or without different stages of T1D. This could be explained by several possibilities: (1) the frequency of T1D-associated T cells may be lower than that of pathogen-specific T cells; (2) antigens involved in T1D pathogenesis, especially those at different stages of T1D, may be more heterogeneous than those in infectious diseases; (3) autoreactive TCRs could be more private (i.e. not common between patients) than those of conventional T cells; and (4) sample sizes studied to date have not been large enough. However, having large TCR data sets produced by next generation sequencing will enable machine learning algorithms to cluster and classify TCR clonotypes. Using these newly developed techniques, even infrequent disease-specific TCRs having less publicity (i.e. commonality) between people may be identified from relatively small numbers of samples. Indeed, some computational TCR classifying methods are now capable of identifying cancer patients responding to immune checkpoint inhibitors (40), and also early stages of cancer can be differentiated from healthy individuals using this type of technique (107, 108). In the next section, we will discuss how to take advantages of the latest TCR clustering/classifying techniques for T1D TCR biomarkers.

Clustering and Classification of TCR Clonotypes

TCR clonotypes recognizing the same peptide-MHC complex often share similar motifs and features. For example, influenza-specific TCRs prefer to use TRAV38-1/TRAJ52/TRBV19/TRBJ1-2 (109–111), and melanoma (MART-1)-specific TCRs often contain an alpha chain with TRAV12-2 (112). Likewise, several features common for islet antigen-specific TCRs have been reported. We discovered that insulin B-chain-specific TCRs tend to use TRAV38-1/38-2 and other Valpha segments having similar motifs in the CDR1 and CDR2 regions (113). Also, it has been shown that a specific motif "SGGSNYKLTF" is contained in the CDR3 region of alpha chains specific to an IGRP peptide (45). More recently, crystal structure analysis of TCRs specific to a hybrid insulin peptide composed of proinsulin and islet amyloid polypeptide (IAPP) demonstrated that motifs in the TRBV5-1 segment commonly interact with amino acid residues in IAPP (92). Our work also indicates that T cell responses to hybrid insulin peptides precede clinical T1D onset (114), making these TCR clonotypes excellent candidates for biomarkers. Thus, autoreactive TCRs share commonalities and similarities, which provide clues to cluster TCRs and stratify those specific to a certain condition.

A number of algorithms to cluster or classify TCR clonotypes have been developed. Each algorithm has advantages and disadvantages as reviewed by others (115, 116), but in respect to TCR biomarker development for T1D, the algorithms can be divided to two groups. First, those that clusters TCRs by assessing similarities of TCR sequences with each other in datasets. Second, those that seek to classify TCRs by identifying similar to known antigen-specific or disease-specific TCR clonotypes. The former algorithms such as TCRdist (111), GLIPH/GLIPH2 (117, 118), ClusTCR (119), and GIANA (108) do not need information about T1D-specific epitopes and TCR sequences beforehand, and thus can be used to predict disease-specific TCR clonotypes that are specifically detected in T1D patients but not in non-diabetic subjects. On the other hand, machine learning-based algorithms that assess similarities to known antigen-specific TCR datasets to predict epitopes, such as DeepTCR (101), DeepCAT (107), TCRmatch (120), and TCRAI (100) need prior information about disease-specific TCR sequences. These algorithms show excellent performance when classifying TCRs specific to the same epitopes that were used to develop the machine learning algorithm but not for those having different specificities. Therefore, large sets of disease-specific TCR sequence information for machine training are necessary to achieve high specificity and sensitivity. Typically these types of algorithms show better performance to detect antigen-specific TCR clonotypes than the clustering-based algorithms, thereby being useful to validate TCR clonotypes once epitopes or disease-specificity are determined.

Alternatively, they can be also used to 'clean up' (i.e. eliminate non-specific TCR clonotypes) TCR datasets that are obtained from multimer-stained T cells or those activated by antigen stimulation (**Figure 1B**).

In any case, it is essential to prepare TCR datasets from a large number of individuals with and without T1D at multiple time points to elicit the best performance by machine learning and clustering algorithms. Typically, diverse datasets rather than large data but from a limited number of samples improve learning efficiency (100). In addition, it is also important to prepare accurate TCR clonotype information to differentiate T1D patients from healthy subjects. There are now several TCR databases available, which accumulate and curate information about TCR sequences along with target peptide-MHC complexes, such as VDJbase (121, 122), IEDB (123), VDJdb (124), iReceptor (125), and McPAS-TCR (126). While these are incredibly useful resources, a proportion of islet-specific clonotypes is still very small, accounting for only ~100 out of tens of thousands of clonotypes, the majority of which are specific to viruses and tumor antigens. Assuming that self-reactive TCR clonotypes are more heterogeneous and rarer compared to pathogen-specific ones, there is a need for higher numbers of clonotypes specific to T1D. Thus, identifying a large set of accurate disease-specific TCR clonotypes will be a key component to achieve successful big data analysis, which will ultimately lead us to establish TCR biomarkers in T1D (**Figure 1**).

PERSPECTIVE

It is still controversial whether T1D patients have distinct islet antigen-specific T cell subsets in the blood compared to healthy individuals. Even in the pancreas, non-diabetic organ donors have preproinsulin-specific T cells in the exocrine compartment, but such antigen-specific T cells accumulate into the islets over the course of T1D progression (127). In the islets, we recently demonstrated that only T1D donors have CD8 T cells highly reactive to preproinsulin (74). Mallone and colleagues also reported that pancreata of T1D donors have a higher number of zinc transporter-8-specific T cells than non-diabetic controls (7). Thus, multiple studies demonstrate that islets of T1D individuals have distinct T cell repertoires from those without diabetes. However, a number of studies indicate that healthy individuals have islet-antigen specific T cells in the blood (7, 113, 128–131), and depending on cell subsets examined, some studies including those looking into pathogenic T cells show that T1D patients have higher numbers of islet-specific T cells, whereas others do not detect differential islet-specific T cells in T1D patients. This controversy could be explained by either (1) detectable numbers of pathogenic T cells in the islets do not leak into the peripheral blood (**Figure 2A**); or (2) pathogenic T cells in the islets do indeed circulate, but because there are already a number of islet-specific (but not harmful) T cells in the circulation, the total numbers of islet-specific T cells (i.e. pathogenic T cells leaked from the islets plus non-pathogenic T cells) are not differentiated enough in the blood of T1D patients from healthy individuals (**Figure 2B**). Given evidence that a portion of T cell repertoires are shared between pancreas, pancreatic lymph nodes, and peripheral blood cells (72), and that TCR repertoires in the islets of T1D organ donors are clonally distinct from those of non-diabetic donors (74), if the latter hypothesis (**Figure 2B**) is correct, islet-derived TCR sequences will be a powerful marker to discriminate pathogenic from physiological T cells, thereby capable of stratifying individuals with active insulitis prior to clinical T1D onset.

To develop practical TCR biomarkers in T1D, a number of obstacles need to be overcome, some of which may be unique to autoimmune diseases. These challenges can be considered from the view of (1) publicity, (2) abundancy, and (3) disease-specificity.

1. Publicity

   It will be important to understand the frequencies of public *vs* private TCR clonotypes that are specific to the T1D disease state, and these likely fluctuate over time during T1D development. Given the genetic risk associated with HLA class II genes, heterogeneity provided by HLA diversity could be smaller than other diseases for TCR clonotypes expressed by CD4 T cells. However, autoreactive T cells, which often bind to peptide-MHC complexes with low affinity, may have a larger TCR repertoire than conventional anti-pathogen T cells, resulting in less commonality. Therefore, frequency of public T1D-specific TCR clonotypes may be low. Strategies that compare TCR repertoires in each individual such as pre and post treatment (40) do not need to consider publicity of clonotypes, and therefore may be more easily applicable to T1D immune intervention studies.
2. Abundancy

   Theoretically, 10^{15}-10^{16} diverse TCR clonotypes can be assembled (12–14); however, a practical TCR repertoire size is estimated to be about 10^{8}-10^{10} per person (15, 17, 18). This indicates that the frequency of target clonotypes is extremely low. However, there is evidence that identical clonotypes are persistently detected from the same individuals over time (44, 81, 93, 132). We believe quantitative resolution of TCRs will need to be increased. This could be achieved by enriching samples before sequencing (e.g. beads enrichment by antigen-specific multimers). Another very attractive approach is to target sequencing to TCRs containing a preferred Vgene segment of interest, thus greatly enhancing the depth of sequencing by analyzing clonotypes that can be obtained for a specific V allele. Blood sample volume needed to quantitatively evaluate frequency of disease-associated TCR clonotypes is another important consideration, which will need to be addressed given that the T1D disease process does begin in young children.
3. Disease-Specificity

   Identification of disease-specific TCR clonotypes is an essential component to develop robust T1D TCR biomarkers. A larger number of TCR clonotypes with higher specificity to the disease that are in place will allow for more sensitive and specific assays. Therefore, the key

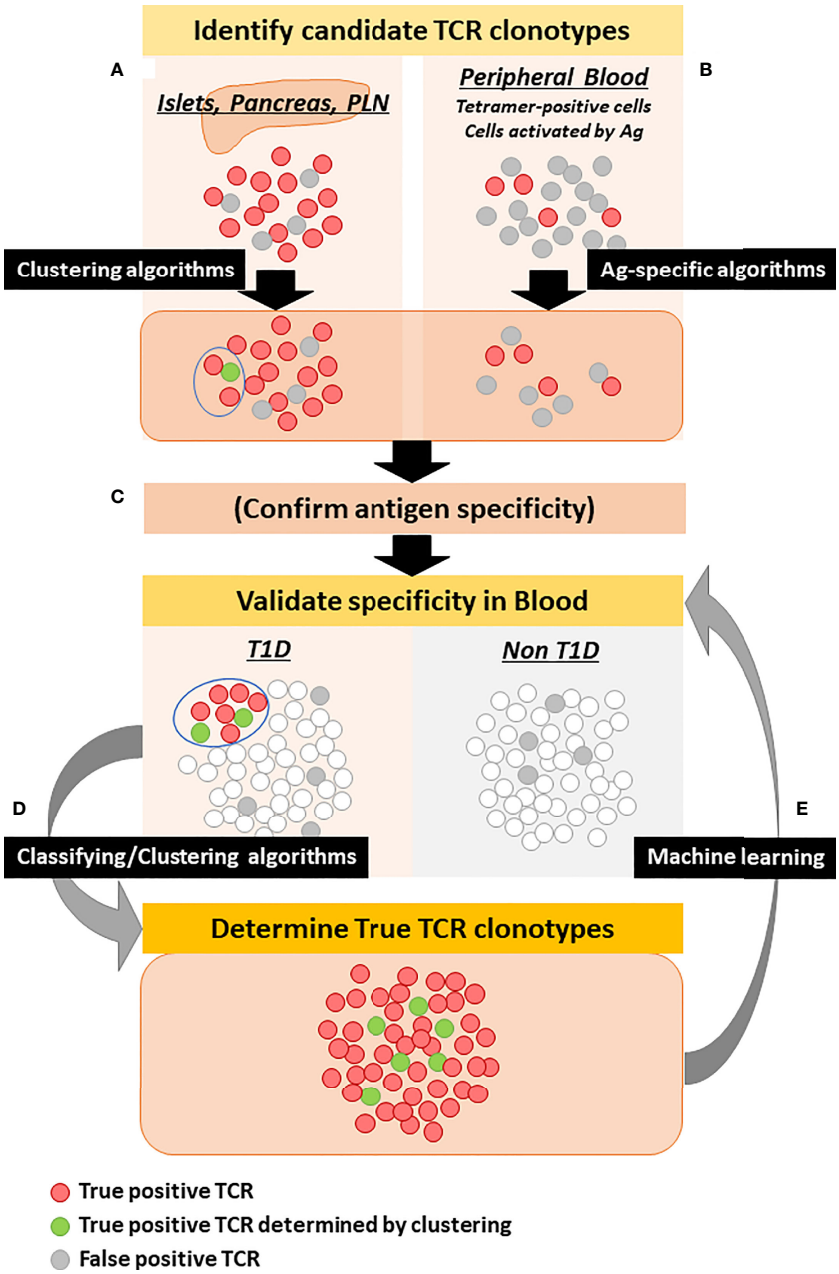

FIGURE 1 | Strategy to determine disease-specific TCR clonotypes. Red and gray circles represent true and false disease-specific TCR clonotypes, respectively. Green circles are true disease-specific clonotypes determined by clustering with known disease-specific TCR clonotypes. **(A)** TCRs detected in the islets, pancreata, and pancreatic lymph nodes, in particular those for which antigen specificity has been determined as well as those that are clustered with known disease-specific TCRs, can be the initial source for disease-specific TCR candidates. **(B)** TCRs detected from peripheral blood T cells enriched by antigen stimulation or peptide-MHC-conjugated multimers are also an initial source. Antigen-specific algorithms can enrich TCR clonotypes that are truly specific to antigens. **(C)** Candidate TCR clonotypes may be assessed for specificity to islet tissues, proteins, and peptides. **(D)** Using classifying algorithms, candidate TCR clonotypes are assessed for frequency in the blood of individuals with and without T1D to determine disease specificity. Simultaneously, clustering algorithms can select additional clonotypes that are clustered with known disease-specific TCR clonotypes. **(E)** TCR clonotypes selected by classifying and clustering algorithms are used for machine learning of antigen-specific algorithms to further determine true disease-specificity.

is how to select such truly disease-specific TCR clonotypes. As illustrated in **Figure 1**, both accumulation of actual TCR datasets produced from individuals with and without T1D and computational big data analysis will facilitate the development of biomarkers. While the majority of TCR big data analysis currently uses only CDR3-beta sequences, it has been demonstrated that inclusion of entire sequence information such as V and J segments, in particular CDR1

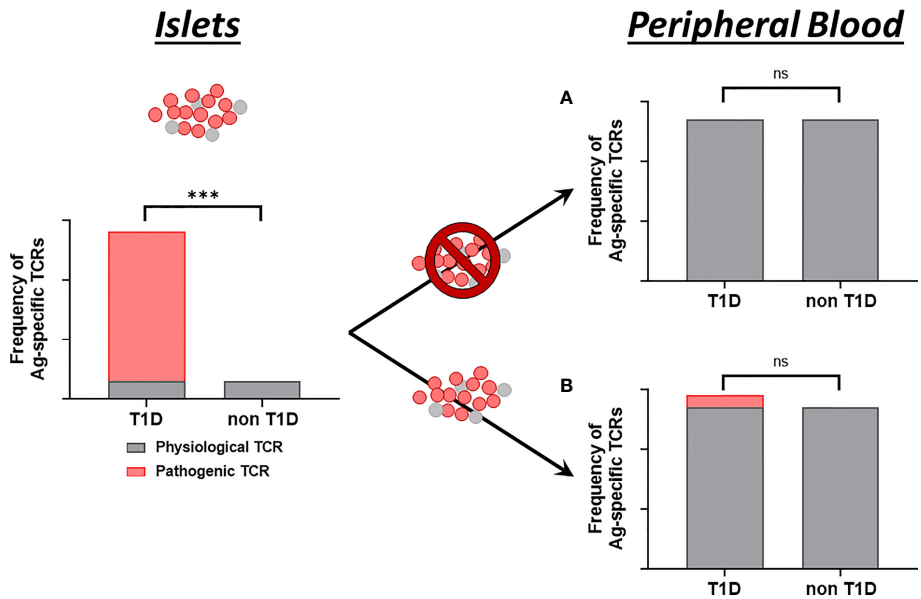

FIGURE 2 | Proposed models of islet-specific T cell detection in the blood. While pancreatic islets contain a certain amount of T cells regardless of disease status (gray bars), only islets of individuals with T1D contain T cells that are highly reactive to islet antigens (red bar). Model **(A)** Leak of T cells from the islets to peripheral blood is limited. T cell repertoires specific to islet antigens are not different between individuals with and without T1D. Model **(B)** There are substantial amounts of islet-specific but not disease-specific T cells in the blood regardless of disease status (gray bars). T cells in the islets do circulate in the blood (red bar), but the total numbers of islet-specific T cells are not different between individuals with and without T1D. Enumerating only T cells derived from the islets can identify individuals having T1D. TCR clonotypes are a distinct property to identify islet-derived T cells. *** significantly different. ns, not significant.

and CDR2 sequences, increases accuracy of classifying TCR clonotypes (100, 120). While the number of T1D-specific clonotypes that have been determined so far is low, evolutions in both TCR sequencing technologies and computational analysis strategies will dramatically impact this effort.

In conclusion, the antigen receptor on disease specific T cells holds promise for a non-cell based biomarker of not only the presence of T1D but disease activity as well. Efforts to define the TCR repertoire within the human pancreas of T1D and non-T1D organ donors is underway with a need to define the antigen specificity and HLA restriction of these identified clonotypes. Those clonotypes that are shared between individuals with T1D, frequent, and circulate from the pancreas and pancreatic lymph nodes to the peripheral blood are prime candidates for deep sequencing and clustering of TCRs using developed computational analyses.

AUTHOR CONTRIBUTIONS

MN and AM wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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