FRESH IDEAS, FOUNDATIONAL EXPERIMENTS: IMMUNOLOGY AND DIABETES

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FRESH IDEAS, FOUNDATIONAL EXPERIMENTS: IMMUNOLOGY AND DIABETES

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EDITORIAL
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Editorial: Fresh Ideas, Foundational Experiments: Immunology and Diabetes

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Keywords: type 1 diabetes, autoimmunity, NOD mouse model, type 2 diabetes, islet transplantation

Editorial on the Research Topic

Fresh Ideas, Foundational Experiments: Immunology and Diabetes

The Fresh Ideas, Foundational Experiments (FIFE): immunology and diabetes research topic is a collection of 13 articles ranging from perspectives, reviews, to hypothesis, and theories all focused on diabetes. The global rise in incidence of Type 1 Diabetes (T1D) does not correlate with genetic drift and indicates that environmental exposures are playing an increasingly significant role. The FIFE:Immunology and diabetes group would like to use this research topic to share their data and ideas to promote collaborations and accelerate the development of novel therapies with the goal of a cure for T1D.

The multidisciplinary FIFE mini-symposium brought together young researchers from across North America investigating various interconnected contributors to T1D onset, progression, interventions, and put forth multiple concepts for further examination. Its members have convened annually for the past 3 years to share their perspective and research updates and establish new collaborations unified by the universally held goal of finding a sustainable, life-long cure. The first perspective in this series by Mouat et al. "Fresh Ideas, Foundational Experiments (FIFE): Immunology and Diabetes 2016 FIFE Symposium" describes the group, its goals, and summarizes the inaugural FIFE mini-symposium held at the University of British Columbia in Canada under the vision and leadership of Dr. Horwitz.

The inaugural FIFE symposium led to 12 additional publications from members of the FIFE collaborative research team. This includes a perspective from Chen et al. entitled "The Role of NOD Mice in Type 1 Diabetes Research: Lessons from the Past and Recommendations for the Future." The authors describe the usefulness of the non-obese diabetic (NOD) mouse for the past 35 years as a primary model for studying autoimmune diabetes. They focus on the similarities to the human disease, polymorphisms, gene perturbations of a disease that targets similar biological pathways, tissues, and islet antigens. They also address the reasons why immune therapies have failed to translate from mice to humans. Finally, they propose new strategies to edit the NOD genome to improve a better understanding of human diabetes.

With a better understanding of the NOD mouse, we next focus on the complex mechanisms and pathways involved in disease pathogenesis. Our journey begins with a review by Newby and Mathews entitled "Type I Interferon Is a Catastrophic Feature of the Diabetic Islet Microenvironment." In this review they provide a detailed understanding of the molecular and cellular pathways resulting in islet beta cell destruction. T1D develops from a complex interaction between genetics, the immune system, and environmental factors. The authors focus on type 1 interferons as the link between these critical pieces and review the evidence supporting the diabetogenic potential of IFNα/β within the islet microenvironment for the development of T1D.

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At the heart of autoimmune-mediated T cell diseases lies the recognition of self-proteins being presented by the human leukocyte antigen (HLA) complex to the T cell receptor (TCR). The next contribution by Bettini and Bettini address this critical interaction in their review: "Understanding Autoimmune Diabetes through the Prism of the Tri-Molecular Complex." The strongest susceptibility alleles for T1D reside within the HLA loci, which supports the role for T cells as the critical drivers of T1D. This review provides a summary of autoimmune T cell development, the significance of the antigens targeted in T1D, and the relationship between TCR affinity and immune regulation.

The era of genome-wide association studies (GWAS) has yielded the discovery of ∼57 independent loci contributing to the overall genetic risk for T1D development. This next review by Wallet et al. is entitled "Isogenic Cellular Systems Model the Impact of Genetic Risk Variants in the Pathogenesis of Type 1 Diabetes." In this review, they provide a comprehensive list of single nucleotide polymorphisms associated with T1D risk and summarize the functional impact of several candidate risk variants on host immunity in the context of T1D. They also discuss the potential for an "isogenic disease-in-a-dish model system" to interrogate the biological role of risk variants, with the goal of expediting precision therapeutics in T1D.

The next article in our series is a review by Wagner entitled: "Overlooked Mechanisms in Type 1 Diabetes Etiology: How Unique Costimulatory Molecules Contribute to Diabetogenesis." CD28 is the classical co-stimulatory molecule while CTLA-4 is the classical inhibitory counterpart. This review is focused on additional co-stimulatory molecules such as TNF-receptors I and II, CD40, mucin, ICOS, and immunoglobulins. Wagner proposes that inflammation driven by interactions between CD40 with CD154 results in the loss of Foxp3 expression and the generation of pathogenic TH40 (CD4+CD40+) effector cells. Thus, targeting the CD40/CD40L pathway creates a potentially new therapeutic avenue for T1D.

The topic for the next review is focused on environmental stressors, namely virus infections, as triggers of T1D in genetically susceptible individuals. The review by Morse and Horwitz, "Innate Viral Receptor Signaling Determines Type 1 Diabetes Onset" focuses on the observation that heritable susceptibility alone cannot explain the rising incidence of T1D. The authors discuss that the recognition of viral antigens via innate pathogen-recognition receptors could trigger inflammatory events which ultimately result in the destruction of insulin-secreting beta cells. They further discuss that activation of innate pathways and inflammatory molecules, including type I and III interferon, can differentially prime the immune system to produce a protective response or a diabetogenic response. The authors conclude by hypothesizing that the increase in incidence of T1D may be due to changes in how the immune system senses and responds to viral antigens.

We close the review sections with discussions of both the immune systems' response to transplanted islets and the health of the islet transplant itself. Barra and Tse discuss "Redox-Dependent Inflammation in Islet Transplantation Rejection." In this review the authors discuss the main challenges associated with transplant rejection and islet viability, thus preventing long-term β-cell function. Redox signaling and the production of reactive oxygen species (ROS) by recipient immune cells and transplanted islets themselves are key players in the demise of the beta cell and contribute to graft rejection. The authors focus on redox signaling, the process in which ROS are generated during graft rejection as well as new strategies to limit or modulate ROS synthesis during islet cell transplantation.

Transplants containing insulin-producing cells are vulnerable to both recurrent autoimmunity and conventional allograft rejection. Burrack et al.'s review "T Cell-Mediated Beta Cell Destruction: Autoimmunity and Alloimmunity in the Context of Type 1 Diabetes" discuss this complex topic. Current immune suppression acts globally, but ideally, a successful approach would limit T cells targeting the transplanted islets. First, they describe the current understanding of autoimmune destruction of beta cells including the roles of CD4 and CD8 T cells and several possibilities for antigen-specific tolerance induction. Second, they outline diabetic complications necessitating beta cell replacement. Third, they discuss transplant recognition, potential sources for beta cell replacement, and tolerance-promoting therapies under development.

The next review steps outside of the autoimmune field to focus on patients with type 2 diabetes (T2D) as the largest population of patients who experience post-sepsis complications and rising mortality in the review by Frydrych et al. "Diabetes and Sepsis: Risk, Recurrence, and Ruination." Patients with T2D have an increased risk of developing infections and sepsis. T2D also worsens infection prognosis and showing increased morbidity and mortality from sepsis. The authors propose that T2D causes a functional immune deficiency that directly reduces immune cell function. T2D patients display diminished bactericidal clearance, increased infectious complications, and protracted sepsis mortality. This comprehensive review explores immune dysfunction including: metabolic regulation, inflammation, molecular pathways, cellular defects, cytokines, and immune modulatory therapies.

In this hypothesis and theory: "The Folate Cycle As a Cause of Natural Killer Cell Dysfunction and Viral Etiology in Type 1 Diabetes" Bayer and Fraker pose an interesting role of natural killer cells (NK) in T1D development. The authors describe a link between inefficient folate metabolism and poor antiviral responses from NK cells to the establishment of chronic viral infections. They hypothesize that defects in the folate cycle within genetically susceptible individuals could lead to immune dysfunction, create a permissive environment allowing for chronic or cyclical latent/lytic viral infections, a dampened NK response, and beta cell death.

The next hypothesis and theory article: "The Four-Way Stop Sign: Viruses, 12-Lipoxygenase, Islets, and Natural Killer Cells in Type 1 Diabetes Progression" by Semeraro et al. outlines a new idea that incorporates early antiviral immune effectors, NK cells, with proinflammatory processes involving 12-lipoxygenase occurring in the pancreatic beta cells. The authors hypothesize that the activation of NK cell lipoxygenase through viral infections could contribute to T1D initiation by affecting the

normal balance of activating and inhibitory NK cell receptors, ultimately leading to autoimmunity and islet destruction.

Finally, the series concludes with a final hypothesis and theory: "Environmental Factors Contribute to β Cell Endoplasmic Reticulum Stress and Neo-Antigen Formation in Type 1 Diabetes" by Marre and Piganelli. This article summarizes the current knowledge regarding endoplasmic reticulum (ER) stress and protein post-translational modifications that can occur in islet beta cells, and it proposes a role for environmental factors in the breakdown of immunologic tolerance to beta cell antigens. The authors describe a number of factors including virus infection, dysglycemia, inflammation, chemical exposure, and ROS synthesis that can lead to ER stress in beta cells, neo-antigen formation, and priming autoreactive T cell responses.

AUTHOR CONTRIBUTIONS

JS and BF wrote the manuscript. All authors edited the final version.

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PERSPECTIVE
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Fresh Ideas, Foundational Experiments (FIFE): Immunology and Diabetes 2016 FIFE Symposium

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The first Fresh Ideas, Foundational Experiments (FIFE): Immunology and Diabetes symposia workshop took place in 2016 and exemplified the active interest of a number of several investigators interested the global rise in the incidence of type 1 diabetes (T1D). This increase does not correlate with genetic drift and indicates that environmental exposures are playing an increasingly significant role. Despite major biomedical and technological advances in diagnosis and treatment, treatments are frequently insufficient as they do not inhibit the progression of the underlying autoimmune response and often fail to prevent life-threatening complications. T1D is the result of autoimmune destruction of the insulin-producing beta cells of the pancreas, and the precise, mechanistic contribution of the immune system to disease pathogenesis and progression remains to be fully characterized. Ultimately, the combinatorial effect of concurrent factors, including beta cell fragility, exogenous stressors, and genetic priming of the innate and adaptive immune system, work together to induce T1D autoimmunity. Thus, T1D is the result of immunological defects and environmental pathogens, requiring the sustained attention of collaborative research teams such as FIFE: I & D with varied perspectives, unified by the universally held goal of finding a sustainable, life-long cure. Herein, the authors provide perspective on various fields in T1D research highlighted by speakers participating in the inaugural FIFE symposium.

Keywords: type 1 diabetes, islet encapsulation, intestinal epithelial cells, self-peptide complexes, T-cell pathogenicity, T-cell metabolism, dendritic cell activity, type 2 diabetes

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INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease of unclear etiology that results in the destruction of the insulin-producing beta cells of the pancreas, causing loss of systemic blood glucose regulation and hyperglycemia, insulin resistance and chronic joint pain (1–3). When left untreated, T1D can lead to defects in wound healing and diabetic retinopathy (4, 5), but on a daily basis, even a person with managed T1D must make significant lifestyle changes to constantly monitor blood glucose and navigate the financial burdens of insulin supplementation, medications and proper diet. Current T1D diagnostics and standard therapeutics typically address hyperglycemia without targeting the underlying autoimmune response and are thus insufficient in predicting prognosis and reducing pathogenesis long term (6–8). Damage to the pancreas is mediated by infiltrating innate and adaptive immune cells that induce pancreatic tissue pathology and disrupt molecular pathways involved in

insulin regulation; however, the early events of disease initiation are still poorly understood, hindering the development of targeted immunological treatments (9–13). The steady increase in the incidence of T1D over time is not accounted for entirely by population genetics, indicating that geographic and environmental agents are important contributors to the pathogenesis of T1D (14–18). Understanding the mechanisms of the pathogenic components and their interactive roles will require the collaboration of many research teams with a variety of perspectives and approaches to tackle central questions in TID research.

Our lab focuses on researching the contribution of viral pathogens to T1D development and pathogenesis. Infection by picornaviruses such as coxsackievirus B (CVB) promotes T1D development through the stimulation of pancreatic inflammation, resulting in the release of islet antigens and the development of autoantigens. CVB antigens are recognized by pattern recognition receptors, which stimulate a type I and III interferon response (19, 20). A multifaceted immune cascade develops, characterized by the activation of innate cells, disruption of regulatory cells, and increased antigen presentation and memory cells. CVB infection is a T1D initiating influence that works in concert with other environmental factors as well as genetic variance. We aim to incorporate a perspective using models with multifactorial autoimmunity triggers by examining the contribution of gut microbiome and virome, pancreatic viral persistence, and T1D-risk genes, to determine impact on inflammation, innate sensing, and loss of regulatory mechanisms leading to onset of T1D.

As scientists work to elucidate the complex and multifactorial nature of T1D, they continue to voice a strong need for collaboration in new and innovative areas of research. With the aim to foster such collaborative endeavors, Dr. Marc Horwitz of the University of British Columbia's Department of Microbiology and Immunology organized the first Fresh Ideas, Foundational Experiments (FIFE): Immunology and Diabetes mini-symposium, named and themed affectionately after Dr. Brian Fife, a cherished member of the JDRF nPOD network. The event centered around immunological T1D research, though this mini-symposium pushed beyond those boundaries by examining shared characteristics with multiple sclerosis (MS), defects in cellular metabolism, immunodeficiencies in type 2 diabetes (T2D), and the contribution of the microbiome to autoimmunity. A variety of approaches and experimental methods were discussed, ranging from therapeutic applications based on innovative biomaterials to translation and complementation of mouse models and clinical data for a more complete understanding of the immunological underpinnings of T1D.

ISLET TRANSPLANTATION USING A NOVEL ENCAPSULATION STRATEGY

To kick-off the symposium, Dr. Hubert Tse of the Department of Microbiology-Comprehensive Diabetes Center, School of Medicine University of Alabama, Birmingham, discussed his group's work on engineering and applying biomaterials for effective transplantation of functional insulin-secreting beta cells in T1D patients (21). A major hindrance to successful islet transplantation is rejection due to reactive oxygen species (ROS)-induced oxidative stress and non-specific inflammation in the pancreatic microenvironment. To address this problem, the Tse lab has constructed a semipermeable, anti-oxidative material which encapsulates and protects the islets from immune destruction, while maintaining interactions between the islets and host microenvironment. The encapsulating material is formed by a layer-by-layer polymerization of anti-coagulant poly(*N*-vinylpyrrolidone) (PVPON) and the natural antioxidant tannic acid (TA), resulting in an ultra-thin, neutral and non-toxic polymeric PVPON/TA capsule (22). Preliminary *in vitro* studies using rat, non-human primate, and human islets demonstrated that PVPON/TA-encapsulated islets were able to sense glucose and secrete insulin. In addition to preserving islet function, the capsules decreased the immunoreactivity of the local microenvironment by reducing effector T-cell infiltration, chemotaxis, and synthesis of pro-inflammatory cytokines and chemokines (23). In the streptozotocin NOD.*scid* mouse model of pancreatic beta cell destruction, transplantation of PVPON/TA-encapsulated islets into the epididymal fat pad restored euglycemia as early as 2 days post-transplantation, with effects lasting up to 30 days. Hyperglycemia was then restored if the fat pads containing the transplanted islets were removed. Overall, the data demonstrate that PVPON/TA-encapsulated islets are viable, functional, and immunoprotective both *in vitro* and *in vivo*.

Rejection of allogeneic islets or pluripotent stem cells persists as the most significant challenge in transplantation treatments necessitating life-long immunosuppression which also reduces the overall functional competency of the transplanted beta cells (24). Rather than utilizing broadly acting immunosuppressive drugs, coating transplanted islets in a nano-polymer allows the islets themselves to be partially immune privileged and escape destruction from autoreactive T cells while maintaining glucoregulatory abilities. However, encapsulation strategies unfortunately also tend to limit oxygen and nutrient diffusion necessary for cell viability and also render the transplant vulnerable to cytokine-mediated toxicity and antibody recognition (25). Consequently, Dr. Tse's work represents inventive progress in an islet transplantation packaging strategy in efforts to preserve the therapeutic effects for long-term treatment success and providing suitable islet microenvironment. However, we feel that a limitation of this project is that it does not address the inflammatory cascade induced by genetic and environmental contributors to T1D, including virus infection. Undoubtedly, treating a disease as complex as T1D will require a multifaceted approach and this research provides significant advancement in modulating local pancreatic immune responses with biomaterials in order to achieve successful islet transplantation in patients with T1D.

THERAPEUTIC USE OF BUTYRATE TO ALTER INNATE EPITHELIAL CELL HOMEOSTASIS

To further the discussion in emerging areas of T1D research, Dr. Shannon Wallet, Associate Professor at the University of Florida's Department of Periodontology, offered insight into the

importance of the intestinal tract in development of autoimmunity. Dr. Wallet proposed that disruption of immune homeostasis in the gastrointestinal tract (GI) tract may elicit autoreactive T-cell development, activation and expansion. To examine the role of the GI tract in T1D, the Wallet group isolated and characterized immune cells in the intestinal crypts of T1D patients (26). They observed a marked expansion of the pro-inflammatory type 1 innate lymphoid cell population as well as an increase in pro-inflammatory cytokines compared to healthy controls. Dr. Wallet hypothesized that intrinsic defects in innate sensing of intestinal epithelial cells (IECs) may be responsible for the inflammation in the gut. IECs isolated from T1D patients expressed higher levels of IL-17c, an autocrine cytokine that increases pro-inflammatory responses in epithelial cells. With the aim of correcting IL-17c signaling dysregulation in the IECs of TID patient, Dr. Wallet examined the influence of administering commensal bacteria-derived butyrate to IECs as a means to promote immune regulation and suppress inflammation. *In vitro* experiments revealed that IECs from T1D patients were far less responsive to butyrate compared to controls. Specifically, butyrate was more effective at increasing the oxygen consumption rate and TSLP (thymic stromal lymphopoietin) production of IECs from control than of IECs from T1D samples. The Wallet lab is continuing to investigate the contribution of innate immune signaling and dysregulation of the GI tract on T1D and the therapeutic potential of butyrate treatment to modify pro-inflammatory IECs.

It is our perspective that an inflammatory cascade is promoted through a variety of dysregulated immune responses that interact and amplify one another. Aberrant innate sensing significantly contributes to disease through a variety of mechanisms and through multiple cell types. Innate receptors that are less experienced in some individuals due to reduced exposure to typical environmental antigens, likely cause an exaggerated or prolonged inflammatory response upon novel recognition. This prolonged inflammatory response in turn contributes to activation of autoreactive B and T cells, some with pancreatic tropism. Type 1 interferonopathies represent an example of how defects in innate sensing can lead to disease. Type 1 interferonopathies, characterized by a dysfunctional production of type 1 interferons, are often associated with autoinflammation and autoimmune phenomena (27, 28). It is the view of our lab that localized type 1 interferonopathies in pancreatic microenvironments caused by environmental, as well as genetic influences, significantly contribute to T1D (29).

Innate sensing can be altered by genetic variation, virus infections, and microbiome dysbiosis; all of which have been implicated in T1D (30, 31). Certain differences in both the gut microbial communities and virome have recently been identified to be correlated with T1D development (32, 33). For instance, butyrate- and acetate-producing bacteria have been associated with protection from spontaneous T1D in non-obese diabetic (NOD) mouse model of T1D (30). Bacterial metabolites, such as butyrate and acetate, can act on various inflammatory pathways to alter immune homeostasis (30, 34). Specifically, stimulation of the innate sensor, TLR5, induces the expression of butyrate receptor GPR43 on IECs enhancing T regulatory responses and modulating inflammation (35). In parallel, Gp43-deficient mice present with heightened inflammatory responses (36). In light of these findings, we believe that innate sensing is essential in priming the immune system during exposure to environmental antigens. As such, we hold that changes in innate immunity/sensing may indeed contribute to altered commensal microbiota as well as influence cellular permeability, all affecting the development of autoimmune disorders such as T1D. As such, Dr. Wallet's research re-emphasizes the importance of innate signaling and commensal microorganisms and their respective influences on disease states.

Our lab has previously exhibited how differences of innate sensing in virus infections can trigger the onset of T1D autoimmunity. Polymorphisms in the interferon induced with helicase c domain 1 (*IFIH1*) gene have been strongly associated with T1D risk among patients (37). The virus sensor melanoma differentiation-associated protein 5 (MDA5) is expressed from *IFIH1* and recognizes ssRNA from viruses like CVB. NOD mice heterozygous for the MDA5 allele and thus expressing roughly half as much of the receptor as WT-NOD are protected from developing T1D following CVB4 infection whereas about 50% of the WT mice become autoimmune within 7 days post-infection (19). These MDA5 heterozygous mice produce a particular type 1 IFN response that appears to be protective for T1D and display an increased regulatory T-cell response. We have found that another ssRNA sensor, toll-like receptor 3 (TLR3), is critical for host defense to CVB4 and NOD mice deficient for TLR3 are highly susceptible to CVB4 infection (38). However, the mice that survive typically become diabetic, indicating that differences in TLR3 signaling may also contribute to T1D development (38). Thus, reduction of MDA5 but not TLR3 signaling is sufficient to down-regulate excessive inflammation that may subsidize autoimmunity. This work further exhibits how modulation of innate receptor activation alters the inflammatory profile and resulting adaptive response that induces or protects from T1D onset.

USE OF TETRAMERS FOR T1D DIAGNOSTICS AND TARGETED THERAPEUTICS

Continuing the focus on immune mechanisms in disease pathology, assistant professor of Rheumatic and Autoimmune Disorders at the University of Minnesota and namesake of this symposium, Dr. Brian T. Fife, discussed the contribution of T cells to autoimmune pathology in T1D. The Fife group works on identifying and targeting autoreactive T cells in T1D using self-peptides and MHC II molecules conjugated in tetramer complexes (pMHCII tetramers) (39). This work aims to identify prediabetic individuals at risk of progressing to clinical disease, and to develop therapeutics against specific autoreactive T-cell subsets.

Using insulin peptide:MHCII tetramers, the Fife group demonstrated that the number of insulin-targeting $CD4^+$ T cells in peripheral blood of T1D patients correlates with insulin autoantibody titers. These findings substantiate the feasibility of using pMHCII tetramers as a tool for early detection of autoreactive T cells in T1D. The Fife lab is currently developing an arsenal of tetramers against various diabetogenic targets, such as PD-1,

which can be multiplexed to either eliminate or induce tolerance in autoreactive T cells (40). Theoretically, coupling the tetramers to toxins could selectively target particular subsets of autoreactive T cells for destruction, aiding in the re-establishment of self-tolerance. The Fife lab is also looking to use tetramers for antigen-specific-coupled cell tolerance, similar to insulin-coupled antigen-presenting cell (APC) therapy as previously published (41). Current efforts in the group are now focused on using pMHCII tetramers and T-cell receptor mimetic peptides as new T1D therapeutics that induces T-cell tolerance. The aim is for pMHCII tetrameric compounds to be used to specifically delete pathogenic T cells in patients.

The ability to identify and distinguish virus-specific and autoimmune-specific T cells is an especially advantageous process that has been universally utilized by many research groups. Tetramer technology has become an incredibly valuable and multifaceted biological tool which in this instance holds a two-sided benefit: not only may this technology be used for diagnostics but it also allows for therapeutics to be precisely delivered to the desired cells and the microenvironments in which they are harbored. Early detection of disease onset or predisposition of autoimmunity provides opportunity for early intervention to preserve beta cell mass and potentially even reverse presence of disease utilizing therapies such as those reviewed by Ludvigsson (42). Creating methods for heightened specificity and efficiency of tetramer identification for very distinct cells allows precise intervention and minimization of non-target destruction. Toxin-coupled tetramers provide opportunity to directly potentiate or eliminate the cell subsets responsible for self-reactivity. Recent work has shown that beta cells secrete neoantigens which further enhance the local T-cell response (43). Therefore, the Fife group is positioned to detail a comprehensive understanding and identification of what types of T1D-related self-antigens are produced, as well as which ones are critically targeted by pathogenic T cells allowing for the ability to provide intervention necessary for inducing antigen-specific T-cell tolerance.

RECENTLY IDENTIFIED Th40 T CELLS PROMOTE AUTOIMMUNITY

Dr. David Wagner, from the University of Colorado, Denver Department of Medicine, discussed the role of CD40 in autoimmune inflammation. The recent discovery that the CD40 co-stimulatory molecule is expressed on T cells, not only APCs as previously thought, led to the hypothesis that $CD40^+CD4^+$ T cells, termed by Wagner as Th40 cells, could contribute to pathogenesis in autoimmunity. Impressively, Dr. Wagner examined the role of Th40 cells in both T1D and MS using murine models and patient data. In a CD40-reporter BDC2.5 T-cell transgenic murine model of T1D, hyperglycemia exacerbated CD40 expression in the pancreas (44). Adoptive transfer of CD40-depleted cell suspensions to NOD mice demonstrated that CD40-expressing cells are necessary and sufficient for the development of TID. Moreover, diabetogenic $CD4^+$ T cells in the periphery of T1D patients expressed high levels of CD40, in contrast to T cells from healthy individuals. To examine the role of Th40 cells in MS, Dr. Wagner used the murine experimental autoimmune encephalomyelitis (EAE) model, an established immune-mediated model of MS. Adoptive transfer of splenic Th40 cells from EAE mice induced EAE in recipient naive mice, demonstrating the pathogenic capacity of Th40 cells (45). These results were then substantiated by the fact that the Th40 population is increased in MS patients irrespective of HLA haplotype, compared to age matched controls. Finally, Dr. Wagner's team examined the therapeutic effects of inhibiting the interaction between CD40 and its ligand, CD154, using a KGYY15-blocking peptide. Blocking the CD40–CD154 interaction reversed hyperglycemia in new onset diabetic NOD mice and improved clinical scores in EAE mice. Importantly, the KGYY15 peptide bound to human T cells and reduced the ability of the T cells to produce IFN-γ. This research indicates that CD40 is a cellular pathologic marker in multiple autoimmune diseases and can be modulated for treatment of disease.

Overall, we feel Dr. Wagner's research further substantiates not only the value of CD40 functionally, but also as a biomarker in viral and autoimmune pathology. Our lab has previously identified that APC expression of CD40 is a mechanism by which viral infection contributes to EAE by diminishing responding regulatory T-cell populations (46). Similarly, we propose that further examination of CD40 on both T cells and APCs in relation to viral infections in T1D onset is worthwhile. When exposed to neo-self-antigens in a transgenic OVA beta cell autoimmunity mouse model, Th40 cells lose the ability to express the immunoregulatory molecule, CTLA-4, as opposed to when in their naive state (47). Furthermore, transfer of antigen experienced CD40-expressing $CD4^+$ T cells are able induce T1D in NOD.*scid* recipients (47). Increased CD40 expression leads to heightened secretion of inflammatory molecules and T-cell activation pushing immune homeostasis toward an inflammatory state instead of a tolerogenic one. Enteroviruses such as CVB have been strongly linked to T1D development; and blocking CD40 engagement in CVB3-induced inflammatory myocarditis has been shown to slow disease progress (48). Accordingly, CD40 may be contributing to an inflammatory state following infection that leads to autoimmunity and can potentially be used as a biomarker for pathogenic T cells. Determination of whether other viruses positively associated with T1D onset may be eliciting CD40-expressing immune cells could be important for understanding how these environmental pathogens are promoting development of autoimmunity.

LAG-3-MEDIATED IMMUNOREGULATION PROTECTS FROM T1D

Given that T-cell overamplification contributes to autoimmune disease etiology, attenuation of the effector functions, activation, and proliferation of diabetogenic T cells may impede the progression of T1D. Dr. Jon Piganelli, from the University of Pittsburgh's Department of Immunology, examines homeostatic factors in relation to cell metabolic profiles to elucidate mechanisms of autoreactive T-cell development and persistence. Cleavage of the MHC II inhibitory receptor LAG-3 is a negative regulatory mechanism of immune cell activation; however, LAG-3 cleavage also

prompts the metabolic transition from oxidative phosphorylation to glycolysis necessary for the activation and proliferation of T cells. Dr. Piganelli hypothesized that lack of LAG-3 results in T-cell overamplification, as fewer T cells are deleted during development and peripheral maturation, resulting in more aggressive autoimmunity. Indeed, LAG-3 knock-out NOD mice experience accelerated T-cell-mediated T1D (49). The group showed that LAG-3-deficient $CD4^+$ T cells exhibit enhanced oxidative and glycolytic metabolism and increased mitochondrial biogenesis, supporting the hypothesis that overactive T cells lacking regulation contribute to T1D. Furthermore, inhibition of LAG-3 cleavage results in decreased T-cell proliferation and activation, as well as inhibition of metabolic switching in antigen-educated T cells. Ultimately, modification of LAG-3 is a potential therapy to prevent and treat effector T-cell-mediated autoimmune diseases such as T1D.

It is our view that virus infection shifts important checkpoints in cell regulation mechanisms in the development of T1D, by increasing local activation and stimulation. Rather than simply removing cell subsets involved in T1D pathogenesis, the work of Dr. Piganelli exhibits how immune cell factors may be targeted and can potentially be programmed to adopt a more tolerogenic state. Recent literature has shown that environment early on in life is important for incidence of T1D in NOD mice and exposure to a "diabetogenic environment" is sufficient to promote incidence (32). It was also determined that composition of certain bacterial pathobionts can induce immunophenotypic changes in mice weaned in this "diabetogenic environment" and harbor B cells in gut-related lymphoid organs which are intrinsically more easily activated by local stimulation (32). Environmental modification of homeostatic cell regulation pathways can necessarily predispose for increased microenvironment inflammation and cell activation that may be sufficient to induce autoreactivity. Therefore, determining pathways which may be safely targeted via drugs and therapeutics could point to effective disease treatments and prevention.

MONOCYTE EXPRESSION OF PTPN22 POTENTIATES T-CELL RECRUITMENT AND ACTIVATION

Type 1 diabetes is traditionally considered a T-cell-mediated disease and as such the bulk of T1D research is focused on the role of T cells (50–52). However, T cells require priming by myeloid professional APCs like dendritic cells (DCs) and macrophages. The upstream interaction between APCs and T cells is the focus of Dr. Mark A. Wallet's research at the University of Florida, particularly the potential molecular mechanisms of DC regulation by the cytosolic phosphatase PTPN22. This protein, a known negative regulator of T-cell signaling, is expressed in DCs as well as monocytes and macrophages. Additionally, studies have shown that a coding variant polymorphism in human PTPN22 is associated with increased risk of T1D (53, 54). In mice, a similar polymorphism in PTPN22 leads to macrophage hyperactivation (55). To study the mechanisms of PTPN22 in regulation of human myeloid APCs, the Wallet team innovatively generated PTPN22-deficient monocytes from induced pluripotent stem cells where PTPN22 expression was ablated using CRISPR/Cas9-mediated gene targeting. When the PTPN22-deficient monocyte-derived DCs were treated with the TLR-stimulator zymosan, there was reduced expression of chemokines involved in recruitment of T cells, including CXCL10. This result indicated that PTPN22 may be involved in the recruitment of autoreactive T cells to the pancreas and enhance progression of T1D. However, lack of PTPN22 had no effect on amount of $CD8^+$ T-cell proliferation or on downstream signaling following zymosan treatment. Meanwhile, the research remains to identify which receptor or receptors are driving CXCL10 and may be regulated by PTPN22. Overall, Dr. Wallet's research shows that PTPN22 regulates the secretion of T-cell-recruiting chemokines by monocytes/DCs, shedding light on potential molecular mechanisms of T-cell priming and subsequent T1D pathogenesis.

We contend that the role of PTPN22 is incredibly complex and more work is necessary to determine how exactly this enzyme impacts T1D pathogenesis and whether genetic variation in *PTPN22* affects viral induction. APC interaction with T cells with regard to antigen presentation, stimulation, and chemical signaling can be detrimental for producing an autoreactive adaptive response to beta cells in T1D. PTPN22 has multiple roles in both the innate and adaptive immunity, affecting myeloid cell activation, T-cell proliferation and effector capacity, and secretion of type 1 interferons (56). The role of PTPN22 in various mouse models of T1D has been controversial (57). For instance, both diminishing and overexpressing PTPN22 were shown to reduce incidence of T1D in NOD mice (58, 59). Using a virus-mediated T1D mouse model (RIP-LCMV), PTPN22-deficient mice had increased incidence of T1D and resulted in an enhanced effector T-cell response to virus (60). Thus, it seems that PTPN22 deficiency positively affects virus-induced T1D but can protect in spontaneous disease. PTPN22 contributes to exhaustion of both $CD4^+$ and $CD8^+$ T lymphocytes and aids in establishment of chronic virus infections (61). We believe that chronic and persistence of certain types of viruses such as CVB are providing low-grade inflammation via interferon production in the pancreatic microenvironment that is triggering self-reactivity resulting in T1D. Dr. Wallet's research further exemplifies how molecular mechanics for cell function can contribute to pathogenesis of T1D and immune disorder.

SEPSIS COMPLICATIONS IN T2D IS A RESULT OF IMPAIRED BACTERIAL CLEARANCE

Dr. Matthew Delano, from the Department of Surgery at the University of Michigan, wrapped up the event by discussing T2D and susceptibility to infection. Dr. Delano approaches immunological diabetes research from the perspective of an acute trauma surgeon who has witnessed an abhorrent number of cases of sepsis among T2D patients. T2D, a disease caused by unresponsiveness to endogenous insulin, functions as an immunodeficiency that predisposes patients for infection (62). Dr. Delano hypothesized that defects in neutrophil function,

previously linked to T2D-associated infections, directly contribute to bacterial persistence and death from sepsis (63). To examine the role of neutrophils in T2D, the Delano lab developed a novel diet-induced obesity (DIO) murine model with septic infection caused by cecal ligation and puncture. Neutrophils and monocytes in septic DIO mice failed to adequately phagocytose invading bacteria, resulting in increased bacterial persistence compared to their lean counterparts. The decreased phagocytic activity was caused in part by reduced ROS production. The group profiled the gene expression of neutrophils and monocytes and identified seven genes that were significantly and differentially expressed between septic DIO mice and lean controls. Most of these genes encoded receptors in pathways for phagocytosis, including particle recognition and engulfment. This work provided evidence that defects in neutrophil and monocyte function in T2D patients could account for persistence and/or susceptibility to sepsis following bacterial challenge. Dr. Delano is looking to target these identified genes to enhance phagocytosis and ROS production by neutrophils and monocytes as a therapeutic approach.

It is our perspective that understanding the contribution of viral infection in T2D is paramount to developing immunotherapies. Sepsis causes significant changes in nearly every type of innate and adaptive immune cells which persist well after septic acute phase and efforts are being made to develop immunotherapies to combat this dysfunction (64). By considering T2D as an immunodeficiency that predisposes patients to secondary infections due to defects in neutrophil function, Dr. Delano's work reestablishes the importance of investigating innate immune processes in disease development and progression, simultaneously proposing a parallel to T1D pathogenesis and defects in innate sensing. Overall, this research emphasizes the necessity of examining other pathogenic exposures and the extent of their effect on disease priming, especially within the context of cross-reactivities and heterogeneous disease presentations.

CONCLUSION

The multidisciplinary FIFE mini-symposium brought together young researchers from across North America investigating various interconnected contributors to TID onset and progression and put forth multiple concepts for further examination. Common themes of interest included autoreactive T-cell expansion and persistence, T-cell activity alteration and loss of homeostatic mechanisms, and environmental exposures, including infections and the microbiota. Immunotherapeutic targets, and methods for delivering treatments to the pancreatic microenvironment or specifying them to specific autoreactive subsets, were proposed. Moving forward, the first 2016 FIFE symposium provided a foundation from which investigators can exchange ideas and form collaborations to advance diabetes research. It is our goal that future FIFE collaborative efforts be planned to provide a positive environment and forum for communication and idea generation with a goal to aid in the prevention and cure of T1D. Next year, we will see you in Gainesville, Florida!

AUTHOR CONTRIBUTIONS

IM helped write sections and edited and organized the manuscript. ZM added perspective to all the sections. ZM, VJ-B, and JA wrote sections and edited. MH edited and oversaw the manuscript.

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The Role of NOD Mice in Type 1 Diabetes Research: Lessons from the Past and Recommendations for the Future

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For more than 35 years, the NOD mouse has been the primary animal model for studying autoimmune diabetes. During this time, striking similarities to the human disease have been uncovered. In both species, unusual polymorphisms in a major histocompatibility complex (MHC) class II molecule confer the most disease risk, disease is caused by perturbations by the same genes or different genes in the same biological pathways and that diabetes onset is preceded by the presence of circulating autoreactive T cells and autoantibodies that recognize many of the same islet antigens. However, the relevance of the NOD model is frequently challenged due to past failures translating therapies from NOD mice to humans and because the appearance of insulitis in mice and some patients is different. Nevertheless, the NOD mouse remains a pillar of autoimmune diabetes research for its usefulness as a preclinical model and because it provides access to invasive procedures as well as tissues that are rarely procured from patients or controls. The current article is focused on approaches to improve the NOD mouse by addressing reasons why immune therapies have failed to translate from mice to humans. We also propose new strategies for mixing and editing the NOD genome to improve the model in ways that will better advance our understanding of human diabetes. As proof of concept, we report that diabetes is completely suppressed in a knock-in NOD strain with a serine to aspartic acid substitution at position 57 in the MHC class II Aβ. This supports that similar non-aspartic acid substitutions at residue 57 of variants of the human class II HLA-DQβ homolog confer diabetes risk.

Keywords: NOD mouse, type 1 diabetes, preclinical, congenic, genetics, gene editing

INTRODUCTION

Since becoming available to the scientific community, the NOD mouse has been used extensively and has provided significant contributions to our mechanistic understanding of autoimmunity and type 1 diabetes (T1D). Indeed, the NOD mouse has been used to understand many facets of human T1D and has been the preferred model for invasive, preclinical/translational studies. While the NOD mouse has a number of critics, this model should be viewed as an important component of a comprehensive approach to understanding T1D. The NOD remains a standout model because

it develops spontaneous T1D with genetic and environmental components that are relevant to the human disease. Further, as recent studies have demonstrated and as we describe below, new protocols to specifically modify single base pairs can generate loci that contain risk alleles that are orthologous to the human on the NOD background. Therefore, the NOD mouse remains a powerful and valuable implement in the investigator's toolbox.

A major strength of the NOD model is the existence of spontaneous autoimmunity and T1D. Similar to the human condition, NOD mice develop autoantibodies (1) and exhibit increases in circulating autoreactive T cells (2, 3) prior to the onset of T1D. The β cell antigens that are targeted are also similar between these species (4). However, in the NOD mouse, the initiating antigen appears to be insulin (1), whereas in human T1D it is thought to result from several initiating antigens (5, 6). These autoimmune phenotypes are followed by the onset of hyperglycemia (7). A progressive loss of β cell function is present in both human and NOD mice suggesting similarities in β cell loss or dysfunction. While hyperglycemia in NOD females and males begins close to 12 and 15 weeks of age, respectively (8), immune infiltration into the pancreatic islets, insulitis, begins much earlier. Pathogenic T cells have been isolated from the islets of 5-week-old NOD mice (9). By 12 weeks of age, insulitis is present throughout the pancreas of NOD mice. A dissimilarity of the diabetes when comparing human and NOD mice is the appearance of insulitis. Studies from the nPOD bio-repository have been critical in defining insulitis in humans where this pathogenic lesion is less severe and less frequent than what can be observed in NOD mice (10). This may result from the fact that the autoimmunity in parental NOD mice is very aggressive and disease onset occurs over an abbreviated timeline (weeks) compared to the decidedly more attenuated onset in humans (i.e., years after the appearance of autoantibodies). Insulitis and T1D incidence in NOD mice can be reduced through genetic modification. While hundreds of variant NOD mice have been made that represent less intense forms of T1D, the idea of improving the NOD as a model for human T1D by decreasing the potency of the autoimmune response remains largely unexplored. The potential of this strategy is discussed below.

Genetics play a significant role in autoimmunity and important similarities exist when equating T1D-risk loci in human and NOD mice. The disease is polygenic in both species with over 50 loci linked to risk in human and NOD diabetes (11). However, a single locus is responsible for the majority of the risk: major histocompatibility complex (MHC) class II. Early papers were critical in establishing that NOD mice encode a T1D-risk MHC haplotype that has important resemblances to the HLA risk alleles in human. Since these publications, genetic and biochemical studies have linked risk to amino acid residue 57. The high-risk DQ2 and DQ8 alleles of human as well as the A^{g7} molecule of the NOD have small polar amino acids substituted for an aspartic acid at position 57. The importance of this amino acid substitution is discussed in detail by Bettini and Bettini in this issue of *Frontiers in Endocrinology* (Co-published in the same edition of FiE). The genetic variations that impart risk in HLA/MHC arise from single-nucleotide polymorphisms that change the amino acid sequence. Similarly, other genes such as *Ctla4* and *mt-Nd2* are linked to risk in both humans and NOD mice. A single leucine to methionine substitution in *mt-Nd2* as well as the human homolog, *mt-ND2*, provides β cells with enhanced resistance to autoimmune destruction (12, 13). While HLA/MHC and *mt-ND2*/*mt-Nd2* represent genes with protein and biochemical differences, these non-synonymous changes in T1D are more the exception than the rule. Only seven of the >50 single-nucleotide polymorphisms associated with T1D arise in coding regions (14). The polymorphism in *Ctla4* of NOD mice results in altered splicing. While the polymorphism in *CTLA4* is not in an identical location, the risk variant is similarly associated with altered splicing of CTLA4 (15–17). Therefore, genes such as *CTLA4* can be modeled in the NOD mouse to aid in understanding the role of non-coding genetic variation in pathogenesis of T1D. Recent advances in genetic editing have further promoted the use of NOD to understand how specific SNPs can affect protein function. Editing of the NOD genome has been used to swap T1D risk or resistance alleles allowing for the role of specific SNPs, such in MHC Class II (described below) or *Ptpn22* (18), in the regulation of autoimmunity to be identified.

Another concept that holds true across species is that T1D onset results from the sum of the genetic parts. In human subjects, T1D risk increases as the haplotype of an individual contains more credible T1D susceptibility SNPs (19, 20). Similarly, by subtracting risk loci from the NOD genome through backcrossing or genetic modification, T1D risk can be altered (11). As discussed in detail below, the NOD represents a powerful tool to study epistasis.

In the current review, we highlight past contributions NOD mice have made to T1D research and outline strategies to better utilize this model in future. Included is an overview of NOD mouse's track record as a preclinical model for developing T1D therapies and a discussion about the impact NOD congenic mice have made to understanding the genetic basis of T1D. Also discussed is a strategy to develop panels of NOD congenic mice from existing congenic stocks to better mimic the spectrum of human autoimmune diabetes subtypes. Finally, we summarize existing and emerging technologies for editing the NOD genome that should greatly enhance the NOD mouse as a research tool, especially for identifying genes that contribute to T1D development.

PRECLINICAL PERFORMANCE OF THE NOD MOUSE

While the NOD mouse has proved useful in many preclinical research areas, significant tension has arisen over the performance of this mouse strain in bench-to-bedside efforts due to a failure to translate therapies developed in the NOD model to humans. The NOD model has been used for at least 30 years to identify agents or protocols that delay, prevent, or reverse disease. In general, investigators apply three approaches: early prevention (treatment is initiated at 3–4 weeks of age), late prevention (begin treatment at 10–12 weeks of age), or intervention after onset of T1D (reversal). Most preclinical successes in NOD have come in

early prevention, where a wide array of agents or protocols can block disease. It should be noted that in most cases the impact of the drug/agent under investigation on autoimmunity (i.e., insulin autoantibodies or the presence of β cell reactive T cells) was not assessed. Further, many of these have seen little to no confirmation by independent laboratories. A recent NIH funded effort to confirm the effects of specific agents was unsuccessful at repeating the majority of the successes that were previously published (21). Late prevention represents a modality that is similar to trials established in humans where autoantibody positive individuals are identified and enrolled, such as the Diabetes Prevention Trial 1 or the recent oral insulin trial (22–24). To date, there has been a failure to translate late prevention successes in the NOD to prevention of human T1D.

At time of writing, very few therapies have resulted in T1D reversal in new-onset NOD mice and fewer still in NOD mice with established disease. Of the agents that have shown benefit, anti-CD3, and the combination of antithymocyte globulin (ATG) and granulocyte-colony stimulating factor (G-CSF) have been used in clinical trials. Preclinical studies using these modalities demonstrated an ability to reverse T1D in 39% of NOD females after onset (25). Multicenter preclinical efforts using anti-CD3 produced similar results, with less than 50% of the treated NOD mice exhibiting long-term T1D reversal (26). The rates of T1D reversal were significantly different when comparing sites, where anti-CD3 efficacy ranged from 10 to 80% among the four locations. Similarly, trials with anti-CD3 resulted in a minority of patients responding to therapy (i.e., preservation of c-peptide responses), yet none of the efforts with anti-CD3 resulted in insulin-free status for the patients (27–29). Likewise, use of ATG + G-CSF in a small multicenter clinical trial (25 total patients: 17 receiving ATG + G-CSF and 8 placebo) established that this combination did not induce T1D-remission but was effective in preventing erosion of β cell function 12 months after treatment (30). The 2-year data for ATG + G-CSF were less promising. At 24 months only 50% of the individuals who received therapy had preservation of β cell function (31). This is similar to the ATG + G-CSF reversal rates in NOD mice (32).

These data provide caution for moving agents forward for clinical trials that have been developed using NOD mice. A recent paper in Science Translational Medicine (33) has called for standards in clinical diagnosis as well as timing of therapy initiation in NOD mice. In preclinical studies, it is well established that NOD mice should be treated immediately after onset of T1D for maximal therapeutic response. Most groups have now established protocols for checking mice every other day for T1D onset allowing for initiation of therapy as soon as 1 day after diagnosis (21, 27, 28). In humans, trials enroll participants much more slowly and this delay in therapeutic administration likely postpones protection of the β cell mass allowing for further β cell loss. Additionally, it is clear that prior to agents or protocols moving to clinical trial there must be independent replication. The lack of a systematic understanding of T1D in the NOD and humans also impacts success. Comprehensive studies in comparative immunology and endocrinology are needed to mechanistically detail T1D reversal in NOD mice.

ROLE OF NOD CONGENIC MICE IN T1D GENETICS

Since researchers first started mapping *insulin-dependent diabetes* (*Idd)* loci by outcrossing NOD mice to mouse strains that do not develop T1D [i.e., C57BL/6 (B6), C57BL/10 (B10), NOR, and C3H], considerable effort has been spent creating recombinant congenic mouse strains to delineate genetic intervals containing diabetes loci and identifying the genes within each interval that are responsible for T1D susceptibility or protection. Several regions have been refined through the generation of subcongenic stocks that encode different subregions of the original confidence interval. These strains have revealed how several of the original *Idd* regions are composed of multiple susceptibility and/or resistance alleles. Notable examples include *Idd3* that was dissected into *Idd3, Idd10, Idd17,* and *Idd18* (34–37), *Idd5* that was dissected into *Idd5.1*, *Idd5.2*, *Idd5.3,* and *Idd5.4* (38–41), and *Idd9* that was dissected into *Idd9.1*, *Idd9.2*, *Idd9.3*, *Idd9.4*, and *Idd9.5* (42–46). While many of the dominant *Idd* regions are now well delineated, relatively few of their underlying genes have been firmly established. This is because validation has been technically challenging, in large part because even small *Idd* intervals often contain large numbers of candidate genes. Slow progress in improving candidate gene identification has led to reduced support for large-scale mouse genetic studies, forcing many in the field to decommission their congenic stocks. A new generation of genetic tools discussed in Section "Strategies for Improving Candidate Gene Identification" may reverse the fortunes of some of these strains. Nevertheless, even without discovering the causative genes, congenic mice have provided valuable insight about the genetic causes of human T1D that no other resource could have delivered. Some of their most important contributions are described below.

Epistasis and Gene–Gene Interactions

Intercrossing congenic stocks has revealed that an individual's disease risk is ultimately determined by the interactive effect of multiple *Idd* resistance and susceptibility loci. The challenge of disentangling these complex networks was taken up by a few courageous groups who, over decades, have detailed how different combinations of disease resistance and susceptibility loci modulate diabetes and various disease sub-phenotypes on the autoimmune-permissive NOD background. The advantage of this approach is that eliminating genetic variability between *Idd* loci allows for the detection of gene-masking and gene–gene interaction effects that are normally concealed in conventional genetic association studies with human subjects as well as mouse studies involving F2 and backcross one generation for segregation analysis (47).

There are several examples of how interactions between individual *Idd* susceptibility and resistance alleles gives rise to graded levels of diabetes on the NOD background (48–51). Among the best characterized is the interplay between the *Idd3* and *Idd5* congenic intervals from C57 strains when introgressed into the NOD genome. Combining *Idd3* and *Idd5* confers almost complete protection from T1D and insulitis on the NOD background (38). Yet, combining *Idd3* with individual *Idd5* subloci results in a spectrum

of diabetes protective effects [reviewed elsewhere (11, 47, 52)]. At one end of the spectrum, *Idd3/Idd5.1* NOD mice were found not more protected against T1D than *Idd3* mice (41). Hunter et al. posited that the lack of protection in NOD-*Idd3/Idd5.1* mice may result from T1D resistance alleles at *Idd3* increasing the expression of CTLA-4 on the surface of $CD4^+$ and $CD8^+$ T cells that may render higher levels of inhibitory ligand-independent CTLA-4 induced by protective alleles at *Idd5.1/Ctla-4* somewhat redundant (41, 53). On the other end of the spectrum, *Idd3/Idd5.1/Idd5.3* and *Idd3/Idd5.3* recombinant congenic strains were found to exhibit T1D resistance equal to NOD-*Idd3/Idd5* mice. The lack of T1D initiation in the presence of severe insulitis observed in the *Idd3/Idd5.1/Idd5.3* and *Idd3/Idd5.3* strains indicates that the interaction between *Idd5.2/Nramp1* and *Idd3* is not important for T1D protection, but does contribute to the marked reduction in insulitis (41, 54). Continued studies of these strains will provide models to address the knowledge gap in additive and synergistic genetic effects.

Gene–gene interactions also exist among the various *Idd5* subregions, including between *Idd5.1* and *Idd5.4*. *Idd5.4* encodes a B10-derived susceptibility allele without a known responsible gene product. *Idd5.4* significantly accelerates T1D in the presence of *Idd5.2* and *Idd5.3*, but has no impact on disease if *Idd5.1* is also present (41). This suggests that *Idd5.4* can neutralize the protective effects of *Idd5.2* and *Idd5.3* and that *Idd5.4* is in turn masked by the protective effects of *Idd5.1*. A plausible explanation for this phenomenon is that immune events regulated by the B10-derived susceptibility allele at *Idd5.4* are counteracted by *Idd5.1/CTLA-4* signaling in one or more cell types. Similar masking effects have been detected among other congenic regions including between *Idd19* and *Idd6* on Chr.6 (49), *Idd19* and *Idd20* on Chr.6 (51), *Idd21.2* and *Idd21.1* on Chr.18 (50), and *Idd14* and *Idd31* on Chr.13 (55).

Evidence for epistatic interactions in humans include a study by Winkler et al. that genotyped 12 non-HLA susceptibility genes (*ERBB3, PTPN2, IFIH1, PTPN22, CLEC16A, CD25, CTLA4, SH2B3, IL2, IL18RAP, IL10*, and *COBL*) in high-risk HLA positive children of parents with T1D that were prospectively followed from birth to the development of autoantibodies and disease (19). An analysis was performed to determine the combinations of genes that most accurately predicted T1D development. The results showed that T1D progression in high-risk HLA carriers was best predicted by a collection of 8 genes (*ERBB3, IFIH1, PTPN22, CLEC16A, CTLA4, SH2B3, IL18RAP*, and *COBL*) rather than all 12 SNPs. These results suggest the presence of gene–gene interactions that mask the effect of individual diabetes susceptibility alleles. Another study searched for interactions between 38 T1D-associated non-HLA loci and different HLA class II genotypes in a large collection of T1D samples (20). It was found that SNPs within two T1D-associated genes, *PTPN22* and *CTLA4*, alter the predicted diabetes risk of various HLA haplotypes, partly confirming earlier reports that the effect of a susceptibility allele at *PTPN22* is greater in individuals expressing low-risk than high-risk HLA class II genotypes (56–58). These and other GWAS studies show how some T1D genes but not others are strongly influenced by gene-gene interactions and masking effects.

Cellular Expression of Diabetes-Associated Genes

Congenic mice offer a powerful tool to determine how different T1D genes modulate diabetogenic immune responses within specific cell types, which cannot easily be accomplished by experimentation with human samples. Previous studies have used a variety of adoptive transfer or bone marrow chimerism methods to observe that T1D genetics regulate immune dysfunction. A good illustration is the use of the B6, B10, or NOR derived *Idd9/Idd11* resistance locus to inhibit diabetes. One set of studies found that complex genetic interactions within *Idd9/11* regulate how B cells contribute to disease by engrafting syngeneic bone marrow and B cells purified from different Chr. 4 subcongenic donors into lethally irradiated B cell-deficient and diabetes-resistant NOD.IgH^{null} mice (59, 60). Diabetes development was then monitored to determine if B cells expressing separate subcongenic intervals from the NOR strain protected recipient mice from T1D compared to standard NOD B cells. The results established that at least four adjacent intervals interactively contribute to how diabetogenic B cells become tolerized or cause T1D, including processes that increase the efficiency of B cell anergy or B cell hyperresponsiveness to B cell receptor stimulation.

We used a similar strategy to show that genes within the *Idd9/11* locus control pathogenic CD4 T cells responses in T1D (61). Lethally irradiated CD4-deficient NOD.$CD4^{null}$ mice were reconstituted with syngeneic bone marrow and $CD4^+$ T cells isolated from NOD.NOR-(*D4Mit31-D4Mit310*)/DvsJ: (NOD-$Idd9/11^{NOR}$) NOD mice congenic for NOR genome on Chr. 4. In this system, transfer of $CD4^+$ T cells isolated from NOD-$Idd9/11^{NOR}$ mice caused less diabetes than $CD4^+$ T cells isolated from NOD. It was also shown that $CD4^+$ T cells from BDC2.5 TCR transgenic mice have a reduced capacity to transfer T1D to immunodeficient NOD.CB17-$Prkdc^{scid}$ (NOD-*Scid*) mice when they express protective alleles at *Idd9* (62). Hamilton-Williams et al. found that $CD4^+$ T cells that express protective B10 alleles at *Idd9.2* and *Idd9.3* suppress the expansion of diabetogenic $CD8^+$ T cells (63). Their approach involved reconstituting NOD-*Scid* mice with purified $CD4^+$ T cells from NOD or NOD.*Idd9* congenic mice co-transferred with CD4-depleted spleen and lymph node cells from NOD donors. After reconstitution, mice were infected with a vaccinia virus encoding the H-$2K^d$-restricted $IGRP_{206–214}$ epitope to measure the expansion of CD8 T cells specific for the islet antigen IGRP. High and low frequencies of IGRP-specific CD8 T cells were detected in mice, respectively, reconstituted with NOD and NOD.*Idd9* CD4 T cells indicating that *Idd9* protective alleles restore tolerance to islet IGRP through CD4 T cells.

Other cell types besides B cells and conventional $CD4^+$ T cells have been found to regulate diabetes through *Idd9*. Regulatory T cells (Tregs) expressing B10-derived *Idd9.1* genes have significantly higher suppressive activity than Tregs from standard NOD mice (64). The *Idd9.1* sub-locus has also been reported to increase the capacity for DCs to engage and potentiate natural killer T cells, which are required for *Idd9*-mediated diabetes protection (65). Reciprocal transfers of NOD and NOD.*Idd9* congenic mouse spleen and lymph node cells into NOD-*Scid*

and NOD.*Idd9*-*Scid* recipients identified that non-lymphoid cells possess some component of *Idd9* protection (63). Another finding was that transplanted islets from NOD-*Idd9* mice are more resistant to destruction by $CD8^+$ T cells, suggesting that an element of *Idd9*-mediated T1D protection maps to insulin-producing β cells (66).

Studies dissecting the effects of *Idd9* and other T1D loci have demonstrated that diabetogenic immune responses develop from a complex interplay of genes in multiple cell types. Further, evidence suggests that different cell types can be affected by a single diabetes locus/gene with sometimes opposing effects on disease. Determining how individual *Idd* loci contribute to T1D by affecting immunoregulatory pathways in specific cells offers a useful strategy for identifying the genes underlying these regions.

Genetic Control of Insulitis

Congenic mice have revealed that non-MHC *Idd* loci can be separated into two classes; one that supports T1D by modulating the virulence of insulitis and/or the intrinsic resistance of β cells to cytotoxic stress, and a second class that supports T1D by regulating diabetogenic immune responses before insulitis occurs (67). In the first class, replacement of individual NOD susceptibility loci with resistance alleles from non-diabetes prone strains reduces the incidence of T1D but has no quantifiable effect on insulitis at the gross histological level compared to NOD mice of the same age. *Idd* loci that fall into this category include *Idd9* where introgression of B10-derived resistance alleles did not alter the cellular composition of insulitis. Instead this locus changed the pathogenic properties of leukocytes that accumulated in islets and shifted cytokine production from IFNγ and TNFα to an IL-4 response (43). The overlapped B6-derived *Idd11* interval also reduces the pathogenic effects of β cell-specific lymphocytes in islet infiltrates without affecting the overall amount of insulitis (44). Another example is *Idd6* where C3H-derived resistance alleles confer protection against T1D but not islet infiltration. However, subtle differences exist in the invading leukocyte populations including that $CD4^+$ T cells and B cells are slightly reduced, which is counterbalanced by an increase in non-lymphoid cells such as macrophages and dendritic cells (68).

Disease protection is highly variable among the second class of non-MHC *Idd* loci where resistance alleles protect against both T1D and insulitis. Some regions including *Idd10/18*, *Idd16,* and *Idd21* cause a mild reduction in pancreatic infiltration but only during the early phases of insulitis (34, 50, 69, 70). Most of these loci confer relatively modest protection against T1D. In contrast, loci such as *Idd3* and *Idd5* that each provide substantial diabetes protection also cause a considerable delay in insulitis, although almost all NOD.*Idd3* and NOD.*Idd5* congenic mice eventually develop significant islet infiltration (34, 38). Other *Idd* loci, including *Idd4* and *Idd13*, appear to change the distribution rather than the amount of insulitis (71, 72). NOD mice expressing either of these loci develop non-destructive peri-ductal infiltrates where invading cells remain mostly confined to the peri-islet zone until well after the age most NOD mice develop diabetes. As discussed above, none of the non-MHC *Idd* loci that block insulitis and T1D are sufficient on their own to substantially reduce islet inflammation. However, almost complete protection can be achieved when individual regions are combined, indicating that genetic interactions exist between specific loci that confer greater protection against islet inflammation than the collective effects of each separate region.

Together, these findings suggest that insulitis among patients is also under complex genetic control and that, in some people, combinations of T1D genes could cause high levels of non-destructive islet inflammation long before the onset of overt disease. In contrast, the degree of insulitis may correlate closely with progression to overt diabetes in patients that carry T1D genes that give rise to more virulent forms of insulitis.

MODELING THE GENETIC DIVERSITY OF HUMAN T1D

A major criticism of the NOD mouse has been that this model represents the equivalent of a single human case of T1D. Consequently, immune modulation protocols developed in the NOD mouse could be limited to a few subtypes of the human disease, which may partially explain why some interventions that have shown promise in NOD mice fail to preserve β cell function in patients (73). Better predictions from mouse models might be possible if future treatment protocols were screened using multicenter efforts with heterogeneous populations of NOD-derived mice to mimic the genetic variation among patients. Such a strategy could employ a panel of NOD-related recombinant congenic strains carrying different combinations *Idd* loci where each strain would express a unique set of genetic variants that give rise to a specific subtype of T1D (41). This is analogous to the different subtypes of T1D that arise in patients from various segregating combinations of susceptibility and resistance alleles. The potential of this strategy is that therapies capable of inhibiting diabetes across a panel of congenic strains are more likely to be successful in genetically heterogeneous humans. There are also advantages to finding treatments that only work in congenic mice with specific combinations of *Idd* loci, including that this could provide valuable information about the cellular and molecular mechanisms through which an immune modulation treatment affects disease. It may also help to identify specific subsets of patients that have less or more potential for responding to a particular immune therapy.

Choosing which congenic mice to include in a future drug testing panel presents a challenge because of the large number of *Idd* loci it is possible to combine. It is logical that strain selection should consider the nature of the immune modulation protocol being tested. For therapies like probiotic treatment and immune suppression protocols, where the mode of action is poorly understood or where multiple cell types and molecular pathways are involved, it may be best to test mice with a diverse array of congenic intervals designed to emulate the genetic variation in human T1D. Some of the NOD-related congenic stocks described in the Section "Epistasis and Gene–Gene Interaction" may be suitable candidates, especially those that develop NOD-like levels of T1D due to introgression of susceptibility loci from non-NOD mouse strains. A more targeted panel could be employed for therapies known to act through particular cellular or molecular pathways. For instance, immune modulation protocols designed to enhance

Tregs, such as low-dose IL-2 and combined ATG + G-CSF therapy, could be tested on congenic mice expressing different allelic variants of *Idd3*, *Idd6*, *Idd9.1*, and *Idd9.3* that each separately affect the suppressive properties of Tregs (64, 68, 74, 75). Another example is antigen-specific immunotherapy where autoantigens could be screened in NOD congenic mice expressing different variants of *Il2/Il21* (*Idd3*) (74, 76), *B2m* (*Idd13*) (72), and *Ptpn22* (*Idd18.2*) (77) that, respectively, modulate T cell activation/effector function, peptide presentation, and TCR signaling. All of these factors contribute to the fate of self-reactive T cells that encounter autoantigen and may affect the outcome of autoantigen immunotherapy.

An obvious drawback to testing diabetes therapies using congenic mouse panels is the additional time and resources involved. Even so, the investment is worthwhile if therapies that are ineffectual in humans could be recognized before progressing to clinical trials. An example of how testing the appropriate NOD congenic strain might have produced a different result to standard NOD mice and predicted the failure of a T1D treatment is low-dose IL-2 therapy, which increases the frequency of Tregs but has not been able to produce positive effects on diabetes in patients (78). A chief reason that this treatment advanced to clinical trials is that low levels of IL-2 potently suppresses T1D development and reverses recent onset T1D in NOD mice, presumably by enhancing Treg function and/or development (79). However, it is possible that NOD mice are particularly sensitive to this type of immune modulation because this strain carries a variant of *Il2* that reduces IL-2 gene expression and Treg function (74). An interesting question is whether the outcome of IL-2 treatment would be different in NOD.*Idd3* mice that express the B6 variant of *Il2* and results in higher levels of *Il2* gene expression (74). The answer might address whether low-dose IL-2 therapy has potential for improving immune regulation and result in enhanced β cell function in patients without an IL2/IL2R signaling deficiency. This is important because it is still unclear whether defects in the IL2/IL2R pathway play a significant role in most cases of human diabetes; although a gene variant of *IL2RA* (CD25) has been associated with T1D risk in people, it is protective but rare (80). Furthermore, the causative gene has yet to be identified for the chromosome 4q27 region containing *IL2* and *IL21* that is linked with T1D susceptibility (81).

Another limitation of testing T1D therapies with NOD congenic mice is that many *Idd* loci strongly suppress diabetes, which will require that some experiments be performed with large numbers of animals to achieve sufficient power. Indeed, only 10–20% of female NOD.*Idd3* mice develop T1D by 30 weeks of age (74, 76, 82). As mentioned above, the unique insights from congenic mice will often justify using strains with very low levels of disease. However, there is also potential to alter the genetic composition of congenic strains in ways that will increase the rate of diabetes. For instance, it may be feasible to use NOD mice heterozygous instead of homozygous for the *Idd3* locus, which develop 40% diabetes (76). These mice still produce more IL-2 than standard NOD mice and would presumably be less sensitive to IL-2 therapy. Another strategy could be to breed T1D-resistant congenic strains with NOD mice carrying congenic intervals that accelerate diabetes. For example, NOD.*Idd3* could be crossed to NOD mice carrying B6 alleles at *Idd18.2*/*Ptpn22* that are more diabetogenic than the corresponding NOD alleles (77).

STRATEGIES FOR IMPROVING CANDIDATE GENE IDENTIFICATION

Although genetic studies using inbred mice are costly because of the large number of mice required, they remain a powerful method of detecting rare T1D susceptibility alleles that are impractical to identify through GWAS analyses, which require tens or hundreds of thousands of human subjects (83, 84). Thus, for the reasons outlined above, the question is not whether pursuing the identity of T1D susceptibility and resistance alleles is worthwhile, but rather how to make this process more efficient by employing a comprehensive approach that utilized both human and mouse systems. Considerable encouragement comes from a new generation of genetic tools that may circumvent many of the most intractable obstacles that traditionally limited the identification of *Idd* candidate genes. Some of these are described in the following sections in the order of their development.

RNA Interference (RNAi)

RNA interference has proven useful for manipulating gene expression in NOD mice without introducing genetic contamination from other strains. This approach is based on a well-established transgenesis methodology that entails the direct introduction of short hairpin RNA (shRNA) containing constructs into NOD zygotes by viral transduction (85, 86). The shRNA-containing constructs are designed to silence genes that impact T1D. shRNA is a sequence of RNA that contains a tight hairpin turn. This structure is cleaved by intracellular machinery into small interfering RNA that knocks down any mRNA bearing a complementary sequence (87). Several companies are developing viral libraries that produce shRNA that integrate into the host genome and ensure stable gene silencing after integration. The silencing cassette can be incorporated into many different types of vectors, including lentiviral, adenoviral, or retroviral vectors. Using the NOD model, RNAi has already provided valuable insight into how expression of the T1D candidate genes *IL17* (88), *PTPN22* (89), *CTLA4* (90), *CLEC16A* (91), *RGS1* (92), and *Slc11a1* (*Nramp1*) (93) contribute to diabetes development. It is conceivable that T1D susceptibility genes can regulate disease progression in an age-dependent manner. Establishment of inducible RNAi has also enabled temporal control of target gene knockdown to determine their functions at different stages of disease progression (94).

Zinc Finger Nuclease (ZFN)

Zinc finger nucleases are fusion proteins containing a sequence-specific DNA-binding zinc finger domain and a nuclease domain (95, 96). Engineered ZFNs specifically recognize and bind a defined target gene sequence within the nucleus of a cell and introduce a double-strand break (DSB) (97, 98). The cellular DNA repair machinery fixes these breaks, most frequently *via* the non-homologous end joining (NHEJ) mechanism resulting in small deletions or insertions of the gene sequence (few to hundreds of base pairs) and disruption (knockout) of the target

gene (97, 98). Injected as synthetic mRNAs, ZFNs typically work at the one-cell fertilized embryo stage, resulting in single-step, whole animal gene disruption, and infrequent mosaics (99). More precise genetic engineering can be achieved as well because a DSB also stimulates DNA repair *via* homology-directed repair (HDR) mechanism if a homologous DNA template is co-introduced into the cell (100). Because ZFN-mediated genetic manipulation can be done directly in NOD embryos, the resulting knock-in or knockout can be generated on a pure NOD genetic background. Thus, it eliminates carryover of closely linked passenger DNA that occurs when the induced mutation is introduced in non-NOD embryonic stem cells (129 or B6) and then the targeted allele is backcrossed onto NOD. This is particularly concerning when targeting genes within known *Idd* regions. To study the role of the *Idd9.3* candidate gene *Tnfrsf9* (encoding CD137/4-1BB), we used ZFN to disrupt this gene directly in NOD embryos (101). The NOD allele of CD137 is hypofunctional when compared to the B10 protein that is expressed within the *Idd9.3* congenic strain (102). Thus, it was thought that T1D development would be accelerated in the absence of CD137. Surprisingly, CD137-deficient NOD mice were less susceptible to T1D, indicating that this co-stimulatory molecule has a diabetogenic role. This conclusion could not have been made with certainty if CD137-deficient NOD mice were created by backcrossing the previously reported knockout alleles generated using 129 embryonic stem cells. We further established an important role of CD137 in promoting the accumulation of β cell autoreactive $CD8^+$ T cells (103). In addition, CD137 had a diabetes protective function when expressed in $CD4^+$ T cells, likely due to the immunosuppressive activity of soluble CD137 produced by Tregs (103).

As discussed earlier, the $H2^{g7}$ haplotype is essential for T1D development in NOD mice. A key component of the diabetogenic $H2^{g7}$ haplotype is the unique Ab^{g7} allele. The Ab^{g7} allele includes five nucleotide polymorphisms resulting in the conversion of two usually conserved proline and aspartic acid residues at positions 56 and 57 to histidine and serine (104). Significantly, the non-aspartic acid substitutions at residue 57 also characterize diabetogenic variants of the human class II HLA-DQβ homolog, such as DQ8 (105). While transgenic analyses have shown both histidine and serine, respectively, at positions 56 and 57 amino acid residues of $A\beta^{g7}$ to be important for T1D progression in NOD mice (106–108), their diabetogenic function has not been tested under a more physiological condition. To further study the role of amino acid residue at position 57 in $A\beta^{g7}$, we created a knock-in NOD strain by replacing the serine with an aspartic acid ($A\beta^{g7}$-S57D). This was achieved by co-injecting Ab^{g7}-specific ZFN coding mRNA and a plasmid construct for HDR into one-cell fertilized NOD embryos, which were subsequently transferred into pseudopregnant mothers, and live-born pups were screened for founders. We successfully established a knock-in NOD stock (formal name: NOD/ShiLtJ-*H2-Ab1*^{em2Ygch}/Ygch) with the precise 3 base pair alteration resulting an aspartic acid at position 57 in the MHC class II Aβ chain. The knock-in allele was confirmed at both the genomic DNA and cDNA levels. We used two different antibody clones (AMS-32.1 and 10-3.6) to determine if MHC class II expression was altered in NOD.$Ab^{g7\text{-}S57D}$ mice. The expression level of MHC class II on B cells and dendritic cells was found to be comparable in wild-type NOD and NOD.$Ab^{g7\text{-}S57D}$ mice when 10-3.6 was used to stain their splenocytes (**Figure 1** and not shown). Interestingly, the level of MHC class II staining was found to be lower on B cells and dendritic cells from NOD.$Ab^{g7\text{-}S57D}$ than those from wild-type NOD mice when AMS-32.1 was used (**Figure 1** and not shown). These results indicate that the aspartic

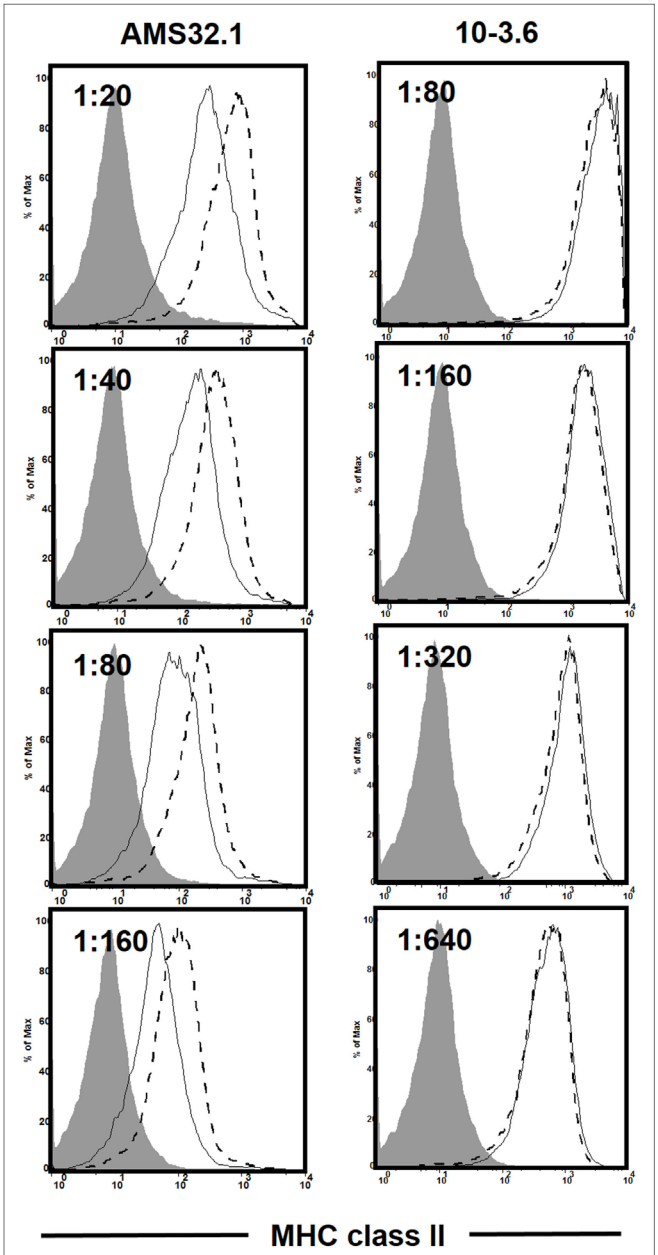

FIGURE 1 | Differential staining of the major histocompatibility complex (MHC) class II molecule in wild-type NOD and NOD.$Ab^{g7\text{-}S57D}$ mice. Total splenocytes were stained with anti-CD11c, anti-CD19, and the indicated I-Ab antibody clone at different titers. Shown is the I-Ab staining on B cells ($CD19^+$ $CD11c^-$) of NOD (dashed line) and NOD.$Ab^{g7\text{-}S57D}$ (solid line) mice. The shaded area is the negative control staining using splenocytes isolated from I-Ab deficient NOD mice (NOD/ShiLtJ-*H2-Ab1*^{em1Ygch}/J, JAX stock no. 027057). Similar results were obtained in two independent experiments.

acid substitution at position 57 in the Aβ chain alters the binding of AMS-32.1, presumably due to a conformational change of the antibody-binding epitope. Strikingly, diabetes development was completely suppressed in homozygous NOD.$Ab^{g7\text{-}S57D}$ mice (**Figure 2**), confirming the importance of the aspartic acid residue at position 57 of the Aβ chain in T1D. The availability of this novel strain will allow studies aimed to understand how diabetogenic MHC class II molecules select and activate β-cell autoreactive CD4 T cells.

Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and CRISPR-Associated Protein 9 (Cas9)

Due to its high efficiency, the CRISPR/Cas9 system has become the top choice when considering gene targeting in a variety of animal models. Similar to ZFN mediated mutagenesis, CRISPR/Cas9 also introduces a DSB, followed by repair through NHEJ and HDR dependent mechanisms (109). Cas9 nuclease is recruited to a specific DNA sequence by a single-guide RNA that can be easily designed using publically available online tools (109). Several groups, including ours, have successfully used the CRISPR/Cas9 approach to disrupt genes directly on a pure NOD genetic background (18, 110–112). The importance of affinity maturation processes of B cells (class switch recombination and somatic mutation) for T1D development in NOD mice was demonstrated by ablation of the activation-induced cytidine deaminase gene (*Aicda*) (111). It was recently shown that IL-2 can indirectly enhance FOXP3 expression through downregulating the level of *Flicr*, a long non-coding RNA (112). The function of Tregs is impaired in NOD mice partly due to reduced IL-2 production by activated T cells in this strain (74). Deletion of *Flicr* decreased accumulation of $FOXP3^{low}$ Tregs in pancreatic islets and suppressed T1D in NOD mice, likely by enhancing the stability and function of Tregs (112). *Ptpn22* has also been targeted in NOD mice using the CRISPR/Cas9 system (18). A nonsynonymous single nucleotide polymorphism resulting in an amino acid substitution (R620W) in human PTPN22 has been linked to numerous autoimmune diseases, including T1D (113).

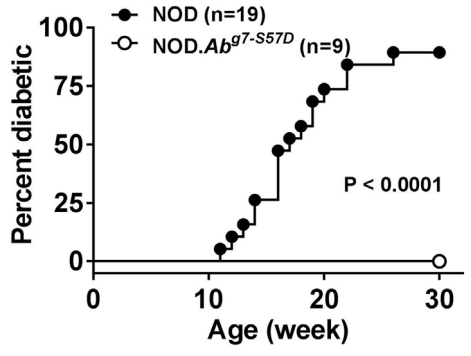

FIGURE 2 | NOD.$Ab^{g7\text{-}S57D}$ mice are completely resistant to type 1 diabetes. NOD and NOD.$Ab^{g7\text{-}S57D}$ mice were monitored for diabetes development weekly for 30 weeks by testing urine glucose. Diabetes onset was defined by two consecutive readings of >250 mg/dl.

Ptpn22 has been identified as a top candidate gene for the *Idd18.2* region in NOD mice. To further study the role of PTPN22 in T1D, the Sherman laboratory generated a *Ptpn22* knockout NOD mice as well as a knockin strain that has the R619W amino substitution to mimic the human variant (18). *Ptpn22* knockout NOD females developed more rapid onset of T1D (18). Similarly, NOD females expressing the *Ptpn22* KI allele (encoding 619W) also developed accelerated T1D (18), providing direct evidence to support the diabetogenic function of this variant.

Testing Human T1D Candidate Genes in NOD Mice

As noted above, the NOD mouse has been criticized for its usefulness as an animal model for human T1D, largely due to disappointing outcomes of clinical trials based on agents that showed therapeutic and/or preventive effects for mouse diabetes. The increased availability of human samples allowing direct examination of pancreata and lymphoid tissues isolated from organ donors at different stages of T1D progression has further decreased the enthusiasm of the NOD model (114). However, it remains a challenge to identify and to mechanistically study T1D susceptibility genes in human. The effect of a single gene on a phenotype is more difficult to detect due to heterogeneity in humans. Many genetic variations associated with T1D have a low phenotypic impact that overlaps when comparing carriers and non-carriers. In addition, human studies are mostly association in nature and strategies that allow investigators to directly analyze the diabetogenic function of a single SNP alone or in combination are limited. The CRISPR/Cas9 system makes it possible to engineer isogenic cell systems that can be used to specifically address the role of a SNP in gene expression and function (115). When combined with the ability to generate patient-derived iPSC and the advance of *in vitro* differentiation of iPSC into insulin-producing β-cells and hematopoietic stem cells, it may be possible to test the function of a SNP in cell types relevant to T1D (116, 117). However, these studies are not likely to overcome the difficulty to understand the course from altered gene expression/function to T1D development, which can only be dissected with *in vivo* experimental systems as disease progression is a consequence of combined effects that a variant elicits in different cell types in a time-dependent fashion. Combinational approaches using both mouse and human experimental systems are thus required to have a comprehensive understanding of the genetic control in T1D. The ability of nuclease based technology to efficiently and precisely modify the genome directly in NOD mice has opened a new door for current and future T1D genetic studies using this model.

Because T1D is a complex disease influenced by a large number of genes and ill-defined environmental factors, the NOD mouse remains an ideal animal model that provides a disease susceptible genetic background to test the diabetogenic function of a human candidate gene. For this reason, we have used both ZFNs and CRISPR/Cas9 systems to target mouse orthologs of human T1D candidate genes nominated by GWAS. As discussed above, the availability of the nuclease based technologies made it possible to do a relatively small scale but focused screening for genes that

can regulate T1D progression in NOD mice. We have successfully targeted more than 40 genes directly in NOD mice. While these studies are still ongoing, the results obtained from this screening will allow us to provide additional evidence to support their roles in human T1D and prioritize them for future mechanistic studies. The eventual goal is to identify a pathway that could be pharmaceutically targeted for clinical translation.

CONCLUSION REMARKS

Despite some shortcomings, NOD mice and NOD-derived recombinant congenic strains provide many advantages for T1D research. As discussed above, the NOD mouse continues to be an important tool for dissecting the genetic control of T1D. As will be discussed below, we also describe T1D research areas where NOD mice and related strains can provide critical information in the next decade.

Previous studies have generated NOD mice transgenically expressing HLA class I and II molecules associated with human T1D (118–121). While HLA class II molecules in NOD mice are not able to promote T1D, expression of the HLA A2.1 allele accelerates diabetes development, providing a model for identifying peptides targeted by A2.1-restricted $CD8^+$ T cells and for testing antigen-specific immunotherapy (122, 123). When combined with various versions of the severe immunodeficient NOD mice (e.g., NSG mice), expression of HLA class I or II molecules in the absence of murine counterparts provides a superior host for primary human T cells and hematopoietic stem cell-derived immune system (124). NSG mice that also express high-risk HLA class I or II molecules have been transplanted with human peripheral blood mononuclear cells or β-cell autoreactive T-cell clones/lines isolated from T1D patients to test the diabetogenic potential of the presumably pathogenic effectors (125–127). Although much progress has been made, overt diabetes has not been observed in HLA class I or II expressing NSG mice transfused with human T cells in various settings. The eventual goal is to reconstitute a T1D prone human immune system that targets β cells derived from the same subject in a mouse for studying "human" T1D. Recent advance in differentiating human iPS cells into functional insulin-producing β-cells and hematopoietic stem cells has brought us one step closer to this goal (116, 117).

Gut microbiome has emerged as an important component that modulates the progression of T1D in both humans and NOD mice (128–134). Longitudinal studies in humans showed that alteration of the diversity and species of gut microbiota preceded T1D onset (133). Studies in NOD mice have shown that manipulation of gut microbiota by means of antibiotics, fecal transfer, or co-housing can either suppress or promote diabetes development (128, 135–138). Collectively, these studies demonstrate that the NOD mouse can provide an excellent experimental platform for understanding the roles of gut microbiota in T1D. Recent studies also suggest that T1D modulation by gut microbiota is not likely to be caused by a single species but rather due to the balance of diverse species within the bacterial community. While it remains to be tested, experiments that utilize germ-free NOD mice reconstituted with fecal samples from T1D patients, at risk individuals, and healthy subjects may provide some information regarding the "good" and "bad" gut bacterial community. This knowledge can then be used to develop methods to alter the gut microbiota for T1D prevention and set the foundation for future clinical trials.

ETHICS STATEMENT

All animal studies were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin under protocol AUA00001863.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Type I Interferon Is a Catastrophic Feature of the Diabetic Islet Microenvironment

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A detailed understanding of the molecular pathways and cellular interactions that result in islet beta cell (β cell) destruction is essential for the development and implementation of effective therapies for prevention or reversal of type 1 diabetes (T1D). However, events that define the pathogenesis of human T1D have remained elusive. This gap in our knowledge results from the complex interaction between genetics, the immune system, and environmental factors that precipitate T1D in humans. A link between genetics, the immune system, and environmental factors are type 1 interferons (T1-IFNs). These cytokines are well known for inducing antiviral factors that limit infection by regulating innate and adaptive immune responses. Further, several T1D genetic risk loci are within genes that link innate and adaptive immune cell responses to T1-IFN. An additional clue that links T1-IFN to T1D is that these cytokines are a known constituent of the autoinflammatory milieu within the pancreas of patients with T1D. The presence of IFNα/β is correlated with characteristic MHC class I (MHC-I) hyperexpression found in the islets of patients with T1D, suggesting that T1-IFNs modulate the cross-talk between autoreactive cytotoxic CD8$^+$ T lymphocytes and insulin-producing pancreatic β cells. Here, we review the evidence supporting the diabetogenic potential of T1-IFN in the islet microenvironment.

Keywords: type 1 diabetes, type 1 interferons, humans, CD8$^+$ T cell, beta cells

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INTRODUCTION

Type 1 diabetes (T1D) results from an autoimmune-mediated attack that specifically targets insulin (INS)-secreting pancreatic beta (β) cells. Through the interactions of β cell antigen-specific T cell receptors (TCR) with MHC-peptide complexes, β cells are destroyed leading to aberrant glucose homeostasis and persistent hyperglycemia. Critical to T1D pathogenesis is the targeted destruction of pancreatic β cells mass by autoreactive cytotoxic CD8$^+$ T lymphocytes (CTLs) (1–6). Although responses in T1D are directed toward autoantigens, the activation of the β cell specific CTLs is expected to be similar to activation of CD8$^+$ T cells observed during a typical response to infectious agents. Following activation, autoreactive CTLs clonally expand, home into the pancreatic islets, and survey the surface of β cells for antigen presented in the context of MHC class I (MHC-I). Recognition of the specific cognate peptide- human leukocyte antigen (HLA) class I complex results in the induction of TCR signaling, formation of the immunological synapse, and targeted destruction of β cells. While the immune system plays a significant role in perpetuating disease pathology, a large body of literature supports the notion that development of T1D is dependent upon a complex network of determinants including those of genetic and

environmental etiologies (7–15). Tissue microenvironments influence immune responses in models of tumor biology and infectious disease. However, this notion remains largely been unexplored in the target tissues of autoimmune diabetes.

Type 1 interferons (T1-IFNs), classically known for interfering with viral infection, have been implicated in the early stages of T1D autoimmunity (16–21). Transcriptome analysis reveals a T1-IFN signature in the peripheral blood of patients prior to the development of autoantibodies (16, 17). Additionally, these cytokines have been identified as being expressed in the pancreata of deceased tissue/organ donors with T1D versus non-diabetic donors (18, 19, 21). GWAS studies reveal several T1D-associated genes that are involved in the production, signaling, and regulation of the T1-IFN pathway (12, 22). Moreover, induction of T1D has been reported in patients receiving T1-IFN therapy for various conditions including hepatitis C, multiple sclerosis, and hairy cell leukemia (23–30) supporting the idea that these cytokines may actively exacerbate T1D progression. Despite the growing evidence for the role of T1-IFNs in T1D, little is known about how these cytokines contribute to the inflammatory environment of the human autoimmune diabetic islet (16, 17, 31–36). This review will consider the current paradigms in the natural history of T1D as well as T1-IFN action while summarizing the published literature regarding a role for T1-IFNs in T1D pathogenesis. Additionally, we highlight the exciting new avenues of research suggesting that T1-IFNs may be a catastrophic feature within the diabetic microenvironment.

SETTING THE STAGE FOR AUTOIMMUNITY: ROLE OF GENETIC SUSCEPTIBILITY

Genetic predisposition constitutes a primary risk factor for the initiation of β cell autoimmunity and can be attributed to the complex interplay of more than 50 genetic loci that may impact immune function, INS expression, and β cell function (11, 37, 38). Identified as the first genetic locus associated with T1D in the 1970s, the HLA region on chromosome 6p21 confers approximately 50% of the genetic risk for disease development (39). This region, also referred to as (it) IDDM1 (it), is highly polymorphic, containing over 200 identified genes that can be categorized as class I, II, or III genes that play an important role in antigen presentation as well as regulation of this process. Particularly, class I and II genes encode the classical HLA cell surface proteins that are involved in presenting antigen to CD8+ and CD4+ lymphocytes, respectively. In fact, the strongest association is found in patients harboring the specific HLA class II haplotypes, DR3-DQ2 (DRB*301-DQB*201) and DR4-DQ8 (DRB*401-DQA*301-DQB*302) with the highest risk seen in DR3/DR4 compound heterozygotes (40, 41). Conversely, strong protection from T1D is observed in individuals with the DQB*602 allele, which is reported in less than 1% of patients with T1D (42, 43). Comparison of high- and low-risk DQ alleles in humans and mouse models reveal key differences in peptide binding, as predisposing alleles contain a substitution of non-charged amino acids (alanine, valine, or serine) for aspartate at position 57, which destabilizes binding of antigenic epitopes (44–46). While most studies assessing HLA risk haplotypes have been carried out in Caucasian individuals, recent efforts have begun to characterize HLA susceptibility in other ethnic groups. For example, HLA genotyping in African American patients found that the African-specific DR9 (DRB1*09:01-DQA1*03:01-DQB1*02:01g) haplotype in combination with DR4 mimics risk for T1D seen in patients with DR3/DR4 heterozygosity in European populations. Alternatively, the African-specific "DR3" haplotype (DRB1*03:02-DQA1*04:01-DQB1*04:02) confers significant protection (47). Future studies in this area should be geared toward understanding HLA risk haplotypes in individuals of diverse ethnic backgrounds. Although not as widely studied, HLA class I alleles, HLA A*24 and HLA B*39, appear to be associated with increased susceptibility for T1D, decreased age of onset, and fulminant β cell destruction (48–50).

Numerous additional loci outside of the HLA region summate the remaining genetic risk for diabetes development, although the individual odds ratios conferred by these regions are modest (11, 12). Several of these genes are thought to influence tolerance mechanisms facilitating the escape of autoreactive T cells into the periphery. For instance, variants within the INS gene are known to modulate thymic INS expression, which comprises about 10% of the genetic risk for T1D and carry an odds ratio of 2.2 (51–53). Extensive mapping of this region associates variable number of tandem repeats in the 5′ promoter of INS with diabetes risk (53–55). Shorter class I alleles [23–63 repeats] predispose for diabetes, while longer class III alleles [140–210 repeats] are protective (55). The number of tandem repetitions determines INS transcription in the thymus through interactions with the autoimmune regulator, AIRE, which is essential for appropriate T cell education and provides strong evidence that central tolerance to INS, the primary autoantigen in T1D, is impaired in patients who harbor this risk variant (56).

Protein tyrosine phosphatase non-receptor type 22 (PTPN22) is another well-known example, as this locus confers the third highest genetic association for T1D and is also known to be a regulator of signaling in a variety of immune cell types including lymphocytes, monocytes, dendritic cells (DCs), and neutrophils (57). Case-control and association studies show that this coding variant causes a non-synonymous substitution from an arginine to a tryptophan (R620W) located within the protein-binding domain of PTPN22. Biochemical studies in lymphocytes demonstrate the PTPN22*W620 allele behaves as a gain-of-function mutant with dampened TCR signaling (58). In contrast, the same variant in myeloid derived cell types is highly controversial with some models demonstrating hyper-responsive DC phenotypes with increased T cell activation while others exhibit reduced function and selective impairment of T1-IFN responses following TLR stimulation (59, 60). How might seemingly paradoxical functions be contributing to onset of T1D? On one hand, diminished TCR signaling by the risk variant could impair central and peripheral T cell tolerance, while reduced T1-IFN production by TLRs may hinder effective clearance of β-cell tropic viruses triggering self-reactivity (61). Studies remain ongoing to determine the full gamut of functional consequences induced by this variant.

Like PTPN22, many T1D-associated genes play multiple roles in immune sensing and signaling especially in response to environmental ques, which supports the hypothesis that genetic risk coupled to permissive environmental determinants collectively contribute to diabetes progression. Diabetogenic viruses signify a highly postulated candidate for initiation and potentiation of islet autoimmunity. Critical for the innate immune response to viral infection are T1-IFNs. Several identified genetic loci for T1D also have prominent roles in the induction and signaling of this pathway, including IFIH1 (rs1990760), TYK2 (rs2304256), and STAT4 (rs7574865) (62–64). TYK2 is a tyrosine kinase involved in proximal TI-IFN signal transduction as well as regulation of IFNAR1 surface expression (65–67). Similarly, STAT4 is a key mediator of T1-IFN signaling essential for the generation of Th1 responses, which contribute to the T cell-mediated pathology observed in diabetes (68, 69). Also associated with several other autoimmune disorders, protective variants for each of these genes is associated with reduced T1-IFN signaling (67, 70). IFIH1 encodes the protein MDA5, a cytoplasmic sensor of viral double-stranded RNA. The non-synonymous SNP found in IFIH1 results in alanine to threonine amino acid substitution at position 946 (A946T) and may diminish ATPase activity of MDA5 activity leading to deranged constitutive provocation of T1-IFN as well as blunted viral sensing (62, 71, 72). Compelling evidence in primary human islets reveals that presence of the homozygous risk allele decreases the autonomous innate response to Coxsackievirus B3 (73). Collectively, these data suggest that the A946T risk variant in IFIH1 may act as a double-edged sword, predisposing β cells to persistent enteroviral infection while concurrently promoting deleterious T1-IFN production in and around the islet microenvironment.

EVOLUTION OF ISLET DESTRUCTION IN HUMAN DIABETES

Human β cells act as quintessential metabolic sensors working to integrate environmental cues for rapid and efficient glycemic control (74). Reports of decreased C-peptide responses and reduced glucose tolerance in autoantibody positive individuals suggest that ongoing inflammation precipitates the deterioration of β cell function prior to diabetes onset (75–77). Additionally, β cells are also widely believed to be active participants in promoting a diabetogenic islet microenvironment. For example, MHC-I is known to be hyperexpressed within the islets of T1D patients, suggesting that β cells may be more visible to infiltrating CTL (1, 20, 21, 78). Increasing data insinuates that signals arising from the islet microenvironment, such as T1-IFNs, could trigger such disease promoting adaptations. Additionally, active inflammatory signals within the islet microenvironment prompt substantial variation in the β cell transcriptome and proteome as well as augmenting the capacity for cytokine and chemokine production by islet or β cells (79).

The conceptual model proposed by George Eisenbarth for the natural history of T1D has shaped theories regarding the evolution of T1D pathogenesis (80, 81). Many facets of this paradigm have been tested and updated over the past 3 decades. The amalgamation of genetic pre-disposition and initiating environmental triggers create the framework for models that describe the insurgence of β cell autoimmunity. Though the nature of the instigating insult is not completely understood, once initiated, active immune-onslaught can be indicated by the presence of autoantibodies and histological detection of the pathognomonic lesion termed insulitis (82). Found in or around the islets, insulitis is a heterogeneous inflammatory infiltrate comprised of T lymphocytes, B lymphocytes, macrophages, and DCs, however $CD8^+$ T cells form the primary constituent [(1, 2, 83) and **Figure 1A**]. First noted by German pathologist Martin Schmidt in the early 1900s, this lesion was not considered a prominent feature of T1D until the landmark paper by Willy Gepts in 1965 where the presence of insulitic lesions were observed in 15/22 recent onset T1D cases (82, 84, 85). Further evaluation of these samples using immunohistochemical techniques and additional data from subsequent studies revealed that inflammation was primarily observed in islets with INS immunoreactivity. Further, in cases with long-standing disease, many islets appear to be devoid of INS containing β cells without active insulitis, alluding to the role of these cells as the inciting antigen in T1D (1, 2, 20, 82, 86–88).

Until recently, efforts aimed at characterizing the nature, composition, and frequency of insulitis have been challenging. This is due to the anatomical inaccessibility of the pancreas for direct study in living subjects as well as a dearth of well-preserved human cadaveric tissues for analysis (84, 89). The inception of the Network for Pancreatic Organ Donors with Diabetes (nPOD) has dramatically advanced our understanding of β cell/Islet autoimmunity (89–92). Moreover, studies of human pancreata have allowed for the emergence of new paradigms in T1D, including the current consensus definition of insulitis, defined as the presence of more than 15 peri- or intrainsulitic $CD45^+$ cells within a minimum of three islets (93). The most comprehensive screening and characterization of insulitis to date was recently described using the nPOD collection where a total of 159 pancreata were screened (61 controls, 18 autoantibody positive cases without a diagnosis of T1D, and 80 T1D cases) (88). Investigators presented confirmatory findings that insulitis is present most frequently in recent-onset patients within INS-containing islets and inversely correlates with disease duration. The presence of adaptive-immune infiltration into the islets of individuals with autoantibodies is a rare event, observed only in individuals with multiple antibodies (94). Additionally, patients with T1D display tremendous heterogeneity in terms nature, distribution, and severity of insulitis in addition to the amount of residual β mass present following diagnosis (88, 95).

A critical cell-to-cell interaction during the development of T1D occurs when β cells and islet-antigen specific CTLs come into contact. Strong evidence has supported a crucial role for $CD8^+$ T cells in T1D. First reported by Bottazzo in 1985, histological characterization of pancreas sections from T1D cases demonstrated that CTL are the most abundant immune cell type found in human insulitis [(20) and **Figure 1A**]. Additional studies have confirmed that $CD8^+$ T cells have a prominent role in T1D as well as recurrent T1D that occurs after transplantation of islets, pancreas (pancreas alone, or SPK recipients) into patients with T1D.

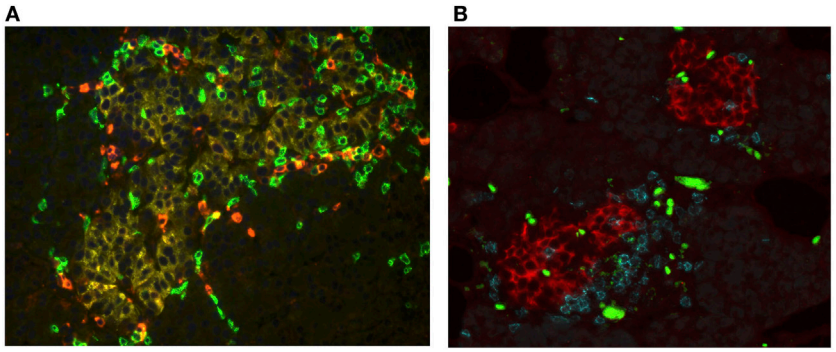

FIGURE 1 | CD8 T cells are the major leukocyte component of the insulitis lesion in type 1 diabetes (T1D) as well as recurrent disease. Pancreatic sections [courtesy of Network for Pancreatic Organ Donors with Diabetes (nPOD)] were examine histologically for the presence of islet invading immune cells. **(A)** Section taken from a pancreas organ donor with T1D (nPOD case 6052). The tissue section was immunofluorescently stained for glucagon (yellow) to identify the islet, CD4 T lymphocytes (red), and CD8 T lymphocytes (green). Within this islet cytotoxic $CD8^+$ T lymphocytes (CTLs) are the predominant T cell type observed. Image courtesy of Martha Campbell-Thompson, DVM/PhD (University of Florida). **(B)** Section taken from a pancreas transplant biopsy, from an simultaneous pancreas and kidney (SPK) recipient who had developed recurrence of T1D. Tissues were stained for insulin (red), CD4 T lymphocytes (blue), and CD8 T lymphocytes (teal). Within the insulitic lesions, CTLs represented the dominant T cell found. Figure shows islets with both CD8 than CD4 T cells, but most islets were primarily infiltrated by CD8 T cells. Green/yellow bright stains represent non-specific fluorescence from red blood cells. Image courtesy of Alberto Pugliese, MD, Francesco Vendrame, MD, and George Burke, III, MD, University of Miami.

Biopsy and histological examination of the transplanted pancreas demonstrate the accumulation of high numbers of $CD8^+$ T cells into INS positive islets in patients who are undergoing active islet autoimmunity (**Figure 1B**).

Regarded as the final executioner in T1D, CTLs mediate direct β cell destruction through the recognition of epitopes from proteins that are selectively expressed in β cells and are presented by these INS-producing cells the context of MHC-I. Following recognition of cognate antigen, CTLs create a close contact with the target β cell by forming an immunological synapse, where several cytotoxic mechanisms are employed to induce death of β cells. These include the induction of molecules involved in the granule exocytosis pathway such as perforin, granzyme, or granulysin as well as increased surface expression of death ligands such as Fas Ligand and TNF-related apoptosis inducing ligand (96–98). The presence of CTLs specific for well-known autoantigens such as IGRP, preproinsulin, and IA-2, have been documented in islets with augmented MHC-I expression (1, 2, 88). $CD8^+$ T cells bearing TCR that are specific for β cell antigens have been detected in circulation of patients. These TCRs imbue CTL with the ability to destroy human β cells *in vitro* (99–102). In patients undergoing recurrent autoimmunity following islet transplantation, autoreactive $CD8^+$ T cells are associated with β cell destruction resulting in graft failure (103). This evidence for an essential role of CTL in T1D in humans is further bolstered by studies in mice. Spontaneous diabetes fails to develop in non-obese diabetic (NOD) mice lacking MHC-I or β2 microglobulin (4, 6), while diabetes onset can be accelerated by adoptive transfer of diabetogenic CTL (104, 105).

Mounting evidence suggests that stimuli from the diabetic islet microenvironment likely contribute to autoreactive CTL-mediated β cell cytotoxicity. For example, using NOD adoptive transfer systems with IGRP-specific NY8.3 $CD8^+$ T cells, it has been demonstrated that $CD8^+$ T cells acquire greater cytolytic capacity and an effector-memory phenotype upon migration into the NOD islet (106–108). As T1-IFNs are linked to increased HLA expression in the pancreatic islets of patients with T1D, suggesting that these cytokines contribute to autoimmune surveillance and promote insulitis. While the effect of T1-IFNs on human islets have only recently begun to emerge, evidence suggests that T1-IFNs are involved in the cross talk between the adaptive immune effectors and the microenvironment of the diabetic islet (16, 17, 31–36, 109, 110).

TYPE 1 INTERFERONS

Type 1 interferons belong to a large family of cytokines that were originally described by Alick Issacs and Jean Lindenmann in 1957 as soluble factors responsible for mediating viral interference following a primary virus exposure (111–113). Since then, this large family of cytokines has been further categorized into three distinct classes that play essential roles in cellular-mediated defense against viral and microbial infections as well as in autoimmunity (113–116). Differing in structural homology and signaling receptor complexes, these categories include the T1-IFNs as well as the type 2 interferon [interferon gamma (IFNγ)] and the recently identified type III IFNs including IFNλ1 (IL-29), IFNλ2 (IL-28A), IFNλ3 (IL-28B), and IFNλ4 (114, 117–121). T1-IFNs signal through the heterodimeric IFNAR1-IFNAR2 receptor [IFNAR] and comprises the largest class of IFN including thirteen IFNα subtypes in addition to IFNβ, IFNε, IFNκ, and IFNω. Though multiple T1-IFN subtypes may appear redundant, these distinct entities display unique binding affinities to the IFNAR that result in diverse functional outcomes with respect to antiviral, immunomodulatory, and growth inhibitory activity (122–128). While all T1-IFN subtypes contain several conserved "anchoring" residues that are important for receptor binding, the contribution of residues flanking these anchor

points determine the overall binding of these polypeptides to IFNAR1/2 (126–130). As such, IFNβ exhibits the strongest interaction with the receptor out of all T1-IFN subtypes (130).

Type 1 interferons represent an early line of defense against viral infection and can be produced by virtually every cell in the body (131–134). Induction of T1-IFNs are initiated by stimulation of pattern recognition receptors (PRRs) that recognize conserved motifs found on viruses, including toll-like receptors (TLR3, TLR4, TLR7, and TLR9), cytosolic RNA helicases (RIG-I and MDA-5), and cytosolic DNA sensors (131, 133, 134). Following activation of these distinct pathways, the adaptor molecules MAVS (cytosolic RNA sensors), STING (cytosolic DNA sensors), TRIF (TLR3/4), and MyD88 (TLR7/8/9) transduce signals that converge on the activation of TBK-1, which phosphorylates IRF-3 leading to transcription of T1-IFN and IRF-7 that engage in a positive feedback loop for amplification of this response (134–136).

Following production, T1-IFNs signal in an autocrine or paracrine fashion through IFNAR. Engagement of the receptor leads to trans-phosphorylation as well as activation of the tyrosine kinases TYK2 and JAK1 that are constitutively associated with the IFNAR subunits, IFNAR1 and IFNAR2, respectively. Signaling downstream of IFNAR can lead to the activation of several pathways that contribute to the widespread range of effects by T1-IFNs depending upon the cell type and the context in which the TI-IFN signal was received (117, 133, 137, 138). Classically, T1-IFN signaling invokes the activation of STAT1-STAT2 heterodimers that rapidly translocate to the nucleus and complex with IRF9 to form the interferon-stimulated gene factor 3 (ISGF3) complex. Formation of ISGF3 leads to binding of the interferon response element (consensus sequence: TTTCNNTTTC) for the transcription of interferon-stimulated genes (ISGs) that mediate a diverse range of functions (117, 133, 139). Alternatively, T1-IFNs are capable of activating all seven members of the STAT family that can manifest as homodimers or heterodimers to induce downstream signaling and transcription. For instance, T1-IFN induced STAT1 homodimers are known to bind IFNγ activated sequences (GAS; consensus sequence: TTCNNNGAA) to initiate proinflammatory programs similar to IFNγ, whereas T1-IFN induced STAT3 homodimers have been reported to interact with the corepressor complex SIN3A to indirectly counteract inflammatory responses (133, 140–142). Utilization of these alternative T1-IFN signaling pathways is partially determined by the expression of individual STAT family members (143). This concept is clearly evident in lymphocytes. The balance between STAT1 and STAT4 dictates T cell responses following T1-IFN exposure (144). This is highly dependent upon STAT4 expression within the T cell, which is initially induced through activation of TCR signaling. This induces a switch from the "classical" anti-proliferative and proapoptotic actions of STAT1 signaling to STAT4 that promotes T cell proliferation, differentiation, and survival (143–145).

In addition to JAK-STAT signaling, several other non-canonical pathways are known to be induced by T1-IFNs. For example, activation of JAK1 and TYK2 after T1-IFN engagement has been shown to induce the PI3K-AKT pathway that leads to activation of mTOR, which leads to downstream control of protein translation, regulation of cellular division, and proliferation, in addition to activation of IKKβ resulting in NF-κB activity (117, 137). In lymphocytes, the MAPK pathway mediates cross-talk between T1-IFN signaling and the TCR complex resulting in growth inhibition (117). While studies are still ongoing to unmask the complex signaling networks induced by T1-IFNs, the ability of these cytokines to induce a wide array of signaling pathways explains their pleiotropic and sometimes paradoxical biological activities.

Type 1 interferons signaling culminates in the induction of a robust antiviral program. Several key components required for T1-IFN signaling, including STAT1 and IRF9, are also well-known ISGs that act to reinforce and amplify the IFN response. T1-IFNs also act to enhance host defense and pathogen detection by increasing the expression of several PRRs involved in viral sensing, expression of 2,5 oligoadenylate synthetase (OAS) that facilitates eradication viral RNA, as well as upregulation of proteins that interfere at various steps of the viral life cycle, including viral entry, replication, and viral egress from infected cells (146).

Type 1 interferons dynamically regulate the actions of innate and adaptive immune cells, including the ability to enhance NK cell cytotoxicity as well as the production of IL-1β and IL-18 by macrophages (147). These cytokines are also well known for directly and indirectly influencing T cell responses that assist in the eradication of invading pathogens or malignant cells (132, 138, 147, 148). IFNα/β promote the differentiation and maturation of DCs by enhancing the expression of MHC-I and II along with costimulatory molecules (CD40, CD80, CD83, CD86, 4-1BBL) required for efficient $CD4^+$ and $CD8^+$ T cell priming (138, 149–151). These cytokines promote trafficking of DCs to lymphoid organs, stimulate expression of adhesion molecules, and induce the secretion of chemoattractant molecules that promote communication between DCs and T lymphocytes (138, 152–154). In line with their effects on DCs, T1-IFNs promote the activity of antigen-exposed $CD8^+$ T cells, by inciting proliferation, enhancing survival, and increasing effector function. Conversely, in antigen-inexperienced $CD8^+$ T cells the T1-IFNs prevent growth and differentiation in an effort to direct a specific T cell response toward the inciting pathogen (138). While T1-IFNs act to implement numerous mechanisms aimed at thwarting the spread of infection, aberrant activation of this pathway, as seen in autoimmunity, can lead to overactivation of immune cells and perpetuation of tissue damage.

T1-IFNs AND PATHOGENESIS OF T1D

Evidence in Humans

The characterization of insulitis in seminal studies by Gepts and Foulis altered the landscape regarding the pathogenesis of T1D to one of an immune etiology. Soon after, it was reported that there was a striking genetic association between the HLA DR locus and T1D onset (155). These findings, along with the notion that class II antigens could be expressed abnormally in other organ-specific autoimmune diseases prompted investigators to hypothesize that altered antigen presentation by pancreatic β cells in T1D might explain activation and infiltration of

autoimmune T cells found within insulitic lesions (156–158). In 1985, Bottazzo et al. reported that residual β cells found in a 12-year-old recent onset donor were selectively positive for HLA-DR. In addition to noting enhanced expression of HLA-DR, this was also the first report to note enhanced HLA class I expression in insulitic islets the same donor (20). Since the aberrant expression of MHC-II molecules could be induced by IFNγ on thyroid follicular cells in autoimmune thyroiditis, it was postulated that IFNγ could be acting in a similar manner to induce this uncharacteristic expression in pancreatic β cells. While subsequent studies showed that interferons were incapable of directly inducing ectopic expression of MHC-II on pancreatic β cells, they were found to be potent inducers of MHC-I expression (21). Subsequent analyses confirmed that MHC-I expression was a prominent phenotype found in patients undergoing islet autoimmunity, especially in normal appearing or inflamed islets containing residual β cells (1, 78, 159–162). Based on the heterogeneity of insulitis in T1D, it was hypothesized that β cells could be actively generating soluble mediators that are capable of acting in a paracrine manner to exert affects within the diabetic microenvironment. IFNα represented a prime candidate, as it was known to induce MHC-I in islet tissue and was known to be produced by a wide range of cells (21). The first report to correlate the presence of IFNα in the islets of patients with recent-onset T1D diabetes was published in 1987. Investigators examined 37 pancreata from cadaveric donors with T1D and found that 34 of 37 samples displayed MHC-I hyperexpression. Further, 97% of patients displaying this feature concurrently exhibited positivity for IFNα by immunocytochemistry (18). Transcript expression of various cytokines, including IFNα, IFNβ, IFNγ, IL-1β, TNFα, were compared in diabetic and control pancreata. Among the panel of cytokines tested, only IFNα displayed a clear and consistent pattern of augmented expression in patients (19).

Additional lines of evidence implicate a pathogenic role for T1-IFNs in human autoimmune diabetes. The presence of β cell-specific autoantibodies signifies the preclinical phase of T1D and serves as an essential biomarker for identifying at-risk individuals (163). Long-term follow up of at risk children enrolled in the BABYDIET and DIPP longitudinal studies reveal T1-IFN inducible signatures in the peripheral blood, which was positively correlated with episodes of upper respiratory infections. The signature was strongest immediately prior to seroconversion and began to decline after the detection of autoantibodies. This time course suggests that activation and production of T1-IFNs may be involved in the early stages of islet autoimmunity (16, 17). In accordance with these findings, IFNα in the plasma of patients with T1D was shown to be elevated when compared to controls (10.1 U/mL; 69.6% positivity vs. 0.4 U/mL, 0% positivity, respectively) and plasmacytoid dendritic cells (pDCs), well known for producing T1-IFNs, were observed in the peripheral blood of new-onset patients during diagnosis (164, 165). Furthermore, enterovirus RNA, particularly Coxsackievirus B, was identified in 50% of patients who displayed positivity for IFNα (165).

The half-life of cytokines within the T1-IFN family is relatively short (IFNα: 4–16 h; IFNβ 1–2 h) and serum levels of IFN begin to decline very rapidly once secreted (166, 167). Due to rapid clearance, detection of IFNs in circulation can prove challenging. To circumvent this, investigators have attempted to use T1-IFN induction pathways, such as Poly(I:C), or the measurement of ISGs in PBMC as surrogate markers for T1-IFN activation when comparing patients and controls (168, 169). For example, patients display a higher basal expression of the ISG OAS, as well as increased sensitivity to IFNα exhibited by maximal induction at lower IFNα concentrations when compared to control subjects (168). T1-IFN production was higher from PBMC isolated from patients with T1D compared to controls, whereas IFNγ production by isolated PBMC in response to concanavalin A was not different between control and T1D patient samples (169). With respect to the IFNα response, there was no correlation to blood glucose levels, HbA1c, age of onset, disease duration, or ICA positivity, which may point to the importance of genes associated with T1D that are involved in signaling of this pathway (169).

Initiation of islet autoimmunity has been noted in individuals following T1-IFN therapy for chronic hepatitis, multiple sclerosis, as well as hematologic malignancies (23, 24). First reported in 1992, T1-IFN-induced autoimmune diabetes was described in a patient with Hepatitis C, who was seropositive before treatment for autoantibodies against both GAD and INS (30). While this complication occurs in a minor subset of patients, half of all cases reporting T1D following IFN therapy were positive for autoantibodies. This suggests that T1-IFNs may precipitate loss of tolerance and self-reactivity in at-risk patients (170). Studies investigating β cell function in these patients suggest that T1-IFNs can reduce INS secretion, impair carbohydrate metabolism during an oral glucose challenge, and induce INS dependency over the course of treatment (171, 172). Patients who incur T1-IFN-induced autoimmune diabetes to not exhibit normoglycemia when T1-IFN therapy is arrested suggesting that in these patients β cell mass is lost to an extent that metabolic control cannot be reestablished.

Evidence in Animal Models of T1D

Animal models have been indispensable for ascertaining knowledge regarding the cellular and molecular events involved in T1D pathogenesis. Likewise, these models have also been instrumental in elucidating how T1-IFNs contribute to diabetes pathogenesis. One example includes the diabetes prone biobreeding rat (BB-DP rat). These animals emulate some pathologic features observed in human diabetes including polygenic inheritance [including the MHC], peripubescent onset, and β cell destruction characterized by mononuclear infiltration (173, 174). Initial studies conducted in this model demonstrate a dose dependent stimulation of IFNα production by Poly(I:C) that correlates with accelerated diabetes incidence and severity (175, 176). Conversely, elevation of serum IFNα in non-diabetes prone Wistar rats did not instigate diabetes, suggesting that T1-IFNs are not pathogenic without an inherent risk for diabetes (175, 176). Additionally, investigation into the natural history of diabetes in BBDP rats revealed spontaneous expression of IFNα in the islets of Langerhans prior to insulitis proposing that induction of T1-IFNs in the islet microenvironment may disrupt self-tolerance in this preclinical model (177).

The NOD mouse model has served as the principal animal model for the investigation of causative mechanisms leading

to autoimmune diabetes (178). Several lines of evidence in the NOD support an association for T1-IFNs in T1D. One of the most striking is the presence of a T1-IFN signature in NOD islets prior to diabetes onset, reminiscent of the signature observed in humans and BBDP rats (31). In 4- to 6-week-old NOD females, T1-IFNs serve as one of the first distinctive signs of pathology in these animals followed by lymphocytic infiltration and synchronized elevation of activation markers in the islet tissue (31). Elevated levels of IFNα and pDC in the pancreatic draining lymph nodes are also reported in 2- to 3-week-old NOD mice (36). This argues that aberrant activation of pDCs, a DC subset that specializes in T1-IFN production, may contribute to the development of this signature, perhaps through ineffective clearance of islet cell debris (36, 179, 180). Moreover, innate sensing by TLRs represents an essential pathway for the stimulation of T1-IFNs. Accordingly, ablation of MDA-5 (encoded by *Ifih1*) in NOD mice results in protection from spontaneous T1D development, while NOD mice carrying a single allele of MDA-5 experience slowed progression and reduced incidence (181). MDA-$5^{+/-}$ animals also displayed protection from Coxsackie B4 virus-induced T1D when compared to MDA-$5^{+/+}$ littermates that developed disease despite being able to efficiently clear the virus (181). Further investigations have revealed that CB4 infection of MDA-$5^{+/-}$ mice resulted in a transient increase in IFNβ that returned to baseline by 7 days postinfection, while IFNβ levels in MDA-$5^{+/+}$ mice remain consistently elevated after infection (181). These data suggest that protective allotypes of MDA-5 may act in a similar manner to tightly regulate IFN production while keeping antiviral defense mechanisms intact (181). Accordingly, stimulation of TLR7, which recognizes ssRNA to promote T1-IFN production, results in accelerated T1D onset in NOD animals, whereas abrogation of TLR9 signaling, important for the response to unmethylated DNA, retards progressive islet destruction (182, 183). Inhibition of T1-IFN signaling through the heterodimeric IFNAR has presented conflicted results. Incidence in NOD and NOD.IFNAR$1^{-/-}$ was indistinguishable, however short-course administration of an IFNAR1 blocking antibody to NOD animals 15–25 postpartum significantly delayed the onset of diabetes (36, 184, 185). Recently, CRISPR-Cas9 deletion of the IFNAR1 subunit in LEW.1WR1 rats, a newly described animal model for T1D, caused delayed onset and frequency of Poly(I:C) induced diabetes (186, 187). Taken together, these data support the idea that coordinated activation of T1-IFN is an early event in autoimmune diabetes but its role in disease progression is likely heavily influenced by the immune response to environmental cues and inheritance of risk/resistance alleles in genes that impact T1-IFN production or signaling.

Utilization of transgenic model systems during the late 20th century provided strong evidence that T1-IFNs may act to accelerate diabetes pathogenesis. Overexpression of IFNα or IFNκ in pancreatic β cells of mice not normally prone to T1D leads to onset of diabetes with severe insulitis, hypoinsulinemia, and diabetes (35, 188). Transgenic mice expressing of IFNβ under the control of the rat INS promoter display various phenotypes depending on genetic background. For example, C57BL6/SJL mice with the RIP-IFNβ transgene do not develop overt diabetes, but display mild hyperglycemia with decreased glucose-stimulated INS secretion and impaired glucose tolerance characteristic of a pre-diabetic state (34). However, overexpression of IFNβ in the islets of other mouse strains that are not prone to developing T1D, including the non-obese diabetes resistant, induced spontaneous diabetes development (34, 189). Moreover, NOD RIP-IFNβ mice had accelerated and fulminant onset of T1D (189). Taken together, these data demonstrate that T1-IFNs can act as a spark leading to autoimmunity but only in individuals that possess an inherent risk for development of T1D. Further, these data demonstrate that T1-IFNs in the islet microenvironment result in deleterious effects on β cell function and viability by promoting islet inflammation.

T1-IFNs ARE MAJOR PLAYERS IN T1D

Although T1-IFNs have been associated with the induction of T1D and have been identified as a consistent component of the islet autoinflammatory milieu, the direct impact of these cytokines on the pancreatic β cell, cytotoxic T-lymphocytes, and other cellular constituents within the islet that facilitate ongoing islet autoimmunity have only recently been studied using human systems (18, 19, 160, 190). The defining feature observed in T1D is the hyper expression of MHC-I in the islets of patients with T1D, suggesting enhanced β cell immunogenicity and increased susceptibility for targeting by CTLs (1, 2, 18, 19, 190). T1-IFN represent a likely candidate within the local microenvironment capable of mediating this effect, as IFNα/β have been shown to directly induce MHC expression on primary human islet cells [**Figure 2** and (21, 109)]. Recent findings by Marroqui et al. demonstrate that IFNα induced HLA is dependent upon canonical T1-IFN signaling, with TYK2, STAT2, and IRF9 being critically required for induction of HLA class I (109). Another notable finding within the islets of new onset T1D patients is elevated levels of the chemoattractant, CXCL10 (191, 192). Touted as a well-known ISG, CXCL10 is induced by IFNα in primary human islets (109). Our laboratory has corroborated these data, showing that exposure of primary islets to T1-IFN results in significant increases in cell surface Class I HLA by flow cytometry as well as increased mRNA expression of MHC-I and CXCL10 by transcriptome analysis (193). Furthermore, we also find upregulation of transcripts critically required for the MHC-I antigen processing and presentation. Enhanced expression of immunoproteasome subunits PSMB8 and PSMB9 (**Figure 2**) along with proteasome activator subunits PSME1 and PSME2 by T1-IFN suggests an increased efficiency of peptide generation under conditions of inflammatory stress and ATP depletion (194–196). Analysis of constituents of the peptide loading complex following T1-IFN exposure reveal a significant increase in TAP1, TAP2, TAPBP, chaperones, and the editing enzyme ERAP1 suggesting increased transport, stable processing, and loading of peptides onto MHC-I within the endoplasmic reticulum (ER) [(196) and **Figure 2**]. Additionally, there is a global augmentation of antigen processing and enhanced surface MHC-I with functional reductions in β cell mass, as priming of β cells with T1-IFN results in enhanced CTL-induced lysis by chromium release assay [(193) and **Figure 2**].

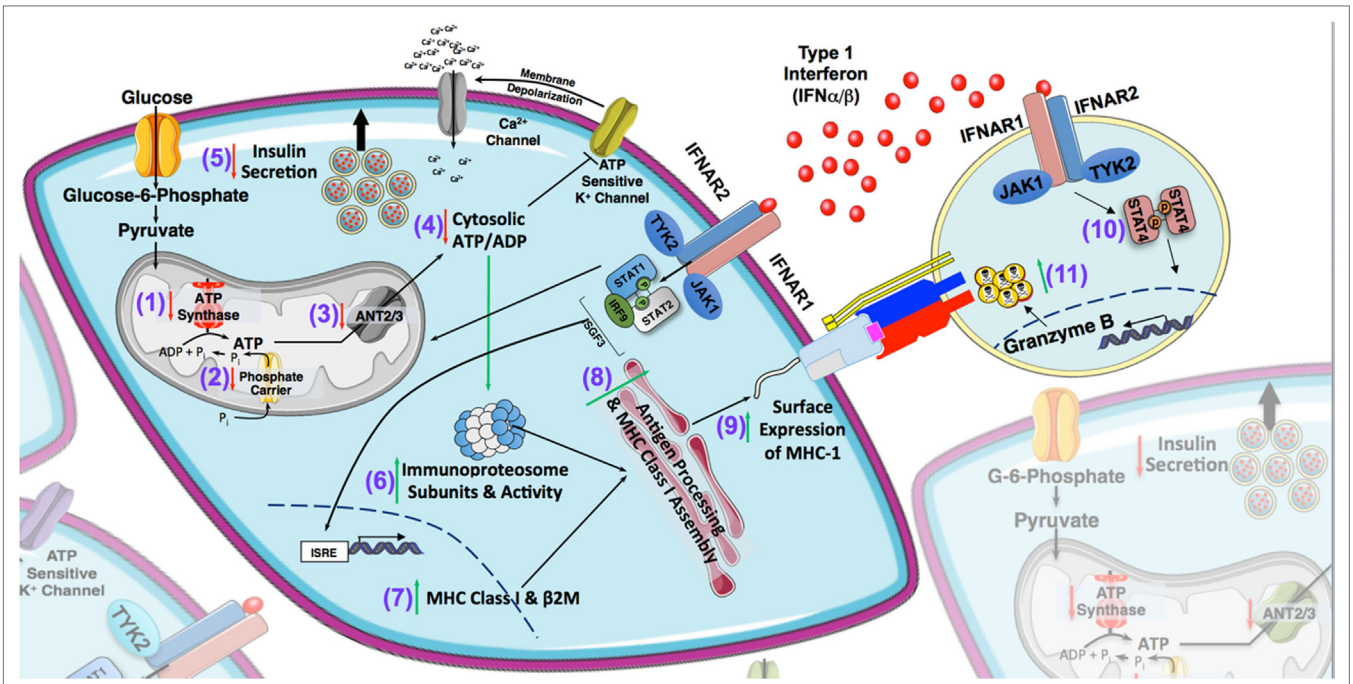

FIGURE 2 | Type 1 interferons (T1-IFNs) are a catastrophic feature of the islet microenvironment in type 1 diabetes (T1D). Based on previous literature and current findings, T1-IFNs are consistently found in the islet autoinflammatory milieu and represent a viable signal that may precipitate diabetogenicity in T1D. With respect to β cells, these cytokines can impair insulin secretory function, possibly through the induction of endoplasmic reticulum (ER) stress as well as by impairing mitochondrial bioenergetics. Whole transcriptome analysis reveals decreased expression of genes involved in the regulation of ATP production and transport, including ATP5A (1), SLC25A3 (2), and SLC25A5/6 (3). Reduction in these transcripts likely lead to decreases in the cytosolic ATP/ADP ratio (4) that is required for glucose-stimulated insulin secretion by β cells (5). T1-IFNs also enhance the autoimmune surveillance of pancreatic β cells through induction of the immunoproteasome (6), *de novo* synthesis of MHC class I and genes responsible for the peptide loading complex (7 and 8), as well as enhanced surface expression of MHC class I (9). This increased capacity for antigen presentation results in a functional ability of cytotoxic $CD8^+$ T lymphocyte (CTL)-mediated β cell destruction, which is further augmented by the ability of T1-IFN to amplify infiltrating CTL cytotoxic capacity through STAT4-induced granzyme B production (10 and 11).

Two recent studies have noted the impact of IFNα on β cells. Using IFNα both groups determined that this cytokine induced the unfolded protein response (UPR) leading to ER stress. However, neither publication reported negative impacts on β cell viability, suggesting that ER stress induced by IFNα did not impact cell death, and there was no reported functional changes (109, 110). While these two studies demonstrate increased expression of markers that signal ER stress, the induction of this response differed in timing and severity, which likely points to differences in experimental design and methodology (109, 110). Indeed, these reports utilized different culture conditions including different media formulations as well as very different time courses of study. For instance, Marroqui et al. revealed an elevated expression of ATF3 and CHOP in primary human islets following 24 h of IFNα (2,000 U/mL) exposure (109). The study conducted by Lombardi and Tomer more widespread induction of the UPR and also assessed INS secretory function in primary human islets and EndoC-βH1 cells after 2 days of exposure to 1,000 U/mL of IFNα. They detected no alterations in glucose stimulated INS secretion, but did correlate the induction of ER stress with reductions in INS content, increased proinsulin to INS ratio, in addition to reduced expression of prohormone convertases, PC1, and PC2 (110). Although ER stress has been a frequently hypothesized explanation for β cell dysfunction in T1D, the idea that IFNα elicits expression of genes involved in the UPR presents a conundrum (197, 198). Previous reports demonstrate that ER stress actually impairs MHC-I expression. These differences in findings of these two recent publications with the discordance of coexisting ER stress and enhanced ER antigen processing highlight the need for a greater understanding of how the numerous signals provoked by T1-IFN alter the β cell in T1D (199–201). Further inspection of metabolic pathways responsible for coordinating INS secretion in β cells by transcriptome analysis revealed a decreased expression ATP5A1, a subunit required for ATP production by ATP synthase; decreased expression of adenine nucleotide translocases 2 and 3 (SLC25A5 and SLC25A6), responsible for regulating mitochondrial ATP export, and decreased expression of the mitochondrial phosphate carrier, SLC25A3 [(193) and **Figure 2**]. A reduction in these genes will likely have major implications on regulation of glucose-stimulated secretion as they directly alter ATP/ADP ratios that are required to trigger islet cell depolarization that leads to release of INS secretory granules.

Another very important component of the islet microenvironment is vascular endothelial cells that facilitate delivery of

oxygen and enable the rapid exchange of nutrients and hormones between the blood and the endocrine pancreas. These cells also act as a barrier to intricately regulate trafficking and extravasation of autoreactive immune cells into the islet microenvironment. Several studies have shown that endothelial cells in and around the islets display an activated phenotype that likely contributes to increased homing and recruitment of autoreactive T cells (202). Immunohistochemical studies examining endothelium within the pancreata of recent onset patients with T1D reveal elevated expression of ICAM-1 as well as hyperexpression of MHC-I and -II (83, 190, 203). Expression of these molecules has also been associated with concomitant expression of IFNα (190). In line with these studies, IFNα is known to directly induce MHC-I and expression of ICAM-1 in endothelial cells, suggesting that these cytokines may increase the capacity for antigen presentation required for autoreactive CTLs to gain entry into the islet (204, 205). Additionally, human pancreatic islet endothelial cells are able to be infected by coxsackievirus B resulting in the production of IFNα, induction of adhesion molecules, and increased interaction with immune cells (206). Mounting evidence suggest that the crosstalk between β cells and the endothelium is important for INS secretory function (207). However, more investigation into the role of T1-IFNs in modulating this interaction is warranted.

It is well known that tissue microenvironments are key determinants in driving local immune responses models of cancer and infectious disease. While armed with the ability to modulate the innate and adaptive arms of the immune system, the impact of T1-IFN within the islet microenvironment has not been fully elucidated. Known to contribute to T cell priming and activation through their effects on DCs, T1-IFNs have been directly shown to mediate DC maturation and migration even in the absence of PPR engagement (208, 209). Specifically, they facilitate the metabolic switch from oxidative phosphorylation to glycolysis through regulation of the transcription factor HIF-1α, inducing the upregulation of MHC-I in these cells as well as costimulatory molecules (208, 209). In the case of the autoimmune diabetogenic microenvironment, the presence of T1-IFNs may act to promote DC immunogenicity skewing toward proinflammatory immune activation in addition to augmenting the function of islet infiltrating immune cells, such as $CD8^+$ T cells. Studies completed in our laboratory suggest that T1-IFN drastically augment cytotoxicity elicited by human islet-reactive CTLs. Extensive characterization of T1-IFN signaling mechanisms within these cells show that these cytokines can induce a remarkably rapid acquisition of effector function through induction and direct binding of pSTAT4 to the promoter of Granzyme B (**Figure 2**). In accordance with studies exhibiting full acquisition of autoreactive CTL effector function within the pancreatic microenvironment, these novel studies implicate T1-IFN as a putative innate signal capable of driving CTL differentiation in the islet (106).

CONCLUSION AND MODEL DETAILING HOW IFNα CAN WREAK HAVOC IN THE DIABETIC MICROENVIRONMENT

Several determinants predict an individual's susceptibility to T1D. It is well appreciated that the immune system plays a critical role in diminishing β cell mass, precipitating the onset of persistent hyperglycemia. Critical to this destruction is the presence of $CD8^+$ T cells within the diabetic microenvironment. These cells enter the pancreas where they directly target and kill β cells through interactions of the TCR with elevated MHC-I expression on β cells. Soluble factors, such as T1-IFNs, act to promote islet autoimmunity. In addition to being linked to the hallmark HLA class I hyper-expression observed in islets of patients with T1D, T1-IFNs are also well known for their wide-ranging effects including modulation of innate and adaptive immune responses, especially in T lymphocytes. However, until now, few studies to date have focused on elucidating how T1-IFN signaling transforms the islet to an environment that promotes diabetogenicity. The work reviewed here demonstrates that T1-IFNs are stimuli that promote dysfunction and increased visibility of target β cells alongside enhanced CTL effector function leading to β cell destruction.

Association of T1-IFN with T1D reported in previous studies together with our current findings makes a strong case that these cytokines play some role in the complexity of the diabetes puzzle (summarized in **Figure 2**). It is likely that a genetic predisposition skewed toward dysfunctional T1-IFN responses create an islet environment permissive to enhanced autoantigen presentation, augmented human β cell-specific cytotoxicity by autoreactive CTLs and resulting β cell dysfunction. While the pleiotropic actions of T1-IFNs are designed to strengthen the immune response to viral pathogens, this response proves detrimental in the case of autoimmunity where the immune response is misdirected toward self and in this way can promote β cell death in T1D.

AUTHOR CONTRIBUTIONS

This manuscript was conceived by BN and CM. Interpretation of data/results and discussion were completed by BN and CM. Manuscript was written and revised by BN and CM.

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REVIEW
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Understanding Autoimmune Diabetes through the Prism of the Tri-Molecular Complex

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The strongest susceptibility allele for Type 1 Diabetes (T1D) is human leukocyte antigen (HLA), which supports a central role for T cells as the drivers of autoimmunity. However, the precise mechanisms that allow thymic escape and peripheral activation of beta cell antigen-specific T cells are still largely unknown. Studies performed with the non-obese diabetic (NOD) mouse have challenged several immunological dogmas, and have made the NOD mouse a key experimental system to study the steps of immunodysregulation that lead to autoimmune diabetes. The structural similarities between the NOD I-A^{g7} and HLA-DQ8 have revealed the stability of the T cell receptor (TCR)/HLA/peptide tri-molecular complex as an important parameter in the development of autoimmune T cells, as well as afforded insights into the key antigens targeted in T1D. In this review, we will provide a summary of the current understanding with regard to autoimmune T cell development, the significance of the antigens targeted in T1D, and the relationship between TCR affinity and immune regulation.

Keywords: T cell, autoimmunity, type 1 diabetes, human leukocyte antigen, regulatory T cell, thymic selection

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INTRODUCTION

Autoimmunity is generally associated with polygenetic susceptibility, while the initial precipitating event is likely triggered by an environmental stressor (1–4). The major alleles associated with most autoimmune disorders are the human leukocyte antigen (HLA), and several alleles are shared among autoimmune conditions (5–8). This suggests that a common T cell-dependent mechanism is the underlying cause of tissue-specific autoimmunity irrespective of the organ or tissue being targeted. Although several hypotheses have been put forth to explain the HLA-mediated susceptibility, the exact mechanisms are still largely unknown. HLA structure selects for a particular peptide sequence motif and can affect the stability of the peptide:HLA complex (9). It is likely that autoimmune epitopes are not efficiently presented within the susceptible HLA molecules during thymic selection, or alternatively are presented with increased stability or at a higher concentration in the target tissue (10). Clearly, HLA allele structure is not the only parameter that might affect the stability of the tri-molecular complex [T cell receptor (TCR)/HLA/peptide], and not all individuals with T1D possess susceptible HLA alleles. Lower level of tissue antigen expression in the thymus, the relative abundance of self-antigen at the tissue site, an increase in immunogenicity of self-peptides either *via* post-translational modifications (PTMs) or molecular mimicry could all influence the stimulatory capacity of peptide:HLA complexes in periphery (**Figure 1**). How these changes in epitope immunogenicity could affect disease development will be discussed in this review.

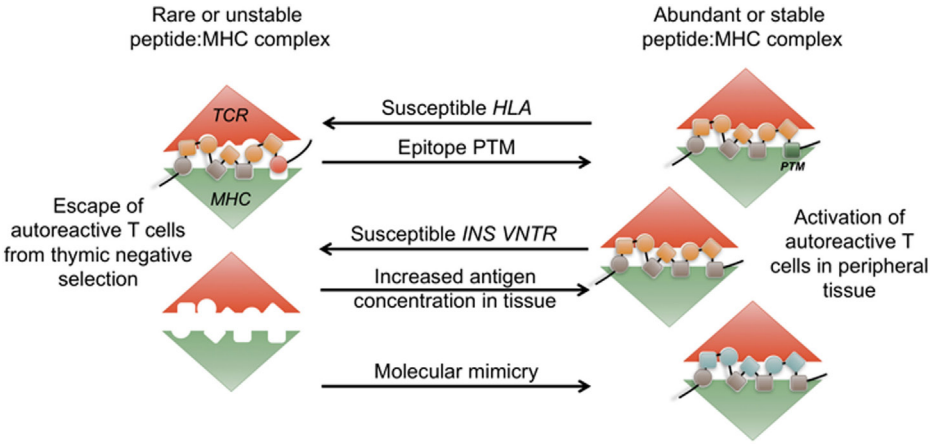

FIGURE 1 | Abundance and stability of the tri-molecular complex at the interface of tolerance and autoimmunity. During thymic development, rare or unstable self-peptide: major histocompatibility (MHC) complexes can lead to escape of autoimmune T cells. Human leukocyte antigen (HLA)-DQ8 and H2-IA^{g7} susceptible alleles form unstable complexes with insulin epitope B:9-23. *INS-VNTR* susceptible allele results in lower level of insulin presentation in the thymus. Post-translational modifications (PTM) of self-epitopes can lead to more stable complexes in periphery. Increase in antigen availability in periphery or presence of structurally similar peptides in the context of infection (molecular mimicry) leads to priming of autoimmune T cells.

The spontaneously diabetic non-obese diabetic (NOD) mouse model has been a useful system for identification of the key mechanisms important in the development of autoimmunity due to its significant similarity to human T1D (11, 12). Nearly 6 years after HLA was first associated with T1D in humans (13, 14), the spontaneously generated NOD diabetic strain was obtained by the Jackson Laboratory from CLEA Japan, where it quickly became an invaluable tool in the etiology of T1D (11, 15). The importance of the major histocompatibility (MHC) locus was originally traced by congenic approach, where MHC locus was introgressed onto the NOD background (16, 17). Further analysis of mice that received a non-NOD MHC class II transgene confirmed the important contribution of I-A^{g7} to diabetes susceptibility (18). Although MHC II confers most of the susceptibility, there are over 50 genetic loci that make up the NOD diabetic phenotype (19). The polygenetic susceptibility of the NOD mouse strain mirrors human disease, and further underlies the complexity of T1D (20). Importantly, the I-A^{g7} MHC II variant has structural similarities with human susceptible DQ8 (DQA1*0301/DQB1*0302) (9, 21, 22). Moreover, many of the antigens targeted in autoimmune diabetes are shared between the two species (19). The similarities of the shallow and positively charged peptide-binding groove characteristic of both human DQ8 and mouse I-A^{g7}, and significant concordance in antigenic targets have made it possible to uncover sequence characteristics of autoimmune epitopes that are relevant to human disease (23, 24). Nevertheless, the precipitating events that lead to T cell priming and beta cell destruction remain unclear (4, 25). While the NOD mouse model has been a prolific tool for mechanistic insight into the many facets of T1D pathogenesis, recent expansion of HLA-humanized mouse models now allow direct interrogation of human autoimmune tri-molecular complex (TCR/HLA/peptide) and its role in loss of self-tolerance.

EVIDENCE FOR T CELL-MEDIATED T1D

A large body of evidence accumulated over several decades has implicated beta cell-specific immune response and, in particular, beta cell-specific T cells as the main drivers of autoimmune tissue damage and development of T1D (12, 26, 27). Progression to disease in humans is associated with islet antigen-specific antibody responses, and T cells specific to islet antigens are found at higher frequencies in T1D patients (28–31). Importantly, both CD4 and CD8 T cells were observed directly in the pancreatic lesions, and islet antigen-specific T cells have been cloned from pancreatic islets of T1D organ donors (32–38). HLA, being the major risk allele, implies that inherent structural differences in HLA and, consequently, TCRs selected on those HLA alleles lead to erroneous T cell reactivity to self (5, 39, 40). While class II HLA alleles confer the majority of the genetic susceptibility, certain class I alleles have been shown to impose a separate risk (41). Multiple antigens are targeted by both CD4 and CD8 T cells in T1D. Beta cell-specific antigens presented by Class II molecules include preproinsulin (PPI), insulinoma-associated antigen (I-A2), glutamic acid decarboxylase (GAD) 65, heat shock protein (HSP)-60, HSP-70, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), and zinc transporter (ZnT8) (42–44). While MHC class I responses display similar wide range of antigenic targets, including PPI signal peptide, IA2, ZNT8, human islet amyloid polypeptide (IAPP), IGRP, and GAD65 (45). The progression to T1D in humans is associated with accumulation of islet antigen antibody reactivity to IAA, GAD65, IA-2, and ZnT8, which mirrors the intra- and intermolecular "antigenic spread" of T cell responses (46, 47). Other non-HLA allelic risk variants are associated with pathways involved in T cell development, activation, and function, further highlighting the importance of T cells are the key drivers of autoimmunity (19).

HLA MECHANISMS OF AUTOIMMUNITY

While the precise mechanisms that lead to loss of tolerance are multifaceted, HLA-DQ8 susceptibility implies that the stability of the tri-molecular complex is an important aspect that underlies autoimmune T cell responses (**Figure 1**). The inbred NOD mouse model that possesses a single susceptible MHC class II allele I-A^{g7} (I-$A^{d}\alpha$/I-$A^{g7}\beta$) has played a vital role in uncovering the mechanisms involved in the development of T1D. The structural similarities characterized by the shallow peptide-binding groove and the positive charge in the p9 peptide-binding pocket present in both I-A^{g7} and human HLA-DQ8 point to a similar mechanism of autoimmune susceptibility (22). The potential mechanisms include altered thymic selection due to peptide:MHC instability, and/or preferential binding and presentation of beta cell neo-antigens formed *via* post-transnational modifications in the periphery (**Figure 1**). Biochemical analyses revealed a propensity for both DQ8 and I-A^{g7} to bind peptides with negatively charged C-terminus (48). In the case of celiac disease, which is also associated with DQ8 susceptibility, gluten peptides targeted in disease have a negative charge at the C-terminus, which results in their stable binding to DQ8 (49). Although this observation suggests that key epitopes targeted in T1D should similarly contain negatively charged residues at p9, most beta cell antigenic epitopes lack this trait. Moreover, the dominant insulin epitope B:9-23 has a positively charged arginine at the C-terminus. Nevertheless, in support of this hypothesis, a mutation of $InsB_{9-23}$ at presumptive p9 to a negatively charged glutamic acid increased the immunogenicity of the epitope and augmented the activation of insulin-specific T cells (50). In addition, a recent study has identified IAPP and Chromogranin A (ChgA) epitopes in beta cells that have been modified by peptide fusion to acquire a negative charge at the C-terminus (35). The modified peptides were significantly more immunogenic compared to unmodified wild-type epitopes. This groundbreaking finding offered a potential explanation for lack of efficient thymic selection under conditions of unstable tri-molecular complex formation in the thymus, followed by priming and activation of autoreactive T cells in response to modified and stable peptide:MHC complexes in peripheral tissue.

THYMIC DEVELOPMENT OF AUTOREACTIVE T CELLS—WHAT IS THE EVIDENCE FOR ALTERED THYMIC SELECTION IN AUTOIMMUNITY?

A body of evidence suggests an important role for altered thymic selection in the development of autoimmunity. Negative selection of autoimmune lymphocytes depends on sufficient amount of self-antigen available for presentation in the thymus, which is regulated by intra-thymic and extra-thymic sources, genetic variation in tissue antigen promoters, and effective antigen presentation on certain HLA alleles (**Figures 1** and **2A**). Normally, tissue-specific antigens are presented by *Autoimmune regulator* (*Aire*) and *Fezf2* expressing thymic medullary epithelial cells (mTECs) to aide in the deletion of self-reactive thymocytes (51, 52). MTECs can also transfer antigens, including beta cell antigens, to thymic resident dendritic cells (DCs), which in turn delete self-reactive T cells (53). Both DCs and *Aire* expressing mTECs are also essential in generating thymically derived $Foxp3^{+}$ regulatory T cells (Tregs), a critical population for the establishment and maintenance of self-tolerance (54, 55). Indeed, there appears to be a correlation between a reduction in DC numbers and residual β cell function in T1D subjects (56), while the NOD mouse exhibits an overall reduction in

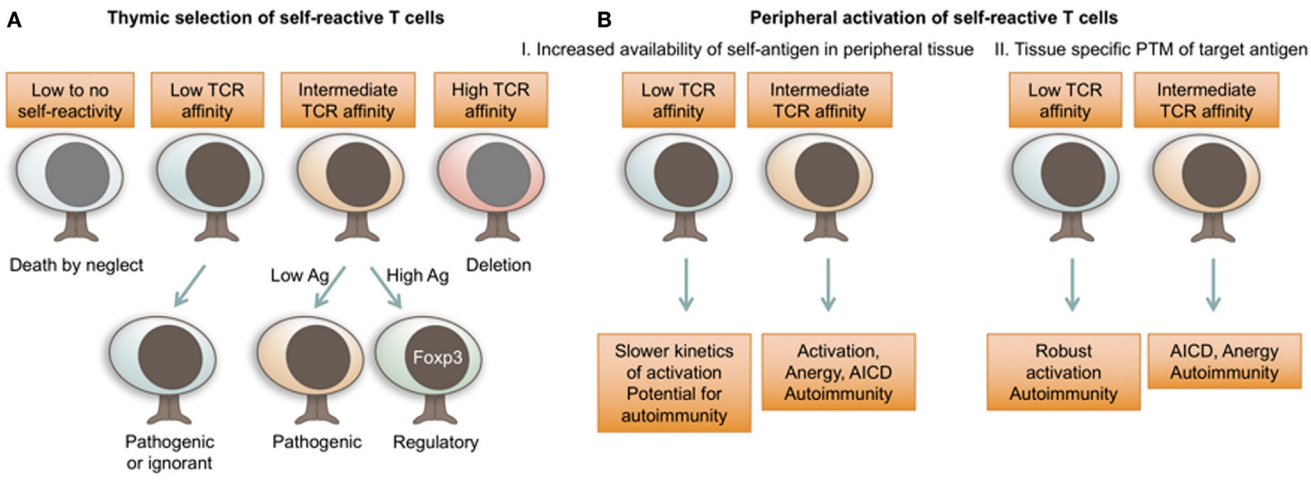

FIGURE 2 | T cell receptor (TCR) affinity for self dictates autoimmune T cell fate decisions. **(A)** TCR affinity for self-ligands and antigen availability dictate thymocyte fate choices during thymic selection. While autoimmune T cells can be selected with a range of TCR affinities, increased antigen availability and relatively stronger self-reactivity will preferentially result in the development of regulatory $Foxp3^{+}$ T cells. **(B)** In peripheral tissues, self-reactive T cells are activated in response to increased concentrations of tissue antigen or highly immunogenic PTM antigens. While autoimmune T cells can possess a range of TCR affinities for self-antigen, lower affinity TCRs are less susceptible to peripheral mechanisms of tolerance. Ag, antigen; AICD, activation induced cell death; PTM, post-translational modification.

DCs (57, 58). These observations suggest a relationship between self-tolerance and the absolute number of DCs present in the thymus and periphery. However, not all peripheral antigens are expressed by mTECs and, therefore, negative selection must also rely on peripheral antigen retrieval and delivery to the thymus by DCs. Importantly, studies have shown that the generation of thymic regulatory T cells by antigen-presenting mTECs and DCs early in life (neonatal) is critical in maintaining tolerance to self (55, 59). The idea of peripheral antigen exposure generating tissue-specific Tregs was elegantly demonstrated by Scharschmidt et. al., where skin colonization of *S. epidermidis* allowed for the development and trafficking of microflora-specific Tregs to the skin. Using sphingosine-1-phosphate receptor antagonist, FTY720, the authors blocked Treg egress and pinpointed the thymus as the main source for Treg development (59).

There is no direct evidence for thymic selection deficiencies in individuals with T1D; however, several key observations suggest that there is a role for altered selection in the development of autoimmune responses to insulin. The level of thymic insulin expression in humans is controlled in part by the polymorphic variable number of tandem nucleotide repeats found in the region proximal to the promoter region of the insulin gene (*INS*–VNTR) (60). It has been shown that VNTR I alleles express 26–63 tandem repeats while the VNTR III carries 141–209 repeats. This difference translates into higher thymic transcript levels for the VNTR III individuals and a threefold to fourfold relative protection from T1D (61, 62). It appears that the number of repeats affects AIRE binding to the insulin promoter region, thus controlling transcriptional regulation of insulin in the thymus (51, 62, 63). In support of alterations in thymic selection, analysis of human peripheral blood from T1D patients and healthy controls revealed that subjects expressing the *INS*-VNTR I (T1D-predisposing) allele displayed elevated frequencies of high affinity proinsulin-specific T cells compared to *INS*–VNTR I HLA–DR4 subjects (64). *INS*-VNTR allelic expression appears to determine insulin reactivity rather than the total number of insulin-reactive T cells, as both VNTR I and VNTR III groups displayed similar total number of insulin-reactive T cells in peripheral blood (64). However, it has only been hypothesized that the differences in thymic insulin expression between VNTR I and VNTR III subjects influence positive and negative selections of insulin-reactive T cells, but this has never been formally demonstrated *in vivo* due to a lack of VNTR mouse models.

The role of thymic insulin expression in the establishment of central tolerance has been addressed in the NOD mouse model by both deletion and overexpression of insulin in the thymus. Deletion of insulin specifically in thymic *Aire* expressing mTECs enhanced diabetes development in both male and female mice (65). In addition, transgenic overexpression of proinsulin, but not GAD65 (66) or IGRP (67), significantly delayed (68) or prevented (69) diabetes progression in NOD mice. However, in these studies overexpression of insulin was targeted to all MHC class II expressing APCs and, therefore, the relative role of central compared to peripheral tolerance was not determined (68, 69). A more recent set of experiments determined that a narrow window of ectopic proinsulin expression in APCs (from birth until weaning) could prevent the development of diabetes in NOD mice (70). This timeframe fits with a previous study that showed organ specific autoreactive T cell escape from the thymus is greatest during the first 10 days of life in NOD mice (71). In the former study by Jhala et. al., protection was due in part to the deletion of insulin-specific T cells, but also the inability of the remaining insulin-specific T cells to respond to cognate antigen in periphery (70). In our recent study, we tested two TCRs (4-8 and 12-4.1, **Table 1**) with defined affinities for $InsB_{9–23}$ for their ability to escape negative selection in the presence of ectopic overexpression of insulin. Surprisingly, we did not observe any increase in thymic deletion of the relatively high (4-8) or low (12-4.1) TCRs, although the increase in insulin expression did protect mice from developing autoimmune diabetes. Protection from disease appeared to be due to an increase in Treg development with a significant increase in thymic, splenic, and pancreas-residing insulin-specific Tregs (72). These findings pose an intriguing possibility that the amount or stability of self-peptide:MHC complexes during thymic selection is more important for Treg development rather than deletion of self-reactive T cells (**Figure 2A**).

Chromogranin A is the only other currently known beta cell antigen necessary for the initiation of autoimmune diabetes in NOD mice; however, expression of ChgA in the thymus has not yet been detected (73). Therefore, tolerance to ChgA may rely in part on transport of antigen by peripheral DCs to the thymus. Whether islet-derived antigens are carried to the thymus to promote islet-specific Treg development has not been explored; nevertheless, the divergent TCR repertoire between islet-infiltrating effector and regulatory T cells suggests a lack of local Treg conversion in favor of thymic lineage being the predominant Treg population in the pancreas (74). The thymic Treg niche was thought to be highly specialized and restricted (75); however, a recent study has demonstrated that the manipulation of either the number of antigen-presenting cells or an increase in antigen exposure within the thymus can expand the Treg niche (54).

While highly self-reactive T cells are removed from the T cell repertoire by negative selection, the quality and the quantity of self-reactive Tregs that develop from the moderately self-reactive thymocyte pool is a critical component of peripheral self-tolerance (**Figure 2A**). This idea is consistent with the observation that healthy individuals possess significant frequencies of self-reactive T cells, but are free from autoimmunity (76, 77). The escape of self-reactive T cells in itself is not just a byproduct of Treg development, but seems to serve an important immunological purpose, since some level of self-reactivity is associated with enhanced responsiveness to foreign pathogens (78–80). It is likely that the fine balance between beneficial self-reactivity and self-tolerance is uniquely perturbed in individuals with a susceptible genetic background. A slight change in thymic antigen expression or the overall stability of the tri-molecular complex could shift the T cell development spectrum toward Treg insufficiency, rather than escape of higher affinity cells. Therefore, the ratio of beta cell antigen-reactive Tregs vs. effector or memory T cells might be a better predictive biomarker of autoimmunity than the overall frequencies of self-reactive cells.

TABLE 1 | Pathogenicity of beta antigen-reactive T cells.

T cell receptor	Restriction	Epitope	Model	Infiltration	% Diabetes	Reference
			Mouse			
Chromogranin A (ChgA)						
BDC2.5	IAg7	ChgA 359–372	Tg/Rg	Insulitis	75/100	(83, 96)
BDC10.1	IAg7	ChgA 359–372	Rg	Insulitis	100	(83)
Insulin						
12.4-1	IAg7	InsB 9–23	Tg/Rg	Insulitis	5/50/72	(82, 83, 103, 104)
12.4-4	IAg7	InsB 9–23	Rg	Insulitis	51	(82)
12.4-4m1	IAg7	InsB 9–23	Rg	Peri-insulitis	–	(82)
8-1.1	IAg7	InsB 9–23	Rg	Insulitis	27	(82)
P2	IAg7	InsB 9–23	Rg	No	–	(82)
1-10	IAg7	InsB 9–23	Rg	Peri-insulitis	48	(82)
4-8	IAg7	InsB 9–23	Rg	Insulitis	59	(82)
3-4	IAg7	InsB 9–23	Rg	Insulitis	21	(82)
G9C8	Kd/Db	InsB 15–23	Tg	Mild insulitis	–	(105)
2H6	IAg7	InsB 9–23	Tg	Prevents diabetes	–	(97)
8F10	IAg7	InsB 9–23	Tg	Insulitis	100	(100)
Glutamic acid decarboxylase (GAD)						
PA17.9G7	IAg7	GAD65 284–300	Rg	no	–	(83)
PA15.14B12	IAg7	GAD65 206–220	Rg	no	–	(83)
PA19.5E11	IAg7	GAD65 206–220	Rg	Peri-insulitis	–	(83)
PA18.10E1	IAg7	GAD65 524–538	Rg	n/d	–	(96)
PA18.9H7	IAg7	GAD65 524–538	Rg	Peri-insulitis	–	(83)
IA4	IAg7	GAD65 217–236	Rg	Peri-insulitis	–	(83)
Protein tyrosine phosphatase-like (IA2)						
Phogrin 13	IAg7	IA2 640–659	Rg	Peri-insulitis	–	(83)
Phogrin 18	IAg7	IA2 755–777	Rg	Mild insulitis	–	(83)
10.23	IAg7	IA2 676–688	Rg	Peri-insulitis	–	(83)
Iselt-specific glucose-6-phosphatase (IGRP)						
8.3	Kd	IGRP 206–214	Tg	Insulitis	33	(95)
Islet amyloid polypeptide (IAPP)						
BDC6.9	IAg7	DLQTLAL-NAAR (Ins-IAPP fusion)	Tg/Rg	Insulitis	56	(35, 83)
Unknown islet antigen						
NY4.1	IAg7		Tg/Rg	Insulitis	72/60/71	(83, 95, 96)
AI4	Db		Tg	Insulitis	100	(98, 102)
			Human			
Glutamic acid decarboxylase (GAD)						
164	DR4	GAD65/67 555–567	Tg	Insulitis	–	(101)
T1D4	DR4	GAD65 115–127	Rg	Mild to no insulitis	–	(99)

TCR PARAMETERS OF T CELL PATHOGENICITY

Autoimmune T cell responses in general, as well as, the T cell population that infiltrates the NOD pancreatic islets, are composed of cells with different T cell lineages, diabetogenic or regulatory capabilities, antigenic specificities, and TCR affinities (10, 81–83). All of these parameters are directly influenced by the TCR (84). Therefore, TCR sequence, specificity, and affinity hold the key to understanding the dynamics of diabetogenic T cell responses during chronic progressive autoimmune disorders, such as T1D. The antigenic specificity of each TCR is dictated by the highly variable CDR3 region found within the α and β chains of the TCR heterodimer. The variability is the result of random genetic recombination events that bring together one of many variable (V) genetic segments with a joining (J) region. The large number of TCR sequences infiltrating an organ, their variability among individuals, and the heterodimeric structure of the TCR has been a significant roadblock in a comprehensive functional analysis of TCRs. In this section, we will summarize the studies that have investigated beta cell-specific TCR parameters for their ability to predict T cell pathogenic potential.

TCR Sequence As a Biomarker of Pathogenicity

One of the main hurdles in the identification and functional analysis of beta cell-reactive T cells in humans with T1D is the breadth of antigens and epitopes that are targeted among affected individuals. Peptide/MHC tetrameric reagents have been the most effective approach to identify T cells with autoimmune potential; however, beta cell-reactive cells comprise a small population of peripheral blood, which makes such approaches technically challenging. Moreover, tetramers detect only the highest affinity subpopulation of T cells specific for a particular epitope, while the majority of autoimmune responder T cells are

often overlooked, as was effectively demonstrated in the mouse model of multiple sclerosis (85). As such, the field is currently lacking sufficient approaches to perform in depth tracking of antigen-specific T cells over time. Recent technological advances in high-throughput sequencing have opened new avenues for tracking self-reactive T cells and could be easily applicable to studies of human tissue-infiltrating T cells (86). A promising biomarker approach could be based on high-throughput TCR sequencing with focus on TCR motifs known to be associated with a specific target epitope. While human CD4 responses have proven to be highly diverse (87, 88), CD8 T cells are generally more clonotypic (86). A recent study was able to identify a public CDR3 motif associated with $IGRP_{265–273}$ specific memory T cells in antibody-positive subjects and individuals diagnosed with T1D (89). Their findings suggest that dominant clonotypes persist in the same individual over time, and some TCR sequences could be shared among individuals. Interestingly, the public TCR motif was also identified in healthy controls, although it was restricted to the naïve T cell compartment. While promising as a potential biomarker, such deep sequencing approaches necessitate knowledge of multiple TCR sequences associated with reactivity to several beta cell antigens.

Antigen Specificity of Pathogenic TCRs

Although, T cells of multiple antigenic reactivities have been isolated from pancreatic islets of T1D donors (35–38), it does not necessitate that these cells are equally pathogenic or are actively involved in beta cell destruction. In order to identify potentially important initiating antigens in T1D, multiple beta cell proteins have been mutated on the NOD background, including IAPP, GAD65, insulin, IGRP, and islet Ag-2 (90–94). Interestingly, only the mutation of insulin and chromogranin resulted in protection against diabetes (73, 94). This suggests that insulin and chromogranin-reactive T cells are either critical for the initiation of autoimmunity, or are necessary for further propagation of the disease and the ultimate destruction of beta cells.

Over the years, pathogenic potential of T cells reactive to various islet antigens was directly assessed in single TCR systems. Multiple mouse and a few human TCRs reactive against various beta cell proteins have been expressed in mice utilizing both transgenic and retrogenic approaches (82, 83, 95–105) (**Table 1**). Importantly, the observed tissue infiltration and spontaneous disease development were highly variable among the antigenic specificities (**Table 1**). Single TCR mice expressing either insulin, chromogranin, or IGRP reactive mouse TCRs developed spontaneous diabetes, supporting the important pathogenic role for these reactivities in autoimmune diabetes. The majority of phogrin (IA2b) and I-A2 reactive mouse TCRs can induce islet infiltration, albeit without overt diabetes. Reactivity to multiple GAD epitopes, however, results in no disease and very limited infiltration for both human and mouse TCRs (**Table 1**). Based on these observations, it is likely that TCRs with select beta cell antigenic specificities are central to disease pathogenesis. Moreover, certain specificities might be important at different stages of disease, while others might not have a pathogenic but rather a regulatory effect, as was observed for GAD-reactive mouse T cells (106–109). Nevertheless, our ability to effectively extrapolate contribution of T cell specificities to disease in a polyclonal multi-antigen specific environment by analyzing their behavior in single TCR systems is limited. NOD mouse models exhibit a single MHC II restriction; therefore, pathogenic responses to antigens presented in alternative susceptible HLA class II or class I alleles might be overlooked. Alternatively, it is possible that inflammation induced by T cells specific for the initiating antigen results in exposure or modification of secondary antigens, leading to pathogenic activation of a distinct repertoire of T cells specific to the newly displayed epitopes.

The molecular determinants of pathogenic TCRs in autoimmunity are still largely unknown. Antigen availability, immunogenic modification of T cell epitopes, and TCR avidity could all shape the responses of beta cell-specific T cells (**Figure 2**). While it is still unclear whether antigen reactivity is an absolute prerequisite for tissue entry, several experimental approaches have shown that T cell accumulation in NOD pancreatic islets is driven by antigen recognition (110–112). The difference in antigen availability could explain relative importance of T cell specificities in the development and progression of autoimmunity. For example, reduced pathogenicity of GAD65-reactive T cells in NOD mouse model might be due to insufficient antigen availability in the pancreas. T cell reactivity to GAD65 and GAD67 can be observed early in NOD mice (113, 114), and antibodies specific for GAD are associated with progression to T1D in humans (115), which suggests a role for GAD reactivity in T1D. However, relative to other beta cell antigens, GAD T cells exhibit reduced pathogenicity in mouse models compared to other antigens (**Table 1**), with only one study showing diabetogenic activity of GAD65-reactive T cells (116). The rather mild pathogenic potential of GAD-reactive T cells in NOD model could be attributed to relatively low levels of both GAD65 and GAD67 expressed in the mouse islets compared to rat or human pancreas (117). In support of this, overexpression of GAD65 under the rat insulin promoter enhanced pancreatic infiltration of GAD-reactive T cells (110). Although, this observation serves as a proof of principle for the importance of antigen availability for islet infiltration, overexpression of GAD65 in polyclonal NOD mice does not result in enhanced insulitis or diabetes (118). Therefore, other parameters in addition to islet antigen availability must regulate T cell pathogenic potential.

TCR Affinity of Pathogenic T Cells

It is logical to assume that TCR affinity for antigen is associated with increased pathogenicity; however, that is not always the case, as we have shown for insulin-reactive TCRs. When eight NOD CD4 T cell-derived TCRs with variable affinity for insulin $InsB_{9–23}$ epitope were compared for their ability to drive spontaneous diabetes, high- and low-affinity T cells were similarly pathogenic (82) (**Table 1**). This is consistent with observations that a polyclonal autoimmune T cell response can encompass a wide range of TCR affinities, and low-affinity T cells are important contributors to the immune response (85, 119). However, it appears that there are certain functional distinctions between high- and low-affinity insulin-reactive T cells. Compared to high-affinity TCRs, low-affinity TCRs were less sensitive to thymic negative selection pressures, exhibited lower frequencies of $Foxp3^+$ T cell

development, and had a reduction in negative regulators of T cell activation (82). Their inability to reach the threshold for engagement of regulatory elements could allow the low-affinity cells to exert effector functions and induce beta cell damage even under relatively low level of TCR stimulation (**Figure 2B**).

PERIPHERAL PRIMING OF AUTOIMMUNE T CELLS

Molecular Mimicry

The mechanisms behind self-reactive T cell priming and ensuing loss of self-tolerance are complex and poorly understood. Autoimmune T cells exhibit a level of reactivity for self-antigens, but are somehow able to escape negative selection in the thymus. In the periphery, these cells encounter cognate self-antigen with enough affinity and in the right context to become activated and cause tissue damage. In the case of T1D, studies have implicated molecular mimicry as a potential trigger, where beta cell-reactive T cells could undergo initial priming and activation in response to structurally similar microbial epitopes (120) (**Figure 1**). While the direct evidence for molecular mimicry as a cause for autoimmunity is lacking, recent work exposing the previously unrecognized propensity of T cells for cross-reactivity reinforces molecular mimicry as a valid hypothesis (87, 121, 122). Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)-reactive CD8 T cells were shown to recognize a transporter protein peptide of *Fusobacteria*. Importantly, activation of IGRP-specific NY8.3 T cells by *Fusobacteria* contributed to enhanced diabetes development (123). Microflora composition in general has been implicated in both human and mouse T1D. In mice, gender hormones influence microbiota and subsequent T1D development (124, 125), while autoantibody-positive children have distinct microbiota signatures (126). It has yet to be seen whether specific microbiota species drive activation of islet-reactive T cells leading to beta cell destruction.

Unusual Orientation of the Tri-Molecular Complex

It is hard to reconcile exceedingly lower reactivity of autoimmune T cells to their cognate antigen, compared to non-self-reactive TCRs, with their capacity to exert significant tissue damage. For example, insulin-reactive TCR 12-4.1 isolated from pancreatic islets of NOD mice exhibits barely detectable reactivity to insulin *in vitro* (82), but causes spontaneous diabetes in 50–80% of mice (82, 103) (**Table 1**). As we alluded to earlier, lower affinity self-reactive TCRs are to some degree resistant to central and peripheral tolerance mechanisms, which might explain their ability to persist in an activated state (82). However, it is still unclear how self-reactive T cells with very low affinity for antigen are capable of causing beta cell destruction and highly penetrant diabetes. It is possible that the inherent unusual TCR structural and signaling characteristics are potential contributing factors that lead to unique responsiveness of autoimmune T cells. Crystal structures of autoimmune TCR:pMHC complexes have uncovered an unconventional docking of self-reactive TCRs on pMHC (127–129). Moreover, self-reactive human and mouse TCRs form unusual disorganized T cell synapses, exhibit slower kinetics of TCR signaling pathways, and yet they are still able to undergo activation and exert effector functions (130, 131). Conceivably, these characteristics allow autoimmune T cell escape from thymic selection, while in the target tissue high level of antigen is sufficient to elicit effector response.

Tissue-Specific PTM of Target Epitopes

In the case of autoimmune T1D, beta cell fragility characterized by increased susceptibility to oxidative and ER stress may be a critical factor in loss of self-tolerance. A consequence of the cellular stress is the altered processing and changes in PTM of proteins. The changes in beta cell epitopes can lead to the generation of tissue-specific neo-antigens that are not expressed in the thymus. T cells specific for neo-antigens can evade mechanisms of central tolerance and initiate an autoimmune response once exposed to PTM antigens in periphery (**Figure 1**). Interestingly, insulin containing granules are highly immunogenic compared to artificially synthesized protein, which suggests some manner of PTM takes place within the NOD beta cell granules (10). In the case of the dominant insulin epitope targeted in the NOD mice ($InsB_{9-23}$), the modification likely affects the MHC-binding residue of the peptide, resulting in stable binding of peptide in a register that is normally unstable and very likely presented at low levels in the thymus (10, 50, 132). In support of this idea, studies have shown that a mimotope of the $InsB_{9-23}$ insulin peptide with a change in the MHC anchor residue (R22E) was highly stimulatory for insulin-reactive T cells, and R22E peptide:MHC tetramers identified insulin-reactive cells within the islet-infiltrating T cell population (50, 133). Just in the last few years, it has been demonstrated that neo-antigenic PTM epitopes can form by fusion of either ChgA or IAPP peptide with a pro-insulin peptide (35). These fusion peptides were highly stimulatory to IAPP- and ChgA-reactive diabetogenic NOD T cell clones, as well as CD4 T cells isolated from the islets of T1D donors (35, 37). While the fusion peptides were identified in beta cells, it is unknown whether their formation is increased during inflammation or ER stress. More recent work has identified immunogenic peptides generated from an alternate insulin reading frame, the translation of which was further increased under ER stress (134). CD8 T cell clones isolated from peripheral blood of T1D subjects and specific for these defective ribosomal products (DRiPs) were able to cause direct beta cell damage *in vitro*, supporting a potentially critical role for DRiPs in T1D. This is yet another PTM mechanism within a mounting evidence for connection between beta cell ER stress and generation of immunogenic PTMs. Nevertheless, it is still unknown exactly to what extent PMT antigen-specific T cells contribute to T1D.

At the moment, we have very little insight into the functional concentration of PTM antigens vs. wild type epitopes presented in the inflamed tissue, the relative frequency of PTM-reactive T cells vs. T cells that recognize the wild-type epitopes, or how these parameters change over the course of chronic autoimmune tissue damage. It is likely that some T cells have a restricted specificity to either PTM or wild-type antigens, while others respond to both with different levels of activation. Addressing these questions

will lead to our better understanding of the triggers that induce autoimmune response, as well as identification of the initiating antigens and the key pathogenic T cell populations. It is currently unknown whether tissue-specific PTM antigens are transported and expressed in the thymus. In order to model how the presence of post-translationally modified peptides in the thymus could alter the selection of insulin-reactive TCRs (4-8 and 12-4.1) that normally escape negative selection, we ectopically expressed the R22E insulin mimetope in bone marrow-derived APCs (72). In the presence of R22E, the high-affinity 4-8 TCR bearing thymocytes were efficiently deleted, while the low affinity 12-4.1 population was affected to a lesser degree, albeit still showing an increase in negative selection based on Annexin V staining. Nevertheless, the ectopic expression of R22E significantly reduced peripheral T cells and halted any islet infiltration in both the 4-8 and 12-4.1 retrogenic mice. These results suggest that unlike expression of wild-type antigen, expression of PTM epitopes in the thymus results in efficient deletion of autoimmune T cells.

Accumulating evidence indicates PTMs as the key to our understanding of autoimmune disease development (35, 135–140). Importantly, T cells specific for PTM GAD65 and ChgA epitopes have been identified in individuals diagnosed with T1D (35, 139). Although the evidence so far is limited, PTM epitope expression is likely restricted to peripheral tissue and is absent from the thymus. While wild-type self-proteins presented in the thymus successfully limit development of high-affinity self-reactive T cells, lower affinity T cells evade central tolerance to be able to respond to PTM antigens in periphery (**Figures 1** and **2B**). Moreover, it is conceivable that the lack of PTM antigen expression in the thymus could lead to holes in the Treg repertoire. While multiple studies have shown that modification of beta cell epitopes increases their immunogenicity, it is unclear what proportion of antigens in the pancreas has been modified. Presumably, relatively low concentrations of immunogenic PTM epitopes are sufficient to prime autoimmune T cells, while presence of wild-type epitope is adequate for propagation of chronic autoimmune response. Further biochemical analyses of the pancreatic beta cells are necessary to identify the predominant PTM epitopes and the stress conditions that lead to their development.

HLA-HUMANIZED MICE TO MODEL T1D ANTIGEN RESPONSES

While we have learned a great deal from the NOD mouse, there are certain limitations to the conclusions and parallels we can draw to human T1D. In order to improve the model, several HLA transgenic mouse strains expressing susceptible or protective alleles have been generated, some of these on the NOD background. Surprisingly, NOD mice expressing susceptible DQ8 or DR4 alleles do not develop spontaneous diabetes (141–143). However, HLA-DQ8 humanized mice do develop spontaneous autoimmune cardiomyopathy (144). Still, both DR4 and DQ8 alleles support the development of beta cell-reactive autoimmune T cells but require an additional trigger to initiate beta cell targeted autoimmunity. When DR4 and DQ8 mice were crossed with transgenic mice expressing B7.1 co-stimulatory molecule on beta cells, both HLA-humanized strains developed spontaneous diabetes (141). The main utility for HLA-humanized mice has been realized by performing systematic identification of the key antigenic epitopes presented on human HLAs (24). Future studies should be extended to assess the *in vivo* functional potential of human autoimmune TCRs specific for key immunogenic epitopes. To date only one beta cell antigen-reactive human TCR transgenic mouse with specificity for GAD65 has been described (101). *In vivo* functional analysis of TCRs, and human TCRs in particular, has been hindered due to limited access to patient samples, labor, and time involved in generating TCR transgenic mice. We have overcome the limitation of TCR transgenic system by utilizing a TCR retrogenic approach that allows rapid functional analysis of multiple TCRs through retroviral gene delivery (110, 145, 146). Using this approach, we have expressed a $GAD65_{115–127}$ reactive TCR isolated from peripheral blood of an individual diagnosed with T1D (99). Although we observed robust development of GAD-reactive T cells in this system, similar to the transgenic expression, we detected a low level of islet infiltration. Future analyses should be expanded to other beta cell protein epitopes targeted in human T1D, including PTM epitopes. The humanized TCR retrogenic approach will allow efficient and relatively high-throughput analysis of autoimmune antigens important in human disease, and can be utilized as a platform for development of antigen-specific immunotherapies. It is likely that many questions pertinent to our understanding of autoimmune T cell development and pathogenicity will be eventually addressed in the context of human susceptible HLA alleles and human TCRs.

CONCLUSION

The biology of low-affinity autoimmune T cells has been perplexing due to the seeming contradiction between suboptimal *in vitro* responses and robust *in vivo* pathogenicity. In many cases, self-reactive autoimmune T cells do not follow the dogma prescribed by studies performed with T cells specific for infectious or model antigens. In addition to unusual TCR:pMHC interactions and downstream signaling, autoimmune antigens themselves can have atypical characteristics. Over the years, it has become clear that antigens targeted in autoimmunity, and particularly in T1D, are often modified versions of self-peptides that are presented during thymic selection. These exceptions to the rule characteristic of autoimmune T cell responses are often centered on the stability of the tri-molecular complex as a master switch from tolerance to autoimmunity.

AUTHOR CONTRIBUTIONS

MB and MLB developed the concept, prepared the figures, and wrote the manuscript.

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REVIEW
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Isogenic Cellular Systems Model the Impact of Genetic Risk Variants in the Pathogenesis of Type 1 Diabetes

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At least 57 independent loci within the human genome confer varying degrees of risk for the development of type 1 diabetes (T1D). The majority of these variants are thought to contribute to overall genetic risk by modulating host innate and adaptive immune responses, ultimately resulting in a loss of immunological tolerance to β cell antigens. Early efforts to link specific risk variants with functional alterations in host immune responses have employed animal models or genotype-selected individuals from clinical bioresource banks. While some notable genotype:phenotype associations have been described, there remains an urgent need to accelerate the discovery of causal variants and elucidate the molecular mechanisms by which susceptible alleles alter immune functions. One significant limitation has been the inability to study human T1D risk loci on an isogenic background. The advent of induced pluripotent stem cells (iPSCs) and genome-editing technologies have made it possible to address a number of these outstanding questions. Specifically, the ability to drive multiple cell fates from iPSC under isogenic conditions now facilitates the analysis of causal variants in multiple cellular lineages. Bioinformatic analyses have revealed that T1D risk genes cluster within a limited number of immune signaling pathways, yet the relevant immune cell subsets and cellular activation states in which candidate risk genes impact cellular activities remain largely unknown. In this review, we summarize the functional impact of several candidate risk variants on host immunity in T1D and present an isogenic disease-in-a-dish model system for interrogating risk variants, with the goal of expediting precision therapeutics in T1D.

Keywords: type 1 diabetes, autoimmunity, induced pluripotent stem cells, gene editing, genome-wide association studies, expression quantitative trait loci

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INTRODUCTION

The combined genetic and environmental factors that result in type 1 diabetes (T1D) are reflected in the heterogeneous clinical presentations of the disease (1). This autoimmune process results from a complex cross-talk between cells of the innate and adaptive arms of the immune system and the target β cells within the islet microenvironment (**Figure 1**) (2). The era of genome-wide association studies (GWAS) has heralded discovery of approximately 57 independent loci conferring some component to the overall genetic risk for the development of T1D (3). This vast discovery effort has reinforced prior notions of an autoimmune basis for disease development and also has shed new light on the

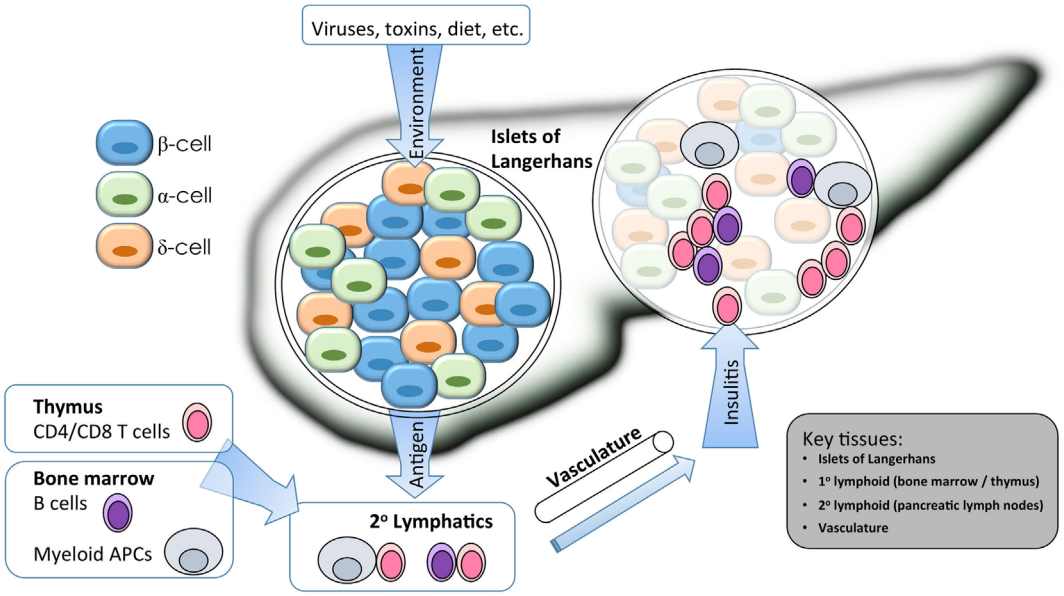

FIGURE 1 | Isogenic modeling facilitates the investigation of multiple cell types important in the pathogenesis of type 1 diabetes (T1D). A combination of environmental and genetic factors influences the overall risk for T1D. Genes conferring risk for T1D may affect the functions of β cells, immune cells, and vascular endothelium. For β cells, risk variants of some genes may alter the response to environmental triggers such as inflammatory or viral sensing, or they may alter the way that β cells cope with stress from bioenergetic demands. For immune cells, gene variants my alter the way that T and B cells are selected in primary (1°) lymphatic tissues during central tolerance, or they may alter several key events that occur during antigen-specific priming and effector differentiation in the peripheral (2°) lymphatics. Immune destruction of β cells requires homing of innate and adaptive effector populations into the pancreatic islets, so alterations to endothelial function could affect disease at this late stage. Isogenic cellular modeling can be applied to complex multifactorial diseases to facilitate a more complete understanding of which genes are expressed in any given tissue/cell type and at which developmental stage they may exert their influence on disease progression.

etiology of T1D, including support for cellular stress within β cells contributing to their demise (4). Despite these advances, there remain numerous questions regarding the mechanisms by which causal gene variants, both individually and in concert, impact immune checkpoints and β cell responses throughout the natural history of the disease. Thus, there remains a critical need in the field to address some fundamental questions regarding the single-nucleotide polymorphisms (SNPs) identified by GWAS including (1) What are the causative variants within any given tag SNP locus? (2) In what cell type(s) and developmental stage(s) are the candidate genes actively expressed? (3) What environmental stimuli modify candidate gene expression or activity? And ultimately, (4) what variants and/or pathways are amenable to therapeutic interventions?

A number of large-scale mechanistic studies to discern the impact of specific genotypes on resulting phenotypes are underway from population-based studies (5). These investigations often utilize clinical material derived from bioresource banks (i.e., genotyped clinical samples capable of recall or recovery from cryopreservation) (6). While promising results have emerged, the number of well-characterized genotype:phenotype interactions remains limited to a small fraction of the putatively identified risk loci. The paucity of functional studies validating causative SNPs can be attributed to a number of challenges including the need to acquire sufficient clinical blood volumes for functional testing, limited access to biological replicates to account for human heterogeneity (particularly with low minor allele frequency variants), and the clear potential for epistatic genetic influences. In sum, these confounding factors constitute a considerable discovery bottleneck limiting human studies by the larger research community.

Immunodeficient mouse models, so-called "humanized" mice, capable of being engrafted with primary human lymphocytes or hematopoietic stem cells (HSCs) have been proposed as a means to fill the translational gap between *in vitro* human studies and clinical trials. These rodent models display full organism level complexity yet can still be manipulated experimentally (7). Despite the powerful tool humanized mice provide when used appropriately, they still present significant constraints as a model system. Mice hold notable differences when compared to human biology, particularly when considering host immune responses in the context of TLR ligands, responses to cytokines and growth factors, and cellular trafficking (8). These factors present challenges in modeling autoimmune T1D in xenogeneic systems, where there are essential homology requirements for full effector function. These requirements include the need for lymphocyte trafficking from circulation to secondary lymphoid organs, auto-antigen priming and activation, and eventual extravasation to target β cells within islets (9). The emergence of induced pluripotent stem cell (iPSC) technologies offers an attractive alternative to humanized mice that allows the interrogation of underlying genetic defects using a vast array of relevant biological tissues and cell types avoiding both allo- and xenogeneic responses.

Isogenic cellular systems constitute a powerful experimental platform for conducting precision gene editing to create a "disease-in-a-dish" model to interrogate multifactorial diseases such as T1D. This methodology provides an opportunity to understand specific molecular mechanisms and pathways in humans to thereby derive rational therapeutics using a precision medicine approach. In this review, we describe some of the emerging technologies for generating and manipulating iPSC-derived cells and tissues to interrogate causative genes and pathways in T1D.

ISOGENIC CELLULAR SYSTEMS

Investigations into the etiopathogenesis of T1D have historically been dominated by studies of peripheral blood. Over the last decade, the Network for Pancreatic Organ donors with Diabetes (nPOD) program has provided essential access to the pancreas and lymphoid tissues from donors with T1D. Emerging studies from this program have already challenged many of the preconceived notions of the disease. Of note, nPOD tissues have highlighted disease heterogeneity across T1D donors and remarkable variability even at the level of adjacent islets within a single T1D donor (10–14). For example, early histological observations from nPOD led Dr. George Eisenbarth to refer to T1D as "vitiligo of the pancreas," in reference to intact insulin-containing islets being observed in close proximity to pseudo-atrophic islets completely devoid of insulin (15). Despite the transformative resource that nPOD provides, donor and programmatic limitations necessitate systematic prioritization of access to tissues. Hence, there is a paramount need within the field to derive cell types from renewable human cellular sources. The capacity for pluripotent and renewable cells to undergo reprogramming to generate immune subsets, endothelial cells, and neuroendocrine lineages will facilitate the modeling of cellular interactions involved in T1D disease pathogenesis (**Figure 2**).

GENETIC SUSCEPTIBILITY IN T1D

The autoimmune destruction of insulin-producing pancreatic β cells in T1D shares complex etiology with a collection of organ-specific disorders (i.e., juvenile idiopathic arthritis, alopecia areata, rheumatoid arthritis, and celiac disease, among others) (3). Though each of these diseases demonstrates unique immunopathologic mechanisms, they all share two common features: specifically, inheritance with a significant genetic contribution coming from the human leukocyte antigen (HLA) region of chromosome 6 and additional genetic risk conferred by loci dispersed throughout the genome (**Table 1**). While no single risk haplotype accurately predicts whether or not a person will develop T1D (or another autoimmune disease), there is clear genetic evidence that T1D is primarily an inherited disease with an autoimmune pathogenesis (**Figure 3**) and with additional poorly defined environmental contributions. Discordant incidence of T1D in monozygotic twins is often cited as evidence for a greater environmental role in T1D (16, 17); however, the early studies likely underestimated the concordance rates. It is now better understood that childhood-onset T1D and latent autoimmune diabetes of the adult (LADA) share overlapping genetic risk (18). Thus, long-term monitoring is essential to capture the total genetic risk for disease development. For example, one study of monozygotic twins found that by the age of 60 years, there was greater than 65% concordance for T1D—i.e., when one twin is afflicted, it is more likely that the other twin will eventually develop the disease (19). In the same study, concordance of autoantibody positivity in the non-diabetic twin was nearly 80%, again supporting the notion of genetic risk controlling the loss of immune tolerance to β cell antigens.

The lack of complete concordance may indicate an additional role for epigenetic and/or stochastic influences due to antigenic receptor gene recombination events. In addition, epidemiological studies support a role for environmental factor(s) influencing disease progression. A number of large consortium studies have been conducted or are currently underway around the world (e.g., TrialNet, TEDDY, DAISY, BABYDIAB, and Pre-Point) to monitor disease progression and potentially, intervene in those identified as being at high-risk for disease development (27–31). From these studies, environmental influences have been reported to affect disease incidence or rate of progression, including enteroviral triggers, lack of protective exposures, and the influence of various components of Westernized diets. Many of these modifying factors impact pathways with associated genetic risk variants (e.g., *Tyk2* and *IFIH1* in response to viral infections), further supporting their potential importance (32). Thus, T1D is principally a genetic disease with environmental exposures influencing progression. These combined influences support the notion of a complex multifactorial disease, yet ultimately beg the question: Why do we not better understand the etiology and pathogenesis of human T1D? Even though the human genome is complex, it is still a finite collection of variables. In principle, utilization of "big data" approaches involving GWAS, biomarker studies, and expression profiling, when paired with robust computational capabilities, should be able to reveal a clear molecular signature, and from this signature, we should be able to progress through reductionist approaches to reveal pathways of disease.

This theoretical solution to the problem of complex autoimmune diseases is hindered by a number of fundamental challenges. Foremost, heretofore there have been no experimental systems available to study individual risk variants in human subjects. For T1D, where approximately 57 different genetic regions confer some portion of genetic risk (immunobase.org, July 2017) (3, 20–26), it is not possible to study one gene at a time without incurring significant epistatic effects from other risk genes. The likelihood of finding two individuals differing at *only* one risk gene (i.e., one person with the protective allele and one person with the risk allele) while having *identical* variants at the remaining 56 risk regions is infinitesimally small. A more practical approach would be to reduce the number of genetic loci being studied to include only those with the largest odds ratios (ORs). Even here, the problem is magnified by the fact that some of the most highly associated risk genes beyond the HLA [e.g., protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*)] have a low minor allele frequency, even among T1D subjects. For North American and European T1D subjects, the frequency of individuals with homozygosity for the risk variant of *PTPN22* (1858T at rs2476601) ranges from 0.6 to 3.7% (33). Moreover,

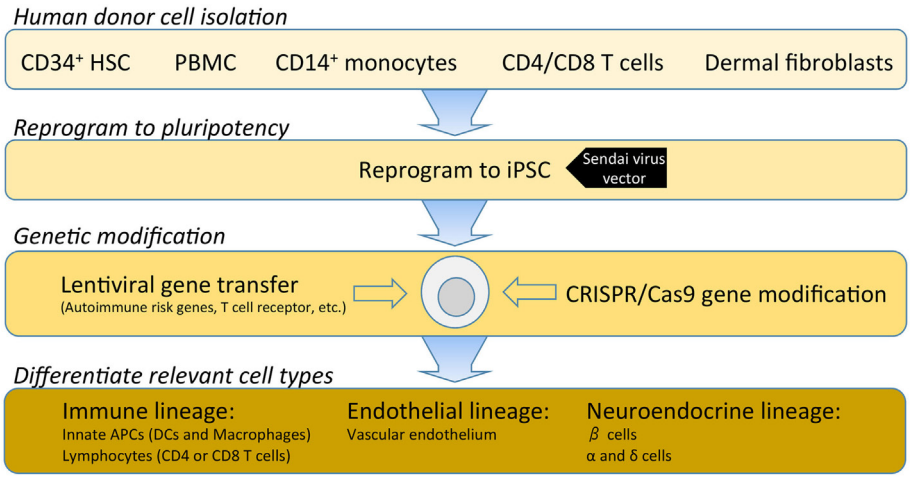

FIGURE 2 | A hypothetical outline for establishing an isogenic disease-in-a-dish workflow. Induced pluripotent stem cell (iPSC) stable cell lines can be generated from several different somatic cell types depending on specimen availability. Traditionally, dermal fibroblasts from skin biopsy were utilized; however, this is being replaced by less invasive samples such as freshly isolated or cryopreserved peripheral blood mononuclear cells (PBMCs). PBMCs can be enriched for various populations such as $CD34^+$ hematopoietic stem cells, $CD14^+$ monocytes, T cells, or reprogrammed as a bulk population. Where a pre-re-arranged T-cell receptor (TCR) is desired, antigen-specific CD4 or CD8 T cells can be used so that iPSC-derived T cells will clonally express the desired TCR with a naive T-cell phenotype. Several commercial platforms for iPSC reprogramming are currently available. Non-integrating Sendai virus vectors provide a safe and efficient means for iPSC reprogramming of human primary cells. Following reprogramming into iPSC, gene modification enables researchers to investigate disease-associated risk variants and/or over-express or knockdown genes to modulate pathways. Once gene modifications are confirmed, validated protocols for differentiation of immune, endothelial or neuroendocrine lineages are utilized to interrogate the specific effects of each gene variant in several disease-relevant cell types.

genes associated with T1D risk encode proteins that cluster within biological processes and/or pathways, posing a considerable challenge when analyzing the impact of a given risk variant.

Currently, 57 genomic regions that are defined by 104 SNPs [some SNPs identify the same linkage disequilibrium block] are significantly associated with T1D according to immunobase.org. The set of 64 T1D candidate gene variants from 57 SNP-tagged regions listed in **Table 1** was analyzed using the Protein ANalysis THrough Evolutionary Relationships gene ontology tool (pantherdb.org) (34, 35). Not surprisingly, pathway analysis revealed a significant enrichment for genes involved in immune processes ($P = 9.9E{-}11$), where 26 of the 64 candidate genes contribute to immune function. The immune system is highly dynamic and integrates signals from antigenic receptors, adhesion molecules/integrins, costimulatory molecules, and cytokine/chemokine receptors. These events in turn lead to signal transduction events that are also significantly enriched as a defined pathway. Based on our analysis, 32 of the 64 T1D candidate genes are implicated in cellular signaling ($P = 9.25E{-}03$) (Data File S1 in Supplementary Material). Considering the role of cross-talk between signaling pathways, it is evident that heterogeneous genetic risk will result in complex downstream effects on cell signaling and functions.

As a specific example of immune signaling pathway cross-talk, we consider one gene that encodes a protein with known effects on cytokine receptor signaling. *SH2B3* encodes a protein phosphatase Lnk that regulates Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling. The risk variant of *SH2B3* (T at rs3184504) encodes a modified Lnk protein where arginine at amino acid 262 is replaced by tryptophan (R262W). Lnk is a regulator of Jak2 signaling in myeloid cells (36–38), and the T1D risk SNP for *SH2B3*/Lnk is associated with altered expression of key elements of IFNγ signaling including signal transducer and activator of transcription 1 (STAT1) (39). Furthermore, the target of Lnk, Jak2, is a cytosolic protein that transduces signals from a variety of cytokine receptors including IL-6, IL-13, G-CSF, IL-12, IL-23, granulocyte-macrophage colony-stimulating factor (GM-CSF), EPO, IL-3, and IL-5 (40, 41). Thus, the specific effect(s) of Lnk^{R262W} upon immune cell function are difficult to predict. Adding to this inherent complexity, additional T1D risk genes/proteins are likely to co-regulate the same pathways as Lnk. For example, at least three T1D candidate genes, *Tyk2*, *SOCS1*, and *IL10*, encode proteins with known roles in modulating JAK/STAT signaling. The interplay of different alleles of each protein will likely modify the effect of Lnk. This example highlights the need for an experimental system that mitigates the epistatic effects of related genes/proteins so that observed phenotypes are attributed to the gene of interest alone.

In addition to the number of variants and overlapping pathways noted above, there are additional layers of complexity at the cellular level. Specifically, it is poorly characterized how a given risk variant may impact function within various innate or adaptive immune subsets. For example, a gene that regulates JAK/STAT signaling in antigen-presenting cells (APCs) such as dendritic cells (DCs) may have an entirely different biological effect in lymphocytes. Moreover, the impact of a gene variant may be combinatorial to multiple cell types that conspire to drive autoimmunity. Furthermore, some genes may affect the β cells themselves, endothelial cells, or other cells such as neurons (42).

The central pathophysiological mechanism of T1D entails at least three major tissue types—immune, endothelial, and

TABLE 1 | Genetic variants associated with type 1 diabetes and other common autoimmune diseases.

Chromosome	Marker	Gene	Feature	Coding variant	Amino acid variation	Additional notes	Region	Other associated diseases
1	rs2476601	*PTPN22*	Exon	Y	R620W		1p13.2	ATD/CRO/JIA/RA/
	rs6679677		3′ region—intergenic	N				SLE/AA/VIT
	rs6691977	*CAMSAP2*	Intron	N			1q32.1	
	rs3024505	*IL10*	3′ region—intergenic	N			1q32.1	CRO/SLE/UC/IBD
	rs3024493		Intron	N				
2	rs35667974	*IFIH1*	Exon	Y	I923V		2q24.2	PSO/SLE/UC/
	rs2111485		3′ region—intergenic	N				IBD/VIT
	rs1990760		Exon	Y	A946T			
	rs11571316	*CTLA4*	5′ region—intergenic	N			2q33.2	ATD/CEL/RA
	rs3087243		3′ region—intergenic	N				
	rs4849135	*ACOXL*	Intron	N			2q13	
	rs478222	*EFR3B*	Intron	N			2p23.3	
	rs9653442	*AFF3*	5′ region—intergenic	N			2q11.2	RA
3	rs113010081	*CCR5* and *CCRL2*	3′ region—intergenic	N			3p21.31	CEL/UC
4	rs2611215	*LINC01179*	5′ region—intergenic	N			4q32.3	
	rs75793288	*CTNNB1*	Intron	N		5′ of ADAD1 and 3′ of IL2	4q27	CEL/CRO/UC
	rs6827756		Intron	N		5′ of ADAD1 and 3′ of IL2		
	rs4505848		Intron	N		5′ of ADAD1 and 3′ of IL2		
	rs17388568	*ADAD1*	Intron	N		3′ of IL2		
	rs10517086	*No gene*	Intergenic—H3K27Ac rich	N			4p15.2	
5	rs11954020	*IL7R*	3′ region—intergenic	N			5p13.2	
6	rs9388489	*CENPW*	Intron	N			6q22.32	
	rs1538171		Intron	N				
	rs9375435		Intron	N				
	rs597325	*BACH2*	Intron	N			6q15	ATD/MS/RA
	rs11755527		Intron	N				
	rs72928038		Intron	N				
	rs924043	*No gene*	Intergenic	N			6q27	
	rs6920220	*TNFAIP3*	5′ region—intergenic	N			6q23.3	RA/SLE/UC/IBD
	rs1738074	*TAGAP*	Exon	N	SYN		6q25.3	CEL/MS
7	rs7804356	*SKAP2*	Intron	N			7p15.2	
	rs4948088	*COBL*	3′ region—intergenic	N			7p12.1	
	rs62447205	*IKZF1*	Intron	N			7p12.2	
9	rs10758593	*GLIS3*	Intron	N			9p24.2	
	rs7020673		Intron	N				
	rs6476839		Intron	N				
10	rs722988	*NRP1*	3′ region—intergenic—H3K27Ac rich	N			10p11.22	
	rs11258747	*PRKCQ*	Exon	N	SYN		10p15.1	
	rs61839660	*IL2RA*	Intron	N			10p15.1	MS/RA
	rs2104286		Intron	N				
	rs12251307	*IL2RA* and	5′ of RMB17 and 3′ of IL2RA	N				
	rs41295121	*RBM17*	5′ of RMB17 and 3′ of IL2RA	N				
	rs7090530		5′ of RMB17 and 3′ of IL2RA	N				
	rs10795791		5′ of RMB17 and 3′ of IL2RA	N				
	rs12416116	*RNLS*	Intron	N			10q23.31	
	rs10509540		3′ region—intergenic	N				
11	rs72853903	*INS*	5′ region—intergenic—H3K27Ac rich	N			11p15.5	
	rs689		Intron	N				
	rs7111341		5′ region—intergenic	N				
	rs7928968		3′ region—intergenic	N				
	rs694739	*BAD*	5′ region—intergenic	N		5′ of CCDC88B and 3′ of PRDX5	11q13.1	CRO/MS/AA

(*Continued*)

TABLE 1 | **Continued**

Chromosome	Marker	Gene	Feature	Coding variant	Amino acid variation	Additional notes	Region	Other associated diseases
12	rs11170466	*ITGB7*	Intron	N			12q13.13	
	rs11171739	*ERBB3*	5′ region—intergenic	N			12q13.2	AA
	rs11171710	*RAB5B*	Intron	N		5′ of IKZF4		
	rs705705	*IKZF4*	3′ region—intergenic	N				
	rs705704		3′ region—intergenic	N				
	rs2292239	*ERBB3*	Intron	N				
	rs3184504	*SH2B3*	Exon	Y	R262W		12q24.13	CEL/CRO/JIA/
	rs653178	*ATXN2*	Intron	N				PBC/RA/AA/PSC/
	rs17696736	*NAA25*	Intron	N				VIT
	rs10492166	*CD69*	3′ region—intergenic	N			12p13.31	
	rs4763879		Intron	N				
13	rs9585056	*GPR183*	5′ region—intergenic—H3K27Ac rich	N			13q32.3	
14	rs4900384	*LINC01550*	5′ region—intergenic	N			14q32.2	
	rs1456988		5′ region—intergenic	N				
	rs911263	*RAD51B*	Intron	N			14q24.1	PBC
	rs1465788	*ZFP36L1*	5′ region—intergenic—H3K27Ac rich	N			14q24.1	
	rs56994090	*DLK1*	3′ region—intergenic	N		Intron of MEG3	14q32.2	
	rs941576		3′ region—intergenic	N		Intron of MEG3		
15	rs12148472	*CTSH*	Intron—splice site	N			15q25.1	CEL/NAR
	rs3825932		Intron	N				
	rs34593439		Intron	N				
	rs12908309	*RASGRP1*	5′ region—intergenic	N			15q14	CRO
	rs72727394		Intron	N				
16	rs4788084	*IL27*	5′ region—intergenic	N			16p11.2	AS/CRO/IBD
	rs9924471		5′ region—intergenic	N		Intron of *SGF29*		
	rs151234	*CLN3*	Intron	N		5′ of *APOBR* and 3′ of *IL27*—K3K27Ac rich		
	rs12708716	*CLEC16A*	Intron	N			16p13.13	MS/PBC
	rs12927355		Intron	N				
	rs193778	*SOCS1*	5′ region—intergenic—H3K27Ac rich	N		3′ of *CLEC16A*, Intron of *RMI2*		
	rs8056814	*CTRB1*	5′ region—intergenic—H3K27Ac rich	N			16q23.1	
	rs7202877		5′ region—intergenic—H3K27Ac rich	N				
17	rs1052553	*MAPT*	Exon	N	SYN		17q21.31	
	rs7221109	*CCR7*	5′ region—intergenic—H3K27Ac rich	N			17q21.2	
	rs2290400	*GSDMB*	Intron	N			17q12	CRO/UC/IBD
	rs12453507		3′ region—intergenic	N				
18	rs763361	*CD226*	Exon	Y	G307S		18q22.2	MS
	rs1615504		3′ region—intergenic	N				
	rs2542151	*PTPN2*	3′ region—intergenic	N			18p11.21	CEL/CRO/UC/IBD
	rs1893217		Intron	N				
19	rs602662	*FUT2*	Exon	Y	G258S		19q13.33	CRO/IBD
	rs516246		Intron	N				
	rs402072	*PRKD2*	Intron	N			19q13.32	
	rs425105		Intron	N				
	rs12720356	*TYK2*	Exon	Y	I684S		19p13.2	CRO/JIA/MS/PBC/
	rs34536443		Exon	Y	P1104A			PSO/RA/IBD
20	rs2281808	*SIRPG*	Intron	N			20p13	
	rs6043409		Exon	Y	V263A			
21	rs11203202	*UBASH3A*	Intron	N			21q22.3	RA/VIT
	rs11203203		Intron	N				
22	rs4820830	*HORMAD2*	Intron	N			22q12.2	
	rs5753037		3′ region—intergenic	N				
	rs229533	*C1QTNF6*	5′ region—intergenic	N		3′ of *RAC2*	22q12.3	
X	rs2664170	*GAB3*	Intron	N			Xq28	

Genes and markers were derived from immunobase.org (3, 20–26). The genes indicated in blue text were imputed from information derived from the University of California Santa Cruz genome browser (genome.ucsc.edu). Amino acid variations (red text) were identified for single nucleotide polymorphism (SNP) variants by downloading the spliced coding sequences from genome browser and translating in SnapGene software. SYN (green text) indicates synonymous variation in an exon. Genomic region and disease information displayed were derived from Immunobase. ATD, autoimmune thyroid disease; CRO, Crohn's disease; JIA, juvenile idiopathic arthritis; RA, rheumatoid arthritis; SLE, systemic lupus erythematous; AA, alopecia areata; VIT, vitiligo; UC, ulcerative colitis; IBD, inflammatory bowel disease; PSO, psoriasis; CEL, celiac disease; MS, multiple sclerosis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; NAR, non-allergic rhinitis; AS, ankylosing spondylitis.

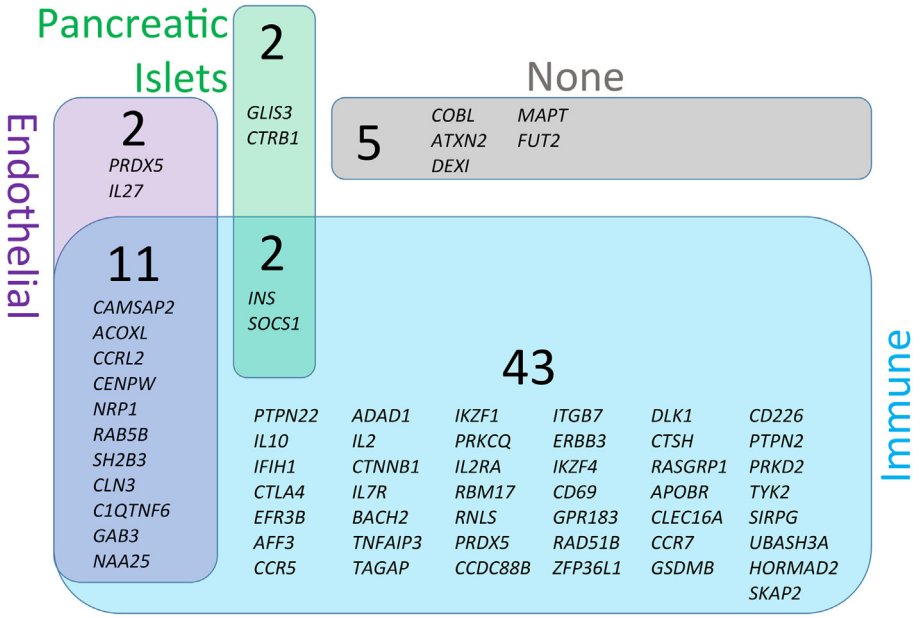

FIGURE 3 | Genetic susceptibility risk variants identified in type 1 diabetes (T1D). The current list of gene regions that have been associated with T1D through genome-wide association studies were collected from the ImmunoBase resource (www.immunobase.org). Individual single-nucleotide polymorphisms (SNPs) corresponding to candidate genes were identified from the ImmunoBase T1D data set. In addition, every SNP tag was queried on the University of California Santa Cruz Genome Browser (GRCh38; genome.ucsc.edu) to identify additional genes in each SNP-tagged region. The complete list of regions and genes are compiled in **Table 1**. Each SNP-associated gene was queried on the GeneVestigator database to identify the top 10 tissues with highest expression of each gene (genevisible.com/search). For each gene it was determined if high-expressing tissues included any one or combination of relevant tissues: immune (blue), endothelial (purple), or pancreatic islets (green). The size of regions in Venn diagram represents the relative abundance of genes expressed in each tissue type. Five genes were not highly expressed by any of the relevant tissue types (indicated as none, gray).

pancreatic (**Figure 1**). To better understand which cells are likely to be affected by each T1D candidate gene, we analyzed all genes from **Table 1** for cell/tissue expression profiles using the online GeneVisible tool (genevisible.com) (43) that queries tens of thousands of curated human gene expression experiments. As seen in **Figure 3**, the majority of T1D candidate genes are expressed most highly in immune cells, but a small number of genes are preferentially expressed in endothelial or pancreatic cell lineages. Notably, 13 genes are highly expressed in multiple lineages. Isolating the effect of candidate genes in relevant cell types should be a goal for the isogenic cellular experimental system described herein.

A number of T1D-associated SNPs encode missense mutations within gene exons, presumably altering protein stability, interactions, or function (**Table 1**); here, the path to dissect the impact of variants on biological processes is straightforward. However, the vast majority of risk loci reside in non-coding regions of the genome and careful studies must be undertaken to first dissect the causative variant(s) from each tag SNP locus and then determine whether any given SNP exerts its impact in a *cis* or *trans* manner to alter gene expression (5). One such study by Ram et al. recently applied a systems genetics approach to dissect the impact of putative risk SNPs on gene expression in purified and activated cell lines. The authors mapped *cis*-acting expression quantitative trait loci (eQTL) and found 24 non-HLA loci that significantly affected the expression of 31 transcripts in at least one cell type from Epstein–Barr virus-transformed B cells and $CD4^+$ or $CD8^+$ T cells (44). They went on to describe an additional 25 *trans*-acting loci that impacted 38 transcripts. Of note, many of the SNPs associated with risk are located within promoter or enhancer regions of their candidate gene (3). These studies provide a framework from which additional mechanistic studies can now be conducted in isogenic cellular systems.

To begin to address these challenges, the research community needs robust platforms to study the effects of individual risk alleles in various cell types under controlled conditions. With the advent of iPSC technologies and genome-editing tools, this once theoretical approach now provides an efficient method to analyze disease mechanisms and identify causal gene variants (**Figure 4**). By creating a disease-in-a-dish experimental platform, we and others have started to dissect the individual contributions of T1D risk genes in specific cell types. Harnessing this information will allow researchers to derive rational therapeutics targeting checkpoints in key pathways.

DEFECTIVE IMMUNE TOLERANCE IN T1D

Autoimmune diseases, including T1D, result from a breakdown in the pathways that maintain a state of immune homeostasis, commonly referred to as immune tolerance (1). The mechanisms controlling this process involve both central and peripheral tolerance mechanisms (e.g., thymic selection and immune checkpoints, respectively). Effective immunity requires the

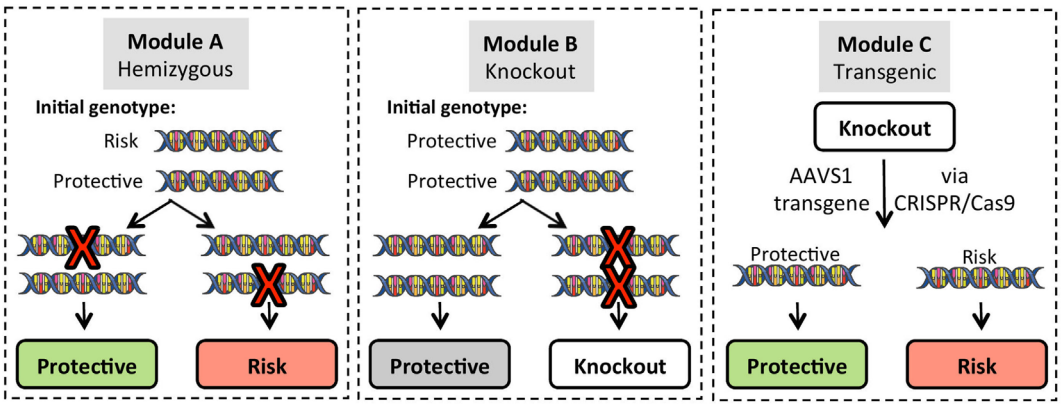

FIGURE 4 | Gene modification strategies for use in induced pluripotent stem cell (iPSC) lines. Three basic strategies can be employed for gene editing. To create single allele homozygous expression (hemizygous) lines, *Module A* targets a single allele of the gene of interest (GOI) in iPSC lines that are heterozygous for the risk variant. Targeting efficiency for hemizygous clones is approximately 20% of green fluorescent protein positive puromycin resistant clones, and allele targeting is random so that either the protective or risk allele can be modified. *Module B* generates complete knockout of the GOI on a background of homozygous protective alleles. *Module C* utilizes GOI-knockout lines to re-express either the protective or risk variant of the GOI using a CRISPR/Cas9 platform that targets integration into the adeno-associated virus integration site 1 (AAVS1) on chromosome 19.

capacity to respond to a vast array of antigens from pathogens, all while functionally limiting host responses to self-tissues and commensal microorganisms. In health, the adaptive immune system consisting of T and B lymphocytes is edited to eliminate portions of the repertoire that acquire somatically re-arranged receptors with high binding affinity for self-antigens through clonal deletion. For T cells, this process is carried out in the thymus under the control of the autoimmune regulator (AIRE) (45). Medullary thymic epithelial cells (mTECs) expressing AIRE are thought to coordinate the expression of a select number of tissue-specific self-antigens (TSAs). These TSAs, when expressed by mTEC work in concert with APCs to eliminate high-affinity autoreactive T-cell receptors (TCRs) through negative selection. The thymus is also the site for the emergence of thymic $CD4^+$ regulatory T cells (tTregs), a population of cells that express the master Treg-transcription factor FOXP3 (46). These tTregs seed the periphery, playing a key role in reinforcing immune tolerance. Rare monogenic mutations in *AIRE* and *FOXP3* result in profound autoimmune conditions, referred to as autoimmune polyglandular syndrome-1 and immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), respectively (47). T1D is a common clinical manifestation of patients presenting with these severe mutations, supporting the essential role for these transcription factors in regulating immune tolerance to β cells. To date, studies of thymic T-cell selection have largely been restricted to animal models. The development of isogenic cellular systems provides a unique opportunity to investigate molecular pathways that shape the human adaptive immune repertoire.

ISOGENIC MODELS FOR STUDYING THYMIC SELECTION

As noted earlier, the HLA region constitutes the major genetic risk locus in T1D (48). While this region has been known to confer risk for over four decades, the exact mechanisms by which variants in HLA influence disease pathogenesis remain poorly characterized. In addition to shaping the T-cell repertoire through the processes of positive and negative selection, the thymic developmental niche controls the composition and relative proportion of naive conventional T cells (Tconv) and tTregs that emerge to form the mature $CD4^+$ T cell population (49). Little is currently known about how high-risk HLA haplotypes (e.g., HLA-DR3/DR4-DQ8) shape the resulting T-cell repertoire, or for that matter, why the HLA-DRB1*15:01-DQA1*01:02-DQB1*06:02 haplotype is so dominantly protective in Caucasian populations (OR ~0.03) (50). A prevailing theory presented by Eisenbarth and colleagues suggests that the key might lie within the tri-molecular complex of HLA class II molecules presenting peptides of insulin (specifically, the insulin B-chain$_{9\text{-}23}$) for recognition by autoreactive TCRs (51). T1D DQ8 risk alleles and I-A^{g7} of the non-obese diabetic (NOD) mouse tend to share non-polar residues in place of Asp at β57 and preferentially bind peptides with acidic side chains in the P9 pocket of the MHC class II binding groove (52). Thus, these molecular interactions within distinct peptide binding pockets may either allow escape of potentially pathogenic autoreactive T-cell clones from the thymus or potentially fail to generate the proper repertoire of protective tTregs capable of maintaining tolerance.

Together with HLA, additional candidate risk genes could also have an impact on thymic T-cell development. Specifically, at least three independent variants within the *INS-IGF2* locus have been associated with risk for T1D (3). This region confers the second highest risk for disease following the HLA locus. Risk associated with the *INS* gene on chromosome 11p15.5 has been most commonly attributed to a variable number tandem repeat locus situated 596 bp 5′ of *INS* (53). Protection from the class III allele has been attributed to a markedly higher level of insulin being expressed within the thymus (54). Insulin has been proposed as a primary or triggering auto-antigen in the NOD mouse model (55) and more recently in human T1D (56). Notably, T cells reactive

to both native and hybrid insulin peptides, insulin conjugated with other β cell antigens, were discovered within the islets of subjects with T1D (56–58). When considered in addition to the dominance of genetic risk conferred by the HLA, these significant observations lend additional support to the dominance of insulin epitopes as a primary auto-antigen in disease pathogenesis.

These reports highlight the need for mechanistic studies to ascertain how susceptibility alleles impact the process of thymic selection. Through the creation of isogenic systems involving human bone marrow progenitors, thymic organoids, mTECs, and APCs, novel avenues can now be explored to investigate genetic control of the human adaptive T-cell repertoire. Key polymorphisms may be altered by gene editing and genes and/or pathways may be "switched" on or off in a temporal fashion by the addition of chemical enhancers or repressors in either T-cell precursors or thymic stroma. Not only will this provide key insight into pathogenic versus regulatory receptors but could also potentially provide an opportunity for the *ex vivo* education of T cells in isogenic thymic organoids for auto- and/or allo-tolerance induction strategies following β cells regenerative or replacement therapies in T1D.

MODELING ANTIGEN-SPECIFIC T-CELL RESPONSES

Type 1 diabetes is most often described as a T-cell-mediated organ-specific autoimmune disease. This notion emanates from seminal experiments including the strong linkage to HLA, early animal model adoptive transfer experiments (59, 60), and the presence of autoreactive memory T cells within the insulitic lesion of organ donors with T1D (12, 14). Studies have been conducted to investigate and monitor autoreactive T cells in peripheral blood mononuclear cell (PBMC) of T1D subjects. To date, none of the commonly employed techniques have approached the sensitivity/specificity and level of standardization observed for autoantibody assays validated by the diabetes antibody standardization program now known as the islet autoantibody standardization program (61–66). We would speculate that the major reason(s) for this inability to identify robust T-cell biomarkers results from both technical limitations of the current assays, along with the inherent biology of T cells. Our data profiling the TCR repertoire in T1D nPOD organ donors demonstrated only modest overlap in high frequency clonotypes between the pancreatic lymph nodes and spleen (as a surrogate of PBMC) (67). This was particularly striking for $CD4^+$ T cells (mean ± SD; 9.2 ± 7.0% of clones shared), with $CD8^+$ T cells demonstrating significantly more TCR-β complementarity determining region 3 amino acid sequence overlap among different tissues (36 ± 21%).

Studies to quantify antigen-specific T cells with ELISpot or MHC-multimer reagents have demonstrated the rare nature of these cells in PBMC (in the range of 1:50,000–1:1,000,000) (68). This presents a number of challenges when trying to identify key auto-antigen targets and peptides important during the natural history of disease. To address this particular limitation, we have co-opted an approach pioneered in the cancer immunotherapy field to generate large numbers of tumor-antigen-specific T cells. Specifically, we have generated lentiviral contructs that express full TCR-α and β chains in multi-cistronic expression cassettes. This technique is effective for redirecting the specificity of primary human Tconv and Tregs as well as $CD8^+$ T cells (69). Recent advances in gene editing and receptor engineering have advanced this field to create programmable circuits for studying T-cell specificity and effector functions (70). Importantly, we have recently employed TCR gene transfer to directly test the cytotoxic activity of glucose-6-phosphatase-reactive $CD8^+$ T cells to target and lyse β-Lox5 cells or primary β cells *in vitro* (71). From a therapeutic perspective, our current efforts demonstrate that human Tregs can be redirected to recognize β cell auto-antigens in the context of DR3/DR4-DQ8 and remain highly suppressive *in vitro* to Tconv recognizing a shared peptide or in a bystander fashion (72). The application of novel single cell/clone analysis platforms, when used in concert with isogenic cellular systems, will allow researchers to quickly move from *in silico* TCR-α/β sequence information to unlimited numbers of antigen-specific T cells to expedite auto-antigen discovery and functional studies.

iPSCs can be used for yet another approach to generate a large number of antigen-specific T cells and to further study mechanisms of thymic selection. A small number of groups have successfully differentiated iPSCs into functional T cells. iPSC derived from a single $CD8^+$ T-cell clone have been re-differentiated into naive and eventually highly functional CTLs (73). This application has emerged as a particularly potent means to not only bolster the number of antigen-specific T cells but also correct the anergic and senescent phenotype common to tumor-infiltrating T cells in cancer, and while early studies were focused on generation of $CD8^+$ CTLs for targeting virus-infected cells (73) or tumors (74), the methods could be adapted to focus on auto-antigen-specific T cells. When iPSC derived from non-T cells (not bearing rearranged TCR genes) are used for T-cell differentiation, a broad diversity of TCR rearrangement events is possible (75).

Differentiation protocols for iPSC-derived T cells require culture on the murine stromal cells line OP9 expressing the Notch ligand protein DL1 (74). The quality of iPSC-derived T cells has been incrementally improved by altering culture conditions, for example activating CD4/CD8 double positive iPSC-derived thymocytes via CD3 to enhance CTL killer activity (76). Today, detailed protocols are available for the differentiation of antigen-specific $CD8^+$ T cells from iPSC (73). To date, advances in single-positive $CD4^+$ T cells have not approached the same progress as CTLs, yet efficient protocols to generate $CD4^+$ T_H-cell populations are expected. For example, advances in deriving human thymic epithelial cells from iPSCs (77) could enhance *in vitro* differentiation of $CD4^+$ and $CD8^+$ T cells by providing the full repertoire of human soluble and membrane-associated growth factors. In addition, iPSC-derived thymic epithelia will enable more precise studies of how disease-associated gene variants impact thymic selection by regulating specific processes such as auto-antigen expression during negative selection. The capacity to grow and differentiate large numbers of isogenic antigen-specific T cells ($>10^9$ cells), without the typical constraints of primary human T-cell clones opens up the potential for gene editing and extensive functional studies. Thus, we are nearing the point where isogenic

iPSC systems can be used to study human T-cell development at a mechanistic level that was previously only attainable in animal models.

MODELING INNATE IMMUNE RESPONSES

Development of auto-antigen-specific T cells requires more than a failure of thymic negative selection. Naive T cells in the periphery must be primed by professional APCs. DCs are specialized APCs with potent abilities to initiate antigen-specific $CD4^+$ and $CD8^+$ T-cell responses. To elicit $CD4^+$ T cells priming, activation, proliferation, and effector function, DCs must first capture antigens via phagocytosis or micropinocytosis. It can be envisioned that this antigen capture in T1D manifests through DCs phagocytosing dead/dying β cells or exosomes derived from β cells.

Several genes associated with T1D risk are expressed in myeloid lineages including monocytes, macrophages, and DCs, and it is likely that at least some of the immune pathogenesis of T1D arises from the innate end of the immune system. Differences in innate immune function could emanate from dysregulated antiviral or type 1 interferon (T1-IFN) responses, altered co-stimulation, changes in antigen acquisition, or enhanced expression of pro-inflammatory cytokines. As an example, a T1-IFN response signature has been observed preceding T1D onset in high-risk populations (32). The NOD Rip-LCMV mouse model corroborates this finding, where IFN-α is critical for progression of T1D (78). Furthermore, some enteric viral infections have been associated with risk for T1D. In NOD mice, rotavirus infection can accelerate T1D in a T1-IFN-dependent manner (79). In humans, a growing number of studies have reported associations between enterovirus infection and T1D (80–84). Thus, genes that regulate the innate response to viruses including T1-IFN expression or signaling could mediate T1D risk by altering innate immune function.

T1D RISK GENES THAT MODULATE ANTIVIRAL IMMUNITY

PTPN22, commonly associated with modifying receptor signaling in T and B cells, is also reported to alter the way that DCs respond to danger signals such as bacterial lipopolysaccharide by modulating TRAF3 signaling and T1-IFN production (85). In lupus, the risk variant of *PTPN22* tagged by rs2476601, the same variant that is associated with T1D (**Table 1**), is associated with altered TLR7-induced T1-IFN production (86).

A major counter-regulator of IFN signaling is the regulatory cytokine IL-10. Indeed, IL-10 is so potent for protection of host cells from CTL-mediated killing that many DNA viruses have evolved viral homologs of IL-10 to protect them from antiviral immunity (87). The T1D risk locus defined by the SNPs rs3024504 and rs3024493 includes *IL10* (**Table 1**). A protective role for IL-10 in murine T1D has been established through transgenic NOD mice that over-express IL-10 or where exogenous administration of recombinant IL-10, plasmid DNA encoding IL-10, or cells expressing IL-10 have been used (88–90). Moreover, *in vitro*, IL-10 protects human islets from the cytotoxic effects of inflammatory cytokines (91).

From the innate arm of the immune system, variant alleles of the T1-IFN receptor downstream signaling protein Tyk2, the cytosolic viral RNA sensor IFIH1 (MDA5), the macrophage lysosomal enzyme cathepsin H, and the phosphatase *SH2B3* are also associated with risk for T1D (**Table 1**). Collectively, these genes along with *PTPN22*, *IL10*, *SOCS1* and potentially others signify a major role for innate immune responses in T1D pathogenesis. Similar to T-cell responses, isogenic systems are critical for understanding how each risk variant affects innate immune function.

ISOGENIC MODELING OF INNATE-ADAPTIVE IMMUNE INTERACTIONS

Innate APCs participate in the initiation of immune responses; however, they also play an important role in sustaining an ongoing adaptive immune response. Interaction of APCs with antigen-specific $CD4^+$ T cells provides bi-directional signals to both cell types. $CD4^+$ T helper type 1 (T_H1) cells are important enhancers of macrophage function. Secreted cytokines (e.g., IFN-γ) and membrane-associated so-stimulatory molecules [e.g., CD40 ligand (CD40L)] expressed by T_H1 cells arm macrophages to more effectively kill microbes or infected cells. In T1D pathogenesis, there are essential roles for T_H1 T cells, IFN-γ, CD40-CD40L, and intra-islet macrophages. Where IFN-γ-secreting T_H1 cells encounter macrophages in the islets of NOD mice, the macrophages become activated and produce inflammatory cytokines and reactive oxygen species that kill β cells (92).

Most human studies of macrophages and DCs rely on two sources of cells—transformed monocytic leukemia cell lines or peripheral blood monocytes isolated from venipuncture. Some studies utilize alveolar macrophages derived from broncioloaviolar lavage or other specialized macrophages that are collected and studied *ex vivo*; however, sample number and size are limiting. PBMCs, while plentiful in number, easy to differentiation into macrophages or DCs, and available from large cohorts due to the low risk of venipuncture, are not ideal for all genetotype:phenotype studies where as discussed above, isogenic systems are key. This is further complicated in monocytes, macrophages and DCs because they are non-dividing cells in culture and generally difficult to modify genetically. iPSCs offer a solution to both problems because they are relatively simple to modify by lentiviral gene delivery or CRISPR/Cas9 and they are effectively immortal in culture. Differentiation of monocytes from iPSC offers the opportunity to study individual T1D risk genes in macrophages and DCs with unprecedented clarity. Protocols for differentiation of iPSC-derived monocytes vary widely from a simple two-cytokine mix of IL-3 and macrophage colony-stimulating factor (MCSF) (93) to a complex mix of cytokines and growth factors (94). Both protocols yield monocytes that can be differentiated into macrophages or DCs using standard conditions (MCSF for macrophages; GM-CSF + IL-4 for DCs), and the differentiated cells retain functional properties of peripheral blood monocyte-derived cells. Thus, isogenic systems now allow researchers to study the effects of a gene variant in either adaptive or innate immune cells alone, but more importantly, we can now determine how T1D risk variants impact innate/adaptive

immune interactions, which are more representative of *in vivo* disease etiology.

THE β CELL AND ISLET MICROENVIRONMENT

While the immune system is thought to serve as the primary pathogenic mediator of T1D, there are events leading up to that cytotoxic cell–cell interaction that must occur to facilitate autoreactive T-cell destruction of β cells. Specifically, autoreactive $CD8^+$ T cells must home from the bloodstream and tether to inflamed endothelium creating firm adhesion contacts, extravasate through the endothelial membrane into the extracellular matrix (ECM), and eventually survey the microenvironment for their cognate antigens presented by HLA class I hyperexpressing islets (95). To completely model the events driving immune destruction of β cells *in vitro*¸ culture systems are needed where both β cells and endothelium can be derived. Extensive research has focused on the differentiation of functional, glucose-responsive, insulin-secreting β cells from human embryonic stem cells (hES) (96–98) as well as iPSC (99–103). Established protocols rely upon multistage culture of pluripotent cells to derive definitive endoderm followed by progressive differentiation of pancreatic endoderm. Often the β cells (or β-like cells) are transplanted to immunodeficient mice where further maturation and functional development continue *in vivo* (104–106). More recently, methods have been developed to convert human fibroblasts into β-like cells by compressing the differentiation protocol so that iPSC reprogramming and differentiation of endoderm occur simultaneously (107). Many of these efforts are being carried out with the eventual goal of replacing β cell mass in T1D patients or utilizing xenotransplantation into humanized mice to model T1D pathogenesis. An alternative is to use β-like cells and immune cells from syngeneic iPSC to model immune destruction of β cells *in vitro*. This process could include endothelial layers (108–110), or ECM barriers that mimic key structures involved in immune homing *in vivo*. The advantage of this specific approach would include the ability to test novel strategies for blocking cellular adhesion, chemotaxis to inflammatory chemokines (e.g., IP-10), as well as potentially blocking degradation of the ECM needed for T-cell migration into the islet microenvironment.

ISOGENIC CELLULAR SYSTEMS: A TOOL FOR EXPEDITING TRANSLATIONAL THERAPIES

The emerging fields of iPSC and isogenic cellular systems, when coupled with genome-editing technologies, hold great potential for elucidating causative genes in complex disorders such as T1D. With at least 57 independent genetic variants contributing to overall risk, the need for experimental platforms to expedite validation of causal variants is paramount to the field of functional genomics. Before starting an iPSC project, a few considerations must be made: (1) What will be the source material for iPSC reprogramming (i.e., risk gene profile)? (2) Which of several available iPSC reprogramming methods will be utilized? and (3) What differentiation protocols are available for the cells of interest?

Each investigator must determine starting cell source and reprogramming method based on available cells and the ultimate research plan. Our group has found that $CD34^+$ HSCs isolated from peripheral blood can be efficiently reprogrammed into iPSCs using Sendai virus (**Figure 5**). This method has a few advantages over the use of PBMCs. First, $CD34^+$ progenitor cells can be isolated from fresh, non-mobilized peripheral blood and expanded *in vitro* (111). Second, the efficiency to generate iPSCs is higher with this approach versus non-sorted PBMC. We have observed that as few as 2,000 isolated $CD34^+$ HSCs yielded several iPSC colonies. Finally, the resultant iPSC will have native genetic configurations at both the TCR and immunoglobulin loci. However, $CD34^+$ cells may not always be the best source of donor material. Where a re-arranged TCR with known antigen recognition is desired, $CD4^+$ or $CD8^+$ T cells from T1D patients could be used. In addition, it is known that T-cell-derived iPSCs differentiate back into T cells more efficiently, putatively due to epigenetic memory of the lineage (73).

In addition to cell type, it is particularly important to know the T1D risk genotype(s) of donor materials. One initiative at the University of Florida Center for Cellular Reprogramming is building an iPSC resource for genomic medicine. Samples from 50 healthy donors (25 males/25 females) with genome-wide SNP typing performed using the ImmunoChip platform are being utilized to generate iPSC lines. The SNP library will include all known T1D risk variants making this cell library and others like it (e.g., the Helmsley Cellular Research Hub, cellhub.org) powerful tools for studying complex genetic traits. Such an iPSC library with SNP database will provide an extremely useful common platform for SNP validation studies in combination with conventional gene editing technologies (**Figure 4**). For example, starting from iPSC clones harboring heterozygous status for a particular SNP (**Figure 4**, Module A), an investigator can obtain SNP hemizygous clones through CRISPR/Cas9-mediated allele targeting. Using such clones, one can study the effect of SNP variations within isogenic conditions in a relatively short timeframe.

Reprogramming somatic cells into iPSC is no longer limited to the investigators who have developed various methods in their own labs. Since the initial discovery of the "Yamanaka Factors" in 2006 where four minimal genes (*Oct3/4*, *Sox2*, *c-Myc*, and *Klf4*) were identified as key iPSC reprogramming factors (112), numerous advancements in reprogramming gene delivery have been made: these include delivery of reprogramming genes as lentiviral transgenes, plasmid DNA, or messenger RNA. Each of these platforms has become commercially available in reprogramming kits so that most labs can reprogram iPSC from a variety of tissues. Our group has found most success with a Sendai virus reprogramming vector. This non-integrating and self-limiting murine parainfluenza virus delivers the four essential iPSC genes in a single polycistronic message (**Figure 5B**) (113–115). Regardless of the method used, iPSCs take on a highly pluripotent phenotype and can be used to differentiate numerous lineages.

We have highlighted earlier progress in differentiating iPSC into key immune, endocrine, and endothelial cell types.

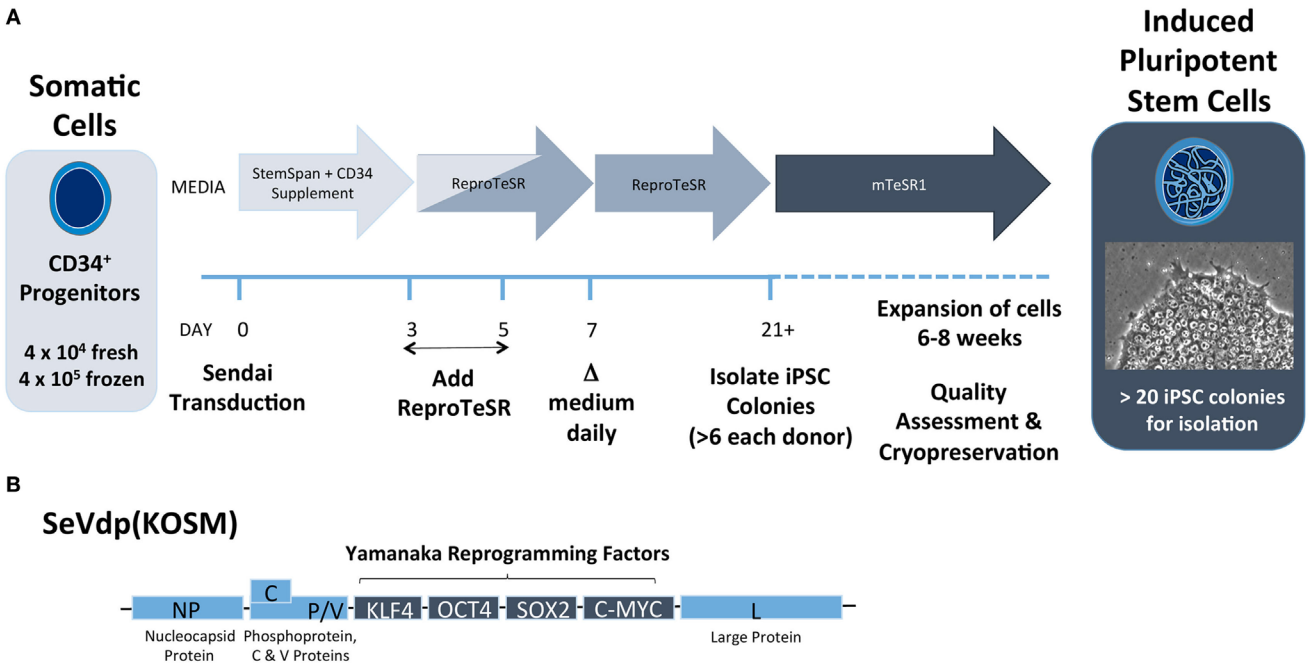

FIGURE 5 | Induced pluripotent stem cell (iPSC) reprogramming schematic from CD34+ cells using Sendai virus. **(A)** CD34+ progenitor cells are transduced with Sendai virus on day 0 and are cultured in feeder-free conditions on Matrigel-coated plates with CD34+ expansion medium (e.g., StemSpan SFEMII plus StemSpan CD34+ Expansion Supplement) for the first 3 days. Medium to support reprogramming (e.g., ReproTeSR) is added on days 3 and 5. Starting on day 7, medium is replaced daily until colonies are sufficiently large to isolate clonally, typically on day 21. After isolation, cells are cultured in PSC maintenance medium (e.g., mTeSR1) for 4–6 weeks of expansion. After expansion, iPSCs undergo assessment for pluripotency and normal karyotype and can be cryopreserved for later use. **(B)** Replication defective Sendai virus (SeVdp KOSM) contains Sendai genes NP (nucleocapsid), P (Phosphoprotein), L (large protein), C protein, V protein, and Yamanaka reprogramming genes KLF4, OCT4, SOX2, and c-MYC.

Without doubt, future applications of this approach will continue to expand as the community derives additional cell types from iPSC progenitor populations. The ability to switch between protective and susceptible variants and effectively turn genes on/off or up/down will allow the reductionist types of mechanistic studies previously only possible in gene knockout or transgenic animal models. One can certainly envision future models employing iPSC that layer increasingly complex admixtures of cells to recapitulate tissue micro-environments complete with multiple endocrine cell types, acinar tissues, microvasculature, and perhaps even innervation. We, along with others, are beginning to print living cells into liquid-like solid matrices allowing for exquisite control of cellular distribution in 3D space (116). The preliminary transcriptional profiles that have emerged from the transition from 2D culture in plastic wells to 3D cell culture have already suggested a distinct gene expression signature, more akin to that extracted from native tissues. Specific investigations using such 3D culture systems together with isogenic cellular models are needed to examine T1-IFN signaling with modulation of IFIH1 and TYK2 risk alleles as well as costimulatory pathways known to confer T1D risk (e.g., CD28/CTLA4 and CD226/TIGIT) (**Table 1**). Moreover, there will certainly be applications to reconstruct immune developmental niches to recapitulate key elements of hematopoietic development in the bone marrow, thymus, and secondary lymphoid organs. Such studies are expected to afford novel drug discovery through identification of new therapeutic targets.

The most obvious applications for stem cells in the T1D field reside in the ongoing need to replace the loss and functional inactivity of endogenous β cell mass that precipitates glucose dysregulation [reviewed in Ref. (117)]. To date, this has been accomplished through both hES- and iPSC-derived insulin-producing β cells. The capacity to model and recreate not only β cells but also functional immune populations will allow the testing of therapies to close the translational loop and prevent recurrent auto- or allo-immune rejection of transplanted β cells. Indeed, this might be accomplished by introducing genes to protect iPSC-generated β cells against apoptosis (e.g., GLIS3) (118) or to shield them from immunological attack, representing key objectives for iPSC-derived treatments in the regenerative medicine space.

Interventional trials to restore or preserve β cells in T1D have largely been driven by individual investigator sponsored trials in the context of larger consortiums (e.g., TrialNet and the Immune Tolerance Network). These efforts have largely taken the form of repurposing clinically approved drugs from other diseases or have been based on preliminary studies generated in the NOD mouse model of T1D. While these efforts are beginning to demonstrate some transient preservation of C-peptide (the serum marker co-secreted in equimolar amounts with insulin), no current therapy has yet resulted in an FDA-approved intervention capable of demonstrating long-term efficacy (119–128). We propose that

additional dose finding studies using human isogenic cellular systems to screen for desired mechanistic outcomes could potentially inform clinical trial agent selection and dosing.

From a patient perspective, the notion of equipoise limits experimental testing of many novel and/or high-risk combinatorial agents. By adopting isogenic cellular systems, those limitations could be mitigated by testing and optimizing prior to trial validations. Moreover, despite some demonstration of efficacy in preliminary trials (e.g., teplizumab, abatacept, alefacept, and ATG with or without G-CSF), no clear marker has emerged *a priori* that effectively predicts clinical responders or non-responders to any particular agent beyond basic cohort demographics of age, residual C-peptide, and disease duration (127–129). The use of isogenic cellular systems and personalized testing could facilitate drug selection and dose optimizations with clearly defined mechanistic readouts (e.g., phosphorylated-STAT5 response following low-dose IL-2) (130–133). Ultimately, the advent of genomic editing and isogenic cellular systems will not only enable a deeper understanding of disease pathogenesis but should also expedite the speed of discovery and clinical translation with the hope of both restoring β cell mass and inducing durable antigen-specific immunological tolerance.

CONCLUSION

The emergence of genomic medicine has accelerated the rate of discovery with regard to the genetic basis of T1D. Multidimensional datasets now make it possible to overlay components of genetic variation, epigenetics, and transcriptional control of gene expression. Unfortunately, the vast number of associated SNPs, heterogeneity in human disease, and limits of clinical resources present a new set of challenges. There remains a paramount need to move beyond discovery of associated SNPs to a deeper understanding of causative variants to elucidate the molecular mechanisms and pathways of disease. The advent of iPSC technologies and precision gene editing now allows researchers to expedite the discovery and validation of these disease-associated variants.

Induced pluripotent stem cell technologies were initially met with great enthusiasm with the prospect of offering the capacity for regenerative medicine applications, including autologous β cell replacement in T1D. While the robustness and efficiency of these approaches will continue to advance, the current technologies exist to derive these cells, enabling researchers to build more powerful models of disease pathogenesis. Specifically, isogenic cellular systems now allow modeling of target β cells, effector T-cell populations, and the innate and stromal components that interact with both the target organ and effector arms of the immune system. The capacity to rapidly derive these highly limited and rare populations at scale, all while targeting genomic loci in a high-throughput manner is expected to expedite functional genomics in a manner heretofore not observed. A detailed understanding of the mechanisms by which gene variants confer susceptibility or protection to disease will undoubtedly identify a number of key immunological lynchpins that can be therapeutically targeted in a rational approach to restore immune tolerance to β cells in individuals with T1D.

AUTHOR CONTRIBUTIONS

MW and TB conceived the content and contributed to the writing and editing of the manuscript. KS and NT contributed to the writing and editing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fendo.2017.00276/full#supplementary-material.

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REVIEW
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Overlooked Mechanisms in Type 1 Diabetes Etiology: How Unique Costimulatory Molecules Contribute to Diabetogenesis

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Type 1 Diabetes (T1D) develops when immune cells invade the pancreatic islets resulting in loss of insulin production in beta cells. T cells have been proven to be central players in that process. What is surprising, however, is that classic mechanisms of tolerance cannot explain diabetogenesis; alternate mechanisms must now be considered. T cell receptor (TCR) revision is the process whereby T cells in the periphery alter TCR expression, outside the safety-net of thymic selection pressures. This process results in an expanded T cell repertoire, capable of responding to a universe of pathogens, but limitations are that increased risk for autoimmune disease development occurs. Classic T cell costimulators including the CD28 family have long been thought to be the major drivers for full T cell activation. In actuality, CD28 and its family member counterparts, ICOS and CTLA-4, all drive regulatory responses. Inflammation is driven by CD40, not CD28. CD40 as a costimulus has been largely overlooked. When naïve T cells interact with antigen presenting cell CD154, the major ligand for CD40, is induced. This creates a milieu for T cell (CD40)–T cell (CD154) interaction, leading to inflammation. Finally, defined pathogenic effector cells including TH40 ($CD4^+CD40^+$) cells can express FOXP3 but are not Tregs. The cells loose FOXP3 to become pathogenic effector cells. Each of these mechanisms creates novel options to better understand diabetogenesis and create new therapeutic targets for T1D.

Keywords: autoimmunity, T cell receptor revision, costimulation, type 1 diabetes, etiology of willful acts

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Medical advances in infectious diseases have been extraordinary, completely changing human history. The polio vaccine, small pox vaccine, and measles vaccine among others changed modern medicine. Success with infectious diseases has influenced and created a generalized approach to most medical problems. Unfortunately, using that approach for autoimmune disease has fared much less well. Diseases like type 1 diabetes (T1D) and multiple sclerosis (MS) have seen unpredictable and steady increases in incidence over the last half-century, with only limited treatment options and no cure options on the horizon (1). World-wide incidence of T1D has doubled over the past two decades (2). Examples of how diabetes is expanding can be described in former Soviet-Bloc countries where T1D incidence was virtually unheard of decades ago, but now has increased substantially in all age groups examined. Incidence in adults is now surprisingly high, but the highest increase was seen in children aged 0–4 years (2). Speculation as to why the incidence rate is so drastically expanding focused on the introduction of Western-Style diet, in other words high-fat, high-glucose

diets (3). Other parameters, such as genetics, could not account for the increases. Like T1D, MS incidence is increasing (4), as is inflammatory bowel disease (IBD) (5), even the more rare Hashimoto's Thyroiditis is seeing steady incidence increase (6). Another alarming trend is autoimmune comorbidities. Once thought to be unlikely, different autoimmune diseases now are being diagnosed in the same patient; MS and T1D (5); MS and IBD (7); rheumatoid arthritis and T1D (8); psoriasis and T1D (9); and alopecia areata and T1D (10). This is partially due to improved diagnostic techniques, and due to longer survival of patients with autoimmune conditions.

Each autoimmune disease is disparate in symptoms and effect; nonetheless, they share immunologic mechanistic similarities. The integral components of autoimmune disease like infectious disease involve classic immune reactions. During an immunological event, foreign antigens, including viruses, bacteria, funguses, etc., are processed and presented as antigens that lead to T and other cell type activation. This process collectively creates an inflammatory microenvironment. Macrophages or dendritic cells (DCs) at the infection site take up the invading pathogen that then is processed. The pathogen is internalized by engulfment or receptor-mediated uptake, and associates with proteasomes to create antigen fragments. The order of antigenicity is protein $\gg$ DNA/RNA > carbohydrate > lipid. There are very few lipid antigens, although CD1-bearing cells are able to present lipids through the CD1 complex to activate T cells (11). Once generated, the antigen associates with MHC/HLA in the Golgi–endoplasmic reticular compartment. MHC + antigen then is exported and maintained on the cell surface. Professional antigen presenting cells (APCs) include B cells, macrophages, and DC that express the class II version of MHC. $CD4^+$ lymphocytes interact with professional APC to create localized inflammation. $CD8^+$ cells interact with MHC-class I type molecules. Under normal immune conditions, $CD8^+$ cells respond to virally infected cells. In autoimmune conditions including T1D and MS, $CD8^+$ cells can play a role in pathogenesis (12, 13). T cells that carry a specific and unique T cell receptor (TCR) recognize and respond to a specific antigen creating localized inflammation. Under septic conditions, the initial response is pro-inflammatory including activation of TH1 and/or TH17 type cells (14). Under non-septic, autoimmune conditions, the pro-inflammatory phenotype also occurs (15–18). In both scenarios, an appropriately expressed TCR is required, the problem being that in autoimmune diseases T cells arise that recognize and respond to self-antigens. The thymic microenvironment is responsible for generating mature T cells with appropriate TCRs; this means generating T cells that respond to foreign/invader antigens but do not respond to self-antigens. There are, however, medical conditions where self-antigen response is desirable. Transformed cells that result in cancers need to be targeted, and self-antigen reactive T cells perform that function. Maintaining the fine-line homeostatic balance, however, becomes the tricky part.

The interaction of TCR and MHC-Antigen is a crucial aspect of immune function, never more so than during autoimmunity. Autoaggressive T cells have been predicted to slip through the selective pressures of the thymus (19). Alternatively, it has been shown that autoaggressive TCRs can be generated in the periphery through a process termed TCR revision (20–23), thereby subverting central tolerance. It is possible that peripheral negative selection occurs and may be dysfunctional in T1D and other autoimmune diseases. TCR revision contributing to autoimmune disease development has been discussed in a previous review (24). A defining feature to peripherally generated, autoaggressive T cells is expression of CD40 (20, 21, 24–28). Relative to CD40 expression on T cells is what function(s) does it perform? A surprising outcome was the potential role as a costimulus molecule (29–31). Relative to the accepted two-signal model for T cell activation (32, 33), costimulation can play a critical, even decisive, role during autoimmunity.

THE ROLE OF COSTIMULATION AND AUTO-AGGRESSION

The necessity of costimulatory molecules for T cell activation was described in the two-signal model by Bretscher (32). Signal 1 is antigen specific, mediated by TCR interaction with an antigen/MHC complex. Signal 2 is antigen independent, mediated by receptor–ligand interactions that occur between the T cell and APC. The TCR/CD3–MHC/Ag complex with the assistance of adhesion and addressin molecules expressed on each cell type act in a velcro-like manner to adhere the cells together during antigen recognition creating what is known as the immunologic synapse (34). Signaling within the synapse is bidirectional with each cell contributing to the others full activation. Therefore, dysfunction involving either cell could result in pathogenesis. From the T cell perspective, Jenkins and Swartz suggested that TCR-mediated signals alone, without appropriate costimulus, results in a permanent non-responsive condition called anergy (35). While stimulatory signals are sent from T cell to APC within the synapse, the type of costimulation toward the T cell often dictates the immunologic outcome.

IMMUNOGLOBULIN "Ig" COSTIMULUS

One of the first described T cell costimulatory molecules was CD28, which turned out to be a member of a subfamily of proteins that includes ICOS and CTLA-4. This subfamily was categorized as "Immunoglobulin-family costimulus" due to the biochemical structure of the proteins. CD28 is expressed on most naïve, activated, and memory T cells; ICOS and CTLA-4 are expressed on activated T cells and subsets of memory cells (36). CD28 and CTLA-4 interact with CD80 (B71) and CD86 (B72) found primarily on professional APCs. ICOS interacts only with B7H expressed constitutively on naïve B cells but expression extinguishes after antigen engagement and IL-4 cytokine exposure (37). CD40 stimulation of B cells restores B7H expression (37). The fact that ICOS is limited to only B7H stimulation provides possible unique signaling outcomes. CTLA-4, unlike its counterparts, plays a role in cell regulation as opposed to cell activation (36). CTLA-4 is constitutively expressed on Tregs and is inducible on effector cells (38), including potential pathogenic effector cells (39). Study of

CTLA-4-mediated tolerance demonstrated interesting outcomes relative to endogenous and exogenous antigens. Using double transgenic mouse models, a more definitive role for CTLA-4 was determined. Mice genetically manipulated to express an ovalbumin peptide directly on islet beta cells, RIPmOVA mice on a BALB/c background, and exposed to OVA-peptide-specific T cells, DO11.TCR-transgenics, only developed diabetes if the cells originated from $RAG^{-/-}$.DO11.TCR.Tg donor mice and if OVA peptide was injected (40). If donor T cells further included a CTLA-$4^{-/-}$ background, then disease occurred independently of injected OVA. Because of CTLA-4's role as potential tolerance inducer, it was considered an ideal candidate for therapeutic development. A CTLA-4 analog was developed as a therapeutic in T1D, but has had only limited success (41, 42).

Given the potential prominence of CD28 signaling, considered crucial for T cell activation, it was assumed that CD28 would be a useful target for controlling autoimmunity. The assumption being that induced anergy, as suggested by Jenkins and Swartz, would prove therapeutic. This rationale applied to T1D and other autoimmune diseases. CD28 and B7 knockout mice were created but the results were unanticipated. $CD28^{-/-}$ and $B7^{-/-}$ mice developed extensive autoimmunity (36, 43–48). One study showed that in the experimental autoimmune encephalomyelitis model of MS, $CD28^{-/-}$ mice developed very rapid, more severe disease when challenged with disease causing MOG antigen (46). If, however, the CD40–CD154 signaling pathway was blocked, no disease development occurred (46). CTLA-$4^{-/-}$ mice likewise experience extensive, systemic autoimmunity (49, 50). Collectively these data suggested that CD28 plays a more prominent role in regulatory T cell development. In fact, CD28 is required for Treg development (43). Rather than CD28 being the "all-purpose" T cell costimulus it was originally thought to be, its part in autoaggressive T cell stimulation comes in to question.

MUCIN COSTIMULUS

Given that the Immunoglobulin family (CD28/ICOS specifically) knockouts still experienced classic T cell responses; the only viable explanation was "other" costimulatory molecules. A series of T cell potential costimulatory molecules was discovered in relation to Asthma that were defined as T-cell-immunoglobulin-domain/mucin-domain (TIM) proteins. The family is comprised of eight members, two of which occur directly on T cells (51). Only TH2 cells express TIM-1 where it plays a pro-regulatory role while TIM-3 preferentially is expressed on TH1, TC1 cells, and DC (52). TIM-3 engagement results in inhibitory signals that lead to apoptosis (52). Polymorphisms within the TIM family were examined in T1D and no positive correlations were discovered (53). The ligand for TIM-3 is galectin-9 (51). NOD mice that were treated with a plasmid encoding galectin-9 were significantly protected from diabetes (54). In another model, galectin-9 treatment induced aggregation and cell death of TH1 cells, selective loss of IFNγ producing cells and suppression of TH1 autoimmunity (55). Treating mice with anti-TIM-3 resulted in increased fatty streak formation and increased atherosclerotic plaque formation in mice (56). The problem with identifying galectin-9 as the ligand for TIM-3 is that galectins are proteins that non-specifically interact with carbohydrates. Galectin-9 for example interacts with β-galactoside sugars on proteins including CD44 (57) and CD40 (58), both of which are associated with pathogenic T cells. In addition, Galectin-9 interaction with CD40 is independent of TIM-3 (58). A study showed that activation of human T cells is not affected by the presence of galectin-9 nor to antibodies to TIM-3 (59). That result is logical given that galectin-9 can bind to any β-galactoside. Given these findings, many of the TIM-3 studies must be reconsidered in relation to autoimmunity. Galectin-9 for example can interact with any β-galoctoside and, therefore, may impact a large number of signaling pathways not just TIM-3.

TNF-RECEPTOR-SUPERFAMILY COSTIMULUS

A perhaps somewhat surprising subgroup of T cell costimulatory molecules involves members of the TNF-receptor-superfamily (TNFRSF). The initial understanding of TNFRSF costimulation was from the perspective of APCs. The early assumption was based on the determination that TNFRSF ligands or TNF-super family members largely are expressed on activated T cells and the receptors, TNF-receptors I and II, and CD40, etc., were first described on APC. TNFα is a pro-inflammatory cytokine produced by TH1 cells and macrophages (60); it occurs both as a secreted cytokine, the major form, and as a cell surface molecule (61). Both the soluble and transmembrane forms of TNFα interact with TNFR I and II (61). CD154, one of the ligands for CD40, is activation induced on T cells where its expression is temporal (18). CD154 also is expressed on APC (62), astrocytes in the CNS (63), and its major source is platelets (64). Like TNFα, CD154 occurs as both a cell surface molecule and a secreted form (65–67). Soluble CD154 is significantly increased in serum of T1D (68), and other autoimmune diseases; it may behave as a highly pro-inflammatory cytokine.

Members of the TNFRSF that act as T cell costimulatory molecules include 4-1BB and OX40. Both OX40 and 4-1BB are activation induced and promote cell survival, potential T cell memory formation, and cytokine production (69–71). In addition to expression on effector T cells, OX40 was detected on $CD25^{+}$, potential Tregs in T1D patients (72). 4-1BB performs similar function on T cells (73, 74). 4-1BB mapped to the *Idd9.3* locus in NOD mouse studies, and reportedly increases IL-2 production and improves CD3 stimulated-activation-outcomes (75–77). These data suggest that OX40 and 4-1BB are more directed toward regulatory outcomes. In that same vein, another TNFRSF member is glucocorticoid-induced-TNF-receptor-protein, GITR known as TNFRSF18. GITR is predominately associated with Tregs (38). Like OX40 and 4-1BB, GITR increases IL-2 production, and improves CD3 activation, developing the MAPK signaling cascade (38, 78). Tregs have been discriminated into innate, those that arise during thymic development (79, 80), and induced, Tregs that are created in the periphery often after exposure to IL-10, GITR expression associates with induced Tregs (38, 79–82).

CD40 (TNSFR5)

Unlike the other TNF-receptor costimulatory molecules on T cells, CD40 acts in a predominant pro-inflammatory manner (18, 27, 31, 58, 83–99). CD40 expression was first described on B cells, and when associated with IL-4, CD40 signals induce antibody class switching. While this action could be involved in autoantibody generation, such function has not been described in T1D or other autoimmune diseases. Like other TNFRSF members, CD40 signals ablate cell death and promote cell survival in B cells, performing similar function in T cells (22, 100). A major problem in understanding the scope of CD40-mediated inflammation has been a gross underestimation of CD40 expression. As studies of CD40 evolved, its expression was identified in numerous cell types. CD40 is expressed on all professional APC, B cells, but also DCs and macrophages. On DCs, it plays a central role in T cell licensing. CD40 engagement on DC switches the DC's interactions with T cells (101). DCs that are high CD40 expressers promote TH1 cell development while CD40-low or CD40-negative DCs favor Treg development (102). CD40 induces iNOS in macrophages (103), thus contributing to the innate immune arm and it induces pro-inflammatory cytokines, including TNFα, IL-1α, IL-1β, and IL-6 (17, 18, 104). CD40 expression has been described on endothelial cells (105); neural cells (106); and surprisingly on islet β cells (107–109). On each of those cell types, CD40 engagement leads to pro-inflammatory cytokine production.

While initially unexpected, CD40 expression occurs on T cells, including $CD4^+$ and $CD8^+$ cells (20–23, 26–28, 31, 39, 58, 100, 110–113). Like OX40 and 4-1BB, CD40 on $CD8^+$ cells is associated with memory cell generation (114). On $CD4^+$ cells, CD40 has been reported on naïve, effector, central, and effector memory cells (29–31), in both murine and human studies. CD40 engagement works independently of CD28 or other costimulatory molecules, inducing predominantly TH1 phenotype cytokines including TNFα and IL-6 (29), as well as GM-CSF and IL-1β (31). CD40 costimulus also induces the TH17 phenotype cytokines IL-17 and IL-21. Interestingly, the TH1 and TH17 cytokines express concomitantly in TH40 cells after CD40 engagement. Because TH40 cells produce both TH1 and TH17 cytokines, post CD40-mediated costimulus these helper cells do not fit the paradigm of either TH1 or TH17 cells, and thus have been termed TH40 cells (20–22, 27, 28, 39, 100, 112, 113).

TH40 CELLS: CD40 SERVES AS A BIOMARKER FOR AUTOAGGRESSIVE T CELLS

When isolated from diabetic or pre-diabetic NOD mice TH40 cells transfer diabetes readily and without any manipulations; thus CD40 constitutes a diabetogenic T cell biomarker (20–22, 26–28, 100). A panel of highly pathogenic, autoaggressive T cell clones, including the well described BDC2.5 and BDC6.9 clones, express CD40 (20, 21, 28). Non-diabetogenic T cell clones including BDC2.4, isolated from the same NOD spleen as BDC2.5 cells, do not express CD40 (28). Primary TH40 cells increase to significantly greater percentages and cell numbers during autoimmunity (20–22, 26, 27, 100). However, like Tregs, some CD40-expressing CD4 cells arise in the thymus (39). In NOD mice that develop spontaneous diabetes, substantial thymic increases in numbers of $CD40^+$ thymocytes were observed (111). Likewise, in a double transgenic, neo-self-antigen model, DO11.RIPmOVA mice, where TCR.Tg T cells that are specific for OVA encounter OVA on thymic medullary epithelial cells, thymic CD40 expressing $CD4^+$ cells were significantly expanded in number (39). The percentage of developing TH40 thymocytes in NOD mice was identical to that of DO11.RIPmOVA mice, suggesting that autoantigen drives the expansion of TH40 cells in the thymus. During T1D, the percentage of TH40 cells expands proportionately with increasing insulitis over time in NOD mice (29). In fact, TH40 cells proved to be diagnostic for T1D. In female NOD mice, 80% develop T1D by 18–22 weeks of age, while only 20–50% of male mice develop disease. Observations reveal that diabetic male NOD had peripheral TH40 cell numbers equivalent to that of diabetic female mice (Wagner Lab observations). Likewise, in NOD female mice that did not develop diabetes, TH40 cell numbers remain at numbers found in non-autoimmune mice. These observations suggest that breach of tolerance involves TH40 cell number expansions.

Primary TH40 cells isolated directly from the pancreatic lymph nodes or spleens of pre-diabetic and diabetic NOD mice transferred progressive insulitis and diabetes to NOD.*scid* recipients (21, 28). $CD40^-$ T cells did not transfer disease, even after removal of Tregs and additional *ex vivo* activation (20–22, 28). T cell CD40 expression is long-lived unlike classic activation molecules, i.e., CD69, CD25, or CD154. Furthermore, classic TCR-mediated activation of naïve $CD40^-$ T cells does not induce CD40 expression. Interestingly, in murine studies, TH40 cells are less susceptible to Treg suppression than non-CD40 expressing T cells (22). TH40 cells are able to express CTLA-4, one of the immunoglobulin family, pro-regulatory molecules. Using the neo-self-antigen model of T1D, TH40 (DO11 TCR^+) cells isolated from diabetic mice did not express CTLA-4, while the vast majority of $CD40^-$ T cells (also DO11 TCR^+) were $CTLA\text{-}4^+$ (39). CTLA-4 is activation induced, requiring TCR engagement (38); therefore CTLA-4 expression may be regulated by CD40-mediated signals as was demonstrated (39). Observations further show that in diabetes prone NOD mice expression of CTLA-4 is deficient (39).

BATTLE OF THE COSTIMULATORY MOLECULES

Clearly, T cells express an array of costimulatory molecules and while each can contribute to activation, the reality is that different costimulators drive the T cell in different directions. CD28, for many years, was considered the central T cell costimulatory molecule. Virtually all *in vitro* T cell stimulation/activation protocols utilized CD28 costimulation. What became a surprise was that CD28 interaction with B71 or B72 drives a more regulatory phenotype leading to production of IL-4, IL-10 and especially IL-2 (**Figure 1**). The role of IL-2 will be discussed further below. One reason that $CD28^-$ only costimulation became universally

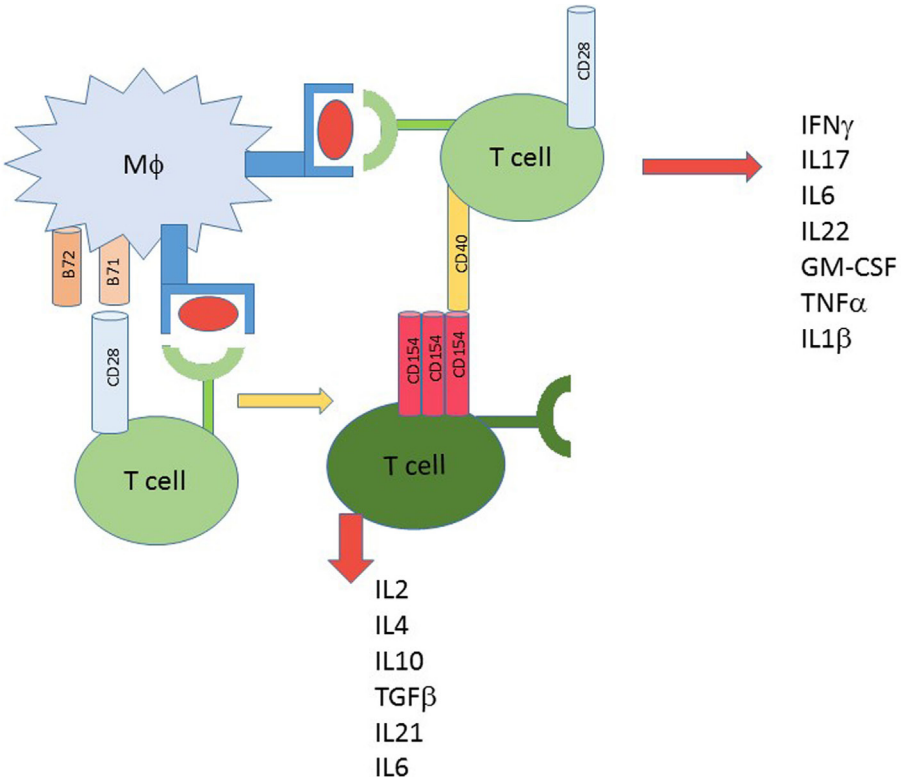

FIGURE 1 | T cell activation alternative methods. Classic 2 signal activation involves T cell interaction with antigen presenting cell. Signal one is antigen dependent and delivered to the T cell receptor by MHC/HLA and antigen. The costimulus can include CD28 interacting with B71 or B72; that outcome creates activation leading to cytokine production. When a source of CD154 is available, including from the newly activated T cells, other T cells receive a different second signal costimulus to produce inflammatory type cytokines. CD28 signals favor more regulatory type cytokines.

accepted as pro-inflammatory is that the coincident response of CD40–CD154 interaction was overlooked. When CD40 is engaged on T cells, independently of CD28 (29–31, 110) or in combination with CD28 (30, 31, 110), a TH1/TH17 pro-inflammatory phenotype results (**Figure 1**). When T cell activation occurs, signals through the synapse lead to induction of CD154 expression (115). Therefore, in the microenvironment a source for CD40–CD154 interaction in addition to CD28, costimulation is developed. In T1D compared to controls, CD40-bearing T cells, TH40 cells in particular, are over represented (20–22, 24, 27–29, 31, 39, 90, 100, 110–112, 116). The overlooked issue is T cell–T cell interactions (**Figure 1**). An activated T cell is an abundant source of CD154 and can, therefore, interact with CD40 on APC, within the immune synapse; but it also can interact with CD40 on TH40 cells. That interaction leads to inflammatory cytokine production (23, 29–31, 39, 58, 110–112), as represented in **Figure 1**. Importantly, the CD40 signal can override the CD28-mediated signal (29, 30), to drive inflammation.

THE UNINTENTIONALLY MISUNDERSTOOD ROLE OF IL-2

IL-2 discovery began from an accidental, but important happenstance involving the kidney bean extract, phytohemagglutinin (PHA). When cells in culture were treated with the extract, there was lymphocyte cellular expansion (117). The expansion was temporary, however. Over time, the substances induced by PHA that caused leukocytes to proliferate were identified as interleukins and the interleukin associated with T cell expansion specifically became known as IL-2. After this discovery, the addition of IL-2 to all T cell cultures was considered essential. To better understand T cells and IL-2 responses specifically promoting autoimmunity, IL-2 knockout mice were generated. The *a priori* hypothesis was that autoimmune disease would be completely negated, since theoretically IL-2 would be the cornerstone T cell survival cytokine. Surprisingly, IL-$2^{-/-}$ mice demonstrated that IL-2 was not the crucial cytokine for T cell development that it was thought to be (118, 119). Effector T cells develop normally in the thymus in the absence of IL-2 (120) and in fact, autoimmunity is abundant and spontaneous in those mice (121). IL-2 is, however, required for Treg development and homeostasis (120). Further study demonstrates that IL-2 promotes Treg development but does not promote pathogenic effector cell development (120). This observation creates unique problems for the vast array of T cell *in vitro* experiments where IL-2 has been added as the T cell costimulus for pathogenic effector cells. Those studies require re-visiting given that IL-2 promotes regulation.

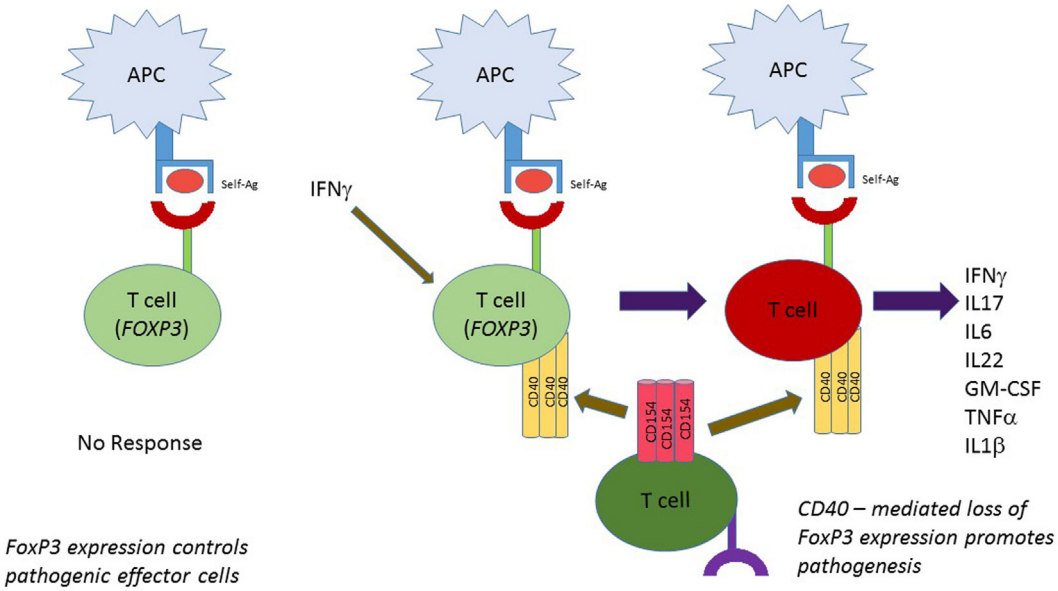

FIGURE 2 | FOXP3 as a static T cell regulator independent of Treg status. Cells other than classic Tregs express FOXP3. In the BDC2.5 T cell receptor transgenic mouse model effector, T cells express FOXP3 but in the inflammatory environment where IFNγ, etc., is produced, CD40 is induced on those cells. CD40 interaction with CD154 leads to loss of FOXP3 expression and full effector status.

ALTERNATE MECHANISMS FOR EFFECTOR T CELL REGULATION: TH40 CELLS EXPRESSING FOXP3

The BDC2.5 T cell clone rapidly and efficiently transfers diabetes to NOD.scid recipient mice (20, 28, 122). When the TCR transgenic (TCR.Tg) version of BDC2.5 was created, only about 22% of mice became diabetic in the time frame considered (123). This outcome was surprising, given the strong diabetogenicity of the BDC2.5 clone. Longitudinal studies revealed a different outcome, however. If BDC2.5.TCR transgenic mice were followed for 45 weeks, disease incidence achieved 100% (111). That finding suggests that eventual breach of tolerance occurs. TH40 cells expand rapidly in the BDC2.5.TCR.Tg mouse model, but disease kinetics are much slower than in classic NOD mice (111). The unexpected finding was that TH40 cells at younger ages contained high levels of FOXP3 (111). In this circumstance TH40 cells were not Tregs; the BDC2.5.TCR.Tg mice maintained classically defined Tregs, $CD4^+CD25^{hi}FOXP3^+$. Tregs but not TH40-$FOXP3^+$ cells performed regulatory functions (111). The disease defining parameter was loss of FOXP3 in TH40 cells. TH40 cells isolated from young animals had high FOXP3 levels and could not transfer diabetes; TH40 cells that rapidly and efficiently transferred diabetes were FOXP3 negative regardless of the age of the donor (111). Not only were TH40 cells disease instrumental, but CD40 itself proved to be a disease master switch. BDC2.5.TCR.Tg mice bred onto the CD40 knockout background did not develop diabetes at any age (111) and equally impressive, those mice did not exhibit insulitis (29). In addition, BDC2.5 T cells isolated from the CD40 KO mice maintained high levels of FOXP3 even after 45 weeks. These findings indicate that when CD40 levels are sufficiently controlled, effector cells are able to express FOXP3. Furthermore, that expression is independent of Treg status. If systemic CD40 levels and CD154 levels are substantially elevated, just as occurs in T1D, then effector cells loose FOXP3 to become pathogenic effector cells (**Figure 2**).

CONCLUSION

Failure of central tolerance cannot be excluded as an autoimmune mechanism. Numerous studies demonstrate that negative selection failure gives rise to peripheral pathogenic T cells. However, the overlooked mechanism of TCR revision also must be considered. This process that increases overall T cell repertoire to pathogens likewise increases risk for autoimmunity. If appropriate selective pressures are not applied in the periphery following TCR revision, then autoaggressive T cells necessarily will arise. For autoimmune disease to commence, additional criteria must be met, including perhaps HLA haplotype, CTLA-4, FOXP3 or other mechanisms of tolerance failure, etc. Within these bounds the role of costimulation is becoming evident as contributory for autoimmunity. The classic CD28 family has been thought essential for driving T cell expansions, but overlooked costimulation, CD40 in particular, is emerging as diabetogenic. TH40 cells become prominent in the NOD mouse model of T1D as well as in human T1D (20, 21, 26, 27). These cells have clearly proven to be important disease drivers. Of significance is that the CD40 molecule itself acts as a highly

pro-inflammatory stimulator (29, 30). When T cell CD40 is directly engaged, T cells produce TH1 and TH2 cytokines; but when CD28 is engaged, T cells produce TH2/Treg cytokines (29, 30). Another concern is that classic tolerance mechanisms driven by Tregs or by tolerance inducing molecules, CTLA-4 and FoxP3 for example, work much less efficiently on TH40 cells derived from autoimmune backgrounds (39, 111). These findings include tolerance dysfunction in human T1D (26, 27). Full understanding of disease mechanisms leading to breach of tolerance in T1D and other autoimmune diseases is a complicated process. No one mechanism alone is causative. Nonetheless a clearer picture of those processes involving TCR development and T cell costimulation is beginning to emerge.

AUTHOR CONTRIBUTIONS

DW conceived and wrote the manuscript.

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REVIEW
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Innate Viral Receptor Signaling Determines Type 1 Diabetes Onset

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Heritable susceptibility of the autoimmune disorder, type 1 diabetes (T1D), only partially equates for the incidence of the disease. Significant evidence attributes several environmental stressors, such as vitamin D deficiency, gut microbiome, dietary antigens, and most notably virus infections in triggering the onset of T1D in these genetically susceptible individuals. Extensive epidemiological and clinical studies have provided credibility to this causal relationship. Infection by the enterovirus, coxsackievirus B, has been closely associated with onset of T1D and is considered a significant etiological agent for disease induction. Recognition of viral antigens *via* innate pathogen-recognition receptors induce inflammatory events which contribute to autoreactivity of pancreatic self-antigens and ultimately the destruction of insulin-secreting beta cells. The activation of these specific innate pathways and expression of inflammatory molecules, including type I and III interferon, prime the immune system to elicit either a protective regulatory response or a diabetogenic effector response. Therefore, sensing of viral antigens by retinoic acid-inducible gene I-like receptors and toll-like receptors may be detrimental to inducing autoreactivity initiated by viral stress and resulting in T1D.

Keywords: type 1 diabetes, autoimmunity, innate immunity, toll-like receptors, RIG-I-like receptors, MDA5, type I and III interferon, coxsackievirus B

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INTRODUCTION

Characterized by the destruction of the insulin-secreting beta cells of the pancreas and subsequent loss of blood glucose regulation, type 1 diabetes (T1D) is an autoimmune disorder whose onset is triggered by a combination of both genetic and environmental factors. Virus infections, vitamin D deficiency, dietary antigens, and disruption in the gut microbiota all have been implicated in eliciting T1D development in genetically susceptible individuals (1–4). Significant evidence suggests a strong causal association between genes involved in host–virus interactions and susceptibility to

Abbreviations: CVB, coxsackievirus B; DC, dendritic cell; dsRNA, double-stranded RNA; GWAS, genome-wide association studies; IFIH1, interferon induced with helicase C domain 1; IFN, interferon; IFNAR, type I interferon receptor; IFNλR, type III interferon receptor; IRF7, interferon regulatory factor 7; LCMV, lymphocytic choriomeningitis virus; MDA5, melanoma differentiation-associated protein 5; MYD88, myeloid differentiation primary response gene 88; NOD mice, non-obese diabetic mice; pDC, plasmacytoid dendritic cell; poly I:C, polyinosinic:polycytidylic acid; PRR, pattern-recognition receptor; RA, rheumatoid arthritis; RIG-I, retinoic acid-inducible gene I; RLR, RIG-I-like receptors; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; ssRNA, single-stranded RNA; T1D, type 1 diabetes; TLR, toll-like receptor; T_{REG}, regulatory T cell; VP1, viral protein 1.

T1D. Using genome-wide association studies (GWAS), single nucleotide polymorphisms (SNPs) and gene variants conferring risk for T1D have been identified in multiple sites including the *interferon induced with helicase C domain 1* (*IFIH1*), *HLA class II*, *CTLA-4*, *insulin*, and *PTPN22* genes (5–7). The precise mechanisms leading to a loss of self-tolerance experienced in T1D are not adequately understood. Virus-mediated activation of T1D has been proposed to be caused by several different processes including direct islet infection, increased exposure to self-antigens which may have been previously sequestered, bystander activation, and molecular mimicry (8, 9).

Natural drift of genetic predisposition cannot adequately explain why the incidence of T1D has increased approximately 1.8% annually from 2002 to 2012 worldwide (10, 11). The concordance rate for T1D among monozygotic twins is about 35% by age 60, signifying significant contributions from environmental factors ultimately leads to the onset of autoimmunity (12). Indeed, epidemiological evidence indicates a link between virus infections and development of T1D as well as multiple other autoimmune disorders, including multiple sclerosis (MS), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA). Studies have demonstrated geographical and seasonal differences, as well as disease outbreaks, correlate with increased incidence of T1D (11, 13–17).

Upon virus infection, initial innate sensing likely primes genetically susceptible or protected individuals for an effector or regulatory immunological response, respectively (18). Therefore, signaling from pattern-recognition receptors (PRRs) that identify pathogen-associated molecular patterns (PAMPs) associated with certain viruses could determine whether infection will promote T1D induction. The production of interferon (IFN) from this PRR–PAMP interaction is a prominent immunological response for defense of virus infections. All three types of IFN, type I (IFN-α, -β, -ε, -κ, and -ω), type II (IFN-γ), and type III (IFN-λ1, -2, -3, and -4), stimulate the production of pro-inflammatory molecules from the interferon-stimulated genes (ISGs) to induce a strong antiviral state to prevent spreading of the infection to surrounding cells and also to establish an adaptive immune response (19, 20). Accordingly, alterations in signaling stemming from PRR activation represent the foundational mechanisms leading to T1D development by producing an IFN signature which is conducive for autoimmunity.

INNATE VIRAL RECEPTORS

Genome-wide association studies indicate heritable differences in viral receptors and their related genes influence T1D susceptibility. Functional diversity of innate PRRs due to genetic variants may push the immune homeostasis toward an imbalance between pathogen hypersusceptibility and autoimmunity. In conjunction with an inherent variation, several different viruses have been implicated in causing inappropriate responses leading to T1D (4, 21). Among these viral candidates, enteroviruses such as coxsackievirus B (CVB) have been the most notable etiological agent attributed to T1D (22–24).

Dependent on the signals received from PRRs, innate immune cells including dendritic cells (DCs) macrophages, monocytes, natural killer cells and innate lymphoid cells can contribute to establishing either an effector inflammatory response or a more tolerogenic response by secreting cytokines, chemokines, and through priming of naïve T cells. While cross-reactivity of lymphocytes due to homology between viral and endogenous antigens and have been proposed in the establishment of T1D, non-specific immune stimulation causing persistent and low-grade inflammation are more likely underlaying the cause of pathogen-induced triggering of autoimmunity (25). The scale of an immune response is reliant on tightly regulated activation and inhibitory signals which may tip into an exaggerated or improper response causing the loss of self-tolerance (26).

Innate immunity and PRRs represent the first line of defense to coordinate the immune system for pathogen clearance and sets the stage for ensuing cellular and molecular pathway activation. The initial inflammatory state established with innate recognition of viral products induces beta cell damage and is then followed by apoptotic events and an effector T lymphocyte response killing the beta cells. Therefore, placing emphasis on the PRRs is critical for understanding the pathogenesis of autoimmune diabetes. There are three primary families of PRRs involved in detecting viral products: toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide oligomerization domain-like receptors (27). Summarized in **Figure 1**, this review will focus on the contribution of RLRs and TLRs to T1D following engagement with their respective viral PAMPs.

RLRs IN T1D

The RLR family consists of RIG-I, melanoma differentiation-associated protein 5 (MDA5), and Laboratory of Genetics and Physiology 2 (LGP2), which are cytosolic receptors that recognize pieces of viral RNA from picornaviruses, flaviviruses, and paramyxoviruses (28). RLRs primarily bind viral replication intermediates [i.e., double-stranded RNA (dsRNA)] in infected cells and promote recruitment of transcription factors and adaptor molecules to restrict virus replication and prevent spread to other cells. Among a diverse range of effects, activation of MDA5 and RIG-I induce a potent type I and III IFN expression which go on to stimulate antiviral gene expression and increase antigen presentation (29, 30). LGP2 can bind short pieces of dsRNA and acts as a negative regulator for both RIG-I and MDA5; however, it lacks N-terminal caspase activation and recruitment domains necessary for signaling (31).

Expressed from the *IFIH1* gene, MDA5 is a cytosolic helicase which binds long viral dsRNA to induce a type I and III IFN response (18, 32). MDA5 has been identified as an important part of the host immune response to CVB and is necessary for preventing early replication of the virus and potentiating tissue damage (33). Various SNPs in the *IFIH1* gene have been found to confer either greater or reduced susceptibility for the onset of T1D (7). These SNPs likely alter the expression and activation of MDA5 when challenged with pathogenic stress. The A946T

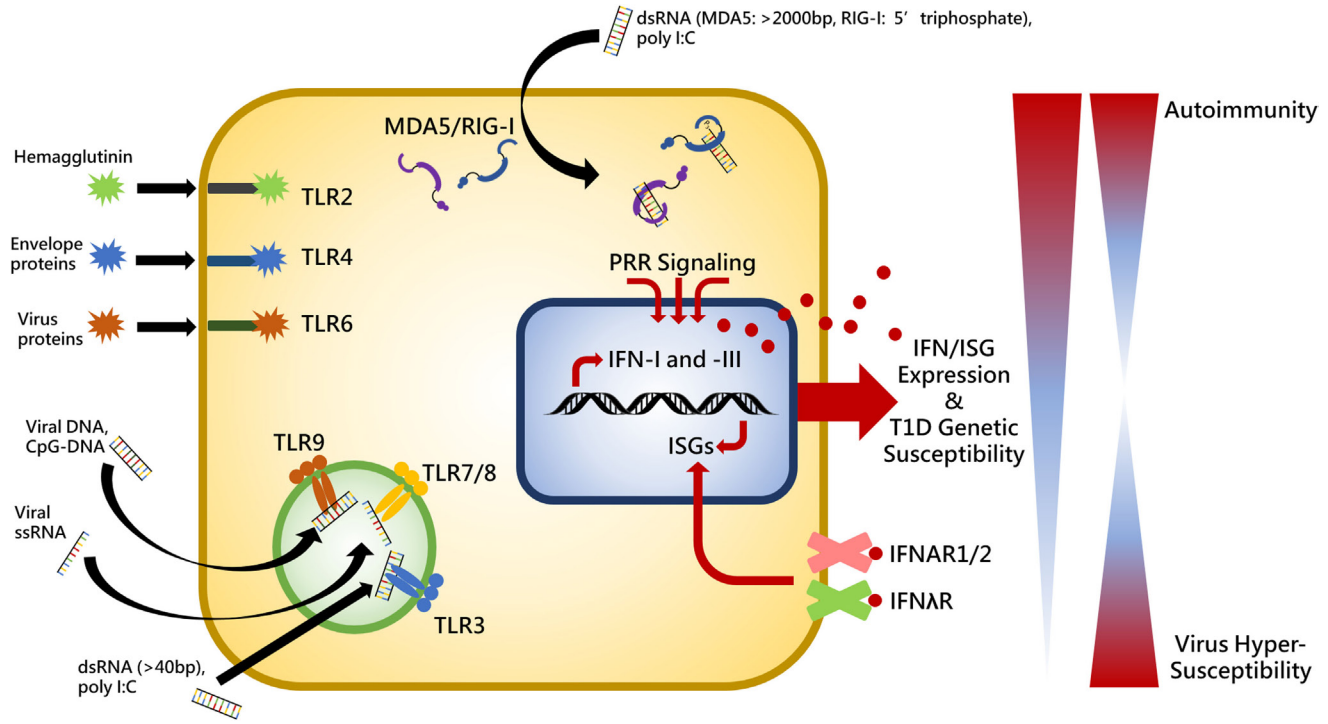

FIGURE 1 | Summary of toll-like receptor (TLR)- and RIG-I-like receptors virus-associated ligands and the relationship between interferon (IFN) expression, genetic susceptibility, and autoimmunity. Upon ligand binding, cytosolic MDA5 and RIG-I receptors induce activation of the adaptor molecule, VISA (also called MAVS, IPS1, and CARDIF), endosomally located toll-like receptor 3 (TLR3) recruits TRIF (also known as TICAM), and TLR2, -4, -6, -7, -8, and -9 interact with myeloid differentiation primary response protein 88 (MYD88) in order to provide IFN expression stimulation in the cell nucleus. IFN induces expression of various interferon-stimulated genes (ISGs) which perform positive feedback on IFN genes. IFN is released from the cell to establish an antiviral state in surrounding cells and act in an autocrine and paracrine manner by binding to its cell surface receptors, IFNAR1/2 and IFNλR. Individuals exhibiting heightened genetic susceptibility to type I diabetes (T1D) can have increased basal and pathogen-elicited expression of IFN causing the immune system to skew toward a self-reactive state. Conversely, significantly diminished IFN expression would render a host unable to mount a proper response to virus infection. Thus balance of receptor stimulation between autoimmunity and virus hypersusceptibility is tightly regulated and pathogenic stimuli which exacerbates inflammation in genetically susceptible individuals may result in loss of tolerance.

(rs1990760) mutation in *IFIH1* has been implicated in the development of multiple autoimmune diseases, including T1D, SLE, and MS (34, 35). Individuals which exhibit loss-of-function SNPs on even a single allele in RLR genes including E627* and I923V in MDA5 generally benefit from protection to T1D (35, 36). Hyperexpression or constitutive activation of MDA5 by mutagenesis has been shown to promote development of type I interferonopathies including SLE and Aicardi-Goutières syndrome (37–39). Diabetic patients which are heterozygous for the A946T SNP have a more robust ISG expression and immune response to CVB challenge when compared to healthy controls, potentially signifying an enhanced ability to promote IFN and ISG signal transduction during infection (40, 41). Accordingly, mutations in *IFIH1* causing gain-of-function are associated with hyperexpression of both IFN-I and -III (32, 39). Gorman et al. recently found that mice homozygous for the 946T variant as well as mice simultaneously exhibiting two *IFHI1* risk alleles (843R and 946T) have increased basal activation of *IFIH1*-related genes, enhanced protection from encephalomyocarditis virus infection, increased incidence of autoimmunity, and are inherently more sensitive to self RNA ligands (42). These mutations conferring T1D risk may be altering the homeostatic intensity of inflammatory molecule expression and/or the kinetics of target binding and activation—causing ligands to produce more potent or prolonged IFN responses. For example, the E627* mutation in MDA5 causes loss of a portion of the protein's C-terminal region and consequently forfeiture of dsRNA ligand binding (36). The A946T risk variant is also associated with heightened sensitivity to IFN-α in SLE patients so this mutation may allow receptors to become more easily activated (43). This evidence supports the notion that pathogen-mediated T1D is likely similar to the described type I interferonopathy disorders.

Our lab has previously exhibited the importance of MDA5 signaling by demonstrating reduced expression of the receptor can be protective for T1D. Non-obese diabetic (NOD) mice which were heterozygous for a null *IFIH1* allele (MDA5$^{+/-}$) and expressed roughly half as much MDA5 as wild-type NOD mice were shown to have decreased incidence of spontaneous disease

(18). More importantly, upon CVB4 infection, these heterozygous mice were completely protected from diabetes onset while about 50% of homozygous NOD mice carrying a full complement of *IFIH1* developed T1D within 7 days of infection. MDA5 knockout mice were also completely protected from spontaneous T1D onset; however, they were highly susceptible to virus. Compared to homozygous mice, the MDA5$^{+/-}$ mice displayed a specific type I IFN response characterized by a large spike in IFN-β occurring three days post-infection. It appears this particular IFN signature provides a succinct signal from IFN-β that is sufficient to clear the virus without inducing autoimmunity. Furthermore, MDA5$^{+/-}$ mice had decreased CD4$^+$ and CD8$^+$ effector T cells as well as a robust CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (T_{REG}) response that suppressed IFN-γ-producing CD4$^+$ T cells, thereby preventing T1D.

TLRs IN T1D

Toll-like receptors are broadly expressed PRRs in both immune and non-immune cells which detect microbial- and viral-associated PAMPs (44). Upon recognition of pathogenic and/or foreign material, TLRs influence a number of immunologic mechanisms including activation and maturation of antigen-presenting cells (APCs), antibody production, downregulating T_{REG} responses, and inducing a pro-inflammatory environment through secretion of various cytokines and chemokines (45). Each of the TLRs may be stimulated with endogenous DNA or RNA antigens produced during cell death that may be a result of virus infection (46). However, those specifically recognizing viral-associated ligands: toll-like receptor 3 (TLR3), TLR7, TLR8, and TLR9 (and to a lesser extent TLR2, TLR4, and TLR6), have all been implicated in having a role in the diabetogenic potential of certain viruses (45, 47).

Toll-Like Receptor 3

Binding short pieces of dsRNA, TLR3 is an endosomal receptor heavily expressed in classical DCs and a variety of epithelial cells (47). Unlike all other TLRs, TLR3 is MYD88-independent and instead utilizes the adaptor molecule TRIF for signal transduction following activation (44). The dsRNA mimetic polyinosinic:polycytidylic (poly I:C) is recognized by TLR3 and has been shown in various mouse studies to either protect or induce and increase severity of T1D depending on dose and administration (48–50). NOD mice deficient for TLR3 have high mortality from CVB4 infections and the few that survive develop T1D (51). However, in some instances, TLR3-KO NOD mice can show less severe insulitis as well as some reduced susceptibility to T1D induction following CVB4 infection, but experience no difference in spontaneous disease development (52). TLR3 signaling within resident macrophages is critical for antiviral host defense to CVB4 as well as altering marginal zone B cell composition in NOD mice (50, 51). This indicates that enhanced TLR3 activation may participate in T1D development as a result of virus infection. Certain polymorphisms in the *TLR3* gene have shown to be associated with increased risk of T1D and more aggressive pathology (rs3775291 and rs13126816) while others impart protection (rs5743313 and rs11721827) (53).

Toll-Like Receptors 7 and 8

Expressed in the endosome, TLR7 and TLR8 recognize single-stranded RNA (ssRNA) while TLR9 is typically activated by binding unmethylated CpG DNA ligands from DNA viruses and microbial pathogens (54). TLR9-KO NOD mice have significantly lower rates of spontaneous diabetes, reduced activation of diabetogenic CD8$^+$ cytotoxic T cells (CTLs), and elevated expression of the immunosuppressive marker CD73, particularly on T_{REG} cells (55–57). Thus, activation of TLR9 induces a less tolerogenic immunological state that contributes to the pathogenesis and acceleration of T1D.

Using rat insulin promoter mice expressing lymphocytic choriomeningitis virus glycoprotein (LCMV-GP), researchers have shown that LCMV infection produced IFN-α *via* stimulation of TLR3 and TLR7; this in turn increased the expression of MHC class I molecules in the insulin-secreting beta cells of the pancreas (58). This mechanism, where TLR-mediated expression of IFN-α upregulates MHC-I in the islets, was shown to be vital for the diabetogenic potential of LCMV and subsequent progression toward an overt autoreactive response. LCMV-GP-specific CTLs in the pancreas were unable to cause disease without hyperexpression of MHC-I (58). Stimulation of TLR7 in conjunction with CD40 activation of DCs can induce diabetogenic CTLs in the pancreatic lymph nodes of NOD mice to promote onset of autoimmunity (59). Even the repeated topical administration of a TLR7 agonist, imiquimod, is sufficient to promote T1D development while inhibition using IRS661 can significantly decrease onset (59). TLR7 signaling in plasmacytoid DCs (pDCs) primes B and T cell activation *via* IFN-I secretion in rotavirus infections; however, inhibition of TLR7 is able to block this process from occurring and prevent acceleration of T1D following infection (60). The role of TLR7 and TLR8 in promoting autoimmunity has also been indicated in CVB3-induced self-reactivity toward myocardial tissue (61).

Environmental Inducers of TLRs

Previously, therapeutics for T1D prevention and treatment in the past have been primarily aimed at modifying or suppressing the adaptive immunity. Today, a shift in perspective of clinical methodology points toward targeting innate components to tolerize early pathogen-stimulated mechanisms as an effective strategy. Bednar and colleagues demonstrated that a TLR4-agonist monoclonal antibody, TLR4–MD-2, was able to halt and reverse fulminant T1D by inducing APC tolerance to pathogen in NOD mice (62). TLR4 is typically activated by lipopolysaccharides and other microbial products; however, envelope proteins from viruses including CVB can also stimulate its activation (63). Although it is uncertain whether CVB interaction with TLR4 is involved in T1D.

The natural route of enteroviral infection is through the gut, where the biodiversity of bacteria, viruses, fungi, and other microorganisms are significant mediators of immune homeostasis and autoimmunity (64). Accordingly, stimulation of the

innate immunity and signaling on the mucosal surfaces from environmental pathogens could be detrimental to T1D onset. All TLRs other than TLR3 use the adaptor molecule MYD88 for signal transduction. Deletion of MyD88 in specific pathogen-free NOD mice confers resistance to diabetes (65). T1D resistance through loss of MYD88 is attributed to disruption in the gut flora since these MYD88-KO mice develop autoimmunity when housed in germ-free facilities (65). A "balanced signal hypothesis" has been suggested where microbiota-derived stimulation of TLR4 signaling through the adaptor molecule, TRIF, provides a tolerogenic effect on T1D pathogenesis, while TLR2 signaling promotes diabetogenesis (66). Commensal virus communities likely contribute similarly to innate stimulation through PRR recognition of viral ligands. A recent study has determined the intestinal virome is significantly altered prior to onset of autoimmunity in T1D-susceptible children (67). The overall diversity of the gut virome is reduced preceding disease development and certain viruses, such as *Circoviridae* and various bacteriophages, are significantly associated with either negative or positive T1D risk (67). This signifies a complex host–microbiome–virome relationship contributes to T1D and further studies are necessary to understand how these interactions alter disease and inflammation to skew genetically susceptible individuals toward either a protective or disease-causing state.

T1D DISPLAYS INTERFERONOPATHY-LIKE QUALITIES

Type I IFN

Pattern-recognition receptor activation and signaling remain the predominant inducer for IFN signatures that can protect as well as portend onset of not only T1D, but are also typical of rheumatic disorders such as SLE and RA (68, 69). The synergistic effects of type I and III IFNs are significant mediators for the adaptive immune system that promote lymphocyte maturation and mediate antigen presentation (19, 70). Accordingly, the IFN expression elicited by PRR activation is essential to autoimmune development. As such, it has been proposed that virus infections including CVB may be inducing localized interferonopathy-like characteristics within the islet microenvironment to trigger autoreactivity (71). Islets from patients recently experiencing onset of T1D exhibit heightened expression of certain ISGs in the islet and peri-islet regions in a manner which is similar to islets infected with virus (72). Knocking out the type I IFN receptor (IFNAR) in the T1D-susceptible rat strain, LEW.1WR1, protects from T1D, reduces insulitis, and delays onset following poly I:C or virus challenge (73). Originating with PRR stimulation, aberrant activation of pDCs and genetic mutations in the IFN signaling pathway likely contribute to the IFN signature evident in T1D induction (74).

Transient upregulation of type I IFN can be seen in genetically predisposed children preceding the seroconversion of T1D-related autoantibodies (75). Nearly all cells produce and respond to type I IFN; however, pDCs secrete a considerable amount of systemic IFN-α. Indeed, the secretion of IFN-α through TLR7- and TLR9-stimulated pDCs in the PLN of NOD mice is critical for onset of T1D (76). Blocking IFN-α signaling through IFNAR1 of young NOD mice (2–3 weeks old) significantly delays onset and incidence of diabetes as well as promote secretion of immunoregulatory cytokines, IL-4 and IL-10, in splenic $CD4^+$ T cells (76). Treating human islet cells with IFN-α *in vitro* triggers endoplasmic reticulum stress which disrupts insulin production by hindering the conversion of proinsulin to insulin signifying a potential mechanism by which IFN-α may be prompting development of T1D (77). Using a neutralizing antibody against IFN-α or using a specific agonist for S1PR1, an immune regulatory receptor which mediates IFN-α autoamplification, protects T1D onset in a *Rip*-LCMV mouse model by limiting the infiltration of autoreactive T cells into the islets and by inducing expression of tolerogenic receptor genes, such as *Pdcd1*, *Lag3*, *Ctla4*, *Tigit*, and *Btla* (78). This immunomodulation is able to prevent the autoreactive T cells from harming the insulin-secreting beta cells thus preserving the glucoregulatory function of the pancreas. Accordingly, the progression from prediabetes to full-onset disease requires signaling from IFN-α.

The transcription factor, interferon regulatory factor 7 (IRF7), is constitutively expressed in pDCs and is expressed in most other cells upon IFNAR activation (79). IRF7 is involved in signal transduction from MYD88-dependant endosomal TLRs (TLR7, TLR8, and TLR9) as well as RLRs to trigger IFN gene expression. A study by Hienig et al. used rat tissues to elucidate the IRF7-driven inflammatory network (IDIN) to relate that genetic mapping with known viral response genes and disease GWAS (80). It was determined that an rs9585056 SNP (on chromosome 13q32), located in the orthologous human genes controlling IDIN, was significantly associated with susceptibility to T1D and promoted expression of the IRF7-driven signaling network. Similar to gain-of-function mutations in *IFIH1*, this type of genetic predisposition would cause vigorous antiviral engagement resulting in an IFN and immune response which may be more pathogenic than the actual virus.

Type III IFN

While all nucleated cells respond to type I IFN, the type III IFN receptor (IFNλR) is primarily only expressed on pDCs and epithelial cells including pancreatic islet cells. Type III IFNs bind to the IFNλR consisting of dimer of IFNLR1 and IL10R2 domains. There is significant overlap in signaling pathways and activation between IFN-III and IFN-I; however, non-redundant roles for IFN-III in host antiviral responses exist (70, 81). Islets from humans exhibiting a protective *IFHI1* rs1990760 (946A/T) polymorphism produce an increased IFN-III response following CVB3 infection, likely through IRF-1 signaling, when compared to individuals with a risk-associated genotype (946T/T) (32). It is uncertain whether this additional expression of IFN-λ has protective qualities or whether it is simply a compensatory mechanism for lower IFN-I signaling from MDA5. However, IFN-λ-treated DCs are able to promote the specific proliferation of T_{REG} cells *in vitro* and IFN-λ treatment has been exhibited to improve pathology of RA in mice by reducing inflammatory neutrophils (82, 83). Collectively, this signifies IFN-III may be

contributing to diabetes pathogenesis and should be further studied.

TIMING IS IMPORTANT FOR THE VIRAL ETIOLOGY OF T1D

Acute versus Persistent Infections

With regards to T1D induction, it is unclear whether virus infections are being sustained or acute infections are initiating mechanisms which go on unregulated even after viral clearance. If the virus becomes persistent, remnants of the infection linger within tissue-specific microenvironments to provide continuous stimulation of innate receptors to produce chronic inflammation as illustrated in **Figure 2**. Some picornaviruses such as Theiler's murine encephalomyelitis virus have been shown to persist in certain tissues to provide sufficient inflammation to drive autoimmunity (84). Conversely, acute infections may be priming the host and establishing events which direct an autoreactive effector response. Initial infections may be activating pathways which proceed with incessant positive feedback likely due to genetic differences which result in functional variations in innate compsonents, receptor activation/deactivation, and/or signaling pathway elements.

Coxsackievirus B is a positive sense ssRNA virus with a tropism for the pancreas, heart, and liver; however, it does not appear to establish cytolytic infections of pancreatic beta cells (85). Rather, CVB is likely promoting a pro-inflammatory environment within the pancreas to elicit autoimmunity (86). Infecting human islets with CVB3 induces potent expression of type I and III IFN, MDA5, RIG-I, and TLR3 along with a variety of inflammatory cytokines (32). Clinical evidence suggests some individuals who develop a loss of tolerance against insulin early in life (1–3 years) have an impaired capacity to mount a sufficient defense against the enterovirus viral capsid protein, viral protein 1 (VP1), that may cause an inability to sufficiently clear CVB following infection (87). Correspondingly, persistent pancreatic cell CVB4 infections have been shown to induce epigenetic changes by promoting production of dysregulated microRNAs targeting T1D risk genes (88).

Prolonged viral infections may be providing sustained activation of PRR signaling for the expression of IFN-I, IFN-III, and inflammatory cytokines, leading to a robust lymphocyte response and induction of autoimmunity. Low-grade enterovirus infections have been demonstrated to be established and maintained within the pancreatic islets of patients recently experiencing onset of T1D (3–9 weeks prior) but rarely in healthy controls (89). Persistent enteroviral presence has also been detected in the gut mucosa of T1D patients, however, viral genomes seem to be maintained in the absence of viral protein expression or production of infective particles (90). It is not clear whether defective replication is allowing production of the viral genes without assembly of virus particles or if virus components are simply persisting in the tissue after infections. However, conformational differences and modification of viral PAMPS may be dictating innate signaling. Stem-loop structures in long dsRNA are preferentially recognized by MDA5, while 5′ di- or triphosphate motifs on dsRNA are sensed by RIG-I (28, 91). Deletion or alterations

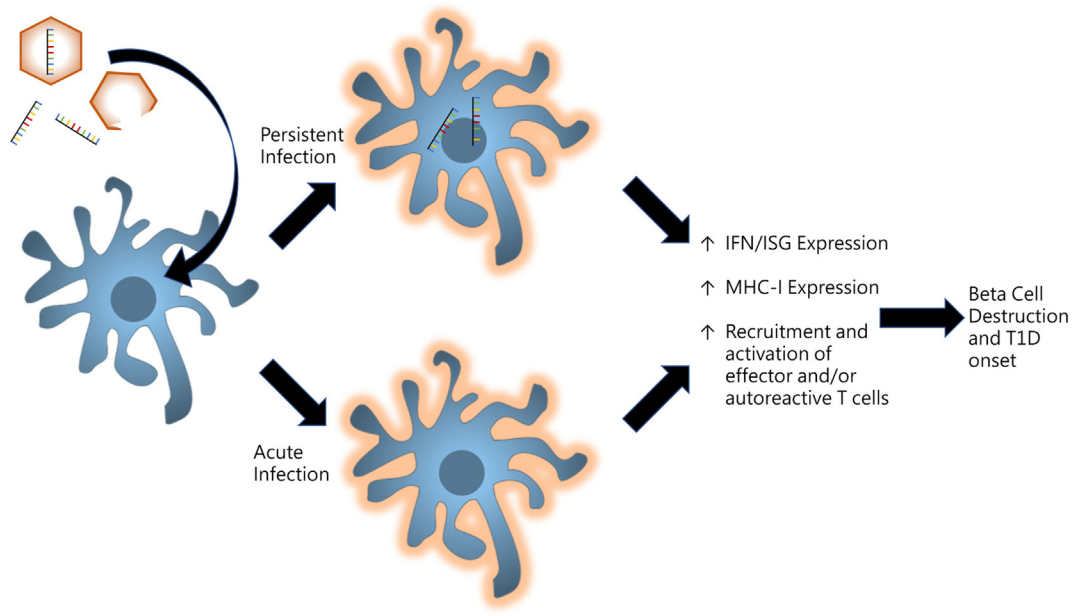

FIGURE 2 | Model for persistent versus acute infections in pancreas for promoting type I diabetes (T1D). Virus infections induce proinflammatory signaling in resident and infiltrating antigen-presenting cells of the pancreas. Persistent infections whereby viral products including enteroviral 5′ terminally deleted double-stranded RNA are maintained in tissue microenvironments to produce low-grade inflammatory signaling. Alternatively, acute infections may result in unregulated positive feedback mechanisms which produce chronic inflammation even after the virus is cleared.

in the structural composition of viral genomic PAMPs may be altering innate receptor signaling. CVB is known to persist in myocardial tissues following naturally occurring deletion of 5′ end terminal genomic sequences resulting in reduced virus replication and loss of cytopathicity (92–94). A recent study has shown that 5′ terminally deficient CVB persists also in the pancreas of NOD mice for at least several weeks following an acute wave of infection (95). The 5′ end of the CVB genome includes a cloverleaf-like tertiary structure which may be favorably sensed by MDA5; however, it is unknown exactly how innate sensing is affected with these terminal deleted viruses. Persistence of modulated enteroviruses may be providing sustained innate activation for prolonged inflammatory responses in and/or around the islets that result in the loss of self-tolerance in T1D. Alternatively, loss of structurally relevant motifs for RLR sensing may actually render receptors like MDA5 unable to bind the modified dsRNA ligands.

Temporal Determinants of T1D-Related Virus Infections

A systematic review compiling and analyzing clinical studies over approximately the last two decades, found that individuals had about 10 times higher odds of having enterovirus infections before or during onset of diabetes or prediabetes when compared to controls (24). Patients experiencing fulminant T1D directly following suspected enterovirus infection had strong expression of MDA, RIG-I, and VP1 in the islets when compared to T1D and non-diabetic control patients (96). Furthermore, mononuclear cells which infiltrate the pancreata of patients experiencing fulminant T1D had high expression of TLR3 and TLR4 (96). A recent study by Laitinen et al. screening systematically collected blood samples from birth through seroconversion for T1D-related autoantibodies and progression to clinical T1D, found that children were at higher T1D risk if infected with CVB1 (97). However, the patient was protected if exposed to either CVB3 or CVB6 prior to CVB1. Phylogenetic similarity between CVB1, CVB3, and CVB6 indicates the possibility that cross-protection between highly related enterovirus serotypes may be occurring. Additionally, CVB1 infection has been often followed by the appearance of islet autoantibodies about 6 months later (98, 99).

Characterizing temporal relationships between infection and autoimmunity onset are incredibly intricate due to the incredibly multifactorial nature of the pathogen, the hosts, and the disease. The timing of pathogen exposure and an individual's age likely has crucial impact on immunological development (100). It was recently determined that weaning pups from a colony of NOD mice with low incidence of T1D in a "diabetogenic environment" (i.e., with a colony of NOD mice with high T1D incidence) is able to transfer rates of diabetes development by adapting similar gut microbiota and promoting development of B cells in the mesenteric lymph nodes which are inherently more easily activated (101). This transmittance is only evident when the mice are weaned together, as this environmental exposure does not affect rate of diabetes onset when mice are co-housed starting at 3 weeks of age. Mustonen et al. performed a clinical analysis among children with HLA-dependent T1D genetic risk (exhibited DR3-DQ2 and/or DR4-DQ8 haplotypes) in Finland, Estonia, and Russian Karelia to determine disease trends in T1D susceptibility (102). Children who exhibited seroconversion of T1D-related autoantibodies had their first infection earlier and overall had more infections in the first year of their lives especially in the respiratory tract. Furthermore, those which progressed to T1D had twice as many infections in the first 3 years of their lives than non-diabetic children. It can be questioned, however, whether inherent susceptibility to T1D also confers lower tolerance to pathogens or whether the children experienced onset of T1D due to stress of the frequency of infections they experienced. A report from The Environmental Determinants of Diabetes in the Young study has confirmed that young children experiencing recent respiratory infections withstand a heightened risk of developing T1D-related autoimmunity; however, more work is necessary to determine specific viral agents present in the preceding months before autoantibody seroconversion (103).

While enteroviruses remain the most likely candidate for T1D onset, numerous other viruses have been shown to have roles in promoting or protecting T1D (104). Links between many viruses, however, seem to be more circumstantial and less evident. For instance, a study was performed examining the spatio-temporal exposure of viruses using geographical disease incidence rates in France and relating that data with mapping of T1D patient residences and the timing of the patients' T1D onset (105). This analysis indicates a positive correlation between summer diarrhea and influenza-like infections at 1–3 years of age with eventual development of T1D while there was negative relationship between varicella. Additionally, evidence suggests autoreactivity in NOD mice may be induced as a consequence of an immunological response against endogenous retrovirus-secreted microvesicles in the islets (106). Recently, a study using high-throughput proteomic profiling of antibodies in new-onset T1D patients found serum antibodies exhibit a significant reactivity against Epstein–Barr virus viral antigens (107). Ultimately, a multifactorial and heterogenous contribution from multiple environmental agents is likely for T1D pathogenesis.

CONCLUSION

The increased incidence of autoimmunity witnessed in developed nations likely signifies a deleterious shift in pathogenic environment especially early in life. This may be due to modern alterations in the host–pathogen paradigm developed over milleniums of co-evolvement. Epidemiological studies have not indicated an emergence of infections that could adequately explain such a significant increase in autoimmunity. Thus, it is likely caused by an alteration in how individuals respond to environmental and pathogenic stressors. One rationalization for this change, the "hygiene hypothesis," states that a reduction in pathogenic and environmental antigen exposure particularly during development has caused the immune system to produce over-exaggerated responses resulting in increased rates of autoimmunity. Decreased interaction with typical environmental antigens has fostered inexperience by innate host receptors, causing over-sensitization and improper stimulation of inflammatory pathways.

Environmental induction of T1D *via* viral infection may essentially require a "perfect storm" of immune reactivity where genetic susceptibility allows PRR signaling to render a target organ susceptible to attack by self-reactive lymphocytes. A balance of signaling by different receptors including RLRs and TLRs is providing opposing forces to simultaneously promote and inhibit autoimmunity and certain environmental stressors may be sufficient to tip that balance toward autoimmunity by inducing pro-inflammatory signaling. Ultimately, the timing, pathogenesis, and target site of infection influences the likelihood of antigen-non-specific bystander activation of autoreactive B and T cells. Understanding these pathways may hold a high degree of therapeutic potential to block onset of autoimmunity by mediating antigen exposure, developing relevant vaccines, and managing molecular pathogenesis mechanisms which confer disease development.

AUTHOR CONTRIBUTIONS

ZM and MH conceptualized, wrote, and edited the manuscript. ZM created the figures.

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REVIEW
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Redox-Dependent Inflammation in Islet Transplantation Rejection

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Type 1 diabetes is an autoimmune disease that results in the progressive destruction of insulin-producing pancreatic β-cells inside the islets of Langerhans. The loss of this vital population leaves patients with a lifelong dependency on exogenous insulin and puts them at risk for life-threatening complications. One method being investigated to help restore insulin independence in these patients is islet cell transplantation. However, challenges associated with transplant rejection and islet viability have prevented long-term β-cell function. Redox signaling and the production of reactive oxygen species (ROS) by recipient immune cells and transplanted islets themselves are key players in graft rejection. Therefore, dissipation of ROS generation is a viable intervention that can protect transplanted islets from immune-mediated destruction. Here, we will discuss the newly appreciated role of redox signaling and ROS synthesis during graft rejection as well as new strategies being tested for their efficacy in redox modulation during islet cell transplantation.

Keywords: redox signaling, reactive oxygen species, type 1 diabetes, islet transplantation, immune rejection, immunology, encapsulation

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INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by chronic inflammation where self-reactive immune responses selectively target and destroy β-cells within the pancreas. In a majority of patients, insulin therapies can help regulate the rapid variations in blood glucose levels that result from this autoimmune attack, however, this is not a cure and for a relatively large number of patients, exogenous insulin treatment is not enough for them to maintain stable blood glucose levels (1). A major hurdle for insulin therapy is the ability to optimally sense and respond to glucose fluctuations as rapidly or precisely as a living β-cell. Therefore, the constant struggle to achieve

Abbreviations: APC, antigen-presenting cell; CITR, Collaborative Islet Transplant Registry; CTLA-4, cytotoxic T lymphocyte associated antigen-4; DAMPs, danger-associated molecular patterns; DCs, dendritic cells; Del-1, developmental endothelial locus-1; ECs, endothelial cells; ER, endoplasmic reticulum; Gpx-1, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; HIF, hypoxia-inducible factor; HO-1, heme oxygenase-1; H_2O_2, hydrogen peroxide; IBMIR, instant blood-mediated inflammatory reaction; IFN-γ, interferon gamma; IL-1β, interleukin 1 beta; iNOS, inducible nitric oxide synthase; JNK, Jun N-terminal protein kinase; LbL, layer-by-layer; MCP-1, monocyte chemoattractant protein-1; MDSCs, myeloid-derived suppressor cells; MnSOD, manganese superoxide dismutase; MOMP, mitochondrial outer membrane permeabilization; MSCs, mesenchymal stem cells; NAD(P)H, nicotinamide adenine dinucleotide phosphate; NOD, non-obese diabetic; NOX, NAD(P)H oxidase; $O_2^{\bullet-}$, superoxide; $^{\bullet}OH$, hydroxyl radical; PAMPs, pathogen-associated molecular patterns; PARP, poly (ADP-ribose) polymerase; PRRs, pattern recognition receptors; PVPON, poly(*N*-vinylpyrrolidone); RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TA, tannic acid; Teff, effector T cell; TF, tissue factor; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor alpha; Treg, regulatory T cell; T1D, type 1 diabetes; UPR, unfolded protein response; VEGF, vascular endothelial growth factor.

efficient glucose homeostasis still persists. The death of these vital insulin-secreting cells within the islets of Langerhans and the resulting glucose dysregulation leaves patients at risk for developing serious life-threatening complications including cardiovascular disease, neuropathy, and renal failure (2).

Poor glucose control and the prevalence of secondary risks associated with T1D have lead researchers to investigate alternative treatment options for these patients. Unfortunately, in humans, there are currently no adequate metrics to detect these diabetic patients before the onset of autoimmunity. Usually, the presentation of symptoms, such as fatigue, extreme thirst, polyuria, or weight loss prompts a visit to a health professional, and only then does the presence of autoantibodies in their blood lead to their diagnosis. However, even at the time of diagnosis these patients still have some functioning β-cell mass remaining. Attempts have been made to delay or reverse the severity of T1D after diagnosis and to prevent further β-cell loss by utilizing immunotherapies to dampen autoreactive responses. A few of these therapies include the inhibitory cytotoxic T lymphocyte associated antigen-4 (CTLA-4)-Ig to block effector T cells (Teffs) (3), anti-CD20 therapy to deplete functional B cell responses and increase regulatory T cell (Treg) responses (4), and interleukin 1 beta (IL-1β)/IL-1R antagonists including anakinra and canakinumab to neutralize inflammatory signaling cascades including MAPK and NF-κB pathways (5). Clinical trials utilizing these treatments in early onset T1D patients displayed variable efficacy for maintaining higher C peptide levels with less reliance on exogenous insulin, however, any benefits were only temporary, and treatment was not successful in halting the progression of disease (3–5). The persistent challenges in early detection and the minimal effectiveness of immunotherapies have lead to a search for alternative treatment options to restore the functionality of insulin regulation in individuals after the destruction of β-cells has already occurred. One such attractive therapy is islet transplantation.

Islet cell transplantation is a more permanent alternative to exogenous insulin therapies with fewer long-term complications. By restoring functional β-cells into these patients, they can more accurately modulate their blood glucose levels and diminish the risks associated with glucose fluctuations. Unfortunately, as with any other organ or tissue transplant, immune-mediated graft rejection as well as an initial loss in islet graft viability induced by oxidative stress and inflammation continue to pose challenges for the long-term success of this strategy. There is also damage associated with the recurrence of autoimmunity toward the graft in the T1D patient as well as islet-specific risks of immunosuppression. In addition to the low survivability of the islet graft, other barriers to widespread utilization of this therapy include the sensitivity of islets toward hypoxia, redox-associated mechanical and chemical damage during isolation, and poor viability and islet yield from human cadaveric donors (6, 7). Subsequently, efficient human islet transplantation normally requires more than one cadaveric donor per recipient and some patients require consecutive transplants to prolong adequate blood glucose regulation (8).

Despite the hurdles that still need to be overcome, islet transplantation has come a long way in the last three decades. Prior to the late 1990s, islet transplantation into human patients had very little success in maintaining euglycemia and preventing hypoglycemic events, with few patients being insulin-independent beyond 1 week after transplantation (9). Since the 1980s, digestive enzymes and a mechanical shaking process known as the Ricordi method have been used to isolate islets (10, 11). Then, in 2000, a group led by Dr. James Shapiro at the University of Alberta published what would come to be known as the Edmonton protocol (12). This small clinical trial followed seven patients beyond 1 year after intraportal islet transplantation. The Edmonton protocol revolutionized the way human islets were isolated by utilizing xenoprotein-free isolation media and transplanting the purified islets directly after isolation, eliminating the dangers of islet culture. The islets were infused into the portal vein and utilized a novel combination of immunosuppression including sirolimus, low dose tacrolimus, and daclizumab, a monoclonal antibody that recognizes CD25. All recipients attained insulin independence for nearly 5 months after transplantation. This marked a profound improvement in patient outcomes compared to previous reports, and the protocol was soon adopted worldwide. In the nearly two decades since the Edmonton protocol was reported, advancements in our understanding of islet biology, islet graft viability, methods to protect islets following isolation, *in vitro* culture, and islet transplantation has improved. According to the 2016 Collaborative Islet Transplant Registry 9th Annual Report, 50% of recipients maintain insulin independence beyond 1 year posttransplantation, and around 20% of islet transplant recipients are insulin-independent after 5 years.

Ultimately, one challenge that still persists is the harmful side effects of immunosuppressive drugs to the patient as well as the islet graft (13). These anti-rejection medications inhibit the adaptive immune response; however, most of them do not protect the graft from redox-mediated destruction or direct autoimmune inflammatory interactions. In fact, the use of corticosteroids and tacrolimus can cause serious adverse effects including diabetogenicity and elevated extracellular reactive oxygen species (ROS) production in the islets themselves (14–17). It has been shown that immunosuppression with tacrolimus, sirolimus, and anti-IL-2R can even promote the proliferation of autoreactive memory T cells due to a chronic increase in serum IL-7 and IL-15 levels (18), potentially leading to a recurrence of autoimmunity. Tacrolimus and sirolimus have also been shown to impair mitochondrial calcium uptake and ATP production (19, 20), which are key steps in the glucose responsiveness of β-cells (21, 22).

Although the mechanisms that contribute to autoreactive immune responses in T1D and islet transplantation are not fully understood, what has become clear is the significant impact inflammation and oxidative stress can have on immune responses, β-cell function, and β-cell survival. Genetic attenuation of superoxide $\left(O_2^{\bullet-}\right)$ synthesis in the non-obese diabetic (NOD) mouse model through a point mutation in the nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase (NOX) complex can influence innate and adaptive immune responses necessary for spontaneous diabetes progression (23–25). The inability to produce superoxide through the NOX complex highlights the important role of ROS generation and inflammation in disease progression, induction of β-cell death, and β-cell dysfunction (26). The generation of free radicals is not inherently a detrimental

biological process, as ROS control apoptotic pathways within the cell, and the NOX complex is involved in eradicating microbial infections. While both of these responses are vital to cellular turnover and health, elevated ROS levels can influence cellular proliferation, survival, and the induction of inflammatory signaling cascades to mediate cellular damage (27). The dysregulation of ROS synthesis in an autoimmune setting can contribute to inappropriate activation of the immune system to recognize healthy tissue as foreign. This problem is particularly dangerous if an elevated level of ROS production overwhelms antioxidant defenses, which can result in oxidative stress, ROS-mediated damage, and eventual cell death (28).

In the context of islet transplantation, the role for redox signaling is even more vital due to the relatively low levels of native antioxidant defenses within the β-cell including superoxide dismutase (SOD), catalase, and glutathione peroxidase (Gpx-1), leaving them highly susceptible to ROS-mediated damage (6, 7). The impact of redox signaling within the context of islet destruction is twofold. The presence of oxidative species such as hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{\bullet -}$) can impact glucose sensing within the β-cell (29), but they can also serve as a third signal to promote the maturation and expansion of β-cell-specific autoreactive T cell subsets (30–32). These autoreactive immune responses can initiate the destruction of β-cells though either the induction of apoptosis using the FAS pathway or by necrosis through the release of pro-inflammatory cytokines, perforin, granzyme B, and ROS (33, 34).

As scientists begin to appreciate the role of ROS in mediating inflammation and promoting transplant rejection, dissipating oxidative stress is a prime target for immunotherapies during islet cell transplantation to reduce islet vulnerability, boost patient outcomes, and prolong insulin independence (35). One proposed method to address these persistent challenges is to target the production of these reactive species during different stages of islet transplantation. The hope is that attenuating the redox status of the islets themselves or the surrounding microenvironment will promote islet function and prolong graft viability without the need for toxic immunosuppressive drugs.

IMMUNE MECHANISMS INVOLVED IN ISLET TRANSPLANTATION REJECTION

Islet transplantation into patients with T1D comes with a risk for alloimmune responses as well as recurrent autoimmunity. Both responses can utilize redox signaling to facilitate their damaging effects on the islet graft. During allogeneic graft rejection, host immune responses can become activated through either direct or indirect recognition of donor tissues (**Figure 1**). Direct graft recognition involves the interaction of donor tissue-resident antigen-presenting cells (APCs) and host T cells through an MHC-mismatch interaction (36, 37). Indirect recognition involves the processing of donor graft peptides by host APCs to stimulate host T cells through the corresponding MHC interactions. Both direct and indirect recognition pathways require the involvement of co-stimulatory molecules to trigger and activate T cell responses. To understand why these aberrant signaling pathways and the corresponding redox responses are vital at various stages of islet transplantation, it is necessary to acknowledge the interplay between redox signaling and inflammatory responses. While others have examined certain specific pathways in great depth (38, 39), this review will highlight pathways involved in redox-dependent inflammation.

Direct Recognition and Redox Signaling

In allogeneic transplantation, the direct recognition pathway involves donor APCs interacting with host effector CD4 and CD8 T cells to facilitate contact-mediated allograft rejection (40, 41). During islet transplantation, the direct recognition pathway stimulates a cellular rejection response in which direct killing of the islet graft by T cells is the primary endpoint (37). Several studies indicate that there are two requirements to execute direct islet allograft recognition and damage: the production of interferon gamma (IFN-γ) by T cells (42) and the initiation of apoptotic pathways through perforin and/or the use of Fas/FasL interactions between activated T cells and target tissues (43). Both of these mechanisms involved in islet allograft destruction have redox-dependent components that are intimately connected to their inflammatory responses.

The production of IFN-γ as well as other inflammatory mediators by APCs and by T cells is a tightly controlled process. One key regulator of inflammatory cytokine production involves the redox status of intracellular thiols. Reduced glutathione (GSH) is the most abundant free thiol in mammalian cells and is an important regulator of multiple cellular processes (44, 45). During stress conditions, GSH is oxidized into glutathione disulfide, leading to the activation of damage responses within the cell including the unfolded protein response (UPR) and apoptosis (45). Dendritic cells and macrophages with elevated levels of intracellular GSH produce more IFN-γ than those with low intracellular GSH levels (46, 47). This increased inflammatory profile by APCs can skew T cell responses through the synthesis of T cell polarizing cytokines such as IL-12 (47) and in an autocrine fashion to further promote APC activation (48). Once macrophages are activated, they produce large amounts of ROS as well as IFN-γ and IL-1β (38). These inflammatory signaling molecules aid in macrophage killing of target pathogens or facilitate islet graft destruction by mediating phagocytosis of β-cell debris.

Interferon gamma, tumor necrosis factor alpha (TNF-α), and IL-1β not only perpetuate damaging innate and adaptive immune responses but they also interact with their cognate cytokine receptors on the surface of the β-cell. Engagement of these β-cell surface receptors can initiate the activation of the RAS signaling cascade (49). Through a string of downstream phosphorylation events, the RAS pathway leads to the activation of MAPK and Myc, which can enter the nucleus and induce the transcription of genes involved in cell division, survival, and the production of inflammatory mediators (50). This pathway is redox-mediated through mitochondrial hydrogen peroxide activation of the Jun N-terminal protein kinase (JNK), which activates MAPK and stress pathways to further propagate inflammatory cytokine synthesis and apoptotic cell death (51).

The presence of ROS such as superoxide and hydrogen peroxide can also play a role during contact-dependent damage

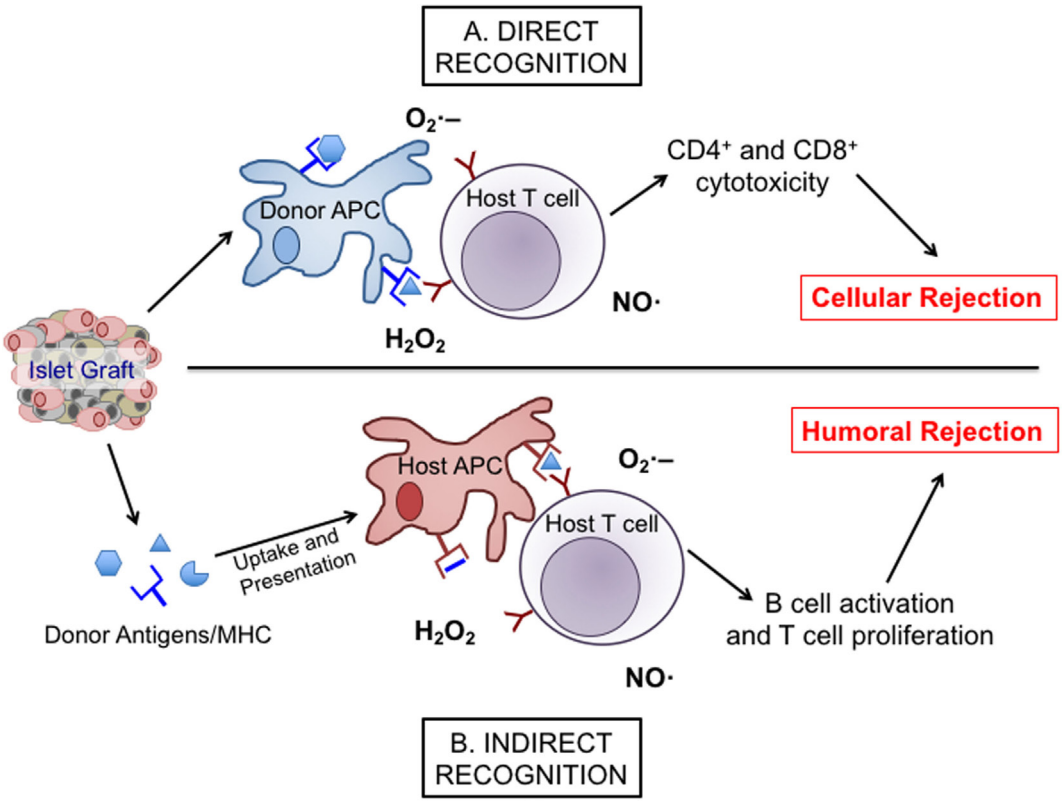

FIGURE 1 | The direct and indirect recognition pathways of islet allograft destruction. After transplantation, islet-resident antigen-presenting cells (APCs) from the donor graft can present directly to host T cells with stimulatory signals provided by reactive oxygen species, such as $O_2^{\bullet-}$, $^{\bullet}OH$, and H_2O_2 to promote the expansion and activation of alloreactive cytotoxic T cell subsets. This stimulation can then activate inducible nitric oxide synthase (iNOS), NAD(P)H oxidase, and mitochondrial oxidative pathways within the T cell to produce more nitric oxide (NO), hydrogen peroxide, and superoxide, eventually leading to cellular rejection *via* the direct pathway **(A)**. Alternatively, islet antigens can be shed into the surrounding environment following transplantation to be engulfed and presented by host APCs and then indirectly presented to host T cells. Through co-stimulation and the release of $O_2^{\bullet-}$, $^{\bullet}OH$, H_2O_2, and NO, those APCs promote a classical antibody response involving the activation and expansion of alloreactive T cells and humoral rejection **(B)**.

within the islet graft since the maturation of CD8 T cells to become cytolytic is a redox-dependent process (31). As a consequence of CD8 T cell activation by donor APCs, contact-mediated production of perforin and granzyme B by cytotoxic lymphocytes can permeabilize cells within the islet graft (52). Once these toxic molecules engage and enter the cell membrane, they initiate caspase-signaling cascades, which lead to either the direct initiation of cellular apoptosis through caspases-3 and -7 or to the cleavage of pro-apoptotic Bcl-2 family member, Bid, by caspase-8 (53). Bid then binds to the mitochondrial membrane and activates mitochondrial outer membrane permeabilization, stimulating the release of cytochrome *c* to kill the cell (54).

Redox regulation of apoptotic pathways has many facets that are detailed elsewhere (45), however, it is important to note that the primary role for ROS during apoptotic cascades involves intrinsic pathways. Mitochondria inside the β-cell can initiate stress-induced production of superoxide, which can then be converted into hydrogen peroxide and hydroxyl radicals ($^{\bullet}OH$) through the Fenton reaction (55). These radical ions are potent inducers of further redox-mediated DNA and protein damage inside the cell (56) and can lead to further apoptosis or inflammatory processes that negatively impact the viability of the graft.

Indirect Recognition and Redox Signaling

Indirect recognition of allogeneic transplants involves the interaction between host APCs with host T cells. The host APC will process and present graft antigens on MHC II molecules to activate host CD4 T cells (40, 41). The indirect recognition pathway can promote two major forms of immune responses: the humoral B cell/antibody response through the interaction between CD4 follicular helper T cells with naïve B cells and the continual activation of innate responses, namely macrophage-associated killing (37).

The transition of a B cell to a terminally differentiated plasma cell requires various cellular and metabolic changes, some of which have redox components. H_2O_2 is involved in B cell receptor signaling and activation (57). In addition, as a B cell transitions to a differentiated plasma cell, the endoplasmic reticulum (ER) undergoes drastic reorganization and expansion. During ER stress, a host of ER-based enzymes generate ROS as byproducts, leading to multiple changes in antibody production, i.e., the switch to IgM (58) and proliferation within these newly formed plasma cells (59, 60). Specifically, it has been demonstrated that oxidation of Keap1, a negative regulator of the antioxidant response, allows for the nuclear internalization of Nrf2 and transcriptional

activation of various target genes involved in B cell differentiation and antioxidant defenses (61).

After differentiation, antibodies produced by activated plasma cells can bind to the islet graft and activate the complement system to induce apoptosis within the target cell and facilitate islet graft destruction by cytotoxic lymphocytes through Fc binding (62). Antibody responses by B cells are not the only redox-dependent mechanism that can contribute to islet graft destruction. B cells are also capable of producing inflammatory cytokines including IL-6 that is redox regulated (63, 64). When a B cell receives co-stimulation through the CD40 surface receptor, the cross-linking of this receptor leads to the generation of ROS and subsequent activation of JNK pathways, resulting in the increased secretion of IL-6 into the surrounding environment (65, 66). IL-6 interacting with the cognate IL-6R can promote activation and proliferation of other immune cells by signaling through JAK2. This protein can initiate the MAPK cascade described above, or interact with STAT3, forming the JAK/STAT complex (67, 68). STAT3 is vital for optimal activation and effector function in T cells because it can directly enter the nucleus and initiate the transcription of inflammatory genes, or activate the NF-κB pathway and affect the cell cycle (69, 70). Therefore, redox regulation of B cell responses including antibody production and secretion of inflammatory cytokines can perpetuate damaging T cell responses to further destroy the islet graft.

ROLE OF FREE RADICALS AND PRO-INFLAMMATORY MEDIATORS INVOLVED IN ISLET CELL TRANSPLANTATION REJECTION

The interplay between free radicals and inflammatory molecules modulates β-cell dysfunction and death during multiple stages of purification from the pancreas and transplantation. Islets are sensitive to hypoxic stress or damage signals that occur during isolation and culture including pro-inflammatory cytokines and free radicals. Stress or damage caused by hypoxia, cold ischemia, and reperfusion can activate downstream inflammatory cascades including the NF-κB signaling pathway (71–73). After transplantation, immune effector cells including macrophages, neutrophils, B cells, and T cells migrate to the transplant site and target the islet graft for destruction by releasing pro-inflammatory cytokines, antibodies, and free radicals (74–76). Understanding the redox-dependent signaling pathways during islet isolation and following transplantation is vital to the development of novel interventions to improve transplantation success and prevent β-cell dysfunction.

Redox Signaling in Islet Isolation and Culture

Pancreatic islets in their natural setting have rather high oxygen tension, with islets receiving more than 15% of the total pancreatic blood supply (77). This massive influx of blood and nutrients plays a key role in their rapid ability to regulate glucose levels, however, linked to their relatively low levels of antioxidant defenses, it also leaves them highly susceptible to ROS-mediated damage (6, 7). In addition, this sensitivity also further exacerbates their susceptibility to oxidative damage during isolation when they are deprived of that elevated oxygen supply, leaving them in a hypoxic state (78–80).

To separate islet cells from the surrounding tissue of the pancreas, harsh digestive enzymes and mechanical separation techniques are utilized to break down exocrine tissue while leaving the islets mostly intact. These methods, while efficient, induce a level of oxidative and mechanical stress as vascularization and in turn, the nutritional stores of the islets are severed (81). This detachment from the extracellular matrix leaves islets reliant on passive diffusion to survive the isolation and transplantation process (82, 83). Consecutive days incubated under hypoxic conditions *in vitro* can have serious impacts on islet function and survival. The increase in hypoxia and oxidative stress within *in vitro* cultured islets can induce DNA damage and the peroxidation of proteins and lipids (84, 85). Mitochondrial-derived stress can cause larger islets to develop a necrotic core as less oxygen is able to diffuse to the cells in the center (86) as well as impacting insulin secretion through stress-associated decreases in mitochondrial Ca^{2+} uptake (87). Islets compensate for the low availability of oxygen in culture by upregulating transcription factors including hypoxia-inducible factors, which induce transcription of multiple genes including toll-like receptors (TLRs) and genes involved in vascular endothelial growth factor (VEGF) signaling (88, 89). Hypoxic conditions can also activate NF-κB to induce the upregulation of inducible nitric oxide synthase (iNOS) and monocyte chemoattractant protein-1 (MCP-1) expression (71), which can have significant impacts on local inflammation after the islets are transplanted.

Strides have been made in recent decades in an attempt to combat oxidative stress with the advent of less damaging enzymatic digestion methods (90) and isolation techniques to improve cadaveric human islet yield (91), but challenges persist that motivate researchers to find alternative strategies to dampen oxidative stress and hypoxia. One method to protect islets from redox-mediated damage following isolation is to increase expression of detoxifying or antioxidant enzymes. Under normal circumstances, redox scavengers are upregulated in response to inflammatory signals released from cells during times of damage or stress. Unfortunately, the low levels of these scavenging enzymes in islets make it difficult for them to combat redox stress. A few of these key enzymes include SOD, manganese superoxide dismutase (MnSOD), and Gpx-1, antioxidant enzymes that are present at lower levels in islets than in other rodent tissues (7). Therefore, increasing endogenous antioxidant defenses or supplementing with exogenous scavengers could protect isolated islets from oxidative stress.

Strategies to Dampen Redox-Mediated Damage in Isolated Islets

Mechanical and metabolic stress during islet isolation can significantly reduce the number of viable islets available for transplantation. To combat this early loss in islet mass, various

groups have attempted to protect purified islets by targeting the redox mechanisms underlying sources of cellular stress. There are two primary techniques utilized to dampen oxidative stress and redox-mediated damage in purified islets: either supplementing culture media with exogenous redox scavengers like MnSOD or genetic manipulation of the islets themselves.

The first method to decrease the oxidative damage that islets endure in culture is to treat with antioxidants after isolation. The activation of both NF-κB and poly (ADP-ribose) polymerase pathways contribute to islet damage during the isolation process (92, 93). Dissipating oxidative stress through the use of a SOD mimetic can decrease NF-κB activation, reduce the production of inflammatory MCP-1 and IL-6 by human islet cells during stress conditions, and reduce NO_2^- and $O_2^{\bullet-}$ production by macrophages (93, 94). This same antioxidant demonstrated protection from STZ-induced apoptosis during *in vitro* human islet cultures as well as prolonged islet allotransplant survival in MHC-mismatched mouse models after purified islets were cultured in the presence of the SOD mimetic (95). Systemic administration of the SOD mimetic through the use of sustained release pellets prolonged the viability of allogeneic islet grafts by reducing immune migration to the site of transplantation. The reduction in inflammation and increase in graft viability observed in the above studies is a key step in protecting islets from oxidative stress produced by immune cells and can promote long-term survival of an islet graft. Similarly, a naturally occurring antioxidant from the extract of Chinese bayberries, cyanidin-3-*O*-glucoside (C3G), was shown to increase expression of heme oxygenase-1, Bcl-2, and survivin, antioxidant, and anti-apoptotic regulators that protect islet cells from oxidative stress *in vitro* (96, 97). In addition, C3G treatment of isolated islets prior to transplantation demonstrated prolonged graft survival with fewer islet numbers required to induce euglycemia when transplanted either under the kidney capsule or into the hepatic portal vein (97). The use of soluble antioxidants, while somewhat protective, is a short-term treatment option, and once these islets are transplanted, they are still susceptible to immune-mediated damage. Genetic modifications of purified islets may provide a more permanent solution and supply antioxidant protection that can persist long after transplantation.

Viral transduction of isolated islets to overexpress antioxidant genes provides benefits to islet survival not only during *in vitro* culture but also following transplantation. Transgenic mice overexpressing SOD and Gpx-1 within islets displayed a marked resistance to redox-mediated damage *in vitro* and improved glycemic control after transplantation under the renal capsule of syngeneic mouse recipients (98). One group genetically altered isolated murine islets to overexpress MnSOD and found that upon transplantation into immunodeficient recipients, the transgenic islets displayed a marked delay in graft failure following adoptive transfer with diabetogenic T cells (99). Similarly, transfection of islets with a lentiviral vector containing thioredoxin, an ROS scavenger, reduced the toxic effects of H_2O_2 *in vitro* and prolonged graft viability after transplantation into the kidney capsule of spontaneously diabetic NOD mice (100). Other groups have also shown protective effects of glutamylcysteine ligase and SOD overexpression on islet function (101, 102), further supporting the important role of antioxidant defenses and oxidative stress for the maintenance of islet graft function.

These studies have focused on the treatment of islets after isolation, however, in human islet isolation, another hurdle also exists. Most human islets isolated for transplantation are obtained from cadaveric or brain dead organ donors. Unfortunately, these donor conditions create an elevation of inflammatory and redox-mediated damage to human tissues that can negatively impact islet yield. Therefore, it is not surprising that human islet transplant recipients can require three or more donors to obtain sufficient islet equivalents for a single transplant (103). If human islets will continue to be used for transplantation, the state of the donor before isolation cannot be ignored. One group found that treatment of brain dead rats with exendin-4, a glucagon-like peptide-1 analog that acts to increase insulin secretion and decrease glucagon production (104), served to prevent islet viability loss induced by brain death-related inflammation as well as increasing glucose-stimulated insulin secretion of these isolated islets (105). In addition, exendin-4 has also been shown to reduce hypoxia-related islet injury, reduce oxidative stress, and improved both syngeneic and xenotransplantation survival in mouse transplants (106).

While dissipation of oxidative stress during isolation and culture is important to improve islet yield, viability, and function from donors for transplantation, there are numerous other redox-dependent insults transplanted islets have to withstand to delay graft failure including immune-mediated inflammation. Therefore, defining how the two arms of the immune system facilitate islet transplant rejection, graft failure, and synergize with oxidative stress is important to prolong the survival of transplanted islets into patients with T1D.

Acute Responses and Redox Signaling After Islet Transplantation

Following transplantation, islets are susceptible to acute mechanisms of stress that lead to the loss of a large portion of islet cell mass and function (107). One such stress includes ischemia reperfusion injury as these islet cells are placed back into living tissue. The rapid influx of a multitude of nutrients as well as soluble factors not seen in culture media induces an inflammatory response involving oxidative stress known as the instant blood-mediated inflammatory reaction (IBMIR). This reaction is a nonspecific response by the innate immune system that causes robust coagulation and immune infiltration into and around the islets (88, 108), which leads to the induction of cellular apoptotic signaling pathways and internal activation of oxidative stress within the β-cell.

During allogeneic transplantation into the hepatic portal vein, IBMIR-associated responses cause an instantaneous activation of complement pathways that can lead to thrombosis and significant loss of the islet graft (109, 110). One of the major initiating factors in this response is the expression of tissue factor (TF) by islet endothelial cells (ECs). This factor can lead to the activation of thrombin, platelet activation, and secretion of other inflammatory mediators that can perpetuate inflammation and induce macrophage-directed killing (111–113). Once this cascade has

begun, upward of 60% of the islet graft is lost within the first few days (82, 107). This local inflammatory cascade at the site of transplantation can induce tissue-resident macrophages to produce superoxide and hydrogen peroxide to damage surrounding tissues (114, 115). The outflow of these reactive molecules can directly lead to DNA strand breakage and peroxidation of proteins or lipids while also activating a number of signaling pathways shown to induce apoptosis in the vulnerable islet graft (116). Not only can local redox signaling originating from the site of transplantation during IBMIR impact islet survival but also the functionality and glucose responsiveness of the β-cells. Therefore, targeting this reaction immediately after islet transplantation is a good technique to prevent the early loss of islet mass.

Another key innate immune mechanism of inflammation during this early stage of islet transplantation is the release of danger signals known as danger-associated molecular patterns into the extracellular space. These danger signals are highly pro-inflammatory, being recognized by pattern recognition receptors (PRRs) on innate immune cells as well as by epithelial cells (117). One key subset of PRRs are TLRs, which recognize specific pathogen-associated molecular patterns, including lipopolysaccharide, dsRNA, flagellin, and unmethylated CpG. Signaling through these TLRs can lead to downstream activation of MyD88, a myeloid differentiation adaptor protein that plays a key role in signal transduction associated with the activation of immune responses (118, 119). Once activated, MyD88 can initiate NF-κB-dependent transcription, one of the major transcription factors involved in the inflammatory response toward the islet graft (120). One group found that inhibition of MyD88 dimerization with small molecule TJ-M2010-6 in NOD mice displayed reduced onset of diabetes, inhibited insulitis, and suppressed T cell activation (121).

Targeting Redox-Mediated Acute Responses After Islet Transplantation

There are multiple ways to target these acute interactions and prolong graft survival. One of the major stages of IBMIR is coagulation and platelet aggregation around the islet graft. To diminish this damaging reaction, one group utilized α-1 antitrypsin, a serine protease inhibitor to reduce IBMIR coagulation and cytokine-induced inflammation in human islets transplanted into the portal vein of NOD.*scid* mice (122). Administration of α-1 antitrypsin reduced TF expression by the islets, inhibited neutrophil infiltration, and protected islet grafts from IBMIR-mediated damage. Another strategy involves developmental endothelial locus-1 (Del-1), an endothelial-derived homeostatic factor that has anti-inflammatory properties due to its involvement in leukocyte adhesion (123, 124). The overexpression of Del-1 reduced leukocyte-platelet aggregation, which protected islets from IBMIR-associated damage (124). Alternatively, targeting the production of TF by the islets themselves can be inhibited by nicotinamide treatment (113).

The damage from IBMIR can cause an outflow of cytokines from these early immune effectors that can activate redox-sensitive signaling cascades within the islets. These redox-dependent pathways induce apoptosis within the islet graft and compromise insulin secretion from β-cells (125). In human islets, it has been shown that IFN-α can participate in the early stages of T1D progression by triggering ER stress responses to reduce insulin production (126). This change in insulin production was linked to a functional delay in the rate of proinsulin to insulin conversion within the ER. The role of oxidative stress in this ER response has also been investigated. In particular, it has been suggested that the production of iNOS and nitric oxide (NO) within isolated islets after cytokine exposure can lead to the activation of UPR within the ER (127, 128). During times of cellular stress, misfolded proteins can accumulate within the ER lumen. When this build up occurs, it can cause damage to cellular functions as well as disrupt cell division and survival (129). The UPR cascade is designed to protect the cell by increasing protein degradation, upregulating transcription of protein folding machinery, and reestablishing proper ER function. However, if the UPR is incapable of compensating for the amount of cellular stress, such as in the case of chronic inflammation following islet transplantation, the cell can undergo apoptosis. This has been shown to occur during spontaneous T1D progression in the NOD mouse model. ER stress responses were demonstrated to precede the onset of hyperglycemia in the NOD mouse, establishing a link between redox signaling, ER stress, and the early wave of islet dysfunction seen in T1D models (130). Pre-diabetic NOD mice displayed β-cells with fewer secretory granules and a more fragmented ER when compared to β cells from diabetes resistant mouse strains.

It has been shown that UPR defects in β-cells from both animal models of T1D as well as from human patients can contribute to the pathogenesis of autoimmune diabetes (131). Blocking UPR hyperactivation through the use of tyrosine kinase inhibitors such as KIRA8 and imatinib displayed reductions in ER stress-induced apoptosis and even reversed autoimmune diabetes in the NOD mouse (132, 133). In addition to autoimmune diabetes, targeting the UPR during ER stress may be a potential therapeutic target to delay islet transplant rejection. Negi et al. provided evidence of ER stress being implicated in the high degree of human islet loss during isolation and during the early posttransplantation period when these islets were engrafted into a chronic hyperglycemic environment (134). To circumvent ER stress and apoptosis, one group found that pre-treatment of human islets with glial cell line-derived neurotrophic factor reduced ER stress and improved graft function after transplantation into the kidney capsule of diabetic immunodeficient mice (132, 133, 135).

In addition to ER stress, one of the major problems during islet transplantation is the early loss of functional insulin-producing cells due to hypoxia-related injury (107, 136). Redox reactions are tightly linked to hypoxic and reoxygenation conditions as the cellular electron transport chain of the mitochondria become damaged (136, 137). Due to the intimate link between mitochondrial function and insulin secretion, the mitochondrial stress induced by hypoxic or reoxygenation conditions can induce β-cell dysfunction. It has been demonstrated that even transient exposure to H_2O_2 can reduce β-cell glucose responsiveness by upward of 40% long after the stress has been removed (138). This decrease in responsiveness correlated with increased mitochondrial ROS and decreased mitochondrial biogenesis, solidifying the link between

internal sources of oxidative stress and β-cell dysfunction. In addition, dissipating mitochondrial ROS through antioxidants such as MitoTempo or Mitoquinone can protect β-cells from oxidative damage and increase insulin responsiveness in diabetic conditions (139). Not only can mitochondrial ROS impact insulin secretion but it can also induce further DNA and protein damage (55). Therefore, potentially reducing mitochondrial ROS-mediated damage through the use of redox regulators could protect islets from this initial loss of functional β-cell mass in the few weeks after implantation (85).

To address this problem of reoxygenation, researchers have attempted to use gene delivery or co-culture methods to promote revascularization of these islets in the days after transplantation, thereby limiting oxidative damage and loss of early graft function. One popular method to achieve this is the use of VEGF. This particular growth factor gained attention due to its limited ability to cause secondary side effects as compared to cytokines like transforming growth factor-β (140). Using an adenovirus-based delivery system, the induction of elevated levels of VEGF in human islets resulted in a protective effect from TNF-α and IFN-γ induced apoptosis (141). Researchers also found that the addition of VEGF promoted revascularization in human islets transplanted under the kidney capsule of mouse recipients by promoting the growth of new blood vessel formation (142). Co-expression of VEGF and an IL-1R agonist demonstrated suppressive effects on cytokine- and consequently redox-mediated necrosis and apoptosis (143). Therefore, combinatorial therapies including VEGF expression and IL-1β-dependent signaling blockade demonstrate promise in maintaining stable islet engraftment and function.

Transplantation studies targeting these early immune responses demonstrate some protection for the islet graft; however, the islet graft is still susceptible to immune-mediated damage from adaptive immune effectors. Therefore, targeting one pathway may not be sufficient to prevent redox-mediated islet destruction. Dissipation of only one subset of free radicals provided a modest protective effect and negligible improvement in islet function (144, 145). Perhaps a more comprehensive blockade of redox signaling mechanisms during islet transplantation would improve the duration of islet viability. Support for this hypothesis is demonstrated through the use of cell-permeable catalytic antioxidants, which are effective in delaying streptozocin-induced islet cell death, and decreasing the synthesis of inflammatory cytokines and free radical production by immune cells (31, 94, 95). Treatment of rat islets with metallothionein, a broad antioxidant involved in a wide array of protective stress responses (146), can restore and maintain euglycemia after subcutaneous islet transplantation, a result not seen in untreated control transplants due to the challenge of revascularization under the skin (147). An even more drastic effect was seen with the use of fusion proteins combining metallothionein and SOD to target multiple sources of free radical damage, leading to improved graft survival of syngeneic transplantation models in mice (148).

Taken together, redox-meditated destruction of islet cell grafts can be initiated and perpetuated by a multitude of different signaling pathways induced by immune cells and β-cells. With its multiple intersections with inflammatory responses as well as the production of redox molecules such as an increase in iNOS expression and production of $O_2^{\bullet-}$, the NF-κB pathway has become a major target for therapeutic intervention (**Figure 2**). Using two common NF-κB inhibitors, withaferin A, which inhibits IKKβ and NEMO complex formation (149), or an analog of resveratrol, which blocks the phosphorylation and subsequent nuclear localization of the p65 NF-κB subunit (150), Kanak et al. found that NF-κB blockade reduced the release of C-peptide and proinsulin as well as the production of pro-inflammatory cytokines and chemokines including TNF-α, MCP-1, IL-8, and IL-6 in *in vitro* human islet and blood co-cultures (88). The use of a natural NF-κB inhibitor, withaferin A, is another good example. The addition of withaferin A induced a decreased expression of five key inflammatory genes, *RANTES* (CCL5), *IP10* (CXCL10), *MIG* (CXCL9), *IL1B*, and *NOS2* when islets were cultured in the presence of a cytokine cocktail as compared to controls (151), indicating a strong anti-inflammatory response in addition to a reduction in redox mediators involved in this vital signaling cascade.

While the blockade of this vital pathway has shown some potential at reducing inflammatory responses, understanding the functional and redox-dependent mechanisms involved in activating these pathways at various stages of islet transplantation is critical to understanding the immune-mediated pathology of islet cell destruction and graft failure. With these overlapping mechanisms in mind, it is not surprising that targeting a single pathway may not be efficacious in eliminating the challenges facing the field of islet cell transplantation. One example is the use of imatinib, which hinders the non-receptor tyrosine kinase c-Abl. This drug was initially used to treat chronic myeloid leukemia; however, several clinical trials also demonstrated improvement or reversal of diabetes phenotypes (152, 153). In animal models, imatinib demonstrated protection from both spontaneous and drug-induced islet death and dysfunction (154–156), and when investigated further, imatinib treatment of human islets demonstrated a decrease in islet inflammation following cytokine exposure (157). There has also been some data indicating that imatinib treatment may be capable of reversing autoimmune diabetes in NOD mice by blunting the ER stress responses within pancreatic β-cells (132, 133). With these biological roles, researchers believed imatinib would be a potent inhibitor of redox-mediated apoptotic pathways. However, when used for *in vivo* transplantation studies, pre-treatment of islet cells or treatment of recipients posttransplantation did not improve islet transplant outcomes (158). The failure of imatinib treatment to protect islet grafts *in vivo* serves as a reminder that oxidative damage and redox signaling is complex and a potent mediator of multiple pathways involved in graft failure.

While the above therapies are promising techniques for the reduction of inflammatory reactions, transplanting antigenic islets and delaying graft rejection into a recipient with established autoimmune diabetes is a herculean task. Not only will there be issues of MHC incompatibility in these allotransplant settings, which will potentially mark the graft for destruction, but also an inherent autoimmune response primed and ready to produce signaling molecules and oxidative species to immediately attack the transplanted β-cells is also present. The development of novel

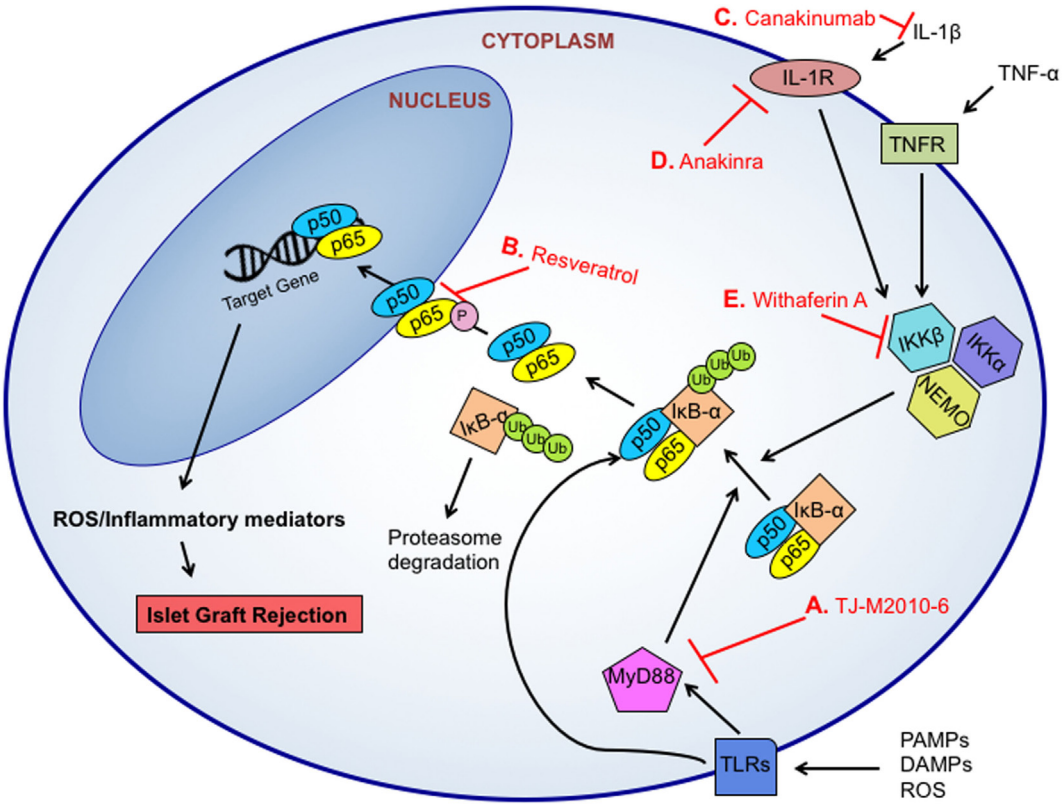

FIGURE 2 | Targeted therapeutic approaches for NF-κB inhibition. NF-κB can induce the transcription of various inflammatory and oxidative molecules to facilitate islet graft rejection. The NF-κB pathway can be triggered by pro-inflammatory cytokine signaling, pathogen-associated molecular patterns (PAMPs)-, danger associated molecular patterns (DAMPs)-, or reactive oxygen species (ROS)-initiated toll-like receptor (TLR)-dependent signals. TLR-signaling activates the MyD88-dependent or MyD88-independent signaling pathways, which results in IKK phosphorylation, IκB-α degradation in the proteasome, and NF-κB (p50/p65) nuclear translocation. Small molecule inhibitors like TJ-M2010-6 can prevent MyD88 activation and IκB-α degradation **(A)**. Resveratrol inhibits the phosphorylation and subsequent nuclear localization of the NF-κB p65 subunit to prevent transcription **(B)**. Signaling from cytokines like interleukin 1 beta (IL-1β) have been targeted through the use of monoclonal antibodies including anti-IL-1 canakinumab **(C)**, which blocks binding of IL-1β to its receptor, and the use of IL-1R antagonist anakinra to block signaling through the receptor **(D)**. The IKK complex is a target for NF-κB inhibition. Specifically, inhibition of IKKβ and NEMO complex formation by withaferin A can prevent the phosphorylation and release of IκB-α **(E)**.

therapies that can efficiently decrease adaptive immune responses involved in graft destruction is necessary if there is hope for diminishing graft rejection without the use of immunosuppressants.

Therapies Targeting Adaptive Immune Rejection of Islet Grafts

There are multiple strategies being investigated to suppress the adaptive immune responses that contribute to islet graft destruction, however, two in particular have gained more attention in the last few decades: islet encapsulation strategies to provide a barrier between the sensitive islet graft and the immune system and co-transplantation methods using accessory cells to dampen inflammatory immune responses. Both seek to provide immunoprotection to the islet graft while maintaining the ability for the β-cells to respond to environmental stimuli.

Current strategies for protecting the islet graft against adaptive immune rejection utilize inhibitors of some of the most commonly utilized pathways for inflammatory responses.

One method to provide protection from immune-mediated damage without perpetual dependency is encapsulation of isolated islets with materials designed to delay immune rejection. These materials have gained attention for their potential to provide an immunoprotective and physical barrier between the immune system and newly transplanted islets. Encapsulation aims to produce a semi-permeable membrane around islet cells that allows insulin and other nutrients access across the membrane while excluding larger proteins like antibodies or interactions with immune cells (159). There are three common methods used for encapsulation: (1) an intravascular device, (2) macroencapsulation, and (3) microencapsulation (160–162).

The first category requires the use of a small chamber containing multiple islets, that is, then directly connected to a blood supply (160), and while this type of intravascular device was capable of restoring euglycemia in mouse models (163), the threat of thrombosis made this method unreasonable for clinical use. Macroencapsulation of islets does not require direct attachment to a blood supply and is more attractive for clinical

application. However, the thickness of these capsules can impede the transfer of insulin, oxygen, and other nutrients, potentially harming the islets and limiting possible transplantation sites (164, 165). More recently, the development of new technologies including the subcutaneous implantation of islets held within a thin membrane-bound device by TheraCyte can protect insulin-producing cells from the immune system and delay islet allograft rejection (166, 167). In addition, a device by ViaCyte utilizing PEC-01 precursor insulin-producing cells and a subcutaneous transplantation site is currently in a phase 1 clinical trial. Finally, microencapsulation is the encapsulation of a single islet, attempting to address the porosity and mass issues that plagued the earlier methods. Reducing the width and the number of encapsulated islets improves porosity and reduces redox-related injury, however, retrieval of transplanted islets is more difficult (161, 168). Finally, alginate is typically used for islet micro- and macroencapsulation, but is also innately immunogenic due to an inability to generate a completely pure form of this algae-derived compound (169, 170).

While each method above has shown some success in restoring euglycemia in animal models and clinical trials (171), the inability to consistently control the size, shape, and thickness of these capsules continue to hamper long-term success. In addition, addressing the issue of reactive species, which may be small enough to cross these semi-permeable membranes, continues to pose a challenge and is a source of much debate (168, 172). In an attempt to address the setbacks associated with these encapsulation materials, the congregation of microspheres containing suppressive materials with or around these islets may provide a solution. Microspheres are specialized structures comprised from thin layers of cross-linked polymers, which can then be optimized for porosity to suit the desired cellular effect (173, 174). Because this method relies less on a bulky shell, microspheres offer the flexibility to address larger issues such as the cellular microenvironment, both within and outside the capsule. For example, the congregation of curcumin, an anti-apoptotic drug containing free radical scavenging capabilities, with the polymer poly(lactic-co-glycolic acid) to form hetero-spheroids can decrease oxidative stress and bolster insulin release in rat islets when used as an encapsulation material (174). This technique allows for a localized release of the redox-modulating drug directly at the site of transplantation without degradation in circulation.

Another novel method of redox-dependent protection that does not compromise size, permeability, or charge of the islets is the use of a layer-by-layer (LbL) polymer ultrathin coating.

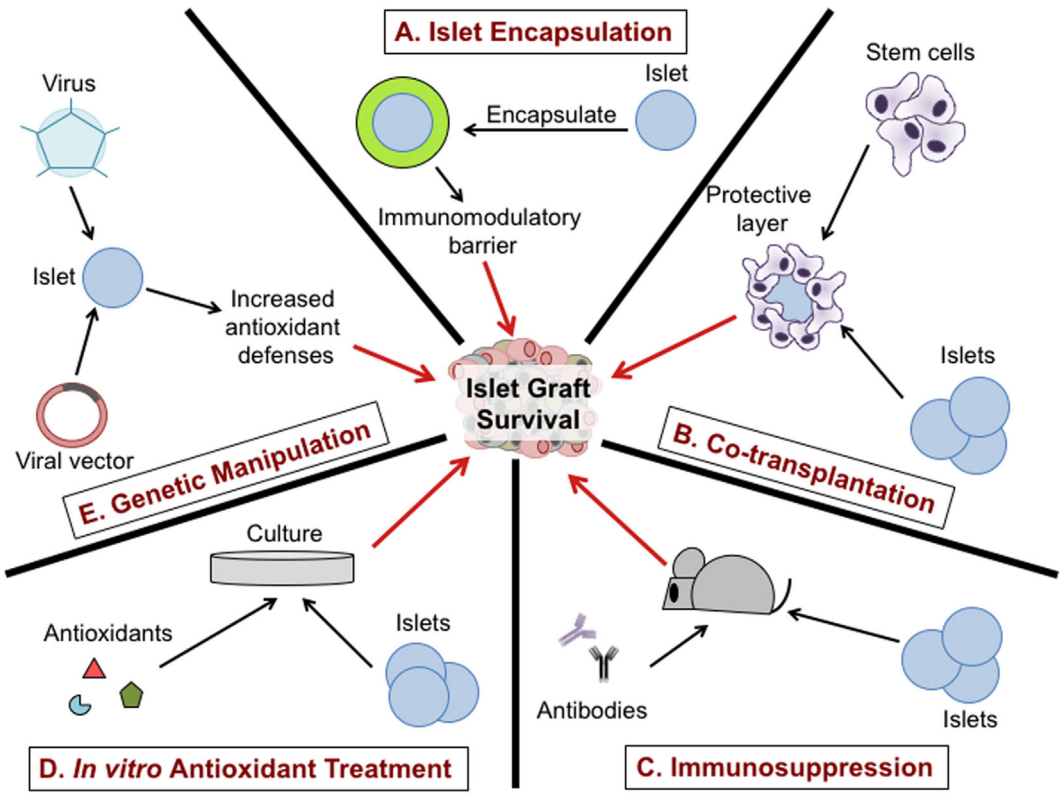

FIGURE 3 | Therapeutic approaches to protect islet graft viability. Encapsulation of purified islets provides a physical and potentially immunomodulatory barrier between the islet graft and the host immune system **(A)**. Co-transplantation of stem cells or regulatory immune cells with the islet graft can reduce immune infiltration to the graft **(B)**. The use of immunosuppressive drugs such as monoclonal antibodies specific for immune cell subsets can suppress inflammatory responses in the host and prolong graft survival **(C)**. Treatment of purified islets with antioxidants during culture can dissipate free radicals involved in islet dysfunction and viability, may enhance engraftment and delay graft rejection **(D)**. Transfection of purified islets using a virus or viral vector to increase antioxidant defenses can promote islet graft viability and prolong survival after transplantation **(E)**.

These nanothin layers allow for the manipulation of surface area, permeability, and bioreactivity of the encapsulation material while still providing protection to the encapsulated islets (175). This technique allows for the potential aggregation of different materials into a single, confluent capsule and opens the door for addressing multiple mechanisms of islet transplant destruction. Furthermore, in contrast to other methods, the LbL technique does not require a priming step for adherence of the biomaterial to the islet surface, which has been shown to be detrimental to the stability and viability of islet cells (176). Instead, these ultrathin coatings rely on hydrogen bonding between the lipid polymer and the lipid bilayer of the membrane to form an anchor point which binds the polymer to the surface of the cell (177).

Utilizing this LbL technique, capsules composed of poly-(*N*-vinylpyrrolidone) and tannic acid (TA), a powerful antioxidant, can suppress the production of IFN-γ and TNF-α, pro-inflammatory cytokines, which are key players in islet cell destruction (178, 179). These capsules can scavenge ROS as well as reactive nitrogen species produced by immune cells, demonstrating their redox-modulation capacity. The TA-containing capsules are also efficacious in suppressing pro-inflammatory chemokine production by innate cells, leading to a decrease in T cell trafficking to the site of inflammation and a decrease in T cell activation (180). By reducing immune cell trafficking, these capsules not only serve as a physical barrier to immune destruction but also serve in a localized manner to suppress immune responses without eliciting global immunosuppression.

Other strategies to reduce early loss of β-cell mass include co-transplantation with accessory cells that can enhance islet function, prevent apoptosis, promote vascularization, and provide immunoprotection, including mesenchymal stem cells (MSCs), ECs, Tregs, and myeloid-derived suppressor cells (MDSCs) (181). MSCs are mesodermal multipotent cells that have self-renewing properties and can be isolated from almost every adult tissue (182). They can surround purified islets in culture due to their strong adhesive capabilities and improve islet graft viability and revascularization in both rodent and non-human primate models of co-transplantation (183–185). MSCs can suppress inflammatory immune responses including the proliferation of cytotoxic T cell subsets in part through NO synthesis and inhibition of STAT5 phosphorylation (186). In islet transplantation, Mohammadi Ayenehdeh et al. demonstrated that congregation of adipose tissue-derived MSCs with isolated islets in a hydrogel could maintain euglycemia for more than 30 days during intraperitoneal allotransplant (187). This prolonged islet survival was in part due to an increase in Treg populations as well as a reduction in the inflammatory cytokines IFN-γ and IL-17A.

Another population being investigated for its resistance to IBMIR reactions is ECs. Co-culture of human ECs with isolated pig islets was shown to prevent IBMIR-mediated islet damage both *in vitro* (188) as well as after co-transplantation into the kidney capsule of diabetic immunodeficient mice (189). Another group found that co-culturing isolated human islets with primary human ECs produced a protective coating that would surround the islets and protect them from IBMIR upon transplantation into the portal vein (190). This co-transplantation strategy also induced a reduction in $CD11b^+$ innate immune cell infiltration into these islet grafts, indicating that the presence of ECs served as an immunoprotective barrier for transplanted islets.

An innate immune cell type that has gained attention for its contact-dependent immunosuppression is the MDSCs. Through their production of superoxide, iNOS, and elevated arginase activity, MDSCs can suppress Teff activation and function while promoting Treg development (191, 192). During allogeneic islet transplantation into the kidney capsule of diabetic mice, co-transplantation of MDSCs increased the presence of Treg cells through the B7-H1/PD-1 pathway (192) and reduced CD8 T cell infiltration by activating iNOS (193).

Regulatory T cells are an immune cell population with the potential to prevent islet graft rejection due to their suppressive effects on immune responses. A recent clinical trial demonstrated that *ex vivo* expansion and subsequent infusion of autologous human Treg cells in 12 patients with newly diagnosed T1D lowered the patients' exogenous insulin requirements and prolonged endogenous islet survival (194). The use of Treg cells also show some promise in animal models of islet transplantation, however, due to the short half-life of expanded Tregs as well as challenges in migration from peripheral blood to the site of engraftment (195), alternative strategies to improve Treg localization have been attempted. The combination of CTLA-4, a key protein receptor that downregulates immune responses, and reparixin, which blocks against inflammatory neutrophil infiltration, resulted in lower serum IFN-γ as well as decreased T cell infiltration into the islet graft after transplantation under the kidney capsule (196). Other groups have investigated the use of fusion proteins and antibodies to promote graft survival. Zhang et al. utilized CTLA-4/Fc and demonstrated reduced local inflammation, a concomitant increase in $Foxp3^+$ Treg cells, and improved engraftment (197). Treg cells have also been utilized in co-transplantation strategies where co-aggregation of syngeneic Treg cells with purified allogeneic islets within an agarose hydrogel displayed prolonged allograft survival after transplantation into the portal vein of mice (198).

CONCLUSION

Due to the comprehensive role of oxidative stress on islet transplantation, targeting redox-dependent inflammatory responses during islet isolation, *in vitro* culture, and after transplantation has the potential to increase islet viability and function (**Figure 3**). Utilization of a broad range of antioxidants including SOD mimetics to dissipate ROS synthesis can prolong islet viability and maintain function during islet isolation (93). The addition of a physical barrier as well as an immune modulator is likely the most promising combinatorial approach to protect islet transplants from immune-mediated rejection. Interventions including islet encapsulation with TA may be efficacious and safer in delaying allograft rejection than global immunosuppressive therapies (12, 15, 162, 180). In an effort to achieve stable islet engraftment in patients following islet transplantation, therapies that specifically target the removal of free radicals and redox-dependent signaling are highly warranted. It is apparent that synergistic interactions between redox biology and immune responses following islet

transplantation are an underrepresented area of research. Future strategies implementing the LbL encapsulation approach in combination with potent antioxidants can enhance the viability and yield of isolated islets, making islet cell transplantation a realistic and curative treatment option for patients with T1D.

AUTHOR CONTRIBUTIONS

JB and HT wrote the manuscript and reviewed/edited manuscript. HT is the guarantor of this review article.

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REVIEW
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T Cell-Mediated Beta Cell Destruction: Autoimmunity and Alloimmunity in the Context of Type 1 Diabetes

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Type 1 diabetes (T1D) results from destruction of pancreatic beta cells by T cells of the immune system. Despite improvements in insulin analogs and continuous blood glucose level monitoring, there is no cure for T1D, and some individuals develop life-threatening complications. Pancreas and islet transplantation have been attractive therapeutic approaches; however, transplants containing insulin-producing cells are vulnerable to both recurrent autoimmunity and conventional allograft rejection. Current immune suppression treatments subdue the immune system, but not without complications. Ideally a successful approach would target only the destructive immune cells and leave the remaining immune system intact to fight foreign pathogens. This review discusses the autoimmune diabetes disease process, diabetic complications that warrant a transplant, and alloimmunity. First, we describe the current understanding of autoimmune destruction of beta cells including the roles of CD4 and CD8 T cells and several possibilities for antigen-specific tolerance induction. Second, we outline diabetic complications necessitating beta cell replacement. Third, we discuss transplant recognition, potential sources for beta cell replacement, and tolerance-promoting therapies under development. We hypothesize that a better understanding of autoreactive T cell targets during disease pathogenesis and alloimmunity following transplant destruction could enhance attempts to re-establish tolerance to beta cells.

Keywords: type 1 diabetes, immunology, autoimmune diseases, transplantation immunology, tolerance induction, T cells, alloimmunity

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INTRODUCTION

Pancreatic beta cells are destroyed by T cells of the immune system, precipitating type 1 diabetes (T1D). Unfortunately, preventing beta cell destruction in at-risk individuals has proven challenging. Despite a working knowledge of genetic risk factors associated with T1D (1), determining specific beta cell targets and preventing beta cell destruction by autoreactive T cells remains elusive. To develop a successful approach to protect beta cells, we must understand how and why T cells are directed to specifically destroy insulin-producing cells in the pancreas while sparing adjacent hormone-producing cells including alpha, delta, and epsilon cells. There may be at least two paths to protect beta cells from T cell-mediated death. The first approach is to control or regulate effector

T cell responses, and the second is to enhance beta cell survival or resistance to T cell-mediated death.

The first section of this review outlines our current understanding of the pathogenesis of autoimmune diabetes. We describe the process by which insulin-producing beta cells are destroyed and contrast the roles of $CD4^+$ and CD8 T cells during autoimmune pathogenesis. We compare T1D pathogenesis in the non-obese diabetic (NOD) mouse to our current understanding of human disease. We also discuss an exciting recent development in the field of autoreactive T cell biology: recognition of neoantigens generated through hybrid peptide fusion or response to neoantigens formed through defective protein translation. Finally, we describe immune tolerance in several forms, including thymic central tolerance, T cell ignorance in the periphery, anergy, and regulatory T cell induction.

The second section of this review briefly describes the necessity for pancreas or islet transplantation to treat severe diabetic complications. With improving glycemic control through insulin injections and continuous glucose monitoring, many T1D individuals live with minimal complications (2, 3). However, some T1D individuals develop life-threatening complications including hypoglycemia unawareness and end-stage renal disease. Unawareness of severe hypoglycemia is a primary indicator for pancreas or islet transplantation and is often combined with kidney transplantation to treat renal failure.

The third section of this review focuses on islet replacement strategies and briefly outlines beta cell regeneration. The two primary avenues for beta cell replacement are transplantation of cadaveric islets or induced pluripotent stem cell (iPS)-derived beta cells. While there has been considerable progress in both strategies, a cure for established T1D must also involve targeted immunotherapy. This approach must inhibit memory autoreactive T cells and naive allograft-reactive immune responses. In the third section of this review, we describe allorecognition, or how T cells "see" transplants, focusing on pancreatic islet transplantation. We describe two categories of allorecognition by T cells in transplant recipients: direct recognition of donor major histocompatibility complex (MHC) molecules and indirect recognition of transplant-derived peptides through recipient MHC molecules. We also discuss the challenges of transplant tolerance in the NOD mouse and human T1D islet allograft recipients. Recent evidence suggests that the presence of autoimmunity acts as an "adjuvant," accelerating and strengthening the conventional alloimmune response.

AUTOIMMUNE DIABETES PATHOGENESIS

Type 1 diabetes is a T cell-mediated autoimmune disease, whereas T2D is the result of peripheral cell resistance to endogenous insulin. The best evidence supporting immune system involvement in T1D are studies reporting lymphocytic infiltrate in the islets of T1D cadaveric donors (4, 5), islet-specific autoantibody production in individuals with T1D (6–8), and identical twin studies in which the twin with T1D rejected islet transplants from their non-diabetic twin (9). Analyses of pancreas sections harvested from individuals with T1D have shown fulminant immune infiltration within individual islets, corroborating a key role for CD4 and CD8 T cells in beta cell destruction (10–12). This is in sharp contrast to pancreas sections from individuals with T2D, who, despite having high levels of systemic inflammatory markers, do not have similar T cell infiltration within pancreatic islets (10–12). Virtually all individuals who develop T1D before the age of 5 years produce insulin-specific autoantibodies (IAAs), suggesting an important role for peptides derived from the insulin molecule in disease pathogenesis (13, 14). Islet autoantibodies are a differential diagnosis marker for T1D versus T2D and arise from autoreactive B cell and autoreactive CD4 T cell interactions. Human leukocyte antigens (HLAs) class II alleles DR4, DQ8, and DQ2 confer the highest genetic risk for T1D in human patients (15). This strong HLA II allele association with T1D suggests that HLA II-restricted CD4 T cells play a key role in disease pathogenesis. CD4 T cells can provide "help" to B cells and stimulate antibody production as noted above, as well as promote responses by effector CD8 T cells, and stimulate islet-resident macrophages (16, 17). With this in mind, autoreactive CD4 T cells represent an active area of research and clinical interest for therapies. Developing antigen-specific tolerance-promoting methods to inhibit autoreactive CD4 T cells is the focus of the first section of this review.

The NOD Mouse Model of T1D

The NOD mouse was first characterized at the Shionogi Research Laboratories in Aburahi, Japan, by Makino et al. (18). The NOD mouse was developed as a sub-strain of the JcI:ICR mouse strain, which was used to study cataract development (18). The NOD strain exhibited very high fasting blood sugar levels but not cataracts and has been an invaluable tool for T1D research. Depending on the colony, 50–90% of female NOD mice develop spontaneous autoimmune diabetes between 10 and 30 weeks of age (19). Generally, diabetes onset in male NOD mice is much less frequent (20% in the same age range); therefore the majority of studies of autoimmune diabetes utilizing this strain of mice use female diabetic mice (20). This review will focus on spontaneous autoimmune diabetes pathogenesis in NOD mice, although other models of beta cell destruction mediated by T cell receptor (TCR) transgenic T cells targeting ectopically expressed antigen such as in rat insulin promoter (RIP) driving lymphocytic choriomeningitis virus (21) RIP-membrane-bound form of ovalbumin (22) or insulin hemagglutinin (23) have contributed extensively to our understanding of T1D and are discussed elsewhere (24). Studies in the NOD mouse demonstrate a strong dependence on MHC class II allele I-A^{g7} and the requirement of CD4 T cells (25), CD8 T cells (26), and B cells (27, 28) for autoimmune diabetes. Interestingly, diabetes-associated MHC II, I-A^{g7} does not precipitate diabetes when expressed in non-autoimmune-prone B6 mice (29), but NOD mice engineered to express MHC class II alleles other than I-A^{g7} are protected from disease development (30). Collectively, these findings suggest that I-A^{g7} is necessary, but not sufficient, for autoimmune diabetes. The roles of CD4 T cells, CD8 T cells, and B cells in diabetes pathogenesis are discussed below.

CD4 T cells are thought to provide help to effector CD8 T cells, stimulate antibody production by B cells, and activate islet-resident M1 macrophages (**Figure 1**). CD4 T cells are required for diabetes development in NOD mice (31), and either depletion

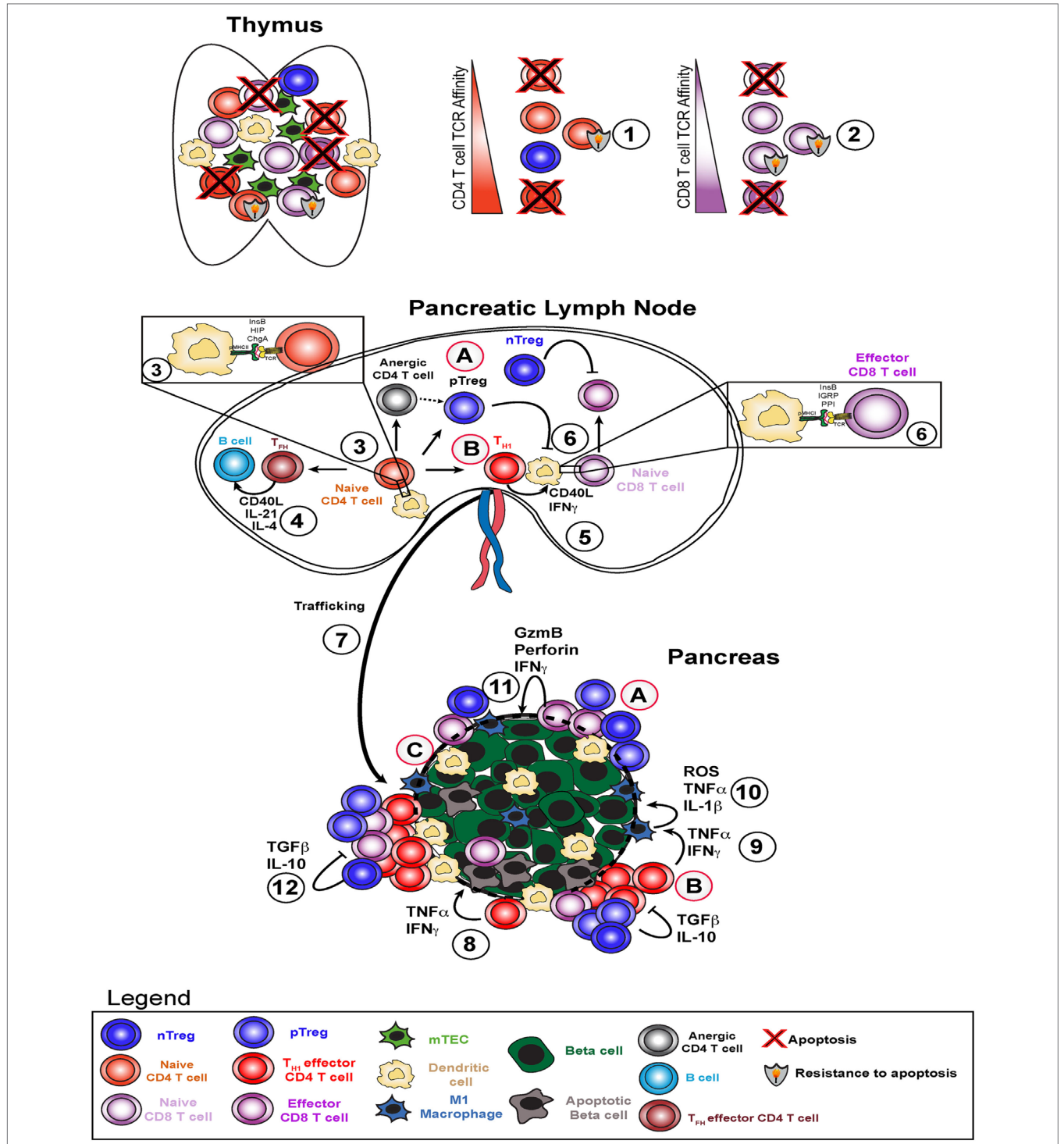

FIGURE 1 | Type 1 diabetes pathogenesis and potential therapeutic avenues. Type 1 diabetes arises due to failure of several key checkpoints. Defective central tolerance (1 and 2) allows islet-reactive CD4 and CD8 T cells to escape the thymus as naive cells and reach the pancreatic lymph node. In the pancreatic lymph node, autoreactive CD4 T cells interact with dendritic cells presenting islet antigen (3) and can become T helper 1 (T_{H1}), T_{FH}, pTreg, or anergic cells. T_{FH} cells help B cells produce high affinity islet-specific antibodies (4). T_{H1} cells activate dendritic cells and enhance antigen presentation to islet-specific CD8 T cells (5) to induce effector CD8 T cell skewing (6). T_{H1} cells traffic to the pancreas (7), secrete pro-inflammatory cytokines interferon gamma (IFNγ) and TNFα, and induce beta cell death (8). T_{H1}-derived IFNγ and TNFα stimulate M1 macrophages in the islets to produce ROS, TNFα, and IL-1β (9), which in turn amplify beta cell death cycle (10). Resulting inflammation leads to increased CD8 T cell infiltration and direct beta cell killing via perforin and granzyme B (11) and attempts by nTregs and pTregs to dampen this response via TGFβ and IL-10 (12). Potential therapeutic strategies include (A) infusion of *ex vivo* expanded (broadly reactive or pancreas-specific) Tregs, (B) re-educating T_{H1} cells through approaches like peptide-linked apoptotic splenocytes, and (C) promoting beta cell-intrinsic expression of defense molecules *in situ* or engineering transplanted beta cells to be more resistant to T cell-mediated attack.

of CD4 T cells (32) or treatment with non-depleting anti-CD4 antibodies prevents diabetes (33). Early research in the NOD mouse model demonstrated that T helper 1 cells transferred to neonatal NOD recipient mice could precipitate diabetes (34). Recent studies in NOD mice and human T1D patients have characterized the diabetogenic CD4 T cells as pro-inflammatory, capable of secreting interferon gamma (IFN-γ) and/or interleukin 17 (35–39). Interestingly, HLA-matched healthy donors may also have CD4 T cells with islet antigen specificity, but in their case, the cell phenotype and functional output is regulatory, with a cytokine profile consisting mainly of IL-10 (35, 36). CD4 T cell targets are peptides restricted to HLA or MHC II and are discussed in further detail below. In human T1D, the available evidence from studies of individual islets from the Network for Pancreatic Organ Donors with Diabetes suggests that beta cell destruction is mediated in large part through direct CD8 T cell contact with beta cells and CD4 T cell-mediated polarization of M1 macrophages (4, 10, 40). CD4 regulatory T cells will be addressed below.

Autoreactive CD8 T cells are activated through interaction with peptides presented by MHC class I and can mediate beta cell death in a contact-dependent manner through perforin and granzyme molecules (**Figure 1**) (41). MHC class I is required for T1D, with some reports suggesting that CD8 T cell/MHC class I interactions are required only early in disease development (42), whereas others have concluded that MHC class I is required late in diabetes pathogenesis (43). Insulin-specific CD8 T cells are key for diabetes onset in both mouse (44, 45) and humans (46). Even though CD8 T cells are required for disease pathogenesis, due to space limitations, the bulk of this review will focus on the biology of CD4 T cells.

Beta cell death can also be mediated through cytokine production by both CD4 and CD8 T cells within pancreatic islets. Pro-inflammatory cytokines such as TNF-α and IFN-γ are directly toxic to beta cells (**Figure 1**) (47, 48). These cytokines also activate macrophages to M1 phenotype and stimulate a positive feedback loop, further increasing cytokine production *in situ* and killing more beta cells (**Figure 1**) (49). In addition, data from mouse and human samples demonstrate that beta cells can express the IFN-γ-inducible chemokine CXCL10, which promotes T cell infiltration and may accelerate beta cell destruction (50, 51). Data from adoptive CD4 T cell transfer model of diabetes in the NOD mouse model suggest that M1 macrophages are required for beta cell destruction in this setting (52). Indeed, it has been demonstrated in the NOD mouse that superoxide production by T cells or macrophages is critical to promote beta cell death and T1D (16) and that loss of superoxide production by macrophages delays diabetes pathogenesis (53). Moreover, transient depletion of islet-infiltrating dendritic cells and macrophages using clodronate-loaded liposomes abrogated T cell infiltration and significantly delayed subsequent diabetes development in liposome-treated mice (54). More recent work has demonstrated a critical role for dendritic cells expressing the Batf3 transcription factor in autoimmune pathogenesis of NOD mice (55). Taken together, these results suggest that antigen presentation to CD4 T cells by dendritic cells and macrophages within pancreatic islets plays a key role in promoting beta cell destruction.

Finally, our current understanding is that B cells act as antigen-presenting cells to both CD4 and CD8 T cells and also produce IAAs (**Figure 1**) (56). Early studies established that NOD mouse production of IAA peaks between 8 and 12 weeks of age and gradually decreases afterward presumably as beta cell mass decreases (57, 58). In addition, >60% of mice which developed IAA at 3–5 weeks of age develop T1D by week 20, while >50% of IAA-positive mice at 8 weeks of age develop T1D by week 20 (57–59). Translating these results to human patients, as pioneered by Eisenbarth (58), autoantibody responses against multiple different T cell antigens are highly predictive of diabetes onset within 12–36 months in human subjects (1, 8, 60). In addition, recent work from Finland has demonstrated that high proportions of children with IAA and/or multiple autoantibodies against beta cell targets at ages younger than 5 years develop T1D (61). As shown by sibling studies (DAISY, TEDDY), the presence of one known autoantibody response confers a moderate risk level, with risk of imminent development of diabetes increasing exponentially with the detection of each additional autoantibody response.

While analogous experiments have not been performed using human autoreactive T cells and human beta cells in an *in vitro* setting or humanized mouse system, studies in the NOD mouse have elucidated potential mechanisms of beta cell destruction in human T1D, in particular key roles for CD4 and CD8 T cells. However, there are important differences between NOD and human T1D. In particular, there is a gender bias in NOD mice, with higher incidence in female than male mice (19, 20). In contrast, human T1D does not show gender bias, unlike other autoimmune diseases. A full account of the physiology behind this discordance is outside the scope of this review, but may include (a) more synchronous T cell infiltration into pancreatic islets in NOD mice than in at-risk human subjects, (b) the potential for a greater dependence on CD8 T cells in diabetes pathogenesis in human disease (10), and (c) confounding effects of multiple concurrent T cell responses in human patients exposed to the "universe" of viral and bacterial pathogens as opposed to inbred specific pathogen-free NOD mouse colonies.

Autoimmune Diabetes Antigens and Neoantigens

Diabetes-relevant antigen targets have been defined through the presence of serum autoantibodies, ELISpot assays, proliferation assays, and mouse studies [reviewed in Ref. (62)]. In mice and humans, some of the B cell and T cell antigen targets of T1D are overlapping, but not identical (63). The majority of autoantigens identified in the NOD mouse are peptides from the insulin secretory granules. At the Barbara Davis Center in the late 1980s, Haskins et al. (64, 65) and Wegmann et al. (66) utilized the NOD mouse to generate a series of pancreatic islet secretory granule-specific autoreactive CD4 T cell lines (67). Chief among these, the BDC2.5 CD4 T cell line has been studied extensively (68). Two key transgenic mouse lines were generated including the BDC2.5 TCR transgenic mouse (69) and the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)-specific CD8.3 transgenic mouse (70). The NOD mouse

has proven to be a useful "work horse" model system for studying the pathogenesis and cellular immunology of spontaneous and adoptively transferred T1D. T cell-mediated destruction of beta cells represents an intricate coordination between innate and adaptive lymphocytes, with CD4 T cells occupying a key node in this network, as described above. CD4 T cell epitopes discovered to date include epitopes derived from the insulin B chain (45), chromogranin A (71), and islet amyloid polypeptide (**Figure 1**) (72, 73). CD8 T cell epitopes include peptides derived from preproinsulin (44, 46), IGRP (70), Zinc transporter 8 (74, 75), and glutamic acid decarboxylase 65 (**Figure 1**) (76). Of particular importance in both the NOD mouse model system and for translation to the human disease is a peptide derived from amino acids 10–23 of the insulin B chain (InsB10:23). This peptide is required for the development of autoimmune diabetes in the NOD mouse (45). Nakayama et al. determined that a single amino acid substitution in a TCR contact site for both CD4 and CD8 T cells conferred complete protection by altering a dominant immune target within the insulin protein (45). Similarly, we determined that insulin-specific T cell responses were critical in the spontaneous mouse model of diabetes (77). We demonstrated that blocking insulin-specific T cell responses could reverse and even cure diabetes in mice. In addition, re-establishing immune tolerance to proinsulin prevents diabetes onset in NOD mice, but re-establishing tolerance to $IGRP_{206\text{-}214}$ does not prevent diabetes in NOD mice (78). Despite these fundamental discoveries, we still do not fully understand antigen hierarchy in T1D patients, likely because multiple different targets may be required for disease in different patients (79).

Exciting recent work from several groups has demonstrated the presence of neoantigens for diabetogenic CD4 T cells. These comprise hybrid peptides or combinations of amino acid sequences derived from two different secretory granule proteins or peptide sequences (80, 81). The frequency of T cell priming events against hybrid peptides during autoimmune pathogenesis is not clear *in vivo*; however, compelling evidence *in vitro* suggests that these cells may play an important role in T1D pathogenesis. It is thought that hybrid peptides are generated exclusively in beta cells and not in the thymus, thus representing "new" targets in the periphery. These targets could be viewed as foreign peptides eliciting a strong immune response. Recent reports also suggest that pancreatic neoantigens could arise from defective ribosomal insulin gene products (DRiPs), which are produced by metabolically stressed beta cells (82). Similarly to hybrid peptides, central tolerance to DRiPs generated by stressed beta cells would be lacking in the thymus. In the presence of inflammation and cell death, T cell responses to such neoantigens would develop in the periphery and could contribute to disease pathogenesis. **Table 1** summarizes known autoantigens in T1D development in human subjects and NOD mice and if they are recognized by CD4 or CD8 T cells in the context of the appropriate HLA/MHC molecule.

TABLE 1 | Beta cell secretory granule-derived auto antigens.

Protein target	NOD mouse and/or human T1D	CD4 and/or CD8 T cells	Reference
(Pre)proinsulin	Mouse and human	CD4 and CD8	(36, 44, 46, 78)
Insulin	Mouse and human	CD4 and CD8	(44, 45, 76, 83)
Defective ribosomal insulin gene product	Human	CD8	(82)
Insulin hybrid peptides	Mouse and human	CD4	(80, 81)
GAD65	Mouse and human	CD4 and CD8	(84–88)
ZnT8	Mouse and human	CD4 and CD8	(74, 75, 89–92)
Islet antigen-2	Human	CD4 and CD8	(93–95)
Phogrin	Mouse and human	CD4	(96–99)
Islet cell autoantigen 69 kDa	Human	CD4	(100–103)
Chromogranin A	Mouse and human	CD4 and CD8	(71, 104, 105)
Islet amyloid polypeptide	Mouse and human	CD4 and CD8	(72, 73, 106, 107)
Islet-specific glucose-6-phosphatase catalytic subunit-related protein	Mouse and human	CD4 and CD8	(70, 78, 108, 109)

Mechanisms of Immune Tolerance

There are four broad categories of immune tolerance that could protect beta cells from destruction by autoreactive T cells. First, negative selection during thymic development culls self-reactive T cells during T cell development. Due to this mechanism, autoreactive T cells generally do not survive thymic development. However, diabetes-associated MHC class I and II alleles facilitate the escape of self-reactive lymphocytes from the negative selection process. This escape could be due to several non-mutually exclusive reasons: low thymic expression of islet antigens (110), poor binding of native (non-transcriptionally modified) islet autoantigens to MHC I/II [as suggested in Ref. (111)], and T cell-intrinsic resistance to apoptosis (112) (**Figure 1**). GWAS studies link allelic variation at the insulin variable number tandem repeat (INS-VNTR) IDDM2 locus with the level of thymic insulin expression and disease development. Protective alleles of the IDDM2 diabetes susceptibility locus promote higher levels of insulin expression in the thymus, which would promote more robust negative selection of insulin-reactive T cells (113). In addition, mice genetically engineered to express lower levels of insulin in the thymus demonstrate correspondingly higher levels of peripheral T and B cell reactivity against insulin (110), and published work indicates that pancreatic lymph nodes of NOD mice contain higher than expected levels of insulin mRNA at 3–5 weeks of age (114). These observations suggest a direct link between the level of extra-pancreatic insulin expression and peripheral lymphocyte reactivity to insulin and point to ineffective negative selection in NOD mice and human patients. Second, immune ignorance occurs if an autoreactive lymphocyte survives thymic development, but does not encounter its cognate antigen in the periphery. The ignorance pathway appears to be an important method for maintenance of B cell tolerance (115). Additional evidence of autoantigen-specific T cell ignorance can be found in the MHC-matched T1D-resistant B6.g7 mouse model (116).

A third mechanism of tolerance is a state of antigen-specific unresponsiveness called anergy. CD4 T cell anergy is defined as expression of folate receptor 4 and CD73 and hyporesponsiveness to TCR stimulation (117). While the majority of insulin-specific CD4 T cells in NOD mice are anergic, this form of tolerance is not sufficient o halt diabetes (**Figure 1**) (116). A fourth mode of immune tolerance relies on thymic-derived and peripheral regulatory CD4 T cells (Tregs) expressing the transcription factor Foxp3 (**Figure 1**). Foxp3 is the master regulator of Treg fate, stability, and suppressive capacity (118). Mutations in the Foxp3 locus (IPEX in humans and Scurfy in mice) lead to multiorgan autoimmunity and demonstrate a non-redundant role of Foxp3 in maintaining tolerance (118). Recent evidence demonstrates that the augmentation of Treg activity specifically within pancreatic islets may ameliorate diabetes pathogenesis in NOD mice (119). This result suggests that promoting Treg activity specifically within the pancreas may be beneficial in human T1D as well. In addition, Tregs can inhibit effector T cells specific for the same or "linked" peptides. "Linked suppression" refers to the ability of regulatory T cells to suppress activation of effector T cells interacting with the same antigen-presenting cell at the time of Treg-APC interaction. This concept was originally demonstrated by Davies et al. (120) and reviewed in Ref. (121) and has been shown to apply to the murine model of multiple sclerosis, experimental autoimmunity encephalomyelitis, as well (122). In addition, this mechanism has recently been shown to apply to a heart transplant model in mice, in which immune tolerance was induced to multiple distinct foreign MHC molecules (123). As such, we speculate that determination of "linked" peptides to promote CD4 T cell tolerance to islet allografts in autoimmune recipients represents a powerful opportunity to prevent islet allograft rejection in autoimmune recipients.

While several hundred protocols have prevented diabetes in NOD mice, very few of these have successfully reversed disease, and none have yet been translated to standard clinical practice (124, 125). Briefly, tolerance-promoting therapies have generally focused on inhibiting autoreactive T or B cells, decreasing inflammation prior to diabetes onset, or some combination of these approaches. In attempts to restore tolerance in the CD4 T cell compartment, we previously used whole insulin protein coupled to apoptotic cells through the chemical cross-linker ethylene carbodiimide, or ECDI (77). This approach reversed T1D in almost half of the treated mice. ECDI-coupled cells have been used in phase I safety trials for multiple sclerosis and have shown a desirable safety profile (**Figure 1**) (126). We predict that this approach could be tested for safety and efficacy in T1D. Adoptive transfer of regulatory CD4 T cells can halt diabetes pathogenesis in mice through inhibition of IFN-γ production by islet-infiltrating CD4 and CD8 T cells and decreased islet infiltration by CD8 T cells (127). These findings were translated to the clinic, with encouraging results. Two separate research groups have demonstrated that deficiencies in IL-2 production (128) or the responsiveness of Treg cells to IL-2 (129) may be related to the development of autoimmune diabetes in NOD mice. Two separate groups have adoptively transferred autologous (self-derived) Tregs into new-onset T1D patients to enhance function of endogenous Tregs (**Figure 1**). A European group isolated and expanded Tregs from T1D patients (130) and then went on to demonstrate preservations of C-peptide in 8 of 12 subjects and reversal of new-onset T1D in 2 patients (131). In addition, a group at UCSF led by Bluestone and colleagues developed a protocol to expand Tregs from T1D patients (132) and then proved safety in phase I clinical trials (133). Several groups have established that Tregs can be isolated, expanded *ex vivo* in the presence of CD3/CD28 stimulation and IL-2, and adoptively transferred into patients (132–134). Transferred Tregs were detectable in blood up to 12 months later, remained phenotypically stable, and had the potential to influence diabetes pathogenesis. Both of these Treg adoptive transfer clinical trials utilized *in vitro* expanded Tregs, not Tregs specific for particular pancreatic target(s). It is not known if targeting particular autoantigens would provide additional protection compared to the current Treg transfer approach. Taken together, these recent clinical trials suggest that adoptive Treg therapy may help preserve residual beta cell mass in new-onset T1D patients. Whether this approach could prevent T1D onset in at-risk individuals is an open question and warrants future investigation.

DIABETIC COMPLICATIONS INDICATING ISLET CELL REPLACEMENT

Type 1 diabetes often results in large swings in blood glucose levels outside the normal physiologic range of 70–110 mg/dl. Studies of 50-year Joslin Medalists indicate that individuals with T1D can live for many decades with minimal or no diabetic complications (2, 3). In addition, recent advances in fast-acting synthetic insulin analogs, continuous glucose level monitoring technology, and early attempts at developing pump-like systems to deliver glucagon suggest that individuals with T1D would continue to see improvements in diabetes management and therefore in quality of life. However, even with adequate clinical control of blood sugar levels, long-term diabetic complications can develop in individuals with T1D. In addition, despite the technical and clinical advances noted above, some individuals with T1D nonetheless have labile blood glucose level control and are susceptible to severe and life-threatening disease-related complications. These chronic complications can affect essentially every organ system and are particularly pronounced in the microvasculature. Diabetes, T1D and T2D combined, is the leading cause of adult blindness [diabetic retinopathy (135)] and end-stage renal failure [diabetic nephropathy (136)], as well as a leading cause of lower-leg amputations [diabetic peripheral neuropathy (137)] and heart disease [diabetic cardiomyopathy (138, 139)]. Perhaps the most debilitating diabetic complication is hypoglycemic unawareness. This occurs when an individual with T1D is not aware their blood glucose levels are dangerously low (<50 mg/dl). This condition can result in seizures, diabetic coma, and, in the most severe cases, death. The development of hypoglycemia unawareness is thought to result from frequent, severe swings in blood glucose levels in some long-term T1D patients. Why hypoglycemia unawareness develops in some individuals but not others with long-term T1D is an open question. One possibility is that, over time, some T1D patients develop autoreactivity against glucagon-producing

alpha cells. Glucagon-reactive CD8 T cells have been identified in NOD mice (140); therefore we speculate that some individuals with T1D may develop autoimmunity against alpha cells over time. Glucagon acts in opposition to insulin, promoting glycogen breakdown in the liver and therefore promoting increased blood glucose levels. If glucagon-derived peptides are associated with inflammation and cell death within the pancreas, existing autoreactive T cells could become primed in pancreas-draining lymph nodes, proliferate, and mediate destruction of glucagon-producing cells. In fact, there is emerging evidence that a small proportion of T1D patients develop antiglucagon antibodies (140). Another possibility is that destruction of autonomic innervation within pancreatic islets (141) leads to impaired communication with the hypothalamus, so that glucagon is not produced when signals are present based on blood glucose levels. If autonomic innervation of pancreatic islets is perturbed in individuals with T1D, the consequence could be a breakdown in communication with the hypothalamus. Interestingly, some T1D but not T2D subjects develop autoantibodies against the neuroendocrine protein tetraspanin7 from sympthatheic nerves within islets (142). In some patients with severe hypoglycemia, both of these scenarios, and others, could lead to impaired glucagon responses to hypoglycemia. Whole pancreas and isolated pancreatic islet transplantation are options to restore blood glucose level homeostasis for individuals with hypoglycemia unawareness. There are two potential sources of pancreatic beta cells for islet replacement, cadaveric (deceased) donors and iPS-derived beta cells (143), both of which are discussed below.

ISLET GRAFT ALLOIMMUNITY

Islet Replacement Strategies

The current clinical strategy to replace the lost beta cell function is through whole pancreas or isolated pancreatic islet transplantation from genetically unrelated cadaveric donors. Because donors are limited, currently only T1D patients with hypoglycemia unawareness are considered for transplantation. This has created great interest in cell culture methods to produce large quantities of insulin-producing cells for transplantation. After more than 10 years of development, the Melton laboratory became the first group to develop a reproducible protocol for iPS conversion to insulin-producing beta cells (143), quickly followed by several other groups (144–146). However, these methods are not yet suitable for large-scale production of patient-specific iPS-beta cells for transplantation studies since individuals require several hundred thousand individual pancreatic islets. Furthermore, a critical limiting factor of a "universal donor" beta cell line is conventional transplant recognition, described below. In addition, unlike whole pancreas or isolated islet transplantation, iPS-beta cells do not replace the lost alpha cell function. Until these challenges are addressed, transplantation from a cadaveric donor will likely remain the preferred approach in combination with immune suppression (147, 148). A recent phase III clinical trial demonstrated improved glycemic control in islet transplant recipients following multisite standardized processing protocols (149, 150). As less beta cell-toxic immune suppression treatments are developed, we can expect transplant function and long-term survival to continue to improve. In the absence of these treatments, transplanted beta cells in autoimmune recipient patients would be subject to at least two categories of T cell responses: (a) autoimmune (islet-specific) responses by T cells (151, 152), and (b) conventional anti-transplant-reactive T cell responses. However, current immune suppression treatments do not promote immune tolerance as described above, must be continued indefinitely after transplantation, and can render the transplant recipient vulnerable to cancer and infectious agents. Therefore, transplant-specific tolerance-promoting treatments are a highly sought after goal in the islet transplantation field.

An alternative to replacing the lost beta cell mass would be to stimulate beta cell regeneration. Beta cell regeneration is based on the premise that if autoreactive T cells are removed or inhibited, existing beta cells could proliferate, alpha cells could convert into beta cells, or islet-resident stem cell populations could proliferate and differentiate into beta cells. There is little experimental evidence to support these suppositions to date. Beta cells are exceptionally metabolically active, continuously producing insulin secretory granules. The less beta cell mass is available to produce insulin, the higher the metabolic stress is on each individual islet. Therefore, the ability to regenerate beta cells from existing beta cells could be a significant hurdle. Another theoretical option to replace lost beta cell mass is to promote trans-differentiation of existing alpha cells into beta cells. Recent evidence from Kim's laboratory at Stanford suggests that alpha cell conversion to beta cells may be feasible (153). However, even if beta cell replacement, alpha cell trans-differentiation, or beta cell regeneration succeed, these strategies do not address the deficiency in alpha cell glucagon production, which precipitates hypoglycemia unawareness, and as such do not represent a complete treatment for this life-threatening diabetic complication on its own. Therefore, whole islet transplantation will remain the clinical standard-of-care over beta cell replacement until these concerns can be fully addressed.

Concurrent Autoimmune and Alloimmune Pathogenesis

There are two separate immune recognition pathways leading to the destruction of transplanted beta cells in the autoimmune recipient. As mentioned above, the first is autoimmunity due to antigen-specific memory T cells. Regardless of the source of beta cells transplanted into an individual with T1D, autoimmune T cells would target cells producing insulin and must be inhibited or removed to facilitate long-term transplant function (154). In contrast, autoreactive T cell targeting of a kidney transplant in a diabetic individual would not likely occur, because there would be no pre-existing kidney-specific memory T cells (154). Alloimmunity is the second major concern leading to the destruction of transplanted beta cells. Transplant-reactive or alloreactive T cell responses can target the genetic differences between the transplant donor and recipient (155). This category of immune response occurs against any organ or tissue transplant, in any individual, regardless of autoimmune disease status (156). Importantly, these transplant-specific responses focus primarily on the HLA molecule of the human transplant or MHC in mouse.

HLA molecules are the most polymorphic loci in the human genome, and each individual expresses multiple alleles of both class I and class II HLA (154–156). All the genetic differences in both alleles are potential antigens and could be targeted by T cells in transplant recipients. The differences in HLA class I are targeted by recipient CD8 T cells, and the differences in HLA class II are targeted by recipient CD4 T cells (156). Ironically, genetic diversity in HLA promotes diverse T cell responses to the same pathogen in different individuals, but unfortunately these genetic differences also promote strong T cell responses against any transplanted organ or tissue. In this section, we describe transplant recognition and alloimmunity separately from autoimmunity.

Transplant Recognition: Direct and Indirect Pathways

Donor-derived MHC (or HLA) molecules are the most prevalent transplant-derived antigen seen by the immune system of a transplant recipient. Transplant recipient T cells can interact with donor MHC molecules in two ways termed direct and indirect recognition (157). Direct allorecognition results from T cell interaction with donor MHC (plus some peptide loaded in MHC), whereas indirect allorecognition results from T cell interactions with recipient MHC (plus peptide derived from donor MHC, or any other transplant-derived protein). It is estimated that 1–10% of CD8 T cells or CD4 T cells will spontaneously respond to allogeneic MHC I or MHC II, respectively [reviewed in Ref. (158)]. In contrast, we hypothesize that the indirect precursor frequency is even smaller. In support of this hypothesis, recent evidence indicates that only 10% of allograft-reactive CD4 T cells in a mouse model of cardiac allograft rejection are indirect, while the remaining 90% are direct alloreactive CD4 T cells (159). Due to the higher precursor frequency for direct allorecognition than indirect allorecognition [reviewed in Ref. (157)], immune suppression protocols appear to hold direct alloreactivity in check. However, indirect recognition, which leads to antibody formation, CD4 T cell reactivity, and complement activation, is not completely inhibited using current immune suppression treatment regiments, as shown by complement deposition and antibody formation in chronic rejection models (160).

Importantly, both CD4 and CD8 T cells in the recipient can interact with donor MHC through either the direct or indirect pathway. The frequency and physiologic relevance of direct and indirect allorecognition varies with the nature of the transplanted organ or tissue. For islet allograft recognition, donor MHC class I and direct interaction with recipient CD8 T cells is a high-frequency event, because all cells in the graft express MHC class I. Since beta cells do not express MHC class II at baseline (161), direct recognition via CD4 T cells may not be as high frequency of an event. However, recent evidence suggests that beta cells may express MHC class II following T cell infiltration (161), which suggests that direct alloreactive CD4 T cells may be critical for anti-islet allograft responses. In contrast, indirect allorecognition by CD8 T cells must be therapeutically addressed to prevent islet allograft rejection (see below discussion of CD154 blockade therapy). **Table 2** summarizes the roles of direct and indirect CD4 and CD8 T cells in islet allograft rejection in the NOD mouse model.

Islet Allograft Tolerance in Non-Autoimmune Diabetic Mice

Unfortunately, islet transplants are subject to both autoimmune disease recurrence and allograft recognition in T1D mice and humans. To remove autoimmunity as a confounding variable from islet transplant tolerance studies, several labs have made use of the free radical generator streptozotocin (STZ) (165–167). STZ induces diabetes due to the relative lack of free radical scavenging enzymes expressed in pancreatic beta cells relative to other cell types (168). Following induction of diabetes with STZ, mice can be transplanted with allogeneic (MHC-disparate) pancreatic islets and treated with candidate transplant tolerance-promoting therapies. In experiments using non-autoimmune diabetic mice, untreated recipients serve as control groups to determine time to normal allograft rejection.

Multiple different general immune suppressive therapies have been tested in preclinical mouse models and are used clinically (168). These therapies can include anti-CD3, antithymocyte globulin, calcineurin inhibitors, mTOR inhibitors, tacrolimus, or mycophenolate mofetil (169). Interestingly, one of the tolerance-promoting protocols, which reversed diabetes, ECDI-coupled splenocytes, can also promote islet allograft tolerance in non-autoimmune mice (170). Of particular interest, monoclonal antibodies to block T cell co-stimulation (or signal 2) have been tested by several groups (165, 171). For example, short-term monoclonal antibody therapy directed against the T cell-expressed co-stimulation molecule CD154 (CD40L) has been shown by several groups (165, 171) to induce long-term

TABLE 2 | Islet allograft recognition pathways and likely players in rejection in autoimmune diabetic recipients.

Direct or indirect	T cells	Target	Precursor frequency in recipients	Fold expansion posttransplant	Sufficient for rejection?	Required for rejection?	Reference
Direct	CD4 T cells	Donor MHC II + transplant-derived peptide	0.1–10% versus individual donor MHC	10–100	Yes	No	(162–164)
Direct	CD8 T cells	Donor MHC I + transplant-derived peptide	0.1–10% versus individual donor MHC	10–100	Yes	No	(162–164)
Indirect	CD4 T cells	Donor-derived peptide loaded in recipient MHC II	Less than 1 in 1,000,000	>100	Yes	Appears likely	(162–164)
Indirect	CD8 T cells	Donor-derived peptide loaded in recipient MHC I	Less than 1 in 1,000,000	>100	Yes	Appears not	(162–164)

(>100 days) islet allograft tolerance across full MHC mismatch donor/recipient pairs (e.g., BALB/c islets transplanted into STZ-treated B6 male mice). This tolerance resides in the CD4 T cell compartment and can be transferred from treated and tolerant mice to naive mice (165). It is controversial whether this therapy induces allo-specific regulatory T cells *de novo* [suggested by Ferrer et al. (172)] or inhibits reactivity of naive alloreactive CD8 T cells through killing mediated by NK cells (173), or if these effects are simultaneous. In addition, the combination of anti-CD154 antibody with other therapies has been highly efficacious, in particular LFA-1 blockade. LFA-1 (CD11a) is an adhesion molecule expressed on most leukocytes, in particular on neutrophils, macrophages, and activated T cells. LFA-1 inhibition appears to delay and/or prevent islet allograft rejection as a single therapy. Similar to anti-CD154-induced transplant tolerance, uniform (100% of mice), long-term (>100 days) tolerance induced by the combination therapy of LFA-1 blockade and CD154 blockade resided in the CD4 T cell compartment and was serially transferable to multiple islet allograft recipients (165). In summary, STZ-induced diabetes represents a useful, non-autoimmune model system to test candidate islet allograft tolerance-promoting therapies. However, the end goal is to induce islet tolerance in autoimmune recipients, such as the NOD mouse.

In islet transplantation studies, "indirect" (recipient MHC-restricted) alloreactive CD4 T cells are key perpetrators of islet allograft rejection (174). As such, we hypothesize that co-transfer of islet antigen-specific Tregs at the time of islet transplantation would inhibit alloreactive T cell responses. Indeed, immune tolerance to antigen-presenting cell-depleted islet allografts in non-autoimmune mice requires CD4 T cells in transplant recipient mice (175). An alternative approach is to promote expression of T cell inhibitory receptor ligands on beta cells prior to transplantation (**Figure 1**). One example of this approach is beta cell expression of Fas ligand, which when combined with the immune suppressive drug rapamycin generated Tregs in recipient mice (176). Another example of this approach is a recent report which demonstrated that enforced beta cell-intrinsic PD-L1 and CTLA4 expression significantly delayed islet allograft rejection in NOD mice (177). In conclusion, whether autoimmunity or alloimmunity drives islet transplant rejection, generation, or adoptive transfer of Tregs or pre-arming transplanted beta cells with co-inhibitory molecules represent two distinct strategies to protect beta cells.

Potential Role for Regulatory CD4 T Cells in the Autoimmune Recipient of an Islet Allograft

Importantly, regulatory CD4 Foxp3$^+$ T cells engage peptides through the indirect antigen recognition pathway. Therefore, therapies that promote the development of transplant-specific Tregs are highly desirable. One long-term goal of the islet transplantation and autoimmunity field is to either deplete "indirect" autoreactive CD4 T cells or re-educate these CD4 T cells to become Foxp3$^+$ regulatory CD4 T cells, while also generating additional "indirect" Tregs specific for transplant-derived antigens. Based on the above considerations for beta cell MHC II expression in the inflamed transplant recipient, we hypothesize that regulatory CD4 T cells specific for donor MHC II would prolong islet allograft survival. In addition, we hypothesize that conventional self-reactive and "indirect" CD4 T cells, which recognize autoantigens through the transplant recipient's MHC class II molecule, would prolong graft survival. In combination, we speculate that adoptive transfer of both autoantigen-specific "indirect" Tregs as well as transplant MHC II-specific "direct" Tregs would synergize to significantly prolong islet allograft survival in autoimmune recipients.

Failure of Islet Transplant Tolerance in the NOD Mouse

Laboratories at the Barbara Davis Center (31), Vanderbilt (178), Harvard (179), University of Massachusetts (180), University of North Carolina (181), the University of Miami (182), and the St. Vincent's Institute in Melbourne (78) have utilized the NOD mouse as a model system to study both autoimmune disease recurrence (rejection of NOD-background islets) or islet allograft rejection (rejection of islet from genetically unrelated donor strains including B6, C3H). Due to its autoimmune disease status, the diabetic NOD female islet transplant recipient is a difficult, but clinically relevant model to test islet transplant tolerance-promoting therapies. Several studies have demonstrated the requirement for both CD4 T cells and CD8 T cells in diabetes recurrence in NOD mice (183, 184). Less data are available in the islet allograft scenario in NOD mice. Due to the sheer number of pancreatic islets required to reverse hyperglycemia and rapid T cell-mediated transplant rejection, diabetic female NOD mice are not frequently used to test transplant tolerance-promoting therapies.

The NOD mouse is an extremely stringent model to test transplant tolerance-promoting therapies. There are vanishingly few examples of long-term transplant tolerance in NOD mice. In particular, the combination of CD154 and LFA-1 in B6 mice resulted in long-term tolerance (180, 185). It is controversial whether this stringency results from resistance to therapeutic intervention in the autoimmune primed/memory T cell compartment, the alloreactive T cell response in NOD mice, or both. Mouse models and human clinical reports have suggested that autoimmune T cells are less susceptible to conventional immunosuppression (151, 185). In addition and in parallel, data from NOD mice support the existence of an accelerated and therapy-resistant anti-allograft T cell response (162). Additional studies in the Bio Breeder rat further suggested that autoimmune T cells are strongly impervious to tolerance-promoting therapy in this animal model of T1D, whereas the anti-allograft response can be made tolerant (186–189). These differences between models, and a lack of peptide-MHC II reagents to separately track both autoreactive and alloreactive CD4 T cells in the same transplant recipient mouse, lead to a lack of consensus in the field and an incomplete understanding of auto- and allo-T cell tolerance, in particular when both immune responses occur simultaneously.

While global immune suppressive treatments promote survival of transplanted beta cells [with the exception of calcineurin inhibitors, which are toxic to beta cells (190)], it is challenging to interpret effects of immune-modulatory therapies on specific T cell populations. Clinically, in the autoimmune recipient of

pancreatic islets, there are at least two concurrent immune responses. As such, a major limiting factor in this analysis is the quality and availability of reagents to reliably and separately track autoreactive and alloreactive T cell responses in human patients. Lack of validated reagents to monitor these responses longitudinally in clinical samples presents a major challenge to interpret therapeutic effects on recurrent autoimmunity versus anti-allograft responses. Lack of reagents to separately assess these two categories of T cell responses in the NOD mouse prevents the development of reagents to preferentially influence either category of T cell response in the preclinical or clinical setting.

CONCLUDING REMARKS

To prevent diabetes onset in the NOD mouse or at-risk human patients, several goals must be achieved. The genetics of T1D risks are well established, but the field lacks a comprehensive panel of peptide-HLA II tetramers to specifically track disease-associated CD4 T cell populations. Several groups (191–193), including our own (194), are working to fill this gap. Reagents to track key pathogenic CD4 T cells, perhaps including hybrid peptide-specific or DRiP-specific CD4 and CD8 T cells, are being developed and validated for clinical use. In addition, predictive biomarkers to measure not only the presence of these autoreactive T cells but also their activation status should be a focus of attention. Real-time monitoring of the activation status of rate-limiting autoreactive T cells is required to measure the efficacy of any tolerance-promoting therapy. Finally, to establish beta cell protection, measurements of beta cell function are required, in combination with assessment of autoreactive T cell biology. Non-invasive imaging methods represent one option (195, 196), but require specialized imaging technology and may not have sufficient sensitivity. More recently, methods such as high-sensitivity C-peptide assays (46, 197) and quantification of demethylated insulin DNA in the circulation (198, 199) could accomplish this beta cell health surveillance goal.

Despite our understanding of diabetes pathogenesis and ever-improving clinical care for individuals with T1D, some individuals develop debilitating diabetic complications that necessitate whole pancreas or isolated islet transplantation. In the autoimmune recipient, two categories of T cell responses must be prevented or inhibited to promote long-term transplant function. Both memory autoimmune T cell responses and nascent T cell responses against polymorphic MHC molecules occur after pancreas, islets, or iPS-beta cell replacement in T1D individuals. Therefore, a thorough understanding of not only autoimmune pathogenesis but also transplant recognition is required to develop methods to protect transplanted beta cells in autoimmune individuals. Intriguingly, Foxp3$^+$CD4 regulatory T cells may represent a path toward developing antigen-specific tolerance in both autoimmunity and transplant recognition. As such, immunotherapies that promote the development of regulatory CD4 T cells in both autoimmune models and transplantation models are highly desirable.

Multiple challenges remain to achieve the elusive goal of preventing islet transplant rejection in autoimmune recipients. Chief among these is to more specifically define the roles of CD4 and CD8 T cells and to determine whether autoimmunity or alloimmunity represents the higher barrier to beta cell transplant survival. Additional challenges to establishing islet allograft tolerance in the autoimmune recipient include (a) determining whether removing MHC from islet allografts would delay transplant rejection, (b) investigating if there is overlap of autoimmunity and alloreactivity on the individual T cell level, as has been shown for viral memory and transplant rejection (200, 201), (c) understanding if an MHC-matched "universal donor" beta cell line would avoid alloimmune T cell responses, (d) determining if beta cells can be induced to express proteins that would protect a transplant, and (e) developing reagents to track "direct" alloreactivity (against donor MHC). We and others are working to determine answers to these and other critical questions. With coordinated work by many dedicated individuals, we anticipate further advancements in our understanding of autoimmune pathogenesis, beta cell biology, and transplant recognition.

AUTHOR CONTRIBUTIONS

ALB, TM, and BTF wrote and edited the review article. TM and BTF created the model figure.

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REVIEW
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Diabetes and Sepsis: Risk, Recurrence, and Ruination

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Sepsis develops when an infection surpasses local tissue containment. A series of dysregulated physiological responses are generated, leading to organ dysfunction and a 10% mortality risk. When patients with sepsis demonstrate elevated serum lactates and require vasopressor therapy to maintain adequate blood pressure in the absence of hypovolemia, they are in septic shock with an in-hospital mortality rate >40%. With improvements in intensive care treatment strategies, overall sepsis mortality has diminished to ~20% at 30 days; however, mortality continues to steadily climb after recovery from the acute event. Traditionally, it was thought that the complex interplay between inflammatory and anti-inflammatory responses led to sepsis-induced organ dysfunction and mortality. However, a closer examination of those who die long after sepsis subsides reveals that many initial survivors succumb to recurrent, nosocomial, and secondary infections. The comorbidly challenged, physiologically frail diabetic individuals suffer the highest infection rates. Recent reports suggest that even after clinical "recovery" from sepsis, persistent alterations in innate and adaptive immune responses exists resulting in chronic inflammation, immune suppression, and bacterial persistence. As sepsis-associated immune defects are associated with increased mortality long-term, a potential exists for immune modulatory therapy to improve patient outcomes. We propose that diabetes causes a functional immune deficiency that directly reduces immune cell function. As a result, patients display diminished bactericidal clearance, increased infectious complications, and protracted sepsis mortality. Considering the substantial expansion of the elderly and obese population, global adoption of a Western diet and lifestyle, and multidrug resistant bacterial emergence and persistence, diabetic mortality from sepsis is predicted to rise dramatically over the next two decades. A better understanding of the underlying diabetic-induced immune cell defects that persist following sepsis are crucial to identify potential therapeutic targets to bolster innate and adaptive immune function, prevent infectious complications, and provide more durable diabetic survival.

Keywords: diabetes, sepsis, septic shock, infections, complications, resource utilization

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INTRODUCTION

The Third International Consensus Definitions for Sepsis and Septic Shock Report defines sepsis as life-threatening organ dysfunction caused by a dysregulated host response to an infection. This is associated with a >10% in-hospital mortality. Septic shock is defined as sepsis associated with profound circulatory, cellular, and metabolic abnormalities. Patients with septic shock have serum

lactate levels >2 mmol/L (>18 mg/dL) and require vasopressors to maintain a mean arterial pressure of 65 mmHg or greater in the absence of hypovolemia. Compared to sepsis alone, it has a much higher in-hospital mortality rate of >40% (1).

Long-term sepsis mortality is abysmal at 60–80%. Despite substantial advances in immune pathophysiology, this number has not considerably improved (2). In intensive care units, sepsis remains the leading cause of death (3). Considering the rapidly expanding elderly population with extensive comorbid burdens, physiological frailty, and immune senescence (4), over the next couple of decades, sepsis mortality is predicted to rise at a frightening rate (5). Just as terrifying are the mounting costs associated with treating septic patients. The United States spends ~$17 billion on sepsis-associated medical care (6).

Despite over 100 therapeutic clinical trials in sepsis, there are no current FDA-approved therapies that improve sepsis survival (7). In contrast, advancements in clinical treatment protocols (8) have resulted in increased in-hospital survival from life-threatening sepsis and organ dysfunction. However, a substantial portion of these in-hospital survivors will then die in the months to years following the acute event. A trimodal pattern of death during and after sepsis has been described. The first peak occurs at several days and is likely secondary to inadequate resuscitation. The second peak occurs at several weeks and is secondary to persistent organ injury and/or failure (9). The late (months to years) deaths comprise the largest mortality group and are speculated to be the consequence of improvements in intensive care medicine that keep elderly and comorbidly challenged patients alive despite persistent immune, physiological, biochemical, and metabolic aberrations (10). In 2008, over 800,000 Medicare patients survived admissions for severe sepsis. This population of survivors is composed of individuals with significant comorbidities that are at risk for hospital readmission (11). Several reports suggest that it is the synergistic effect of patients' advanced age, comorbidities, and persistent organ injury that create this damaging state of ongoing immune dysfunction, immune suppression, catabolism, and inflammation (12–14), leading to long-term sepsis mortality. Patients with Type II diabetes (T2D) are physiologically frail and comprise the largest population of patients who experience post-sepsis complications and rising mortality.

Type II diabetes is a common and devastating disease frequently encountered by clinicians who care for critically ill patients. With increasing globalization of the western diet and lifestyle, the worldwide incidence and prevalence of T2D is approaching pandemic proportions. In the United States, the prevalence has almost doubled from 11.9 million in 2000 to 21.9 million people in 2014, and the incidence has more than tripled from 1980 to 2014 (15). Globally, T2D is no longer a disease of high-income countries. In 2014, an estimated 422 million adults worldwide had T2D, compared to 108 million in 1980. The largest growth in prevalence can be found in low- and middle-income countries (16). From 1980 until 2014, China, India, and United States had the largest T2D patient populations. However, recently, the global share of people with T2D has dramatically increased in India and China while United States share has decreased. As the growth trends in T2D prevalence continue, the number of adults with T2D will surpass 700 million worldwide in the near future (17).

As medical management strategies improve, patients with T2D live longer with their disease. In addition, the increasingly young age at diagnosis results in prolonged exposure to glucolipotoxicity, low-grade inflammation, and increased oxidative stress, creating a metabolic milieu conductive to cancer growth (18). This represents a major public heath challenge. Delayed diagnosis, inadequate follow-up, and suboptimal care of T2D patients predisposes them to develop acute and chronic complications, leading to further burden on the patient, health-care system, and society as a whole (19). A 2012 global systematic analysis of disease and injury epidemiology identified T2D as a leading cause of years lived with disability (YLD), with a 67.2% increase in YLD from 1990 to 2010 (20). Furthermore, T2D has been shown to be a significant cause of mortality. Stokes and Preston performed a cohort study of National Health Interview Survey and National Health and Nutrition Examination Survey participants between 1997 and 2010 and estimated the proportion of deaths attributable to T2D to be 11.5–11.8% (21). These numbers underestimate the burden of T2D, as an estimated one in four people with T2D are unaware that they have the disease (22). As the sedentary, calorie-rich western lifestyle continues to infiltrate the global landscape, T2D will continue to become a more common comorbidity encountered in the hospital setting.

Patients with T2D have an increased risk of developing infections and sepsis. Although a few rare infections such as *Klebsiella* liver abscesses, malignant otitis externa, and emphysematous cholecystitis are strongly associated with diabetic patients, most infections that occur in diabetics are also common in the general population (23). T2D also worsens infection prognosis, with T2D patients showing increased morbidity and mortality from sepsis (24). The combination of increased incidence, prevalence, and life expectancy of individuals with T2D, combined with an increased risk of infections is resulting in a rapidly expanding patient population consuming more medical resources.

Some investigators have refocused their efforts to work on understanding the underlying innate and adaptive immune system derangements that facilitate the development of infectious complications, impair recovery from sepsis, and increase long-term mortality (25, 26). However, little effort has focused on the interplay between T2D, sepsis, immunity, and their impact on overall survival. In this review, we highlight the immune system's interdigitating role in the pathogenesis of T2D and sepsis. We focus on the clinical implications and then explore potential therapeutic interventions available to improve long-term survival in patients with T2D. To combat this pandemic, we hypothesize that disease-modifying therapeutics that have the ability to alter the course of disease have to be utilized, instead of focusing on palliative treatments that merely treat the sequelae of disease. Immune-modulatory therapy has been shown to improve patient survival in cancer, autoimmune diseases, and HIV. However, from these successful therapeutic advances, it has been shown that these therapies need to involve multiple agents, given in combination and introduced at the correct time to dampen disease progression, enhance patient immune responses, and affect host–pathogen interactions. We believe single-agent interventions are the reason why the sepsis

literature is littered with failed therapeutic interventions. Combine the immune aberrations in T2D with the immune dysregulation found in sepsis and there are multiple targets for modulatory therapy. We propose that combinations of tailored interventions that focus on specific immune system perturbations that exist in sepsis and T2D will result in a high probability of success.

IMMUNE DYSFUNCTION IN T2D AND SEPSIS

Type II diabetes is a complex clinical syndrome, depicted by persistent hyperglycemia in the setting of decreased insulin secretion and sensitivity, which results in a compilation of aberrant metabolic changes (24). Key metabolic changes include increased formation of advanced glycation end products (AGEs), activation of protein kinase C isoforms, and increased flux through the polyol and hexosamine pathways (27). These changes lead to increase production of superoxide (28), which activates inflammatory pathways, linking T2D to perturbations of the immune system (28). In addition, individuals with T2D have been shown to have abnormal host responses, including disorders of humoral immunity, defects in neutrophil function, and response of T cells (23, 29, 30). A recent study looking at obese individuals with and without T2D showed that individuals with T2D have specific immunological perturbations compared to metabolically healthy obese individuals, supporting the notion that T2D itself contributes to this identified immune dysfunction (31).

There is considerable clinical evidence that T2D worsens prognosis of pathological infections, with increased mortality from infections and sepsis in patients with T2D (24, 30, 32). This raises the pivotal question: why? The hematopoietic compartment constantly replenishes terminally differentiated innate and adaptive cells that are necessary for wound healing, successful tissue regeneration, and immune surveillance against offending pathogens (9). Sepsis impacts the immune system globally by affecting the lifespan, production, and function of innate and adaptive immune cells, leading to homeostatic perturbations in immune cell repletion (33, 34). In patients with T2D, this homeostasis may be altered secondary to over-nutrition and increased adiposity (35). These metabolic-induced immune perturbations clearly play a substantial role in the increased frequency, severity, and duration of infections (24, 28, 36).

In sepsis, an ongoing debate persists as to whether inflammatory/anti-inflammatory processes or innate/adaptive immune dysfunction are more detrimental to survival (37). Genomic studies on tissue samples from septic and severely injured trauma patients have provided more information (13). These studies have identified an enduring and simultaneous inflammatory and anti-inflammatory state, which is driven by dysfunctional innate and suppressed adaptive immunity. Together, these culminate in persistent organ injury (38), inflammation, and patient death (39, 40). **Figure 1** illustrates how the immune system responds to an acute septic episode. At baseline, patients with T2D have an aberrant immune system. After the initial acute septic episode, T2D patients continue to experience significant morbidity and mortality several months to a year later. We believe that it is the enduring derangements in the innate and adaptive immune system cellular functions that contribute to the long-term morbidity and mortality.

METABOLIC REGULATION OF IMMUNITY

The immune system protects against foreign microbial invaders, maintains optimal tissue homeostasis, and facilitates wound

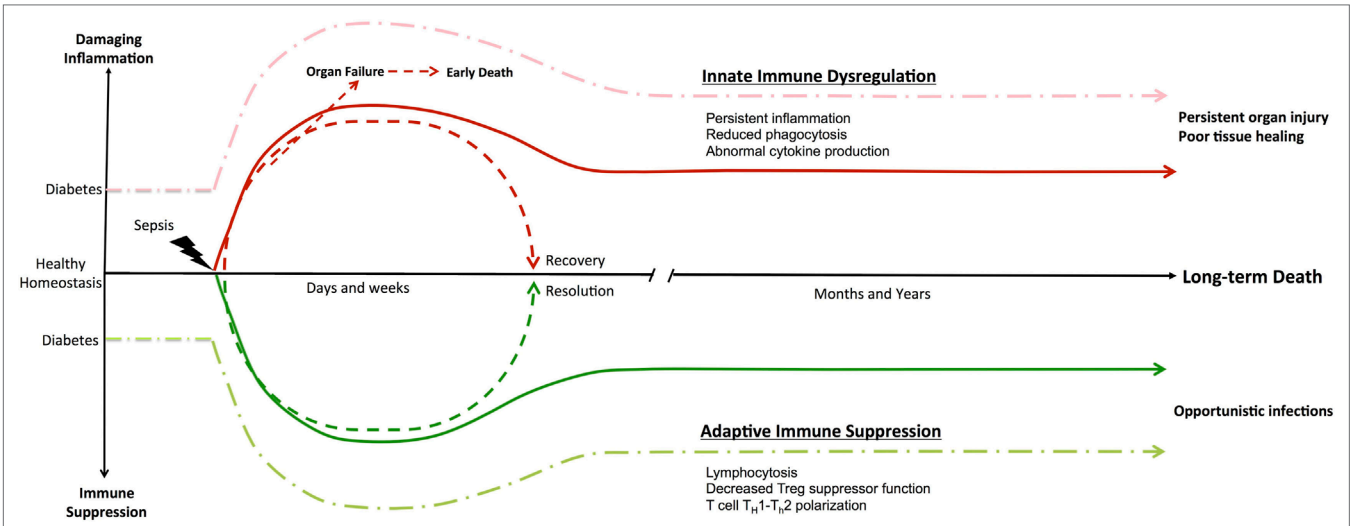

FIGURE 1 | Immune dysregulation in Type II diabetes and sepsis. Diabetes is a functional immune deficiency with chronic inflammation and immune suppression that affects an individuals' overall immune system homeostasis. The development of patient management protocols in sepsis has decreased early organ failure and sepsis mortality, allowing highly comorbid elderly patients to survive the initial insult. Furthermore, sepsis studies have demonstrated an enduring inflammatory state driven by dysfunctional innate and suppressed adaptive immunity that culminates in persistent organ injury and patient death. Subsequently, the highly comorbid elderly patient population that initially survived now experiences significant morbidity and mortality several months to a year later. Multiple hypotheses for these observations exist, with persistent derangements in the innate and adaptive immune system cellular functions as the main contributors to this long-term mortality.

healing. These processes are dynamic in nature, changing to meet the needs of the organism. Most immune responses are fueled by cellular metabolism that is regulated by extracellular signals, which direct the uptake, storage, and utilization of glucose, amino acids, and fatty acids (9). When the organism senses an invading pathogen or tissue insult, the innate immune cells secrete cytokines, chemokines, and inflammatory mediators, which influence the expansion of adaptive immune cells (9). Since immune cells do not store nutrients, immune responses are only upregulated and sustained when there is an increased uptake of nutrients from the surrounding microenvironment. Nutrients provide substrates for ATP, RNA, DNA, and protein synthesis, along with the membranes necessary for the immune cell's proliferation and maturation (41). Over a century ago, it was shown that a successful innate effector response is dependent on glucose metabolism (42), and that mitogen-driven proliferation of adaptive immune cells requires the utilization of extracellular glutamine (43, 44). T2D is a disease characterized by aberrant glucose metabolism. Homeostatic conditions are altered with an environment now characterized by chronic hyperglycemia and an increase in free fatty acids (FFAs) (45). An overall change in glucose metabolism therefore contributes to the immune dysfunction seen in T2D and sepsis.

In homeostatic conditions, immune cells rely on oxidative phosphorylation and β-oxidation as energy sources for ATP production (46). However, after stimulation, leukocytes shift their metabolism toward aerobic glycolysis in a process known as the Warburg effect (47). Subsequently, glycolysis produces cellular energy, followed by lactic acid formation in the cytosol instead of oxidation of pyruvate in mitochondria (48). Upon exposure to lipopolysaccharides (LPS), macrophages demonstrate a shift from oxidative phosphorylation to glycolysis and succinate and induce IL-1β production (49, 50). How T2D affects these processes is unknown, but clearly altering the substrates available for these pathways likely contributes to ongoing immune dysfunction. A better understanding of how hyperglycemic environments affect the metabolic checkpoints that control immune cell function, transition, and maturation is needed. In fact, delineating these pathways may provide targets for modulating systemic inflammation, cellular immunity, and recovery from infectious insults suffered by patients with T2D.

While several investigations have addressed the impact of hyperglycemia on sepsis and trauma outcomes in the critically ill in the ICU (51, 52), there is a paucity of studies that address the complications of T2D during infectious states and sepsis. The studies that do examine the association between T2D and sepsis outcomes are limited in their ability to account for all confounders (53, 54). It has been shown that adequate control of hyperglycemia is associated with improved outcomes and survival in times of critical illness; conversely, too tight of glycemic control has been associated with decreased survival (52). This U-shaped curve between glycemic control and mortality suggest that the ideal glycemic control for T2D patients is at moderately elevated glycemic levels. However, it is unclear that this effect is actually due to moderately elevated glucose levels, instead of confounding variables that lead to both lower glycemic levels and poor outcome (55). Although early glycemic control has been associated with risk reduction in the development of heart disease, hypertriglyceridemia, nephropathy, and cataracts, the biochemical mechanisms responsible for these effects are unknown (56, 57). Therefore, the more important question is: does long-term glycemic control augment immune function, prevent infectious complications, and promote durable survival? Although it makes logical sense that early and improves glycemic control would result in better immune function and reduced infections and sepsis episodes, there are few if any studies investigating this assumption. Moreover, there is a paucity of literature investigating the biochemical and physiological pathways central to immune function that benefit from glycemic control. Much more scientific investigation is necessary to determine the biological effect of glycemic control on immune function to improve long-term T2D survival from sepsis.

INFLAMMATION

Once the host loses local containment of an infection, the body is systemically exposed to microbes, microbial components, and products of damaged tissue. This induces an inflammatory response and initiates sepsis-like responses through the recognition of pathogens and damaged tissue by way of pattern-recognition receptors (PRRs), which are ubiquitous on immune cell surfaces. PRRs are expressed primarily on immune and phagocytic cells and on many types of somatic tissues. Microbial infections are recognized by pathogen-associated molecular patterns (PAMPs), which are expressed by pathogenic and harmless microbes. PAMPs are recognized by PRRs such as toll-like receptors (TLRs), C-type and mannan-binding lectin receptors, NOD-like receptors, and RIG-I-like receptors (9). Proteins and cellular products released by tissue damage are similarly recognized as damage-associated molecular patterns (DAMPs) (58). During sepsis, systemic activation of the innate immune system by PAMPs and DAMPs results in severe and persistent inflammatory responses characterized by an excessive release of inflammatory cytokines such as IL-1β, TNF, and IL-17, collectively known as the "cytokine storm" (38). This unregulated release of inflammatory cytokines occurs over a relatively short period of time (hours or days). Furthermore, instead of stimulating what should be a normal physiological response to an infection, intense complement activation and innate immune cell stimulation enhance an excessive inflammatory response resulting in tissue damage, compromised cellular responses, and molecular dysregulation. The resulting damage incites organ dysfunction and even multiorgan failure (38).

Type II diabetes is an inflammatory disease within itself. In T2D, FFAs bind to TLR2, a receptor for pathogen lipoproteins, and TLR4, a LPS receptor, to activate the innate immune system (59, 60). In addition, there is indirect activation through TLR signaling (61). This elicits the inflammatory pathways activated in sepsis. In addition, AGEs are DAMPs that activate pro-inflammatory pathways.

Several studies also show that the inflammatory response is altered in patients with T2D. For example, mononuclear cells and monocytes have been found to secrete less IL-1 and IL-6

in response to stimulation by LPS, all of which appears to be secondary to an intrinsic defect in cells (29, 62). Although some patients recover from the inflammatory state during an acute septic episode, for unknown reasons elderly patients with significant comorbidities fail to resolve this initial condition. They instead progress to a state of persistent inflammation, immune cell dysfunction, and catabolic metabolism, all of which degrade the immune system's ability to clear infections and heal injured tissues (63). In individuals with T2D, the chronically inflamed environment may play a role. Adipose tissue serves as a site of inflammation (28), with an increase in adiposity being associated with upregulation of genes encoding pro-inflammatory molecules resulting in the aggregation and accumulation of immune cells (64). Macrophages then create a pro-inflammatory loop by forming crown-like structures, which promote differentiation to pro-inflammatory M1 macrophages (28) and the associated pro-inflammatory cytokines. Similar to the environment seen in adipocytes, pro-inflammatory conditions have also been seen in the pancreas. In the pancreas, there is β-cell apoptosis from glucose-induced IL-1β (65), and β-cell dysfunction by lipoapoptosis from FFAs acting as effector molecules (28). This stress-induced β-cell death results in the release of autoantigens and alarmins, which are endogenous molecules released by necrotic cells resulting in stimulation of the immune system through self-antigen presentation (28). This leads to an enhanced adaptive immune response (66).

Given the growing knowledge in the field of metabolic-induced immune dysfunction in T2D, possible interventions that curb inflammation may offer therapeutic benefits in T2D. In sepsis, recent investigations have suggested that therapeutic interventions that curb hyperinflammation, shift catabolism toward anabolism, and bolster immune function may be beneficial in combination, once the initial episode of sepsis has subsided (25, 67, 68). Although in other disease states, such as severe burns, advanced cancers, and autoimmune diseases, combination therapies that reduce inflammation, optimize metabolism, and decrease infections are common-place, in sepsis there currently is no clear plan for the routine use of these or similar strategies (9). Combinations of immune modulators that target affected pathways in T2D and sepsis have the potential to offer clinically significant improvements in overall survival.

MOLECULAR ALTERATIONS IN T2D AND SEPSIS

The pathogenesis of T2D can be described as insulin resistance associated with inactivity, obesity, and aging (69, 70). Initially, the pancreatic islet cells respond to this decrease in insulin-stimulated glucose uptake by increasing cell mass and secretory activity. When functional expansion of the islet β cells fails to compensate for the insulin resistance, insulin deficiency, and subsequent T2D develop. The hypothesized mechanisms behind insulin resistance and islet β-cells dysfunction focus on molecular changes that influence the pathogenesis of T2D. Specifically, most research centers on lipotoxicity, glucotoxicity, oxidative stress, endoplasmic reticulum stress, amyloid deposition in the pancreas, and ectopic lipid deposition in the muscle, liver, and pancreas (70). The contribution of each of these mechanisms remains unclear, but, interestingly, all of these cellular stresses can be caused by over-nutrition (71) and are induced or exacerbated by an inflammatory response (72).

Obesity-induced inflammation is chronic and indolent, differing from the more acute type of inflammation commonly associated with infections (70). Current observations in sepsis show that sepsis-induced organ dysfunction occurs primarily though cellular and molecular dysregulation of signaling pathways, as opposed to gross tissue damage. This may result in multiple organ failure even in the context of preserved cell morphology and in the absence of significant cell injury. Therefore, immune dysfunction in sepsis is associated with molecular alterations that alter cellular phenotype and function. How the molecular changes in T2D and sepsis interact and influence each other resulting in worse clinical outcomes is unclear. Below we outline several important pathways of cellular dysfunction that impact immune function in diabetics and sepsis, illuminating gaps in knowledge, which could influence why patients with T2D have infections that are difficult to treat and are associated with significant morbidity and mortality (70).

Complement Activation

Obesity and elevated insulin levels have been associated with elevations in plasma C3 (73), C5, and C8 (74). These increased levels are likely a result of glycated immunoglobulins activating complement (75). Elevated glucose may then attack the thioester bond of C3, making it functionally deficient and leading to a decreased ability to opsonize bacteria (76). In sepsis models, a robust and consumptive depletion of complement occurs, resulting in a sharp drop in the hemolytic activity of plasma complement and its activation products (77). There is also evidence that sepsis in humans causes shedding of the C5a receptor into plasma, likely due to release of microparticles from neutrophils (78). In addition to complement activation in sepsis, there is well-established evidence that activation of the complement system leads to activation of the clotting and fibrinolytic systems (79), resulting in activation of several clotting factors, including thrombin, which have C3 and C5 convertase activities. These ultimately generate C5a and the terminal membrane attack complex (MAC) (80). The progress in understanding how complement activation increases systemic inflammation, organ failure, and mortality have resulted in the development and randomized phase 2 trial of a C5a inhibitor, CaCP29 (EudraCT Number: 2013-001037-40). This C5a inhibitor has shown great promise despite a historically large field of other failed antibody inhibitors (81).

The fact that glycated immunoglobulins affect complement could obviously play a role in T2D patients having an increased risk of infections. However, it is still unclear why these patients have worse outcomes during septic episodes. One hypothesis is that obese T2D patients have baseline elevations of C5, which then becomes activated by enzymatic cleavage during a septic episode, leading to more MAC generation. To date, there have been no published studies looking at C5a inhibitors in T2D patients with sepsis.

Mitochondrial Dysfunction and Redox Imbalance

Mitochondria are essential for maintaining an adequate supply of ATP for cellular processes. Mitochondria have a significant role in glucose-stimulated insulin secretion from pancreatic β cells (82), with decreases in mitochondrial oxidative activity and ATP synthesis leading to insulin resistance (83, 84). Mitochondrial dysfunction, or direct damage of mitochondria, can trigger cell death pathways through release of mitochondrial cytochrome *c* (9, 85) as well as directly affect the generation of ATP. Not only will the drop in ATP negatively affect intracellular processes and cellular function, such as insulin secretion, but severe lack of ATP can also trigger cellular anergy. In this state, the cell does not necessarily die, but instead acquires a hibernation-like state resulting in tissue dysfunction and organ failure (86).

In addition, hyperglycemia itself has been shown to induce ROS. Obese and insulin-resistant T2D individuals have a hyperglycemic intercellular environment with elevated concentrations of FFAs (87). Hyperglycemia itself has been shown to induce ROS (88, 89) through enzymatic cascades in mitochondria, including activation of NADPH oxidase, uncoupling of NO synthesis, and stimulation of xanthine oxidase (90). Glycated proteins have also been shown to promote ROS formation (91). ROS may then lead to the formation of NLRP3 inflammasomes and caspase 1, which activates the IL-1, pro-inflammatory system (92, 93).

In sepsis, there is generation of excessive amounts of ROS and RNS, which can directly inhibit respiration and damage the respiratory chain components in mitochondria (94–96), leading to mitochondrial dysfunction (9). In addition to this pathway, sepsis-impaired tissue perfusion (due to fluid loss, both intrinsic and extrinsic, as well as reduced vascular tone) leads to tissue hypoxia. Loss of tissue oxygenation significantly impairs oxidative phosphorylation and may trigger cell death pathways (97). In T2D, microvascular dysfunction can lead to local tissue hypoxia. The degree to which local tissue hypoxia propagates cell death and enables ongoing infections in T2D has not been defined.

In both T2D and sepsis, mitochondrial dysfunction and redox imbalance plays an integral role in progression of disease. In human models, cellular ATP levels are correlated with sepsis survival (96, 98). In T2D, changes in cellular ATP levels lead to insulin resistance. In a T2D patient with sepsis, it is unclear if these altered pathways are synergist, antagonistic, or some combination of both. Either way, given the oxidative stress, it seems clear that antioxidant therapies may have a therapeutic role.

Calcium (Ca^{2+}) Homeostasis

Calcium homeostasis in T2D is ubiquitously impaired across tissues, including but not limited to adipocytes, platelets, pancreatic β cells, kidney, and liver (99). The most consistent finding is an increase in intracellular Ca^{2+} levels, leading to tissue-specific dysregulation (99), such as glucose resistance. Glucose homeostasis is determined by the rate of glycolysis, gluconeogenesis, glycogen synthesis, and glycogenolysis, all which are calcium-regulated pathways (100, 101). When intracellular Ca^{2+} increases, glycogen synthase is inhibited causing glucose resistance (102). Clinical trial NCT00436475 examined how Ca^{2+} supplementation impacted pancreatic β cell function, but did not show any significant differences (103, 104).

Hypocalcemia in sepsis, hypothesized to be secondary to defective intracellular calcium homeostasis, is common and correlates with disease-specific scores during critical illness (105). Although systemic Ca^{2+} levels are reduced during sepsis, there are increased cytosolic Ca^{2+} levels, similar to those observed in T2D. These heightened intracellular Ca^{2+} levels lead to elevated inflammatory responses, cellular dysfunction, and can even be cytotoxic (9). In addition, accumulation of Ca^{2+} in organs during sepsis is associated with significant organ dysfunction (106).

Poly(ADP-Ribose) Polymerase 1 (PARP1) and PARP2 Activation

Poly(ADP-ribose) polymerase 1 and PARP2 are enzymes that catalyze poly(ADP-ribosyl)ation of proteins, after being stimulated by DNA strand breaks. PARP activity is therefore viewed as a sensor of DNA damage. PARP1 activation and initiation of the inflammatory response occur simultaneously (107). PARP1 activity upregulates pro-inflammatory gene expression (108), which is attributed to PARP1-induced alterations in chromatin structure and in transcriptional regulation (107, 109). Because PARP1 also directly contributes to cell death in affected tissues (107) it is hypothesized that PARP1 has a role in sepsis-associated immune cell death. Further data to elucidate the role of PARP enzymes suggests they play a role in metabolic regulation by affecting mitochondrial function and oxidative metabolism (9). PARP activation impacts cellular functions by diverse mechanisms. In general, PARP inhibition enhances oxidative metabolism and mitochondrial content. This suggests that reducing PARP activity may prevent metabolic-related diseases such as T2D, which are characterized by impaired mitochondrial function (110).

Inhibitors of PARP1 have been assessed in clinical trials as potential cancer therapeutics, but trials in sepsis and T2D have not been initiated. It is not clear whether inhibition of PARP1 in humans would be beneficial in the case of T2D or sepsis. In addition, the practicality of long-term inhibition without negative effects on genomic stability is unknown (110).

CELLULAR DEFECTS

Below we will summarize the alterations seen in the majority of innate and adaptive immune cells in T2D. Furthermore, we highlight how these cells types are affected by sepsis and try to illustrate how T2D and sepsis together may interact to exacerbate long-term mortality.

Innate Immunity

Endothelium

The endothelium, a single cell semi-permeable barrier, is composed of endothelial cells (ECs), which line all of the vasculature and lymphatic systems in the body. They also play a role in many innate and adaptive immune responses (9). They are one of the first cells to identify invading microbes in the bloodstream *via* endogenous metabolite-related danger signals (111). ECs

express TLR-2 and TLR-4, which enable them to be activated by LPS. Activation subsequently leads to the production of pro-inflammatory cytokines and chemokines. These boost the immune response through recruitment of further immune cells (112). Therefore, ECs function as innate force multipliers, cell mobilizers, and immune regulators by modulating cellular function (113). In addition, ECs also express both MHC I and II molecules, which allow them to serve as antigen presenting cells for T cells by presenting endothelial antigens (112).

Endothelial cells are very sensitive to blood glucose alterations, with hyperglycemia-induced ROS leading to EC damage (114). In T2D, increased concentrations of glucose and FFAs also activate ECs, leading to a pro-inflammatory and pro-thrombotic endothelial phenotype (115). There is increase production of plasminogen activator inhibitor-1, thromboxane, tissue factor, and von Willebrand's factor (vWF), which promotes platelet aggregation and adhesion to the subendothelial layer and the formation of pathological thrombi (116). In sepsis, EC dysfunction is present and manifests as several pathological processes including capillary leak, altered vasomotor tone, and microvascular thrombosis (117). An increased release of pathological quantities of vWF once again promotes platelet aggregation and adhesion to the subendothelial layer and the formation of pathological thrombi. These findings show that ECs are key regulators of the physiological and immune dysfunction seen in both T2D and sepsis. It would make sense that worsened EC dysfunction would be present in a septic T2D patient compared to a septic non-T2D patient given the pathways involved, but how these pathways interconnect is not understood. However, it is clear that EC modulation could be beneficial to improve survival outcomes in septic T2D patient cohorts.

Neutrophils

Neutrophils are the most prevalent and integral cell type of innate function and are critical for containment and eradication of microbes (9). Neutrophil dysfunction has been linked to hospital-acquired infections (118). Neutrophils are the majority cell in bone marrow and are the very first responders to microbial infections sites (119). One important aspect is their capacity to produce pro- and anti-inflammatory cytokines and growth factors, which regulate the inflammatory response (120).

In T2D, neutrophils show defects in almost all functions, including migration to inflammatory sites, phagocytosis, release of lytic proteases, production of ROS, and apoptosis (121). In addition, a study evaluating the release of TNF, IL-1β, and IL-8 from neutrophils in individuals with T2D showed increased amounts of TNF, IL-1β, and IL-8 in both the basal state and after stimulation by LPS. This excessive release may lead to tissue injury and cell death (121), increased susceptibility to invasive microorganisms (122), and impairment of normal wound healing (123).

In addition to microbial eradication by phagocytosis, oxidative burst, and degranulation, it has been shown that neutrophils can eliminate a wide range of microbes by forming neutrophil extracellular traps (NETs) (124). If a system is primed to produce NETs, a process termed NETosis, tissue damage can occur (125). NETosis requires a microenvironment with increased levels of TNF (126), upregulated PAD4 (127), elevated intracellular calcium levels, and fasting serum glucose (128), which are all seen in T2D.

In sepsis, there is delayed neutrophil apoptosis (129), leading to ongoing neutrophil dysfunction. This delayed apoptosis is further complicated by the release of immature band-like neutrophils from the bone marrow that demonstrates clear deficits in oxidative burst (130), cellular migration patterns (131, 132), complement activation ability, and microbial eradication (133). These defective neutrophils play a signification role in the persistent inflammation and immune dysfunction seen in sepsis. These findings combined with TLR signaling deficits, chemokine-induced chemotaxis reductions, altered apoptosis signaling pathways, and neutrophil immune senescence, result in a sundry of functional deficits that endure long after sepsis symptoms have subsided (9). In addition, septic patients have been shown to have elevated NET concentrations compared to healthy controls, and that these increased NET levels were associated with sepsis severity and organ dysfunction (84).

Neutrophils clearly have a role in the immune dysfunction seen in both T2D and sepsis. The increased tendency to form NETs contributes to the pathogenesis of both diseases; however, how or if this contributes to the worsen outcomes in patients with sepsis and T2D is unclear.

Monocytes and Macrophages

Macrophages have important roles in immune response and homeostasis. They play a significant role in phagocytosis, effectively killing microbes, and in clearing apoptotic and necrotic cells. In addition, they secrete pro- and anti-inflammatory cytokines and express MHC-II molecules, allowing them to activate $CD4^+$ T-cells and promote differentiation into T helper subsets (9, 134). Just as important, they play a role in the regulation of glucose and lipid metabolism, and in the inflammation of adipose tissue (135). Macrophages have the ability to display remarkable phenotypic heterogeneity depending on the biological situation (136), leading to the establishment of M1 pro-inflammatory ($CD11C^+$) and M2 anti-inflammatory macrophages. First discovered in adipose tissue (64, 137), it was shown that accumulation of macrophages leads to elevated inflammatory cytokines. In addition, the accumulation of these inflammatory cytokines is associated with insulin resistance. The mechanism behind the accumulation of these pro-inflammatory M1 macrophages is thought to occur through two main processes. First, the adipocytes and resident macrophages secrete increased levels of chemokines, LTB3, MIP, MIF, and MCP-3 to promote recruitment of blood monocytes (138). Once the monocytes arrive to the area, the inflammatory signals within the adipose tissue push the monocytes to differentiate into the pro-inflammatory M1 phenotype.

In sepsis, blood monocytes have endotoxin tolerance, with the reduced ability to release pro-inflammatory cytokines after an LPS challenge (9). This has been suggested to facilitate poor short- and long-term sepsis outcomes (139, 140). Although a sundry of complex mononuclear cell signaling pathways are altered and contribute to the establishment of endotoxin

tolerance, the major implication on monocytes, and to a lesser extent macrophages, is reduced antigen presentation related to diminished HLA-DR cell surface expression (141). In addition, the reduced monocyte capacity to secrete pro-inflammatory cytokines suggest that intracellular signaling has shifted toward the production of anti-inflammatory mediators, which are associated with hospital-acquired, ongoing, and secondary infections that ultimately increase sepsis-associated mortality. Although the mechanisms accounting for monocyte LPS tolerance are not clear, sepsis-induced monocyte epigenetic reprogramming may play a pivotal role in the establishment of LPS tolerance, myeloid anergy, and the overall immune suppressive monocyte phenotype (142). Analysis of human monocyte mRNA clearly shows increased levels of inhibitory cytokine genes and reduced levels of pro-inflammatory chemokine genes (143).

These findings make us question what happens to monocytes and macrophages in T2D individuals with sepsis. At baseline, obese T2D individuals have a shift toward pro-inflammatory macrophages; however, the fate of these recruited macrophages and their contributions to infection eradication remain less studied. Unlike in a resolving acute infection where homeostasis is restored, adipose tissue inflammation fails to resolve naturally (144). When a T2D individual is exposed to an acute infection, it is unclear how monocyte and macrophage populations change and if these changes are affected by the baseline obesity and chronic inflammation.

Natural Killer (NK) Cells

Natural killer cells act as immune complex regulators. NK cells have the ability to destroy target cells spontaneously, without prior exposure, and without MHC restrictions (145). In sepsis, NK cell cytotoxic function is greatly decreased (146) and specific subsets of NK cells are significantly altered. These changes have been associated with increased lethality (147). Recent studies show that individuals with T2D have abnormal NK cell phenotypes, with a significant decrease in NKp46, a NK receptor that recognizes influenza hemagglutinins, and tumor ligand NKG2D, an activating receptor on NK and $CK8^+$ lymphocytes. They also have functional defects with reduced degranulation (148). In T2D patients, it is unknown what happens when these altered NK phenotypes are further affected during a septic episode.

Dendritic Cells (DCs)

Dendritic cells are characterized as conventional DCs (cDCs) or plasmacytoid DCs (pDCs). cDCs secrete IL-12 and are comparable to monocytes. pDCs secrete IFNα and are comparable to plasma cells. cDCs and pDCs have enhanced apoptosis in patients with sepsis, as well as in patients who developed nosocomial infections (9). In T2D, elevated glucose induces a pro-inflammatory cytokine profile in DCs leading to their maturation (149). It addition, hyperinsulinemia promotes DC activation and upregulation of scavenger receptors including SR-A and CD36, a receptor found on many cells including ECs, cardiomyocytes, platelets, monocytes, and macrophages, all which are involved in the macrovascular complications of T2D (150). AGEs, through binding with SR-A, can also induce maturation of DCs (151).

In sepsis, just like monocytes, DCs have decreased HLA-DR expression and secrete increased amounts of IL-10, which is anti-inflammatory. In addition, when DCs are cocultured with T effector cells, T cell anergy in induced and regulatory T cell (Treg) proliferation enhanced, both which correlate with sepsis-induced immune dysfunction (152). A couple of recent investigations have also demonstrated that inhibition of sepsis-induced DC apoptosis or amplification of DC function improves sepsis long-term survival (153, 154). These observations reveal that adaptations in DCs contribute to the pathogenesis of T2D and sepsis and that targeted manipulation of DCs may provide a therapeutic strategy.

Myeloid-Derived Suppressor Cells (MDSCs) and Myelopoiesis

Myeloid-derived suppressor cells are a heterogeneous population of undeveloped myeloid cells. They expand during trauma and sepsis, impede immune responses, and signal through TLR-mediated pathways (155, 156). MDSCs inhibit $CD8^+$ T cell function; however, their impact during sepsis is uncertain. Current literature implies a beneficial role, by focusing on their ability to restore innate immune cell function and surveillance through "emergency" granulopoiesis (132). Prior to MDSC increase, there is a brief period of host vulnerability to secondary microbial infections. This brief period is associated with overall mortality secondary to reduced numbers of bone marrow cells and a reduction in neutrophil and monocyte numbers and function (130). It has also been demonstrated that robust MDSC expansion, *via* augmented granulopoiesis, imparts lasting immunity to secondary and nosocomial infections during sepsis (157). Given these findings, there is mounting interest in exploring myelopoiesis, MDSC expansion, "emergency" granulopoiesis, and hematopoietic stem cell (HSC) production and function (130, 155, 157–159). Due to the importance of efficiently regenerating functioning neutrophils, monocytes, and DCs during sepsis, MDSCs expansion is a necessity to replenish the pool of functional innate immune cells. However, in T2D and obese patients, hematopoiesis and myelopoiesis are significantly altered (9). This observation raises the question as to the combined impact of myelopoietic derangement promoting ongoing infection, depressed wound healing, and increased mortality following sepsis.

It has been demonstrated that HSCs and myeloid lineage expansion all occur through c-KIT-, type-I IFN- (IFN-I), and CXCL10-dependent signaling that involves IFN-I-secreting B cells (158, 159). Impaired HSC proliferation, development, and function in human bone marrow transplant and diabetic models (160) is clearly associated with increased mortality from chronic, secondary, nosocomial infections (161). Humans with altered granulopoiesis ability undoubtedly experience more frequent, severe, and anomalous infections, demonstrating the essential requirement for effective neutrophil production especially in T2D (23). Recently, patients with sepsis have been shown to have persistently increased MDSCs that are functionally immune suppressive. These are associated with adverse outcomes including increased nosocomial infections, prolonged ICU stays, and poor functional status at discharge

(162). On the other hand, overabundant MDSC proliferation may provoke a physiological state of persistent inflammation, such as in adult respiratory distress syndrome, leading to septic patients having poor outcomes (12). Recent work has demonstrated that acute inflammation causes the reduction of peripheral lymphocytes and common lymphoid progenitors in the bone marrow, which has been connected with a profound reduction in the number of osteoblasts (9). The specific contributions of lymphopoiesis, myelopoiesis, and MDSCs to sepsis recovery in T2D populations versus persistent inflammation and catabolism remain poorly understood. However, new insights into these processes and their roles in sepsis resolution and recovery will hopefully present new targets for immune-modulatory therapy to improve sepsis outcomes in T2D cohorts.

Adaptive Immunity

Lymphoid Apoptosis and Immune Suppression

Apoptosis plays a crucial role is tissue homeostasis and the size and duration of immune responses. Once an infection is successfully cleared, activated lymphocytes undergo apoptosis to curtail the immune response. In the periphery, lymphocyte numbers are tightly regulated. Increased lymphocyte apoptosis leads to immunodeficiency, whereas decreased lymphocyte apoptosis leads to cancer and autoimmune diseases (163). Lymphocyte apoptosis is accepted as a critical step in the pathogenesis of sepsis and contributes to septic immunosuppression (164). It has been shown that T2D patients have an overall leukocytosis; however, analyses of these leukocytes show an overall lymphocytosis (163). Given these findings, blockade of lymphocyte apoptosis may have a therapeutic benefit in septic T2D patients.

Gamma Delta T Cells (γδ T Cells)

Gamma delta T cells are a diminutive subset of T cells that have a T cell receptor made up of one γ chain and one δ chain. This uniquely distinct group of T cells exists in the skin, lungs, adipose tissue, peripheral blood, and intestinal epithelium. Once activated, γδ T cells release interferon gamma (IFNγ), IL-17, and other inflammatory chemokines (9).

Obese individuals have a decreased amount of γδ T cells, which is inversely proportionate to body mass index. In addition, the remaining γδ T cells have a reduced ability to secrete IFNγ (165). This is significant because despite obesity being a pro-inflammatory condition, they have a decreased ability to mount an inflammatory response. The number of circulating γδ T cells is also significantly diminished when individuals have an episode of sepsis. Reductions in the γδ T cell population have been correlated with high rates of sepsis lethality (166). These findings suggest that γδ T cells represent a possible target for immune enhancement.

T Helper Cell (Th Cell) Subpopulations

T helper cells assist other cell types with immunological processes. APCs present peptide antigens to $CD4^+$ cells through MHC class II molecules. The $CD4^+$ cells are quickly activated, proliferate, and efficiently secrete cytokines, which modulate adaptive and innate immune responses. Upon activation, $CD4^+$ cells have the capability to differentiate into specialized T cell subsets, including Th1, Th2, Th3, Th17, Th22, Th9, or T follicular helper. These subsets promote monocyte stimulation, B cell differentiation, and cytotoxic T cell activation through cytokine generation and secretion (9, 167).

It is hypothesized that adipocytes upregulate class II MHC molecules and play a direct immunological role in antigen presentation (168). Several clinical studies have shown that there is a decline in naïve $CD4^+$ T cells, as well as an imbalance of $CD4^+$ Th cell subsets toward Th17 and Th22 pro-inflammatory subsets in obese individuals with T2D. This leads to a cytokine-induced hyperinflammatory response leading to further innate immune system activation and response (169). This shift to a pro-inflammatory environment is of significant importance in patients with T2D, as it has been shown that Th cells contribute to the complications associated with T2D, such as coronary artery disease (169).

In sepsis, $CD4^+$ populations undergo apoptosis (13, 170). Compared to individuals who survive an episode of sepsis, in humans who die from sepsis there is more lymphocyte (specifically $CD4^+$ cells) apoptosis. When evaluating the $CD4^+$ cells that survive, there is reduced Th1- and Th2-associated cytokine production both during and long after sepsis subsides (171). In addition, Th17 cytokine production is reduced in sepsis and probably negatively impacts long-term mortality (172). These Th populations play a significant role in both T2D and sepsis. The mechanism by how they contribute is still unclear but it may be that Th cells contribute to the development of the macrovascular complications of T2D, which then contributes to long-term mortality in T2D patients.

Regulatory T Cells

Regulatory T cells are master regulators of the adaptive immune system. They help maintain self-tolerance and suppress responses of effector T cells subsets (9). An appropriate balance of pro-inflammatory (Th1 and Th17) and anti-inflammatory (Treg) cells are critical to maintain homeostasis. In T2D, there is a loss of homeostasis with a decreased amount of Tregs (173, 174). This imbalance is hypothesized to contribute to the clinical complications of T2D (175). Tregs have also been shown to induce M2 macrophage differentiation. Therefore, it has been speculated that the decrease in Tregs in T2D contributes to the known polarization toward M1 macrophages.

During the period of inflammation, such as sepsis and critical illness, Tregs enhance the deleterious effector T cell suppression, which subsequently prolongs recovery and may dispose to increased complications. There is an increased Treg ratio present early after episodes of sepsis, which is either due to an absolute increase in Treg number or effector Th cell loss from apoptosis. It could be that Tregs are not susceptible to sepsis-induced apoptosis (176). The fact that hospitalized patients who died from sepsis and T2D patients both have alterations in their Treg amounts make Treg function a possible therapeutic intervention.

B Cells

B cells are a very diverse immune cell population. Historically, B cell function was thought to only encompass producing

antibodies and plasma cells for long-term antibody responses; however, recent data have focused on the role of B cells in chronic inflammatory disease and sepsis (9). In T2D, TLR ligands activate B cell cytokine production, most significantly IL-8. This pro-inflammatory response then augments T2D patient's B cell inability to upregulate IL-10 production in response to TLR ligands (177). In *ex vivo* studies in both aging and sepsis patients, B cells demonstrated significant reductions in supernatant IgM production, which may explain why older individuals are more vulnerable to Gram-negative bacteria and fungal infection (178). It is unclear what happens to the B cells in elderly patients with T2D during sepsis, but clearly B cell physiology contributes to the worsened morbidity and mortality experienced by this patient cohort.

INFLAMMATION RESOLUTION

As related to infection, inflammation is generally followed by inflammation resolution. In sepsis, compensatory anti-inflammatory pathways are activated shortly after sepsis initiation (37). The hallmark cytokine in these anti-inflammatory pathways is IL-10. IL-10 suppresses IL-6 and IFNγ, while stimulating the production of soluble TNF receptor and IL-1 receptor antagonist (IL-1RA). At the subcellular level, autophagy eliminates DAMPs and PAMPs by packaging pathogen components, damaged organelles, and cellular proteins into vesicles targeted for lysosomal degradation. This results in reduced inflammation and cellular activation (179). After a severe infection, resolution of inflammation involves an interdigitating, complex, and coordinated array of cellular processes and molecular signals. The offending pathogen needs to be eliminated from the host, while damaged tissues, cells, and leukocytes need to be removed. These processes occur through activation of anti-inflammatory pathways with production of IL-10 and transforming growth factor β.

Sepsis differs from obesity and T2D since the latter has persistent inflammation that does not resolve. The secretion of pro-inflammatory adipokines [IL-6, TNF, and monocyte chemoattractant protein-1 (MCP-1)] is increased while the secretion of anti-inflammatory and insulin-sensitizing adiponectin is reduced (180). The formation of pro- and anti-inflammatory lipid mediators is also deregulated in obesity (181). In addition, deficiencies in IL-10 expression or IL-10 receptor signaling results in inflammatory diseases (182, 183). A recent study showed that T2D patients have decreased IL-10 function, through downstream signaling in the IL-10 pathway (184). Moreover, expression of IL-1RA is decreased in β cells from T2D patients, with an IL-1RA being a current FDA-approved therapeutic (185).

IMMUNE SUPPRESSION

Type II diabetes patients have an increased susceptibility to pathological infections. These patients also have some of the worst long-term morbidity and mortality. This is secondary to the inability to eradicate pathological infections. In sepsis, in addition to immune activation, a component of immune suppression concomitantly exists, which enables individuals to develop recurrent, secondary, and nosocomial infections. This leads to worse outcomes and increased long-term mortality (26). The combination of chronic immune suppression from T2D, combined with sepsis-induced immune suppression, leads to innate and adaptive immune system changes that the human body cannot overcome. As illustrated in **Figure 2**, both the innate and adaptive immune systems are affected in T2D and sepsis, altering homeostasis. It is not known how these aberrant pathways interact when they are superimposed. However, we do know that these superimposed pathways lead to worsened morbidity and mortality.

When looking at immune suppression in the innate immune system, there are several key pathways to mention. Neutrophils are essential for bacterial eradication. In T2D and sepsis, neutrophils display defects in chemotaxis and recruitment to sites of infection (186, 187). This leads to the reduced ability to eradicate bacteria (99). T2D-associated hyperglycemia also increases cytosolic calcium in neutrophils, which inhibits the synthesis of ATP leading to reduced chemotactic, phagocytic, and bactericidal activity. The production and release of essential effector molecules, such as ROS and cytokines, is significantly impaired leading to bacterial persistence and the development of infectious complications (133, 187, 188). In addition, T2D is associated with elevated FFAs from dysregulated carbohydrate metabolism, which cause EC dysfunction and pathological cytokine fluctuations (189). In T2D, the antioxidant systems and humoral immunity are also depressed. Furthermore, T2D predisposes patients to micro- and macrovascular comorbidities leading to environments susceptible to infections (190).

In addition to diminished innate function, adaptive immunity is similarly impaired. Splenocytes harvested from deceased sepsis patients demonstrate reduced numbers of $CD4^+$ and $CD8^+$ lymphocytes, due to substantial apoptosis (13). Apoptosis of lymphocytes and APCs (DCs, T cells, and B cells) is considered a hallmark of septic immune suppression (191, 192). Moreover, $CD4^+$ cell loss is associated with a reduced ability to mount immune responses to viral infections after septic insults (193). However, reduced lymphocyte numbers are not just reflective of the risk for viral reactivation following sepsis. Lymphopenia 4 days after the onset of sepsis is associated with the development of secondary infections and is predictive of long-term mortality at 1 year after sepsis (194).

Several studies have examined the link between increased infectious morbidity and T2D. It is hypothesized that T2D patients are predisposed to infection due to impaired neutrophil function, decreased adaptive immune response, and dysfunctional immune cell function through high serum levels of inflammatory mediators (195). The cellular alterations observed in T2D and sepsis combine to create a chronic state of immune suppression, characterized by recurrent, secondary, and nosocomial infectious complications (196). These infectious complications often result in hospital readmissions (197–199) and poor long-term survival (200). Compared to patients without sepsis, sepsis survivors require more antibiotics, have more ICU days, and consume more hospital resources (201). T2D patients are also associated with bacterial pathogens with increased antibiotics resistance, such as MRSA, *Pseudomonas*, and *Acinetobacter*, which are associated with ICU-related mortality (202).

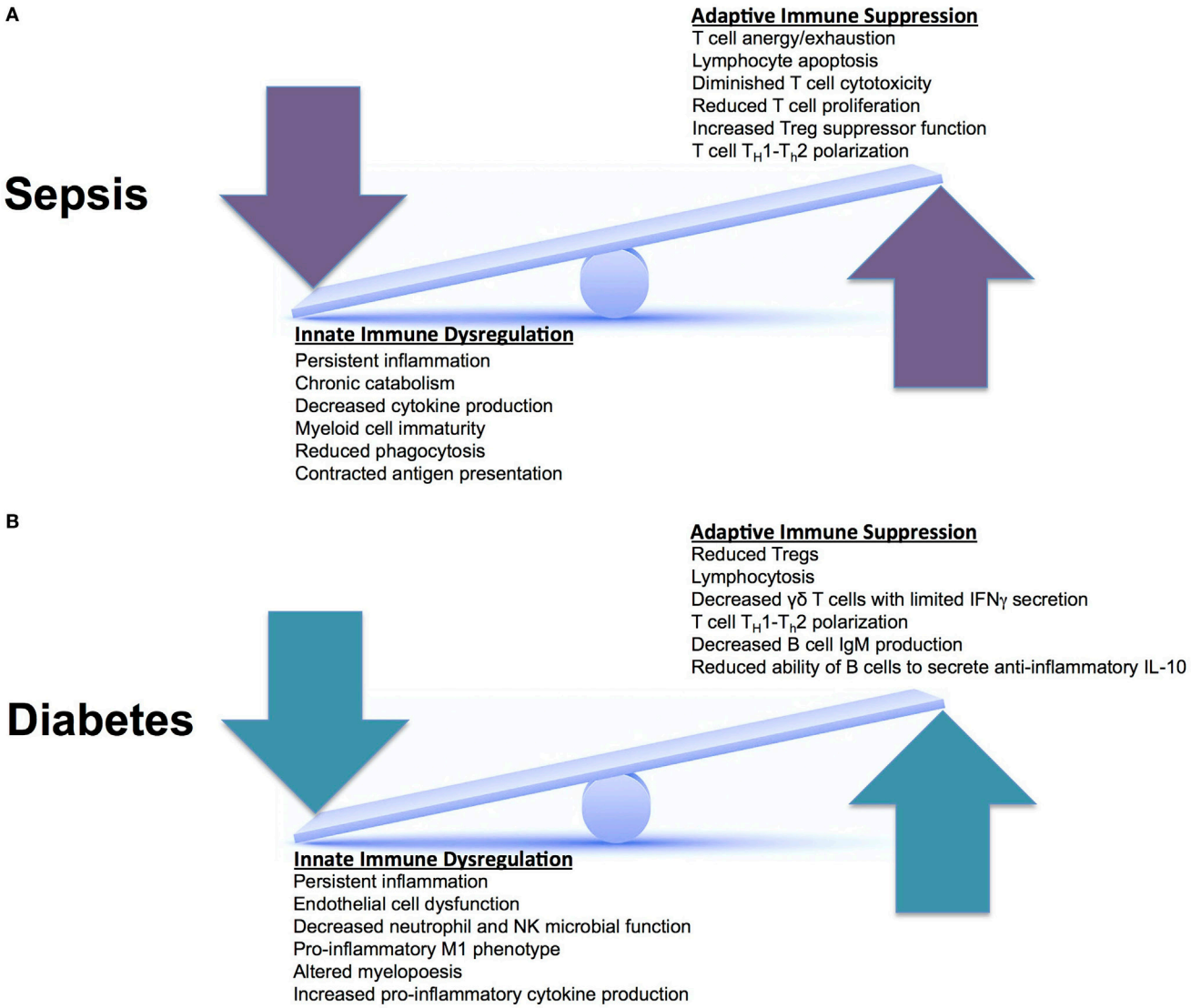

FIGURE 2 | Innate versus adaptive immune responses in sepsis and Type II diabetes. **(A)** During an acute episode of sepsis, the innate and adaptive immune systems are in a constant state of fluctuation. They respond to an invading pathogen and attempt to recover homeostasis after the pathogen is cleared. This continual seesaw effect is thought to drive ongoing inflammation, facilitate organ injury, and enable infectious complications. **(B)** In diabetes, the innate and adaptive immune systems experience chronic derangements secondary to chronic inflammation, also placing these systems in constant flux. When these two systems (sepsis and diabetes) are superimposed, patients have increased morbidity and mortality; however, we do not know why. There are unclear synergistic versus antagonistic changes occurring that leads to worsened immune system perturbations and the inability to return to homeostasis.

It is evident that sepsis induces a pathological state of immune suppression that prompts the development of secondary infections while still in the ICU setting (203). In addition, several reports demonstrate that sepsis survivors and T2D patients experience dramatically higher rates of subsequent infections long after the initial episode of sepsis has abated (204, 205). The increased hospital readmission rates due to infectious complications among T2D patients and sepsis survivors is a sign of ongoing immune suppression and dysregulation that if not corrected, diminishes life quality and durable survival. With the ever increasing, comorbidity challenged, elderly T2D population experiencing persistent inflammation, immune suppression, and immune senescence, the number of T2D sepsis survivors who develop subsequent infections is predicted to rise substantially in the next decades (200, 206).

IMMUNE-MODULATORY THERAPIES IN T2D

Below we will address immune modulators/modulatory pathways that deserve further consideration as disease-modifying therapeutics. These immune modulators, their proposed benefits, and some possible combinations are also listed in **Table 1**.

TABLE 1 | Immune modulators.

Immune modulators, diabetes	**IL-1 inhibition**	**TNF inhibition**	**NF-κβ inhibition**	**Diacerin**	**MCP-1 antagonism**	**IL-6 inhibition**	**Sirtuins augmentation**	**PPAR-γ agonists**
Proposed benefit	↓ acute phase inflammation	↓ risk of developing T2	↓ release of TNF-α, IL-1B, IL-8, and MCP-1	↓ concentrations of TNF-α and IL-1B	↓ monocyte/macrophage migration/infiltration	↓ inflammation	↑ insulin secretion	↓ insulin resistance
	↓ pancreatic β-cell apoptosis		↓ hemoglobin A1c	↑ insulin secretion	↓ insulin resistance		↑ insulin sensitivity	↓ hemoglobin A1c
	↑ insulin secretion		↓ insulin clearane	↑ metabolic control				↓ macrophage concentration
Potential cells affected	**T cells, Lymphocytes**	**Neutrophils, macrophages, endothelial cells**	**T cells, lymphocytes**	**Neutrophils, macrophages**	**Monocytes, Macrophages**	**T cells, monocytes, neutrophils, lymphocytes**	**T cells, monocytes, neutrophils, lymphocytes**	**Macrophages**
Immune modulators, sepsis	**G-CSF**	**GM-CSF**	**IFNγ**	**PD-1 and PD-L1**				
Proposed benefit	↑ neutrophil and monocyte production and release	↑ neutrophil/monocyte production and function	↑ monocyte HLA-DR expression and function	↓ T cell exhaustion				
	↑ myelopoiesis and granulopoiesis	↑ monocyte/lymphocyte cytotoxicity	↓ infection and related complications	↑ lymphocyte proliferation				
		↑ T cell responses	↑ immunity against fungal infections	↑ neutrophil and monocyte cytotoxicity				
		↓ nosocomial infection acquisition		↑ opportunistic infections				
		↓ ventilator days						
Potential cells affected	**T cells, monocytes, neutophils, lymphocytes**	**T cells, monocytes, neutrophils, lymphocytes**	**T cells, monocytes, neutophils, lymphocytes**	**T cells, monocytes, neutrophils**				
Proposed combinations	**PD-1 and MCP-1**	**PD-L1 and diacerin**	**IFNγ and diacerin**					
Proposed benefit	↓ monocyte infiltration	↓ inflammation	↑ monocyte function					
	↑ lymphocyte proliferation	↑ neutrophil and monocyte cytotoxicity	↓ inflammation					
	↑ T cell function	↓ opportunistic infections	↓ fungal infections					
Potential cells affected	**Lymphocyte, T cells, monocytes**	**Neutrophils, monocytes**	**Monocytes**					

IL-1

IL-1 has long been given to patients after transplantation to enhance recovery (207). Since these patients developed symptoms and signs of a systematic inflammatory reaction during treatment, subsequent research focused on blocking IL-1 during sepsis by using anakinra, a naturally occurring IL-1RA. There have been multiple controlled trials of anakinra in human sepsis. In one placebo-controlled trial, there was a reduction in 28-day all-cause mortality, but the results did not reach statistical significance (208). Attention was then turned to focus on antagonism of IL-1 during noninfectious chronic inflammatory diseases, including myeloma and rheumatoid arthritis. IL-1β antagonism is now the standard of therapy in autoinflammatory diseases (209). T2D can be classified as an autoinflammatory disease, with the innate immune system inappropriately activated due to metabolic stress leading to a chronic inflammatory disease (210). IL-1 prevents insulin secretion while promoting pancreatic β-cell death *via* apoptosis (211). In patients with T2D, there is increased expression of IL-1 expression in pancreatic β-cells with subsequent reduction in IL-1RA (212). In these patients, anakinra lowered blood glucose levels and improves β cell secretory function and insulin sensitivity, as well as reducing evidence of systemic inflammation. Just as interesting, after withdrawal of anakinra treatment, improvement in insulin secretion lasted 39 weeks (212), suggesting that the therapeutic effect IL-1 antagonism is long-lasting, perhaps due to interruption of IL-1 autoinduction (213). However, anakinra has a short half-life requiring daily administration to maintain adequate suppression of IL-1β and often causes injection-site reactions, limiting its ability to serve as a long-term therapy option (214). Subsequent studies therefore focused on humanized monoclonal antibodies, Gevokizumab, Canakinumab, and LY2189102, against IL-1β. Gevokizumab improved glycemic control (potentially by restoring insulin production) and reduced inflammation in patients with T2D (210, 215). Given the half-life of around 3 weeks, preliminary studies indicated that monthly or longer administration might be possible. Clinical trial NCT00900146 utilized Canakinumab and showed a numerical reduction in hemoglobin A1C, with a trend toward improved insulin secretion rate (216). LY2189102 improved glycated hemoglobin levels and corrected fasting and postprandial glycemia, as compared to placebo (217). In addition, just like the studies on anakinra, treatment effects were noted to be long lasting, even after treatment was stopped. These trials show the potential therapeutic benefit of inhibiting the IL-1 pathway. To further support this, a current diabetic sulfonylurea medication Glibenclamide has actually been shown as a powerful inhibitor of IL-1β in islet cells (93).

TNF

The role of TNF in insulin resistance and T2D was first observed in 1993 (218). Numerous clinical trials have evaluated the benefits of TNF antagonism but have failed to demonstrate advantageous effects on glucose metabolism (219–221). However, these trials were underpowered, with limited patients over a short amount time, and did not account for inter-individual variations (genetic background, body weight, food intake, and exercise). Trials on TNF for other inflammatory diseases, including Crohn's disease, rheumatoid arthritis, and psoriasis, implicate TNF blockade in altering insulin sensitivity (222, 223). Large cohort studies in patients with rheumatoid arthritis and psoriasis showed that TNF inhibition is associated with a reduction in T2D rates (224, 225). Further clinical trials specifically focusing on T2D with prolonged antagonism of TNF will likely prove to be therapeutically beneficial.

Nuclear Factor-Kappa Beta (NF-κβ)

Lipopolysaccharides from bacterial cell walls and FFAs bind Fetuin-A to activate TLR2 and TLR4, leading to nuclear translocation of NF-κβ, which induces an inflammatory response (226, 227) through the release of TNF, IL-1β, IL-8, and MCP-1 (93). Since 2001, we have known that salsalate, a prodrug form of salicylic acid, can ameliorate T2D *via* inhibition of NF-κβ (228). Multiple trials have been completed to evaluate the potential therapeutic role of salsalate. An initial proof-of-concept study showed improvement in glycemia, decreased C-reactive protein levels, and higher adiponectin in plasma (229). Follow-up studies supported this initial observation (230, 231) with two multicenter, placebo-controlled studies, including clinical trial NCT00799643, showing that salsalate can decrease hemoglobin A1c and improve other markers of glycemic control (232, 233). However, salsalate also reduces the clearance of insulin, and thus lowers glucose concentrations through a non-inflammatory mechanism (229, 232). Metformin, a current widely accepted diabetic drug, has been shown to inhibit release of pro-inflammatory cytokines *via* IL-1β mechanisms by antagonizing NF-κβ in cells of the vascular wall as well as in macrophages (234). Metformin also inhibits the maturation of IL-1β in macrophages (235).

Diacerein

Diacerein is a common medication for inflammatory joint disease. It decreases concentrations of cytokines such as TNF and IL-1β (236, 237). Given the benefits seen in long-term use in inflammatory joint disease, it was hypothesized that diacerein could provide benefit in T2D. The randomized, double-blind placebo-controlled clinical trial NCT01298882 showed increased insulin production and improved glycemic control after treatment with diacerein in patients who were drug naïve. Further studies investigating the mechanism of action and the role it plays in immune dysfunction could reveal a therapeutic role for diacerein in T2D patients.

MCP-1 Antagonism

Monocyte chemoattractant protein-1 (or CCL2) is an essential chemokine active in the migration and infiltration of monocytes/macrophages (238). MCP-1 levels are increased in patients with T2D (239, 240). The gene expression of MCP-1 and its receptor CCR2 is elevated within visceral and subcutaneous adipose tissue of patients with obesity, as contrasted to lean controls (241). In addition, there is increased expression in omental fat with increased macrophage proliferation, when compared with the fat within the subcutaneous tissue (242). CCX140-B is a CCR2 antagonist. A pilot study in patients with T2D showed

that administration of CCX140-B decreased placebo-corrected glycated hemoglobin (93). Multiple studies have shown that downregulation of MCP-1 cooccurs with improvement in the symptoms of T2D. These results implicate a close relationship and support further studies that investigate the role of MCP-1 as a therapeutic target (240).

IL-6

IL-6 is a one of the main cytokines that is responsible for an inflammatory processes and responses. It is produced by macrophages, T cells, osteoblasts, kidney cells, muscle cells, and adipocytes (243). It has pleiotropic effect on glucose metabolism that is dependent on tissue type and the surrounding milieu. Increased levels of IL-6 are associated with obesity, T2D, and cardiovascular disease (244). Under specific conditions, IL-6 may either decrease or enhance insulin resistance, as well as improve glucagon-like peptide-1-mediated insulin section. In the paradigm of inflammation within obesity, it is hypothesized that IL-6 enhances the prevailing inflammation, thus precipitating insulin resistance and leading to further micro- and macrovascular complications (245).

Sirtuins

Sirtuins represent a class of NAD^+-dependent deacetylases that have a wide array of biological functions, one being to coordinate the body's reaction to caloric intake. Sirtuins are associated with metabolic disorders (246) and play a critical role in restoring homeostasis during stress responses (247). Emerging evidence supports that failure to maintain homeostasis during metabolism and bioenergy reprogramming result in acute and chronic inflammatory disease (247). In obesity, there is a decrease in sirtuin 1 levels and activity. This is likely secondary to upregulation of peroxisome proliferator-activated receptor gamma (PPAR-γ) genes that regulate fatty acid uptake and triglyceride synthesis in mature adipocytes (248). Increased sirtuin 1 expression and activation is associated with increased insulin secretion (249). There are substantial data to support that increased sirtuin 1 activity counters obesity, the metabolic syndrome, and T2D with or without obesity (247) making it a desirable therapeutic target.

Peroxisome Proliferator-Activated Receptor Gamma

A current antidiabetic therapeutic group, the thiazolidinediones which include rosiglitazone and pioglitazone, are PPAR-γ agonists. PPAR-γ is a type II nuclear receptor found mainly in macrophages, adipose tissue, and in the colon. These drugs effectively improve insulin resistance and reduce hemoglobin A1c though multiple mechanisms. One mechanism is that they can inhibit pro-inflammatory pathways leading to decreased macrophage concentration in adipose tissue (250, 251). The overall clinical effect from the improved insulin resistance and anti-inflammatory effects of these agents are not clearly defined; however, they reveal multiple mechanistic pathways to further evaluate (252).

IMMUNE-MODULATORY THERAPIES IN SEPSIS

Granulocyte Colony-Stimulating Factor (G-CSF) and GM-CSF

Granulocyte colony-stimulating factor stimulates the production of stem cells, progenitors, and granulocytes (253). Two randomized controlled human trials with recombinant G-CSF were performed to test its effect on neutrophil production, maturity, and overall function. Although an increase in blood leukocyte counts was observed, there was no improvement in 28-day patient mortality (254, 255). This makes one wonder if a longer study therapy or observation time would have changed the investigation outcomes. Given the ongoing and continuous alterations observed in granulocyte production, myelopoiesis, and neutrophil function in T2D and septic patients, prolonged G-CSF administration may be efficacious for improved immune surveillance, infection eradication, tissue regeneration, and survival during sepsis.

GM-CSF is an additional cytokine that enhances stem cells to differentiate into macrophages, monocytes, and neutrophils (256). In one study, ventilator-dependent septic patients who were prescribed GM-CSF during the immune suppressive phase had fewer days on the ventilator and within the ICU (257, 258). Recombinant GM-CSF treatment in septic children improved lymphocyte TNF production and significantly reduced hospital-associated infections (259). Further evidence for GM-CSF therapy from a meta-analysis of over 12 clinical studies using GM-CSF or G-CSF showed that treatment with either reduces infectious complications (260). In light of the fact that 70–80% patients who succumb to sepsis harbor persistent, chronic, ongoing, or secondary infections (13), G-CSF or GM-CSF combined with other immune regulators may bolster immune response and eradicate infection in septic T2D populations, potentially improving overall survival (254, 261).

Interferon Gamma

Interferon gamma is the sole protein within the family of type II interferons. Adequate IFNγ production and signaling is critical for appropriate immune targeting of microbial invaders. IFNγ is also a central inducer of macrophage activation, stimulating class I MHC expression (141). Patients with severe sepsis treated with recombinant IFNγ demonstrate reversal of sepsis-induced monocytic dysfunction, as well as having better overall survival (262). It is important to note that even though the patient population of most trials involving IFNγ were mixed cohorts of severe trauma patients, the largest study reports a clear decrease in mortality due to infections (263). A recent report on severe trauma patients shows that 42 of 63 genes were within the interferon pathway and differentially expressed in patients with uncomplicated versus complicated outcomes. Recombinant IFNγ treatment was also able to partially restore immune metabolic defects associated with immune paralysis in humans after sepsis, further suggesting that IFNγ therapy after sepsis may benefit a multitude of cellular immune functions (264). IFNγ is a very promising agent if it is targeted to specific patient populations, such as T2D patients who

have immune suppression, adaptive immune dysfunction, and chronic inflammation.

Programmed Cell Death Protein-1 and Ligand (PD-1 and PD-L1)

The PD-1 protein is expressed on myeloid lineage cells and most B- and T-lymphocytes, while its ligand (PD-L1) is expressed universally on monocytes, macrophages, epithelial cells, ECs, and DCs (265). Its ultimate effect is inhibitory, reducing $CD8^+$ T cells from proliferating or accumulating in lymphoid organs. PD-1 becomes upregulated during viral infections and cancer states and is associated with "T cell exhaustion from prolonged periods of exposure to self-antigens" (266). Subsequently, patients in septic shock exhibit higher levels of PD-1 and PD-L1 on their monocytes and T-lymphocytes (267). Anti-PD-1 and anti-PD-L1 have demonstrated encouraging results in clinical trials on human with viral infection or cancer (267). Studies have demonstrated that upregulation of granulocyte PD-L1 potentiates lymphocyte apoptosis *via* contact inhibition, which correlates with outcome (268). Given PD-1 and PD-1L's positive effect on adaptive immunity as well as tumor growth, they both could be used as biomarkers of immune suppression from sepsis. They are also potential targets to ameliorate adaptive immune dysfunction or increase overall survival in the long-term (9).

CONCLUSION

Type II diabetes is a disease of altered immunity that results in protracted inflammation, immune suppression, and significant infection morbidity. Clinically, it is obvious that patients with T2D are more susceptible to infections. In sepsis, despite the best goal-directed therapies that control hyperglycemia, administer antibiotics early, and prevent organ damage, T2D patients still have worse morbidity and mortality for reasons that are poorly understood. However, the link between the two appears to be the dysregulated immune pathways. We believe that immune-modulatory therapies that are strategically introduced and influence the interdigitating immune derangements between these two diseases have the potential to substantially improve the overall morbidity and mortality that these individuals experience.

AUTHOR CONTRIBUTIONS

All authors have made substantial contributions to all phases of manuscript development. MD and LF conceived the larger project. FF, KH, and PW helped conceive the focus of the paper. LF drafted the first version, figures, and table, with all authors providing substantive and editorial feedback on multiple revisions. We have all approved the final version prior to submission.

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The Folate Cycle As a Cause of Natural Killer Cell Dysfunction and Viral Etiology in Type 1 Diabetes

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The folate pathway is critical to proper cellular function and metabolism. It is responsible for multiple functions, including energy (ATP) production, methylation reactions for DNA and protein synthesis and the production of immunomodulatory molecules, inosine and adenosine. These play an important role in immune signaling and cytotoxicity. Herein, we hypothesize that defects in the folate pathway in genetically susceptible individuals could lead to immune dysfunction, permissive environments for chronic cyclical latent/lytic viral infection, and, ultimately, the development of unchecked autoimmune responses to infected tissue, in this case islet beta cells. In the context of type 1 diabetes (T1D), there has been a recent increase in newly diagnosed cases of T1D in the past 20 years that has exceeded previous epidemiological predictions with yet unidentified factor(s). This speaks to a potential environmental trigger that adversely affects immune responses. Most research into the immune dysfunction of T1D has focused on downstream adaptive responses of T and B cells neglecting the role of the upstream innate players such as natural killer (NK) cells. Constantly, surveilling the blood and tissues for pathogens, NK cells remove threats through direct cytotoxic responses and recruitment of adaptive responses using cytokines, such as IL-1β and IFN-γ. One long-standing hypothesis suggests viral infection as a potential trigger for the autoimmune response in T1D. Recent data suggest multiple viruses as potential causal agents. Intertwined with this is an observed reduced NK cell enumeration, cytotoxicity, and cytokine signaling in T1D patients. Many of the viruses implicated in T1D are chronic latent/lysogenic infections with demonstrated capacity to reduce NK cell response and number through mechanisms that resemble those of pregnancy tolerance. Defects in the folate pathway in T1D patients could result in decreased immune response to viral infection or viral reactivation. Dampened NK responses to infections result in improper signaling, improper antigen presentation, and amplified $CD8^+$ lymphocyte proliferation and cytotoxicity, a hallmark of beta cell infiltrates in patients with T1D onset. This would suggest a critical role for NK cells in T1D development linked to viral infection and the importance of the folate pathway in maintaining proper NK response.

Keywords: diabetes, natural killer cells, virus, folic acid, folate cycle

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THE CELLULAR FOLATE PATHWAY: ROLE IN ENERGY PRODUCTION, PROTEIN/DNA SYNTHESIS, AND IMMUNE FUNCTION

Figure 1 depicts the cellular folate pathway and the importance of the vitamin (B9) for the maintenance of cellular energy, DNA manufacture and repair, protein production, single-carbon transfers (methylation) and as a co-factor for numerous reactions. It is especially important for rapid cell growth and division, and critical to proper immune function. Particularly related to natural killer (NK) cell function, the production of inosine (**Figure 1A**, red box) is critical in maintaining NK cell cytotoxicity and proliferation in response to pathogens, while the production of adenosine will result in decreased NK cytotoxicity and proliferation, as well as generalized immunosuppression, as evidenced by adenosine deaminase (ADA) inhibitors, such as EHNA and drugs, such as Methotrexate. Literature demonstrates that increased activity of the enzymes associated with energy production (ATIC and GART), shown in **Figure 1A** (purple box) which (1) suppresses the function of ADA and the formation of inosine and hypoxanthine and (2) causes the internalization of the insulin receptor and an excess of intracellular ATP/adenosine (1).

In **Figure 1B**, important protein synthesis and methylation occurs, particularly the regeneration of the disease-associated homocysteine to methionine. Methionine is an essential amino acid critical to the formation of many biologically active proteins and the methylation of other critical products, such as S-adenosyl methionine, an important methyl donor to further methylation reactions. Defects in the folate pathway have been linked to numerous disease conditions, including fetal/infant neural tube defects, homocysteinemia, anemia, cognitive defects, cardiovascular disease, and cancer. It is clear that changes in the folate pathway could significantly impact functions throughout the whole body, including cell energy, protein/DNA synthesis, and, critically, immune function.

In 1992, the WHO recognized that there are over 2 billion people worldwide that suffer from micronutrient deficiencies, such as folate. In order to combat the increasing incidence of health conditions related to these deficiencies, 159 countries implemented a micronutrient/folic acid fortification plan in primarily, processed flour products. The flour fortification initiative became mandatory in these 159 countries in 1996 (**Figure 2**) and was fully implemented by 1998. Intriguingly, this is the same time period that the incidence of diabetes, both Type 1 and Type 2, began an upward trend that significantly exceeded epidemiological predictions (replotted from http://cdc.gov/diabetes/statistics). Superimposed on the graphic of **Figure 2** are results from the NHANES study examining serum folate levels in subjects over the age of 9 at specific time points within that same period (2). In the period from 1988 to 2000, there was a nearly 2.4-fold increase in the median serum folate of all subjects. Another study examining folate and unmetabolized folic acid (UMFA) in 2007–2008 NHANES collected serum samples found UMFA in all subjects with 33.2% of the subjects having

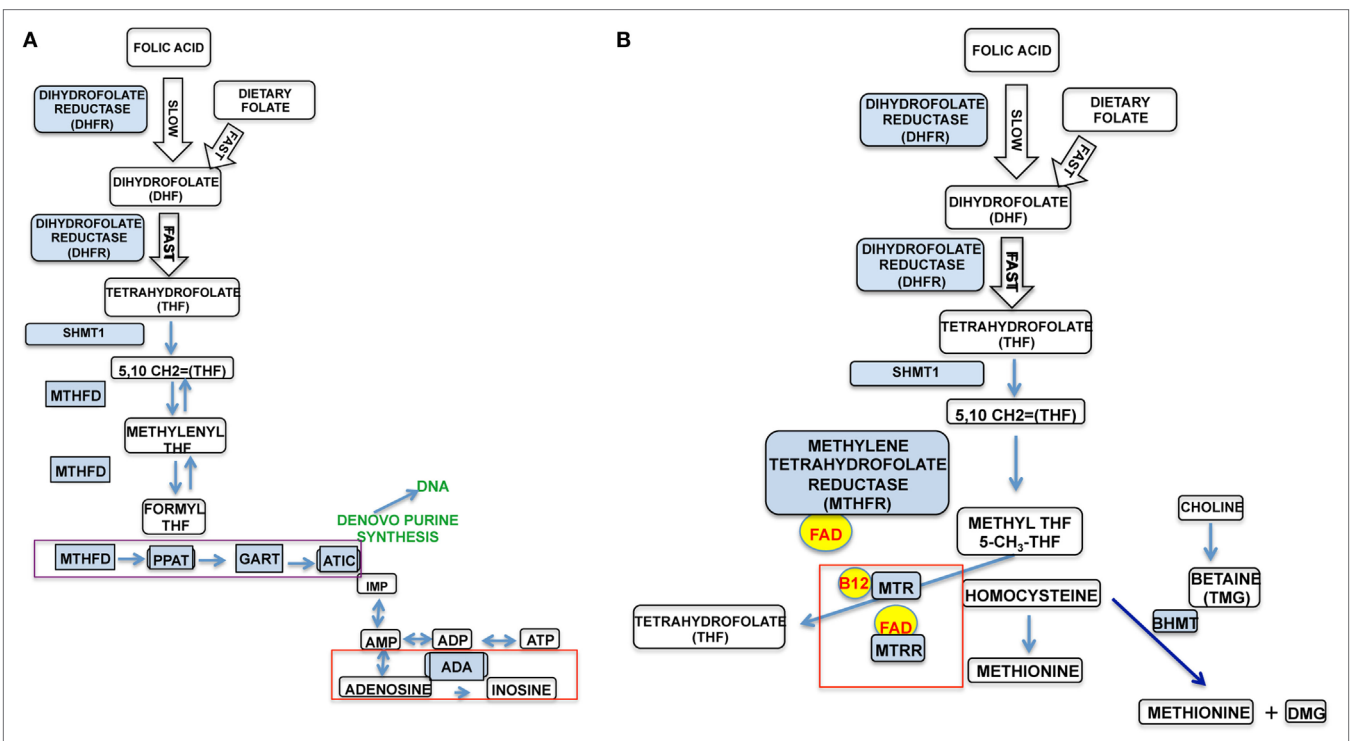

FIGURE 1 | The two major pathways in the cellular folate cycle. **(A)** It details the purinosome, DNA synthesis, cellular energy pathway (ATP synthesis), and immune system modulation. **(B)** It details amino acid synthesis (homocysteine–methionine).

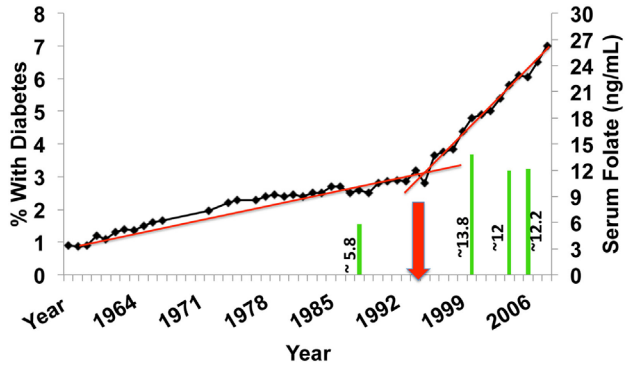

FIGURE 2 | U.S. incidence of diabetes over the past 50 years (type 1 diabetes and T2D) expressed as % of total population. The red arrow depicts the initiation point of mandatory fortification of flour products with folic acid. The green bars are measurements of serum folate levels from NHANES subjects of the corresponding years.

levels greater than 1 nmol/L (3). This increase in folate/folic acid levels supports the idea that micronutrient fortification may be unnecessary in developed countries and indicates a marked consumption of enriched flour products in the U.S. As well, the increase is superimposable on the rate of diabetes increase. Adding to this argument, in developing countries, comparable increases have been observed in autoimmune conditions in the past 10–15 years (4, 5). Given the importance of the folate cycle a broad, population-wide exposure to micronutrient fortification could result in sudden, dramatic increases in unexpected pathologies, such as type 1 diabetes (T1D). There is evidence that in developed countries, where micronutrient deficiencies are much less evident, people may be consuming an excess of folic acid. This excess consumption, in genetically susceptible individuals, might result in adverse health and dysfunction of the innate immune system. As an example, a recent paper demonstrated that excess B vitamin intake, the family containing folate (B9), was correlated with increased obesity and diabetes in the studied populations (6).

Folic acid is a synthetic that works in the same pathways as naturally occurring folates because it is a substrate for the enzyme dihydrofolate reductase (DHFR), segment 1 of **Figure 1**. It is first processed into dihydrofolate (DHF). This reaction is up to 1,300 times slower than the metabolism of non-synthetic folates in the liver of human subjects with an inherent fivefold variation in activity among subjects (7). This is the same pathway in which the immunosuppressant Methotrexate works, through competitive inhibition of DHFR. The inefficient reaction of folic acid and DHFR could potentially mimic this immunosuppressive effect. The inhibition of DHFR by Methotrexate results in dysfunction in the purinosome (**Figure 1A**, purple box) and the accumulation of adenosine (**Figure 1A**, red box), which is immunosuppressive. This is likely due to a slow production of THF, which is the primary substrate for the cell energy/immune modulatory side of the folate cycle. Thus, high levels of folic acid and variations in DHFR activity could result in high levels of UMFA that could adversely modulate NK cell and, furthermore, other immune cell activity. In support of this, malarial infection in mice fed a high folic acid (HFA) diet was associated with decreased NK cell activity, NK cell numbers, and survival; this was not observed in mice fed a control diet (8).

NK CELLS: THE FIRST-LINE DEFENSE AGAINST PATHOGENS

Natural killer cells respond to and directly kill pathogenic invaders. Their name derives from their capacity to cause cytotoxicity in cells that do not properly present the major histocompatibility complex class I (MHC-I), bound with cytoplasmic peptides, to the surveillance of the immune system; either lacking expression, expressing non-self peptides, or hyper-expressing peptides. NK cells lyse target cells directly unlike adaptive cells needing effector differentiation. Mature NK cells reside in the body prepared to respond to invaders. Cytokines, such as INFγ, granzyme, and perforin, are stored in preformed granules and rapidly released upon NK cell activation. This is different from cells of the adaptive system requiring post-activation gene transcription to achieve effector status.

In addition to direct killing, NK cells are involved in the strength and finely tuned control of adaptive immune responses. In recent studies, it has been shown that NK cells control both effector and suppressive activities of downstream responses, including those of activate or kill antigen-presenting cells and regulatory T cells, cytotoxic T lymphocytes (CTLs) T-helper (Th) cells and B-cells (9–13). This is done through direct killing or by signaling through cytokines, such as TNF-a, IFN-y, and others. The role of NK cells is the modulation of cytotoxic $CD8^+$ T lymphocyte response is to control aberrant/chronic inflammatory responses avoiding unchecked cell/tissue destruction. In the context of autoimmunity and T1D, defects in NK cell function and number could play a bigger role in the observed $CD8^+$ CTL infiltration of beta cells and the chronic destruction of self-tissue than originally thought. In a 2012 study by Ehlers et al., autoimmune diabetes was ameliorated by NK-cell-mediated destruction of $CD8^+$ CTLs in the NOD model (13). In our preliminary work, there are stark differences in NK cell populations in NOD mice compared to age/sex-matched control strains, such as C57Bl/6 and NOR mice; particularly, at the time prior to disease onset. In the Ehlers study, incubation of conventional NK cells with IL-18 resulted in an increase in a CD117 positive subset that had a direct lytic activity against the CTLs in PD-1/PD-L1-dependent manner suggesting the importance of NKs and, likely, specific subsets of NKs in adaptive immune responses in disease development. In a 2010 study by Olson et al., it was demonstrated that NK cells, reduced GVHD in an animal model by inhibiting alloreactive response by inducing apoptosis and reducing IFN gamma production by cytotoxic T-cells (14). Although NK subpopulations were not studied, we would propose that the NK cells responsible for this intricate control of the T-cell response are likely the mouse equivalent of the CD56 bright, $CD117^+$ population observed in humans.

Natural killer cells constantly circulate through the blood monitoring the classical MHC-I, or human leukocyte antigen A, B, and C (HLA-A, HLA-B, and HLA-C). If classical HLA

self-antigens are properly presented, the effectors will also encounter HLA-E resulting in the inhibition of the cytotoxic activity. The non-classical human leukocyte antigen HLA-E has a specialized role in cell recognition by NK cells. HLA-E is expressed on the cell surface after binding a restricted subset of peptides, primarily those derived from leading sequence signal peptides of HLA-A, -B, and -C, and, most importantly, the non-classical Class I, HLA-G. NK cells recognize the HLA-E peptide complex and produce an inhibitory effect on the cytotoxic activity of the effectors to prevent cell lysis.

Over the past 20 years, research into NK cells has greatly expanded and analyses that typically was limited to bulk NK cells identified as $CD3^-$, $CD56^+/CD16^-$ or $CD56^{dim}/CD16^+$, has expanded to include other important clusters of differentiation for subpopulation analysis, such as CD11b, CD27, CD57, CD7, CD69, and others. Importantly, subpopulations have been classified in terms of strong cytotoxic capabilities ($CD56^{dim}/CD16^+$, $CD11b^+$, $CD27^-$, and $CD57^+$) to the highly suppressive decidual phenotype ($CD56^+/CD16^-$, $CD27^-$, and $CD11b^-$) (15–19). This has expanded the understanding and research into the role of NK cells in numerous autoimmune pathologies, including T1D.

VIRUSES MODULATE THE INNATE IMMUNE SYSTEM THROUGH PREGNANCY TOLERANCE MECHANISMS

One of the primary roles of NK cells is combatting viral infection through direct killing of infected cells and recruitment of adaptive responses, including memory responses, to prevent reinfection with re-exposure. Many viral pathogens have developed the ability to disrupt Class I and Class II presentation in order to avoid recognition of their antigens by the immune system (20–22). This is likely the reason why higher species evolved the adaptive immune system, as the innate system was inadequate to defend against the varied number of pathogens encountered and their capability to mutate. It is also likely that some viruses survived by exploiting pathways that impart tolerance in the placenta during pregnancy. When the innate system is functioning properly, there is a balance of effector and suppressive subpopulations of NK cells that recognize invaders through unique receptor mechanisms with both activating and inhibitory pathways. The inhibitory pathways recognize MHC-I antigen expression and preferentially shut down the effector subpopulation. If the Class 1 molecules are not present or if non-self-antigens are presented, the activating pathway is initiated and the unrecognized entity is destroyed.

During pregnancy, the mother's innate immune response, particularly that of NK cells is dampened through placental hyperexpression of membrane-bound HLA-G and elevated plasma levels of the circulating soluble isoform (23–26). The soluble form recruits decidual NK cells to the decidua forming a immunoprotective layer around the fetus, while the membrane-bound isoform disables circulating NK effector cells by inducing apoptotic signaling and reducing cytotoxicity (24, 27). In addition, HLA-G serves to stabilize the membrane presentation of HLA–E to NK effectors another potent inhibitor of cytotoxicity. This induces cytokine and chemokine secretion conducive to tolerance induction in the placenta. HLA-G is constitutively expressed in several tissues within the adult body. Initially described in trophoblast cells of the placenta, it has subsequently been found in thymic epithelial cells, erythroblasts, corneal cells, mesenchymal stem cells, and most intriguingly, pancreatic islet beta cells (28–33). Through the flexibility of the effector and the suppressive subpopulations of NK cells, the innate immune system provides mechanisms for threat removal and self-protection, much like the two arms of the downstream adaptive immune responses. It is clear why such mechanisms, if assumed by pathogens, could be utilized to escape detection and allow for unchecked persistence in a host. This long-term escape of a virus from the innate immune response could lead to ineffective viral clearance and presence in tissues normally uninfected by pathogens. This, in turn, could result in an aggressive and unchecked adaptive immune response resulting in the destruction of self-tissue, characteristic of all autoimmune conditions. The constitutive expression of pregnancy/immune modulatory factors, such as HLA-G on some somatic cells would provide an immune-privileged site for viral evasion, even from the moment of fetal development. This hypothesis would also help to explain the disparity in the female-to-male ratio of autoimmune pathologies, as every month, when a woman menstruates, she is temporarily immunosuppressed in preparation for implantation. This has been demonstrated in studies of NK cells during both pregnancy and the menstrual cycle (34–36). The one unique exception to this rule in autoimmune pathologies is T1D, where the age of onset is earlier than other autoimmunities, frequently earlier than puberty, and the female-to-male ratio is approximately 1:1. Given the constitutive expression of HLA-G on the surface of beta cells, this is easily explained as beta cells could be an immune-privileged site for viral infection (31).

Some viruses (and other somatic invaders, such as specific cancers) implement pregnancy mechanisms to avoid detection by the innate immune system. Particular whole families of viruses have the ability to lie dormant for years, integrated into the host genome, in a latent ("lysogenic") phase of their life cycle. This behavior is characteristic of members of the Herpes and Coxsackie virus families. Members of these viral families utilize, much like cancer cells and trophoblast cells, the host's own signaling pathways to disable the innate immune system. Particularly, HLA-E and HLA-G are modulated by viruses, such as Epstein–Barr virus (EBV), cytomegalovirus (CMV), parvovirus-B19 (Parvo B19), herpes simplex virus type 1, and RABV26 (37–45). Many of these viruses force surface expression of HLA-E, typically occurring only with self-peptide recognition, strongly inhibiting the innate effector population (46–50). These findings have broad implications in clearance of viruses from host tissues and hint at a potential etiology for the development of many disease conditions. Viruses have been suggested as a causative agent in many autoimmune pathologies, including MS, T1D, Sjogren's syndrome, rheumatoid arthritis, Crohn's disease, and systemic lupus erythematosus (51–64).

Important to the theory of viral etiology and recent increases in prevalence of autoimmune pathologies, including T1D, is the passage of viruses through gametes. Originally thought to only occur with endogenous retroviral infections, there is growing

evidence that other viruses can be passed in gametes by means of episomal latency (65, 66). In episomal latency, viral genes are stabilized as both linear and lariat structures floating in the cytoplasm or the nucleus, without integrating into the genome. While this makes them more susceptible to viral defenses and cellular enzymes, there is the possibility that avoidance of entering the nucleus and integration with nuclear domain 10 thereby avoiding activation of interferon is beneficial to their survival and propagation. Coupled with our proposed environmental weakening of innate immune defenses, persistent viral infections cycling through latent and lytic phases and ineffectively cleared could progress to an aberrant immune response and development of autoimmune pathologies, including T1D.

VIRUSES, NK CELLS, AND T1D

A role for viruses as a cause for T1D has been controversial and hotly debated for decades but recent findings, the result of improved detection strategies and strong collaborative efforts, are increasing the likelihood that viruses have a greater role in the disease etiology than initially thought. Specific viruses, including but not limited to the Coxsackie family (B4, B6, and B1), the Herpes family (HHV-6, EBV, and CMV), and others (e.g., Parvo B19) have all been implicated in autoimmune disease development (45, 52, 55, 58, 60, 63, 64, 67, 68). Early discordant results were due to methodological issues (sample size, sampling frequency, assay sensitivity, and biology of viral infections) and have now been resolved through research networks and standardized protocols. Recent studies have linked genetic factors that influence T1D risk with viral infection (55, 60, 69–71). Others have demonstrated enteroviral infection can occur in beta cells resulting in cell death, in the case of acute lytic infections, and dysfunction, with more chronic infection. Acute and chronic viral responses in predisposed individuals could trigger chronic islet autoimmunity. Histological pancreatic specimens from a UK cohort of new-onset T1D patients were examined and found to have viral antigens and markers of inflammation in islets containing insulin-positive cells. This was found at a significantly higher frequency in this cohort compared to non-diabetic subjects of similar age and sex. Other clinical study data associated T1D with antibody responses to certain viral strains. One group recently utilized a high throughput immuno-proteomics methodology as a screening tool examining responses to seven viruses associated with T1D most frequently in the historical literature (72). Antibody responses to 646 viral antigens associated with the seven viruses were assessed in 42 long-standing patients relative to 42 sex and age-matched controls. Antibody response to EBV, a member of the Herpes family, was found to be significantly higher in case versus control subjects in both sex and age groups. There was also a trend toward earlier EBV infection in the case subjects. This platform is an example of improved detection methodologies that are helping to unravel the association of viruses with T1D and other autoimmune conditions. EBV is a virus that has been demonstrated in the literature to disable and suppress NK cells efficiently. This supports the idea that viruses could suppress innate response leading to unmodulated $CD8^+$ T-cell responses.

Improper viral clearance and manipulation of innate response had been suggested in the development of T1D in other papers (68). This group examined donor pancreata from 6 T1D patients and 26 controls, performing histopathological analysis of the tissues looking for viral infection and lymphocyte infiltration. In 3 of the 6 T1D patients, Coxsackie B4 infection was detected through positive staining of viral capsid protein (VP1) and then DNA extraction and sequencing of infected regions. In the same patients, the islet infiltrates comprised primarily NK cells. The islets cells in this group were intact and had positive staining for insulin. In the other 3 T1D patients, no virus was observed and infiltrates were NK free and represented mainly by $CD8^+$ T cells. In this group, the islets were undergoing degranulation and destruction/apoptosis. It is unlikely that at the time of death, all three patients had ongoing Coxsackie B4 infection or that the cause of death was fulminant Coxsackie infection. Rather, these data offer additional compelling evidence for inefficient clearance of an enterovirus highlighted by subclinical/latent infection and the presence of nearby NK cells. Because subpopulation analysis was not performed, it is unclear whether those NK cells had cytotoxic function or were regulatory. Viruses, much like tumors, can recruit regulatory NK cells that are much like T-regulatory cells and are immunosuppressive in their function. The observed NKs could quite possibly be from this unique population; they could also be dysfunctional NK effectors. This speaks to the study by Ehlers et al., where $CD117^+/CD56^{bright}$ NK cells, a known regulatory phenotype, destroyed $CD8^+$ CTLs associated with diabetes onset in NOD mice. As further evidence for viral manipulation of innate immune function in T1D, a 2009 paper by Tanaka et al. (73) described MHC Class 1 hyperexpression in islet cells also positive for VP1 associated with enterovirus infection and coexpression of CXCL10 and IFN gamma. This demonstrates a persistent battle between viral suppressive mechanisms and cellular chemokine/cytokine secretion recruiting immune response to the site of viral infection (73).

Despite these findings, the role of NK in T1D is not completely understood. This is likely because the majority of prior literature has examined bulk NKs ignoring multiple subsets with important and differing immunological functions (15, 18, 74). It is well established that NK dysfunction plays a role in the pathogensis of T1D. As an example, T1D patients and NOD mice have defective NKG2D signaling which is important in activation during viral response. This is present irrespective of disease duration. In addition, NK cells in T1D patients have been shown to have defective responses to IL-2 and IL-15, lipopolysaccharide, and reduced cytotoxicity and improper, often elevated, IFNγ secretion (75). At disease onset, it has been shown that the effector population, $CD56^{dim}CD16^{pos}$, is reduced (76–78), again suggesting manipulation by viral entities or an environmental factor adversely affecting the NK effector subpopulation.

In the NOD mouse, similar trends have been observed. In one study, NK infiltration into the pancreas of NOD mice was observed before T-cell auto-reactive infiltrates (74). These NKs displayed a more immature phenotype and reduced proliferative capacity, suggesting a dysfunction and turn over similarly observed by our group in long-standing and at-risk clinical patients. These could be equivalent to the suppressive subset observed in humans that

inhibit DCs and CD8+ CTLs. One subset of these cells produced IFN-γ spontaneously, suggesting an ongoing response, perhaps to a pathogen, such as a virus. This suggests the presence of some pathogen and an NK dysfunction/pathogen clearance problem that ultimately results in an amplified T-cell response due to aberrant IFN-γ, inability of regulatory NK cells to balance CD8+ CTL response and eventual autoimmunity leading to β-cell destruction.

In our preliminary work, the lymphocytes of 26 control, 12 long-standing T1D, and 7 recent T1D onset (<2 years) subjects were analyzed for NK cell frequency and subpopulation type. Total NK cells and NK effectors were compared among the three groups using non-parametric Kruskal–Wallis analysis followed by Dunns *post hoc* testing. Our preliminary data in T1D patients with long-standing disease provide evidence for a significant defect of both bulk NKs and the same NK effector phenotype ($CD3^-$, $CD14^-$, $CD19^-$, $CD66b^-$, $CD7^+$, $CD56^{dim}$, $CD16^+$, $CD27^-$, $CD11b^+$; expressed as % of total lymphocytes). Moreover, we find a similar defect in at-risk autoantibody positive subjects, suggesting diminished NK effector populations and activity before diabetes diagnosis that may be an important component of the disease pathogenesis (**Figure 3A**). Of note, this observation holds when long-standing T1D patients were compared to age/sex-matched control subjects using the non-parametric Mann–Whitney *U* test. (**Figure 3B**; $P = 0.0026$). Importantly, no significant correlation was found between subject age or sex and NK status.

Natural killer dysfunction in the literature is shown to lead to chronic, subclinical infection from many viruses that have high prevalence in the general population (46, 56, 79, 80). This is the result of an inefficient clearance that is further exacerbated by the ability of these viruses to manipulate NK cells. This combination of critical defects might lead to the hyper-inflammatory adaptive cell response observed in patients, which in those with HLA variants predisposing to T1D could lead to the triggering of islet autoimmune responses and the chronic destruction of pancreatic β-cells (81). Given the major role of NK cells in the innate immune system and their interplay with the adaptive system, modulating the activity and function of downstream role players, such as NK-T cells, $CD8^+$ cytotoxic lymphocytes, and T-regs, it is not an unreasonable proposition that NK cells may have a much bigger, upstream function in the etiology of T1D and many other autoimmune pathologies (13, 81). Recently, it has been demonstrated that NK cells have memory capabilities and with secondary exposures to pathogens increased IFN-γ secretion (82, 83). This suggests that the aberrant IFN-γ secretion observed in T1D and the hyper-inflammatory adaptive response drive by $CD8^+$ CTLs could be the result of a cyclical response to lytic and lysogenic viral phases. This would correlate with the relapsing and remitting cycles characteristic of multiple sclerosis as well. Defective NK function and receptor activation is critical to maintenance of innate/adaptive balance and proper immune function, as evidenced in a recent study by Cook et al. where NK dysregulation lead to amplified aberrant responses, cytokine storm, and death (81). This further supports the potential greater role for NK cells in T1D development.

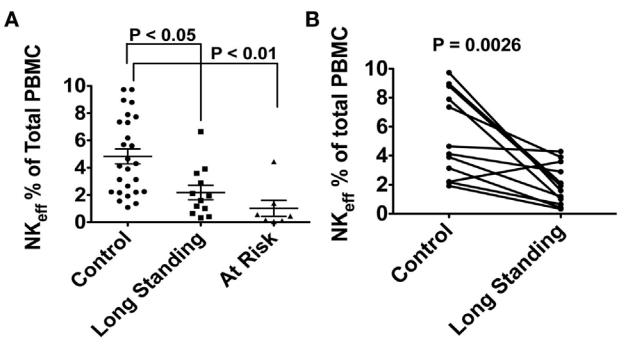

FIGURE 3 | **(A)** Differences in natural killer (NK) effector cell population expressed as % of total lymphocytes in 26 control subjects, 12 long-standing type 1 diabetes (T1D) patients and 7 multiple autoantibody positive at-risk subjects. **(B)** Randomized age/sex-matching sub-analysis between control subjects and long-standing T1D patients. No correlation was found between NK effector population and either age or sex. Peripheral blood samples from the subjects in this study were obtained after obtaining written informed consent. The study was reviewed and approved by the University of Miami Institutional review Board (protocol 1995-0119).

FOLIC ACID, NK VIRAL RESPONSE, AND T1D: TYING IT ALL TOGETHER

Normal response to a primary viral infection occurs in three distinct phases. The first is an early, non-specific response characterized by fever, inflammation, and the production of interferons (Type 1: alpha, beta, epsilon, kappa, and omega, produced by fibroblasts and monocytes; Type 2: gamma, NK cells, and Th cells). There is a third interferon type with a role in specific types of infections, but primarily 1 and 2 are critical in both regulating, signaling, and activating the viral response. NK cells play a major role in this early response actively lysing cells recognized as non-self through the production/secretion of granules containing granzyme B and perforin. In addition, NK cells are intertwined with the activation and regulation of dendritic cell (DCs) activity in a positive feedback loop. They can directly activate DCs, dependent on TNF alpha and IFN gamma secretion. In turn, activated DCs can then further stimulate NK activity by secreting IL-12, IL-15, and IL-18. NK cells also regulate DC antigen presentation by actively lysing immature DC cells while sparing mature/active DCs. NK effectors then work to directly lyse virally infected cells while DCs circulate to stimulate adaptive responses through either the T-cell receptor mediated MHC Class II antigen presentation pathway ($CD4^+$ Th cells) or MHC Class I antigen presentation pathway ($CD8^+$ CTL cells). In addition to controlling DCs and antigen presentation, the NK cells of the regulatory phenotype ($CD56^{bright}$, $CD117^+$) modulate $CD8^+$ CTL activity to balance responses through acquisition of a lytic phenotype and destruction of the $CD8^+$ CTLs. In the aforementioned work of Dotta et al., where NK cells were observed in T1D post-mortem pancreatic sections of islets with no evidence of T-cell infiltrate but the presence of VP1, this might be explained by viral manipulation of innate responses. Recruitment of these $CD56^{bright}$, $CD117^+$

cells of a regulatory phenotype with lytic capacity would prevent $CD8^+$ CTLs from tissue destruction through direct lysis of the infiltrating cells. This is further supported by the observation of islets containing $CD8^+$ CTL infiltrates with no VP1 and no NK cells. A defect in NK number and activity, particularly in this $CD56^{bright}$, $CD117^+$ population would also adversely affect antigen presentation as immature DCs would not be targeted as effectively. This could lead to improper or excessive presentation, a hallmark of T1D, and amplified $CD8^+$ CTL responses.

In our preliminary data (**Figures 3A,B**), we observed a drop in bulk NKs in long-standing and at-risk T1D subjects relative to controls and in the effector population responsible for viral clearance. This could have a twofold consequence. The decreased number of NK effector cells would lead to persistent viral infections and improper innate response. Over time, we hypothesize that the adaptive response is still activated through constant cycling of viral activation and latency. Adaptive $CD8^+$ CTLs are activated and respond to the sites of persistent infection. Given the drop in bulk NKs, we theorize there is also a shortage of the $CD56^{bright}$ regulatory cells that function to keep balance in this adaptive response. This likely leads to the infiltration and destruction of beta cells. The temporal progression to T1D onset is highly variable likely due to age of exposure to or reactivation of viruses, innate immune status at time of infection/reactivations, and exposure to environmental factors, in this case our proposed, folic acid. The folate pathway is instrumental in the production of molecules that fuel the activation and suppression of the immune response. Potential dysfunction in several segments of folic acid metabolism, detailed in the next paragraphs, could have direct impact on proper immune function and lead to T1D and T2D, if uncorrected.

Dysfunction in DHFR (**Figures 1A,B**) could result in dysfunction in the purinosome enzymes, resulting in increased intracellular adenosine and in turn an immunosupressive effect similar to that imparted by Methotrexate. Furthermore, this would cause a decreased level of THF, the substrate for both sides of the folate cycle, through the formation of 5,10 methyltetrahydrofolate (5,10-CH2 = THF); this would also result in defects in the homocysteine to methionine reaction and the associated enzymes. In critical support of this contention, metabolomic studies showed that plasma methionine was significantly lower in children at-risk for T1D compared to age-matched controls (84). A potential defect in the folate pathway is one explanation for this. In similar metabolomic studies examining differences between diabetic and non-diabetic NOD mice, pathway analysis indicated a deficiency of methionine in diseased animals, coupled with significant differences in several NK cell pathways, apoptosis pathways, purine and pyrimidine pathways and the DNA replication pathway, all important components of the folate pathway. This lack of THF would likely lead to increase in Betaine S-homocysteine methyltransferase (BHMT) activity, a redundant enzyme in the homocysteine to methionine reaction and a concomitant decrease in methionine synthase (MTR, MTRR) activity (**Figure 1B**, red box). A study that examined hypomethylation in diabetic rats relative to age/sex-matched controls reported significantly higher levels of BHMT activity and significantly lower methionine synthase activity, further suggesting folate pathway defects in diabetes (85).

When DHFR activity is suboptimal, it is possible that THF, normally utilized by the two major components (**Figures 1A,B**) of the folate pathway, is not produced properly. This would result in aberrant increased purinosome activity (PPAT, GART, and ATIC) and dysfunction in ADA, as in immunosuppression with Methotrexate, as shown in a 2006 paper (86). Increased activity within this complex has several effects, including dyslipidemia, internalization of the insulin receptor, and suppression of ADA (6). In normal metabolism, these are necessary biofeedback sensors. In a dysfunctional state, this results in an accumulation of intracellular ATP and adenosine, both adverse to proper cell function. Intriguingly, insulin receptor internalization is an established characteristic of both Type 1 and Type 2 diabetes. An excess of adenosine and a significantly lower level of ADA in lymphocytes, present with Methotrexate use, is a potent immune-suppressor of NK cell cytotoxicity and all immune cell function.

The production of inosine is stopped through the suppression of ADA, which also decreases NK cytotoxicity and proliferation. Intracellular ATP also increases. Studies have shown that high levels of intracellular ATP are a biofeedback signal driving apoptotic pathways (87, 88). This, would in turn, result in elevated serum extracellular ATP (eATP) released from cells undergoing apoptosis and through signaling pathways. eATP, at sufficiently high concentrations is a well-established signaling molecule that drives cascades of inflammation through cellular P2 receptors and is indicated in both acute and chronic/autoimmune inflammatory pathologies (89). Preliminary data from our own group show a significant increase in eATP at the onset of diabetes in NOD females relative to earlier time points in disease progression and to non-progressing NOD females. Increased intracellular ATP in NK cells leading to increased eATP could be one possible explanation for the observed deficit in NK number in T1D subjects relative to age- and sex-matched controls.

The upsurge in diabetes prevalence seen in **Figure 2** includes patients diagnosed with Type 2 DM. Although associative, this increase could also be related to consumption of folic acid, given the multiple cellular functions mediated by the folate cycle. This is supported by a recent paper that demonstrated that excess B vitamin intake, the family containing folate (B9), was correlated with increased obesity and diabetes in the studied populations, although not attributed to any particular B vitamin (6). As folic acid is critical in development and its deficiency is correlated with increased incidence of neural tube defects during fetal development, it is one of the primary micronutrients in baby formula and prenatal vitamins. Folic acid and its derivatives are co-factors for the majority of cellular single-carbon reactions, including DNA methylation. Disruption of the folate cycle could, therefore, result in epigenetic changes from conception, onward.

Our preliminary data suggest that folic acid negatively affects glucose metabolism and confers a phenotype of insulin resistance both *in vitro*, in muscle cell lines and in animals supplemented with increased doses of folic acid. L6 rat myoblasts were cultured and differentiated into an insulin-responsive myotubular phenotype for 7 days (with and without increasing concentrations of folic acid) and utilized for insulin-mediated glucose uptake assays. The results are shown in **Figure 4**. With the exception of cells exposed to 25 and 50 μm folic acid, the cells displayed

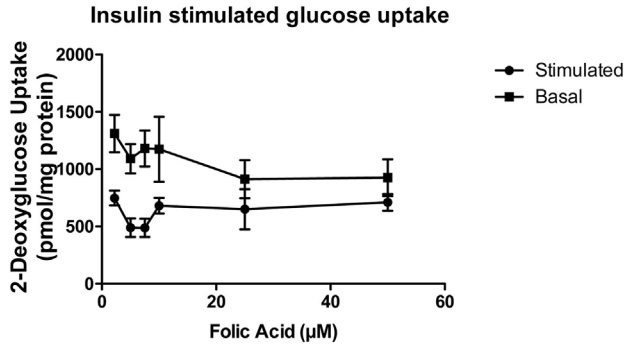

FIGURE 4 | Insulin-stimulated glucose uptake of L6 myotubular cells exposed to sequential dosing of folic acid ($n = 4$). Insulin-stimulated glucose uptake was significantly different from basal uptake in all doses of folic acid (lowest dose 2.2 μM present in media) except 25 and 50 μM. Differential uptake decreased in a dose-dependent fashion.

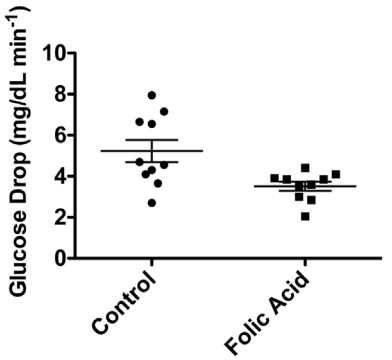

FIGURE 5 | Decrease in blood glucose (mg/dL/min) in C57BL/6 mice after IP injection of insulin. Ten mice were supplemented with high-dose folic acid in their drinking water for 12 weeks prior to the insulin tolerance test. Blood glucose decrease was significantly slower in the group fed high-dose folic acid ($P = 0.0125$, non-parametric Mann–Whitney test).

significant differences ($P < 0.05$) between basal and stimulated glucose uptake. Significance decreased in a dose-dependent fashion at concentrations greater than 7.5 μm FA (multiple paired *t*-test, $n = 4$ per group). This suggests that prolonged exposure of these muscle cells to elevated levels (>4× basal) of folic acid results in metabolic dysfunction indicative of an insulin resistant phenotype.

Female C57BL/6 mice ($n = 10$ per group) were randomly assigned to two groups, control or HFA. HFA was solubilized in drinking water with dosing based on the average daily water consumption described in the literature (90). The RDA for mouse intake of folic acid per the American Institute of Nutrition is 2 mg/kg. The average water intake in C57BL/6 is 6.67 mL. The HFA group received 20× the standard dose, or 40 mg/kg, per other published studies (91). The supplementation with HFA began at 5–6 weeks of age and studies were performed at 20 weeks of age. Over the first 20 min after administration of insulin (3 U/kg body weight), the control group had a significantly faster decrease, shown in **Figure 5**, in plasma glucose levels relative to the group receiving the HFA treatment (5.23 ± 1.72 versus 3.51 ± 0.69 mg/dL/min; $P = 0.0125$, two-tailed non-parametric Mann–Whitney test). These data suggest an insulin-resistant phenotype related to high folic acid (HFA) intake.

The cause of NK cell dysfunction and its increased prevalence among the population not only of the United States but also worldwide remains undiscovered. All of the findings detailed above suggest an environmental factor that still eludes researchers despite many suggestions over the years ranging from heavy metals to chemical toxins and including viruses, more recently. These are likely secondary to a primary causal agent that adversely affects cellular metabolic pathways, protein synthesis, proliferation, and immune function. It is our hypothesis that many of these pathologies can be tied to the synthetic form of vitamin B9, folic acid, as all of the critical cellular functions listed above are directly modulated by the cellular folate pathway.

The folate cycle is critical in the maintenance of numerous cellular pathways and is an important site of cellular one-carbon metabolism/methylation. Given the importance of methylation in phenotypic expression through mechanisms of epigenetic modification, the folate cycle may have a role in the etiology of multiple pathologies. As it broadly affects all cells in the body, it can adversely impact multiple systems by slowing cellular metabolism reactions, accumulating unwanted reaction byproducts and disrupting homeostasis. As our hypothesis suggests, this could result in immune dysfunction leading to viral reactivation, improper antigen presentation and cytokine production, and unmodulated/unbalanced adaptive-heavy, $CD8^+$ CTL and B-cell autoimmune responses in subjects with genetic predisposition. Therefore, folic acid could have a role in the development of T1D, along with many other autoimmune pathologies. The defects in insulin-mediated glucose metabolism that we have observed in our preliminary data suggest that folic acid could be a contributor to the recent upsurge of dyslipidemia, insulin resistance, obesity, and T2D. It is clear that there is strong need for further research into the folate cycle, its metabolites and the role these cellular pathways may have in the maintenance of immune function, metabolism, and general health status. Importantly, research into the innate system, critical in viral immune response, should be an area of greater focus as the evidence for a viral etiology is growing. The innate system evolutionarily precedes and is the upstream initiator of most adaptive responses including those that result in beta cell destruction in T1D. Improper innate function is likely the cause of downstream adaptive abnormalities that are the subject of the majority of immunological research in T1D.

If folic acid is indeed an environmental contributor to autoimmune and metabolic pathologies, as increasingly suggested, further research could tie this important cellular pathway to multiple disease etiologies and to conditions resulting from chronic innate immune deficiency such as cancer. It is encouraging to think that a simple dietary change may positively affect some of these conditions but the caveat is that anything that simply restores the innate effector function may result in strong NK-driven responses to viruses that lead to cytokine storm and further autoimmunity, if viruses are indeed a causal agent (81). This could be one possible explanation for the autoimmunity observed with cancer immunotherapies as enhanced responses to cancer could awaken

innate responses once the immunosuppressive strategies of tumors are removed with cell destruction (92). Ideally, therapies to treat autoimmune conditions, particularly T1D, should not be immunosuppressive as this could lead to viral spread and the development of long-term pathologies, such as cancer. Rather, increased research into anti-viral strategies and gradual restoration of the innate balance to prevent catastrophic inflammatory responses (cytokine storm) might have better long-term outcome than current clinical trials.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of HIPAA regulations from the University of Miami Leonard M Miller School of Medicine Institutional Review Board with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the University of Miami Leonard M Miller School of Medicine Institutional Review Board (protocol 1995-0119). For animal studies: this study was carried out in accordance with the recommendations of the Guidelines for the Care and Use of Laboratory Animals from the National Research Council Institute for Laboratory Animal Use and AAALAC. The protocol was approved by the University of Miami Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

CF and AB were responsible for the experimental design, data collection and analysis, literature review, writing, and editing of the manuscript.

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HYPOTHESIS AND THEORY
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The Four-Way Stop Sign: Viruses, 12-Lipoxygenase, Islets, and Natural Killer Cells in Type 1 Diabetes Progression

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Natural killer (NK) cells represent an important effector arm against viral infection, and mounting evidence suggests that viral infection plays a role in the development of type 1 diabetes (T1D) in at least a portion of patients. NK cells recognize their target cells through a delicate balance of inhibitory and stimulatory receptors on their surface. If unbalanced, NK cells have great potential to wreak havoc in the pancreas due to the beta cell expression of the as-yet-defined NKp46 ligand through interactions with the activating NKp46 receptor found on the surface of most NK cells. Blocking interactions between NKp46 and its ligand protects mice from STZ-induced diabetes, but differential expression non-diabetic and diabetic donor samples have not been tested. Additional studies have shown that peripheral blood NK cells from human T1D patients have altered phenotypes that reduce the lytic and functional ability of the NK cells. Investigations of humanT1D pancreas tissues have indicated that the presence of NK cells may be beneficial despite their infrequent detection. In non-obese diabetic (NOD) mice, we have noted that NK cells express high levels of the proinflammatory mediator 12/15-lipoxygenase (12/15-LO), and decreased levels of stimulatory receptors. Conversely, NK cells of 12/15-LO deficient NOD mice, which are protected from diabetes development, express significantly higher levels of stimulatory receptors. Furthermore, the human NK92 cell line expresses the ALOX12 protein [human 12-lipoxygenase (12-LO), related to mouse 12/15-LO] *via* Western blotting. Human 12-LO is upregulated in the pancreas of both T1D and T2D human donors with insulin-containing islets, showing a link between 12-LO expression and diabetes progression. Therefore, our hypothesis is that NK cells in those susceptible to developing T1D are unable to function properly during viral infections of pancreatic beta cells due to increased 12-LO expression and activation, which contributes to increased interferon-gamma production and an imbalance in activating and inhibitory NK cell receptors, and may contribute to downstream autoimmune T cell responses. The work presented here outlines evidence from our lab, as well as published literature, supporting our hypothesis, including novel data.

Keywords: coxsackievirus infections, islets, natural killer cells, 12-lipoxygenase, type 1 diabetes

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INTRODUCTION

Autoimmune destruction of the pancreatic beta cells leads to the development of Type 1 diabetes (T1D). The number of T1D cases is on the rise, with the relative risk for developing the disease ranging from 0.1% [no family history, protective human leukocyte antigen (HLA)] to up to 70% (monozygotic twin with susceptible HLA), and is dependent largely upon genetic susceptibility

(1). Importantly, the strongest genetic link to the development of T1D is the expression of certain HLA haplotypes. Class II HLA genes, especially DR3, DR4, and DQ8, are the strongest links; however, HLA Class I molecules also play a role in diabetes development (2, 3). Expression of both Class I and Class II molecules is the largest contributing factor in determining the immune response to a given pathogen, as peptides are processed and presented to T and natural killer (NK) cells *via* the proteins of the major histocompatibility complex (MHC) locus (4). Therefore, these molecules play a key role in directing immune responses, be they beneficial or detrimental. However, the genetic contributions to T1D development are unable to fully account for the increased rates, supporting the idea that environmental factors play a role in the development of T1D. Furthermore, susceptible siblings of T1D patients who are closely monitored frequently show signs of autoimmunity in the form of autoantibodies prior to metabolic dysfunction. Many believe, based on this evidence, that development of full-blown diabetes requires multiple insults to the system in order to manifest itself.

Patients with T1D currently depend upon treatment options that are limited to methods that replace the deficit in insulin production, either *via* injection or transplantation [reviewed in Ref. (5), in press]. While technological advances have helped improve these methods, they still do not provide a cure for the disease. Therefore, determining the mechanisms leading to immune damage of pancreatic beta (β) cells, and treatments to maintain β cell mass, are of the utmost importance.

Recently, perceptions of T1D development have evolved, with a greater attention being paid to islet inflammation as an important event propagating autoimmunity and further loss of β cell mass (6–8). Debates persist as to whether islets are independently inflamed prior to the autoimmune response or the autoimmune response brings about the islet inflammation. One of these recent studies described the incorrect processing of the insulin protein that led to the generation of abnormal peptides recognized by circulating $CD8^+$ T cells in T1D patients (8). This line of evidence certainly points to β cell defects contributing to diabetes pathogenesis; however, this study does not address what might cause β cells to produce this incorrectly processed protein. One study in non-obese diabetic (NOD) mice has suggested that incorrect protein processing in these mice causes an increase in endoplasmic reticulum (ER) stress, and results in the development of autoimmunity (9). Given the lack of complete concordance among monozygotic twins, many believe external environmental factors, such as viruses, strongly influence the development of islet inflammation leading to T1D. Trying to understand all of these data in concert brings researchers in the field to ponder the chicken and egg scenario. Are either islets or immune cells in susceptible individuals causing the initial insults that spark diabetes development, or does an environmental factor trigger the disease? Do we see signs of virus infections in patients with T1D because the infection is what precipitates diabetes development, or are patients with diabetes more susceptible to developing virus infections because of defects in their bodies' defense systems? With the data that are currently available, the order of events in the precipitation of T1D is unclear.

A NEW HYPOTHESIS

As we gather more evidence, it is becoming clear that we must look at the integrated physiology to fully understand the mechanism(s) of T1D development. Here, we outline an idea that incorporates early antiviral immune effectors, NK cells, with proinflammatory processes involving 12-lipoxygenase (12-LO) occurring in the pancreatic beta cells. We hypothesize that the activation of NK cell 12/15-LO (*Alox15,* in mice) or 12-LO (*ALOX12,* in humans) through environmental triggers, such as Coxsackievirus infection, contributes to T1D initiation by affecting the normal innate immune interplay between NK cells and islets, which primes downstream autoimmune responses leading to islet destruction. This may occur, in part, due to the effects of inflammation (including 12-LO) on the balance of NK cell receptor expression (10). Below, we will describe the evidence supporting this hypothesis, beginning with one of the suspected environmental triggers, enteroviruses.

DIRECT EVIDENCE FOR VIRUS INFECTIONS IN T1D

Over the past fifty years, there has been accumulating evidence linking viruses, and the patients' responses to these viruses, to the initiation of T1D. This idea that viruses contribute to the initiation and development of T1D was first introduced in the 1960s (11, 12). This is, of course, difficult to fully pinpoint, as the infection may occur long before disease onset, and scientists with access to human pancreas tissues are granted only a snapshot of the patient's final day as their window into the disease process. Additionally, as mentioned previously, it is unclear whether or not patients susceptible to developing T1D are also more susceptible to developing virus-mediated infections in the pancreatic islets, which might increase the viral signature in the islets of patients with T1D. Therefore, this might not be a causal relationship, but merely coincidental. Since we cannot directly test whether viruses initiate T1D in humans, researchers have used animal models to test this theory. NOD mouse models have been used to show that Coxsackievirus B1 and B4 (CVB1, CVB4) infection speeds diabetes pathogenesis (13), and is dependent upon host sensors of virus (14–16). Others have shown that these effects are highly age dependent, as infection at before 10 weeks of age can prevent diabetes development (17, 18). Additionally, studies of immunodeficient mice engrafted with human islets have shown that human islets can become infected with CVB4, which causes direct damage to the β cells, and results in diabetes. Gene expression profiles of these infected islets indicated significant increases in genes related to the Type 1 interferon (T1-IFN) pathway, as well as genes related to ER stress (19). While these data support the idea of viruses contributing to diabetes development, they do not answer the question about which occurs first: islet dysfunction or immune activation.

To address the role of virus infections in human T1D, groups such as Persistent Virus Infection in Diabetes Network and the Network for Pancreatic Organ Donors with Diabetes—Viral Working Group (nPOD-V) have approached the question with

great coordination across multiple platforms (20) assessing the same donor samples (PCR, immunohistochemistry, proteomics, and ISH). These team science efforts have yielded results estimating that Coxsackievirus infections might contribute to diabetes development in over 50% of cases (21, 22). While certainly not causal, pancreas tissues, and specifically β cells, from T1D donors have been found to express viral VP1 proteins more frequently than non-diabetic (ND) donors (23, 24). These studies continue to progress, generating a wealth of information from human donor samples.

Mechanistically, enteroviruses can infect *via* a fecal/oral route, thereby implicating intestinal involvement during the infection process. Mounting evidence has shown a role for the gut microbiome as a contributing factor in autoimmune diabetes development. Viruses and microbiota are known to interact with one another, and shape the response of both parties, which may influence the development of T1D (25). Recent human studies of closely matched control and T1D experimental groups demonstrate both increased inflammation in the duodenum of T1D patients (26), and direct detection of enteroviruses (27). In the first study, donors were tested for markers of inflammation using histological techniques and PCR array, indicating significant inflammatory processes in T1D donors, including increased macrophage numbers in the duodenum of T1D (26). In the second study, T1D donors were much more likely to have markers of enterovirus infection than control donors independent of HLA haplotypes, as tested by *in situ* hybridization and histological techniques (27). This work could not conclude whether T1D patients were more susceptible to the virus infections, or the infections are persistent. Coxsackievirus infection of β cells with strains B1 (28) and B4 (13) may occur *via* β cell expression of the Coxsackie Adenovirus Receptor (29, 30) following viral migration from the duodenum to the pancreas through the common bile duct or affiliated vessels (27). Pursuant to the role of islet inflammation following environmental insult, *in vitro* studies indicate that infection could lead to ER stress in β cells, contributing to islet dysfunction that activates the autoimmune response (9, 31). Alternatively, the infection could also directly activate immune responses that become uncontrolled due to inherent immune defects. Until imaging of live T1D patients affords the ability to detect virus infection in real-time, other experimental avenues must be explored, including the use of cultured islets and mouse models.

INDIRECT EVIDENCE FOR VIRUS INFECTIONS IN T1D

While viruses themselves may be difficult to detect in our snapshot views of human T1D, there is ample "circumstantial" evidence that exists in the form of immune cells and mediators. Both mouse models and organ donors with T1D have provided clear evidence that islet inflammation is a key hallmark of this disease. Immune cells infiltrate the islets, albeit at different intensities, in both species. Many patients show signs of adaptive immunity against the pancreatic islets in the form of autoantibodies and islet-specific T cell clones.

Beyond cellular responses, cytokines and chemokines also contribute to islet demise and can stem from both innate and adaptive responses. T1-IFNs have recently gained more respect as effectors in the development of T1D (see the review by Newby and Mathews in this issue). Indeed, virus infections are strong stimulators of T1-IFN production, which leads to a subsequent upregulation of MHC Class I expression, another hallmark of T1D (32).

Downstream of this response, numerous proinflammatory cytokines and chemokines have been detected in patients with diabetes (33, 34). One of these, IFN-gamma (IFN-γ), has been shown to play an important, albeit controversial, role in T1D pathogenesis (7, 35). While absence of the cytokine itself leads to delayed disease development (36), absence of the receptor protects against the development of insulitis (37). IFN-γ has many points at which it can act in the development of T1D, from altering MHC/HLA expression on involved cells to altering endothelial cell function and signaling to immune cells to activate cytotoxic effectors (37). Diminished IFN-γ responses can prevent the recruitment of insulitic T cells, as well as their ability to respond to antigens, which might prevent diabetes progression. However, increased IFN-γ production by $CD4^+$ T cells can actually contribute to the resolution of $CD8^+$ T cell responses (35). While it is appreciated that $CD4^+$ T cells contribute to the IFN-γ production during T1D pathogenesis, this does not exclude the idea that NK cells may be the first producers of IFN-γ present in the islets. Interestingly, IFN-γ is also frequently detected following virus infections, and is used by the immune system to combat viral replication. Given these data, it is unclear whether IFN-γ is serving in a proinflammatory capacity or an unsuccessful attempt at tolerance induction during the development of T1D (35).

NK CELLS AND THEIR ROLE IN T1D

Natural killer cells are large granular lymphocytes that are considered part of the innate immune system. While they do not react as quickly as neutrophils and macrophages against invading pathogens, they mount a response more quickly than do T cells from the adaptive arm of the immune system. NK cells are known as key players in fighting off both tumor cells and virus-infected cells. Despite their small number (only 5–10% of leukocytes in the spleen and 1–6% in peripheral blood) (38), NK cells are powerful cytolytic effectors. Upon stimulation by a variety of cytokines, including T1-IFNs and IL-12 (39), NK cells utilize several different mechanisms to lyse their targets: the combination of perforin and granzymes, signaling through death receptors (i.e., Fas/FasL), and antibody-dependent cellular cytotoxicity leading to either apoptosis or necrosis (40). NK cells can also produce potent cytokines, such as IFN-γ and TNF-α (41). IFN-γ production by NK cells might also serve an antigen presenting capacity (42–44), which, along with their potent cytokine production abilities, would give them the power to stimulate immune responses downstream of their own activation.

In order to recognize their targets, NK cells have developed an intricate system of check and balances. As NK cells are expected to determine aberrant "self" cells (tumors and virus-infected cells), they must be able to distinguish which cells are healthy, and

which are not. NK cells respond to virus infections in both mouse and man (4) through signaling mechanisms involving a delicate balance of inhibitory and stimulatory receptors expressed by NK cells. Normal expression of MHC Class I molecules (HLA in humans) send "self" signals to NK cells, inhibiting lytic responses (45). Virus infection can lead to the downregulation of MHC Class I molecules on the surface of infected cells. While this prevents $CD8^+$ T cells from responding to viral peptides, it also diminishes the inhibitory signal transmitted to NK cells [reviewed in Ref. (46)]. Subsequently, stimulatory signals to the NK cell are able to override inhibitory signals, leading to lysis of affected cells. In some instances, including during infection of pancreatic islets, viruses push the cellular machinery into overdrive and promote Type 1 IFN production (47), causing hyperexpression of the MHC Class I molecules (32). To circumvent this tactic, NK cells utilize receptors that recognize the upregulation of ligands for the natural cytotoxicity receptors, like NKp46, on the surface of infected cells (48, 49). Thus, NK cells can become "licensed to kill" through several mechanisms that allow them to detect alterations in MHC Class I molecules, as well as increased expression of stimulatory ligands, making them versatile effectors during virus infections (50).

Typically, T1D is thought to be dominated by autoimmune T cell responses; however, growing evidence suggests that NK cells are also involved (51). NK cells take up residence throughout the body (52), providing immune surveillance and protection against viruses wherever they enter the body. NK cells are plentiful in the intestines as compared to other organs (53), comprising 20–40% of Intestinal Epithelial Lymphocytes in healthy children (54), as compared to about 10% of the blood and spleen. This provides NK cells ample opportunity to respond to Coxsackievirus infections, as well as others (*Salmonella, Toxoplasma gondii,* other parasites, viruses, and bacteria) (55), transmitted *via* the fecal/oral route. Paired with the evidence of increased inflammation in duodenum of T1D patients, these data support our hypothesis. Furthermore, NK cells have been detected in the pancreas of both diabetic mice and humans. In mice, the cells appear shortly after macrophages (56). In human pancreatic samples, although NK cells are not frequently detected (57), they have been found in insulitic lesions, and show indications of having a protective effect (58). When one considers the frequency of NK cells in lymphocyte-rich organs (5–10% of leukocytes in the spleen), and also accounts for the number of cells required to define insulitis in humans [six or more $CD3^+$ cells in at least three islets (59)], then perhaps it is not surprising that NK cells are rarely detected in donor samples. Alternatively, it is possible that NK cells may prime the pancreatic environment for the entry of diabetogenic T cells, and subsequently depart. As we only have access to one time point for each human donor, we cannot currently distinguish these hypotheses. However, the use of mouse models made aid in this differentiation.

Natural killer cells themselves have recently been directly implicated in the development of T1D through additional expression quantitative trait loci analysis following genome-wide association studies, which further suggests that NK cells play a key role in T1D pathogenesis (60). Interestingly, this study indicates that NK cells may impact T1D development more than $CD8^+$ T cells. The carefully designed and executed study is limited to only 105 Japanese subjects, which might not apply to other ethnic backgrounds. However, it is comprehensive, and provides a solid approach for other ethnic backgrounds to be tested. Another data set investigating NK cell phenotypes from patients with T1D showed that NK cells from these patients express significantly reduced levels of activating receptors on their surface as compared to healthy controls (10).

It is unlikely that NK cells act independently in T1D development. Macrophages recognize environmental signals, and have been shown to enter pancreatic islets at 3–4 weeks of age in NOD mice (61, 62). Macrophage production of IL-12 and IL-18 can strongly activate NK cells, which are found in the pancreas of diabetes-prone NOD mice as early as 4 weeks of age (56, 63), to produce high levels of IFN-γ (64). Indeed, serum levels of both IL-12 and IL-18 are higher patients with T1D (65, 66), and IL-18 has been shown to participate in T1D pathogenesis of NOD mice (67). Despite a defect in IL-15 signaling in NOD mice (68), which affects NK cell development and function, others have shown in IL-15-deficient mice that increased IL-12 signaling may allow NK cells to overcome this deficit when faced with pathogenic stimuli (69, 70). The early appearance of NK cells in the pancreas may enable them to activate diabetogenic T cell responses.

Perhaps most importantly, NK cells can directly interact with pancreatic islets through expression of ligands for the NK activating receptors NKG2D and NKp46. Both of these receptors have been implicated in NK-mediated self-aggression in human NK cells that can be triggered by signaling through NKG2D and NKp46 (71). The NKG2D ligand, RAE-1 (72), is one of these ligands. Some speculate that NKG2D ligands cause down-modulation of the receptors, thereby making the NK cells less active (73); however, others have failed to validate this hypothesis (74). They instead showed that differential expression of NKG2D ligands did not hinder NK cytotoxicity through methodical assessment of receptor and ligand levels using genetic tools to dictate the alteration of expression.

Pancreatic islets also broadly express ligands for the NKp46 natural cytotoxicity receptor. NKp46 is a Type I transmembrane protein with two extracellular Ig-like domains followed by a short stalk region, a transmembrane domain containing a positively charged amino acid residue, and a short cytoplasmic tail (75, 76). However, the cellular ligands for NKp46 have not been identified or characterized. The only NKp46 ligands identified so far are the hemagglutinin of influenza virus and the hemagglutinin-neuraminidase of parainfluenza virus (48), suggesting a role for sugars in NKp46 ligand recognition. Studies of NKp46 ligands have utilized the NKp46 Fc chimeric protein in flow cytometry and histological techniques to examine expression over time in the islets of mice and humans (63). Functional studies from the same group showed that blockade of NKp46 receptor/ligand interactions protects against streptozotocin-induced diabetes (63). Although these studies have assessed expression over time, differential expression of the NKp46 ligands in human ND controls versus T1D donor samples has not been tested.

We recently studied ND, autoantibody positive (AAb+), and T1D donor samples from nPOD in order to determine whether there were expression differences in NKp46 ligands

using the NKp46 Fc chimeric protein. **Figure 1A** highlights representative images from donors with different health statuses. Islet images from two ND, three AAb+, and three T1D donors were analyzed by NIH Image J to quantify the density of NKp46 Fc staining (red) within the islet area as determined by glucagon staining (**Figure 1B**). Islets from T1D donors frequently retain alpha cell mass longer than insulin-positive beta cell mass (77, 78). Therefore, we used glucagon staining to more accurately determine the islet area for each donor in order to calculate the intensity of NKp46 Fc staining in islets. Interestingly, NKp46 Fc levels were significantly higher in the AAb+ donors as compared to ND donors. Although the difference was not statistically significant when comparing T1D and AAb+ donors, there was a trend toward higher expression in the AAb+ donors. These data suggest that NKp46 ligands are upregulated during the development of T1D, and diminish as the islet health and mass decrease over the course of the disease. While this may be the result of ongoing immune responses in these tissues, insulitis has only been detected in one each of the AAb+ and T1D donors. General characteristics of donors tested are listed in **Table 1**.

Mechanisms of NK cell action in diabetes are not well understood, and phenotypic differences in NK cells residing in different tissues may confound the results reported to date (40). Increased expression of stimulatory receptors on NK cells has been reported in both diabetic mice (79) and humans (80), while others maintain that a lack of NK cell activation contributes to

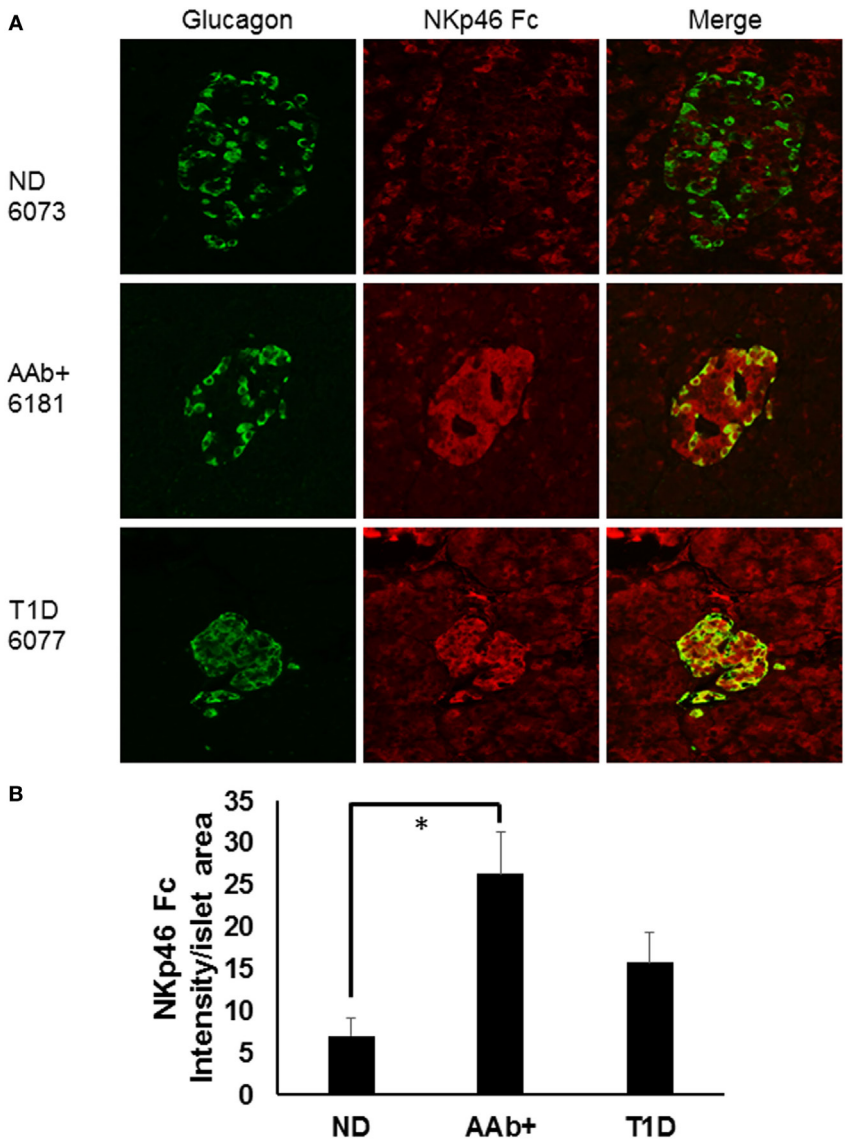

FIGURE 1 | Human islets express NKp46 *ligands*. **(A)** Representative images comparing donor pancreas tissues from Network for Pancreatic Organ Donors with Diabetes (nPOD) biorepository samples. Sections were stained with antibodies against glucagon (green) and the NKp46 Fc chimeric receptor (red). **(B)** Quantification of NKp46 Fc staining per islet area. Density of NKp46 Fc staining was determined for each islet area. Islets for each donor were assessed, and donors of the same group were averaged. $N = 2$ for non-diabetic (ND); $N = 3$ for both autoantibody positive (AAb+) and T1D. $^*p < 0.05$ by one-way ANOVA.

TABLE 1 | Donor profiles for NKp46 ligand staining.

nPOD case #	Donor type	Age	AAb+	Diabetes duration (years)	Insulitis
6048	ND	30	—	—	N
6073	ND	19.2	—	—	N
6151	AAb+	30	GADA	—	N
6181	AAb+	31.9	GADA	—	N
6197	AAb+	22	GADA, IA2A	—	Y
6077	T1D	32.9	mIAA	18	N
6083	T1D	15.2	mIAA	11	N
6088	T1D	31.2	mIAA, GADA, IA2A, ZnT8	5	Y

diabetes development (10, 68, 73, 81). This could be due in part to the type of analysis, as genomic studies do not always translate to protein expression. Several groups have studied NK cells in NOD mice. Both found that murine pancreatic NK cells exhibit a different phenotype from those found in the spleen and lymph nodes and have increased proliferative capacity (56, 82); however, there is not a consensus on levels of IFN-γ production, as one indicates lower levels *ex vivo*, but normal levels *in vivo* (82). Depletion of NK1.1$^+$ cells in NOD.NK1.1 congenic mice did not significantly affect disease onset, but the depletion protocol also removed NK/T cells (82). These tissue-specific phenotypic differences may also alter the detectability of the NK cells residing within the human pancreas using standard methodologies, but this has not yet been studied in humans. NOD mice have been shown to have a defect in IL-15 production, which contributes to NK cell dysfunction (68). IL-15 is required for NK cell maturation, and although NOD mice are not completely IL-15 deficient, they do show impaired NK cell development. Taken in concert with other systemic alterations in the NOD strain, the impact of this IL-15 defect has not been fully explored. As mentioned previously, NK cells functionally adjust to the absence of IL-15 by responding to IL-12 and IL-18 in order to produce IFN-γ (83), and by responding to pathogenic stimuli in the presence of IL-12 (69).

THE PROINFLAMMATORY MEDIATOR, 12-LO, IN T1D

12-Lipoxygenase [(12-LO) gene name *ALOX12S* in humans; 12/15-lipoxygenase (12/15-LO), gene name *Alox15* in mice] converts arachidonic acid to the proinflammatory 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) through a 12-S-hydroperoxy-eicosatetraenoic acid (12-HPETE) intermediate (84, 85). IL-12 signaling downstream of 12(S)-HETE production (86, 87) activates STAT4 [reviewed in Ref. (88)], contributing to additional inflammation. IL-12 signaling through STAT4 strongly activates NK cells and T cells and is also known to be a strong contributor to autoimmune conditions in general [reviewed in Refs. (89, 90)]. In addition to T1D, 12/15-LO has been implicated in many inflammatory processes, including cancers (91, 92), asthma (93), and Type 2 diabetes (94).

Several lines of evidence indicate a critical role for 12/15-LO in the pathogenesis of T1D. It has been shown that deletion of STAT4 signaling molecules, which are downstream of 12/15-LO activation, in NOD mice protects the NOD strain from developing T1D (95). Subsequently, we published that the NOD mouse line congenic for the global *Alox15* (NOD-*Alox15*^{null}) deletion is >98% protected from developing spontaneous

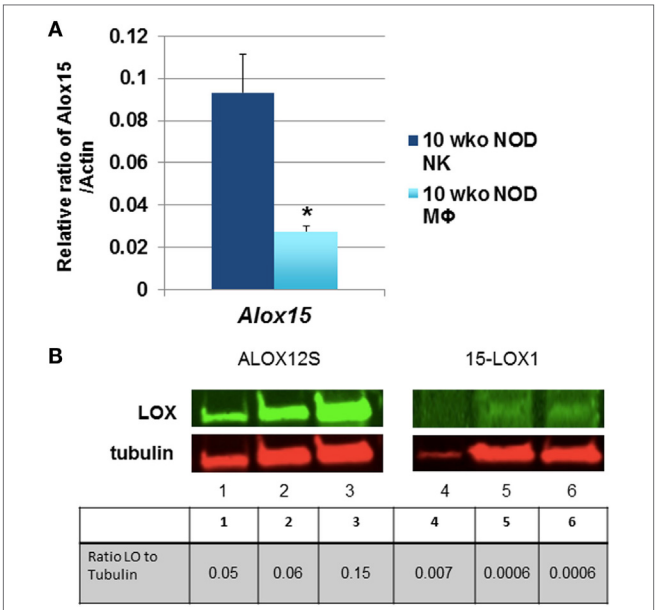

FIGURE 2 | Natural killer (NK) cells express 12/15-lipoxygenase (12/15-LO). **(A)** Murine non-obese diabetic (NOD) natural killer cells express Alox15. mRNA levels of the *Alox15* gene were tested in NK cells from NOD mice. These levels were compared to thioglycollate-induced peritoneal macrophages from 10-week-old NOD mice using the relative ratio of *Alox15/Actb*. *$p < 0.05$ using a two-tailed Student's *t*-test to compare NK vs. macrophages in age-matched NOD mice, $n = 6$ mice per group. **(B)** 12-Lipoxygenase (12-LO) protein expression in human NK92 cells. The human NK92 cell line was tested for protein expression of 12-LO by western blotting. ALOX12S expression was most abundant in the cell line, which is the most abundant form found in human islets. Lanes 1 and 4 are nuclear proteins from the two pooled NK92 samples; lanes 2 and 5 are cytoplasmic proteins from one NK92 sample; lanes 3 and 6 are cytoplasmic proteins from different NK92 samples.

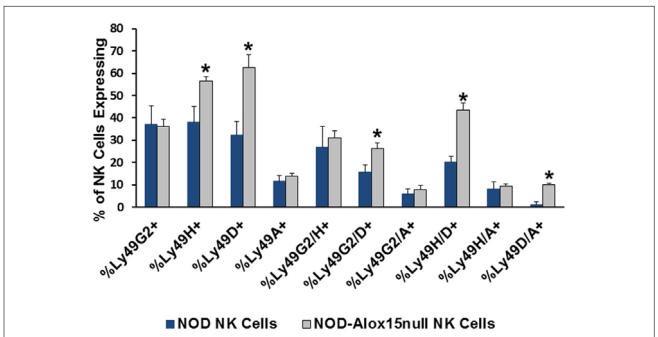

FIGURE 3 | Pancreatic lymph node natural killer (NK) cell expression of NK cell markers. Ly49 receptors determine which targets NK cells recognize and respond to during interactions with potential targets. Several of the expression patterns are altered in the absence of *Alox15,* with *Alox15*^{null} cells expressing higher proportions of the activating Ly49 receptors [*$p < 0.05$ using a two-tailed Student's *t*-test to compare receptor expression in non-obese diabetic (NOD) vs. NOD-*Alox15*^{null} NK cells for each receptor or receptor pair]. $n = 4$ mice/group.

T1D (61). This line boasts a narrow congenic region delineated through extensive microsatellite mapping, and shows significantly reduced disease incidence (~2%). To understand the origin of the protection seen in these mice, wild-type NOD mice were tested for their expression 12/15-LO in islets, macrophages, and lymphocytes. Islets and macrophages expressed the enzyme in appreciable amounts, while lymphocytes had either low or undetectable amounts (96). Additionally, *Alox15*^{null} mice have been shown to express decreased levels of IL-12 (96) and IL-18 (97), which are cytokines that contribute to NK cell IFN-γ production (83). Since publication, this strain has been shipped to additional vivaria and maintained this phenotype. Subsequent studies of human islets have also indicated 12-LO expression under inflamed conditions (98, 99), which feeds into the detrimental cycle of inflammation.

Natural killer cell expression of 12/15-LO has not been extensively studied, although historical data suggest that NK cells expressed a member of the lipoxygenase family (100). Using more modern information and methods, we discovered that both mouse and the human NK92 cell line express 12/15-LO and 12-LO, respectively (**Figure 2**). Surprisingly, this expression of 12/15-LO in freshly isolated NOD mouse NK cells is significantly higher (3.3-fold more) than that seen in thioglycollate-induced NOD macrophages (61, 96). Since NK cells are closely related to T cells, we expected that expression levels would be similar to those seen in T cells, which is almost undetectable (96). While

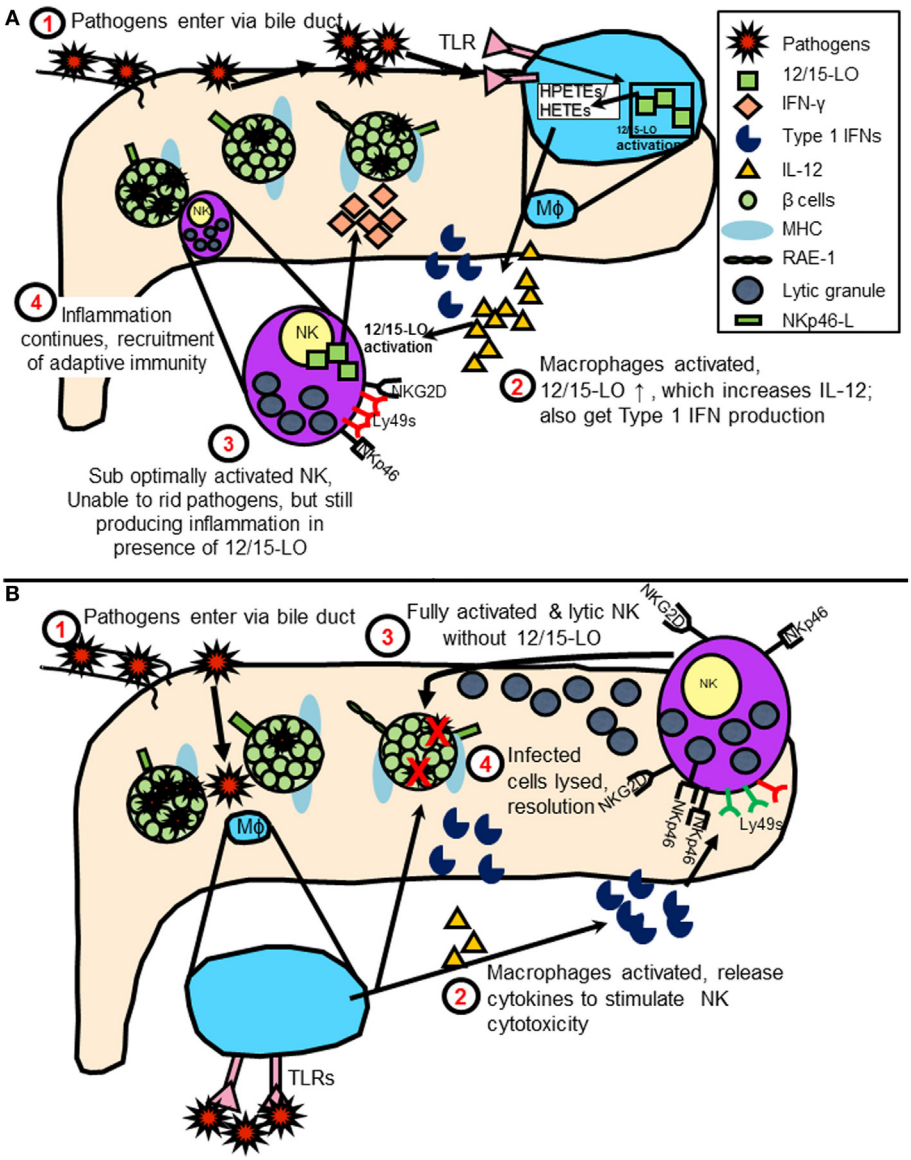

FIGURE 4 | **(A)** Model of natural killer (NK) cell activation leading to type 1 diabetes (T1D) in the presence of 12/15-lipoxygenase (12/15-LO) following virus infection. IL-12 production stimulates NK interferon-gamma (IFN-γ) response, feeds cycle of 12/15-LO activation and inflammation. **(B)** Model of NK cell activation in absence of 12/15-lipoxygenase following virus infection. NK cells respond to macrophage T1 IFN production following TLR signaling by using lytic mechanisms to rid virus-infected cells. Reduced inflammation prevents T1D development.

investigating the downstream effects of 12/15-LO expression in our NOD-*Alox15*^{null} mice, we found that the pancreatic lymph node NK cells of the NOD-*Alox15*^{null} mice had an increased percentage of NK cells expressing activating markers (**Figure 3**), which are similar phenotypically to peripheral blood NK cells from ND human controls (10). Taken together, it appears that activation of 12/15-LO, which leads to increased IL-12 levels (96), increases the inflammatory nature of the NK cells, presumably through the 12/15-LO pathway. We are currently testing whether NK cells are better able to resolve infections without contributing to the chronic inflammatory milieu in the absence of 12/15-LO.

Several additional lines of evidence suggest that NK cells are strongly influenced by 12/15-LO activity and that they have the capacity to play an important role in the development of diabetes. As mentioned above, activation of 12/15-LO leads to IL-12 production [reviewed in Ref. (94)], which activates the STAT4 signaling cascade that is required for NK cells to respond functionally (101–103), including production of IFN-γ (104, 105). This combination can exacerbate T1D, although IL-12 can also trigger the activation of different cytokine pathways in the absence of IFN-γ (106). The involvement of IL-12 in T1D pathogenesis is not without controversy. Deletion of the IL-12p40 subunit, which can heterodimerize with either IL-12p35 to form IL-12 or the p19 subunit to form IL-23, did not protect against T1D development (107). This may be due to the effect of inhibiting both IL-12 and IL-23 generation simultaneously, although IL-23 had not been discovered at the time these results were published. Subsequently, the same group published work indicating that administration of exogenous IL-12 exacerbated diabetes development (106). Importantly, when key molecules in the 12/15-LO pathway (i.e., 12/15-LO or STAT4), upstream of IL-12, are disrupted in NOD mice, diabetes is prevented (61, 95).

A NEW MODEL OF T1D DEVELOPMENT

By bringing NK cell expression of 12/15-LO into the equation of diabetes initiation following virus infection, one can envision a model in which duodenal NK cells encounter some sort of pathogen- or virus-infected cells, such as Coxsackievirus-infected cells. As the pathogen is transmitted through the bile duct or related vasculature on the way to the pancreas, NK cells and macrophages are alerted. Under normal conditions, NK cells and macrophages quickly dispense of the virus, removing only the infected cells. In a patient susceptible to developing diabetes, the interactions are altered, perhaps due to activation of 12/15-LO either by the virus directly or due to increased stress placed upon the beta cells upon infection (19). This leads to abnormal interactions between the innate immune cells with islets expressing NK cell ligands and prevents the resolution of the infection. 12/15-LO activation is known to feed into a vicious cycle of chronic inflammation, which in this instance, may be perpetuated by macrophages and NK cells (shown graphically in **Figure 4**). As mentioned earlier, in individuals with susceptible HLA haplotypes, or in mice (in was left out inadvertently) with susceptible MHC haplotypes, chronic inflammation signals the diabetogenic T cells to join the fight. This leads to significant islet destruction. Both HLA Class I and II molecules strongly influence the T cell responses in humans, as they dictate the ability of the T cells to recognize and react to autoantigens during the T cell development process, as well as in the periphery (108). This autoreactivity can be precipitated by stress placed on the islets, perhaps due to inflammatory processes. Such stress may lead to HLA Class II-mediated recognition of hybrid insulin (109) or posttranslationally modified (110) peptides by CD4$^+$ T cells, thereby breaking peripheral tolerance to neoantigens and furthering the disease progression.

CONCLUSION

We have hypothesized that activation of NK cell 12/15-LO (or 12-LO, *ALOX12,* in humans) contributes to T1D initiation by affecting the normal innate immune interplay between NK cells and islets, which primes downstream autoimmune responses leading to islet destruction.

At best, our current understanding of T1D initiation is murky. It is appreciated that there is a role for cells of the mucosal-associated lymphoid tissues, including NK cells, and it is quite likely that infectious initiation of T1D would occur through fecal-oral routes. However, T1D progression also requires the presence of macrophages, which produce 12/15-LO. Following the appearance of the macrophages in the pancreas of NOD mice, NK cells, which also produce 12/15-LO, are found. This process in humans has not yet been delineated, and, therefore, it is unclear if NK cells are migrating from the gut to the pancreas following an infection, or if they are recruited by other means. Macrophages have both the ability to respond to virus infections through TLR signaling, as well as activate NK cells through IL-12 and Type 1 IFN production. It is known that IL-12 production in macrophages is increased following 12/15-LO activation and that IL-12 signaling can feedback into the 12/15-LO signaling cascade (84). NK cell 12/15-LO is then a target for activation following IL-12 stimulation. IL-12, in concert with IL-18, is also known to drive IFN-γ production by NK cells (83). Both IL-12 and IL-18 are increased in mouse models of T1D (67, 96), as well as in patients with T1D (65, 66), and both are increased in the presence of 12/15-LO (96, 97). NK cell-derived IFN-γ could aid in expanding the effector T cell population (111). Conversely, in the absence of 12/15-LO, normal IL-12 levels [as generated through TLR signaling (112)] and Type 1 IFNs from activated macrophages might play a stronger role in influencing pancreatic NK cell function, leading to upregulation of activating receptors, optimal cytotoxic activation, and clearance of viral pathogens with minimal residual inflammation.

Moving forward, it is important to understand mechanisms by which environmental factors might spark the activation of 12-LO in NK cells, macrophages, and islets, leading to the development of T1D. While some pieces of this puzzle remain to be placed, there is striking evidence that our hypothesis and model is possible. Many of the remaining questions can be answered in part by the use of novel global and conditional knock-outs of 12/15-LO on the NOD background in experiments with Coxsackievirus infections. By understanding the order in which these events occur, we will be better able to design selective therapies that might prevent the disease development and progression without resorting to global immunosuppression.

METHODS

Mice

Female NOD/ShiLtJ (NOD) mice were ordered from Jackson Laboratory (Bar Harbor, ME, USA); global NOD-*Alox15*^{null} mice (bred on-site at EVMS) were housed in SPF conditions and treated in accordance with the AAALAC and IACUC guidelines at the Eastern Virginia Medical Center. Mice were euthanized by asphyxiation with CO_2. Blood glucose levels were assessed following euthanasia at 4 and 10 weeks of age. Spleens, lymph nodes, and islets were removed.

Cell Isolations

Natural killer cells were isolated from spleen using cell isolation kits from Stem Cell Technologies (Vancouver, BC, Canada) per the manufacturer's instructions. Purity of the isolated populations was assessed by flow cytometry (see below) after staining with antibodies against cell surface markers including anti-CD3, anti-CD19, anti-NKp46, and anti-CD11b. Cells were generally 85–90% pure.

Flow Cytometry

Cells isolated from the pancreatic draining lymph nodes of NOD and NOD-*Alox15*^{null} mice at 10 weeks of age were stained with antibodies against cell surface markers for T (CD3), B (CD19), and NK cells (NKp46, Ly49A, Ly49G2, Ly49D, Ly49H). Gates were determined by using fluorescence minus one controls. All antibodies were purchased from Biolegend (San Diego, CA, USA).

qRT-PCR

mRNA was isolated from indicated cells and tissues using the RNeasy Kit from QIAgen (Germantown, MD, USA), and used to generate cDNA for use in qRT-PCR assays as described (96). 12/15-LO expression in mouse cells was assessed by a SYBR green protocol, and compared to a newly available *Alox15* Taqman probe (Thermofisher Scientific, Waltham, MA, USA). Expression was tested in two independent experiments using five mice per group for each experiment.

Western Blotting

Cell lysates from the NK92 human NK cell line was used as a source of proteins to measure level human 12-LO levels using the Odyssey LI-COR system (Lincoln, NE, USA) as previously described (61). Nuclear and cytosolic proteins were fractionated and tested separately. Duplicate samples were stained with antibodies recognizing tubulin, ALOX12S, and ALOX15-1.

Immunofluorescence

Formalin-fixed, paraffin-embedded tissues sections from human pancreas tissues (obtained through nPOD) were stained as described with antibodies to glucagon (DAKO, Copenhagen, Denmark), and the NKp46 Ligand (using the NKp46 Fc chimera, R&D Systems, Minneapolis, MN, USA) as described previously (48), with a modification using tyramide amplification (Perkin Elmer, Waltham, MA, USA) to amplify NKp46 Fc staining. Islet area was determined, and the intensity of NKp46 Fc staining within the islet area was calculated using NIH Image J. Data are expressed as density of NKp46 Fc staining per islet area for ND, autoantibody positive, and T1D donor ($n = 3$ donors per group), which is calculated with the following equation: [Integrated density − (area of selected cell × mean fluorescence of background readings)]/total islet area = average fluorescence per islet.

Statistical Procedures

Statistically significant differences were determined by the use of Student's *t*-test where appropriate, or ANOVA followed by *post hoc* testing. Significant differences in all cases were determined by $p < 0.05$.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "Principles of laboratory animal care" (NIH publication no. 85–23), AAALAC, and IACUC guidelines at the Eastern Virginia Medical Center. The protocol was approved by the IACUC at Eastern Virginia Medical Center. The studies of the nPOD human donor tissues were considered to be exempt and deemed "Non-Human Subjects Research" by the Eastern Virginia Medical School Institutional Review Board due to the nature of the donors. All donors are deceased and de-identified.

AUTHOR CONTRIBUTIONS

MM conceived the hypothesis outlined. MS and MM wrote the manuscript. MM, LG, and MS contributed to experimental design and data analysis. MS and LG critically reviewed the manuscript prior to submission.

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HYPOTHESIS AND THEORY
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Environmental Factors Contribute to β Cell Endoplasmic Reticulum Stress and Neo-Antigen Formation in Type 1 Diabetes

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Type 1 diabetes (T1D) is an autoimmune disease in which immune-mediated targeting and destruction of insulin-producing pancreatic islet β cells leads to chronic hyperglycemia. There are many β cell proteins that are targeted by autoreactive T cells in their native state. However, recent studies have demonstrated that many β cell proteins are recognized as neo-antigens following posttranslational modification (PTM). Although modified neo-antigens are well-established targets of pathology in other autoimmune diseases, the effects of neo-antigens in T1D progression and the mechanisms by which they are generated are not well understood. We have demonstrated that PTM occurs during endoplasmic reticulum (ER) stress, a process to which β cells are uniquely susceptible due to the high rate of insulin production in response to dynamic glucose sensing. In the context of genetic susceptibility to autoimmunity, presentation of these modified neo-antigens may activate autoreactive T cells and cause pathology. However, inherent β cell ER stress and protein PTM do not cause T1D in every genetically susceptible individual, suggesting the contribution of additional factors. Indeed, many environmental factors, such as viral infection, chemicals, or inflammatory cytokines, are associated with T1D onset, but the mechanisms by which these factors lead to disease onset remain unknown. Since these environmental factors also cause ER stress, exposure to these factors may enhance production of neo-antigens, therefore boosting β cell recognition by autoreactive T cells and exacerbating T1D pathogenesis. Therefore, the combined effects of physiological ER stress and the stress that is induced by environmental factors may lead to breaks in peripheral tolerance, contribute to antigen spread, and hasten disease onset. This Hypothesis and Theory article summarizes what is currently known about ER stress and protein PTM in autoimmune diseases including T1D and proposes a role for environmental factors in breaking immune tolerance to β cell antigens through neo-antigen formation.

Keywords: type 1 diabetes, β cell, environmental factors, endoplasmic reticulum stress, posttranslation modification, neo-antigen, autoimmunity

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INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease in which insulin-producing pancreatic islet β cells are targeted and destroyed by autoreactive immune cells. Autoimmune recognition of β cell antigens leads to decreased β cell mass and to the subsequent decline of insulin-mediated regulation of glucose levels in the blood. Eventually, too few β cells remain to meet the demand for

insulin to maintain normal blood glucose levels. This insufficient insulin secretion leads to chronic hyperglycemia and T1D.

Type 1 diabetes is strongly associated with a genetic predisposition to autoimmunity that is conferred by single-nucleotide polymorphisms (SNPs) and gene variants found at many genetic loci. In particular, SNPs and variants in genes associated with both the innate and adaptive branches of the immune system cause failures of central and peripheral tolerance that eventually lead to autoimmune targeting of β cells. Of these loci, polymorphisms in the major histocompatibility complex (MHC) locus are most strongly associated with T1D onset (1–3). MHC proteins are crucial to central tolerance, because the antigens they present during T cell development in the thymus determine which T cells survive selection. This process directly shapes the mature adaptive immune repertoire. Strongly autoreactive T cells should be deleted upon encountering self-antigen presented by MHC during selection (4), but in individuals expressing MHC polymorphisms associated with autoimmunity, autoreactive T cells successfully mature and exit the thymus (5, 6). If peripheral tolerance mechanisms also fail, these autoreactive T cells become activated when they encounter β cell antigens in pancreatic lymph nodes. This autoimmune response destroys pancreatic β cells and ultimately causes T1D.

To better understand the processes by which the autoimmune response leads to T1D, and to identify the β cell proteins that are targeted by autoreactive T cells, researchers have studied the non-obese diabetic (NOD) mouse. These mice develop a spontaneous autoimmune diabetes that is similar in many ways to the human disease. These similarities include genetic susceptibility at the MHC locus and other immune-related loci, intra-islet infiltration of autoreactive immune cells as disease progresses, and ultimate β cell destruction (7–9). The β cell autoantigens identified using this murine model include preproinsulin (10), glutamic acid decarboxylase 65 (GAD65) (11), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (12), chromogranin A (CHgA) (13), islet amyloid polypeptide (14), zinc transporter 8 (15), and 78 kDa glucose-regulated protein (GRP78) (16) (**Table 1**). Subsequent studies confirmed the relevance of these autoantigens to human T1D (17–23) (**Table 1**). In addition, several additional autoantigens have been identified in humans but not yet confirmed in NOD mice, including tyrosine phosphatase-like insulinoma antigen 2 and IA-2β (also known as phosphatase homolog of granules from rat insulinomas) (24, 25), and islet cell autoantigen 69 (26) (**Table 1**).

The immunogenicity of these β cell autoantigens has long been attributed to failures in the mechanisms that govern immune tolerance to self-peptides. While this likely remains true, seminal studies conducted by several laboratories demonstrated that many of these β cell peptides undergo posttranslational modification (PTM). These studies propose that aberrant PTM of these β cell proteins generates so called "neo-antigens" that are then recognized as non-self by immune cells (16, 19, 23, 27–32), hastening the break in tolerance and exacerbating immune targeting and destruction of β cells. However, most of these studies did not explore the cellular processes that lead to PTM of these proteins in the context of β cell function and biology.

To address this question, our laboratory demonstrated that endoplasmic reticulum (ER) stress in the β cell leads to the activation of PTM enzymes and the modification of β cell proteins, which in turn leads to increased recognition of these β cells by diabetogenic T cells (32). ER stress in the β cell originates from various sources. For instance, the normal function of β cells (to produce and secrete insulin) causes ER stress (32–42). We demonstrated that this inherent physiological ER stress is sufficient to activate PTM enzymes and to generate β cell immunogenicity (32) (**Figure 1**). In addition, many of the environmental factors associated with T1D onset such as viral infection (43–48), chemicals (32, 49–51), reactive oxygen species (ROS) (52–55), dysglycemia (56), and inflammation (57–59) may cause β cell ER stress (**Figure 1**). Therefore, any of these environmental factors has the potential to enhance autoimmune targeting of β cells through the generation of ER stress- and PTM-dependent neo-antigens (32, 60, 61). However, the mechanisms by which these factors

TABLE 1 | β Cell autoantigens identified in murine and human T1D.

Autoantigen	Species	Reference
Preproinsulin	Mouse	(10)
	Human	(20)
Glutamic acid decarboxylase 65	Mouse	(11)
	Human	(17)
IGRP	Mouse	(12)
	Human	(22)
Chromogranin A	Mouse	(13)
	Human	(19)
Islet amyloid polypeptide	Mouse	(14)
	Human	(18)
Zinc transporter 8	Mouse	(15)
	Human	(21)
78 kDa glucose-regulated protein	Mouse	(16)
	Human	(23)
IA-2, IA-2β	Human	(24, 25)
Islet cell autoantigen 69	Human	(26)

Abbreviations: Aire, autoimmune regulator; APC, antigen-presenting cell; ATF6, activating transcription factor 6; ATP, adenosine triphosphate; Ca^{2+}, calcium; CHgA, chromogranin A; DRiP, defective ribosomal product; EAE, experimental autoimmune encephalomyelitis; ER, endoplasmic reticulum; GAD65, glutamic acid decarboxylase 65; GFP, green fluorescent protein; GRP78, 78 kDa glucose-regulated protein; IA-2, tyrosine phosphatase-like insulinoma antigen 2; IAPP, islet amyloid polypeptide; ICA69, islet cell autoantigen 69; IGF-2, insulin-like growth factor 2; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; IFNγ, interferon gamma; IP_3R, inositol 1,4,5-trisphosphate receptor; IRE1, inositol-requiring protein 1; JNK, c-jun N-terminal kinase; MAP, mitogen-activated protein kinase; MHC, major histocompatibility complex; mTEC, medullary thymic epithelial cell; NET, neutrophil extracellular traps; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NOD, non-obese diabetic mouse; PAD2, peptidylarginine deiminase 2; PDI, protein disulfide isomerases; PERK, protein kinase RNA (PKR)-like ER kinase; Phogrin, phosphatase homolog of granules from rat insulinomas; PTM, posttranslational modification; ROS, reactive oxygen species; RyR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPases; SNP, single-nucleotide polymorphisms; T1D, type 1 diabetes; Tgase2, tissue transglutaminase 2; UPR, unfolded protein response; ZnT8, zinc transporter 8.

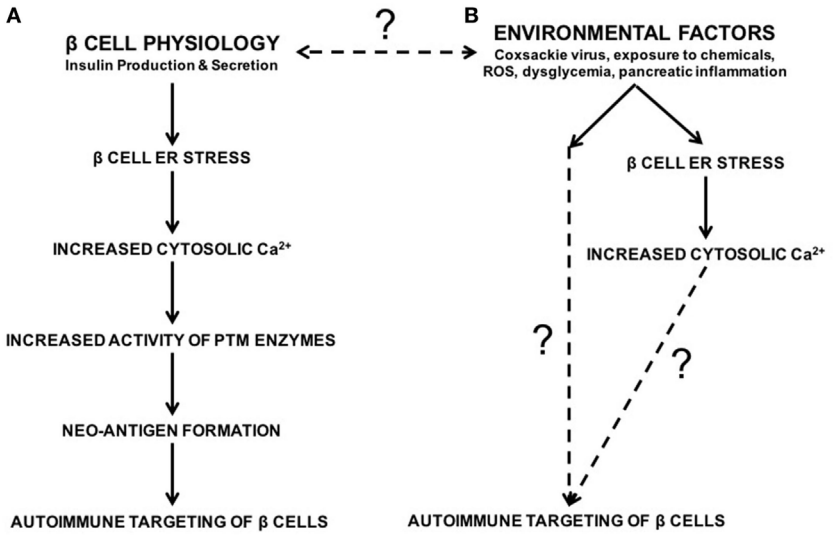

FIGURE 1 | The roles of β cell physiology and environmental favors in the autoimmune targeting of β cells in type 1 diabetes (T1D). **(A)** Normal β cell secretory physiology causes inherent endoplasmic reticulum (ER) stress, which in turn results in a release of Ca^{2+} from the ER into the cytosol. We have previously demonstrated that ER stress and its Ca^{2+} efflux lead to increased activity of Ca^{2+}-dependent posttranslational modification enzymes, formation of neo-antigens, and β cell immunogenicity (32). **(B)** In addition, many environmental factors are associated with T1D onset, such as viral infection, exposure to chemicals and reactive oxygen species, dysglycemia, and pancreatic inflammation. Although the mechanisms by which these factors lead to autoimmune targeting of β cells remain unknown, these environmental factors all cause β cell ER stress and Ca^{2+} efflux. Whether the ER stress and Ca^{2+} efflux caused by these environmental factors contributes to T1D onset, and whether this ER stress cooperates with physiological ER stress to generate neo-antigens, remain unknown.

hasten T1D onset, and whether the ER stress they cause cooperates with that caused by β cell physiology, remain unknown (**Figure 1**).

Here, we review what is known about β cell ER stress, neo-antigen formation, and the progression to pathology in T1D. We also review the role that the environmental factors associated with T1D may play in exacerbating β cell ER stress. Finally, we discuss the evidence supporting our novel hypothesis that environmental factors converge with β cell physiology to increase ER stress above a putative threshold. According to our "threshold hypothesis," ER stress must be sufficiently severe or prolonged to allow for the generation of PTM-dependent neo-antigens. We hypothesize that the convergence between β cell physiology and exposure to environmental factors increases ER stress above this threshold, leading to neo-antigen formation, β cell immunogenicity, and ultimately to the onset of T1D.

ER STRESS AND THE UNFOLDED PROTEIN RESPONSE (UPR)

The ER is primarily responsible for the proper folding and modification of proteins that are membrane bound or destined for secretion. Therefore, the ER lumen contains the molecular chaperones and the environment necessary for protein folding and PTM, including sufficient levels of adenosine triphosphate, an oxidizing environment to support disulfide bond formation, and millimolar concentrations of calcium (Ca^{2+}) (62). Proteins that are folded and modified properly exit the ER and are shuttled to their intended intra- or extracellular locations. However, proteins that become misfolded cannot exit the ER lumen. The accumulation of misfolded or aberrantly modified proteins causes ER stress.

Endoplasmic reticulum stress induces the UPR, which functions in two main modes: the adaptive UPR and the terminal UPR (63, 64). The adaptive UPR occurs early in ER stress and functions largely to alleviate ER stress and restore normal cellular homeostasis through three signaling cascades, each of which begins with the activation of protein sensors of stress in the ER membrane (65). First, protein kinase RNA (PKR)-like ER kinase (PERK) activates a signaling cascade that inhibits mRNA translation and reduces the protein burden in the lumen of the ER (66, 67). Second, activating transcription factor 6 signaling leads to increased production of new molecular chaperones to aid with the folding of accumulated misfolded proteins (68). And third, the signaling pathway initiated by inositol-requiring protein 1 increases expression of chaperones for protein folding and of proteins involved in lipid synthesis to increase ER volume (69, 70). Together, these branches of the UPR work to facilitate the proper folding of proteins that have accumulated, and also reduce the entrance of additional non-chaperone proteins into the ER lumen. In these ways, the adaptive UPR acts to allow the ER to return to normal homeostasis.

Although the adaptive UPR aims to protect the cell from the negative effects of ER stress, ER dysfunction that is excessive or extended may overcome these cytoprotective mechanisms. Under these conditions, the terminal UPR activates proapoptotic processes (71–73) leading to death of the affected cell. However, long before apoptosis pathways are activated, even temporary ER stress and the adaptive UPR may have important consequences for cellular function and physiology.

All cells undergo periods of increased protein production, which increases the ER burden, leading to misfolding or aberrant modification of nascent proteins, and ultimately to ER stress and UPR activation. However, secretory cells, due to their normal physiology, are uniquely susceptible to ER stress. These cells must produce not only the proteins necessary for normal cellular maintenance, but also the proteins to be secreted and the proteins that comprise the secretory pathway itself. Even with a larger ER volume and greater numbers of chaperones to account for this increased demand (74), the secretory function of these cells leads to significantly increased ER burden and stress.

Like other secretory cells, β cells undergo naturally high levels of ER stress due to their normal physiological role of insulin production and secretion (32–42). Indeed, increased ER stress, and its consequences for protein folding, occurs as a direct consequence of glucose sensing (37, 38). In response to increased glucose concentrations, β cells upregulate the translation of preproinsulin by 50-fold, reaching a production rate of one million molecules of preproinsulin per minute (75). These one million molecules of preproinsulin inundate the ER lumen for folding and the formation of three disulfide bonds per molecule, causing tremendous ER stress. Under these conditions, many of the insulin molecules produced by β cells become misfolded or incorrectly modified (75) (**Figure 2**). In addition to this inherent ER stress due to normal physiology, β cell ER stress may rise due to exposure to the environmental factors that are associated with T1D onset (32, 43–61) (**Figure 1**). Under these circumstances, β cell ER stress may rise above physiological levels.

Heightened β cell ER stress does not necessarily activate the terminal UPR or suggest β cell exhaustion or impending death as observed in some models (63, 76–80). Rather, β cells exhibit naturally high ER stress very early and activate the adaptive UPR long before β cell death. In a study using a reporter mouse in which green fluorescent protein is expressed with the activation of the UPR, the pancreas exhibited the highest ER stress of all tissues examined, and did so as early as day 16 of life (81). In spite of the observed ER stress and UPR activation, these mice (on the C57Bl/6 background) never succumbed to loss of β cell mass and diabetes (81). These data confirm that high levels of β cell ER stress does not necessarily activate the terminal UPR and lead to β cell failure and death. Indeed, the β cells in most individuals resolve ER stress through the proper function of the adaptive UPR and therefore maintain healthy and functional β cell mass throughout their lifetimes. Therefore, β cell death is not the only consequence of ER stress. We hypothesize that lower and more transient ER stress, and the activation of the adaptive UPR, may have consequences for β cell function and for the autoimmune targeting of β cells much earlier.

ER STRESS AFFECTS Ca^{2+}-DEPENDENT CELLULAR FUNCTIONS

In addition to its role in the folding and modification of new proteins, the ER contains the largest store of intracellular Ca^{2+} and is an important organelle for regulating Ca^{2+} concentrations,

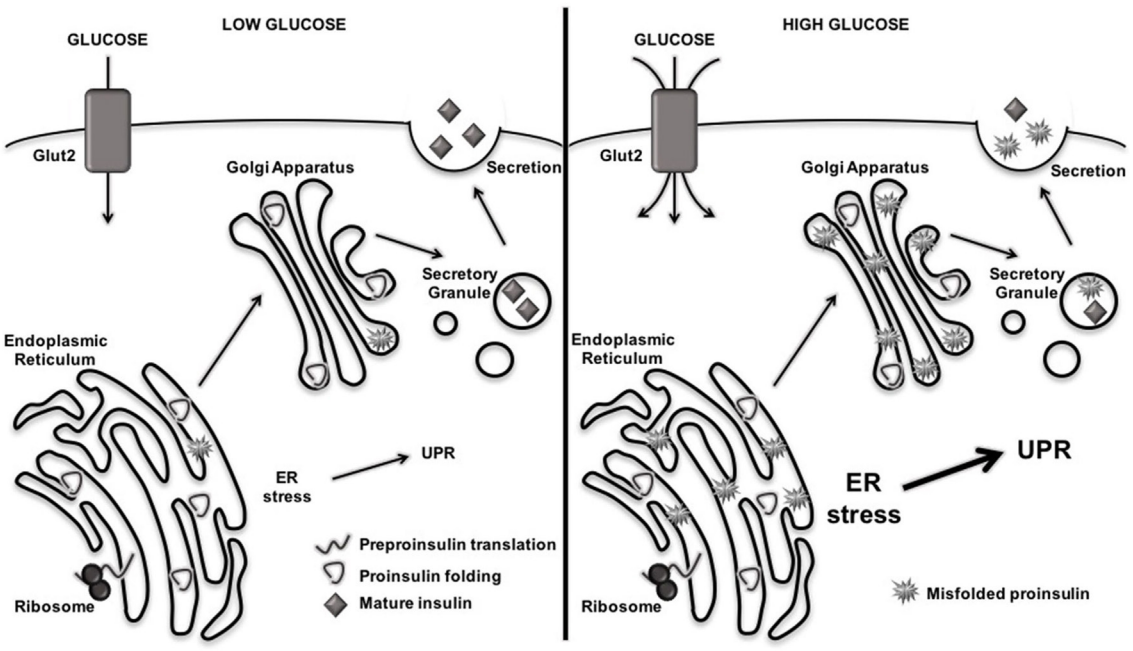

FIGURE 2 | Rising blood glucose increases misfolding of proinsulin and endoplasmic reticulum (ER) stress levels. When blood glucose levels are low, preproinsulin is translated, properly folded and modified in the ER, and secreted as mature insulin into the extracellular space. When blood glucose levels rise, β cells increase production of preproinsulin, flooding the ER lumen with one million molecules per minute that require folding and disulfide bond formation. This increased protein burden in the ER leads to misfolding of proteins and aberrant posttranslational modification, which further exacerbates ER dysfunction and activates the unfolded protein response (UPR).

and therefore Ca^{2+}-dependent processes, throughout the cell (82). One consequence of ER stress is the release of Ca^{2+} from the ER lumen into the cytosol. This Ca^{2+} efflux has important consequences for cellular physiology.

In the ER, millimolar concentrations of Ca^{2+} are necessary for proper protein folding and modification (62). Indeed, molecular chaperones that assist in protein folding and protein disulfide isomerases that facilitate the formation of disulfide bonds depend on these high Ca^{2+} concentrations (83, 84). These high concentrations of Ca^{2+} are maintained by sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCA) pumps in the ER membrane that actively transport Ca^{2+} from the cytosol into the ER. When Ca^{2+} leaves the ER lumen during ER stress, the function of these proteins also decreases, further inhibiting protein folding and modification and contributing to greater ER dysfunction (85).

In the cytosol, Ca^{2+} is required for the regulation of normal cellular processes such as metabolism, vesicular trafficking, protein secretion, mRNA transcription, and apoptosis (86). To achieve the necessary cytosolic concentrations, Ca^{2+} is released from the ER by the ryanodine receptor and inositol 1,4,5-trisphosphate receptor channels. Under conditions of ER stress, the efflux of Ca^{2+} from the ER lumen increases cytosolic concentrations above normal physiological levels. This increased cytosolic Ca^{2+} can be deleterious for cellular function. For example, increased cytosolic Ca^{2+} can initiate apoptosis through activation of caspase-dependent cell death pathways (87, 88) or mitochondria-dependent pathways (89–92).

It is clear, then, that ER stress greatly affects Ca^{2+}-dependent cellular functions. While the adaptive UPR works to relieve ER stress, cytosolic Ca^{2+} still increases before ER homeostasis is regained. β cells, which are particularly susceptible to ER stress, are therefore also prone to the dysregulation of cellular processes following even a temporary efflux of Ca^{2+} from the ER. In addition, the environmental factors associated with T1D onset also lead to increased cytosolic Ca^{2+} (32, 43–61) (**Figure 1**). Therefore, we propose that the combination of physiological ER stress and that derived from environmental factors, even if transient, may have consequences for β cell health and function.

ER STRESS ACTIVATES CYTOSOLIC PTM ENZYMES

Transient ER stress and increased cytosolic Ca^{2+} concentrations can activate cytosolic Ca^{2+}-dependent enzymes, including those that mediate PTM. Activation of these PTM enzymes can have significant implications for proteins being folded in the ER. In particular, two such PTM enzymes reside in the cytosol and are activated during the ER stress Ca^{2+} flux: tissue transglutaminase 2 (Tgase2) and peptidylarginine deiminase 2 (PAD2).

Tissue transglutaminase 2 is ubiquitously expressed and resides in the cytosol (93). When activated, Tgase2 translocates to several intracellular compartments (94), including the ER (95–97) and secretory granules (98) to modify proteins through two mechanisms (99): first, Tgase2 crosslinks proteins through the formation of ε(γ-glutamyl) isopeptide bonds between glutamine and lysine residues, and second, Tgase2 mediates the deamidation of glutamine residues. Tgase2 plays important roles in the regulation of apoptosis (100, 101), gene expression (93, 102, 103), and cellular adhesion and wound healing (104–107). Of relevance to T1D, Tgase2 is expressed in and functions in β cells (32, 60).

Of the five mammalian PAD isoforms, PAD2 is the most widely expressed and is the isoform expressed in the pancreas (108). PAD2 also resides in the cytosol (109), and, similar to Tgase2, activated PAD2 is recruited to various subcellular compartments for the modification of proteins (110). PAD2 mediates the conversion of arginine to citrulline. This amino acid conversion alters the overall charge and hydrophobicity of the protein (111), causing changes in protein folding and conformation (112). PAD2 plays roles in many cellular functions, including the negative regulation of nuclear factor kappa-light-chain-enhancer of activated B cells activation (113), cytoskeleton disassembly (114), and in the formation of neutrophil extracellular traps (115).

While Ca^{2+}-dependent activation of these enzymes is necessary for normal cellular function, these enzymes also contribute to pathology in many diseases.

PTM GENERATES NEO-ANTIGENS IN AUTOIMMUNE DISEASES

Protein PTM is necessary for cellular viability and function. However, autoantigens in many different autoimmune diseases such as celiac disease (116), collagen-induced arthritis (117), multiple sclerosis/experimental autoimmune encephalomyelitis (118–121), rheumatoid arthritis (122–127), and systemic lupus erythematosus (128–131) contain PTM, suggesting that these modifications may contribute to breaks in tolerance that exacerbate disease.

Central tolerance is established during T cell development in the thymus. In the thymus, medullary thymic epithelial cells (mTECs) express peptides normally found in peripheral tissues through the function of autoimmune regulator (132–134). When these peptides are presented to developing T cells in the context of MHC molecules, T cells that respond too strongly to these self-peptides are deleted and are thus absent from the mature T cell population (4, 135–137). However, if self-proteins undergo PTM in peripheral tissues, as in the autoimmune diseases listed above, these proteins may be processed and presented differently by peripheral antigen-presenting cells (APCs) than by the mTECs (138). If such modified epitopes were not expressed and presented by mTECs, T cells that recognize these modified epitopes escape negative selection and are present in circulation as mature T cells. When these T cells encounter these neo-antigens in peripheral tissues, they become activated and lead the autoimmune targeting of peripheral tissues.

As with all peripheral tissues, peptides from β cell proteins, including insulin and insulin-like growth factor 2, are presented by mTECs to developing T cells in the thymus (5, 6, 139–142). However, the presence of T cells in the periphery that recognize islet proteins and target β cells suggests the failure of crucial tolerance mechanisms. This failure in central tolerance mechanisms may be explained by the growing body of literature that

abnormal PTM increases the immunogenicity of β cell peptides in both murine and human models of T1D (**Table 2**). These studies have demonstrated that some β cell proteins undergo various modifications including oxidation (28, 143), Tgase2-mediated crosslinking by isopeptide bond (19, 29), Tgase2-mediated deamidation (30–32), PAD2-mediated citrullination (16, 23, 31), the formation of hybrid peptides (144, 145), and the formation of a defective ribosomal insulin gene product (146). Furthermore, the neo-antigens formed by these PTM elicit stronger immune responses than the native proteins (16, 19, 23, 28–31, 143, 145), suggesting an important role for these neo-antigens in precipitating disease onset. These findings have been of great importance to the understanding of T1D pathogenesis, because these studies identified novel autoantigens that are targeted in T1D. However, the mechanisms by which these neo-antigens arise in β cells was not examined.

β CELL NEO-ANTIGENS ARISE DURING ER STRESS

To begin to elucidate how PTM neo-antigens might arise in β cells, our laboratory examined the consequences of β cell ER stress for β cell immunogenicity, since β cells inherently undergo high levels of ER stress (32–42, 60, 81). To do so, we used a model system of β cell recognition by diabetogenic BDC2.5 $CD4^+$ T cells. These particular T cells were chosen because they recognize a Tgase2-modified peptide of CHgA (29) and secrete interferon gamma (IFNγ) when they encounter their PTM-dependent antigen.

Our studies demonstrated that, in primary murine islets, ER stress induced by thapsigargin [a widely accepted chemical inducer of ER stress (96, 147, 148)] contributed to heightened cytosolic Ca^{2+} concentrations, increased Tgase2 activity, and increased β cell immunogenicity (32). In fact, murine islets undergoing ER stress elicited greater IFNγ secretion from BDC2.5 T cells (32) and by all other β cell antigen-specific T cells examined (**Figure 3**), suggesting a role for Ca^{2+}-dependent PTM in immunogenicity of many other β cell antigens. This increased immunogenicity was dependent upon both Ca^{2+} and Tgase2-mediated PTM, since chelation of cytosolic Ca^{2+} or decreased expression of Tgase2 reduced this consequence of ER stress (32). These data show that β cell ER stress leads to β cell immunogenicity through Ca^{2+}-dependent PTM of endogenous proteins.

Since ER stress is inherent to β cell physiology and function (32–42, 60), we hypothesized that ER stress induced by normal physiology [e.g., dynamic glucose sensing and secretory function (33–42, 60)] may be sufficient to cause Ca^{2+}- and PTM-dependent β cell immunogenicity. Indeed, a murine insulinoma (NIT-1) that exhibited low ER stress and immunogenicity was exposed to physiological milieu by transplantation into NOD.*scid* mice. After transplant, these cells exhibited insulin secretion, ER stress, Tgase2 activity, and immunogenicity (32). These data confirm that β cell physiology and insulin secretion contributes to the autoimmune targeting of β cells (60).

Many groups have demonstrated an increase in β cell ER stress long before β cell death and T1D onset (79, 81, 149, 150). In fact, relief of ER stress has been proposed as therapeutic opportunity for preventing β cell death and maintaining euglycemia (63, 80, 151, 152). However, most researchers conclude that ER stress leads to β cell death through the terminal UPR and activation of apoptosis pathways (76, 77, 80). Ours was the first study to demonstrate that normal, physiological β cell ER stress and the adaptive UPR contribute to T1D through the formation of β cell neo-antigens. In doing so, we became the first to propose a mechanism by which β cell neo-antigens (**Table 2**) may occur (**Figure 4**).

TABLE 2 | Posttranslational modification (PTM)-mediated neo-antigen formation in type 1 diabetes.

Autoantigen	PTM	Reference
Proinsulin	Oxidation	(28, 143)
	Formation of hybrid insulin peptides	(23, 144, 145)
Chromogranin A (WE14)	Crosslinking/isopeptide bond	(19, 29)
Preproinsulin	Deamidation	(30)
Islet cell autoantigen 69	Deamidation	(30)
Zinc transporter 8	Deamidation	(30)
Phosphatase homolog of granules from rat insulinomas	Deamidation	(30)
IA-2	Deamidation	(30)
IGRP	Deamidation	(30)
Glutamic acid decarboxylase 65	Citrullination	(31)
	Deamidation	(30, 31)
78 kDa glucose-regulated protein	Citrullination	(16, 23)
Insulin	Defective ribosomal product	(146)

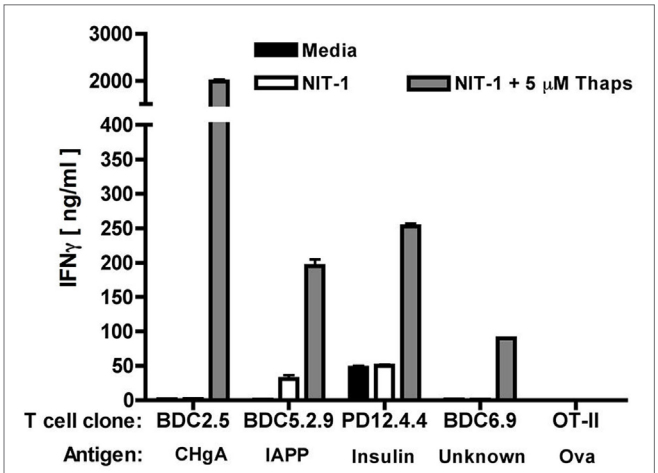

FIGURE 3 | Endoplasmic reticulum (ER) stress increases the immunogenicity of several β cell autoantigens. The immunogenicity of NIT-1 insulinoma cells treated with 5 μM thapsigargin or control for 1 h was assessed by T cell assay. Briefly, T cells (2×10^4), NOD.*scid* splenocytes as antigen-presenting cells (4×10^5), and NIT-1 cells as antigen (1×10^3) were combined in 200 μl in triplicate in 96-well flat-bottom tissue culture plates and incubated at 37°C for 72 h. T_H1 effector function was determined by measuring interferon gamma (IFNγ) secretion by enzyme-linked immunosorbent assay. Data are mean IFNγ secretion ± SD and are from one representative experiment of three independent experiments. For all specificities examined, NIT-1 cells undergoing ER stress elicited higher effector responses from the T cells, suggesting that ER stress contributes to the modification and greater immunogenicity of each of these proteins.

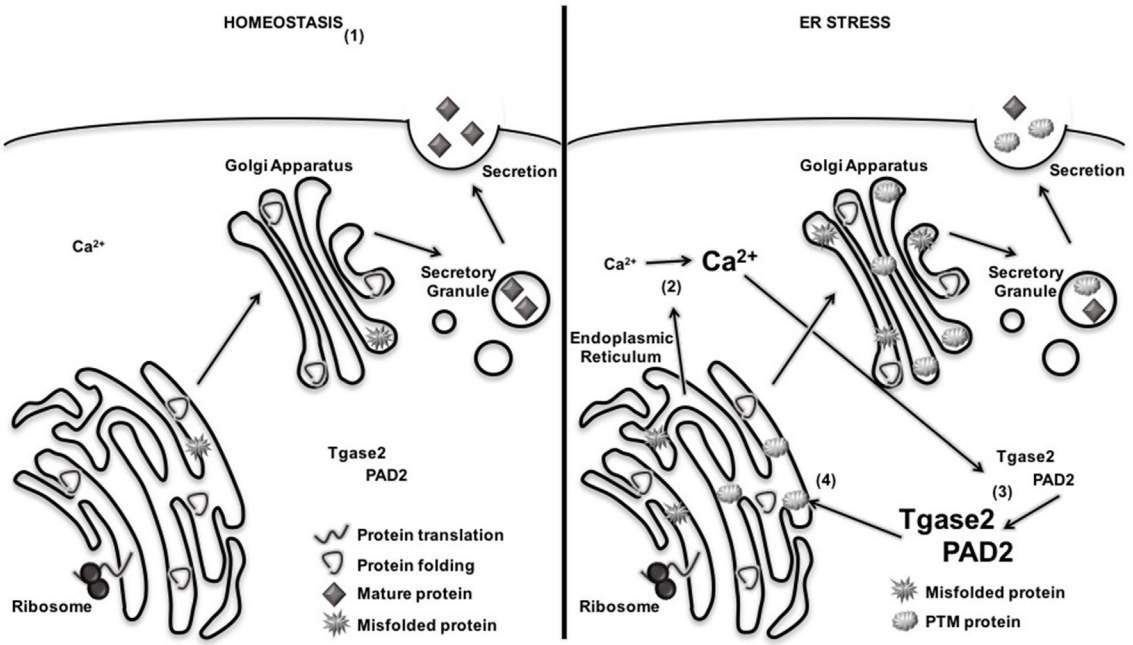

FIGURE 4 | Endoplasmic reticulum (ER) stress increases the activation of Ca^{2+}-dependent posttranslational modification (PTM) enzymes and the formation of PTM-dependent β cell neo-antigens. (1) Under homeostatic conditions, proteins are translated, folded, and packaged into secretory granules. Cytosolic Ca^{2+} and PTM enzyme activity remain low. (2) During β cell ER stress, Ca^{2+} stores are released from the ER, increasing cytosolic Ca^{2+}. (3) Increased Ca^{2+} concentrations activated Ca^{2+}-dependent enzymes tissue transglutaminase 2 (Tgase2) and peptidylarginine deiminase 2 (PAD2). (4) Active PTM enzymes modify nascent proteins. If presented to autoreactive T cells by antigen-presenting cell, modified β cell proteins break tolerance and facilitate immune recognition of β cells.

β CELL IMMUNOGENICITY REQUIRES A THRESHOLD OF ER STRESS

Endoplasmic reticulum stress occurs along a gradient. The burden of unfolded proteins in the ER lumen can vary from mild to severe, resulting in varying degrees of ER dysfunction and stress. This variance in levels of ER stress has important implications for the cellular consequences of ER stress. As discussed earlier, the strength and duration of ER stress-induced UPR signaling is a major factor in determining whether the adaptive UPR or terminal UPR is initiated (63, 64). One explanation may be that the severity and duration of ER stress affects the strength of the Ca^{2+} efflux from the ER lumen and determines whether cytosolic Ca^{2+} concentrations cross a putative threshold. Differences in cytosolic Ca^{2+} concentrations may significantly alter PTM enzyme activity, neo-antigen generation, and β cell immunogenicity.

This "threshold hypothesis" is further supported by literature that demonstrates that Tgase2 and PAD2 remain largely inactive in the cytosol, and activation requires significantly increased concentrations of cytosolic Ca^{2+}. In fact, the activation of both enzymes requires Ca^{2+} concentrations up to 100-fold higher than what is necessary for normal cellular physiology and function. Therefore, these enzymes generally become activated only under conditions of cellular distress or dysfunction, such as ER stress (96, 97, 108, 109, 147, 153, 154). Since these PTM enzymes require particular levels of cytosolic Ca^{2+} to become activated, it follows that a particular level of ER stress must be achieved before PTM-dependent neo-antigen formation can occur.

Previous work in our laboratory examined whether varying levels of ER stress lead to different degrees of β cell PTM-dependent immunogenicity. NIT-1 cells were incubated with increasing doses of thapsigargin, which increases ER stress and cytosolic Ca^{2+} by inhibiting the SERCA pumps that transport Ca^{2+} from the cytosol into the ER. As expected, thapsigargin induced ER stress and UPR activity in a dose-dependent manner (**Figure 5A**). The immunogenicity of these cells was examined by the BDC2.5 T cell clone, and T cell effector function was measured by IFNγ as previously described (32). Only the highest dose of thapsigargin elicited detectable IFNγ secretion from the T cells (**Figure 5B**). Therefore, although lower doses of thapsigargin induced ER stress, the stress (and consequences thereof) at these lower doses was not sufficient to result in β cell immunogenicity.

Tunicamycin is another chemical inducer of ER stress that blocks the initial steps of glycoprotein synthesis in the ER and thus increases the burden of unfolded proteins in the ER lumen (148). Increasing doses of tunicamycin increased ER stress in NIT-1 cells (**Figure 6A**), but to lesser degrees compared with thapsigargin (**Figure 5A**). Also, as with lower doses of thapsigargin, the lower ER stress induced by tunicamycin was not sufficient to elicit effector responses from BDC2.5 T cells (**Figure 6B**). Together, these data serve as further evidence that a particular threshold of ER stress must be reached to achieve PTM-dependent β cell immunogenicity.

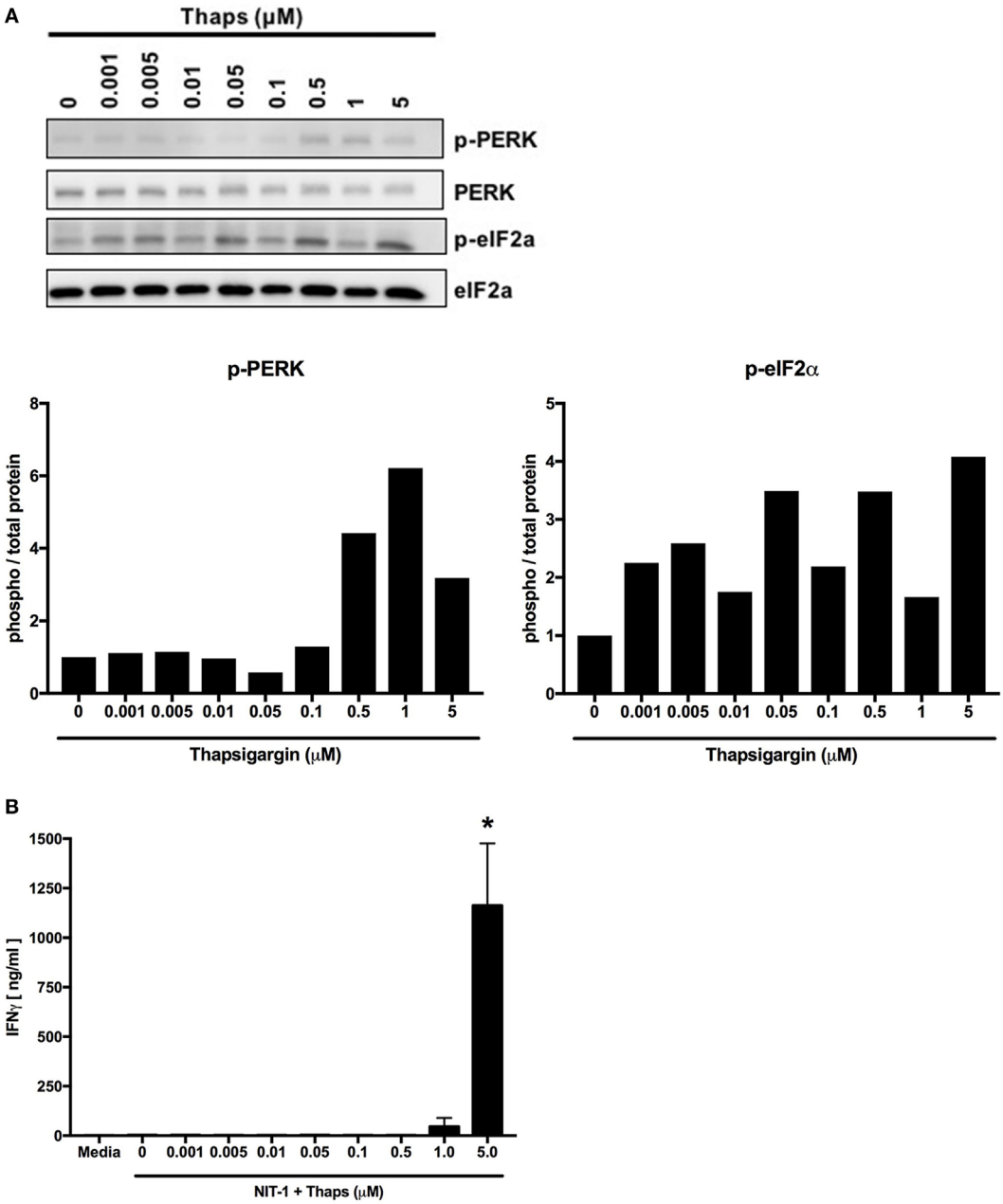

FIGURE 5 | Endoplasmic reticulum stress must increase above a threshold to induce posttranslational modification-dependent immunogenicity. **(A)** NIT-1 insulinoma cells were incubated with increasing concentrations of thapsigargin for 1 h and washed extensively. Cell lysates were analyzed for the phosphorylation of UPR proteins—protein kinase RNA (PKR)-like ER kinase (PERK) and eIF2α. Data are representative of two independent experiments. Densitometry data are phosphorylation levels normalized by total protein and relative to that in control (0 μM) treated cells. **(B)** The immunogenicity of NIT-1 cells treated with increasing concentrations of thapsigargin for 1 h was measured by BDC2.5 T cell assay. Data are mean interferon gamma (IFNγ) secretion ± SEM. *$p < 0.05$.

ENVIRONMENTAL FACTORS ASSOCIATED WITH T1D INDUCE HEIGHTENED β CELL ER STRESS

Every pancreas undergoes ER stress (32, 81), but this stress does not lead to T1D in every individual. In fact, even in those with a genetic predisposition to autoimmunity, T1D may never occur (155) or may occur much later than expected (156, 157). These observations suggest that environmental factors may precipitate disease onset. Indeed, T1D onset is associated with several environmental factors such as viral infection (43–48), chemicals (49–51), ROS (52–55), dysglycemia (56), and inflammation (57–59). Although these environmental factors are thought to exacerbate the autoimmune targeting of β cells and hasten

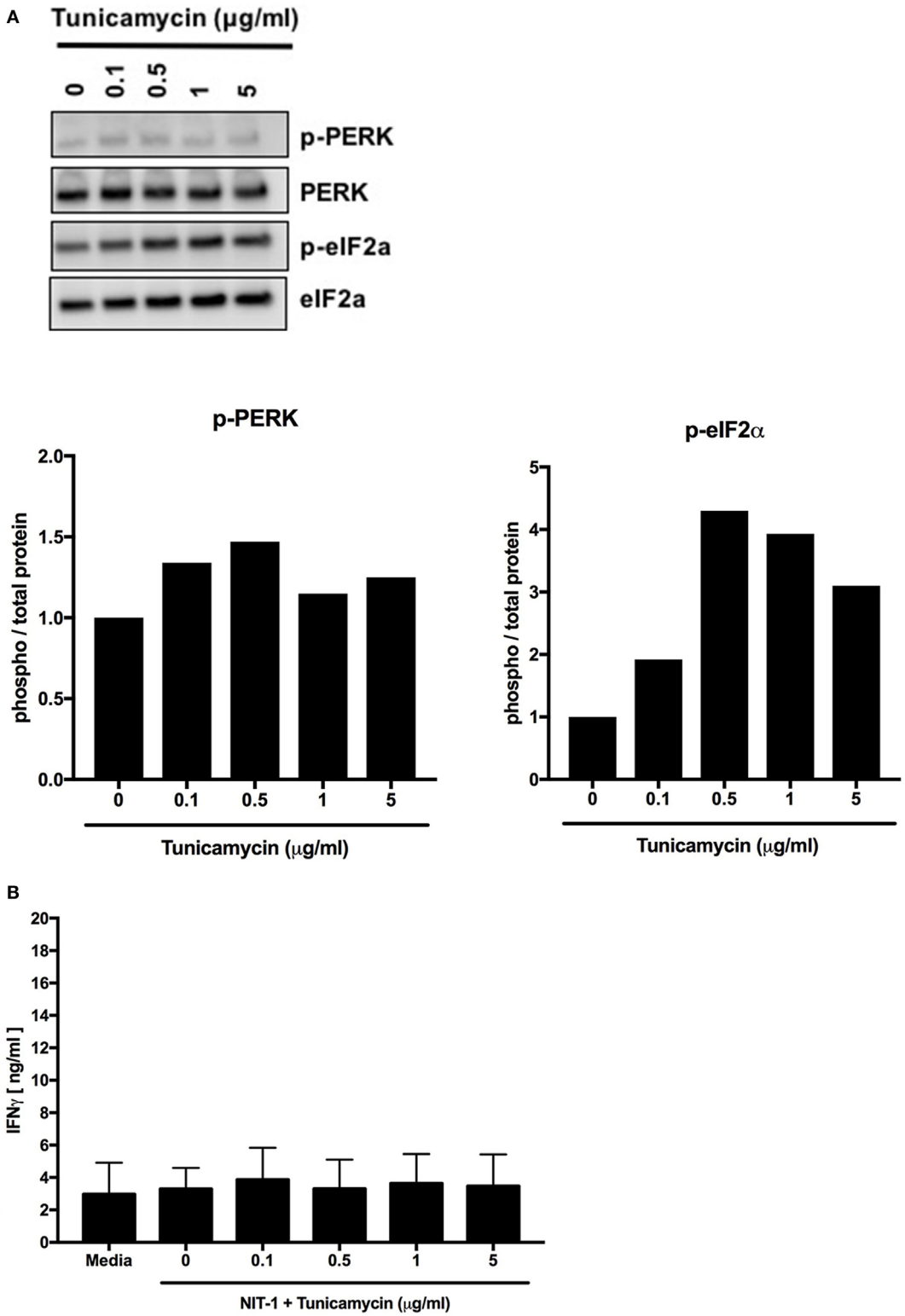

FIGURE 6 | Endoplasmic reticulum stress below a threshold does not induce posttranslational modification-dependent immunogenicity. **(A)** NIT-1 insulinoma cells were incubated with increasing concentrations of tunicamycin for 4 h and washed extensively. Cell lysates were analyzed for the phosphorylation of UPR proteins—protein kinase RNA (PKR)-like ER kinase (PERK) and eIF2α. Data are representative of two independent experiments. Densitometry data are phosphorylation levels normalized by total protein and relative to that in control (0 µg/ml) treated cells. **(B)** The immunogenicity of NIT-1 cells treated with increasing concentrations of tunicamycin for 4 h was measured by BDC2.5 T cell assay. Data are mean interferon gamma (IFNγ) secretion ± SEM.

disease onset, the mechanisms by which these environmental factors advance pathology, and whether these factors contribute to PTM-mediated neo-antigen formation, remain unknown (**Figure 1**).

As discussed earlier, β cell ER stress and Ca^{2+} flux into the cytosol must cross a threshold before Tgase2 and PAD2 can modify β cell proteins to generate neo-antigens and elicit effector responses from diabetogenic T cells. While β cell physiology causes ER stress (32–42) and this ER stress can, under some circumstances generate neo-antigens and immunogenicity (32, 60) (**Figure 1**), the discrepancy in disease onset in those genetically predisposed to autoimmunity (155–157) suggests that this physiological stress alone may not be sufficient to generate neo-antigens. Interestingly, each of the environmental factors associated with T1D also lead to an increase in β cell ER stress and cytosolic Ca^{2+}.

Coxsackie Virus

Coxsackie virus infection is associated with T1D onset. Recent onset T1D patients have viral RNA in their pancreas and higher titers of antibodies against Coxsackie virus (158, 159). Also, Coxsackie virus infection accelerates disease onset in NOD mice with established insulitis (46, 160–162), suggesting a role for Coxsackie virus in breaking immune tolerance. Studies with BDC2.5 TCR transgenic NOD mice attributed this acceleration to activation of bystander immune cells (46). These data provide a strong link between pancreatic viral infection and broken tolerance. Since BDC2.5 T cells do not recognize a viral protein (29) but rather modified CHgA, activation of BDC2.5 T cells in these mice suggests that Coxsackie virus infection may lead to PTM of endogenous β cell proteins and neo-antigen formation. Indeed, viruses cause neo-antigen generation and exacerbate pathology in other models of autoimmunity (163).

Moreover, Coxsackie virus protein 2B disrupts the ER membrane (164–166), releasing Ca^{2+} from the ER into the cytosol and causing ER stress. We have shown that β cell ER stress contributes to neo-antigen formation and immunogenicity (32). Therefore, it is plausible that Coxsackie virus may raise β cell ER stress and cytosolic Ca^{2+} concentrations above the levels attributed to normal physiology, increasing neo-antigen production through Ca^{2+}-dependent PTM.

Exposure to Chemicals

Exposure of β cells to chemicals such as alloxan and streptozotocin cause the loss of insulin secretion and β cell death (167). For each of these chemicals, β cells experience DNA damage, protein ADP ribosylation (168), and ROS generation (169–171), all of which ultimately lead to apoptosis and significant loss of β cell death. However, before apoptosis pathways are activated, ADP ribosylation and ROS cause misfolding and accumulation of nascent proteins in the ER lumen. As discussed earlier, the accumulation of misfolded and abnormally modified proteins leads to ER stress and release of Ca^{2+} into the cytosol (172, 173).

Reactive Oxygen Species

Reactive oxygen species, which have the potential to cause irreversible damage to cellular proteins and organelles (174–176), are generated both during normal β cell function (52) and when β cells are exposed to other insults such as Coxsackie virus (177–179). Although antioxidant defenses work to prevent ROS-mediated damage, β cell mitochondria express very low levels of antioxidant enzymes (180–182), making these cells particularly susceptible to ROS-mediated damage. When ROS exceeds the capacity of the cell to scavenge these species, oxidative stress leads to β cell death (183, 184) and ultimately to T1D (52, 54, 180, 185–190). However, before the loss of β cell mass, ROS leads to oxidative

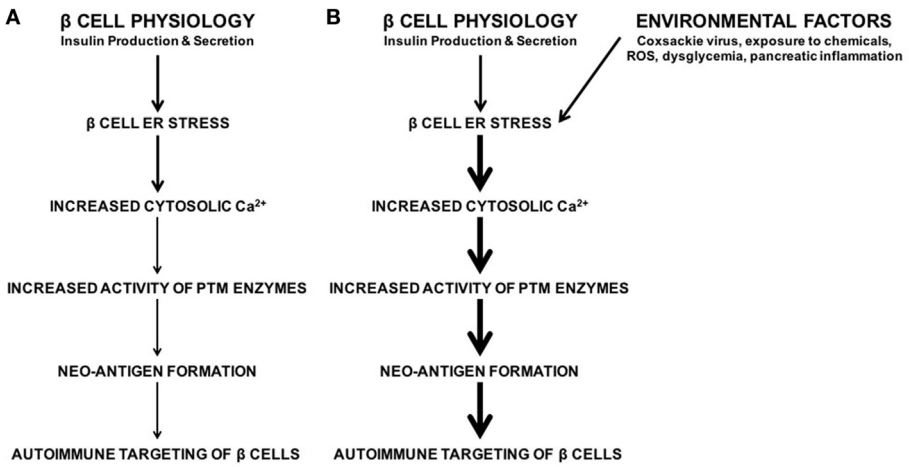

FIGURE 7 | Model. **(A)** Normal β cell secretory physiology causes inherent endoplasmic reticulum (ER) stress, which in turn results in a release of Ca^{2+} from the ER into the cytosol. This ER stress and Ca^{2+} efflux lead to increased activity of Ca^{2+}-dependent posttranslational modification (PTM) enzymes, formation of neo-antigens, and β cell immunogenicity (32). However, neo-antigen formation and immunogenicity due to inherent physiological ER stress may not be enough to cause type 1 diabetes (T1D). **(B)** The environmental factors associated with T1D onset cause β cell ER stress and Ca^{2+} efflux. The ER stress induced by these environmental factors cooperates with the physiological ER stress to raise cytosolic Ca^{2+} concentrations above a threshold to activate PTM enzymes, generate neo-antigens, cause autoimmune targeting of β cells, and precipitate T1D onset.

modification of proteins and lipids (191), and to the release of Ca^{2+} from the ER into the cytosol (192–194). Therefore, ER stress and Ca^{2+} efflux caused by ROS may lead to protein PTM and the formation for neo-antigens in β cells.

Dysglycemia

As discussed earlier, increased glucose sensing by β cells during times of dysglycemia increases insulin production and secretion (75). Normal insulin secretion raises β cell ER stress (32–42), but when blood glucose rises too high, or the hyperglycemia is too prolonged, so called "glucotoxicity" further enhances β cell ER stress. At later stages of T1D, ER stress induced by glucotoxicity is thought to be a major contributor to β cell death through the terminal UPR. However, fluctuation in blood glucose levels as β cell mass is gradually lost may also induce the adaptive UPR. In this way, glucotoxicity may, long before β cell death, contribute to Ca^{2+}- and PTM-dependent neo-antigen formation and therefore to autoimmune targeting of β cells.

Inflammation

As autoreactive immune cells infiltrate the islets to target their antigens, these activated immune cells secrete pro-inflammatory cytokines. In addition, β cells themselves release additional pro-inflammatory cytokines during viral infection (195), and cellular stress (196). These inflammatory mediators initiate signaling cascades in the β cells. For example, pro-inflammatory cytokines activate NF-kB in β cells, which inhibits the expression of other transcription factors necessary for normal β cell function (197). Also, inflammatory cytokines activate c-jun N-terminal mitogen-activated protein kinase signaling, which is associated with ER stress and Ca^{2+} release (198, 199). Finally, inflammatory cytokines reduce SERCA expression, effectively preventing the return of Ca^{2+} from the cytosol to the ER and further exacerbating ER stress (197, 200). Therefore, pancreatic inflammation may lead to β cell neo-antigen formation and exacerbate autoimmune targeting of β cells.

Therefore, we hypothesize that the ER stress generated by these environmental factors may converge with the stress caused by normal physiology to allow cytosolic Ca^{2+} to cross the necessary threshold to activate PTM enzymes and generate neo-antigens long before the terminal UPR initiates apoptosis pathways. In this way, ER stress-mediated neo-antigen formation may be a common mechanism by which these environmental factors augment autoimmune targeting of β cells and hasten T1D onset.

CONCLUSION

Type 1 diabetes is caused by the autoimmune targeting and destruction of pancreatic β cells. The autoreactive immune cells target many β cell proteins (**Table 1**) when central and peripheral tolerance fail. The mechanisms by which tolerance fails are still being elucidated, but a growing body of literature demonstrates that β cell peptides modified by Ca^{2+}-dependent PTM elicit stronger responses from autoreactive T cells than their native counterparts (16, 19, 23, 28–31, 143, 145). However, the mechanisms by which these β cell peptides become modified during β cell physiology is only beginning to be explored (32, 60).

We have previously demonstrated that β cell ER stress leads to PTM-dependent immunogenicity (32). Although this ER stress may be derived from the natural secretory physiology of the β cell (32–42), inherent, physiological ER stress alone may not sufficient to precipitate T1D onset even in those individuals harboring a genetic predisposition to autoimmunity (155–157). We therefore propose a model in which β cell ER stress leads to neo-antigen formation and immunogenicity of β cells when this ER stress reaches a critical threshold. The ER stress induced by the environmental factors associated with T1D may combine with physiological ER stress to raise cytosolic Ca^{2+} above this putative threshold, allowing for the activation of PTM enzymes and the generation of PTM-dependent neo-antigens (**Figure 7**). This convergence of with physiological stress may explain how environmental factors hasten T1D onset.

It is important to note that, although physiological and environmental factor-derived ER stress likely occurs in the β cells of all individuals, autoimmunity predominantly occurs in the context of genetic predisposition to autoimmunity. For patients who express the MHC molecules that predispose them to autoimmunity, β cell neo-antigens generated during ER stress are presented by these MHC molecules and activate the T cells that escaped negative selection during development. The activation of these T cells ultimately leads to the autoimmune destruction of the β cells and to T1D onset. However, in those without this MHC predisposition, β cell ER stress may still result in the modification of β cell proteins without leading to disease. In these patients, these neo-antigens may not be presented by APC or may not be recognized if autoreactive T cells are correctly deleted from the repertoire during negative selection in the thymus. Therefore, β cell ER stress and the subsequent neo-antigen formation likely still require genetic predisposition to autoimmunity to lead to T1D.

Our model proposes a "threshold hypothesis" according to which cytosolic Ca^{2+} must cross a particular threshold to allow for the generation of PTM-dependent β cell neo-antigens. Additional studies are necessary to confirm the cooperation between physiological ER stress and that derived from exposure to environmental factors to reach this threshold. These studies will further advance our understanding both of how neo-antigens are formed in the β cell and the mechanisms by which environmental factors hasten disease onset. Such studies may reveal novel opportunities for therapeutic intervention to prevent or delay T1D onset in at-risk patients.

MATERIALS AND METHODS

Mice

Mice were bred and housed under specific pathogen-free conditions at Rangos Research Center of Children's Hospital of Pittsburgh of University of Pittsburgh Medical Center. All experiments were approved by Institutional Animal Care and Use Committee of the University of Pittsburgh.

Cell Culture

The NIT-1 insulinoma cell line was a gift from Clayton Mathews (University of Florida) and were maintained at 37°C in a 5% CO_2

humid air incubator, in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Mediatech), 10 mM HEPES buffer (Gibco), 4 mM L-glutamine (Gibco), 200 µM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 61.5 µM β-mercaptoethanol (Sigma-Aldrich), and 100 µg/ml gentamicin (Gibco).

$CD4^+$, MHC class II-restricted BDC2.5, BDC5.2.9, PD12.4.4, and BDC6.9 T cells were a gift from Kathryn Haskins (University of Colorado). T cell clones were maintained in supplemented DMEM as described previously (32, 201–203).

OT-II splenocytes were harvested and prepared in supplemented DMEM as described previously (204–208).

Induction of ER Stress

NIT-1 cells were cultured in 25 cm^2 tissue culture flasks (Greiner Bio-One) with various concentrations of thapsigargin or control for 1 h at 37°C or with various concentrations of tunicamycin or control for 4 h at 37°C. Before downstream analysis, the cells were washed extensively (50,000× original volume) to remove residual thapsigargin or tunicamycin, and removed from the flask with 0.05% trypsin–EDTA (Gibco).

T Cell Assays

T cells (2×10^4), NOD.*scid* splenocytes as APC (4×10^5), and antigen (1×10^3 dispersed NIT-1 cells) were combined in 200 µl supplemented DMEM in triplicate in 96-well flat-bottom tissue culture plates (Greiner Bio-One) and incubated at 37°C for 72 h. T_H1 effector function was determined by measuring IFNγ secretion by enzyme-linked immunosorbent assay (ELISA).

Splenocyte Assay

OT-II splenocytes (1×10^6) were combined with antigen (1×10^3 dispersed NIT-1 cells) in 200 µl supplemented DMEM in triplicate in 96-well flat-bottom tissue culture plates (Greiner Bio-One) and incubated at 37°C for 72 h as described previously (204–208). T_H1 effector function was determined by measuring IFNγ secretion by ELISA.

Enzyme-Linked Immunosorbent Assay

Interferon gamma from T cell assays was measured with murine IFNγ ELISA antibody pairs (BD Biosciences) as described previously (32, 202–204, 208). Absorbance was measured at 450 nm with a SpectraMax M2 microplate reader (Molecular Devices). Data were analyzed with SoftMax Pro (Molecular Devices).

Preparation of Cell Lysates

Cells were lysed by sonication in 50 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM NaF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 2 mM Na_3VO_4, and 1 mM PMSF. Protein concentration was determined by bicinchoninic acid protein assay (Thermo Fisher Scientific).

Western Blotting

Lysates were separated by SDS-PAGE with 10% polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked in 5% BSA in TBST for 1 h, and probed with antibodies to phosphorylated PERK (Cell Signaling Technology; 1:200), phosphorylated eIF2α (Cell Signaling Technology; 1:1,000), total PERK (Cell Signaling Technology; 1:1,000), and total eIF2α (Cell Signaling Technology; 1:1,000) overnight at 4°C. Membranes were washed and incubated with HRP-conjugated goat anti-rabbit (Cell Signaling Technology; 1:2,000) for 1 h. Chemiluminescence was detected with Luminata Crescendo Western HRP Substrate (Millipore) and analyzed with Fujifilm LAS-4000 imager and Multi Gage Software (Fujifilm Life Science).

Statistical Analysis

For ELISA, data are mean IFNγ secretion ± SD or SEM (as indicated). For Western blotting, data are representative of two experiments. Densitometry data are phosphorylation levels normalized by total and relative to that in control-treated cells. Statistical significance was determined by Student's *t*-test, and statistically significant differences are shown for $*p < 0.05$.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

AUTHOR CONTRIBUTIONS

MM and JP contributed to the composition of this manuscript.

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