GENETIC BASIS OF TOLERANCE INDUCTION DEFECTS UNDERLYING THE DEVELOPMENT OF AUTOIMMUNE PATHOLOGIES

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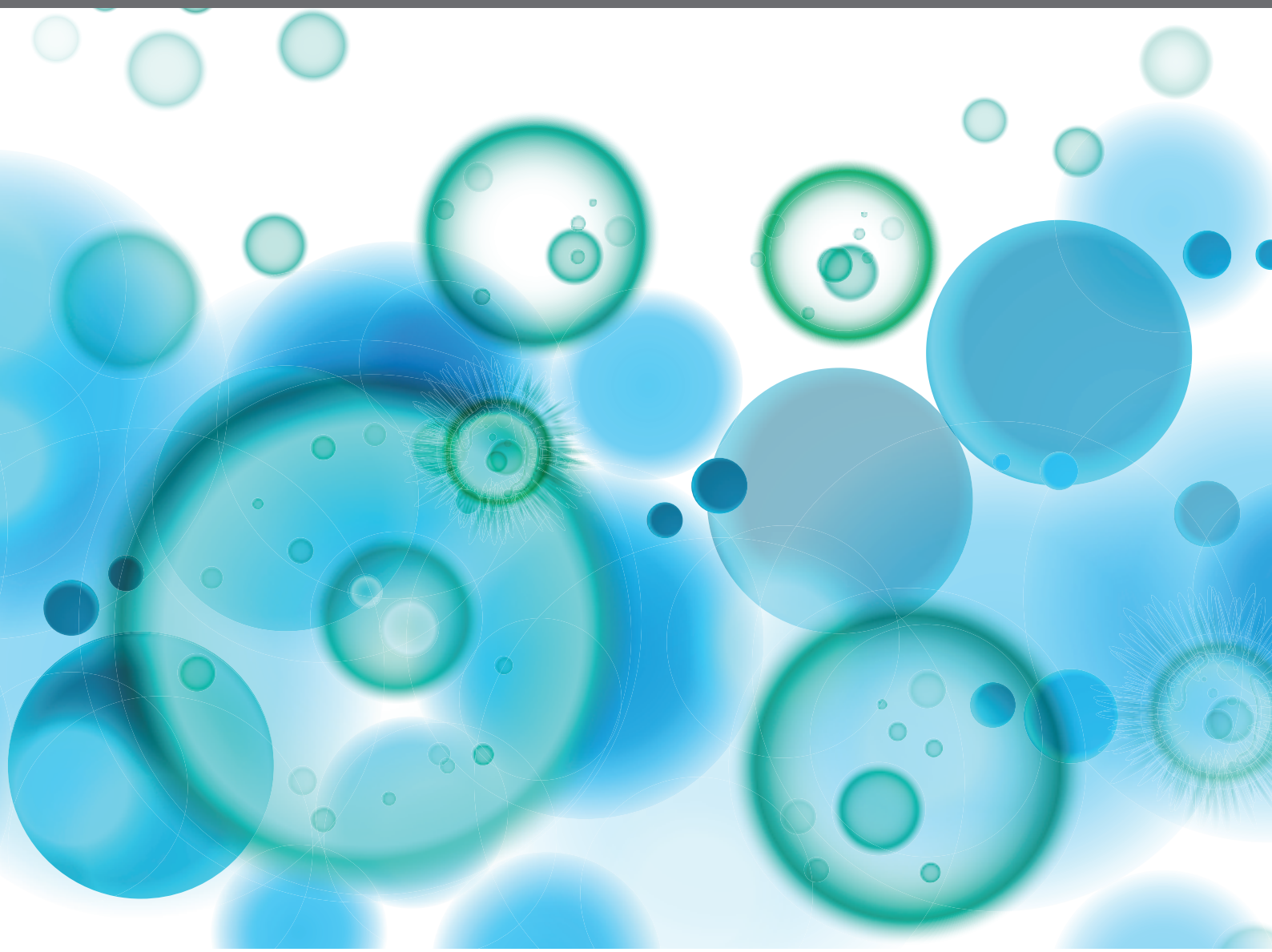

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GENETIC BASIS OF TOLERANCE INDUCTION DEFECTS UNDERLYING THE DEVELOPMENT OF AUTOIMMUNE PATHOLOGIES

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Editorial: Genetic basis of tolerance induction defects underlying the development of autoimmune pathologies

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Editorial on the Research Topic
Genetic basis of tolerance induction defects underlying the development of autoimmune pathologies

In our initial call for review articles for the Research Topic "*Genetic Basis of Tolerance Induction Defects Underlying the Development of Autoimmune Pathologies*" we envisioned four areas of interest for this Research Topic: Area 1 - the genetic basis for tolerance induction defects underlying any single or multiple autoimmune disease states; Area 2 - the controversy concerning how well some animal models, in particular mice, inform the basis of various autoimmune pathologies in humans; Area 3 - how gene variants disrupt Treg/Breg development or activity resulting in autoimmune disease states; and Area 4 - genes x environment interactions contributing to various autoimmune states.

For anyone involved in autoimmunity research, it is not surprising to find the usual suspects in the three review articles that fall under Area 1 of this Topic: MHCs/HLAs, PTPN22, PTPN2, CTLA4, IFIH1 etc. The editors are grateful to the contributing authors for presenting these common players from differing perspectives using both overlapping and unique sources. Here, we would like to call out discussed allelic variants unique to each of the reviews. Kissler's "*Genetic Modifiers of Thymic Selection and Central Tolerance in Type 1 Diabetes*" presents a section on *TAGAP* which may be of interest for those studying thymic migration of developing T-cells. Hocking and Buckner's "*Genetic basis of defects in immune tolerance underlying the development of autoimmunity*" presents a section on *PADI2* and *PADI4* variants in rheumatoid arthritis (RA). *PADI2* and *PADI4* are enzymes responsible for the conversion of arginine to citrulline. Recent work has indicated that post translational modifications

like citrullination can cause the creation of neoepitopes in type 1 diabetes [T1D) (reviewed in (1, 2)]. Finally, Gootjes et al.'s "*Functional Impact of Risk Gene Variants on the Autoimmune Responses in Type 1 Diabetes*" has a section dedicated to CD226. Researchers investigating the CD226 versus TIGIT axis may find this section of interest. Additionally, much of this section focuses on a particular CD226 associated SNP (rs763361) which has been implicated in multiple autoimmune disorders.

T1D researchers have long had to balance the power of the NOD mouse for dissecting the genetic and cellular contributions to T1D with the difficulty in clinical translation [reviewed 19 years apart in (3, 4)]. Three review articles submitted to this Research Topic loosely fall into the parameters of Area 2. Two of the most common complaints of the NOD mouse are: 1) The ease in preventing T1D development and 2) additional autoimmune manifestations beyond T1D. Aubin et al.'s "*The NOD Mouse Beyond Autoimmune Diabetes*" focuses on these other autoimmune manifestations, especially in the context of experimental manipulations that render the strain T1D-resistant. This review argues for the utility of the NOD as a model for understanding a diverse range of autoimmune disorders. Harley et al.'s "*Polygenic autoimmune disease risk alleles impacting B cell tolerance act in concert across shared molecular networks in mouse and in humans*" focuses on the use of risk-gene network analyses impacting B-cell tolerance utilizing T1D and SLE as models. This review showcases how network analyses can pinpoint where monogenic and polygenic versions of these diseases overlap, as well as the extent and areas human and murine disease networks may be similar or different. Additionally, the authors provide multiple possible explanations for the translation gap between mouse and humans with heavy focus on the limited environmental diversity presented to experimental mouse colonies versus the great variability in patients (Topic Area 4). Finally, Rojas et al.'s "*The long and winding road: From mouse linkage studies to a novel human therapeutic pathway in type 1 diabetes*[1]" bridges Topic Areas 1, 2 and 3. Much of this review focuses on the still ongoing journey of identification of a T1D-susceptibility gene in NOD mice to developing and testing a putative future clinical therapeutic.

Two review articles in this Research Topic focus on the biology of Tregs with a special emphasis on their stability. Roach and Morel's[2] article "*Genetic Variations Controlling Regulatory T Cell Development and Activity in Mouse Models of Lupus-Like Autoimmunity*" focuses on this topic in the context of SLE. One section that may be of particular interest delves into the genes that regulate Treg metabolism and how this may affect their functionality. Raugh et. al.'s "*Nature vs. nurture: FOXP3, genetics, and tissue environment shape Treg function*" provides a deep dive into the biology of Tregs covering diverse topics from their heterogeneity, to the genetic, epigenetic, and non-coding RNA control of the development and activity of these cells. Topic Area 4 is also touched upon, as the role of microenvironmental cues, such as microbiome, is briefly covered. Finally, this review discusses how these areas impact possible Treg-based therapy development.

Finally, while we set out to focus solely on review articles for this Topic, two primary research articles were submitted that the Editors felt sufficient to include within the scope of our original goals for this Topic. Di Lorenzo et al.'s "*Natural history of type 1 diabetes on an immunodysregulatory background with genetic alteration in B-cell activating factor receptor: A case report*" details the identification of a clinical case of T1D and common variable immunodeficiency in a patient with a low T1D-risk score putatively caused by the monoallelic H159Y mutation in *TNFRSF13C* (BAFFR). Zhong et al.'s "*Herpesvirus entry mediator on T cells as a protective factor for myasthenia gravis: A Mendelian randomization study*" utilized Mendelian Randomization to identify two SNPs (rs1886730 and rs2227313) in *TNFRSF14* associated with herpesvirus entry mediator (HVEM) expression on T-cells and protection from myasthenia gravis. This adds to the growing body of evidence on the role of HVEM - BTLA interactions in modulating autoimmune diseases, just recently reviewed in (5).

Together, the articles in this Research Topic provide an up-to-date overview on genetic contributions to immune tolerance pathways and autoimmunity.

Author contributions

JJR, LM, Y-GC, DVS were all Topic Editors for this Research Topic and contributed to the preparation of this Editorial. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

Publisher's note

1 Yi-Guang Chen, a co-author on this editorial, is also a co-author on this cited review.

2 Laurence Morel is also a co-author on this editorial.

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MINI REVIEW
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Genetic Modifiers of Thymic Selection and Central Tolerance in Type 1 Diabetes

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Type 1 diabetes (T1D) is caused by the T cell-driven autoimmune destruction of insulin-producing cells in the pancreas. T1D served as the prototypical autoimmune disease for genome wide association studies (GWAS) after having already been the subject of many linkage and association studies prior to the development of GWAS technology. Of the many T1D-associated gene variants, a minority appear disease-specific, while most are shared with one or more other autoimmune condition. Shared disease variants suggest defects in fundamental aspects of immune tolerance. The first layer of protective tolerance induction is known as central tolerance and takes place during the thymic selection of T cells. In this article, we will review candidate genes for type 1 diabetes whose function implicates them in central tolerance. We will describe examples of gene variants that modify the function of T cells intrinsically and others that indirectly affect thymic selection. Overall, these insights will show that a significant component of the genetic risk for T1D – and autoimmunity in general – pertains to the earliest stages of tolerance induction, at a time when protective intervention may not be feasible.

Keywords: type 1 diabetes, Genome Wide Association Study (GWAS), thymic selection, autoimmunity, mouse model

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INTRODUCTION

Type 1 diabetes (T1D) is caused by the autoimmune destruction of pancreatic beta cells that produce insulin. The etiology of T1D has been investigated for more than 50 years (1). Animal models for autoimmune diabetes have been available for more than 40 years (2). And yet, the precise events that lead to beta cell autoimmunity remain incompletely understood. It is clear that T lymphocytes are key drivers of beta cell killing, as evidenced by genetic data, histological observations and mechanistic studies. However, a discrete trigger for beta cell autoimmunity, if it exists, is still being searched for. Environmental factors undoubtedly play a role in sensitizing individuals to type 1 diabetes. Both commensal microbes and viral infections have been implicated in diabetes etiology (3, 4). Not surprisingly, disease risk is also heavily modulated by genetic variants. The most prominent genetic risk factor for T1D is the highly polymorphic MHC region, driven by several high-risk HLA haplotypes (5, 6). In addition, a significant number of non-HLA genetic loci contribute to the heritable component of diabetes risk (7, 8). Linkage and association studies in the pre-genomic era uncovered the first non-HLA risk variant for T1D in the *Insulin* locus (9). This was followed a decade later by a risk variant in the *CTLA4* gene (10). In the early 2000's, a

handful of additional risk gene variants were discovered that included the *CD25*, *PTPN22* and *IFIH1* loci (11–13). In 2007, the results from the first genome wide association study (GWAS) conducted for an autoimmune disease revealed a much vaster landscape of risk variants for T1D across the genome (14). Subsequent GWAS with increasing statistical power have now brought the total of T1D-associated loci to more than 60 (7, 8, 15). Many GWAS for other autoimmune diseases followed the first T1D GWAS. A key insight from these association studies was that a large number of risk loci are shared between immune-mediated diseases. Only a minority of disease-associated genetic variants appear to be specific for T1D, while the majority seem to pertain more broadly to the risk of autoimmunity overall. This feature of shared genetic risk between diseases pointed to the fact that many disease variants impact basic immune regulatory mechanisms. Despite the enormous progress that GWAS have enabled in our understanding of disease genetics, it has been challenging to conclusively ascribe a function to individual disease variants. Notwithstanding, functional studies have highlighted the potential role of several T1D candidate genes in fundamental aspects of immune tolerance. This review will highlight several genes associated with the risk of T1D that impinge on the selection of the T cell repertoire in the thymus.

The development of T cells entails the migration of T cell progenitor cells from the bone-marrow into the thymic cortex, where the cells mature through several stages of $CD4^-CD8^-$ (double-negative or DN) thymocytes into $CD4^+CD8^+$ (double-positive or DP) cells. These DP thymocytes undergo a process of positive selection conditional on productive antigen-receptor interactions with thymic antigen presenting cells that include thymic epithelial cells (TECs). Positively selected cells further mature into single-positive (CD4SP or CD8SP) T cells that go on to migrate into the thymic medulla, the compartment where most of the negative selection takes place and that curates the T cell repertoire to eliminate highly self-reactive clones. Fully mature thymocytes that have undergone selection in the cortex and medulla then enter the circulation to become part of the immune surveillance machinery. The process of thymic selection that bars many, though not all, self-reactive clones from exiting the thymus is a key component of central tolerance – a protective quality control that occurs in a central location prior to mature T cells interacting with other cells throughout the body.

Defects in central tolerance lead to autoimmunity. In the most severe cases, single gene mutations can cause multiple immune pathologies. For example, mutations in the *FOXP3* gene prevent the induction of functional regulatory T cells in the thymus that are critical to the control of immunity. As a result, individuals with a mutant *FOXP3* allele develop the Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome that includes type 1 diabetes (16, 17). Another example is the *AIRE* gene whose disruption diminishes the expression of tissue-restricted antigens (TRAs) within the medullary thymic epithelium (18). TRA expression is necessary for the deletion of tissue-reactive T cell clones during thymic selection. Patients with deleterious *AIRE* mutations develop Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED) that presents with multiple pathologies, often including type 1 diabetes (19, 20). The monogenic diseases IPEX and APECED are extreme examples of pathologies that arise as a consequence of defective central tolerance.

In this review, we will discuss more common gene variants associated with autoimmune disease including T1D. These common variants cause a much more subtle perturbation of central tolerance. However, even minor effects contribute to the overall risk of autoimmunity when compounded with other defects in immune tolerance.

GENETIC MODIFIERS OF THYMOCYTE FUNCTION

Among the genes implicated in disease risk, several pertain to antigen receptor or cytokine receptor signaling. Changes in stimulatory cues that thymocytes receive during thymic selection significantly impact their developmental trajectory.

The first two examples of genes that modify thymocyte signaling encode the phosphatases PTPN2 and PTPN22. Both belong to the protein tyrosine phosphatase non-receptor family and impact key signaling events involved in the positive selection of thymocytes.

PTPN2

The first GWAS for autoimmunity identified the single nucleotide polymorphism (SNP) *rs2542151* located 5.5kb upstream of the *PTPN2* on chromosome 18p11 (14, 21). A subsequent study further dissected this region and associated two intronic SNPs in the *PTPN2* gene with T1D (22). Both these SNPs are in strong linkage disequilibrium with *rs2542151*. Because *PTPN2* is the only gene in this region, it emerged as the strongest causal candidate for this particular disease association.

PTPN2 encodes a phosphatase, and its expression is not restricted to immune cells. In fact, like many T1D-associated genes, PTPN2 is pleiotropic and affects the function of multiple cell populations including beta cells (23, 24). The phosphatase PTPN2 attenuates receptor signaling by desphosphorylating either receptors directly (e.g. InsR, EGFR), or their signaling transducers (e.g. SRC family kinases, JAKs, STATs). Most relevant to thymocyte development, PTPN2 decreases T cell receptor signaling *via* dephosphorylation of FYN and LCK, but also STAT5 phosphorylation that mediates IL-2 signaling.

Changes in PTPN2 function were shown to modify thymocyte development, positive selection and thymic lineage commitment of αβ TCR versus γδ TCR T cells (25, 26). Together, these effects have implications for the functionality of the T cell repertoire. Exactly how *PTPN2* variants skew T cells towards autoreactivity is difficult to dissect owing to the gene's role in both thymocyte development, in the function of mature peripheral T cells and in the biology of multiple other cell populations relevant to T1D.

PTPN22

PTPN22 is another tyrosine phosphatase associated with T1D (12), but in this case, expression is restricted to lymphocytes.

PTPN22 encodes the lymphoid-associated phosphatase LYP that interacts with several mediators of antigen receptor signaling, including LCK, ZAP70 and TCRζ. Of interest, the genetic variant associated with T1D is located in the coding region of *PTPN22*. This is unusual because most disease-associated variations are intergenic or intronic, making it difficult to study their function. In contrast, the effects of the *PTPN22* disease-associated allele has been studied in more detail and have even been replicated in rodent models using genetic engineering.

The risk variant is a C to T substitution at position 1858 of the coding region, effecting an amino acid change in the protein sequence of LYP (R620W). This mutation has a direct implication for LYP function. The R620W substitution was shown to disrupt LYP's interaction with a kinase, CSK, that negatively regulates phosphatase activity (12). This would be consistent with the first functional description of the risk variant that suggested a gain of function (27). The precise effect of the R620W mutation has been debated, however. Researchers modelled this mutation in mice by introducing an equivalent R619W substitution in PEP, the mouse ortholog of LYP (28). Data from this model initially suggested that mutant PEP was prone to faster degradation. This interpretation was later disputed and the preponderant hypothesis remains that the risk variant of *PTPN22* is a gain-of-function allele (29).

Additional studies in *Ptpn22* knockout and knockdown animals showed that the loss of PEP increased the frequency of regulatory T cells (Tregs) and suggested that animals were protected against autoimmunity (30, 31). These data were supported by the observation that the disease variant of *PTPN22* was associated with the frequency of circulating Tregs in human (32). Notably, *Ptpn22* deficiency increased the frequency of Tregs in the thymus (30), and this could relate to increased TCR signaling in the absence of the phosphatase that skews thymocytes towards a Treg transcriptional program. Extra-thymic effects of *Ptpn22* variation were also observed in both T and B lymphocytes, as could be expected given the phosphatase's role in antigen-receptor signaling (29, 33). Ultimately, it is difficult to establish with certainty which immune cell population is most affected by *PTPN22* variation. Notwithstanding, the risk variant of *PTPN22* has a strong effect on thymic selection, with implications for the effectiveness of central tolerance.

IL2RA

IL2RA encodes the high-affinity α chain of the IL-2 receptor and is also known as CD25. IL-2 signaling is critical to T cell development and function. Significantly, IL-2 is pivotal in the lineage commitment of Tregs in the thymus (34). Tregs are a key modifier of disease risk, and a target of experimental therapies for autoimmune disease. For example, low-dose IL-2 administration has been shown to expand Tregs in both humans and animal models (35, 36), where IL-2 therapy is a potent therapy of autoimmune diabetes.

IL2RA risk variants diminish IL-2 signaling (37, 38). This effect can be predicted to diminish both the development and maintenance of a functional Treg compartment that relies on IL-2 signaling both in the thymus and periphery. Complete IL-2 deficiency does not prevent T cell development (39) but causes severe inflammatory disease including colitis (40). While T1D-associated *IL2RA* variants lead to much more subtle changes in signaling, the gene has a central impact on immune regulation, starting with the generation of Tregs in the thymus.

TAGAP

The signals that developing thymocytes receive and that direct their fate are tightly regulated. Part of this regulation relies on the spacial segregation of cues that guide positive selection versus negative selection. The first stages of thymocyte maturation occur in the thymic cortex. Once DP cells have been positively selected, they migrate into the thymic medulla to interact with a variety of antigen presenting cells that include medullary TECs (mTECs) presenting TRA for negative selection. The migration of thymocytes from the cortex to the medulla depends on both chemokines and adhesion molecules (41). *TAGAP*, the candidate gene for a genomic region associated with multiple immune diseases including T1D (15), plays a key role in releasing thymocytes from their cortical niche and allowing migration into the thymic medulla (42). This was demonstrated in a study of *Tagap* deficient mice, where thymocytes that recently underwent positive selection as measured by their expression of CD69 were retained in the thymic cortex. *Tagap* was found to mediate plexinD1 signaling that releases β1 integrin-dependent adhesion in the cortex (42). PlexinD1 is upregulated on the surface of positively selected thymocytes, allowing its ligand, sema3a, to facilitate chemotaxis towards the thymic medulla (43). A decrease in *TAGAP* expression diminishes the propensity of thymocytes to migrate from the cortex into the medulla. Longer dwell times in the cortex may allow maturation of the cells in an environment where they do not undergo the stringent negative selection imposed onto them in the medulla. This would lead to deficient tolerance induction by failing to delete autoreactive clones or to select Tregs that depend on interactions with medullary antigen presenting cells. Changes in the selection of thymocytes were observed in *Tagap* deficient mice, pointing to a role for this T1D risk gene in central tolerance (42).

The genes described above all relate to thymocyte-intrinsic pathways involved in the responsiveness to extracellular cues. Gene variants associated with T1D modify the sensitivity of thymocytes to TCR stimulation, cytokine stimulation and to chemotactic cues. Together, these effects can significantly redirect the fate of developing T cells with autoreactive potential and diminish either their deletion or their inclusion into the Treg compartment. Next, we will discuss disease risk genes that operate extrinsically by modifying the antigenic landscape that thymocytes navigate during selection.

GENETIC MODIFIERS OF ANTIGEN PRESENTATION

A key function of the thymus is the selection of a TCR repertoire that is functional (through positive selection) and not harmful (through negative selection). Negative selection in particular relies on the presentation of antigens that T cell may

encounter in various tissues (44). Many of these antigens are encoded by genes whose expression is restricted to specialized cell types. Most relevant for type 1 diabetes is insulin, one of a key antigens driving beta cell autoimmunity. More generally, tissue restricted antigens (TRAs) need to be presented within the thymus to allow central tolerance to take effect against these gene products. TRA presentation in the thymic medulla relies on three components. First, the antigen itself needs to be expressed within the thymus. Second, the antigen processing machinery needs to generate peptides from this antigen. Third, MHC molecules need to be present that are able to bind particular peptides so they can be presented on the cell surface. All three of these steps are subject to genetic control, as illustrated by T1D-associated genes discussed below.

INS

A genomic region that included the insulin gene was the first non-HLA locus associated with T1D almost 30 years ago (9). The disease-associated haplotype encompasses a variable number of tandem repeats (VNTR) region 5' of the *INS* gene. Initial analyses of the effect of the different VNTR alleles, termed class I, II and III based on their length, described a very small change in insulin expression associated with this polymorphism in fetal pancreas (45). How this subtle change would impact disease risk was unclear. Upon replication of this finding, Todd and colleagues speculated that the polymorphism may impact insulin expression in the thymus, rather than in the pancreas itself (46). Two studies published back to back in 1997 corroborated this hypothesis. The two independent papers reported that the protective haplotype that contains class III VNTR increased insulin expression in the thymus by 2-3 fold (47, 48).

In support of the hypothesis that VNTR alleles affected central tolerance, mouse models provided evidence that thymic expression of insulin had a strong effect on disease risk (49, 50). Unlike humans, mice harbor two insulin genes on separate chromosomes. While *Insulin 2* is expressed in both thymus and pancreas, *Insulin 1* is only expressed in beta cells. Deleting *Insulin 2* does not cause insulin insufficiency, because *Insulin 1* is fully functional and able to regulate glycemia on its own. However, in the absence of *Insulin 2*, thymic tolerance against insulin is severely impaired and the risk of diabetes is increased. While direct evidence in human for insulin's role in central tolerance is lacking, it is also known that thymic *INS* expression is dependent on *AIRE* (51), whose deficiency leads to multiple pathologies that include T1D. Together, these observations support a key role for thymic insulin expression in establishing central tolerance to beta cell antigen.

CLEC16A

TRA expression in the thymus is not sufficient in itself to ensure presentation of relevant peptides. Antigens need to be processed prior to being loaded onto MHC molecules for presentation on the surface of thymic epithelial cells or hematopoietic antigen presenting cells. One of the pathways involved in intracellular antigen processing and delivery to MHC compartments is autophagy (52). While MHC class I peptides are typically generated by the proteosome, MHC class II antigens rely on lysosomal degradation pathways. In this context, autophagy can shuttle endogenous proteins towards the lysosomal compartment for digestion and subsequent loading onto MHC class II molecules. The importance of autophagy for central tolerance had first been demonstrated by Klein and colleagues (53). TECs have remarkably high levels of constitutive autophagy. Disruption of autophagy in thymic epithelium caused multi-organ inflammation, indicative of defective central tolerance. This study demonstrated the importance of autophagy for antigen presentation in the thymic epithelium whose TRA expression is indispensable to central tolerance.

CLEC16A was shown to modulate autophagy, and the first indication of the gene's function came from *Drosophila* studies where the *CLEC16A* ortholog *Ema* was implicated in the endolysosomal pathway (54), with subsequent data indicating a role in autophagy (55). Knockdown of *CLEC16A* was found to be highly protective in the NOD mouse model for type 1 diabetes (56). In this study, protection was not derived from *Clec16a* deficiency in immune cells but rather from gene knockdown in thymic epithelium. The loss of *Clec16a* diminished TEC autophagy and had repercussions for thymic selection and for the reactivity of the T cell repertoire (56).

CLEC16A is a prime example of a gene whose function is not obviously related to immune function. Yet, many cellular pathways contribute to robust thymic function that is critical for the establishment of central tolerance. It is likely that other T1D-associated genes whose function is not yet well characterized could affect immune tolerance in similarly unexpected ways.

MHC Region

The final component of antigen presentation is the MHC molecule itself, encoded by HLA genes on Chromosome 6. When a TRA is expressed and processed into peptides suitable for MHC loading, the repertoire of peptides that are presented on the surface of thymic antigen presenting cells depends not only on the pool of peptides available but also from the binding preference of different HLA alleles. The HLA locus is the strongest genetic determinant for T1D risk (57). A handful of HLA haplotypes confer very high risk, while a few haplotypes are protective (58, 59). It is difficult to ascertain the stage at which HLA polymorphism most impacts pathogenesis, because MHC molecules are required throughout the lifetime of T lymphocytes. MHC/peptide complexes are required for T cell selection in the thymus, T cell maintenance in the periphery, and for the initiation of T cell responses in secondary lymphoid organs by antigen presenting cells bearing MHC class I and class II molecules.

The strongest T1D association in the HLA region derives from the HLA-DQ haplotype that encode MHC class II molecules. HLA-DQ2 (linked to HLA-DR3) and HLA-DQ8 (linked to HLA-DR4) are the most significant determinants of disease risk. Both haplotypes increase risk on their own, particularly in homozygous individuals. But their effect is even stronger in combination (when both HLA-DQ2 and -DQ8 are present) (60). This synergy is thought to be caused by trans-heterodimers, where the alpha and beta chains of the two

different alleles (DQ2 and DQ8) are combined to form an alpha/beta heterodimer different from either DQ2 or DQ8 cis-heterodimers (61). One possible explanation for the high risk conferred by these particular HLA heterodimers is their preferential binding of peptides from beta cell antigens in a manner that is ineffective to enforce central tolerance yet sufficient to drive an immune response in the pancreas. Evidence for this mechanism lead to the hypothesis that peptide-HLA interactions in the low affinity range may be more likely to promote autoimmunity than high affinity binding peptides (62). Consistent with this notion, the T cell receptor of several $CD4^+$ autoimmune T cell clones bind peptide-HLA complexes in unconventional, suboptimal conformations (63). While this weaker binding may derive in part from the TCR structure itself, the data support an overall model where the strength of interaction between autoreactive clones and their cognate peptide-HLA complexes is pivotal in bypassing negative selection. Therefore, the structure of HLA molecules, dictated by their genetic sequence, is central to the development of autoreactive clones in the thymus.

The same principles apply to MHC class I required for thymic selection of $CD8^+$ T cells. Again, the structure of MHC class I molecules determines the pool of peptides that can be presented to developing thymocytes and the avidity of the TCR-MHC/peptide interactions at play during selection. MHC class I molecules are encoded by HLA-A, -B and -C genes. Rigorous analyses of the MHC region have shown that both HLA-A and HLA-B polymorphisms associate with the risk of T1D independently of the major effect of the MHC class II region (64). Subsequent experiments where the high-risk alleles HLA-B*39 or HLA-A*02 were expressed in transgenic mice devoid of endogenous MHC class I showed that these MHC alleles significantly changed the selection of the TCR repertoire (65). These data lend further support to a model where particular HLA alleles promote the thymic selection of an autoreactive repertoire prone to causing T1D.

CONCLUSIONS

The list of disease-associated regions described in this brief review is not exhaustive, and other type 1 diabetes risk gene variants are likely to affect thymocyte selection by a variety of mechanisms. The examples cited above illustrate the wide range of mechanisms by which gene variations can modify T cell selection. Some risk genes operate cell intrinsically to desensitize thymocytes to negative selection or to diminish their likelihood of adopting a regulatory program. Other risk variant act extrinsically to shape the MHC/peptide landscape that fine-tunes the TCR repertoire and directs thymocytes to different selection trajectories. Most of these risk loci, whether they mediate intrinsic or extrinsic effects, have pleiotropic effects that can not only span the lifetime of a T cell but also alter the biology of other immune cell types. The result is a complex interplay of changes at many stages of immune function. To dissect individual components and to ascribe causal function to single gene variants remains exceedingly difficult and uncertain. Notwithstanding, our understanding of the genetics of autoimmunity and of T1D in particular have made great strides in the past 15 years. We now have a better grasp of the many fundamental changes in immune development and function that underlie autoimmunity. Defective central tolerance is almost certainly an important prerequisite for autoimmune diabetes, and one that is subject to genetic control by common variants.

When GWAS for T1D were first performed, they held much promise to yield new insight into disease etiology. In addition, there was hope that new knowledge of risk genes would lead to the rational design of novel interventions. This optimism has been significantly dampened by the realization that 1) identifying exact causal variants for disease-associated regions is often very difficult, 2) the precise functional contribution to pathogenesis of the many risk genes is still largely unresolved, and 3) pleiotropic effects of many causal variants would decrease the specificity of an intervention that targets these T1D risk genes. In the context of central tolerance, an additional challenge is that many of the changes described herein occur early in development, with long-lasting effects for immune function. Targeting thymic selection for disease prevention is possible in experimental models. This was shown by intrathymic islet transplantation in very young NOD mice (66, 67). However, this approach is unlikely to be effective at late pre-diabetic stages or in newly diagnosed patients, where islet autoimmunity is already established and ongoing. Notwithstanding, the development of an immune intervention for T1D based on disease genetics remains an enticing idea. Ultimately, only a better understanding of causal gene variants can help turn this idea into a testable clinical intervention.

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REVIEW
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The NOD Mouse Beyond Autoimmune Diabetes

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Autoimmune diabetes arises spontaneously in Non-Obese Diabetic (NOD) mice, and the pathophysiology of this disease shares many similarities with human type 1 diabetes. Since its generation in 1980, the NOD mouse, derived from the Cataract Shinogi strain, has represented the gold standard of spontaneous disease models, allowing to investigate autoimmune diabetes disease progression and susceptibility traits, as well as to test a wide array of potential treatments and therapies. Beyond autoimmune diabetes, NOD mice also exhibit polyautoimmunity, presenting with a low incidence of autoimmune thyroiditis and Sjögren's syndrome. Genetic manipulation of the NOD strain has led to the generation of new mouse models facilitating the study of these and other autoimmune pathologies. For instance, following deletion of specific genes or *via* insertion of resistance alleles at genetic loci, NOD mice can become fully resistant to autoimmune diabetes; yet the newly generated diabetes-resistant NOD strains often show a high incidence of other autoimmune diseases. This suggests that the NOD genetic background is highly autoimmune-prone and that genetic manipulations can shift the autoimmune response from the pancreas to other organs. Overall, multiple NOD variant strains have become invaluable tools for understanding the pathophysiology of and for dissecting the genetic susceptibility of organ-specific autoimmune diseases. An interesting commonality to all autoimmune diseases developing in variant strains of the NOD mice is the presence of autoantibodies. This review will present the NOD mouse as a model for studying autoimmune diseases beyond autoimmune diabetes.

Keywords: NOD mice, polyautoimmunity, thyroiditis, neuropathy, biliary disease

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HIGHLIGHTS

1. The Non-Obese Diabetic (NOD) mouse as a model of multiple autoimmune diseases
2. Congenic and transgenic NOD mice represent relevant models of human pathologies
3. Spontaneous occurrence of autoimmune thyroiditis, neuropathy and biliary diseases
4. The NOD mouse can be used to study polyautoimmune phenotypes

INTRODUCTION TO THE NON-OBESE DIABETIC MOUSE STRAIN

Since its first description by Makino et al. in 1980 (1), the Non-Obese Diabetic (NOD) mouse strain represents the only mouse model that spontaneously develops autoimmune diabetes (2–4). The NOD strain is originally derived from Cataract Shinogi (CTS) mice, an inbred subline of the outbred ICR mouse strain, which develop cataracts (1, 2). In an effort to generate a mouse model for insulin-dependent diabetes, CTS mice with either low or high fasting glucose levels were further interbred. Eventually, mice from the 'normoglycemic' colony presented with diabetic symptoms, namely polyuria and glycosuria. These mice were selected for breeding, establishing the original NOD mouse colony (1, 5). Importantly, the autoimmune diabetes pathology in NOD mice shares several characteristics with human type 1 diabetes (T1D) (1, 3, 6, 7). For instance, the major histocompatibility class (MHC) locus is a defining autoimmune diabetes susceptibility factor in both mice and humans, with a common amino acid substitution in an MHC class II gene (4, 8). Studying the NOD mouse has considerably improved our understanding of this autoimmune disease, facilitating the identification of genetic variants contributing to disease susceptibility, of various immune cells causing pancreatic β-cell destruction, and of environmental contributors to disease susceptibility (6, 7, 9, 10). For further information on the use of NOD mice in dissecting the pathophysiology of autoimmune diabetes, the readers are referred to the following reviews on the topic (2–4).

This review will instead focus on the other organ-specific autoimmune diseases that spontaneously develop in NOD mice as well as in genetically manipulated NOD mice. Specifically, several NOD congenic mice and NOD genetic knockout mice are protected from autoimmune diabetes. In these diabetes-resistant mice, other autoimmune diseases spontaneously arise, such as autoimmune thyroiditis, autoimmune polyneuropathies, and autoimmune biliary disease. The use of the NOD mouse and its variants to study polyautoimmune syndromes will also be discussed. While autoantigen-specific T cell responses are a critical part of the pathology in autoimmune diabetes (6, 11), this review will more broadly discuss the presence of immune cells in the target tissues as well as the presence of autoantibodies in variants of the NOD mouse model, for each autoimmune pathology.

AUTOIMMUNE THYROID DISEASE

Autoimmune thyroid disease (AITD) includes Hashimoto's thyroiditis, Graves' disease (autoimmune hyperthyroidism), neonatal Graves' disease, and postpartum thyroiditis (12). All forms of AITD are characterized by the presence of immune infiltrates (in variable amounts) in the thyroid gland and particularly by the presence of IgG class autoantibodies directed towards specific thyroid autoantigens, namely thyroglobulin, thyroid peroxidase (TPO), and the thyrotropin receptor (TSHR) (13). Of note, while some of these autoantibodies are present in the serum of many individuals with normal thyroid function, the presence of TPO is significantly associated with thyroid disease (14). Interestingly, the prevalence of AITD is more frequent in people living with T1D (PWT1D) than in the general population (15–18). Based on the study of Hwang et al., the prevalence of thyroglobulin and TPO thyroid autoantibodies in PWT1D is around 30% (19), whereas the prevalence in the general population is approximately 10% (14).

The NOD Mouse as a Model of Autoimmune Thyroiditis

As in PWT1D, NOD mice can develop spontaneous autoimmune thyroiditis (SAT). In NOD mice, the cumulative incidence at one year ranges from ~5% to ~15% (20, 21). In both humans and mice, an iodine-rich diet accelerates the development of the disease (21, 22). The iodide excess is toxic for thyroid cells by a mechanism involving oxidative stress (23). This parallel between humans and mice highlights the relevance of the NOD mouse model for understanding autoimmune thyroiditis pathology (24). However, there are limits associated with the use of the NOD mice for the study of SAT. For one, NOD mice have a high incidence of autoimmune diabetes, especially in females where it reaches 70 to 90% by 30 weeks (25). This presents a challenge when attempting to isolate the immunological factors that specifically drive SAT independently of the immune response to autoimmune diabetes. In addition, the incidence of SAT is low in NOD mice in absence of an iodine-rich diet (20), such that very large cohorts of mice must be used to characterize the progression of the pathophysiology. Currently, an autoimmune diabetes-resistant genetic derivative of the NOD mouse model, the NOD.$H2^{h4}$ congenic mouse, is more commonly used to study SAT.

NOD.$H2^{h4}$ congenic mice were originally generated to determine the impact of the MHC class II locus on diabetes and insulitis development (26, 27). Specifically, the NOD.$H2^{h4}$ bears the thyroiditis-prone $H2^{h4}$ MHC locus from the B10.A(4R) mouse strain, composed of H-$2K^{k}$ and H-$2D^{b}$ for MHC class I, and I-A^{k} for MHC class II (26) (**Figure 1**). In contrast to the low ~5% to ~15% incidence of SAT in NOD mice, 50%-70% of NOD.$H2^{h4}$ congenic mice develop SAT (21, 26, 28). Moreover, subjecting the mice to an iodine-rich diet enhances the severity and the incidence of thyroid lesions in both NOD and NOD.$H2^{h4}$ congenic mice, which can reach an incidence of nearly 100% in both strains (21, 22, 24, 28–30).

While NOD.$H2^{h4}$ mice develop SAT, this strain is completely protected from diabetes onset (21, 26, 28). This suggests that the progression to diabetes is not necessary for SAT development, and that the break of tolerance towards thyroid autoantigens is favored by the $H2^{h4}$ MHC haplotype, while the $H2^{g7}$ MHC haplotype is necessary for diabetes onset. This observation also revealed that the organ-specific autoimmune susceptibility determined by the NOD genetic background can be shifted to other organs by modification of different genetic loci. In other words, different MHC loci in NOD mice can predispose to different organ-specific autoimmune diseases. Studies in families with T1D and AITD also revealed a strong genetic link to the MHC class II locus (17, 31, 32). Specifically, the MHC

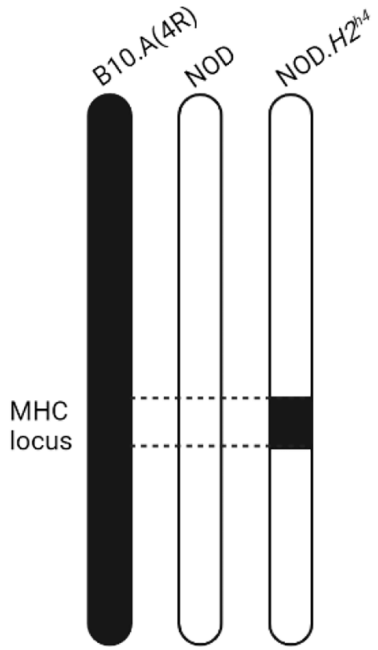

FIGURE 1 | The NOD.$H2^{h4}$ congenic mouse model. Representation of the mouse chromosome 17 from the parental B10.A(4R) (left), parental NOD (middle), and congenic NOD.$H2^{h4}$ (right) strains. By backcrossing NOD mice to B10.A(4R), the thyroiditis-prone $H2^{h4}$ MHC locus from chromosome 17 of the parental B10.A(4R) mouse has been selected at each backcross generation to replace in the NOD-derived $H2^{g7}$ MHC locus, resulting in the NOD.$H2^{h4}$ congenic mouse.

class II haplotype DR3-DQB1*0201 is a risk haplotype shared by both T1D and AITD (17, 31), while HLA-DR3 is specifically linked to T1D susceptibility (31, 32). Therefore, in both mice and humans, the MHC locus shifts the autoimmune response towards given target organs.

To assess the contribution of the MHC locus in thyroiditis development in mice, a comparative study was done using the NOD.$H2^{h4}$ mouse and the NOD.$H2^{k}$ mouse (28). The primary difference between the NOD.$H2^{h4}$ and NOD.$H2^{k}$ mice is the presence of I-E MHC class II molecule in the $H2^{k}$ locus (26, 28). After exposure to an iodine-rich diet, the extent of the autoimmune thyroiditis and the levels of thyroglobulin and TPO autoantibodies were higher in the NOD.$H2^{h4}$ mice than in the NOD.$H2^{k}$ mice, in which TPO antibodies were essentially absent (28). This suggests that variants in the MHC locus between these two mouse strains influence the thyroid autoantibody profile (28). Consequently, the NOD.$H2^{h4}$ mouse, which develops both thyroglobulin and TPO autoantibodies, is arguably the most representative mouse model of human AITD pathology (28).

Immune Cells Infiltration Within the Thyroid Gland

One of the key characteristics shared between AITD in humans and SAT in the NOD.$H2^{h4}$ mouse is the infiltration of immune cells within the thyroid gland. The recruitment and migration of lymphocytes in this gland is supported by adhesion molecules expressed on endothelial cells (33). Of interest, whereas NOD mice express high levels of ICAM-1 on thyrocytes, CBA/J, A/J, BALB/c, and C57 mice show little to no expression of ICAM-1 (33). The high expression of ICAM-1 on thyrocytes driven by the NOD genetic background (and thus also present in NOD.$H2^{h4}$ mice), is a genetic risk factor to SAT. ICAM-1 promotes the recruitment of immune cells into the thyroid, which then target specific thyroid autoantigens (33).

In the NOD.$H2^{h4}$ mouse model, as in people living with AITD, the thyroid immune cell infiltrate is predominantly composed of $CD4^{+}$ and $CD8^{+}$ T cells, B cells, macrophages, natural killer cells, and dendritic cells (34). Still, in humans, information regarding the kinetics of the infiltration within the thyroid is limited. To better understand the kinetics of thyroid cell infiltration, Bonita et al. took advantage of the NOD.$H2^{h4}$ mouse model (34). They show that the immune cell infiltration in the NOD.$H2^{h4}$ thyroid begins with $CD4^{+}$ T cells, followed by $CD8^{+}$ T cells and macrophages, and finally by B cells (34).

$CD4^{+}$ and $CD8^{+}$ T Cells

T cells are part of the adaptive arm of the immune response and self-reactive T cells are necessary and sufficient for onset and

progression of many autoimmune diseases (35–38). Elimination of $CD4^+$ and $CD8^+$ T cells completely prevents thyroiditis development by suppressing thyroid infiltration and thyroid autoantibody production in the NOD mouse, even on iodine-supplemented diet (24). This suggests that T cells are necessary for SAT (24). In addition to promoting thyroid autoantibody production by B cells (30, 34), $CD4^+$ T cells are also required for the maintenance of inflammation in the thyroid gland (30). IFN-γ, secreted by $CD4^+$ T cells, damages thyrocytes (30, 34) and induces the expression of MHC class II and adhesion molecules on thyrocytes, ultimately resulting in the recruitment of other immune cells, such as $CD8^+$ T cells, macrophages, B cells, and plasma cells (30, 34). $CD8^+$ T cells also contribute to disease progression by secreting cytokines, namely IFN-γ and TNFα (34), and by mediating perforin/granzyme-dependent lysis of thyrocytes, resulting in severe damage to the thyroid gland (34).

iNKT Cells

Invariant Natural Killer T (iNKT) cells have first been identified as an unusual T cell population expressing both T cell receptors (TCR) and the NK markers (NK1.1, NKG2D, and Ly49) (39–41). iNKT cells recognize antigens by the non-polymorphic MHC class I-like molecule CD1d (39–41). These cells exhibit a wide array of immunological functions such as the production of chemokines and cytokines, cytolytic activity, and activation and recruitment of other cell types (39, 41). Of interest, an indirect pathogenic role of iNKT cells has been suggested in autoimmune thyroiditis (42). Sharma et al. generated two iNKT cell lines derived from NOD.$H2^{h4}$ splenocytes (42). After stimulation with thyroglobulin, these iNKT cell lines produce cytokines such as IFN-γ, TNF-α, IL-2, IL-4, and IL-10 (42). The adoptive transfer of thyroglobulin-stimulated iNKT cell lines enhanced autoimmune thyroiditis in NOD.$H2^{h4}$ mice fed with an iodine-rich diet (42), suggesting that iNKT cells enhanced autoimmune thyroiditis in NOD.$H2^{h4}$ mice. In addition, it was reported that the spleen of NOD.$H2^{h4}$ mice contains more iNKT cells than BALB/c mice (43), suggesting a link between iNKT cell abundance and SAT susceptibility. With the availability of CD1d-tetramers allowing to quantify iNKT cells more precisely, we revisited this concept. In contrast to the previous report (43), we observed a higher percentage and number of iNKT cells in the spleen of BALB/c mice when compared to NOD.$H2^{h4}$ mice (**Figure 2**). Further studies are required to understand the true implication of these cells in the development of autoimmune thyroiditis.

Regulatory T Cells (Treg) and T-Helper (Th) Cells

Tregs are immunomodulatory cells that prevent autoimmune responses and thus could be used as a therapeutic in autoimmune diseases (44). Accordingly, a depletion of $CD25^+$ Tregs before subjecting the mice to an iodine-rich diet increases the severity of thyroiditis in NOD.$H2^{h4}$ mice (45), suggesting an important role for Tregs in the control of autoimmune thyroiditis.

Apart from Tregs, other Th subsets differentiated from naïve $CD4^+$ T cells include Th1, Th2 and Th17, which are primarily distinguished based on the expression of specific transcription factors and their cytokine profile (46). Th1, Th2 and Th17 respectively express T-BET, GATA-3 and RORγt and secrete IFN-γ, IL-4 and IL-17 as their prototypical cytokine (47). In NOD.$H2^{h4}$ mice, the presence of IFN-γ in the thyroid before the onset of lesions suggests that Th1 cytokines may play an important role in the initiation of autoimmune thyroiditis (30, 34). In addition, Th2 cytokines, such as IL-4 and IL-13, are maximal after thyroid lesions develop suggesting that these cytokines are involved in the late chronic phase of the disease, maintaining the thyroid inflammatory response (30). Moreover, NOD.$H2^{h4}$-IFN-$\gamma^{-/-}$, NOD.$H2^{h4}$-IFN-$\gamma R^{-/-}$, and NOD.$H2^{h4}$-IL-$17^{-/-}$ are resistant to the development of thyroiditis (48, 49), suggesting that both Th1 and Th17 profiles contribute to the pathology (50).

Of interest, there is an interplay between Tregs and Th cells in immune responses (51). This holds true in susceptibility to thyroiditis. Indeed, while both NOD.$H2^{h4}$.IL-$17^{-/-}$ and NOD.$H2^{h4}$ IFN-$\gamma R^{-/-}$ mice are resistant to thyroiditis, depletion of $CD25^+$ Tregs induces thyroiditis in NOD.$H2^{h4}$.IL-$17^{-/-}$ mice but not in NOD.$H2^{h4}$ IFN-$\gamma R^{-/-}$ mice (50). This suggests that Tregs may more effectively control Th1-driven thyroiditis than Th17-driven pathology. Altogether, these observations point to a key role for Th cells in the development and progression of thyroiditis. Knowing that Th subsets facilitate the humoral response (46), they may effectively contribute to autoantibody production in thyroiditis.

B Cells

By producing antibodies, B cells can provide immune protection against infections (52). However, B cells can also have pathogenic roles in autoimmune diseases by producing autoantibodies, by promoting immune complexes deposition, antibody dependent cell cytotoxicity (ADCC), and as antigen-presenting cells (APCs) (53). Indeed, B cells are important players in SAT in the NOD.$H2^{h4}$ mouse. This is exemplified in the NOD.$H2^{h4}$-$\mu^{-/-}$ mouse, devoid of B cells, as well as in NOD.$H2^{h4}$ mice treated with anti-IgM or anti-CD20 antibodies, to deplete B cells (54–56). In these models, B cell depletion results in a decrease in the severity of thyroid lesions, as well as undetectable levels of thyroid autoantibodies (54–56). Further characterization of B cells in NOD.$H2^{h4}$ mice revealed that expression of costimulatory molecules, such as CD80 and CD86, is increased on B cells following SAT onset (56). In addition, these B cells produce proinflammatory cytokines such as TNF-α and IL-6 (56). By providing costimulatory signals and secreting proinflammatory cytokines, it was suggested that B cells act as APCs, promoting the activation and expansion of autoreactive T cells (54–56). This model proposes a central role for B cells in autoimmune thyroiditis *via* their involvement in the activation of pathogenic T cells and their production of autoantibodies (**Figure 3**). Incidentally, B cells are essential for the development of Graves' disease in which hyperthyroidism is directly caused by thyroid stimulating antibodies that target the TSHR (13).

Production of Thyroid Autoantibodies

In addition to immune infiltration, the breakdown of tolerance towards thyroid autoantigens is shared between autoimmune thyroiditis in humans and NOD.$H2^{h4}$ mice, as shown by the

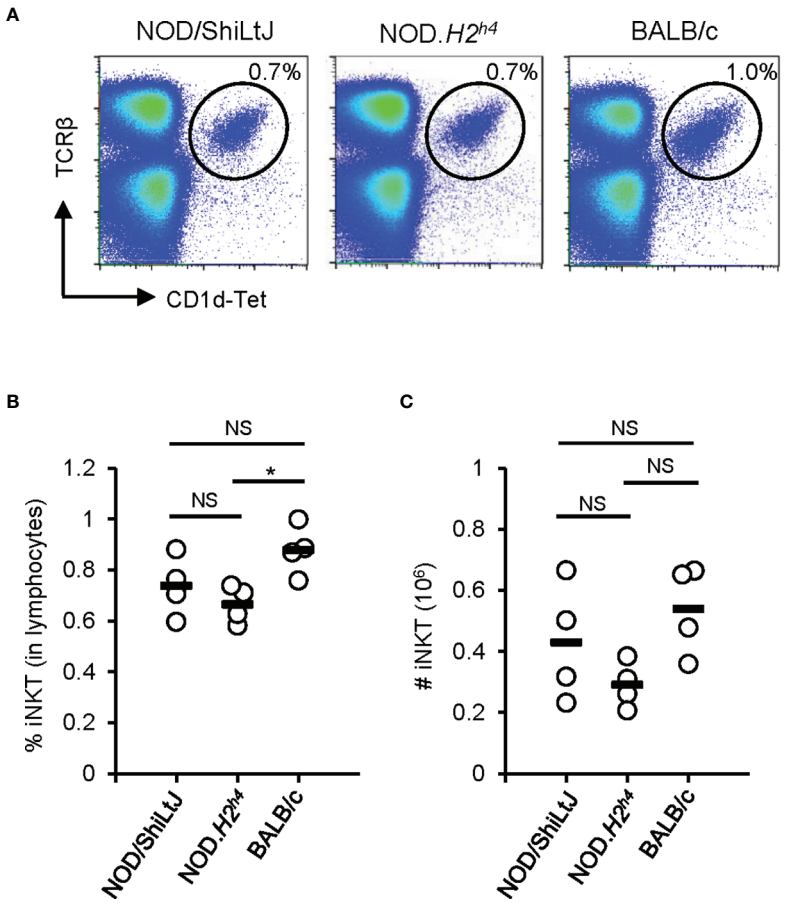

FIGURE 2 | iNKT cell abundance in the spleen. Spleen of NOD/ShiLtJ, NOD.*H2*^{h4}, and BALB/c mice were stained with antibodies to TCRβ and with CD1d tetramer (NIH Tetramer Core Facility). Data was acquired on BD FACSCanto II flow cytometer and analyzed with FlowJo. **(A)** Representative flow cytometry profiles of iNKT (TCRβ^{+}CD1d-Tet^{+}) cells in the spleen of NOD/ShiLtJ, NOD.*H2*^{h4}, and BALB/c mice. **(B)** Compilation of the percentage of iNKT cells in the spleen of NOD/ShiLtJ, NOD.*H2*^{h4}, and BALB/c mice (n = 4). **(C)** Compilation of absolute number of iNKT cells in the spleen of NOD/ShiLtJ, NOD.*H2*^{h4}, and BALB/c mice (n = 4). One-way ANOVA tests were performed for statistical analysis. Non-significant, NS; *P*-value > 0.05, and *; *P*-value = 0.03.

presence of autoantibodies. In both species, the major thyroid autoantigens are thyroglobulin (13), the predominant component of the thyroid gland, and TPO; both thyroglobulin and TPO are involved in the process of thyroid hormones synthesis (57). The break of tolerance towards these two thyroid autoantigens can be explained by their immunogenicity (13). For example, the abundance and size of the thyroglobulin and TPO proteins promote the generation of a large pool of peptides, which can be presented on MHC to T cells (57). In mice, thyroglobulin autoantibodies appear first followed by TPO autoantibodies (28, 29), suggesting that thyroglobulin is one of the first targeted autoantigens (57). The NOD.*H2*^{h4} mouse, when exposed to iodine-supplemented diet, develops thyroglobulin-antibodies of subclasses IgG1 and IgG2b (30). IgG2b thyroglobulin antibodies correlate with thyroid lesions and could therefore represent a biomarker for predicting thyroiditis (29). Of interest, treating NOD.*H2*^{h4} mice with blocking antibodies to PD-1 and to CTLA-4 markedly enhances thyroiditis and autoantibodies to thyroglobulin and TPO (58).

In humans, antibody levels to thyroglobulin and TPO are twice as high in women than in men, with reported values of 15.2 U/ml in women vs 7.6 U/ml in men for thyroglobulin and 17 U/ml in women compared to 8.7 U/ml in men for TPO (14). However, in NOD.*H2*^{h4} mice, the levels of thyroglobulin antibody levels are higher in males than females (22), whereas TPO antibody levels are higher in females than males (22). Thus, the presence of TPO antibodies in NOD.*H2*^{h4} mice more closely resembles the situation in humans than the presence of thyroglobulin antibodies (22). Of note, autoantibodies to thyroglobulin or to TPO in NOD.*H2*^{h4} mice are species specific, and do not cross-react with human thyroglobulin or human TPO (30, 57). Importantly, most humans with autoantibodies to thyroglobulin and TPO are euthyroid. Hypothyroidism is only manifest after extensive thyroid lymphocytic infiltration and thyroid tissue damage depletes the substantial thyroid hormone reserves and overwhelms the capacity of TSH to restore thyroid function (14, 59). Consequently, like NOD.*H2*^{h4} mice, most patients with autoantibodies to thyroglobulin and/or TPO have subclinical disease (14). It

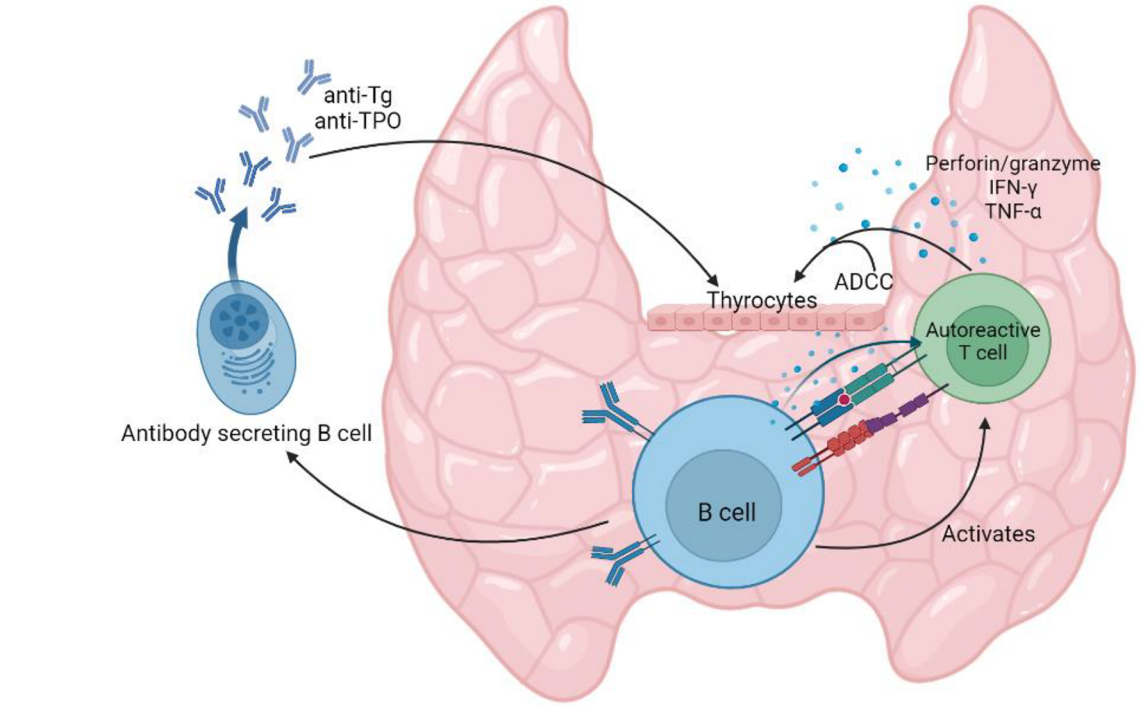

FIGURE 3 | The central role of B cells in autoimmune thyroiditis. By producing anti-thyroglobulin (Tg) and anti-TPO autoantibodies, by activating autoreactive T cells, and by promoting antibody dependent cell cytotoxicity (ADCC), B cells play an important role in the onset and the progression of autoimmune thyroiditis in the NOD mouse.

should be emphasized that autoantibodies to thyroglobulin and particularly to TPO are markers of thyroid lymphocytic infiltration and are a risk factor for the development of hypothyroidism (60, 61). Of note, as for autoimmune diabetes, the presence of autoantibodies directed towards thyroid antigens reflects an ongoing humoral response. Yet, the direct pathogenic potential of these autoantibodies has not been clearly demonstrated, except for thyroid stimulating antibodies that target the TSHR in Graves' disease (13). To that effect, transgenic expression of the human TSHR A-subunit at low levels in the thymus enables hTSHR/NOD.$H2^{h4}$ females, exposed to iodine-supplemented diet, to develop stimulating antibodies to the TSHR, the hallmark of Graves' disease (62). These TSHR antibodies stimulate cAMP production by human-TSHR-expressing cells in a bioassay. However hTSHR/NOD.$H2^{h4}$ mice do not develop hyperthyroidism, because the antibodies target human TSHR and do not cross react with the mouse TSHR (63).

Overall, the NOD.$H2^{h4}$ mouse has presented itself as an invaluable mouse model for the study of AITD and manifestations of this disease, such as immune cell infiltration and autoantibody production; these traits are similar to those observed in people living with AITD. The NOD.$H2^{h4}$ mouse strain therefore represents an excellent animal model for the dissection of the mechanisms leading to AITD (30) and for the investigation of potential therapies against autoimmune thyroiditis (21, 22, 28, 45, 64). Moreover, manipulation of this mouse model has revealed that thyroiditis results from complex immune responses, where T cells are necessary for disease progression. Still, the humoral arm of the immune response plays a clear role in this pathology, as the presence of autoantibodies precedes disease diagnosis and eliminating B cells dampens the pathology. There is also evidence to support a role for B cells in antigen presentation to T cells. All of these traits are reminiscent of autoimmune diabetes progression in NOD mice, suggesting a parallel between the organ-specific immune mechanisms leading to these two pathologies.

NEUROPATHIES

In the general population, the prevalence of neuropathy, also called peripheral neuropathy, is around 2% and increases with age up to 8% in people older than 55 years old (65). Peripheral neuropathy is characterized by damage to the axon or myelin of a neuron (66). In contrast, polyneuropathy (PNP) describes a pathology where several nerves of the peripheral nervous system are damaged, such as sensory, motor, and/or autonomic nerves (66). PNPs, with a prevalence of ~5% to 8% (67), are the most common type of peripheral nervous system

disorder and are caused by various factors, such as chronic alcoholism, chemotherapeutic drugs, genetic factors, and vitamin deficiency or overdose (66, 67). In Europe and North America, diabetes remains the most common cause of PNP, with diabetic patients representing from 30% to 66% of all PNP cases (65–67). Notably, more people are affected by diabetic neuropathy (DN) than all other types of PNP, including Charcot-Marie-Tooth, Guillain-Barré syndrome, and chronic inflammatory demyelinating polyneuropathy (65–67). Indeed, DN affects from 200 to 600 individuals per 100 000 people each year, whereas the prevalence is less than 15 in 100 000 individuals for all other PNPs combined (66).

DN is a painful disease defined by loss of sensory function and sensation of numbness, prickling, or burning in the distal lower extremities (66, 68). In people living with diabetes, the exact cause of these neuropathic symptoms is unknown, but some hypotheses involve metabolic, neurovascular or autoimmune pathways (69–71). The more common hypothesis suggests that chronic elevation of glucose level in the blood of people living with diabetes leads to redox imbalance and ultimately to oxidative stress (71, 72). This oxidative stress leads to glycation and oxidation of proteins, as well as dyslipidemia characterized by low levels of high-density lipoprotein cholesterol and high levels of total cholesterol, triglycerides, and low-density lipoprotein cholesterol. Dyslipidemia reduces blood flow and nerve perfusion, possibly resulting in neuropathic symptoms (71).

It is estimated that around 50% of people living with diabetes will develop DN (68). Concomitant with the increase in diabetes prevalence, the prevalence of DN is also increasing (73) but remains similar between PWT1D (11–50%) and people living with type 2 diabetes (PWT2D) (8–51%) (68). The incidence of DN is higher in PWT2D (6,100 per 100,000 person-years) than in PWT1D (2,800 per 100,000 person-years) (68). This difference between prevalence and incidence occurring in PWT1D and PWT2D could be due to several factors like differences in the age of diabetes development (68).

The NOD Mouse as a Model of Autoimmune Neuropathy

As for PWT1D, NOD mice are also prone to develop autoimmune damage to the nervous system. Indeed, autoimmune reactions occurring in NOD mice can shift from the pancreatic islets towards nervous tissues after inhibition or disruption of costimulatory pathways, cytokines, or transcription factors that are important in the maintenance of immune tolerance. Here we will discuss some genetically modified NOD mice that develop autoimmune neuropathy and therefore represent a tool for the study of this disease.

Disruption of Immune Tolerance Leading to Autoimmune Neuropathy

T cell activation requires three different signals: signal 1; TCR signaling *via* recognition of peptides presented by MHC, signal 2; costimulatory molecules, and signal 3; cytokines (74, 75). As mentioned, T cells are necessary for autoimmune diabetes progression in NOD mice. In trying to understand how T cells contribute to autoimmune diabetes, various NOD mouse models where genetically engineered to specifically target signal 1, 2 or 3. Altering either signal 1, 2 or 3 in NOD mice appears to shift the pancreatic β cell-specific autoimmune response towards the nervous system (**Figure 4**).

AIRE Transcription Factor: An Indirect Impact on Signal 1

The transcription factor AIRE promotes the ectopic expression of tissue-restricted antigens in the thymus (76, 77). The presentation of these self-antigens allows for the negative selection of self-reactive thymocytes and favors the generation of Tregs that mediate peripheral tolerance (78–80). The NOD.Aire$^{GW/+}$ mouse has a dominant G228W mutation in the gene coding for AIRE causing a partial loss of function, such that expression levels of tissue restricted antigens is reduced by 10% relative to NOD mice (81, 82). Of interest, the NOD.Aire$^{GW/+}$ mouse shows a decrease in the thymic expression of myelin protein 0, one of the major autoantigens of the peripheral nervous system, representing more than 50% of the peripheral myelin protein content (83, 84). In addition to revealing that myelin protein 0 expression in the thymus is regulated by AIRE, it also explains the loss of tolerance to this protein in the NOD.Aire$^{GW/+}$ mouse (82, 83). Indeed, the partial loss of AIRE function in NOD.Aire$^{GW/+}$ mice promotes the escape of myelin protein 0 self-reactive T cells into the periphery, which target nervous system elements but also pancreatic tissue (82). This results in the development of autoimmune peripheral neuropathy, similar to human chronic inflammatory demyelinating polyneuropathy, as well as autoimmune diabetes (82, 83). Therefore, a slight shift in the abundance of self-antigen expression in the thymus of NOD.Aire$^{GW/+}$ mice indirectly impacts signal 1, by not providing sufficient self-antigen presentation to the developing thymocytes. This, in turn, allows for the escape of self-reactive T cells, some of which target the nervous system, causing peripheral neuropathy.

Costimulatory Pathways: Signal 2

Costimulatory molecules are expressed at the surface of immune cells and enhance the intracellular signal provided by signal 1. CD28 is the prototypical costimulatory molecule for naïve T cell stimulation (85, 86). It is constitutively expressed on T cells and binds to the CD80 and CD86 receptors expressed on APCs (87). ICOS, a member of the CD28 family, is expressed on activated T cells and binds ICOSL on APCs (88). The interaction of ICOS with ICOSL and/or of CD28 with CD80 and CD86 triggers a co-stimulatory signaling cascade, which facilitates T cell activation (87). Of relevance, these costimulatory pathways are involved in autoimmunity (88, 89).

To define the involvement of ICOS and CD28 costimulatory pathways in T1D, genetic deletion of ICOS, ICOSL or CD86 was performed in NOD mice. Interestingly, NOD.ICOS$^{-/-}$, NOD.ICOSL$^{-/-}$, and NOD.CD86$^{-/-}$ mice are all protected from diabetes, suggesting an important role for these costimulatory pathways in autoimmune diabetes (90, 91). However, autoimmune neuropathies spontaneously developed in all of these strains (90, 91). Specifically, the NOD.ICOS$^{-/-}$ and NOD.ICOSL$^{-/-}$ mice show neuromuscular autoimmunity

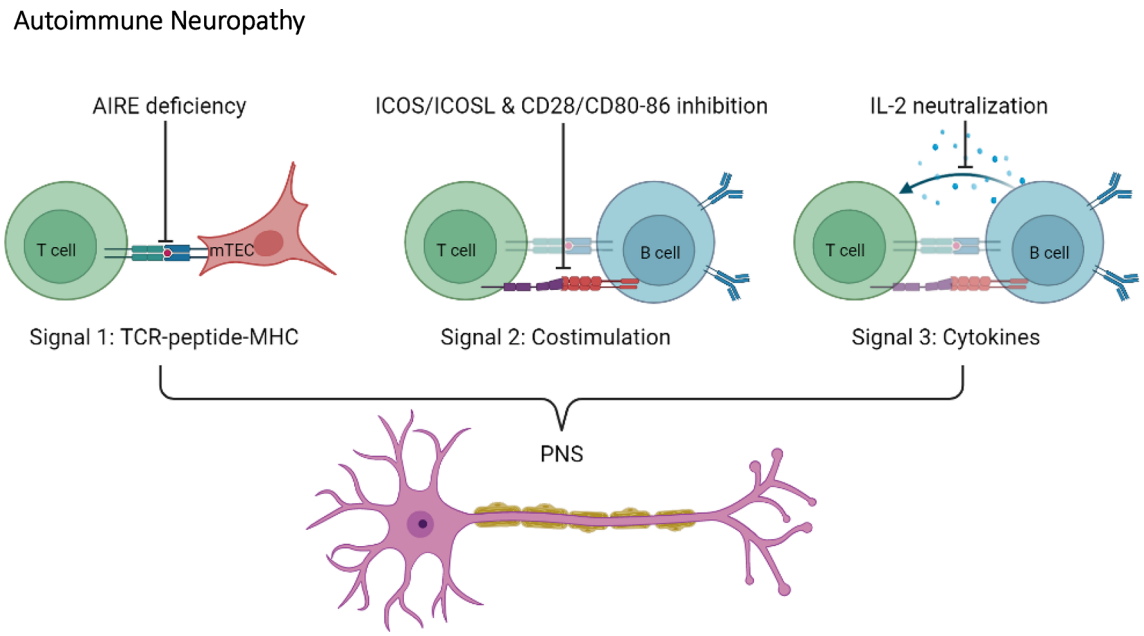

FIGURE 4 | Disruption of T cell activation signals induce autoimmune neuropathy in the NOD mouse. Autoimmune neuropathy can be induced in the NOD mouse after modulation of one of the three T cell activation signals. More specifically, mutations in AIRE transcription factor expression in medullary thymic epithelial cells (mTECs) indirectly impact signal 1. In addition, disruptions in co-stimulation signals, such as ICOS/ICOSL and CD28/CD80-86 (signal 2), or neutralization of IL-2 (signal 3) induce autoimmune neuropathy in the NOD mouse.

characterized by hind leg paralysis and immune infiltration of T cells, macrophages and granulocytes in the peripheral and central nervous system (PNS, CNS), including peripheral nerves, sensory ganglia, muscles, brain, and spinal cord (91). The reason for the autoimmunity deviation from the pancreas to nervous tissue in the NOD.ICOS$^{-/-}$ and NOD.ICOSL$^{-/-}$ mice remains unknown (91). Analogously, the NOD.CD86$^{-/-}$ mouse develops a spontaneous autoimmune peripheral polyneuropathy (SAPP) (90). And, as for NOD.Aire$^{GW/+}$ mice, NOD.CD86$^{-/-}$ mice display a break of tolerance towards myelin protein 0, the dominant autoantigen in the peripheral nervous system (83, 84). The reasons for the shift in target organ for the autoimmune response may be explained, in part, by the fact that CD86 genetic deletion leads to overexpression of CD80 on myeloid dendritic cells infiltrating the peripheral nerves (90, 92). The overexpression of CD80 on these APCs leads to activation of myelin-specific T cells, myelin sheet destruction and SAPP development (90). In addition, disruption of the CD28 costimulatory pathway leads to a reduction in Treg number, which could ultimately enhance susceptibility to SAPP (93). Exploiting genetically modified NOD mice will help dissect how disruptions in signal 2 facilitate a shift in the autoimmune response towards a different target organ. This is especially important when considering therapeutic approaches that target these pathways, to avoid treatment of T1D that would instead lead to the development of neuropathy.

Although autoimmune diabetes and neuropathy are characterized by different manifestations, the genetic factors promoting these two diseases on the NOD genetic background partially overlap. The H2^{g7} MHC haplotype of NOD mice not only plays an important role in autoimmune diabetes but is also necessary for the development of autoimmune neuropathy in the NOD.CD86$^{-/-}$ mouse (94). This suggests that the H2^{g7} haplotype promotes self-reactivity against various organs. The genetic susceptibility overlap can also be attributed to non-MHC loci. For example, diabetes resistance loci were introduced in NOD.CD86$^{-/-}$ mice to generate NOD.CD86$^{-/-}$*Idd3/5* and NOD.CD86$^{-/-}$*Idd3/10/18* congenic mice. These congenic mice are completely protected from both autoimmune diabetes and neuropathy (94). Thus, genetically modified NOD mice allow to study mechanisms as well as genetic factors promoting the development of autoimmune neuropathy.

Cytokines: Signal 3

T cell activation is modulated by the presence of cytokines, which represent the third signal for T cells activation (75). Unbalanced cytokine production is deleterious and may lead to the development of autoimmunity (95–97). A key cytokine in modulating T cell function is IL-2; it facilitates the proliferation of T cells and is involved in immune tolerance by allowing the homeostatic maintenance of Tregs (98–100). Similar to NOD mice with targeted disruption of costimulatory molecules, autoimmune peripheral neuropathy has been described in NOD mice deficient in IL-2 (98). While intraperitoneal injection of anti-IL-2 monoclonal antibodies in NOD mice accelerates diabetes onset, it also induces the development of autoimmune peripheral

neuropathy in more than 50% of the treated mice (98). This neuropathy is characterized by ataxia and paralysis of the limbs due to demyelination of the peripheral nerves (98). Anti-IL-2 treatment in NOD mice enhances autoimmunity by reducing Treg number, their activation, and their suppressive function (98). Of interest, IL-2 is one of the key candidate genes in the *Idd3* susceptibility locus (101, 102). *Idd3*, and thus IL-2 variants, may generally predispose NOD mice to autoimmune diseases by altering the function and development of Treg cells (98).

Altogether, genetic manipulations leading to alterations in T cell signal 1, 2 or 3 in NOD mice can shift the immune response from pancreatic β cells towards the nervous system. This break in T cell tolerance allows for infiltration of autoreactive T cells in the peripheral nerves, which ultimately leads to the production of autoantibodies targeting myelin protein 0 by self-reactive B cells (84). Of note, autoantibodies targeting myelin protein 0 have also been found in serum from individuals diagnosed with Guillain-Barré syndrome and chronic inflammatory demyelinating polyneuropathy (103, 104).

Production of Autoantibodies Targeting Nervous System Antigens

Pancreatic islets are surrounded by cells of the autonomous nervous system (105). In addition, pancreatic β-cells and neuronal cells share some autoantigens such as GAD, ICA515, and the neuronal type III intermediate filament protein, peripherin (105, 106). These autoantigens of the pancreatic nervous system are targeted by islet-infiltrating autoreactive T cells as well as autoantibodies (105). The production of autoantibodies against pancreatic nervous system antigens occurs in the early phase of diabetes and could explain certain neurological pathologies occurring in the prediabetic stage in humans and mice (105). In addition, B cell producing peripherin autoantibodies have been isolated directly from the pancreatic islets of NOD mice (106). Altogether, these observations point to a potential cross-reactive autoimmune response to both pancreatic β cells and neuronal cells, resulting in the production of autoantibodies as a reflection of an ongoing humoral immune response, which likely contributes to the pathology.

To specifically study the impact of peripherin-specific B cells in diabetes and neuritis, a BCR-transgenic mouse model (NOD-PerIg) was generated (106). In this mouse, B cells express the H and L chain Ig transgene from the peripherin-specific hybridoma clone H280, isolated from the pancreas of NOD mice (107). Compared to non-transgenic NOD mice, NOD-PerIg mice develop early onset diabetes, with an expansion of diabetogenic T cells, revealing an important association between the pancreas and the nervous system (107). Genetic manipulation of B cell responses in the NOD mouse has identified a clear link between autoimmune diabetes and neuropathy. This link between autoimmune diabetes and neuropathy has also been observed in non-NOD mouse models of autoimmune diabetes (108).

In sum, as for thyroiditis, manipulating the NOD mouse has informed us on cellular processes and genetic pathways linking autoimmune diabetes to peripheral neuropathies. As multiple immune characteristics are shared between autoimmune neuropathy in NOD mice and humans, the genetically modified NOD mice described above continue to be useful to improve our knowledge on autoimmune neuropathy, as well as the connection between the pancreas and the nervous system.

AUTOIMMUNE BILIARY DISEASES

Primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), and IgG4-associated cholangitis (IAC) represent the three main forms of autoimmune biliary diseases (ABD) (109, 110). All ABD share specific symptoms such as bile duct obliteration and cholestasis, characterized by a strong reduction of bile flow (111–114). Here we will focus on the most common form of ABD which is PBC, with an overall prevalence of ~19 to ~40 cases per 100 000 individuals depending on the geographic location (110). PBC is a chronic autoimmune cholestatic liver disease most frequently observed in middle-aged women (115), and is characterized by lymphocytic infiltration of the liver portal tracts, destruction of the epithelial cells of the intrahepatic bile duct, and serologic hallmarks of antimitochondrial autoantibodies (AMA) (116). Notably, 90-95% of people living with PBC (PWPBC) will develop AMA; these autoantibodies long precede clinical symptoms of PBC, often for many years, and yet represent one of the three criteria for the definitive diagnosis of PBC (117).

The NOD Mouse as a Model of ABD

In an attempt to understand the contribution of genetic loci linked to autoimmune diabetes susceptibility in NOD mice, the congenic NOD.c3c4 mouse carrying resistance alleles on chromosomes 3 (*Idd3*, *Idd10*, *Idd17*, *Idd18*) and 4 (*Idd9.1*, *Idd9.2*, *Idd9.3*), was generated (118, 119). The NOD.c3c4 mouse does not show signs of autoimmune diabetes (119), but about half of the female and a quarter of the male mice spontaneously develop a fatal form of ABD (118). Similar to human PBC, NOD.c3c4 mice exhibit lymphocyte infiltration in the liver, production of autoantibodies, biliary obstruction, and finally liver failure leading to death (118–120). Of interest, the NOD.c3c4 strain was the first mouse model of human PBC (119).

T Cell Infiltration in the Liver

In NOD.c3c4 mice, abundant T cell infiltration can be observed in the liver, with $CD4^+$ and $CD8^+$ T cells primarily located in the biliary epithelium (119). $CD4^+$ T cells in the liver produce pro-inflammatory cytokines such as IFN-γ and IL-2 (119). Importantly, antibody-mediated depletion of T cells leads to a significant reduction in disease onset in NOD.c3c4 mice (119). Moreover, transfer of $CD4^+$ T cells from a NOD.c3c4 mouse to a lymphopenic NOD.c3c4-*scid* mouse is sufficient to induce ABD development (119). Altogether, these observations demonstrate that T cells are necessary and sufficient for ABD in NOD.c3.c4 mice.

The role of T cells in ABD development has also been investigated in a new congenic mouse model of PBC, the NOD.ABD mouse, derived from the NOD.c3c4 mouse (120). This congenic subline, with shorter resistance loci on chromosomes 3 and 4 than those in NOD.c3c4 mouse, develops ABD as well as autoimmune diabetes (120). This

suggests that these two autoimmune diseases are not mutually exclusive in the NOD.ABD congenic mouse model. Of interest, the development of both T1D and PBC has also been reported in humans (121). The NOD.ABD mouse model develops a similar form of ABD as the NOD.c3c4 mouse characterized by common bile duct (CBD) dilation, immune cell infiltration, and biliary epithelial proliferation resulting in cyst formation (120). Of note, NOD.ABD mice show an accumulation of central and effector memory $CD8^+$ T cells in the liver, which effectively produce IFN-γ and TNF-α (120). Additionally, the transfer of NOD.ABD $CD8^+$ T cells alone or with $CD4^+CD25^-$ T cells into NOD.c3c4-*scid* mice promotes ABD development in these recipients, suggesting an important role of autoreactive $CD8^+$ T cells in ABD (120). Overall, studies in NOD.ABD and NOD.c3c4 congenic mice highlight an important pathogenic role of T cells in ABD development.

Production of Autoantibodies in ABD

As mentioned above, the presence of autoantibodies, particularly of AMA, is a strong serologic hallmark of disease, with 90-95% of PWPBC presenting with these autoantibodies (117, 122). In NOD.ABD mice, AMA were shown to bind the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), part of the mitochondrial 2-oxoacid dehydrogenase complexes (120). While PDC-E2 is a ubiquitous autoantigen expressed in all nucleated cells in the body, in PWPBC, only bile duct epithelial cells are targeted (119). The reason for the specific targeting of bile duct epithelial cells is unclear; it suggests that other components are at play, and that the presence of AMA may be secondary to tissue destruction. Of interest, anti-PDC-E2 antibodies are present in both NOD.c3c4 and NOD.ABD mouse models of PBC (119, 120). However, the proportion of NOD.ABD mice presenting with these autoantibodies is rather low, and, in contrast to PWPBC, anti-PDC-E2 antibody-positive mice increases with disease severity and age (120). Still, anti-PDC-E2 antibodies appear before detectable liver immune cells infiltration in both PWPBC and NOD congenic mice (119, 122).

In addition to anti-PDC-E2 antibodies, antinuclear antibodies (ANAs) and anti-Smith antibodies (anti-Sm) are also observed in the sera from PWPBC but at a lower incidence (48% of PWPBC develop ANAs vs 24% for anti-Sm) (123). Notably, the presence or absence of ANAs and anti-Sm varies among the different NOD mice congenic for chromosomes 3 and/or 4. In contrast to NOD and NOD.ABD mice which do not develop ANAs and anti-Sm autoantibodies, these autoantibodies are found in the serum of NOD.c3c4 and other congenic lines (118, 120). Further investigation of congenic sublines suggests that the *Idd9.3* locus is sufficient for ANAs and anti-Sm autoantibody production (120, 124). Within the *Idd9.3* locus, there is a candidate gene encoding for CD137 (4-1BB), an inducible costimulatory molecule on T cells (124, 125). A three amino acid difference in CD137 between NOD and B10 mice results in a lower CD137 costimulatory signal in NOD mice (124, 126). This may explain why NOD mice carrying non-NOD alleles at this locus show an increased production of autoantibodies, *via* the enhanced CD137-mediated costimulation between T cells and B cells (124, 126).

Overall, the NOD.c3c4 congenic mouse is a relevant model of PBC; it shares significant characteristics with PBC including key aspects of the humoral autoantibody response (127, 128) (**Figure 5**). In addition, NOD.ABD congenic subline allows to investigate the relationship between ABD and autoimmune diabetes. These NOD congenic mice further allow the identification of relevant and possibly clinically targetable molecular pathways for the development of new treatments.

POLYAUTOIMMUNITY IN NOD MICE

The term polyautoimmunity is used to describe the presence of more than one autoimmune disease in the same individual (129–131). For instance, a given NOD mouse can simultaneously present with multiple autoimmune diseases, such as autoimmune diabetes and thyroiditis (20, 132). The polyautoimmunity does not need to include autoimmune diabetes. In fact, NOD.$CCR7^{-/-}$ mice are protected from diabetes, but develop multiple autoimmune phenotypes, including immune infiltration in the thyroid, sciatic nerve, lung, stomach, intestine, uterus, and testis, among others (133). Notably, the thyroid pathology in these mice most closely resembles the primary hypothyroidism observed in humans (133). In addition, autoimmune diabetes-resistant NOD.$H2^{h4}$ and NOD.$H2^{h4}$-IFN-$\gamma^{-/-}$ $CD28^{-/-}$ mice spontaneously develop thyroiditis and Sjögren's syndrome (SS) (134–136). While these findings further highlight the remarkable autoimmune-prone background of the NOD mouse, we will mostly focus our discussion to polyautoimmune phenotypes that include autoimmune diabetes.

In addition to autoimmune diabetes and thyroiditis (20, 132), NOD mice can present with both autoimmune diabetes and SS (137–143). T1D and SS can also co-occur in humans, with up to 55% of PWT1D exhibit symptoms of SS, such as keratoconjunctivitis sicca (dry eyes) and xerostomia (dry mouth) (144). SS is a chronic autoimmune exocrinopathy disorder characterized by lymphocyte infiltration and progressive damage to the exocrine glands, mainly the lacrimal and salivary glands (145, 146). These damages lead to decreased tears and saliva secretion, which ultimately result in keratoconjunctivitis sicca and xerostomia (145, 146). SS is notably defined by important B cell alterations of the humoral immunity which result in a polyclonal B cell activation and antibodies production (135, 147). In fact, one of the main hallmarks of SS is the presence of lymphocyte infiltration in the exocrine glands which formed organized lymphoid structures called ectopic follicles (148). In these ectopic follicles, all subsets of B cells are present, including antibody-secreting B cells which produce pathogenic antibodies that are useful for SS diagnosis (135, 148). These autoantibodies, which target non-organ-specific antigens, are Rheumatoid factor, anti-double stranded DNA, ANA, anti-Ro, and anti-La (147–149). Of note, the presence of anti-Ro and anti-La is a criterion for SS diagnosis (149). A study in NOD.$H2^{h4}$ mice reveals that anti-Ro and anti-La appear before the development of ectopic follicles in the salivary gland whereas antibodies to double stranded DNA only develop after the appearance of ectopic follicles (148). These observations are consistent with anti-Ro and anti-La being the hallmark of SS and particularly as markers identifying patients in the active stage of the disease. SS is most prevalent in women aged between 30 to 60 years

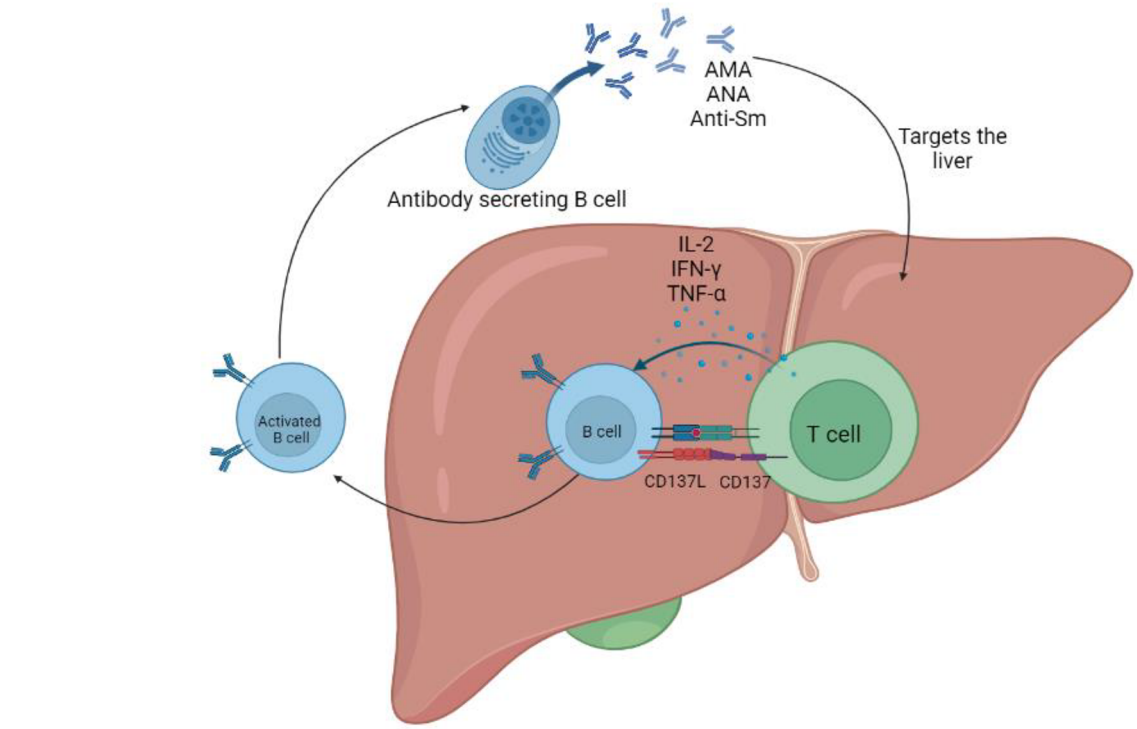

FIGURE 5 | The immunopathogenesis of autoimmune biliary disease. In the NOD congenic mice, T cells play an important role in ABD by activating B cells which will eventually produce AMA, ANA, and anti-Sm autoantibodies.

old, with a female to male ratio from 20:1 to 9:1 (137, 146, 150). In NOD mice, as in humans, the development of SS seems to be influenced by sex hormones because SS in NOD mice is significantly higher in females than males (137).

Another example of polyautoimmune traits present in humans is T1D and multiple sclerosis (MS) (151). MS is an autoimmune inflammatory disease of the central nervous system characterized by autoimmune responses against the protective myelin sheaths around nerve fibers, leading to severe and progressive neurological impairment (152). PWT1D have a 3 to 20 times higher risk of developing MS compared to the general population (151, 153–156). In addition, these two autoimmune diseases share some genetic and environmental susceptibility factors (151). Exposure to vitamin D seems to protect against the onset of MS and T1D (157). Interestingly, immune responses against pancreatic islet have been observed in people with MS, and, conversely, PWT1D show immune responses against central nervous system antigens (158). In addition to T1D and MS polyautoimmunity, T1D can also be observed in association with other autoimmune diseases such as AITD and DN in the same individual (15–18, 68).

The polyautoimmunity observed in NOD mice allows investigation of the mechanisms underlying this complex trait. Indeed, polyautoimmunity in NOD mice can be exacerbated by genetic manipulation and/or modulation of immune functions (**Figure 6**). For instance, targeting PD-1, AIRE, IL-2 or performing thymectomy in NOD mice promotes polyautoimmunity, as discussed below.

PD-1 Driving Polyautoimmunity

PD-1, which is coded by the *Pdcd1* gene, is an immunoreceptor involved in the regulation of peripheral tolerance by inducing and maintaining T cell clonal anergy and homeostatic control of B cells and myeloid cells (159–163). The interaction of PD-1 with its ligands, PD-L1 and PD-L2, suppresses immune responses like autoimmunity and sustained inflammation (163, 164). As such, PD-1 deficiency on the NOD genetic background accelerates the onset and incidence of autoimmune diabetes, with an onset at 5 weeks instead of 12-17 weeks in NOD mice, and an incidence reaching 100% by 10 weeks (164). Sialadenitis is also accelerated and more severe, with significantly greater pathological scores at 6 weeks of age in NOD.*Pdcd1*$^{-/-}$ relative to NOD mice (164). Sialadenitis is an inflammation of the salivary glands caused by an increase in the activation and effector functions of autoreactive T cells (165). The NOD.*Pdcd1*$^{-/-}$ mouse, which presents a rapid onset of both autoimmune diabetes and sialadenitis, can thus be used to study polyautoimmunity. The early onset of autoimmune diabetes in the NOD.*Pdcd1*$^{-/-}$ mice is in part due to early and severe insulitis, resulting in rapid destruction of pancreatic β-cells (164). As for NOD mice, insulitis and autoimmune diabetes in NOD.*Pdcd1*$^{-/-}$

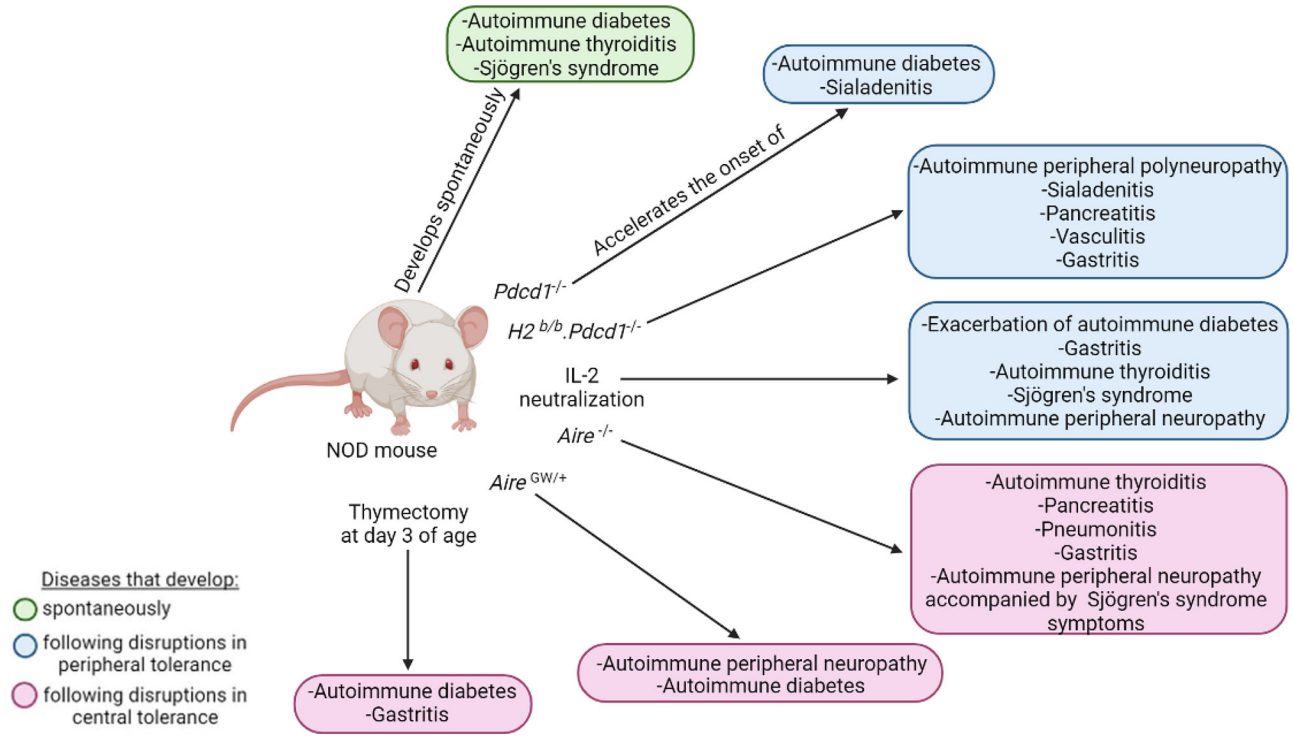

FIGURE 6 | Polyautoimmunity in the NOD mouse. The NOD mouse can spontaneously develop autoimmune diabetes, autoimmune thyroiditis, and Sjögren's syndrome (green). Disruptions in peripheral (blue) and central tolerance (pink) exacerbate or induce a wide spectrum of other organ-specific autoimmune diseases.

mice is dependent on the $H2^{g7}$ MHC locus. Indeed, as for NOD-*H2*$^{b/b}$ mice (26), NOD-*H2*$^{b/b}$.*Pdcd1*$^{-/-}$ mice are completely protected from insulitis and autoimmune diabetes (160, 166). This indicates that the $H2^{g7}$ haplotype is absolutely required for autoimmune diabetes development, even in NOD.*Pdcd1*$^{-/-}$ mice (26, 160). Rather than developing autoimmune diabetes, the NOD-*H2*$^{b/b}$ mice develop SS (141, 167–169), whereas the NOD-*H2*$^{b/b}$.*Pdcd1*$^{-/-}$ female mice are polyautoimmune; they develop spontaneous peripheral polyneuropathy, sialadenitis, pancreatitis, vasculitis, and gastritis (160, 166). This polyautoimmunity is likely due to a break in T cell tolerance as a consequence of a disruption of the PD-1 pathway (160). To identify the genetic factors that drive this polyautoimmune phenotype, Jiang et al. performed a genetic linkage analysis between NOD-*H2*$^{b/b}$.*Pdcd1*$^{-/-}$ and C57BL/6.*Pdcd1*$^{-/-}$ mice (166). They identified 14 non-MHC quantitative trait loci linked to these autoimmune traits (166). These studies highlight the relevance of using genetically manipulated NOD mice to study polyautoimmunity to identify additional genetic variants linked to autoimmune diseases (160, 166).

AIRE Transcription Factor as a Prototypical Factor Causing Polyautoimmunity

As mentioned above, the AIRE transcription factor has an important role in maintaining self-tolerance and preventing autoimmunity (78, 170–173). The polyautoimmune syndrome resulting from AIRE mutations is a rare autosomal recessive disease called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) or autoimmune polyendocrine syndrome type-1 (174–176). In addition to developing chronic mucocutaneous candidiasis, hypoparathyroidism, and primary adrenal insufficiency, people living with APECED also develop several organ-specific autoimmune manifestations including T1D, autoimmune thyroiditis, gastritis, and hepatitis (170, 177, 178). As in people living with APECED, AIRE deficiency in mice from various genetic backgrounds, including NOD.*Aire*$^{-/-}$ mice, have circulating autoantibodies targeting multiple organs and lymphocytic infiltration in various tissues, representing a good model for APECED studies (178, 179). Of note, NOD.*Aire*$^{-/-}$ mice are protected from autoimmune diabetes but exhibit thyroiditis, pancreatitis, pneumonitis, gastritis, and autoimmune peripheral neuropathy accompanied by the development of some SS symptoms (146, 167, 180). As mentioned above, the NOD.*Aire*$^{GW/+}$ mouse is also polyautoimmune in that it develops autoimmune peripheral neuropathy and autoimmune diabetes (82, 83). Thus, both the NOD.*Aire*$^{-/-}$ and NOD.*Aire*$^{GW/+}$ mice are relevant models to study polyautoimmunity.

To study the impact of the humoral response in polyautoimmunity, Gavanescu et al. compared NOD.*Aire*$^{-/-}$ and NOD.*Aire*$^{-/-}$μMT mice, and showed that the lack of B cells in AIRE-deficient mice strongly reduces autoimmune manifestations such as organ inflammation (179). In addition,

depleting B cells in a variant of the NOD.*Aire*$^{-/-}$ model significantly reduced inflammation and destruction of the pancreas (179). These results suggest that B cells contribute to APECED pathology and that anti-B cell therapies could help alleviate symptoms in people living with APECED (179).

IL-2 and Polyautoimmunity

Polyautoimmunity is also observed in NOD mice treated with IL-2 neutralizing antibodies (98, 166). These mice show an exacerbation of autoimmune diabetes and develop a wide spectrum of organ-specific autoimmune diseases such as gastritis, thyroiditis, SS, and peripheral neuropathy (98). This is likely due to the fact that IL-2 neutralizing antibodies broadly reduce Treg number, as well as their suppressive functions (98–100, 181).

Thymectomized NOD Mice Develop Polyautoimmunity

As for PD-1 deficiency, AIRE mutations, and IL-2 neutralization, thymectomy (Tx) performed at three days of age (d3-Tx) in NOD mice leads to the development of polyautoimmunity (182). While d3-Tx in NOD mice does not impact autoimmune diabetes onset, it concomitantly results in autoimmune gastritis development (182). Autoimmune gastritis is a $CD4^+$ T cell-mediated disease mainly characterized by lymphocytic infiltration in the gastric mucosa and the production of autoantibodies against the parietal cell H+/K+ ATPase proton pump (183–185). The BALB/c mouse is particularly susceptible to autoimmune gastritis (183). D3-Tx BALB/c mice develop autoimmune gastritis that closely resembles human disease and for which the pathologic score is higher than in d3-Tx NOD mice (183). Interestingly, susceptibility loci linked to autoimmune gastritis, namely *Gasa1* and *2*, are located on mouse chromosome 4 (184, 185) and overlap with the *Idd11* and *Idd9* loci, respectively (183–185). This suggests a strong genetic association between autoimmune gastritis and diabetes (186). Notably, the prevalence of autoimmune gastritis in PWT1D is 3-to-5-fold higher than in the general population (187). The polyautoimmunity developing in Tx mice is thus relevant to autoimmune gastritis and diabetes.

Overall, disturbances in various components affecting T cell tolerance exacerbates polyautoimmunity in NOD mice, providing clues to the development of polyautoimmunity and potentially revealing therapeutic targets to alleviate the severity of the pathologies.

THE NOD MOUSE AS A RELEVANT TOOL BEYOND SPONTANEOUS AUTOIMMUNITY

Unarguably, the NOD mouse model is a useful tool to study autoimmune diabetes. By genetic manipulation, derivatives of the NOD mouse model represent relevant spontaneous models for multiple human autoimmune pathologies. However, one cannot ignore other highly relevant uses of the NOD mouse model. For one, intravenous injection of pertussis toxin in NOD mice induces the development of experimental autoimmune encephalitis (158). This new induced model exhibits phases of remission, and closely mimics clinical and histopathological properties of MS; it may help to determine the genetic and environmental factors that promote the progression of MS (158). In addition, following injection of heat-killed bacillus Calmette-Guérin, NOD mice develop a non-organ specific autoimmune rheumatic disease similar to SLE (188–190), creating yet another relevant induced model to study the progression of a human pathology.

Apart from autoimmune diseases, the NOD strain has been used for studying human cells. Indeed, due to a polymorphism in CD172a, the NOD strain allows for better engraftment of human hematopoietic cells than other mice (191, 192). The strong interaction between the CD172a protein on the NOD macrophages and CD47 on human cells leads to a negative regulation of macrophage phagocytosis (193). Engraftment of human cells is typically performed in NOD.SCID, NOD.Rag$^{-/-}$, NOD.SCID.IL2Rγ$^{-/-}$ or NOD.Rag$^{-/-}$.IL2Rγ$^{-/-}$ mice, deficient in various components of the adaptive immune system, to further facilitate xenogeneic engraftment (194–199). Additional NOD mouse models are constantly being created to enhance human cell engraftment or to study specific diseases (200–206). For instance, the Human Immune System (HIS)-DRAGA (HLA-A2.HLA-DR4.Rag1$^{-/-}$.IL-2Rγc$^{-/-}$.NOD) mouse, grafted with human epithelial cells expressing the human angiotensin-converting enzyme 2 (hACE2) receptor in their lungs, was generated for COVID-19 research (207). Following immune reconstitution with human HLA-matched hematopoietic stem cells and intranasal infection with SARS-CoV-2, the HIS-DRAGA mouse exhibits T cell infiltration in the lungs and develops the different forms of severity of COVID-19 disease, as seen in the human population (207). The HIS-DRAGA mouse strain provides an important model for studying SARS-CoV-2 infection, as well as the immune responses generated against this virus, and can be used to test potential therapeutics and vaccines (207).

CONCLUSION

The NOD mouse remains one of the best models to study T1D. It is useful to study autoimmune susceptibility as well as genetic and cellular factors contributing to breakdowns of immune tolerance. Genetic manipulation of the NOD mouse has generated excellent models for studying spontaneous organ-specific autoimmune diseases other than diabetes such as thyroiditis, neuropathies, ABD and even polyautoimmunity. The manifestation of these autoimmune diseases in the NOD variant strains share many characteristics with human diseases, particularly immune cell infiltration in the targeted organ and a strong humoral response involving the generation of autoantibodies. Although pancreatic β cells have been shown to be particularly fragile in NOD mice (208), the exact reasons why the NOD mouse develops autoimmune diabetes whereas genetically modified NOD mice spontaneously develop autoimmune responses to other target organs remain unknown. In addition to organ-specific autoimmune diseases,

disturbances in peripheral or central tolerance in NOD mice lead to polyautoimmunity, providing key information on the importance of these immune tolerance mechanisms for maintaining health. All in all, the NOD mouse, along with the several NOD congenic mice and NOD genetic knockout mice that have been generated over the years, represent indispensable tools in research that may be exploited for applications much broader than the study of type 1 diabetes. With their close parallel to various human autoimmune pathologies, these models should be exploited to increase our understanding of these specific pathologies as well as to design and test novel therapeutics.

AUTHOR CONTRIBUTIONS

A-MA wrote the first draft of the manuscript and prepared most of the figures. FL-V contributed to the first draft of the manuscript and prepared some figures. RC analyzed the data for **Figure 2**. HA generated the data for **Figure 2** and revised the final version of the manuscript. SM supervised HA and revised the final version of the manuscript. SL supervised A-MA and FL-V and revised the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Functional Impact of Risk Gene Variants on the Autoimmune Responses in Type 1 Diabetes

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Type 1 diabetes (T1D) is an autoimmune disease that develops in the interplay between genetic and environmental factors. A majority of individuals who develop T1D have a HLA make up, that accounts for 50% of the genetic risk of disease. Besides these HLA haplotypes and the insulin region that importantly contribute to the heritable component, genome-wide association studies have identified many polymorphisms in over 60 non-HLA gene regions that also contribute to T1D susceptibility.

Combining the risk genes in a score (T1D-GRS), significantly improved the prediction of disease progression in autoantibody positive individuals. Many of these minor-risk SNPs are associated with immune genes but how they influence the gene and protein expression and whether they cause functional changes on a cellular level remains a subject of investigation. A positive correlation between the genetic risk and the intensity of the peripheral autoimmune response was demonstrated both for HLA and non-HLA genetic risk variants. We also observed epigenetic and genetic modulation of several of these T1D susceptibility genes in dendritic cells (DCs) treated with vitamin D3 and dexamethasone to acquire tolerogenic properties as compared to immune activating DCs (mDC) illustrating the interaction between genes and environment that collectively determines risk for T1D. A notion that targeting such genes for therapeutic modulation could be compatible with correction of the impaired immune response, inspired us to review the current knowledge on the immune-related minor risk genes, their expression and function in immune cells, and how they may contribute to activation of autoreactive T cells, Treg function or β-cell apoptosis, thus contributing to development of the autoimmune disease.

Keywords: type 1 diabetes, risk gene variants, immunoregulation, Tregs, tolerogenic dendritic cells, vitamin D

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INTRODUCTION

For several decades the incidence of Type 1 diabetes (T1D) has been increasing worldwide (1). This disease is characterized by the infiltration of immune cells in the islets of Langerhans (2, 3) ultimately leading to the loss of insulin producing β-cells with insulin replacement as the only available option to prevent fatal outcomes in all patients. Curative treatments are lacking for several reasons, one being that the events in humans leading to a selective β-cell dysfunction and loss is

hard to detect. Although the analyses of fresh and cryopreserved tissues in the past decade, enabled by the nPOD initiative (www.JDRFnPOD.org), have significantly contributed to our understanding of the important local players in the process (2–5), many questions remain. So far, adaptive immune cells are indisputably involved in the β-cell destruction by their tissue specificity. The clinically approved therapies modulate immunity in general, the more preferable antigen-specific immune therapies show promising results but are not ready for general clinical application (6).

While autoimmune T1D is not completely inherited and environmental factors show a significant contribution to the pathogenesis (7), certain genetic polymorphisms do critically increase the predisposition for T1D (8). Polymorphisms in HLA and insulin (INS) regions were first described and contribute strongly to the disease risk (9, 10). Later, genome-wide association studies (GWAS) have identified many additional SNPs in so called non-HLA risk genes, which show a small but clear individual contribution to the increased risk for T1D (11). When included in a cumulative score (T1D-GRS), it significantly improved the capacity to discriminate T1D from T2D or healthy subjects, and to discriminate monogenic from autoimmune T1D (12–14). The exact functional contribution of many of these SNPs to the T1D-GRS remains to be characterized. We have observed a positive correlation between the non-HLA genetic risk, in addition to, but independently of HLA, and the intensity of the peripheral autoimmune response in T1D patients (15). Indeed, many of the associated T1D risk genes are controlled by lymphoid enhancers or involved in immune networks (11, 16). Our studies focusing on the differential transcriptome of tolerogenic (tolDC) versus inflammatory dendritic cells (mDCs) showed that a tolerogenic modulation of monocytes by $1,25(OH)_2$ vitamin D3 (VitD3) induced a stable change in the expression of sets of these non-HLA risk genes (17, 18), inspiring a hypothesis that quantitative and/or qualitative effects of the SNPs on the related gene products may reflect in a change of the immune regulatory vs. an immune activating balance. Here, we aim to review the knowledge of functional consequences of T1D risk SNPs on the regulation, expression and function of linked risk genes and further contemplate how this may impact the functionality of the effector vs. regulatory T cells, changing the balance between immune activation and suppression in the pancreas that is critical to attenuate chronic inflammation and an uncontrolled damage of insulin producing β-cells.

IMMUNOPATHOGENESIS OF T1D, HOW MUCH DO WE ACTUALLY KNOW?

The exact order of immune events that cause human T1D has not been established. Hurdles such as that circulating blood cells poorly represent what is going on in the human pancreas, as well as the inability to directly analyze the target organ have significantly delayed our understanding of this autoimmune disease. Literature supports different scenarios describing the initiating events, involving an altered thymic selection of T cells that recognize β-cell antigens, viral infections that mark β-cells as the targets for destruction, enhanced expression of neo-antigens by β-cells due to cell stress, or an increased sensitivity of β-cells to inflammation (19–21). In all cases, β-cells seem critically involved in the process while the (auto)immune system is essential to execute the destructive insult resulting eventually in disease (22). The proposed initiating events are not mutually exclusive and likely cause the exposure of β-cell (neo-)antigens, which are taken-up by antigen presenting cells and presented to T cells in the context of high-risk human leukocyte antigen (HLA) molecules. The 'first hit' could occur when such presentation of β-cell antigens activates a destructive autoimmune response that may inflict some damage in the pancreatic islets but the disease is delayed as long as the immune regulation keeps the autoimmunity in check. The 'second hit' may occur when a regulatory checkpoint is bypassed such as upon an anti-cancer stimulating immunotherapy by check point inhibition or when the stressed (or infected) islets of Langerhans start releasing cytokines and chemokines, attracting immune cells where they target and eliminate functional β-cells to the point of no return.

T1D patients donating tissues for research helped to collect a significant evidence that T1D pathogenesis can follow different individual scenarios but also that mechanisms described in animal models are not all or not just as strongly present in the human immune system. For example, the infiltration of immune cells around the islet of Langerhans, designated as insulitis, in which activated CD4+ T cells control macrophages to induce killing of the β-cells by cytokines and reactive oxygen species, is clearly evident in mice (23) but not evident in human pancreas (24, 25). Cytotoxic CD8+ T cells are essential for the ultimate destruction of β-cells, while their antigen specificity varies between patients (4, 26). Hypothetically, techniques that discriminate relevant antigens and cells in the target tissue, allowing to separate primary immune aggressors from those only guilty by association will help solve this puzzle. Such bystanders may be the autoantibodies, which role in the immunopathology of human T1D is proved dispensable although they represent a good biomarker of an ongoing and in time often increasingly complex autoimmunity (27, 28). These antibodies can be found months to years before the clinical symptoms (29), help an early diagnosis of the disease and may prove valuable to identify individuals that will benefit from new curative treatments.

Time will tell whether the knowledge on the specificity of T1D autoantibodies to insulin (INS), 65 kDa glutamic acid decarboxylase (GAD65), insulinoma-associated protein2 (IA2) and zinc transporter 8(Znt8) (30) has helped or derailed the investigation of the β-cell specific targets of T cell autoimmunity (26, 31). More recent findings point to alternative transcripts and (neo-)antigens created by stressed or damaged β-cells, which are normally invisible to the immune system, as more likely to drive the T-cell mediated pathogenic destruction (21, 32–37). The contribution of the originally described antigens could be different, namely to secure immune regulation through a negative selection of high-affinity autoreactive T cells (38), or

to establish peripheral tolerance through low-affinity self-peptide recognizing regulatory T cells (Tregs) (39, 40). The existence of autoantibodies may hence be a sign of a regulation 'gone wrong' as a consequence of a genetically imprinted or environmentally caused impaired T cell selection, effector activation or reduced Treg function, such as demonstrated in T1D patients (41, 42).

THE IMPACT OF MAJOR T1D RISK GENES ON IMMUNE CELLS

Certain genetic polymorphisms associate with a higher risk to develop an autoimmune disease, which is most often expressed as an odds ratio (OR) that measures the strength of association between carrying a gene variant X (exposure) and development of T1D (outcome) (43). Specific HLA haplotypes and SNPs in the insulin gene (INS) strongly increase the odds to develop T1D and are hence designated as major susceptibility genes (44, 45). The HLA region was first associated with the risk of developing T1D, which is in line with a critical role of HLA in shaping the adaptive immunity (46). In the population of Caucasian origin, more than 90% of patients that develop T1D before puberty are carriers of one or both HLA haplotypes, namely HLA-DR3/DQ2 (DRB1*0301-DQA1*0501-DQB1*0201) or HLA-DR4/DQ8 (DRB1*0401-DQA1*0301- DQB1*03020 (47). In fact, heterozygotes carriers of both DRB1*03 and DRB1*04 carry up to 40 times higher risk to develop T1D than individuals with other HLA genotypes (48, 49). This synergic effect is likely caused by the formation of highly susceptible *trans*-encoded HLA-DQ (α1, β1) heterodimer molecules (48, 50), which efficiently bind and present β-cell derived peptides, increasing the number of different peptides that could trigger a pathogenic CD4+ T cell responses (51). Furthermore, the risk variant specific epigenetic modulation of the HLA expression could contribute to the disease pathogenesis (52).

A stable HLA molecule on the cell surface, however, does not exist without a peptide. Hence the contribution of HLA should be considered in combination with antigens/peptides that they present. The so far well-established β-cell antigens that are targeted by both B and T cell responses are INS, GAD65, IA2 and Znt8 but the list of target antigens is increasing (34, 35, 53). Of the β-cell proteins targeted as autoantigens, only SNPs in the INS gene are associated with an increased risk for T1D. The increased risk was first attributed to the polymorphism in variable number of tandem repeats (VNTR) in the insulin promotor (54, 55), determining the differential insulin expression between thymus and islets and leading to a faulty selection of the autoreactive T cells in thymus. While this may explain a part of the association, alternatives have been also explored, one being that other SNPs in the 3' UTR of the INS gene (rs3842752 (56) and rs3842753 (57)) actually functionally contributes to the increased risk. Namely, these SNPs are expressed when an alternative translation start in the INS mRNA is used, creating a new protein sequence called INS-DRIP. Interestingly, a few T1D patients carrying the protective allele (C-H) demonstrated no autoreactivity to INS-DRIP unlike the carriers of the susceptible (R-P) version (36). Which insulin-related SNP is causal and whether the increased risk is a consequence of the expression of 3'SNPs in INS-DRIP or it reflects the 5' INS promotor polymorphism remains unresolved, given the strong linkage disequilibrium between the 5'and 3' regions of INS, and the exact underlying mechanism is currently under investigation.

Despite the critical role of CD8+ T cells, the contribution of HLA class I molecules to the disease propensity is less obvious and affected by the high linkage disequilibrium between HLA class I and II genes. For instance, 50-70% of T1D patients carry HLA-A2 (0201), which turns this HLA class I allele as the most frequent amongst patients; yet, this variant is also present in 30-40% of the general population, affecting the statistical significance. HLA-B*39 has been identified as single HLA class I allele standing out in its association with T1D, but this variant is relatively rare (58). In our view, this indicates a more important role of HLA class II and antigen presentation in establishing and control of the immune regulation than in the actual β-cell destruction.

MINOR T1D RISK SNPS WITH A FUNCTIONAL IMPACT ON IMMUNE CELLS

For many risk genes variants, there is still insufficient understanding of whether and how they functionally impact the initiation and progression of the autoimmune process causing T1D. The functional outcomes of the coding T1D risk variants have been reviewed recently (59), and a fine mapping of the 10 known susceptibility regions combined with functional analyses provided further insight in potentially causal missense and non-coding SNP variants (60). Many of these risk genes were differentially expressed in dendritic cells upon tolerogenic modulation (17, 18). Hence, we here consider the functional roles in immune regulation of the minor T1D risk genes as such or when influenced by the SNP. We mainly focus on the genes for which functional data on human cells are available to allow a discussion on the consequences of the causal SNPs for the autoreactive T cell activation, Treg function or β-cell apoptosis that may support the autoimmune disease (**Table 1** and **Figure 1**).

PTPN22

Protein tyrosine phosphatase, non-receptor type 22 (*PTPN22)* encodes protein Lymphoid-tyrosine phosphatase (LYP) (81). The *PTPN22* allele C1858T has a single amino acid substitution R620W (arginine to tryptophan; rs2476601, OR= 1.890), and has been associated with T1D, Hashimoto's thyroiditis, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), vitiligo and Graves disease (82). The linkage with several autoimmune diseases and the observation that individuals with this variant are protected from pulmonary

TABLE 1 | Risk gene variants associated with T1D (discussed in this review). For each gene variant the variant ID, risk allele frequency and odd ratio are presented.

Gene	Variant ID (RSID)	OriginalPub.*		Frequency**	Odds Ratio	Assoc. p-value	Publication***
HLA class II	DRB1* 04:05-DQA1*03:02-DQB1*03:02				11.370	4.000 x 10^-5	Erlich H et al., 2008 (61)
	DRB1* 04:01-DQA1*03:01-DQB1*03:02				8.390	6.000 x 10^-36	Erlich H et al., 2008 (61)
	DRB1* 03:01-DQA1*05:01-DQB1*02:01				3.640	2.000 x 10^-22	Erlich H et al., 2008 (61)
	DRB1* 04:02-DQA1*03:01-DQB1*03:02				3.630	3.000 x 10^-4	Erlich H et al., 2008 (61)
INS	rs689	(62)	A –> T	T: 68%	2.256	2.161 x 10^-135	Inshaw JRJ et al., 2021 (63)
	rs3842752	(56)	G –> A	A: 20%	0.600	2.310 x 10^-14	Reddy et al., 2011 (56)
	rs3842753	(57)	T –> G	G:70%	0.580	2.180 × 10-32	Howson et al., 2009 (57)
PTPN22	rs2476601	(64)	A –> T	T: 9%	1.890	1.000 x 10^-100	Onengut-Gumuscu S et al., 2015 (11)
PTPN2	rs1893217	(65)	A –> G	G: 15%	1.210	1.200 x 10^-15	Onengut-Gumuscu S et al., 2015 (11)
IFIH1	rs2111485	(66)	A –> G	G: 57%	1.171	1.892 x 10^-10	Forgetta V et al., 2020 (67)
	rs1990760	(68)	C –> T	T: 57%	1.180	2.000 x 10^-11	Todd JA et al., 2007 (69)
	rs3747517	(66)	T –> C	C: 71%	1.700	6.000 x 10^-4	Liu S el al., 2009 (70)
	rs13422767	(70)	G –> A	A: 15%	1.799	1.000 x 10^-4	Zurawek M et al., 2015 (71)
CTLA4	rs231775	(72)	A –> G	G: 37%	2.000	1.000 x 10^-2	Goralczyk A et al., 2018 (73)
	rs5742909	(74)	C –> T	T: 8%	1.500	2.000 x 10^-2	Chen S et al., 2019 (75)
	rs3087243	(69)	G –> A	A: 44%	0.840	7.400 x 10^-21	Onengut-Gumuscu S et al., 2015 (11)
IL2RA	rs11594656	(76)	T –> A	T: 77%	1.220	1.920 x 10^-28	Lowe CE et al., 2007 (76)
	rs2104286	(77)	T –> C	C: 24%	0.880	2.100 x 10^-2	Espino-Paisan L et al., 2011 (78)
	rs12722495	(79)	T –> C	C: 8%	0.620	1.740 x 10^-30	Smyth DJ et al., 2008 (79)
	rs61839660	(76)	C –> T	T: 9%	0.620	2.800 × 10^–39	Onengut-Gumuscu S et al., 2015 (11)
CD226	rs763361	(69)	C –> T	T: 48%	1.120	1.000 x 10^-9	Plagnol V et al., 2011 (80)

*Data in this table has been collected using the database on https://platform.opentargets.org for type 1 diabetes mellitus. Genetic associations were selected as data type and Immune system as pathway types. Per gene variant the odds ratio is derived from the study listed in the OT Genetics Portal. *The original paper reporting the association between the risk variant and T1D. **Frequency of a risk allele in the world. ***Publications have been cited which reported the OR and p-value in the table.*

tuberculosis or cancer (83, 84), suggests a role in promoting effector responses at cost of immune regulation (85–88). LYP protein inhibits T cell and B cell activation by dephosphorylation of tyrosine residues in Src family kinases. The interaction between C-terminal Src kinase (CSK) and the P1 motifs on LYP are important to regulate the inhibitory activity of LYP. Next to lymphocytes, LYP plays a role in the control of activation and migration of innate myeloid cells (monocytes, macrophages, DCs and neutrophils) (89–91).

The molecular consequences of the PTPN22 mutation and the impact on T1D risk have been discussed extensively before (92, 93). The debate regarding the impact of the T1D risk variant on T cells remains as the results support both gain-of-function and loss-of-function as a mechanism (94). Following the gain-of-function hypothesis, the R620W mutation blunts the TCR signaling allowing autoreactive thymocytes to escape selection (95). The same group reported later that R620W is located in the P1 motif and prevents the binding of LYP to CSK (96), directing towards a loss-of-function hypothesis that may affect TCR signaling and certainly applies for the regulation of LFA-1 signaling. A deletion of *Ptpn22* in mice, mimicking the loss-of-function, demonstrated increased Treg levels (97) which is in line with a study that shows a direct correlation between having the *PTPN22* R620W variant and elevated circulating Treg frequency in humans (98). Recently, Perry et al. showed a higher expression of *PTPN22* in Tregs than in conventional T cells (Tconv) at rest irrespective of the genotype, but a lower impact of the risk variant on the suppression of distal TCR signaling in both subsets and permitting a stronger proliferation of Tconvs. The consequences for Tregs in this study were less evident implying a differential contribution of *PTPN22* risk variant to Tconv and Treg (99).

In myeloid cells, *PTPN22* is involved in the downstream signaling of TLR4, TLR7/8, NOD2 and cytokine receptors (reviewed in (92)). In this case, LYP does not work as a phosphatase but promotes TRAF3 ubiquitination and TLR-induced upregulation of type I interferons (IFNs). The *PTPN22* R620W variant failed to support this type I IFN response (100). Additionally, antigen presenting cells with the *PTPN22* R620W variant are more sensitive to NLRP3 and secrete more IL-1b in response to TLR-stimulation (101). Combined with the dampened type I IFN signal, this could affect the response of myeloid cells to infections (102) and the subsequent activation of T cells.

PTPN2

Protein Tyrosine Phosphatase, Non-Receptor Type 2 (*PTPN2*) is ubiquitously expressed, including β-cells and hematopoietic cells. PTPN2 takes part in a broad range of signaling pathways regulating the response to hormones, cytokines and inflammation (93, 103–105). The β-cells upregulate PTPN2 in response to cytokines or polyI:C (mimicking viral infection) (106, 107). Judging by the effects of knockdown in primary rats and human β-cells, which exacerbated cytokine induced pro-apoptotic signaling *via* STAT1, JNK1, and BIM and enhanced apoptosis, PTPN2 plays a protective and anti-apoptotic role in β-cells (106–108). The risk SNP rs1893217 (OR=1.210) is an intronic non-coding variant which may contribute to the sensitivity of β-cells to immune- or virus-mediated apoptosis (107).

The risk variant of *PTPN2* is associated with decreased PTPN2 expression in CD4+ memory T cells and reduced IL-2 receptor signaling *via* STAT5 phosphorylation, which correlated with

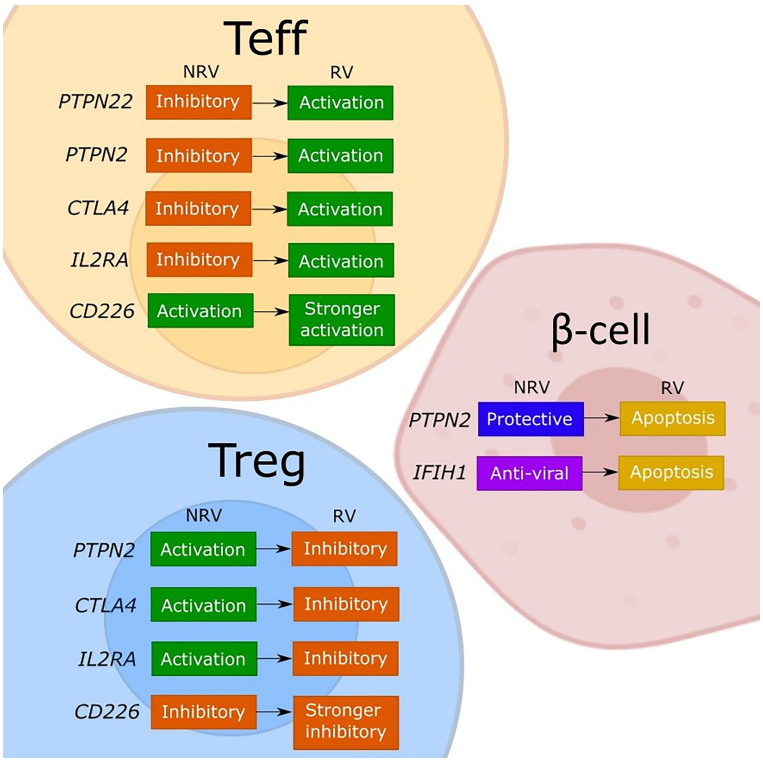

FIGURE 1 | Model of the discussed effects of T1D risk variants on cellular functions. The figure depicts our interpretation of the consequences for effector T cell (Teff), regulatory T cell (Tregs), and β-cells of the described or assumed change in the gene function caused by a T1D risk variant (RV) as compared to the non-risk variant (NRV) SNP as discussed in the manuscript. While the LYP protein normally controls the effector T cells by a downstream signaling inhibition, the risk variant (rs2476601) induces a change in PTPN22 that promotes Teff responses. The functional effects of PTPN22 remain unclear. The PTPN2 protein plays an anti-apoptotic role in β-cells and controls T cells *via* IL-2, which may favor Tregs due to a strong sensitivity to IL-2. Indirectly, a good activity of Tregs keeps the effector T cells under control. The *PTPN2* risk variant (rs1893217) causes a decrease in *PTPN2* expression and contributes to the sensitivity of β-cells to immune- or virus-mediated apoptosis. The risk variant also reduces IL-2 receptor signaling, which decreases FOXP3+ Tregs in T1D patients, and thus dysregulating Treg function. The *PTPN2* deficiency (mimicking the rs1893217 variant) results in increased Teff proliferation. The MDA5 (encoded by *IFIH1*) normally functions to activate stress- and anti-viral response, and by increasing the activity of MDA5, the risk variant (rs1990760) increases the basal IFN-I production leading to β-cell apoptosis. CTLA-4 functions normally to promote Treg function and inhibit Teff activation. The risk variant for *CTLA4* (rs231775) results in decreased expression of CTLA-4 on T cells, releasing the control of a Teff cell activation and reducing the suppressive Treg potency. The *IL2RA* risk variants impair the expression of CD25 and thus the IL-2 response and with the associated lower FOXP3 expression impacts primarily Tregs and their suppressive function. The resulting reduced Treg potency will indirectly release the control on Teff promoting the activation. The CD226 is an activating T cell molecule that promotes the inflammatory activity of Teff and reduces the suppression of Tregs. The *CD226* risk variant (rs763361) results in an isoform of CD226 with increased activity, which further increases Teff and CD226+ Tregs, thereby further reducing the overall suppressive capacity of Tregs.

reduced FOXP3 expression in Tregs (109) suggesting that PTPN2 indirectly modulates IL-2 responsiveness in T cells and thus can work independent of the susceptible *IL2RA* gene variant. This dysregulation of Tregs can contribute to the faulty maintenance of autoreactive T cells and B cells and thus sustain the vicious circle of uncontrolled autoimmune response (93). Indeed, antigen-specific effector T cells (Th1 and Tfh) in *Ptpn2* deficient mice show increased proliferation (110). Cell cultures of human myeloid cells showed that a loss of *PTPN2* enhances IFN-g, IL-6 and MCP-1 secretion (103), implicating PTPN2 in the regulation of inflammation through antigen presenting cells as well.

IFIH1

Interferon Induced with Helicase C Domain 1 (*IFIH1*) encodes for melanoma differentiation-associated gene 5 (MDA5). MDA5 is a cytoplasmic receptor for double stranded RNA (dsRNA) and detects viral RNA (106, 111, 112). Detection of dsRNA will activate a cascade of antiviral responses in the innate immune system by the production of IFN (113, 114). There are four SNPs in the *IFIH1* gene (rs1990760, OR=1.180; rs3747517, OR=1.700; rs2111485, OR=1.171; and rs13422767, OR=1.799) which are associated with T1D (70, 71). Variants rs2111485 and rs13422767 are located in an intergenic region of the 2q24 locus (13–23 kb 3′ of *IFIH1*), but it is not known whether the DNA sequences in this region act as a transcriptional silencer or enhancer. Winkler et al. showed that children at risk and islet-autoantibody positive with the rs2111485 variant genotype progressed faster to T1D (115). The contribution of other SNPs in the disease progression was not validated in this study. Variants rs1990760 and rs3747517 are located within the binding site of transcription factors and could therefore influence the expression of *IFIH1* (116).

Human PBMCs and cell lines with the *IFIH1* rs1990760 variant (coding an amino acid substitution A946T) have heightened basal and ligand-triggered IFN-I production (117). This SNP was thus characterized as a gain-of-function variant with a capacity to protect the carriers against specific viral challenges while promoting the risk for autoimmune diseases. This confirmed a hypothesis based on the results from previous studies in healthy individuals carrying the rs1990760 variant and animal models (118, 119), that this variant enlarges the risk for autoimmune disease by increasing the basal activity of IFN-stimulated genes through the recognition of self-dsRNAs without the need for a concomitant viral challenge.

MDA5 activation in DCs mediates cell maturation, increasing antigen processing and presentation through the expression of MHC class I chemokine receptors and co-stimulatory molecules (120), thus promoting the activation and expansion of inflammatory T cells (119). Hence, a heightened MDA5 activation can support the induction of autoimmunity *via* agitated DCs presenting the islet antigens to T cells in a pro-inflammatory rather than an anti-inflammatory context.

Next to the viral or cytoplasmic dsRNA, mitochondrial dsRNA released after β-cell stress could trigger the production of proinflammatory cytokines in individuals carrying the *IFIH1* risk variants (70, 121). Namely, the normal processing of the transcribed mitochondrial genome increases under stress causing a leakage of the mitochondrial dsRNA remnants into the cytosol (121, 122), where MDA5 recognizes these as damage-associated molecular patterns (DAMP). Hence, metabolic stress in β-cells that causes mitochondrial dysfunction might also contribute to the heightened IFN response and apoptosis of β-cells (123).

CTLA4

The Cytotoxic T-Lymphocyte Associated Protein 4 (*CTLA4*) genes encodes a transmembrane co-receptor expressed on the surface of T cells. CTLA-4 functions as a negative regulator of T cell activation which can mediate T cell regulation or apoptosis by interacting with B7, a co-stimulatory molecule present on antigen presenting cells (124–127).

Genetic studies on *CTLA4* in T1D have been focusing on three gene variants: the A49G SNP (rs231775, OR=2.000) in exon 1, the SNP rs3087243 (OR=0.840) which is in high linkage disequilibrium with the dinucleotide (ATn) repeat in the 3'-untranslated regions (UTR) and the coding C318T SNP (rs5742909, OR=1.500) in the *CTLA4* promotor (128).

The first SNP rs231775 is in exon 1 at position 49 from A to G (A49G) of the *CTLA4* gene (129, 130). Meta-analysis of 76 studies showed that the rs231775 variant is more prevalent in T1D patients with Caucasian and South Asian origin and is associated with Type 2 Diabetes (T2D) in East Asians and South Asians (75). The A49G SNP causes the amino acid replacement of threonine to alanine and influences the posttranslational modification of CTLA-4. These modifications result in an inefficient CTLA-4 glycosylation and decreased expression of CTLA-4 on T cells, leading to uncontrolled T cell activation, including the autoreactive T cells (75, 131). The rs231775 variant was also associated with reduced production of soluble CTLA-4 (sCTLA-4), which can inhibit T cell proliferation by binding/blocking B7 (132). This has been confirmed in *Ctla4* KO NOD mice (133), in which the posttranscriptional silencing of sCTLA-4 reduced Treg potency and accelerated T1D onset. Interestingly, sCTLA4 suppressed proliferation of committed islet autoreactive T cell clones isolated from the blood of T1D patients in a dose-dependent manner, but it was unable to suppress naïve alloreactive T cells in an MLR (134), indicating a differential role for sCTLA4 in the control of memory versus primary immune responses.

The second SNP rs3087243 affects the size of dinucleotide (AT)n repeats in the 3'-UTR and the *CTLA4* mRNA stability through a post-transcriptional control (135), influencing the rate of translation (38, 136, 137). De Jong et al. showed that autoreactive T cells with long variants of the (AT)n repeat in the 3'-UTR region have reduced *CTLA4* mRNA levels (138), thus variations in the length of (AT)n repeats influence *CTLA4* expression contributing to the development of T1D. Also a rare genetic variation (rs13384548) within the 3'-UTR of the *CTLA4* mRNA disrupted the miR-302a* binding site reducing the capacity to control *CTLA4* mRNA (139).

The SNP rs5742909 in the *CTLA4* promotor region cause a C to T mutation at position 318. Individuals carrying the minor 318T allele have a higher promotor activity than the 318C allele, resulting in an increased expression of CTLA-4 by T cells (140). While this suggests that the C to T transition increases a regulatory function, the consequences for the T cell response and the effect of this gene variant on the development of T1D is not clear yet.

IL2RA

The protein IL-2Rα (CD25) is a high-affinity subunit of the IL-2 receptor that forms a complex with IL-2Rβ- and γ-chain to activate intracellular signaling upon interaction with IL-2 (141). IL-2RA is constitutively expressed on Tregs and can be induced upon activation in other (effector) T cells (142). Polymorphisms in the genes encoding for the IL-2 receptor, *IL2RA* (rs2104286, rs61839660, rs10795791, and rs41295121) and *IL2RB* (rs743777), are associated with T1D (69, 76, 143, 144). DNA methylation at CpGs (−373 and −456) within the promotor of the *IL2RA* gene was slightly higher in T1D patients than in controls (142), indicating that epigenetic changes in the *IL2RA* promotor might participate in the *IL2RA* risk allele for T1D. Indeed, methylation at CpG-373 was correlated with 16 SNPs in the IL2RA gene, both with the protective alleles (rs12722495, rs2104286, rs61839660) and the susceptible allele at rs11594656 (**Table 1**) (11, 69, 145, 146).

Regarding the functional consequences for T cells, Dendrou et al. showed that individuals with the SNP rs12722495 (OR=0.620) had a higher CD25 expression on CD4+ memory T cells, while the carriers of the SNP rs2104286 (OR=0.880) showed a lower CD25 expression on naïve CD4+ T cells,

compared to the non-carriers (147). They further demonstrated that individuals with the protective variant (rs12722495) consistently had higher proportion of activated IL-2 producing $CD69^+$ $CD4^+$ memory T cells compared to individuals with a susceptible allele, supporting the hypothesis that cells with a higher surface CD25 are more responsive to IL-2R mediated activation (147). This is consistent with the earlier observed defects in IL-2 production in T1D patients (148, 149). Cerosaletti et al. challenged the view that expression levels of CD25 functionally contribute to the susceptibility and showed a reduced signaling from IL-2R (measured by a phosphorylation of STAT5) in CD4+ CD25hi T cells of T1D patients and healthy individuals carrying the rs2104286 risk haplotype (150). The unexpected higher expression of CD25 on naive Tregs in T1D patients and healthy controls with the rs2104286 risk haplotype compared to the carriers of the protective variant, was not explained in this study. The rs2104286 haplotype also correlated with increased soluble IL-2RA levels, suggesting that shedding of the IL-2RA may account for the reduced IL-2R signaling in these individuals. Alternative hypothesis explaining the protective effect of the SNP rs12722495 and the contribution of polymorphisms in IL-2R-pathway in general was through the effects on nTregs (151). Given their constitutive expression of CD25 and a strong sensitivity to IL-2, lower IL-2 signaling measured by the STAT5 phosphorylation reduces the IL-2 response, impacting the FOXP3 expression and thereby affecting the inhibitory function of Tregs (151, 152).

The SNP rs61839660 (OR=0.620) is located within the *IL2RA* gene and it is a non-coding causal SNP variant for T1D (11, 60). This SNP is co-inherited with a so-called group-A protective T1D haplotype that also includes the rs12722495 (153). Interestingly, a rare variation in the group-A haplotype causing the loss of the protective allele only at SNP rs61839660 was sufficient to counteract the high *IL2RA* mRNA and surface CD25 expression (153). The mechanistic studies revealed that the minor SNP variant reduces the *IL2RA* enhancer activity (154, 155), which is stimulation-responsive causing a delay in CD25 expression upon T cell activation, and that a deletion of this enhancer diverted the effective Treg polarization in mice (155).

Monocytes-derived and myeloid DCs express CD25 both as a surface-bound and soluble molecule when stimulated with prostaglandin E2 (PGE2) (156). Also, tumor-associated DCs co-express CD25 and the inhibitory molecule IDO (156). In our hands, tolDC express lower *IL2RA* mRNA and lack the surface-bound CD25 compared to mDCs (17). We did not measure whether tolDC also release less soluble CD25. Taken together, the surface-bound CD25 may enable mDCs to catch IL-2 and use it to stimulate T cells, while the soluble CD25 molecule could work to block IL-2 and help the regulation of T cell responses (157). The contribution of *IL2RA* risk variants to the DC function has not been investigated. As the effects described so far in T cells predominantly impact the downstream IL-2R signaling and DCs do not express other two proteins of the IL-2R complex, the functional contribution of genetic polymorphisms in *IL2RA* is more likely to show through the surface expression or production of soluble IL-2RA than to impact DC differentiation.

CD226

CD226 or DNAX-accessory molecule-1 (DNAM-1) is a transmembrane receptor expressed on T cells, NK cells, NKT cells, platelets and a subset of B cells (69, 158), and aids their activation and differentiation through co-stimulation (159). The inhibitory counterpart of CD226 is T cell Immunoreceptor with Ig and ITIM domains (TIGIT), which is a negative regulator molecule expressed in Tregs and NK cells (160). TIGIT binds CD155 on DCs, driving them towards a tolerogenic phenotype. Disturbance of the TIGIT/CD226 axis could therefore contribute to the development of autoimmunity (161).

The SNP rs763361 (Gly307Ser, OR=1.120) in the *CD226* gene is associated with multiple autoimmune diseases, such as T1D, multiple sclerosis (MS), autoimmune thyroid disease, RA, SLE and systemic sclerosis (162). This SNP results in a missense mutation at position 307 (glycine to serine) and is located in two intracellular phosphorylation sites of the protein (residue 322 and 329) (159, 163). The SNP rs763361 may alter RNA splicing by disrupting splice site enhancers or silencers, resulting in an isoform of CD226 with altered function (69, 159, 163) and increased CD226 activity in T cells (164).

Indeed, Gaud et al. showed that *in vitro* anti-CD226 and anti-CD3 co-activation of human primary CD4+ T cells of individuals carrying the rs763361 risk variant induces enhanced p-ERK (164). The ERK pathway regulates T cell activation and differentiation. The rs763361 variant is associated with skewing to Th17 and Th17.1 cells after stimulation *in vitro* (164). Indeed, T1D patients carrying the rs763361 risk variant had greater frequency of GAD antibody and low C-peptide levels, reflecting a more aggressive disease pattern in a Brazilian population (165). Wallace et al. observed that the rs763361 risk variant correlated with reduced CD226 mRNA levels in monocytes and which could reduce cell activation and thus alter the interactions between monocytes and lymphocytes (166). When *Cd226* was deleted in NOD mice, this decreased disease incidence and insulitis as compared to WT mice (167), but the deletion also increased the number of CD8+ thymocytes and splenocytes. The CD226 deficient CD8+ T cells showed decreased reactivity to the β-cell specific antigen IGRP, from which Shapiro et al. concluded that CD226 plays a role in the development of T1D by modulating thymic selection and affecting activation of CD8+ T cells (167). The effect of the rs763361 risk variant has not been studied in human CD8+ T cells or Tregs. The majority of human Tregs highly express TIGIT, but a Treg subset co-expresses CD226 (168). These CD226+ Tregs were associated with reduced suppressive capacity. Hypothetically, the rs763361 variant, which increases CD226 activity in T cells, will increase the proportion of CD226+ Tregs and thereby reduce the overall suppressive capacity of Tregs. Studying further the expression and function of CD226 in

humans is needed for a better understanding of whether the rs763361 risk variant contributes through T cell activation only or also by affecting the interaction between monocytes and lymphocytes.

TOLEROGENIC MODULATION OF DENDRITIC CELLS AND THE IMPACT ON THE MINOR RISK GENES

Gene expression can be changed by genetic engineering or using bioactive small molecules, for which aim the specific targeting of the scarce autoreactive T cells seems difficult. The targeting through DCs seems more viable and allows also antigen-specific immune modulation (169). The active form of VitD3 functions as a transcription factor upon binding to the vitamin D receptor (VDR) (170), creating a complex that binds with retinoid-X receptor (RXR) to enable the attachment to vitamin D response elements (VDRE) (171, 172). The VDR complex has a large effect on more than 3000 target genes, which includes forty-seven transcription factors and thus leaving hardly any immune pathway unaffected by VitD3 (173). This natural immunomodulator influences the development and function of T cells, B cells and monocytes (172, 174, 175), and controls the ability of the immune system to dampen inflammation. In two independent studies we found that about a third of the transcripts encoded by non-HLA T1D risk genes were differentially expressed between inflammatory mDCs and VitD3-derived tolDCs (17, 18). Interestingly, only five of these genes were also reported as direct targets of VDR (170), leaving others to an indirect control by VDR-targeted transcription factors. Of the direct VitD3-targets, the expression of *ORMDL3*, *SH2B3*, *IKZF1*, *PTPN2* and *IFIH1* genes was lower in tolDC while RAC2 and PTPN22 were higher in tolDC than in the inflammatory mDCs (17, 18).

The protein encoded by ORMDL3 is an enzyme involved in sphingolipid synthesis and lipid metabolism without a clear function in the immune response but interestingly the T1D patients who were the carriers of the linked polymorphism (rs12150079) showed a lower intensity of autoreactive T cell responses in T1D (11, 60, 176). The *SH2B3* encodes LNK (lymphocyte adaptor protein) that takes parts in several signaling pathways controlling the hematopoiesis, cytokine and integrin signaling and cell migration (177). The functional consequences of the T1D risk variant (rs3184504) that causes a missense mutation remain speculative (11, 59, 146), one study using human cells that reports an augmented lymphocyte proliferation that correlates with the predisposing gene variant (178) Interestingly though, a recent study shows that the T1D risk-gene variant associates with a reduced mortality from sepsis in individuals with a European decent and suggest based on a mouse model that augmented phagocytosis and myelopoiesis may be underlying mechanisms (179). The gene *IKZF1* codes for the transcription factor Ikaros (180), and the associated SNPs (rs10277986, rs62447205) are protective for T1D (11). How these SNPs affect the expression or function of Ikaros has not been described. Ikaros is a regulator of dendritic cell differentiation and immune homeostasis, and *IKZF1* deficiency causes less inflammatory cytokines secretion by human monocytes (181), which is in line with the observed lower expression in our tolDCs. Finally, *RAC2* encodes a protein from a Rho family of GTPases involved in cytoskeletal reorganization (e.g. needed for phagocytosis) but the effect of the described SNP variant (rs229533) increasing the risk for T1D is still unknown (11).

CAN A MODEL BASED ON AN INTEGRATED VIEW ON THE GENETIC RISK HELP US TREAT PATIENTS?

In our view, the polymorphisms in immune genes as are discussed in this review can influence both immune activation and regulation through a change in gene expression or in function (**Figure 1**). The consequences may differ between the cell types depending on the expression level or an implicated cellular function of a given gene. Indeed, by changing the expression of a target protein, some risk variants cause different functional effects in conventional T cells, Tregs or β-cells, depending on the implicated cellular function (**Figure 1**). For most of the evaluated genes, both the non-risk variant and the risk variant show opposing functional consequences in conventional T cells compared to Tregs. Namely, the risk genes for which a non-risk variant supports immune regulation (*PTPN2*, *CTLA4* and *IL2RA*) are indeed activating for Tregs and work to suppress the effector T cells. The risk-variant SNPs of these genes change the function in the same manner irrespective of the cell type but the end result differs so the lower expression and signaling through PTPN2, CTLA-4 or IL-2RA will simultaneously impair the function of Tregs and release the tight control of the effector T cell. Similarly, the activation-promoting function of CD226 in effector T cells, enhanced by the risk-SNP, suppressed the inhibitory function of Tregs. Further, based on the regular function of LYP (PTPN22) to control the post-TCR signaling events, the activating contribution of the risk mutation in Teff is evident but the consequences for the human Tregs remain to be confirmed. The risk-SNP induced modulation of *PTPN2* and *IFIH1* will increase β-cell apoptosis, which increases the antigen release, and thus contribute to the development of T1D. In DCs the *PTPN22* risk variant fails to promote upregulation of type I IFN which might result in diminished human host-protecting responses when dealing with viral infections. The *PTPN2* risk variant may dysregulates the production of inflammatory cytokines and thus the maintenance of immune tolerance by DCs. The *IFIH1* risk variants causes an increased IFN response, stimulating antigen presentation, while the *IL2RA* risk variant may inhibit the capacity of DCs to suppress T cell proliferation and cytokine production. In summary, the functional consequences of the causal T1D-risk variants have been extensively investigated and seem to paint a clear picture regarding the individual contributions but it is difficult from this information to consider how the polymorphisms may

cumulatively modify the cell interactions that promote the impaired β-cell specific immune responses in individuals with high T1D risk-scores.

At the moment, no therapies have been developed that target *PTPN22*, *PTPN2*, *IFIH1* and *CD226*. Therapies targeting *CTLA4* (CTLA-4-Ig, abatacept) has been proven as safe and efficient to inhibit naïve T cell activation and therefore this approach is more selective namely inhibiting only T cell responses as compared to general/broad immunosuppression. The safety of abatacept as a subcutaneous (SC) and intravenous (IV) therapy is evaluated in RA (182, 183) and T1D patients, reporting no therapy-specific adverse effects (184, 185). Abatacept treatment showed good efficacy in prevention complete loss of β-cell function in T1D patients as is shown by preservation of C-peptide levels and insulin sensitivity improvement (184, 186, 187).

To compensate for the impaired IL-2 signaling due to lower expression or dysfunction of the IL2R gene, specifically in Tregs, ultra-low-dose IL-2 therapy has been tested, thus avoiding potential toxic effects of systemic IL-2. A phase II study has already been completed to determine the optimal IL-2 dose to use as a treatment in T1D patients (188). Participants did not exhibit severe adverse effects, a minimal NK cell expansion was observed after IL-2 treatment and no detrimental changes in glucose metabolism were observed, guaranteeing the safe use of IL-2 (188). Also, there was a dose-dependent increase in Tregs in all patients, and the low dose of IL-2 upregulated CD25 and FOXP3 expression on Tregs while CD4+ T effector memory cells were unchanged (189). Even though the latter trial showed some interesting effects of IL-2 therapy, the insulin secretion was not measured so the clinical efficacy of this therapy (i.e. on β-cell preservations) could not be determined. A newer alternative to avoid the influence on effector T cells uses the IL-2 mutant proteins (190), which has advanced to the clinical testing in GvHD (ClinicalTrials.gov Identifier: NCT03422627), though it remains a systemic antigen-independent approach.

Even though these therapies targeting *CTLA4* and *IL2RA* seem promising, it remains to be seen whether they are beneficial for all patients or only those carrying the affected variant, and to what extent such therapies may influence the immune system and health in general given the generic and pleiotropic effects of IL-2. It is tempting to investigate whether therapies targeting specific pathways in which a risk gene of interest is involved or epigenetic targeting of a single of multiple risk gene variants will be more beneficial.

A treatment that simultaneously tackles multiple changes in the risk genes allowing a correction towards the non-risk variant function could potentially aid as curative intervention. The natural immunomodulator VitD3 seems a good candidate since it reduced the expression of *IKZF1*, *PTPN2*, *IL2RA*, *CD226* and *IFIH1* while increased *RAC2*, and *PTPN22* in tolDC as compared to mDC. Considering the effects of the discussed risk-SNPs in these genes, the modulating action of VitD3 could counteract the immune-activating effects of risk-mutations in *IL2RA*, *CD226*, *IFIH1* and *PTPN22* while supporting the protective effects of *IKZF1*.

The potential clinical benefit of the treatment with Vitamin D, has been recognized earlier. The initial trial with VitD3-modulated tolDC in T1D patients confirmed safety and the clinical benefit of the treatment remains to be tested (191). VitD3 modulates T cells (172, 175, 192, 193) and a trial testing the combined treatment of T1D patients with VitD3 and GAD antigen did not show significant change overall but a particular β-cell preservation in individuals with the HLA DR3-DQ2 haplotype (194). Trials testing vitamin D supplementation (195–200), showed some clinical benefit such as improving diabetes control (HbA1c or insulin dose), reducing complications (195, 196), some indications of β-cell protection or immune regulation (197, 198), none of the study monitored the clinical and immunological effect simultaneously. Finally, VitD3 can contribute to T1D prevention since early postnatal VitD3 administration seems to protect from T1D (199), even though reduced circulating VitD3 levels do not increase T1D risk (200).

CONCLUSIONS AND PERSPECTIVES

Understanding the role of genetic risk-variants in the T1D pathogenesis can have important implications for better understanding disease pathogenesis and heterogeneity, as well as the development of specific/selective disease intervention strategies. Models have been generated suggesting that different T1D risk-loci contribute to successive pathogenic checkpoints, which detection could allow timely and appropriate modulation of the autoimmunity and increase the chance for curative interventions. The mechanisms involved in the immunotherapy of cancer (201, 202), teach us about genetic variants that increase the risk for the development of autoimmune disease but positively impact the survival after cancer treatment (203). Hence, polygenic risk scores (204) not only help to predict disease but also to predict when a specific patient is more or less likely to respond to immunotherapy directed at the involved pathways.

The genetic risk score could allow early identification of individuals who will develop T1D allowing earlier curative interventions. Butty et al. studied the frequency of non-HLA risk alleles among individuals at risk of developing T1D (DTP-1 trial), of which about one-third progressed to the clinical disease (205). They concluded that immune risk gene variants more likely condition the initial development of autoimmunity, resulting in a detectable auto-Ab response, but less critically contribute to the events leading to disease onset (205). Hence immune modulation therapy makes more sense prior to the onset of autoimmunity, which will be possible when a prediction of T1D is improved. The recently reported improved cumulative risk score (T1D-GRS2) that includes 67 SNPs (all HLA-DQ haplotypes, non-DR-DQ loci within the HLA region and non-HLA loci) indeed enabled a sensitive discrimination of T1D from T2D and controls (13), but also improved the prediction of future T1D in infants. Still, around 10% of all infants would have to be monitored to capture 77% of future T1D cases. In this study it

remains unclear whether successive application of the HLA-score, followed by a non-HLA score would have further improved the prediction sensitivity. Importantly, this GRS failed to predict T1D in patients with different ethnicities, underscoring the need to study all-inclusive cohorts (206).

Alternatively, fine mapping genetic studies of previously known autoimmune loci will also help to find relevant genetic variants with strong effect on the development of T1D (11, 60, 207). The availability of large human whole-genome sequencing data sets, also allows detecting rare SNPs with large effect size on complex traits (208, 209). Forgetta et al. recently discovered three novel risk gene variants in large human whole-genome sequencing data sets of T1D patients (67). Hence, studying the human whole-genome sequencing data might lead to the discovery of gene variants, which will give a better understanding of the genetics behind the development of T1D and possibly predict therapy responses.

Taken together, current literature only partially explains the functional implications of the risk-SNPs to the development of autoimmunity in T1D. The efficient in-depth analyses of the immune response that can detect and monitor low-frequent autoantigen-specific cells and a better understanding of immune tolerance are needed to investigate and understand the functional contributions of genetic polymorphisms in different cells of the immune system. The same polymorphism can have opposing functional consequences depending on the cell in which the linked gene is expressed. Gaining insight into how the human genetics impacts functional immunity is therefore important to allow discrimination of relevant and treatable targets and for selecting proper immunotherapy strategies with the most benefit for patients or individuals at risk of developing T1D.

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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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Genetic Variations Controlling Regulatory T Cell Development and Activity in Mouse Models of Lupus-Like Autoimmunity

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Immune homeostasis is a constant balancing act between effector T cells and regulatory T cells defined by *Foxp3* expression, the transcription factor that drives their differentiation and immunosuppressive activity. Immune homeostasis is altered when Treg cells are not generated or maintained in sufficient numbers. Treg cells rendered unstable by loss of *Foxp3* expression, known as ex-Treg cells, gain pro-inflammatory functions. Treg cells may also become dysfunctional and lose their suppressive capabilities. These alterations can cause an imbalance between effector and regulatory subsets, which may ultimately lead to autoimmunity. This review discusses recent studies that identified genetic factors that maintain Treg cell stability as well as preserve their suppressive function. We focus on studies associated with systemic lupus erythematosus and highlight their findings in the context of potential therapeutic gene targeting in Treg cells to reverse the phenotypic changes and functional dysregulation inducing autoimmunity.

Keywords: regulatory T cells, Foxp3, autoimmunity, lupus, genetics

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INTRODUCTION

Regulatory T cells maintain immune homeostasis and prevent autoimmune diseases by limiting the responses of proinflammatory and autoimmune T cells. Several subsets of Treg cells have been characterized, among which the classical Foxp3$^+$ CD4$^+$ T cells, referred to here as Treg cells, play an essential role. The mechanisms by which these cells maintain immune homeostasis involve inhibitory cytokines, cytolysis, and metabolic disruption of effector T (Teff) cells (1). Treg cells are defined by the stable expression of *Foxp3, a* forkhead/winged helix transcription factor, and high levels of the high affinity interleukin-2 receptor (IL-2R) α chain (CD25) on their surface (2), which are the main genes required for Treg cell development, maintenance, and function (3). There are two major types of Treg cells: thymus Tregs (tTregs) that develop in the thymus, and peripheral Treg (pTregs) cells that are generated in peripheral sites. In addition, studies have been conducted on induced Treg (iTregs) cells that are induced *in vitro* by T cell receptor (TCR) activation in the presence of TGFβ (4). Treg cell stability, i.e. the maintenance of their transcriptional program, is indispensable for the preservation of their function. Furthermore, unstable or "ex-Treg" cells induce inflammation not just by a lack of suppression but also in a direct manner by secreting inflammatory cytokines (5).

DEFECTIVE NUMBER OR FREQUENCY OF TREGS LEAD TO AUTOIMMUNE DISEASES

Numerical and/or functional Treg anomalies contribute to autoimmune diseases such as type 1 diabetes (6), rheumatoid arthritis (7), and systemic lupus erythematosus (SLE) (8). The absolute number of circulating Treg cells is decreased in SLE patients with active disease as compared to healthy controls (9–13). The number of Treg cells was shown to have a strong inverse correlation with SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) scores, showing the lower numbers of Treg cells corresponding to increased disease severity (11, 12). Controversially, other studies have reported increased (14–16) or similar (17, 18) Treg cell numbers in SLE patients as compared to healthy controls. The discrepancy between these studies has been attributed to different definitions and gating strategies for Treg cells, including the fact that expression of Foxp3 alone is not a reliable marker for human Treg cells, further complicating analyses of their function and stability (12, 19). Variations in the treatment regimen with immunosuppressive drugs may also contribute to the large variations in relative Treg cell frequencies in SLE patients. Dysfunctional Treg cells have also been reported in SLE patients (8), including the expansion of a Treg population with a low CD25 expression (20).

This review discusses recent studies that have identified intrinsic genetic factors maintaining Treg cell stability as well as preserving their suppressive function. We focus on studies associated with systemic lupus erythematosus pathogenesis, or with a lupus-like phenotype, and we highlight their findings in the context of potential therapeutic gene targeting in Treg cells to reverse the phenotypic changes and functional dysregulation inducing systemic autoimmunity. The many studies that have reported gene targeting affecting Treg cells in other autoimmune diseases such as arthritis, uveitis or experimental autoimmune encephalomyelitis are not included in this review. Additionally, other studies that have reported deletions of specific genes in other cell types, such as dendritic cells, that affect Treg cell development and function are also not mentioned in this review.

SINGLE GENE DETERMINANTS OF TREG CELL HOMEOSTASIS

Scrufy mice do not produce Treg cells due to a mutation in *Foxp3*, causing them to develop a severe inflammatory disease with autoimmune components, including lupus-like manifestations (21). A large number of studies have now defined *Foxp3* as the master regulator of Treg cell differentiation and functions (3). A recent study has shown that *Foxp3* sustained expression is also necessary to maintain Treg functions once they have differentiated (22). Reverse genetic approaches have identified several genes that control Treg cell number, stability and/or functions through *Foxp3* expression, and whose deficiency or overexpression lead to autoimmunity or lupus-like manifestations.

Negative Regulators of *Foxp3* Expression

The AP1 transcription complex is comprised of a network of heterodimers formed by proteins of the Jun, Fos, ATF, and MAF families. Fos/Jun dimers promote the expression of *Foxp3* through direct binding to its promoter in response to TCR signaling (23). Within this transcription complex, Fos-like 2 (*Fosl2*) inhibits Treg development in a cell-intrinsic manner (24). *Fosl2* transgenic mice develop spontaneous autoimmunity and systemic inflammation with disease phenotypes resembling that of Treg-deficient IPEX patients and scurfy mice. On the other hand, mice lacking *Fosl2* in $CD4^+$ T cells display less severe disease phenotypes. Mechanistically, Fosl2 interrupts Treg development by repressing the expression of *Foxp3* as well as that of other genes involved in Treg differentiation or function (24).

NFIL3 (Nuclear factor Interleukin 3 regulated, also known as E4 binding protein 4, E4BP4) represses numerous genes and regulates diverse biological processes (25, 26). In the immune system, NFIL3/E4BP4 has a vital role for many cell types including Th1, Th2, NKT and Treg cells by regulating the plasticity of cytokine production (27, 28). Treg cells are the T cell subset with the lowest *Nfil3* expression, and its overexpression attenuated the suppressive ability and stability of these cells (29). Not only does NFIL3 binds directly to the *Foxp3* promoter reducing *Foxp3* expression, but it also downregulates the promoter activity of Treg hallmark genes such as *Icos*, *Tnfrsf18*, *Ctla4*, and *Il2ra*, in both Foxp3-independent and dependent pathways (29). Accordingly, *Nfil3*-deficiency in T cells increased *Foxp3* expression, but decreased the frequency of *Foxp3*-expressing follicular regulatory T (Tfr) cells, resulting in an expansion of follicular helper T (Tfh) cells and the production of autoantibodies (30). Tfr cells are a specialized subsets of tissue Treg cells that work to constrain the activity of Tfh cells and germinal center (GC) B cells with whom they share the Bcl6 transcription factor (31). A decreased relative frequency of Tfr cells has been correlated with disease activity in SLE patients (32). NFIL3 expression was increased and its phosphorylation was decreased in $CD4^+$ T cells from patients with SLE with a positive correlation to disease activity (30). These alterations were associated with the characteristic expansion of Tfh cells in SLE. It would be of great interest to follow up this study with an analysis of the impact that NFIL3 increased expression and decreased phosphorylation has on Treg and Tfr cell numbers and functions in SLE.

Positive Regulators of Foxp3 Expression

NF-kβ is one of the multi-molecular complexes that interacts with Foxp3 to control Treg cell transcriptional programs and biology. c-Rel, one of its subunits activated by TCR signaling, supports tTreg development and *Foxp3* expression by binding to its promoter and one of its regulatory non-coding sequences (CNS3) (33). NF-kβ maintains the stability of mature Treg cells by preventing them from converting into effector-like T cells through mechanisms involving IKKα and IKKβ kinases, which are upstream activators of the NF-kβ pathway (34, 35). Foxp3 forms a complex with Rel-A, one of the most abundant NF-kβ

subunits in conventional T cells, and with other transcription factors including Helios and p300, leading to its full functionality as a transcriptional activator (36). Foxp3-Cre mediated depletion of Rel-A in established Treg cells resulted in defective effector Treg cells that led to the development of an autoimmune syndrome characterized by a massive T cell activation, immune infiltrations of several tissues, as well as the production of inflammatory cytokines, and autoantibodies (36, 37). Furthermore, Rel-A deficient Treg cells were unstable and lost *Foxp3* expression becoming ex-Tregs expressing high amounts of proinflammatory cytokines IFNγ and TNFα (36).

Bcl10 is a gene in the Carma1-Bcl10-Malt1 (CBM) signaling complex that controls NF-kB and MAPK activation in T cells following TCR activation (38). Bcl10 is necessary for the development of Treg cells and their suppressive function. *Bcl10*-deficient Treg cells converted in proinflammatory effector T cells secreting IFNγ, leading to a fatal systemic autoimmunity (39). This indicated that Bcl10-mediated NF-kB activation is required for Treg cell development and function. Previous studies have reported that HIF1-α directly binds to the IFNγ promoter in VHL-deficient Treg cells, a model described later in the text, provoking an increased IFNγ production and impairing Treg cell function (40). This phenotype is also displayed in *Bcl10*-deficient Treg cells (39).

Sclerostin domain-containing protein 1 (SOSTDC1) is selectively expressed in Tfh cells (41), which secretes this factor once they have lost the ability to help GC B cells (42). SOSTDC1 deficiency greatly reduced the generation Tfr cells, which in turn enhanced humoral immunity against viruses (42). Mechanistically, SOSTDC1 inhibits the canonical WNT-β-catenin pathway (43), which in turn inhibits Treg cell differentiation (44, 45). It should be noted that an autoimmune phenotype was not reported in these mice. This implies that although the differentiation of tTreg cells into Tfr cells was impaired, Treg cells themselves were functional and the effect of SOSTDC1 secreted by Tfh cells is confined to the GCs. Inhibition of Tfr cell differentiation in SOSTDC1-deficient mice was mediated by the stabilization of β-catenin (42). As a negative feedback loop, late-stage Tfh cells secrete SOSTDC1, which commits Treg cells in the GC to the Tfr fate by blocking WNT stimuli. Uncontrolled WNT-β-catenin signaling plays a role in autoimmune diseases (46), which may be due, at least in part, to defective Treg and Tfr cell differentiation.

GENES REGULATING TREG CELL FUNCTION AND STABILITY THROUGH THEIR METABOLISM

Mammalian target of rapamycin corresponds to two kinase complexes, mTORC1 and mTORC2, which function as a central metabolic checkpoint. The functional links between metabolism and effector functions has been dissected in T cells, in which the integration by mTOR of the stimulatory signals and the energy status of the cells plays a critical role (47). Treg cells display diminished activity of the mTOR pathway as compared to Teff cells (46, 47), and increased mTOR activity negatively affects the generation and function of Treg cells (48–51). However, mTORC1 deficiency profoundly impairs Treg development and function (52). Mechanistically, mTORC1 enables cholesterol synthesis and lipid metabolism that are triggered by IL-2 signaling, both for which being required for Treg cell proliferation and the upregulation of suppressive molecules. mTOR signaling is required for the generation and function of both tTregs and pTregs, and its Foxp3-driven deletion impairs mitochondrial metabolism and oxidative phosphorylation, which is the main source of energy in Treg cells (53). Accordingly, Treg-specific deletion of the mitochondrial transcription factor *Tfam* severely impaired Treg suppressive functions (53). A recent genome-wide CRISPR/Cas9 screen combined with *in silico* analyses of protein-protein interaction networks identified novel regulatory modules that mediate mTORC1 signaling in Treg cells (54). The requirement for the expression of *Sec31a* and *Ccdc101*, two key genes in these modules, was validated when their deficiency in Treg cells impaired their suppressive functions and led to inflammatory phenotypes. SEC31A promotes mTORC1 activation by interacting with the GATOR2 component SEC13 to protect it from SKP1-dependent proteasomal degradation. Therefore, SEC31A expression is necessary to maintain mTORC1 activation in Treg cells. On the other hand, CCDC101 is a member of the SAGA complex, a potent inhibitor of mTORC1. Therefore, CCDC101 limits the expression of glucose and amino acid transporters and maintains a relative metabolic quiescence that characterizes Treg cells. *Ccdc101*-deficiency impairs Treg cells by unleashing an overreactive mTORC1. Additionally, Lamtor1, a lysosomal scaffold protein for mTORC1 is also important for Treg cell survival. Mice with Lamtor1-deficient Treg cells develop severe autoimmunity showing that Lamtor1 is a vital intrinsic factor for Treg suppressive functions, but not for their development and survival (55).

PP2A is a serine-threonine phosphatase composed of a catalytic C subunit $PP2A_c$, a scaffold A subunit $PP2A_A$ and a regulatory B subunit $PP2A_B$ (56). PP2A is highly expressed in Treg cells, and mice with a Treg-specific deletion of a member of the $PP2A_A$ subunit developed multi-organ autoimmunity with similarities to the scurfy phenotype (57). This indicated that PP2A activity is required to maintain Treg cells. $PP2A_A$-deficiency increased mTORC1 activity in Treg cells, resulting in enhanced glycolysis and oxidative phosphorylation (57), a phenotype that was reversed by a treatment with mTOR inhibitor rapamycin. Therefore, PP2A activity is necessary to prevent mTORc1 overactivation, a process essential for suppressive function of Treg cells. In addition, $PP2A_c$ is required for Treg cell to function by preventing the loss of expression of the IL-2Rβ chain, enabling IL-2 signaling (58). PPP2R2D is a regulatory subunit of PP2A whose expression is increased in T cells from patients with SLE. Mice with PPP2R2D-deficient T cells developed a reduced systemic autoimmunity in response to TLR7 activation (59). Furthermore, PPP2R2D-deficiency enhanced the suppressive function of Treg cells,

which was supported by an increased IL-2 transcription in conventional T cells, a process that is negatively regulated by PPP2R2D (59). Therefore, PPP2R2D regulates Treg cells through PP2A in a cell-extrinsic manner (IL-2 secretion from conventional T cells), as opposed to PP2A controlling Treg function through mTORC1 in a cell-intrinsic manner.

HIF-1α and HIF-2α are two master transcription factors responsible for the physiological responses to hypoxia (60). Under normoxic conditions, prolyl hydroxylase domain proteins (PHD2/PHD3) hydroxylate HIF-1α and HIF-2α allowing for their recognition by von-Hippel Lindau tumor suppressor (VHL)-containing E3 complex, ubiquinating the transcription factors for proteasomal degradation. This process is interrupted under hypoxic conditions, allowing the accumulation of HIF-1α and HIF-2α (61). In immune cells under normoxic conditions, the expression of HIF-1α can also be increased by mTOR activation (62) and induce glycolysis (63). Germline *Hif1a*-deficiency promoted the differentiation of Treg cells over Th17 cells (64, 65). Mechanistically, HIF-1α promotes Foxp3 degradation by the proteasome (64). Germline *Hif1a* deficiency also inhibited glycolysis in favor of mitochondrial metabolism, which promoted Treg cell differentiation (65). Interestingly, *Hif1a*-deficiency in established Treg cells (through Foxp3-Cre mediated deletion) did not impair Treg cell function (66). This indicated that HIF-1α regulates Treg cell differentiation but not their maintenance and function. *Hif2a*-deficiency in established Treg cells impaired their suppressive activity despite normal *Foxp3* expression (66). Moreover, *Hif2a*-deficient Treg cells showed an enhanced secretion of IL-17 (66). Importantly, patients with SLE and associated lupus nephritis have increased numbers of IL-17-producing Treg cells in their peripheral blood (67). These studies demonstrate a complex crosstalk between HIF-1α and HIF-2α in Treg cells in which HIF-1α prevents their differentiation and HIF-2α stabilizes their function.

VHL-deficiency in Treg cells impaired their suppressive activity and stability leading to massive inflammation (40). VHL-deletion induced a HIF-1α-mediated expression of glycolytic enzymes in Treg cells that promoted Th1 differentiation. Moreover, HIF-1α directly activates the *Ifng* promoter. These results contrast with the lack of phenotype resulting from direct deletion of *Hif1a* in Treg cells (59), and suggest that HIF-2α constitutive expression in VHL-deficient Treg cells is likely to play a role.

Serine/Arginine-rich splicing factor 1 (SRSF1) is the prototype member of the highly conserved serine 1 arginine (SR) family of RNA-binding proteins (68). SRSF1 expression was decreased in the T cells of SLE patients with severe disease showing an overactive T cell phenotype (69). Deletion of SRSF1 in T cells led to systemic autoimmunity and lupus nephritis that was associated with mTOR activation in T cells (70). Treg-specific deletion of SRSF1 also led to systemic autoimmunity with Treg cells losing their suppressive function and producing proinflammatory cytokines (70). As with pan-T cell deletion, SRSF1-deficient Tregs displayed a highly glycolytic metabolism and mTOR activation.

TREG CELL REGULATION IN SPONTANEOUS MOUSE MODELS OF LUPUS

Many studies have documented alterations in Treg numbers and functions in spontaneous mouse models of lupus (71). Multiple mechanisms are responsible for these phenotypes, with a major contribution of the inflammatory milieu created by cytokines such as Type 1 IFN and IL-6. Whether the genetic susceptibility that drives lupus pathogenesis in these models affects intrinsically Treg cells, at least in part, is less understood. The frequency of Treg cells varies across a wide range in mice and humans without pathogenic consequences (72). NZW mice do not develop autoimmunity, but their genome contains lupus susceptibility genes that are revealed when combined with other genomes such as NZB or BXSB (73). NZW mice present a low frequency of Treg cells, which was found to be cell-intrinsic and due to a low Foxp3 expression leading to a poor stability of the Treg program (72). Although NZW Treg cells express a distinctive transcriptional profile, it could not be attributed to a single genetic defect. Therefore, the NZW Treg phenotype is likely to be supported by a complex polygenic inheritance, similar to lupus susceptibility as a whole in NZW-derived strains (74). However, we propose that these intrinsically defective NZW Treg cells become pathogenic when combined with other immune defects induced by alleles from lupus-prone strains.

The (NZB x NZW) F1-derived NZM2410 strain is a model of lupus in which an analysis of genetic susceptibility has been conducted, and genes regulating T cell function have been identified (74). NZM2410 mice carry three major susceptibility loci associated with lupus nephritis, *Sle1*, *Sle2*, and *Sle3* (75). Congenic strains carrying separately each of these loci on a non-autoimmune C57BL/6 (B6) background present distinct autoimmune endophenotypes that correspond in combination to the lupus phenotype of the parental strain (76). *Sle1* had the strongest linkage to lupus nephritis and its expression is necessary for the development of autoimmunity in NZM2410 mice (77). *Sle1* regulates the function of T cells (78) in a cell-intrinsic manner (79), and it decreases the number and function of Treg cells (78). *Sle1* corresponds to at least three sub-loci, *Sle1a, Sle1b*, and *Sle1c* (80). Within *Sle1a*, genetic linkage analysis identified an interacting locus *Sle1a1* responsible for expanding the number of activated $CD4^+$ T cells while reducing the frequency of pTreg cells (81). *Sle1a1* only contains one functional gene, *Pbx1* (81), a transcription factor required for mammalian organogenesis (82). Pbx1 is required for the development of B cells and the function of hematopoietic stem cells (83, 84), but its function in T cells had not been characterized. *Sle1a1* corresponds to the overexpression of the truncated splice isoform Pbx1-d over Pbx1-b, the normal isoform, in T cells (85). Pbx1-d lacks both the DNA-binding and HOX-binding domains and functions as a dominant negative (86). The mouse and human PBX1 proteins share complete homology, and PBX1-D was found more frequently in the $CD4^+$ T cells from SLE patients than healthy controls (85).

Furthermore, PBX1-D expression in human CD4+ T cells is associated with defective Treg cells (87). Mice overexpressing Pbx1-d in T cells replicated the phenotypes of B6.*Sle1a1* congenic mice as previously mentioned (88). Pbx1-d transgenic overexpression in T cells impaired iTreg differentiation as well as the induction or maintenance of pTreg cells in a cell-intrinsic manner (88). On the other hand, Pbx1-d overexpression in $CD4^+$ T cells expanded Tfh cell differentiation (88). These results suggest that Pbx1 regulates the balance between Treg and Tfh cells, and that Pbx1-d contributes to autoimmunity by tilting the balance in favor of Tfh over Treg cells. This impaired Pbx1-d-mediated T cell homeostasis has consequences on lupus associated atherosclerosis, with chimeric atherosclerosis-prone mice carrying Pbx1-d expressing T cells developing more severe lesions than mice carrying Pbx1-b expressing T cells (89). Furthermore, there is evidence that dyslipidemia and Pbx1-d expression synergized to impair Treg cell functions. The mechanism by which Pbx1 and its dominant negative Pbx1-d isoform regulate T cell function has not been established yet. Interestingly, Pbx1 directly upregulates NFIL3 expression (90), and NFIL3 regulates the expression of Foxp3 and other Treg-associated genes (29). A disruption of the Pbx1/NFIL3 axis is therefore a potential mechanism by which Pbx1-d may alter the Treg/Tfh cell balance in favor of autoimmunity.

Within the *Sle1c* locus (91), recombinant congenic analysis mapped an activated $CD4^+$ T cell phenotype to the *Sle1c2* sub-

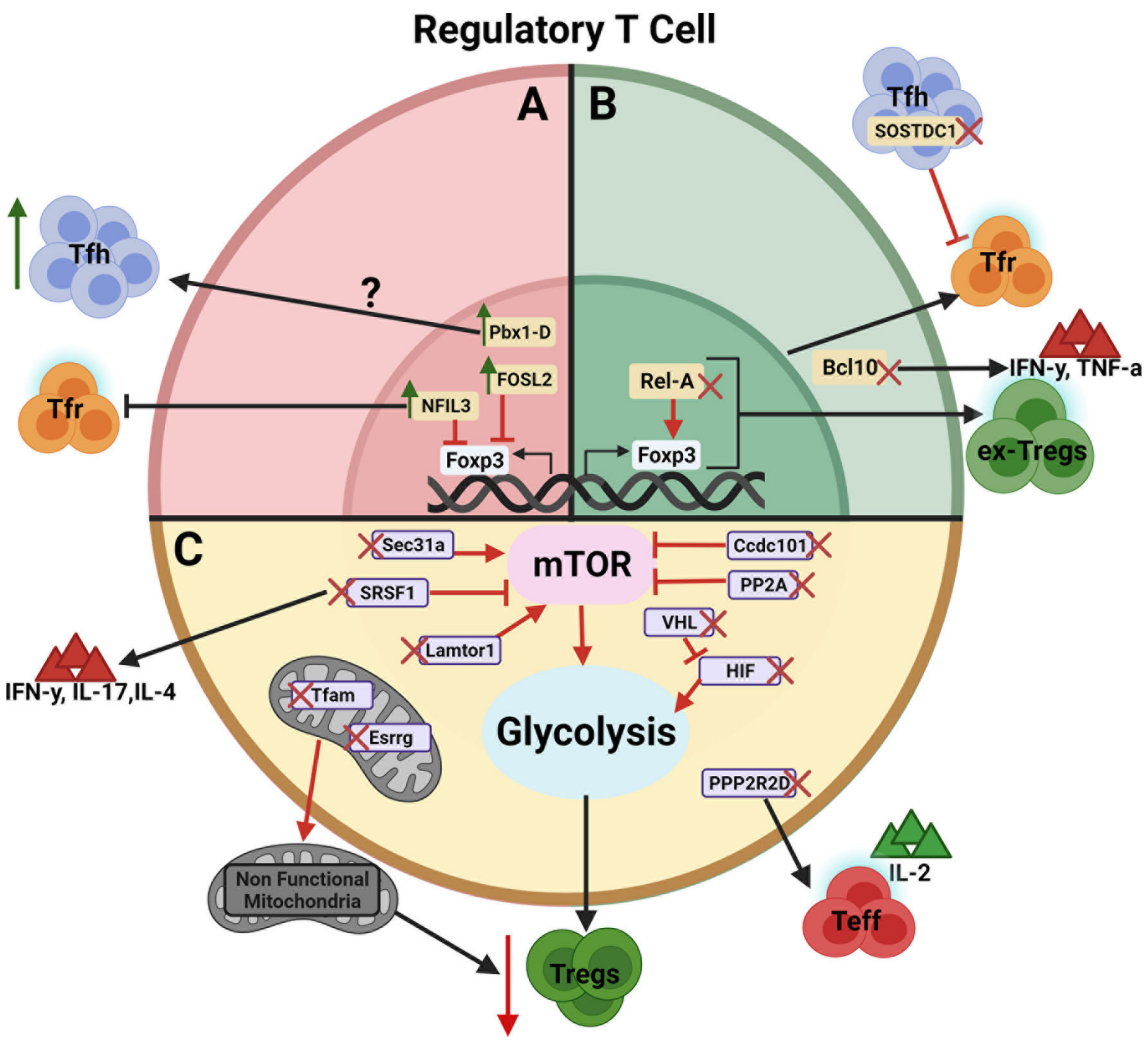

FIGURE 1 | Schematic view of genes regulating Treg cell development, function, and/or stability. The genes are presented according to their effect on Treg cells. **(A)** Negative regulators whose over expression leads to an expansion of Tfh cells or inhibition of Tfr cells (Green arrows indicates gene overexpression).? indicates that the Pbx1-d direct target is unknown. **(B)** Positive regulators whose deletion leads to the inhibition of Tfr cells or the generation of ex-Treg cells producing IFNγ and TNFα (Red X indicates gene deletion). **(C)** Genes regulating Treg cells through their metabolism by way of mTOR, glycolysis and/or mitochondria metabolism leading to decreased immunosuppressive activity. Red arrows between genes and their target indicate an enhancing effect with expression of the target being decreased by the gene deletion. Red blocked arrows indicate an inhibitory effect with expression of the target being increased by the gene overexpression. Figure created with BioRender.com.

locus and the estrogen-related receptor gamma (*Esrrg*) gene it contains (91). *Esrrg* is essential in maintaining mitochondrial metabolism through activation of oxidative phosphorylation, the electron transport chain and ATP production in multiple cell types (91), but its function in T cells was unknown. *Esrrg* expression is reduced in the $CD4^+$ T cells of B6.*Sle1c2* congenic mice, in association with altered mitochondrial functions and a decreased mitochondrial mass (91). This phenotype is consistent with that of $CD4^+$ T cells of SLE patients in which mitochondrial defects have been described (92). *Esrrg* deletion in Treg cells altered the expression of genes involved in mitochondrial and Treg programs (93). This led to impaired suppressive function as well as differentiation into Tfr cells, which allowed for greater Tfh cell and humoral responses. These results suggest that the hypomorph *Esrrg* lupus susceptibility allele contributes to autoimmune pathogenesis by reducing the metabolic fitness of Treg cells.

CONCLUSION

In summary, several genes have been identified as being responsible for sustaining the differentiation, function, and stability of Treg cells. The most common approach has been reverse genetics. Only a few Treg-specific studies have been conducted, but continued analyses of selective gene knockouts or overexpression models could advance our knowledge of novel genes that negatively or positively control Treg cells. However, CRISPR/Cas9 screens such as the one recently performed for mTORC1 activation in Treg cells (54) are likely to accelerate the speed of discovery and uncover novel genetic pathways through a less biased evaluation than classical reverse genetic approaches. The dissection of genetic susceptibility in a spontaneous mouse model of lupus has identified two genes that directly impact Treg cell homeostasis. So far, genetic loci associated with human lupus susceptibility, or susceptibility to other autoimmune diseases, have not been linked with Treg phenotypes. It is therefore unknown if allelic variations directly impacting Treg phenotypes confer autoimmune susceptibility in human populations.

The majority of genes that have been identified to regulate Treg cells either directly control Foxp3 expression or their cellular metabolism (**Figure 1**). Treg cells are highly sensitive to mTOR activation, requiring "just the right amount" for optimal differentiation and suppressive function. Several genes have been identified in mice to maintain this "Goldilocks" homeostasis. The maintenance of mitochondrial metabolism or glycolysis, which is partially under mTORC1 control, is also required by Treg cells. It is predicted that other metabolic genes are also involved, and *in silico* analyses of protein-protein networks may be useful in pinpointing critical nodes in these networks.

Adoptive Treg cell therapies are being evaluated in clinical trials for autoimmune diseases and transplantation (94). The identification of regulatory networks that ensure their stability hand functions has great translational potentials to maximize these approaches. This knowledge could also benefit efforts to deactivate Treg cells in the tumor microenvironment to potentiate immunotherapies. This will require a comprehensive validation of these genetic pathways in human Treg cells, although the restraints of the read-out to *in vitro* suppression greatly limit the scope and the interpretation of these translation studies.

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TR and LM wrote the review. All authors contributed to the article and approved the submitted version.

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The long and winding road: From mouse linkage studies to a novel human therapeutic pathway in type 1 diabetes

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Autoimmunity involves a loss of immune tolerance to self-proteins due to a combination of genetic susceptibility and environmental provocation, which generates autoreactive T and B cells. Genetic susceptibility affects lymphocyte autoreactivity at the level of central tolerance (e.g., defective, or incomplete MHC-mediated negative selection of self-reactive T cells) and peripheral tolerance (e.g., failure of mechanisms to control circulating self-reactive T cells). T regulatory cell (Treg) mediated suppression is essential for controlling peripheral autoreactive T cells. Understanding the genetic control of Treg development and function and Treg interaction with T effector and other immune cells is thus a key goal of autoimmunity research. Herein, we will review immunogenetic control of tolerance in one of the classic models of autoimmunity, the non-obese diabetic (NOD) mouse model of autoimmune Type 1 diabetes (T1D). We review the long (and still evolving) elucidation of how one susceptibility gene, *Cd137*, (identified originally *via* linkage studies) affects both the immune response and its regulation in a highly complex fashion. The CD137 (present in both membrane and soluble forms) and the CD137 ligand (CD137L) both signal into a variety of immune cells (bi-directional signaling). The overall outcome of these multitudinous effects (either tolerance or autoimmunity) depends upon the balance between the regulatory signals (predominantly mediated by soluble CD137 *via* the CD137L pathway) and the effector signals (mediated by both membrane-bound CD137 and CD137L). This immune balance/homeostasis can be decisively affected by genetic (susceptibility vs. resistant alleles) and environmental factors (stimulation of soluble CD137 production). The discovery of the homeostatic immune effect of soluble CD137 on the CD137-CD137L system makes it a promising candidate for immunotherapy to restore tolerance in autoimmune diseases.

KEYWORDS

NOD, T1D (type 1 diabetes), t cell, treg cells, CD137, CD137L

Introduction

Autoimmune diseases (ADs) are a chronic and clinically heterogeneous group of diseases affecting up to 5% of the world population (1, 2), and their incidence is rising (3). Different ADs share risk factors (e.g., environmental and genetic) and immunological mechanisms (4). A single autoimmune disease may manifest with autoantibodies of diverse organ specificities (i.e., latent polyautoimmunity) (5–7). Polymorphisms in *HLA-DRB1, HLA-DQB1, CD226, PTPN22, STAT4, GPR103, TNFAIP3*, and *LRP1/STAT6* are associated with multiple ADs (8, 9), including systemic and organ-specific ADs (10). Therefore, the study of autoimmunity is complex and requires the analysis of multiple genes with diverse immunological effects.

A commonality among ADs is the failure to control peripheral autoreactive T cells, and most ADs exhibit dysfunctional T regulatory cells (Tregs) (11). This T cell population constitutively and highly expresses CD25 (IL-2 receptor α chain) (12), and more specifically, Tregs express the transcription factor Forkhead box P3 (FOXP3) (13–15). The clinical relevance of FOXP3 was demonstrated in patients with the immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (16). More than 70 mutations in *FOXP3* have been described in these patients (17), and they exhibit a high frequency of polyautoimmunity, such as autoimmune thyroid disease, autoimmune cytopenia, or type 1 diabetes (T1D) (18). Polymorphisms in other genes implicated in Treg function, such as *IL2RA* and *CTLA4*, have also been associated with the development of endocrinological and rheumatic ADs (19, 20). This evidence highlights the crucial role of Tregs in the disrupted immune homeostasis characteristic of autoimmunity.

The current management of ADs is centered on immunosuppression. Multiple non-specific immune-suppressive therapies are used to ameliorate autoreactivity/tissue damage (i.e., methotrexate, leflunomide). More recently, antibody-based therapies target specific molecules or cells involved in the immune response (i.e., anti-CD20 for depleting B cells) (21). However, these approaches have a major undesired effect: increased susceptibility to infections. Recently, new therapeutics focusing on Tregs have emerged. For example, administration of IL-2 in patients with systemic lupus erythematosus (SLE) ameliorated disease *via* the expansion of Tregs without an increased risk of infection, and low dose IL-2 therapy is being investigated in T1D (22–25). Restoring Treg function might treat autoimmunity while reducing the risk of life-threatening adverse effects. However, abnormal Treg function and conversion of Tregs to pathogenic Th17 cells are complications in Treg therapeutics (26–28).Thus, deeper knowledge of Treg biology is needed.

T1D is one of the most common ADs in children, characterized by the autoimmune destruction of insulin-producing β cells (29). T1D incidence is increasing rapidly, implying increasing environmental factors interacting with genetic risk loci (*HLA* and *non-HLA* genes) (29–31). Antigen-presenting cells (APCs) initiate pancreatic inflammation by producing inflammatory cytokines such as TNF-α (32, 33). The presentation of pancreatic antigens by APCs then leads to the activation of autoreactive $CD4^+$ and $CD8^+$ T cells, which perpetuate insulitis and the destruction of β cells (34, 35). Treg failure to maintain peripheral tolerance of these autoreactive T cells due to Treg dysfunction is critical in the persistence of inflammation and islet destruction (36).

Phase 1 clinical trials on early-onset T1D showed that the administration of autologous expanded $CD4^+CD25^+CD127^-$ Tregs was associated with a reduced requirement of exogenous insulin and preservation of β-cell function, with this effect persisting for up to 1 year after infusion without severe adverse reactions (37, 38). In a similar study, adult patients showed stable levels of C-peptide and insulin use for up to 2 years (39). However, this Treg strategy would necessitate periodic re-transfusions of Tregs to maintain the immune response, and autologous transplantations of Tregs may be difficult in low-income settings. In addition, these studies are in their infancy (i.e., phases 1 and 2), and the estimated magnitudes of the effect of these approaches were low. Thus, other strategies are needed to boost the peripheral Treg response to restore homeostasis.

The NOD strain and its implications for T1D research

The non-obese diabetic (NOD) mouse, which spontaneously develops autoimmune T1D, has long served as a model to delineate both genetic and immune mechanisms of T1D and its treatment. This model was established in 1980 by Makino et al. (40) and emerged from breeding a mouse strain that spontaneously developed cataracts (i.e., CTS strain) (41). Two groups of mice emerged: males with glucose intolerance but without glucosuria, later known as the non-obese non-diabetic (NON) strain, and females with polyuria, ketoacidosis, and glucosuria, subsequently known as the NOD strain (41). Histological examinations of NOD mice demonstrated lymphocyte infiltration in pancreatic islets (insulitis), as well as a decrease in the number of β-cells and islet size (Figure 1A) (40).

Abbreviations: ADs, Autoimmune diseases; APCs, Antigen-presenting cells; CD, Cluster of differentiation; CD137L, CD137 Ligand; *Foxp3*, Forkhead box P3; *Idd*, Insulin-dependent diabetes loci; IPEX, Immune dysregulation polyendocrinopathy enteropathy X-linked; mCD137, Membrane-bound CD137; NK, Natural killer; NOD, Non-obese diabetic; NON, Non-obese non-diabetic; sCD137, Soluble CD137; sCTLA4, Soluble CTLA4; SLE, Systemic lupus erythematosus; SNPs, Single nucleotide polymorphisms; T1D, Type 1 diabetes; Tregs, T regulatory cells.

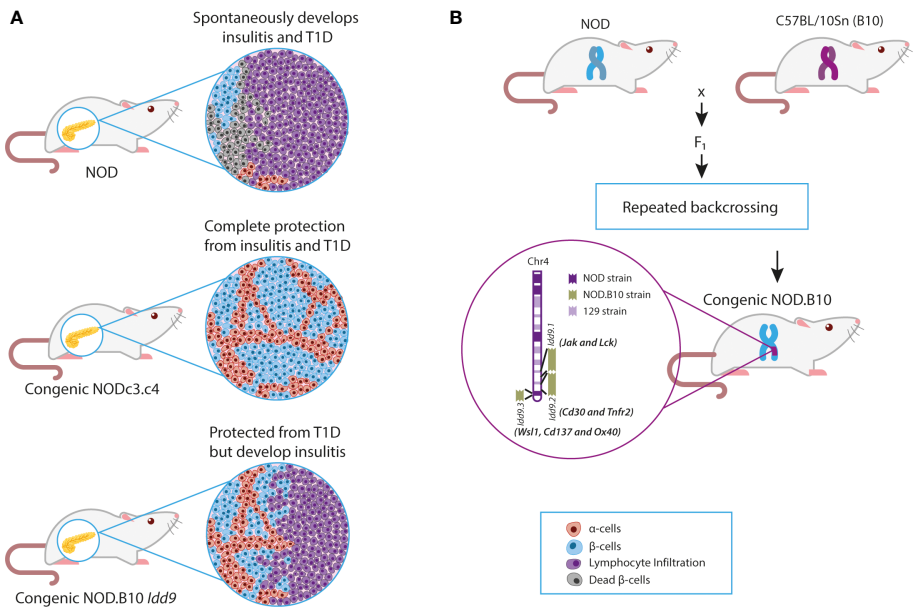

FIGURE 1
NOD and NOD Congenic mice. (A) Effects of congenic intervals on the clinical and histological phenotypes of NOD mice. (B) Breeding of NOD and B10 mice to produce congenic mice with Idd9 regions. Chr, Chromosome; NOD, Non-obese diabetic; T1D, Type 1 diabetes.

Typically, 80% of female NOD mice develop insulitis at three weeks and T1D at ~20 weeks (42). The $H2^{g7}$ MHC haplotype essential for T1D development in NOD mice has the unique I-A allele (I-A^{g7}). I-A^{g7} encodes histidine and serine at positions 56 and 57 instead of the two usually conserved proline and aspartic acid residues found in other mouse strains (43). The diabetogenic variants of the human class II HLA-DQβ homolog also have non-aspartic acid substitutions at residue 57 (44). The genetic association of both MHC class I and class II with disease supports the pathogenic role of $CD8^+$ and $CD4^+$ T cells in the destruction of β-cells in humans and mice (45–48). Multiple autoantigens are targeted by autoreactive T cells (e.g., GAD, insulin, or HSP) (49). However, while the MHC II I-A^{g7} is a major susceptibility allele, it is not sufficient for the development of diabetes, as shown by complete T1D resistance in B10 mice expressing I-A^{g7} molecules (50). In B6 congenic mice expressing I-A^{g7}, circulating T cells can react with the same β-cell autoantigens as in NOD mice; however, no autoimmunity results. These B6.G7 congenic mice confirm the importance of non-MHC genes in controlling autoimmunity in NOD genetic background (51, 52).

In addition to $CD4^+$ and $CD8^+$ T cell autoreactivity, Tregs are involved in suppressing the development of T1D in NOD mice. $CD4^+CD25^+$ Treg cell depletion at critical time points can accelerate T1D progression (53). Ablation of essential proliferative or co-stimulatory signals required for Treg cells, such as IL-2 or CD28, exacerbates T1D (54). NOD Treg quantity and functional capability are reduced, and increasing NOD Treg cell activity can prevent diabetes (55–57). These studies suggested that in addition to the crucial role of T cell autoreactivity, immunological pathways related to Tregs could be genetically determined in the NOD model. Further studies showed shared susceptibility genes affecting Treg function between mice and humans for T1D (e.g., *IL2* and *CTLA-4*) (58–60). Thus, the study of NOD Treg function and control may allow the implementation of novel therapeutics in humans. These considerations highlight the significance of identifying *non-HLA* genes implicated in immune regulatory function, Treg function and development, and T1D pathogenesis (60). Identifying genes in the B6/B10 genetic background that can control autoimmunity has thus been a major goal in this field.

Immunogenetic studies of NOD and Human T1D and translation to novel therapeutics

Before the advent of whole-genome sequencing, many non-*HLA* genomic regions associated with T1D were discovered by linkage analysis of the NOD genome (61–63). Identified genetic regions were confirmed to play a role in T1D pathogenesis through the construction of congenic mice (64). Congenic mice were constructed by introgression of resistant insulin-dependent diabetes (*Idd*) loci/regions onto the NOD background. Backcrossing of NOD with B6 (C57BL/6J), B10 (C57BL/10Sn),

and other T1D resistant strains demonstrated that over 30 murine recessive *Idd* loci were associated with protection from spontaneous diabetes (65) (Figure 1B). These studies allowed the classification of *Idd* intervals into two groups: those that confer both insulitis and diabetes resistance and a second group that protects against T1D but has no effect on insulitis (62) (Figure 1A). For example, the *Idd3* locus on chromosome 3 was implicated in the protection from insulitis and T1D, whereas the *Idd4* locus on chromosome 11 did not protect from insulitis but prevented T1D (61). It suggested that genes within these regions exhibited differential effects on T1D development (i.e., T cell migration, cytotoxicity, or Treg function). The next step was to identify and confirm candidate genes within the introgressed genetic regions. This confirmation process ultimately has taken decades of work and the development of new technologies (e.g., whole-genome sequencing, CRISPR).

One of the first identified non-*HLA* candidate genes encodes interleukin 2 (IL-2). IL-2 is located in the *Idd3* region and has profound effects on T cell and Treg function, and was thus a good candidate gene for T1D (66). NOD produces an altered IL-2 protein compared to the protective B10 allele, with a shortened tandem repeat sequence encoding a poly-glutamine stretch, plus an extra four amino acid insert, in the N-terminal coding region of IL-2 (62). These immunogenetic studies uncovered evidence of multiple genes with multiplicative effects on the immune response. For example, the *Idd3/Idd5* double congenic mice, comprising the *Il2* and *Ctla4* candidate genes, were completely protected from T1D, whereas when studied alone, only ~20% and ~50% rates of protection were observed, respectively (60, 67, 68).

Genetic studies in the mouse were compared to human T1D genetic studies, and marked similarities were uncovered. The genetic architecture of mouse and human T1D is remarkably similar, with variants affecting multiple immune genes and pathways in common between both species, including *IL-2*, *IL-2* receptor, *CTLA-4*, *IL-10*, the *HLA* region, *PTPN22*, and *IL-7R* (69, 70) For example, single nucleotide polymorphisms (SNPs) in the human homologous *Il2* region were also associated with T1D susceptibility, identifying the IL-2 pathway as potentially shared in the pathogenesis of disease in both species (71). The NOD *Il2* gene variant resulted in decreased production of IL-2, and elegant engineering of *Il2* gene haplodeficency reproduced the NOD effect and resulted in functionally deficient Tregs (71). Low dose IL-2 therapy increased Tregs in mouse models, and this led to human trials of low dose IL-2; however, while this boosted human Treg numbers, it did not affect T1D outcome in initial trials (72). A variety of approaches have tried to optimize immune modulation effects *via* IL-2. IL-2 induced *in vitro* expansion of Tregs is one approach that was effective in NOD mice, tying the IL-2 immunogenetic effects to the enhancement of Treg deficiencies in T1D (73). Large numbers of Tregs are needed for human trials, and *in vitro* expansion may overcome some of the deficiencies of earlier Treg trials (74). Clinical trials in human T1D are ongoing with low-dose IL-2 therapy (75) and Treg therapy (76) which have built upon these earlier results.

CTLA-4 is another critical immune molecule with variants identified in mice and humans. The mouse locus (*Idd5*) was noted to overlie the orthologous human *IDD12* locus (67). T1D susceptibility was subsequently mapped to a non-coding region of human CTLA-4 that resulted in lower levels of the CTLA-4 soluble splice variant; the mouse gene also demonstrated alterations in CLTA4 splicing (77). Human trials targeting CTLA-4 with a soluble form that blocks T cell activation appear promising (78). These therapies may be effective even though the human disease demonstrates remarkably different patterns of insulitis than the mouse, with much less exuberant immune infiltrates (79). The difference in β cell immune infiltration may explain why prevention of diabetes NOD is very easily achieved, whereas, in humans, prevention trials have until recently failed. One successful approach to the prevention of human T1D has been achieved using anti-CD3 antibodies, which preferentially target CD8 effector cells (80). Notably, anti-CD3 antibodies were discovered in NOD mice to reverse established disease (not simply prevent disease), demonstrating the usefulness of therapeutic trials of acute T1D in NOD mice. Overall, these examples illustrate the rich insights and potential therapies resulting from T1D immunogenetic studies. The latest large-scale study identified 78 genetic regions linked to T1D (including 36 novel loci) and confirmed the strong association with immune function and potential for clinical therapeutics (81). Thus much more work can be done to apply immunogenetic studies to novel therapeutic pathways.

Our labs have been investigating immunogenetic control of T1D, initially using NOD and NOD congenic mice, for over 20 years. In the rest of this review, we will detail the lengthy investigation of the immune effects of the *Idd9* genetic region and our studies which demonstrated that *Cd137* is the essential T1D susceptibility gene in this region. These studies have revealed many surprises about the function of an *Idd* gene in T1D immunology and have ultimately led to novel immunotherapy based on the immune function of CD137.

The role of *Idd9* and its main candidate gene, *Cd137*, in NOD T1D

After identifying the *Idd9* region in linkage studies, the Wicker group constructed congenic mice with the B10 *Idd9* region introgressed onto the NOD background. The B10 *Idd9* region prevented the onset of spontaneous diabetes in NOD mice (less than 5% of female mice developed T1D) (82). However, most mice still developed insulitis caused by T cells expressing CD30, producing high amounts of IL-4 (82) (Figure 1A). This confirmed that genes associated with lymphocyte infiltration were outside the *Idd9* interval but

suggested that some genes within this region halted autoimmunity.

This hypothesis was validated in double congenic mice comprising B6 (*Idd3*, *Idd17*, *Idd10*, and *Idd18*) from chromosome 3 and B10 (*Idd9*) regions (also known as the NOD.c3c4 strain). NOD.c3c4 mice were completely protected from diabetes, and only 10% of mice developed insulitis (82) (Figure 1A). This confirmed that spontaneous diabetes is a complex trait in which the epistasis of multiple genes (*HLA* and non-*HLA*) is critical for its development, but it also suggested that the *Idd9* interval contained genes associated with T cell activation and modulation.

The *Idd9* region, a 48 cM interval, was fine-mapped into three intervals (i.e., the *Idd9.1, 9.2*, and *9.3*), with seven candidate genes (i.e., *Jak1, Lck, Cd30, Tnfr2, Cd137, Wsl1*, and *Ox40*) (66). The *Wsl1, Cd137*, and *Ox40* were initially proposed as candidate genes within the *Idd9.3* locus (82). However, B6 *Wsl1* did not exhibit sequence variations compared to NOD, and *Ox40* was subsequently found to be located outside of the *Idd9.3* region and was excluded as a candidate gene (82). Thus, *Cd137* remained the key candidate for T1D protection within the *Idd9.3* locus. Jumping ahead 15 years, it was recently confirmed by using combined congenic mapping and nuclease-based gene targeting that *Cd137* is the susceptibility gene within the *Idd9.3* locus critical for modulation of T1D (82, 83).

Cd137 is located at 1.217-Mb of the *Idd9* locus (i.e., *Idd9.3*) (84), and *Idd9.3* conferred ~40% protection for T1D (82). Analysis of coding variants demonstrated two synonymous SNPs in NOD vs. B10 *Cd137*: a valine to alanine substitution at position 24 and leucine to proline substitution at position 211 (near the transmembrane domain). There is also alanine insertion in NOD between amino acids 174 and 175 (82) (Figure 2A). These structural modifications suggested that CD137(4-1BB) could be hypofunctional in NOD mice (82). *Cd137 (4-1bb or Tnfrsf9)* codes for two CD137 isoforms: membrane-bound (mCD137) and soluble (sCD137) forms (Figure 2B) (85). Membrane mCD137 is mostly found on $CD4^+$ and $CD8^+$ T cells, whereas the sCD137 is produced by Tregs (86). The ligand for both isoforms, CD137L, is coded by *Tnfsf9* on chromosome 17 and is expressed on APCs and activated T cells (86).

Since the NOD *Cd137* SNPs suggested that mCD137 was hypofunctional compared to the NOD.B10 strain (82), Cannons

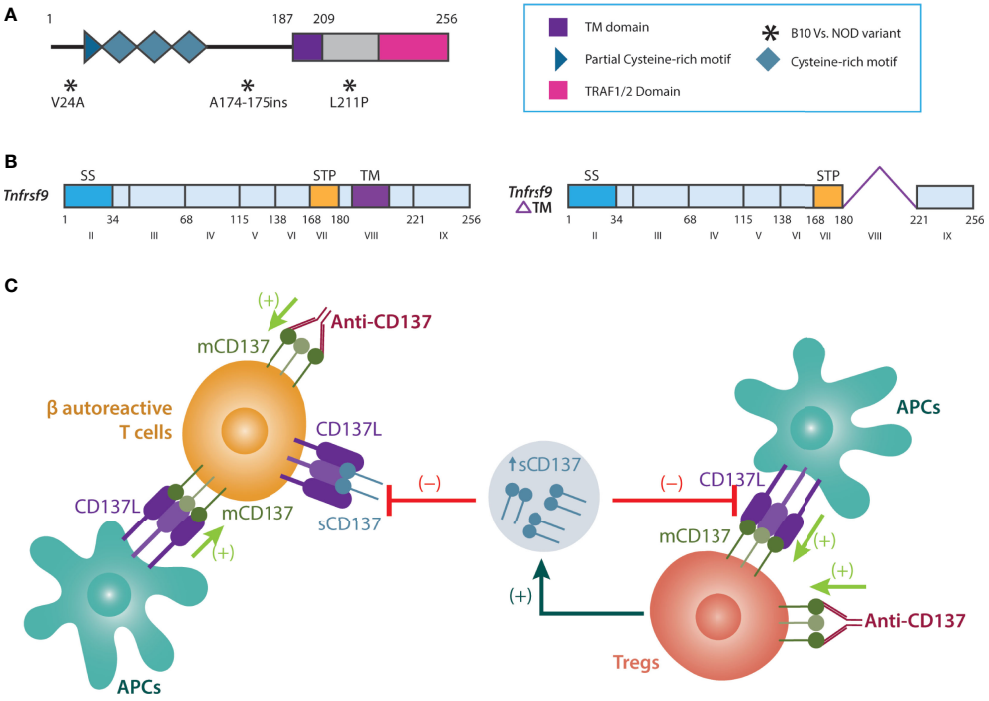

FIGURE 2
Biology and function of sCD137. **(A)** Non-synonymous SNPs of NOD vs. B6 *Cd137*. **(B)** membrane vs. soluble (alternatively spliced) CD137. **(C)** Tregs produce sCD137 with a dimeric structure. sCD137 induces altered CD137L signaling in APCs and autoreactive T cells (compared to membrane CD137), reducing inflammation and damage in the pancreas. In contrast to sCD137, although anti-CD137 antibodies activate Tregs (a strong immune regulatory effect), they may also increase autoreactive T cell survival and proliferation, thus perpetuating inflammation and autoimmunity. APCs, Antigen-presenting cells; CD137L, CD137 Ligand; mCD137, Membrane-bound CD137; NOD, Non-obese diabetic; sCD137, Soluble CD137; SS, signal sequence; STP, Ser/Thr/Pro-rich; T1D, Type 1 diabetes; TM, Transmembrane domain; Tregs, T regulatory cells.

et al. (84) evaluated T cells activation and proliferation to test this hypothesis. They confirmed that NOD and NOD.B10 mice showed similar mCD137 expression after stimulation with anti-CD3 in Th1 and Th2 culture conditions. However, when T cells from NOD mice were costimulated with CD137L, they proliferated less and produced a reduced level of IL-2 than T cells from mice carrying the B10 allele of *Cd137*. This strongly suggested that the NOD SNPs lead to a hypo-functional mCD137 protein, which could play a role in T1D pathogenesis. Over the last 15 years, our work has begun to delineate the complex immune biology of CD137 and CD137L in T1D.

CD137 and CD137L: A double-edged sword in autoimmunity

CD137 is a glycoprotein belonging to the TNF receptor superfamily, and the membrane form is expressed on activated $CD4^+$ and $CD8^+$ T lymphocytes (87), Tregs (88–94), and natural killer (NK) cells (95). CD137 is also constitutively expressed on a subset of Tregs (93). CD137L (4-1BBL), its ligand, belongs to the TNF superfamily and is expressed on activated APCs such as macrophages, B cells, and dendritic cells (96–98). Activated T cells also upregulate and express CD137L (99). mCD137 has no intrinsic enzymatic activity in its intracellular domain and functions by binding TRAF1 and TRAF2 adaptor proteins that enhance K63 polyubiquitination processes in the CD137 signalosome (100, 101). CD137L trimerization, in response to interaction with mCD137, causes mCD137 receptor clustering and TRAF-mediated activation of the ERK, JNK, p38, NF-kB, and MAPK intracellular signaling pathways, resulting in cell activation, proliferation, and T cell survival (102–109). Notably, signaling in the CD137:CD137L pathway is bidirectional: both the receptor and ligand signal into their respective cells (110, 111). This bidirectional signaling adds an additional layer of complexity to the analysis of the biological function of the pathway.

The effects of CD137 in T cell biology are diverse but with specific implications for inflammation and immune regulation. TCR-induced proliferation and cytokine production were enhanced after T cells were stimulated with agonistic anti-CD137 antibodies (also known as 3H3), independent of B7-CD28/CTLA-4 interactions (112, 113). mCD137 signaling results in NF-kB activation that promotes the expression of antiapoptotic genes encoding Bcl-xL and Bfl-1 (114, 115) and mitochondrial function and biogenesis, which improves T cell survival (116, 117).

mCD137 has a prominent role in $CD8^+$ T-cell costimulation, influencing cytotoxicity in an IL-2-independent manner. Furthermore, $CD8^+$ T cells produce a greater amount of IFN-γ after mCD137 activation (118). *In vivo* experiments showed that knockout mCD137/CD137L mice exhibited a reduced memory $CD8^+$ T cell response to viruses (119–121) and decreased T cell survival (122). These findings pointed to a costimulatory involvement of mCD137 in long-lasting memory T-cell activation and enhancement of cytotoxicity and founded the basis for CD137-based therapies for cancer (85, 123–126). In contrast to these effects, when knockout mice were stimulated with CD3, T cells showed hyperresponsiveness, which indicated an additional immunosuppressive role of CD137 (105).

The expression of CD137L on APCs is increased at sites of inflammation *in vivo* (127, 128). Activating APCs by CD137L upregulated B7-1 and B7-2, and increased IL-6 and IL-12 secretion (127). CD137L is upregulated on activated T cells, and CD137L signaling is critical for $CD8^+$ T cell survival *via* STAT3- and FAS-mediated pathways (129). $CD4^+$ T cell activation can also be modulated by CD137L-expressing APCs (*via* APC CD137L signaling through T cell CD137) that stimulate IL-2 and IL-4 T cell production (112, 113).

This data established that CD137L on APCs affects the cytotoxic immune response and is critical for the survival of $CD8^+$ and $CD4^+$ T cells. This also confirmed that inhibition of mCD137 or CD137L might reduce inflammation *via* $CD8^+$ T cells but may at the same time also affect $CD4^+CD25^+$CD137-expressing Tregs. Indeed, *Cd137* is upregulated by *Foxp3* (130). CD137 is expressed by Tregs infiltrating the islets in T1D, suggesting an immunoregulatory role for $CD137^+$ Tregs (131). Thus attempting to modulate CD137 or CD137L action on T effector cells could potentially decrease immunosuppression *via* Tregs, illustrating the intricacies of this pathway and the potential for double-edged effects.

Type 1 regulatory T (Tr1) cells are another type of regulatory T cell characterized by the production of IL-10 and lack of constitutive *Foxp3* expression (132). Despite the evidence of CD137L mRNA expression after stimulation (133), it is unknown whether these cells also exert their suppressive function by sCD137 or their role in NOD mice during T1D pathogenesis. Since *Cd137* is upregulated by *Foxp3* (130), Tr1 cells may not produce large quantities of sCD137. Further studies of this cell subset and their involvement in the mCD137/CD137L axis are warranted.

Agonistic anti-CD137 antibodies induced the proliferation of $CD4^+CD25^+$ Tregs with the maintenance of their suppressive activity (92). Interestingly, the effects of agonistic anti-CD137 antibodies are diverse and dependent on the target and the disease. Activating $CD8^+$ T cells by anti-CD137 antibodies in cancer models leads to tumor cell elimination. In sharp contrast, in models of autoimmunity, e.g., murine models of SLE (134, 135), experimental autoimmune encephalomyelitis (136), collagen-induced arthritis (122, 137), Sjögren's syndrome-like sialadenitis (138), and inflammatory bowel disease (139), anti-CD137 antibody treatment leads to immunoregulation and disease amelioration. For example, anti-CD137 administration in the SLE murine model reversed disease and reduced autoantibody production (i.e., dsDNA antibodies) and

immune complex deposition (135). Induction of T cell anergy by anti-CD137 antibodies might play a role in some of these models (135, 140).

Anti-CD137 antibodies prevented T1D *via* Treg expansion but accelerated T1D in the absence of Tregs

Since *Cd137* was a candidate gene in T1D, we started our investigation of the role of CD137 in T1D with agonistic anti-CD137 antibodies. We showed that anti-CD137 antibodies in NOD mice prevented the development of T1D but did not ameliorate insulitis, which is consistent with the findings of residual insulitis in NOD congenic mice protected from T1D by the B10 *Idd9.3* region (93). We found that anti-CD137 expanded $CD4^+CD25^+$ Tregs, and their transfer to NOD-*scid* mice completely prevented T1D (93). However, T1D progressed more rapidly when NOD-*scid* mice were treated with anti-CD137 after pathogenic $CD4^+$ and $CD8^+$ T cell transfer in the absence of Tregs. Therefore, in the absence of Tregs, mCD137 stimulation could potentially potentiate pancreatic destruction *via* $CD8^+$ T cytotoxicity. This is similar to the effect of anti-CD137 administered in the context of autoimmune thyroiditis, which worsens the disease (141).

Due to this dual effect, activation of effector T cells in acute autoimmunity may prohibit the use of agonistic CD137 antibodies in clinical autoinflammatory states, including T1D, because activated T cells have upregulated mCD137 in these settings. In contrast, CD137 antibodies in non-inflammatory states (e.g., pre-diabetes) might prevent autoimmunity since it targets Tregs constitutively expressing mCD137 without activating T cell effector cells. This dual effect led us to look for alternate ways to therapeutically target the mCD137/CD137L pathway in T1D.

We turned our attention to sCD137, which is formed by alternative splicing (99, 142) (Figure 2B), and exists as a dimer (143). sCD137 was found in the supernatants of splenic and bone marrow-derived dendritic cells (144). Murine sCD137 differs from humans. In mice, only the exon coding for the transmembrane domain of CD137 is spliced out, whereas, in the latter, two splice variants are observed (145). sCD137 is preferentially secreted by $CD4^+$ T cells, whereas $CD8^+$ T cells express higher amounts of mCD137 (146). We found that the major source of sCD137 is $CD4^+CD25^+CD137^+$ Tregs (94).

Spliced variants are critical for the modulation of immune response (147). The induction of alternative splicing is poorly understood but may occur as a response to environmental signals. In autoimmunity, splicing also occurs in the modulation of immune responses (147). Changes in the immunological environment (i.e., T cell autoreactivity and pro-inflammatory milieu) induce the production of sCD137 by Tregs. We have demonstrated that activating Treg cells increases the production of sCD137 by Tregs in mice and humans (148). Thus, inflammatory environmental changes may partly explain the origin of spliced variants of CD137 from Tregs as a homeostatic response to ameliorate inflammation. A similar process is seen with soluble CTLA4 (sCTLA4), a spliced variant of membrane-bound CTLA4 mainly produced by $Foxp3^+$ Tregs (149). sCTLA4 suppresses early T-cell activation by preventing the interaction of CD80/CD86 with the costimulatory receptor CD28 (150). In addition, it inhibits IFN-α, IL-2, IL-7, and IL-13 production while activating TGF-β and IL-10 release (151). Silencing sCTLA-4 mRNA by RNA interference accelerated the onset of T1D in NOD mice and impaired the ability of Tregs to downregulate dendritic cell costimulation (149). Both spliced variants, sCTLA4 and sCD137, may be critical for effective Treg function in the pathogenesis of T1D.

What is the role of sCD137? Our hypothesis was that sCD137, similar to sCTLA4, functions as a negative feedback mechanism to downregulate immune response mediated by mCD137 and CD137L (85, 152). sCD137 reduces the production of IL-10 and IL-12 from activated splenocytes (146). In addition, T cell proliferation and IL-2 release were inhibited when sCD137 was administered to these cells (153). These initial reports clarified the effects of CD137 in different conditions (i.e., cancer and autoimmunity) and suggested that the sCD137 was the missing link in understanding the dual effects of the mCD137/CD137L axis.

To confirm the role of CD137 in the *Idd9.3* locus, we evaluated the function (immunosuppressive effects) and quantity of $CD137^+$ Treg cells in NOD.*Idd9.3* congenic mice (94). When compared to NOD mice, the NOD.*Idd9.3* strain had significantly higher percentages of $CD4^+CD25^+CD137^+Foxp3^+$ Tregs in the thymus and spleen, and the numbers increased with age. This supported the hypothesis that the hypofunctional NOD CD137 allele led to decreased Treg survival, consistent with the known effects of mCD137 on cell survival. $CD137^+$ Tregs showed superior immunosuppression compared to $CD4^+CD25^+CD137^-$ Tregs, directly showing an effect of CD137 on Treg function. Thus, increased numbers of CD137 Tregs, mediated by the protective allele, led to increased overall suppressive capacity. Importantly, $CD137^+$ Tregs showed suppressive capability in an independent contact assay. This supported our continued focus on the possible immunosuppressive role of sCD137 in T1D.

sCD137 is produced by Tregs and inhibits T cell autoreactivity in a paracrine fashion

We first confirmed that sCD137 was mainly produced by $CD4^+CD25^+CD137^+$ Tregs and in a higher amount in NOD.*Idd9.3* congenic mice (94). Next, we demonstrated that

sCD137 primarily exists as a ~55 kDa homodimer under non-reducing and a ~35 kDa monomer under reducing conditions (143). The existence of sCD137 as a dimer, rather than as a trimer as described for mCD137, suggested a structural reason for how sCD137 might suppress T cell function while mCD137 activated T cell function (143). Next, we showed that the administration of recombinant sCD137 to NOD mice prevented diabetes and reduced insulitis by preserving insulin$^+$ islets (143). Since CD4$^+$CD25$^+$CD137$^+$ Tregs inhibited T cells in a contact-independent manner (94), we evaluated the role of sCD137 in T cell inhibition. We demonstrated that sCD137 inhibited activated T cells by binding to CD137L (143). In addition, sCD137 can directly stop the proliferation of effector CD4$^+$CD25$^-$CD137$^-$ T cells in the absence of APCs, and without inducing cell death (143) (Figure 2C).

In addition to the crucial role of sCD137 in immunosuppression, additional reports suggest that mCD137, like other costimulatory molecules, has a nonredundant role in maintaining the pathogenic activity of β cell-autoreactive T cells in NOD mice. We found that, compared to wild-type mice, T1D development is reduced in NOD.*Cd137*$^{-/-}$ and their T cells are less capable of inducing T1D in NOD.*Rag1*$^{-/-}$ recipients (154). This, at first, seemed contradictory to our data on the immunoregulatory properties of CD137$^+$ Tregs and sCD137. As sCD137 produced by Tregs is suppressive, evaluating the distinctive role of mCD137 in CD4$^+$ and CD8$^+$ T cells was crucial. Isolated T cells from NOD and NOD.*Cd137*$^{-/-}$ mice were transferred into NOD.*Rag1*$^{-/-}$ recipients. The T cell adoptive transfer studies revealed that CD137 expression in CD8$^+$ T cells was required to develop T1D in NOD mice, but CD137 expression in CD4$^+$ T cells was diabetes-protective (155). Specifically, CD137 expression in CD4$^+$ Tregs is important for their T1D suppression function. We further demonstrated that CD137 cell-intrinsically stimulates the accumulation and proliferation of autoreactive CD8$^+$ T lymphocytes within the islets, pointing to a role of mCD137 on the diabetogenic activity of CD8$^+$ T cells. However, sCD137 suppressed the proliferation of CD8$^+$ T cells. These experiments supported the concept that the T1D protection conferred by the *Idd9.3* locus is mediated through the production of sCD137 by Tregs.

As the sCD137/CD137L interaction is implicated in the modulation of effector CD8$^+$ T cells, clarifying the role of CD137L in the immunomodulation of T1D is essential. CD137L-deficient NOD mice were shown to exhibit less insulitis and delayed onset of T1D (156). Interestingly, CD137L expression on myeloid APCs appeared to be necessary for the survival of β-cell–autoreactive CD8$^+$ T cells and T1D progression, but CD137L has no effect on the formation or homeostasis of Foxp3$^+$ Tregs (156). It remains to be determined if mCD137 in Tregs modulates their function and whether Tregs capable of producing sCD137 but not mCD137 are sufficient to suppress T1D.

sCD137 induces T cell anergy and can act therapeutically to halt acute autoimmunity

It is relatively easy to prevent T1D in NOD mice, and a much more stringent target is the reversal of actual acute T1D. Thus we treated NOD mice with new-onset T1D with recombinant sCD137 (148). This experiment confirmed that sCD137 could not only prevent T1D but also halt acute T1D and avert the development of end-stage diabetes. In effectively treated mice, β cell immunohistochemistry revealed considerable preservation of insulin$^+$ β cells and a rise in insulin$^+$ islets (148). In this setting, T cells showed downregulation of mTORC1, developed an anergic phenotype (reversed by IL-2), as well as the ability of sCD137 to suppress antigen-experienced and activated memory T cells. CD8$^+$ effector memory cells also showed a reduction in the production of inflammatory cytokines in the presence of sCD137 (i.e., IFN-γ) (148).

In human pediatric T1D patients, we found low levels of sCD137 compared to non-diabetic age-matched controls during acute flares (hospital admission for hyperglycemia). We also confirmed that human Tregs were the primary source of sCD137 (148). Furthermore, human peripheral activated CD4$^+$ T cells were inhibited by sCD137. These results were analogous to those in NOD congenic strains, supporting the notion that these murine models are useful and relevant to affecting the autoimmune phenomena driving human T1D. This evidence showed that sCD137 is associated with autoimmunity in T1D humans, and low sCD137 could be a biomarker in T1D. Further studies are required to confirm the role of sCD137 in reverting established destructive insulitis and the pathways associated with this phenomenon.

Surprisingly, sCD137 is reported to be increased in patients with rheumatoid arthritis (145, 157, 158), and multiple sclerosis (159); and the levels were directly correlated with the severity of the disease (157). Increased levels of sCD137 could be a homeostatic attempt by Tregs to modulate inflammation in these conditions. However, it also raises the possibility that in the presence of a substantial inflammatory substrate, the stoichiometric ratio of sCD137 to CD137L could be reduced, thus reducing the efficacy of sCD137 or possibly indicating that higher sCD137 doses would be required. New strategies improving the half-life and potency of sCD137 could be critical to enhancing their therapeutic effect in human autoimmunity.

Summary and prospects

Linkage studies and the construction of congenic mice allowed the identification of candidate genes with implications for the pathogenesis of T1D. The cumulative evidence suggests

that *Cd137* and its coding isoforms are crucial in the development of T1D, and the CD137-CD137L pathway is a good target for therapeutic modulation. Treg-generated sCD137 modulates the mCD137/CD137L axis, reduces insulitis, and halts T1D in the NOD mouse. The ability to effectively halt acute T1D with exogenous sCD137 is an exciting development with attractive therapeutic potential. Prevention studies in humans are difficult to implement, and those attempted so far have failed (160). Therefore, treating acute disease is a more appealing strategy, but the current landscape of approved therapeutics is limited. The use of antibodies to target the CD137-CD137L axis is appealing; however, while anti-CD137 antibodies are protective in some models of autoimmune diseases due to activation of Tregs, they can also enhance $CD8^+$ T cell killing activity in the absence of Tregs. sCD137, on the other hand, only acts to suppress $CD4^+$ and $CD8^+$ T cell activation and may therefore be safer than an anti-CD137 based approach. In human studies, low levels of sCD137 during T1D flares, and the inhibition of activated $CD8^+$ T cells *in vitro* after sCD137 stimulation, supports its further translational use. Soluble CD137 suppresses autoreactive $CD8^+$ T cells through induction of anergy. However, little is known about the activity of sCD137 on innate immunity. The mechanistic role of sCD137 on CD137L-expressing myeloid APCs should be explored to determine if there will be lasting effects on innate immune function. In addition, it is unknown whether mCD137 on Tregs drives their differentiation to a more robust inhibitory phenotype. This could have therapeutic implications, particularly for the pharmacokinetics and pharmacodynamics of human sCD137.

Author contributions

MR, LH, WZ, YG, WR wrote, reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Genetic basis of defects in immune tolerance underlying the development of autoimmunity

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Genetic variants associated with susceptibility to autoimmune disease have provided important insight into the mechanisms responsible for the loss of immune tolerance and the subsequent development of autoantibodies, tissue damage, and onset of clinical disease. Here, we review how genetic variants shared across multiple autoimmune diseases have contributed to our understanding of global tolerance failure, focusing on variants in the human leukocyte antigen region, PTPN2 and PTPN22, and their role in antigen presentation and T and B cell homeostasis. Variants unique to a specific autoimmune disease such as those in PADI2 and PADI4 that are associated with rheumatoid arthritis are also discussed, addressing their role in disease-specific immunopathology. Current research continues to focus on determining the functional consequences of autoimmune disease-associated variants but has recently expanded to variants in the non-coding regions of the genome using novel approaches to investigate the impact of these variants on mechanisms regulating gene expression. Lastly, studying genetic risk variants in the setting of autoimmunity has clinical implications, helping predict who will develop autoimmune disease and also identifying potential therapeutic targets.

KEYWORDS

genetic variants, autoimmunity, immune tolerance, HLA, PTPN2, PTPN22, INS-VNTR, PADI

Introduction

Development of autoimmunity and progression to autoimmune disease occurs on a continuum with the complex interplay of genetic factors and environmental factors over time (Figure 1). Genetic risk variants and epigenetic alterations predispose to loss of immune tolerance and the subsequent development of autoantibodies, tissue damage, and onset of clinical disease. Environmental factors are less understood but are thought

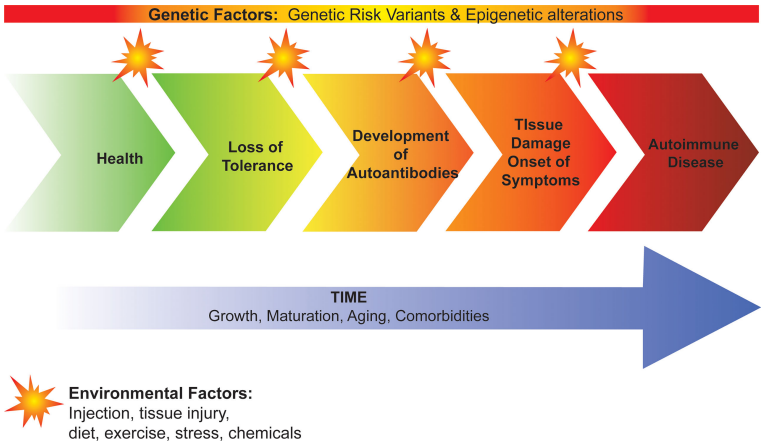

FIGURE 1
Development of autoimmunity and progression to autoimmune disease. Genetic factors predispose to loss of immune tolerance and the subsequent development of autoantibodies, tissue damage, and onset of clinical disease. Environmental factors act as triggers that initiate and promote disease progression. Growth, maturation, aging and comorbidities contribute to the rate of progression from loss of tolerance to autoimmune disease.

to act as triggers that initiate and promote disease progression. To date, viral infection, tissue injury, diet, and stress have all been implicated in this process suggesting that there may be a "threshold effect" involving multiple triggers rather than a single trigger for autoimmunity. Time is also important with growth, maturation, and aging tuning the rate and direction of disease progression. In this review, we focus on the role of genetic variants, specifically how they contribute to failed immune tolerance in autoimmunity. We describe how they have enabled us to identify the molecular and cellular mechanisms underlying immune tolerance. We also provide an update on how genetic variants have helped predict disease development and have facilitated the identification of new therapeutic targets for treatment and prevention of autoimmune disease, including in the setting of personalized/precision medicine.

Role of genetics in development of autoimmunity

Immune tolerance is defined as the state of unresponsiveness to molecules that have the potential to induce an immune response and ensures that the immune system does not mount a response against self-antigens. Importantly, failure of tolerance contributes to induction of autoimmunity (reviewed in (1)). Tolerance is achieved through both central and peripheral tolerance mechanisms (reviewed in (2)). Central tolerance occurs in the thymus for T lymphocytes and the bone marrow for B lymphocytes and acts primarily through negative selection by eliminating immature T and B lymphocytes that recognize self-antigens (2). Peripheral tolerance takes place after the T and B lymphocytes leave the primary lymphoid organs. Mechanisms through which tolerance is maintained in the periphery include: apoptosis, anergy, and regulatory T cell (Treg)-mediated suppression (1, 2).

Studies of monogenic disorders have been critical to understanding tolerance mechanisms (Figure 2). For example, autoimmune polyendocrine syndrome type-1 (APS-1) caused by mutations in the gene autoimmune regulator (*AIRE*) has provided key insight into central tolerance (3). Specifically, AIRE expression by medullary thymic epithelial cells promotes the display of tissue-specific antigens to developing T cells, a key step in negative selection of autoreactive T cells. This lack of central tolerance results in the development of multiple autoimmune diseases including type 1 diabetes (T1D), hypothyroidism, adrenal insufficiency, alopecia, and vitiligo. Conversely, studying both autoimmune lymphoproliferative syndrome (ALPS) and immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome have increased our understanding of mechanisms of peripheral tolerance. ALPS caused by mutations in the first apoptosis signal receptor (*FAS*) gene demonstrates how failed apoptosis drives autoimmunity (4). IPEX syndrome, a multi-organ autoimmune disease from birth, caused by mutations in the transcription factor forkhead box P3 (*FOXP3*) that result in either a lack of Tregs or impaired Treg function, highlights the importance of Tregs in maintaining peripheral tolerance (5). Other rare monogenic forms of autoimmunity are also instructive including LPS-responsive and beige-like anchor protein (*LRBA)* deficiency, *CD25* deficiency and signal transducer and activator of transcription 3 (*STAT3)* gain-of-function, all of which impair Treg cell function (6, 7).

The majority of autoimmune diseases are polygenic and genome-wide association studies (GWAS) have identified

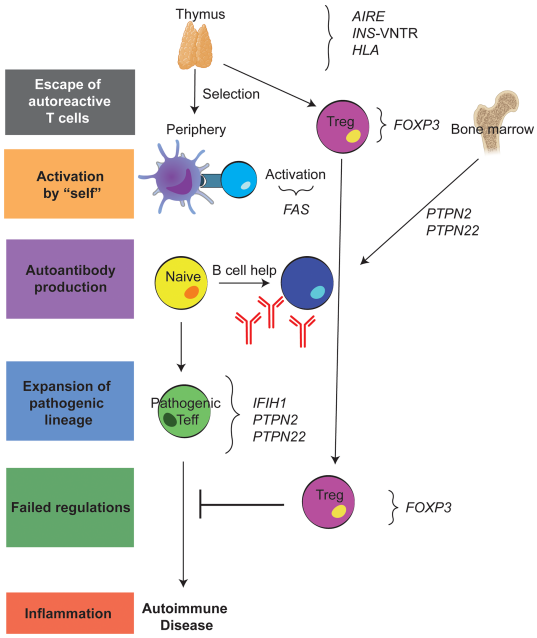

FIGURE 2
Genetic variants underlying failed immune tolerance and development of autoimmunity. Schematic showing where each of the discussed variants contribute to the loss of tolerance both in the thymus and the periphery.

genetic variants shared across multiple autoimmune diseases as well as variants unique to specific autoimmune diseases. Both types of variants have been informative providing insight into the signaling pathways and immune cell types involved in induction and maintenance of tolerance. The shared variants have been most instructive for our understanding of the global tolerance failure underlying autoimmunity whereas disease-specific variants have been more useful defining disease-specific immunopathology. However, defining the functional impact for both shared and disease-specific variants remains challenging since an individual variant may be expressed in multiple immune cell types at different developmental stages and/or at discrete phases of the immune response and may also be influenced by environmental factors. In addition, growing evidence indicates that genetic risk variants synergize with each other to promote autoimmunity (8, 9).

Understanding interactions between genetic risk variants is also important for the development of polygenic risk scores to predict disease susceptibility and disease progression and inform treatment options. In type 1 diabetes (T1D), these scores are being used to predict progression of islet autoimmunity and development of clinical disease in the at-risk population (10, 11). More recently, a combined risk score for T1D has been developed that integrates genetics, autoantibodies, and clinical factors (12). Genetic risk scores for predicting clinical outcomes are also being investigated in the setting of rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). In RA, a polygenic risk score has recently been developed to predict severity of radiographic progression (13) and in SLE, a high genetic risk score was associated with organ damage and renal dysfunction (14).

Shared genetic variants across autoimmune disease

Over time multiple approaches have been undertaken to identify the genetic underpinnings of autoimmunity. These studies included targeted assessments of families with autoimmunity as well as case control association studies of candidate genes. These approaches successfully identified genes with a strong association with autoimmunity, including the HLA locus (reviewed in (15)) and the coding variant PTPN22 (16). The sequencing of the human genome and development of GWAS chip led to the ability to screen large numbers of affected and unaffected individuals. This allowed the identification of common variants that associated with risk for autoimmunity.

A key observation from the initial GWAS studies was that many genetic risk variants are shared across autoimmune diseases (17, 18). Notably, these shared genetic variants highlight the vital role of antigen processing and presentation, T cell activation, cytokine signaling, as well as innate sensing mechanisms in induction and maintenance of immune tolerance

(19, 20). The breadth of information on the many genetic variants associated with autoimmunity is beyond the scope of this review. Instead, we will focus on two protein tyrosine phosphates, PTPN2 and PTPN22, due to their association with multiple autoimmune diseases and the evidence of their role in multiple aspects of immune tolerance, while also discussing human leukocyte antigen (HLA), the region most strongly linked to autoimmunity (Figure 2).

Human leukocyte antigen class II alleles

The HLA region, a large polymorphic region on chromosome 6, encodes HLA Class II molecules, which function to present processed antigens to CD4 T cells (reviewed in (15)). The HLA Class II molecules are heterodimers composed of an alpha and beta chain expressed on the surface of antigen-presenting cells. Importantly the HLA Class II molecules contain a peptide-binding groove that allows formation of a trimolecular complex between the HLA Class II molecule, its bound peptide and the T cell receptor on T cells. The HLA Class II region has the strongest genetic association with human autoimmune diseases (15), underscoring the importance of antigen presentation for immune tolerance. HLA class II alleles are primarily associated with autoimmune diseases characterized by autoantibodies such as T1D, RA and SLE (15, 21–23). Notably, the HLA locus is highly polymorphic, and the allelic associations differ across autoimmune diseases suggesting that HLA is also involved in the tissue specificity of the immune response. Additionally, HLA alleles may be associated with protection as well as risk. T1D is an example where both are seen, HLA DR4, DR3 and DQ0302 are each associated with disease, whereas DQ0602 is protective (23). HLA alleles are also associated with disease characteristics. For example, HLA-DRB1 alleles encoding the shared epitope a "shared" motif (QKRAA, QRRAA or RRRAA in positions 70–74 of the DRB1 chain) that is found on DR1 and DR4 alleles associated with a distinct subset of individuals with RA, specifically those who have anti-citrulline antibodies (ACPA) or ACPA+ RA (reviewed in (24)). It is also important to note that the region linked to HLA risk on chromosome 6 includes additional genes with immunologic significance, and there is growing evidence that they too may impart risk for autoimmunity (25, 26).

PTPN2

The *PTPN2* gene encodes protein tyrosine phosphatase non-receptor type 2, which has a regulatory role in a variety of signaling pathways including T cell receptor signaling, IL-2 signaling, and JAK/STAT signaling (27). There are three autoimmune disease-associated variants in the PTPN2 gene shared across T1D, Crohn's disease, and RA: rs2542151 in the coding region and rs1893217 and rs478582 both in the non-coding region (27). The rs1893217 variant is associated with decreased PTPN2 mRNA levels in human T cells (28). Carriers of the rs1893217 variant also show impaired T cell responses to IL-2 as measured by pSTAT5 (28) and the rs478582 variant is associated with reduced stability of Tregs (29, 30). In murine models, PTPN2 expression is linked to T cell lineage commitment (31), proliferation and survival (32), and Treg stability (33). Yet as a broader understanding of the impact of altered PTPN2 expression is gained, its role in autoimmunity has extended beyond T cells. In murine models, PTPN2 has been shown to negatively regulate IL-21 signaling and B cell responses (34), in humans the PTPN2 risk variant rs1893217 is associated with the loss of B cell anergy (35). PTPN2 is also important in dendritic cell-mediated immune tolerance, partial depletion of PTPN2 in dendritic cells (DCs) results in spontaneous inflammation, altered immune cell composition, increased accumulation of conventional type 2 DCs (cDC2) in organs, and expansion of IFNγ-producing effector T cells (36). Notably, the variants of PTPN2 associated with autoimmunity are quite common (the minor allele frequency (MAF) of rs1893217 risk = 0.1196) and the increase in risk is modest (the odds ratios for T1D and Crohn's are 1.3 and 1.25 respectively (27)) indicating that the risk variants contribute through modest alterations in multiple aspects of immune regulation.

PTPN22

The rs2476601 variant in the coding region of the protein tyrosine phosphatase non-receptor type 22 (*PTPN22)* gene is one of the most strongly associated risk variants shared across autoimmune diseases including RA, T1D, and SLE (37). PTPN22 is notable for its role across multiple immune cell types including lymphocytes, natural killer (NK) cells, neutrophils, monocytes, macrophages, and DCs (37). In T and B cells, PTPN22 regulates antigen receptor signaling (38), making it a major focus of studies investigating its role in autoimmunity risk. The minor allele of the rs2476601 variant is associated with autoimmunity and has a thymine substituted for a cytosine at nucleotide 1858 (*PTPN22* C1858T) resulting in a change from arginine (R) to tryptophan (W) at amino acid position 620. This amino acid change results in modest alterations in the function of PTPN22 but importantly alters the character and function of immune cells. Examples of this include alterations in the composition of the B cell compartment and increases in polyreactive and autoreactive B cells in $PTPN22^{620W/W}$ carriers, indicating a failure of B cell tolerance (39, 40). Notably, murine modeling of this variant recapitulates autoimmunity and confirms that a multiplicity of mechanisms is involved in this process (41, 42). Human studies have also shown that the rs2476601 variant influences T cell maturation including

increased CD4 memory T cells (43) and an increase in Th1 cells (44). In addition, this variant impacts TCR signaling, although the jury is still out as to whether it is a gain- or loss-of-function mutation, and this is likely dependent on context (37). This PTPN22 variant has also been recently implicated in cDC2 homeostasis because expression of the orthologous polymorphism in mice lead to expansion of cDC2 (45). Thus, similar to PTPN2, the PTPN22 variant which is broadly associated with autoimmunity, likely does not confer risk through one pathway, but through a combination of modest alterations, that lead to failures in tolerance checkpoints in both T and B cell compartments and promote the development of pathogenic responses.

Synergy between autoimmune disease-associated variants

Dissecting how autoimmune disease-associated variants interact with each other to promote susceptibility to autoimmunity is a critical next step for understanding how genetic variants contribute to the loss of tolerance. Although, this is challenging to do, it is possible using well-defined cohorts controlled for the genotypes of interest, and/or crossing knockin mouse models expressing the variants of interest. CRISPR/cas9 genome editing is also being utilized to express the variants of interest in primary human immune cells (46–48). Here, we highlight two studies investigating the interactions between genetic variants in the setting of autoimmune disease. The first analyzed a large cohort of individuals with RA and determined that there was synergistic interaction between the *PTPN22* s2476601 variant and the *HLA-DRB1* shared epitope alleles in participants who were positive for both antibodies to cyclic citrullinated peptides and antibodies to citrullinated α-enolase (49). Interestingly, the combined effect of the *PTPN22* s2476601 variant and the HLA-DRB1 shared epitope alleles was further enhanced by smoking (49), underscoring the importance of gene-environment associations for the development of autoimmunity. The second study crossed knockin mice to investigate the interaction between the rs1990760 variant in *IFIH1* and the rs2476601 in *PTPN22* (8). The *IFIH1* variant rs1990760 is associated with risk of T1D, SLE, RA, and multiple sclerosis (MS) (50) and results in an amino acid change from alanine to threonine at position 946 in the C-terminal of the interferon-induced helicase C-domain containing protein 1 (IFIH1 also known as MDA5). IFIH1 is a pattern recognition receptor for dsRNA that induces a type I interferon response to RNA viruses (51). In both humans and mice, the IFIH1 rs1990760 variant acts as a gain-of-function mutation that increases the interferon response (8). When both the *IFIH1* rs1990760 and *PTPN22* rs2476601 variants were introduced into a murine model of T1D, an additive effect was observed with increase in the rate and time to onset of diabetes (8). These studies are examples of the interaction across genetic variants and indicate such interactions may amplify disease risk.

Variants unique to a disease reveal disease-specific immune alterations

There are also genetic variants that are only associated with a single autoimmune disease. Interestingly, these variants typically target a pathway or process that is unique to the underlying pathogenesis of disease such as the antigen targeted in autoimmunity. As noted above, the HLA locus is associated with many autoimmune diseases, but the associated alleles may differ- arguing that the link at this level may be specific to the autoantigen being targeted. Other variants that are disease-specific and associated with specific antigen targets include the insulin variable number of tandem repeats (*INS*-VNTR) variant associated with T1D and the peptidylarginine deiminase (*PADI*) 2 and 4 variants associated with RA.

INS-VNTR

The polymorphic insulin gene variable number of tandem repeats (*INS*-VNTR) is associated with the proinsulin gene promoter region. Variants in this region, specifically the VNTR III haplotype, are associated with a 3- to 4-fold relative protection from diabetes (52). This haplotype is associated with elevated expression levels of proinsulin in the thymus (53, 54) and a decrease in the frequency of high avidity pro-insulin-specific $CD4^+$ T cells in comparison to the diabetes susceptibility haplotype VNTR I (55). Thus, in a manner similar to the AIRE mutation that limits expression of self-antigens in the thymus, this genetic risk variant may act by specifically impeding the expression of pro-insulin in the thymus resulting in a tissue-specific failure of central tolerance which can contribute to the development of pathogenic proinsulin-specific T cells and ultimately the development of T1D.

PADI2 and *PADI4* variants

Genetic variants in *PADI2* and *PADI4* have been associated with ACPA positive-RA (56), although these associations appear to be strongest in Asian populations (57). The functional impact of these variants is still unclear yet the role of peptidylarginine deiminases (PADs) in RA makes this association of particular interest. *PADI2* and *PADI4* encode (PADs) 1 and 4 respectively, enzymes that catalyze the post-translation conversion of arginine to citrulline by calcium-dependent deamination (58). Given that ACPA are present in 80% of individuals with RA, PADs are likely to play a central role in disease pathogenesis due to their ability to generate citrullinated proteins. This is further

supported by the presence of PAD2 and PAD4 in the synovial fluid of patients with RA (59–61). In addition, PADs are involved in immune cell processes implicated in autoimmunity, including neutrophil net formation (netosis) (62) and anti-PAD4 antibodies have been detected in patients with RA and are associated with disease severity (63). Also intriguing is a recent study reporting an association with *PADI4* variants in Caucasian individuals who smoked and carried risk alleles for both HLA-DRB1*04 and PTPN22 (64). A potential explanation for this synergy is the discovery that PTPN22 interacts with and inhibits PAD activity, but the PTPN22^{620W} risk variant (rs2476601) disrupts this interaction leading to enhanced citrullination and netosis (65).

Future directions

The next frontier in the field of autoimmune disease-associated genetic variants is understanding the functional impact of non-coding variants located in regulatory regions of the genome. New discovery opportunities are now possible due to advances in approaches to interrogate the 3-dimensional architecture of the genome including chromatin conformation capture techniques and increasingly sophisticated profiling methods integrating epigenetics, transcriptomics, and proteomics. In addition, there have been substantial improvements in the assays used to elucidate the function of a variant with massively parallel reporter assays (MPRA) and CRISPR-Cas genome editing facilitating high throughput screening. These approaches are now being applied to autoimmune disease-associated variants. A CRISPR activation screen identified a risk variant in an enhancer region of the IL2RA gene (47) and more recently MPRA was used to prioritize approximately 18,000 autoimmune disease associated-variants based on how they perturb regulatory elements in T cells (66). Expression quantitative trait (eQTL) analysis has also been helpful in linking non-coding variants to nearby genes (67). Two recent studies applying single cell eQTL analysis to T cells highlighted the importance of both activation state and cell type on the effects of autoimmune disease-associated variants (68, 69).

Another priority is identifying genetic variants that are associated with either disease progression or response to treatment. This is an emerging field, but the power of this approach has been demonstrated by a study comparing good and poor prognosis in Crohn's disease (70). Notably, variants were identified that were specifically associated with prognosis rather than susceptibility (70). Screening for genetic variants that influence response to therapy has also been limited, but there have been some genetic associations identified for response to TNFα blockade in RA. The biggest challenge for any of these studies is defining the cohort given the heterogeneity with respect to stage of disease and the therapies administered. This type of work will require strong collaborative efforts to assess clinical outcomes for large numbers of patients with studies of mechanistic outcomes; large undertakings with important potential to improve the way we provide healthcare to individuals at risk for and with autoimmune diseases.

Clinical implications

Identification of genetic variants has been important for the development of immunotherapies aimed at achieving immune tolerance. For example, knowing the HLA haplotype is crucial for many antigen-specific therapies including peptide immunization and engineered Treg cell therapy. Genetic variants in the IL-2 signaling pathway such as those in *IL2RA* and *PTPN2* also need to be considered for IL-2-mediated therapy. Likewise for the IL-6 pathway where single nucleotide polymorphisms in the IL-6 receptor may influence the response to IL-6 blockade therapies. Tyk2 inhibitors are now in clinical trials, with initial encouraging results in psoriasis with the potential to be extended to other autoimmune diseases, particularly those associated with protection from the loss-of-function variant, including SLE and MS (71). Collectively, these studies underscore the value of autoimmune-associated genetic variants for development of personalized/precision medicine for the prevention and treatment of autoimmune disease.

Author contributions

AH collected literature and wrote the review. JB conceived, collected literature, and wrote the review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

J.H.B. is a Scientific Co-Founder and Scientific Advisory Board member of GentiBio, a consultant for Bristol-Myers Squibb and Hotspot Therapeutics, and has past and current research projects

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Herpesvirus entry mediator on T cells as a protective factor for myasthenia gravis: A Mendelian randomization study

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Background and objectives: Myasthenia gravis (MG) is a T cell-driven, autoantibody-mediated disorder affecting transmission in neuromuscular junctions. The associations between the peripheral T cells and MG have been extensively studied. However, they are mainly of observational nature, thus limiting our understanding of the effect of inflammatory biomarkers on MG risk. With large data sets now available, we used Mendelian randomization (MR) analysis to investigate whether the biomarkers on T cells are causally associated with MG and further validate the relationships.

Methods: We performed a two-sample MR analysis using genetic data from one genome-wide association study (GWAS) for 210 extensive T-cell traits in 3,757 general population individuals and the largest GWAS for MG currently available (1,873 patients versus 36,370 age/gender-matched controls) from US and Italy. Then the biomarkers of interest were validated separately in two GWASs for MG in FIN biobank (232 patients versus 217,056 controls) and UK biobank (152 patients versus 386,631 controls).

Results: In the first analysis, three T-cell traits were identified to be causally protective for MG risk: 1) CD8 on terminally differentiated $CD8^+$ T cells (OR [95% CI] = 0.71 [0.59, 0.86], P = 5.62e-04, adjusted P =2.81e-02); 2) $CD4^+$ regulatory T proportion in T cells (OR [95% CI] = 0.44 [0.26, 0.72], P = 1.30e-03, adjusted P =2.81e-02); 3) HVEM expression on total T cells (OR [95% CI] = 0.67 [0.52, 0.86], P = 1.61e-03, adjusted P =2.81e-02) and other eight T-cell subtypes (e.g., naïve CD4+ T cells). In particular, HVEM is a novel immune checkpoint on T cells that has never been linked to MG before. The SNPs on the TNFRSF14 *per se* further support a more direct link between the HVEM and MG. The validation analysis replicated these results in both FIN and UK biobanks. Both datasets showed a concordant protective trend supporting the findings, albeit not significant.

Conclusion: This study highlighted the role of HVEM on T cells as a novel molecular-modified factor for MG risk and validated the causality between T cells and MG. These findings may advance our understanding of MG's immunopathology and facilitate the future development of predictive disease-relevant biomarkers.

KEYWORDS

Mendelian randomization, myasthenia gravis, GWAS, HVEM, T cell

Introduction

Myasthenia gravis (MG) is an autoimmune disease that mainly affects the postsynaptic membrane at the neuromuscular junction. Fatigability and weakness in skeletal muscles are the representing clinical features. Immune dysregulation in MG mainly involves malfunctioned T cells, autoreactive B cells, and autoantibody production (1). Autoantibodies that were against postsynaptic membrane components mainly consist of the anti-acetylcholine receptor (AChR), anti-muscle specific kinase (MuSK), and lipoprotein-related protein 4 (LRP4) antibodies (2).

The thymus is a gland where T cells differentiate and mature. The removal of thymus (thymectomy) brought long-term benefits by improving the clinical outcome in thymomatous and non-thymomatous MG patients (3, 4). In the immunological pathogenesis of AChR-associated MG, the thymus releases AChR autoreactive T cells to activate peripheral AChR-directed B cells (5). Besides, chronic inflammation maintained by circulating T helper 17 (Th17) cells, autoantibody production promoted by follicular T (Tfh) cells, and impaired rebalancing function of regulatory T (Treg) cells contribute to the MG exacerbation (6). In contrast, $CD8^+$ T cells were involved in MG pathogenesis, and there are relatively very few studies investigating the exact correlations (7). Current studies on T cells and MG were mainly conventional and observational.

Mendelian randomization (MR) uses genetic variants as the exposure proxy of the exposure to examine the causal effect of that exposure on the outcome (8). The correlations between genetic variants and MG have been explored in several genome-wide association studies (GWASs) and human leukocyte antigen (HLA) haplotype analysis, by which T-cell relevant genes, including CTLA4, TNFRSF11A, PTPN22, and the HLA haplotypes, have been implicated in the pathogenesis of MG (9–12). With now available large data sets, MR analysis may be an elegant tool to explore the novel biomarkers from T cells with causal impacts on MG risk, which has rarely been performed in this field.

We hypothesized that molecules in peripheral T cell traits have direct causal effects on MG risk. A two-sample MR study was performed to determine this causal relationship by leveraging extensive T-cell traits from 3,757 general population-derived individuals and the largest GWAS on MG with 1,873 patients and 36,370 age- and gender-matched healthy controls. The results were further replicated in both FIN biobank with 217,288 individuals and UK biobank with 386,783 individuals. This study may establish causal links between the T-cell relevant molecules and MG development.

Materials and methods

Data sources

The current study applied a two-sample MR method to analyze causal relationships between 210 T-cell traits and MG. The data sources were chosen from studies with publicly available summary GWAS data, and detailed information about different GWAS datasets is displayed in Table 1. The extensive T-cell traits (listed in Supplementary File 1) were derived from the SardiNIA project composed of GWAS data from 3,757 general population individuals who are native to the central east coast of Sardinia, Italy (13). These T-cell traits included subtypes in the T-cell panel (double negative, double positive, $CD4^+$, $CD8^+$), regulatory T (Treg) panel, maturation stages (central memory/effector memory/terminally differentiated), and cell marker expression levels on different T cells. As a primary analysis, the MG data were sourced from the currently largest meta-GWAS conducted in the US and Italy (1,873 patients versus 36,370 age/gender-matched controls) (11). Only anti-acetylcholine receptor antibody-positive (AChR+) MG patients were enrolled in this study, and patients with positive test results for antibodies to muscle-specific kinase (MuSK+) were excluded from the enrollment. In the secondary analysis, the validation datasets include FIN Biobank (https://gwas.mrcieu.ac.uk/datasets/finn-b-G6_

TABLE 1 GWAS datasets used in this Mendelian randomization (MR) study.

Dataset	Phenotype/ variable	First author (year)	Sample size (cases/ controls)	Population	Sex	Phenotype ascertainment
Exposure 1	210 kinds of T-cell traits and markers	Orrù (2020)	3,757	Sardinian (Italy)	57.0% female	Normal individuals' peripheral blood was antibody-stained and processed for flow cytometry
Outcome 1	Myasthenia gravis	Chia (2022)	38,243 (1,873/ 36,370)	US and Italian	47.2% female	Patients diagnosed in myasthenia gravis clinics: characteristic fatigable weakness and electrophysiological and/or pharmacological abnormalities and confirmed by the presence of anti-acetylcholine receptor antibodies
Outcome 2		Fin biobank	217,288 (232/ 217,056)	Finnish	Mixed	Self-reported phenotype (myasthenia gravis subtype information are not applicable)
Outcome 3		UK biobank	386,783 (152/ 386,631)	UK	Mixed	Self-reported phenotype (myasthenia gravis subtype information are not applicable)

MYASTHENIA/) (232 patients versus 217,056 controls) and UK Biobank (http://www.nealelab.is/uk-biobank) (152 patients versus 386,631 controls). The MG phenotype was ascertained from participants' self-reported questionnaires; information of MG subtypes is not applicable. All original studies obtained ethical approval and informed consent from the participants.

Instrument selection

For selecting the most unbiased and representing instrumental genetic variables, a series of quality control steps were conducted to determine eligible instrumental SNPs (Figure 1). First, significant SNPs associated with exposures with genome-wide significance ($P < 5 \times 10^{-8}$) and minor allele frequency (MAF) > 0.01 were selected. Second, given that many SNPs may locate adjacently in linkage disequilibrium status in a GWAS, we performed a clumping process ($R^2 < 0.001$, window size = 10,000 kb) using European reference samples from the 1000 genomes project and retained only the SNP with the lowest P-value. Third, exposure SNPs were extracted in the outcome GWAS summary data. If a particular exposure SNP was not present in the outcome GWAS, then a proxy SNP in linkage disequilibrium with the exposure SNP (minimum LD r-squared value 0.8) was used. Fourth, the exposure and outcome SNPs were harmonized, by which ambiguous SNPs in which the effect allele cannot be determined were removed. Palindromic SNPs were specifically checked in original datasets to avoid unwanted reverse effects. The strength of the genetic instrument was evaluated by F-statistics, and a weak instrument with F-statistic < 10 was removed. The calculation of the F statistic is $F = R^2(n-k-1)/k(1-R^2)$, where R^2 represents the exposure variance explained by the instrumental SNPs, n is the sample size, and k represents the number of instrumental variables (14). These stringently selected SNPs were used as the instrumental variables for the subsequent two-sample MR analysis.

Two-sample MR analysis

Different MR methods were used to estimate the causative effect of exposure variables on the outcome accordingly. The Wald ratio method was used when only one instrumental SNP was available, and the inverse variance weighting (IVW) method was used when more than one SNP was presented. All causal estimates were converted to odds ratios (ORs) for the outcome which was a dichotomous phenotype. For exposure with more than three SNPs available, sensitivity analyses were performed

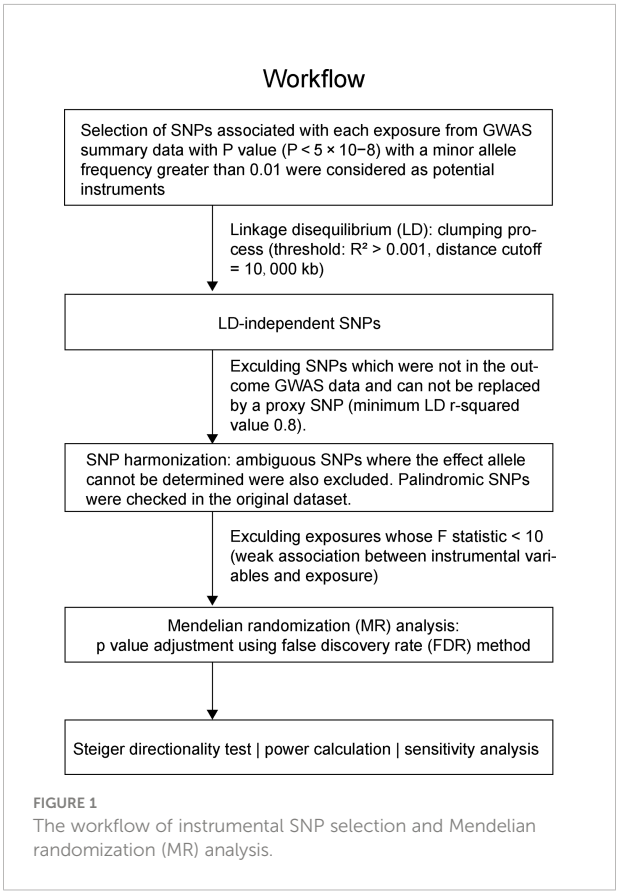


FIGURE 1
The workflow of instrumental SNP selection and Mendelian randomization (MR) analysis.

using different MR methods which hold different assumptions at the cost of reduced statistical power, including weighted median (15), weighted mode (16), simple mode, MR Egger regression (17), and MR-PRESSO (18). The Steiger directionality test was performed in those significant results to validate whether the assumption that exposure causes outcome is valid (19). For exposures with less than three instrumental SNPs, pleiotropy analysis was performed using the PhenoScanner database to query additional associated traits found in previously published GWASs (20). Finally, statistical power for each exposure was calculated with a two-sided type-I error rate $\alpha = 0.05$ (21).

MR assumptions

Three core instrumental variable assumptions for this study were specifically considered: 1) Relevance: instrumental SNPs are associated with the exposure of T-cell signatures. The genetic bases for T-cell functions and subtypes have been fully investigated, and genetically engineered T-cell immunotherapies have provided remarkable clinical success (22). We also calculated the F-statistic for each T-cell signature, and only those instrumental SNPs with F-statistic > 10 were considered qualified. 2) Independence: there is no confounder between the instrumental SNPs and the outcome. Only genetic data sourced from European ancestry and both-sex populations were used in this study to avoid common confounders due to demographic variety. 3) Exclusion restriction: instrumental SNPs affect the outcome exclusively through their potential effects on the exposure T-cell signatures. The pathological mechanisms of how irregulated T cells cause MG have been explained in the introduction. To identify potential horizontal pleiotropy, we also searched the PhenoScanner database to find other impacts that might be caused by those instrumental SNPs.

Statistical analysis

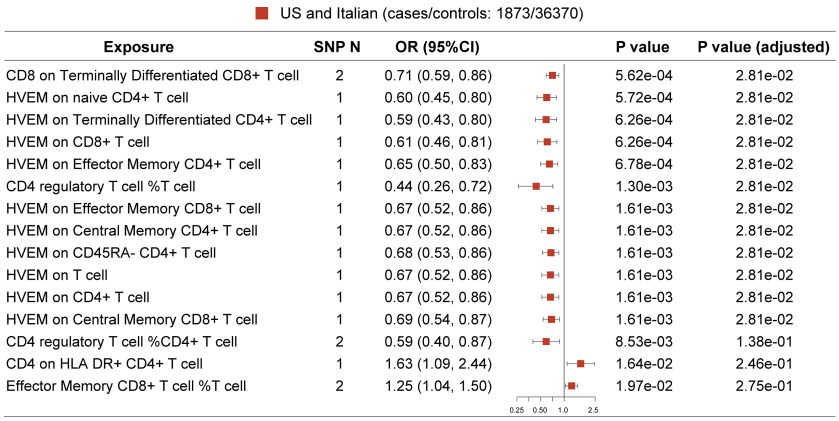
We performed the MR analyses in the R, version 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria), with the TwoSampleMR package (23). Other packages used for processing data and generating figures include Tidyverse, Rsnps, and Forestplot. Since exposures (T-cell traits) were repeatedly compared with each outcome (MG), the P-values were adjusted by the false discovery rate (FDR) method.

Results

The detailed characteristics of the instrumental SNPs associated with 210 T-cell traits (SNP n = 630) used in this study are displayed in Supplementary File 1. The MR findings between them and the outcome in each dataset are displayed in Supplementary File 2. The pleiotropy analysis results for those significant results are displayed in Supplementary File 3.

Primary analysis: The US and Italian cohorts

In the primary analysis, after FDR adjustment, the top 15 significant variables are as specifically displayed in Figure 2. All selected instrumental variants showed strong F statistics (median 223.24, IQR 1167.90) with the exposure, and the



Exposure	SNP N	OR (95%CI)	P value	P value (adjusted)
CD8 on Terminally Differentiated CD8+ T cell	2	0.71 (0.59, 0.86)	5.62e-04	2.81e-02
HVEM on naive CD4+ T cell	1	0.60 (0.45, 0.80)	5.72e-04	2.81e-02
HVEM on Terminally Differentiated CD4+ T cell	1	0.59 (0.43, 0.80)	6.26e-04	2.81e-02
HVEM on CD8+ T cell	1	0.61 (0.46, 0.81)	6.26e-04	2.81e-02
HVEM on Effector Memory CD4+ T cell	1	0.65 (0.50, 0.83)	6.78e-04	2.81e-02
CD4 regulatory T cell %T cell	1	0.44 (0.26, 0.72)	1.30e-03	2.81e-02
HVEM on Effector Memory CD8+ T cell	1	0.67 (0.52, 0.86)	1.61e-03	2.81e-02
HVEM on Central Memory CD4+ T cell	1	0.67 (0.52, 0.86)	1.61e-03	2.81e-02
HVEM on CD45RA- CD4+ T cell	1	0.68 (0.53, 0.86)	1.61e-03	2.81e-02
HVEM on T cell	1	0.67 (0.52, 0.86)	1.61e-03	2.81e-02
HVEM on CD4+ T cell	1	0.67 (0.52, 0.86)	1.61e-03	2.81e-02
HVEM on Central Memory CD8+ T cell	1	0.69 (0.54, 0.87)	1.61e-03	2.81e-02
CD4 regulatory T cell %CD4+ T cell	2	0.59 (0.40, 0.87)	8.53e-03	1.38e-01
CD4 on HLA DR+ CD4+ T cell	1	1.63 (1.09, 2.44)	1.64e-02	2.46e-01
Effector Memory CD8+ T cell %T cell	2	1.25 (1.04, 1.50)	1.97e-02	2.75e-01

FIGURE 2
MR result in primary analysis (US and Italian patients). SNP N, number of SNP. The top 12 ranked T-cell traits by P value show protective effect on MG risk after false discovery rate (FDR) adjustment.

powers of all MR analyses were relatively large (median 1.00, IQR 0.03), as shown in Table 2. We identified three T-cell traits of interest which had protective effects on the risk of MG: 1) CD8 on terminally differentiated $CD8^+$ T cells (OR [95% CI] = 0.71 [0.59, 0.86], P = 5.62e-04, adjusted P =2.81e-02); 2) $CD4^+$ Tregs proportion in T cells (OR [95% CI] = 0.44 [0.26, 0.72], P = 1.30e-03, adjusted P =2.81e-02); 3) HVEM on total T cells (OR [95% CI] = 0.67 [0.52, 0.86], P = 1.61e-03, adjusted P =2.81e-02) and other eight T-cell subtypes (naive $CD4^+$ T cells, terminally differentiated $CD4^+$ T cells, $CD8^+$ T cells, effector memory $CD4^+$ T cells, CD4 regulatory T cells, effector memory $CD8^+$ T cells, central memory $CD4^+$ T cells, $CD45RA^-$ $CD4^+$ T cells). The Steiger directionality test showed that all results conformed to the right exposure to outcome direction.

Among them, no exposure has instrumental SNPs of more than 2. Then the Wald ratio or IVW methods were used to conduct the MR analysis, and no proxy SNP was used in these exposures. Two instrumental SNPs (rs2571390, rs2523887) for exposure "CD8 on terminally differentiated $CD8^+$ T cell" were not located on any known genes. Three SNPs corresponding to HVEM expression levels on T subsets were located on the HVEM encoding gene, TNFRSF14 perse (rs1886730, rs2227313), and a non-coding RNA gene LOC100996583 (rs2182176). One SNP was related to the exposure "CD4 regulatory T cell %T cell" and was located on splicing factor 45 encoding gene RBM17 (rs1571025). Notably, HVEM is a novel immune checkpoint that has never been linked with MG before. The SNPs found on the TNFRSF14 *per se* indicate a more direct link between the HVEM on T cells and MG.

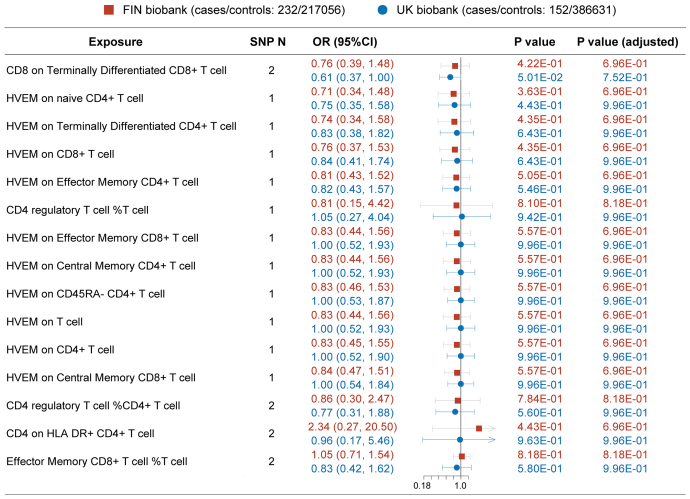
Secondary analysis: Validations in FIN and UK biobanks

Since MG is a rare autoimmune disease with a low prevalence (around 12 per 100,000 population) (24), and another GWAS dataset with a large sample size of patients was not available, hence we conducted this replication in publicly available FIN and UK biobanks (Figure 3). Before P-value adjustment, the exposure "CD8 on Terminally Differentiated $CD8^+$ T cell" in the UK biobank barely reached significance in MR analysis (OR [95% CI] = 0.61 [0.37, 1.00], P =5.01e-02), while after FDR adjustment, all results in both datasets showed a similar protective tendency with the primary analysis but did not

TABLE 2 Detailed MR result in the primary analysis (the US and Italian cohorts).

Exposure	Method	SNP N	OR	r2.exposure	r2.outcome	P value (adjusted)	Power	F statistic	Correct causal direction	Steiger pval
CD8 on terminally differentiated CD8+ T cell	Inverse variance weighted	2	0.71	4.21E-02	3.11E-04	2.81E-02	0.83	82.46	TRUE	4.12E-23
HVEM on naive CD4+ T cell	Wald ratio	1	0.60	3.30E-02	3.11E-04	2.81E-02	0.98	128.21	TRUE	8.12E-09
HVEM on terminally differentiated CD4+ T cell	Wald ratio	1	0.59	5.61E-02	6.11E-04	2.81E-02	1.00	223.24	TRUE	5.59E-14
HVEM on CD8+ T cell	Wald ratio	1	0.61	6.83E-02	6.11E-04	2.81E-02	1.00	275.09	TRUE	3.59E-17
HVEM on effector memory CD4+ T cell	Wald ratio	1	0.65	4.15E-02	3.01E-04	2.81E-02	0.96	162.47	TRUE	5.15E-11
CD4 regulatory T cell %T cell	Wald ratio	1	0.44	2.00E-02	5.41E-04	2.81E-02	1.00	76.69	TRUE	2.25E-11
HVEM on effector memory CD8+ T cell	Wald ratio	1	0.67	2.42E-01	1.56E-03	2.81E-02	1.00	1196.25	TRUE	4.36E-67
HVEM on central memory CD4+ T cell	Wald ratio	1	0.67	2.55E-01	1.56E-03	2.81E-02	1.00	1286.80	TRUE	6.24E-72
HVEM on CD45RA- CD4+ T cell	Wald ratio	1	0.68	2.75E-01	1.56E-03	2.81E-02	1.00	1423.66	TRUE	3.66E-79
HVEM on T cell	Wald ratio	1	0.67	2.43E-01	1.56E-03	2.81E-02	1.00	1208.15	TRUE	1.00E-67
HVEM on CD4+ T cell	Wald ratio	1	0.67	2.66E-01	1.56E-03	2.81E-02	1.00	1362.65	TRUE	5.93E-76
HVEM on central memory CD8+ T cell	Wald ratio	1	0.69	2.96E-01	1.56E-03	2.81E-02	1.00	1578.89	TRUE	3.11E-87
CD4 regulatory T cell %CD4+ T cell	Inverse variance weighted	2	0.59	3.81E-02	5.76E-04	1.38E-01	0.99	74.38	TRUE	1.77E-22
CD4 on HLA DR+ CD4+ T cell	Wald ratio	1	1.63	1.32E-02	1.50E-04	2.46E-01	0.66	50.04	TRUE	4.35E-08
Effector memory CD8+ T cell %T cell	Inverse variance weighted	2	1.25	2.65E-02	1.56E-04	2.75E-01	0.33	51.15	TRUE	1.71E-17

R2.exposure and R2.outcome represent the phenotype variance which can be explained by the corresponding instrumental SNPs.



Exposure	SNP N	OR (95%CI)	P value	P value (adjusted)
CD8 on Terminally Differentiated CD8+ T cell	2	0.76 (0.39, 1.48)	4.22E-01	6.96E-01
		0.61 (0.37, 1.00)	5.01E-02	7.52E-01
HVEM on naive CD4+ T cell	1	0.71 (0.34, 1.48)	3.63E-01	6.96E-01
		0.75 (0.35, 1.58)	4.43E-01	9.96E-01
HVEM on Terminally Differentiated CD4+ T cell	1	0.74 (0.34, 1.58)	4.35E-01	6.96E-01
		0.83 (0.38, 1.82)	6.43E-01	9.96E-01
HVEM on CD8+ T cell	1	0.76 (0.37, 1.53)	4.35E-01	6.96E-01
		0.84 (0.41, 1.74)	6.43E-01	9.96E-01
HVEM on Effector Memory CD4+ T cell	1	0.81 (0.43, 1.52)	5.05E-01	6.96E-01
		0.82 (0.43, 1.57)	5.46E-01	9.96E-01
CD4 regulatory T cell %T cell	1	0.81 (0.15, 4.42)	8.10E-01	8.18E-01
		1.05 (0.27, 4.04)	9.42E-01	9.96E-01
HVEM on Effector Memory CD8+ T cell	1	0.83 (0.44, 1.56)	5.57E-01	6.96E-01
		1.00 (0.52, 1.93)	9.96E-01	9.96E-01
HVEM on Central Memory CD4+ T cell	1	0.83 (0.44, 1.56)	5.57E-01	6.96E-01
		1.00 (0.52, 1.93)	9.96E-01	9.96E-01
HVEM on CD45RA- CD4+ T cell	1	0.83 (0.46, 1.53)	5.57E-01	6.96E-01
		1.00 (0.53, 1.87)	9.96E-01	9.96E-01
HVEM on T cell	1	0.83 (0.44, 1.56)	5.57E-01	6.96E-01
		1.00 (0.52, 1.93)	9.96E-01	9.96E-01
HVEM on CD4+ T cell	1	0.83 (0.45, 1.55)	5.57E-01	6.96E-01
		1.00 (0.52, 1.90)	9.96E-01	9.96E-01
HVEM on Central Memory CD8+ T cell	1	0.84 (0.47, 1.51)	5.57E-01	6.96E-01
		1.00 (0.54, 1.84)	9.96E-01	9.96E-01
CD4 regulatory T cell %CD4+ T cell	2	0.86 (0.30, 2.47)	7.84E-01	8.18E-01
		0.77 (0.31, 1.88)	5.60E-01	9.96E-01
CD4 on HLA DR+ CD4+ T cell	2	2.34 (0.27, 20.50)	4.43E-01	6.96E-01
		0.96 (0.17, 5.46)	9.63E-01	9.96E-01
Effector Memory CD8+ T cell %T cell	2	1.05 (0.71, 1.54)	8.18E-01	8.18E-01
		0.83 (0.42, 1.62)	5.80E-01	9.96E-01

FIGURE 3
MR result in secondary analysis (FIN and UK Biobanks). Before P value adjustment, only the first ranked exposure "CD8 on terminally differentiated CD8$^+$ T cell" barely showed significance in the UK biobank dataset. However, after FDR adjustment, no exposures reach significance, but the tendencies of which are basically in accordance with the primary analysis (as protective factors). This can be explained by the low powers in all analysis due to paucity in patients.

reach significance. In the FIN biobank, the CD4$^+$ regulatory T cell% T cell OR [95% CI] is 0.81 [0.15, 4.42], and HVEM on overall T cells is 0.83 [0.44, 1.56]. In the UK biobank, the CD4$^+$ regulatory T cell% T cell OR [95% CI] is 1.05 [0.27, 4.04], and HVEM on overall T cells is 1.00 [0.52, 1.93]. This may be due to the much lower power in the FIN (median 0.09, IQR 0.04) and UK biobanks (median 0.03, IQR 0.04), as shown in Tables 3, 4. Still, the Steiger directionality test showed that all results were consistent with the same exposure to outcome direction.

Discussion

This is the first MR study exploring the causal effects of risk factors on MG to the best of our knowledge. MR uses genetic variants as instrumental variables, fixed at conception, to conduct causal inferences about the impact of modifiable risk factors, which can overcome some types of confounding (25). This study was reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology Using Mendelian Randomization (STROBE-MR) Statement (26). Our primary analysis extensively evaluated the causality between T-cell traits and MG, and three protective factors were identified in our study.

The first trait is the higher CD8 expression on terminally differentiated CD8$^+$ T cells, the most mature CD8$^+$ T cells residing in the periphery. Previous studies found that CD8 expression levels were lower in CD8$^+$ T cells of chronic graft-versus-host disease and terminally differentiated effector memory T-cell (TEMRA) autoimmune lymphoproliferative syndrome (27, 28). CD8 is a coreceptor for the antigen-presenting process when activating T cells, and its downregulation on tissue-resident T cells has been postulated as a natural desensitization mechanism for prolonged antigen activation (29), which is common in the context of MG *per se* and its comorbidity with other autoimmune diseases (30). Higher CD8 expression levels on terminally differentiated CD8 + T cells represent an inert activated status. These inert CD8+ T cells are less likely to be activated by MG-related autoantigens, hence a less likely inclination to develop MG.

The second protective trait is a higher proportion of CD4$^+$ Tregs, which is in accordance with previous studies. Previous GWASs on MG have identified the correlations between variants in genes (e.g., CTLA4 and PTPN22) with MG risk, which directly modulates the proportion or function of CD4$^+$ Tregs (9, 11). Biological evidence from experimental autoimmune MG (EAMG) models has explained the potential mechanisms in which CD4$^+$ Tregs suppressed the abnormal proliferation of T effector cells in response to MG-related antigens (31, 32). Our MR analysis validated the causality between CD4$^+$ Tregs and MG, which supported the hypothesis that individuals with more CD4$^+$ Tregs would be less likely to develop MG.

Interestingly, the third protective trait is the higher HVEM expression on various T-cell subtypes. HVEM, which belongs to the tumor necrosis factor receptor (TNFR) superfamily, has been recognized as a novel immune checkpoint in recent years (33). HVEM is expressed primarily on immune cells and functions as a ligand to activate the B- and T-lymphocyte attenuator (BTLA)

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TABLE 3 Detailed MR result in the secondary analysis (FIN biobank).

Exposure	Method	SNP N	OR	r2.exposure	r2.outcome	P value (adjusted)	Power	F statistic	Correct causal direction	Steiger pval
CD8 on terminally differentiated CD8+ T cell	Inverse variance weighted	2	0.76	4.21E-02	7.75E-05	6.96E-01	0.13	82.46	TRUE	4.58031E-25
HVEM on naive CD4+ T cell	Wald ratio	1	0.71	3.30E-02	2.28E-05	6.96E-01	0.15	128.21	TRUE	5.39004E-10
HVEM on terminally differentiated CD4+ T cell	Wald ratio	1	0.74	2.81E-02	1.67E-05	6.96E-01	0.12	108.40	TRUE	1.07591E-08
HVEM on CD8+ T cell	Wald ratio	1	0.76	3.41E-02	1.67E-05	6.96E-01	0.12	132.69	TRUE	2.30064E-10
HVEM on effector memory CD4+ T cell	Wald ratio	1	0.81	4.15E-02	1.23E-05	6.96E-01	0.10	162.47	TRUE	1.89862E-12
CD4 regulatory T cell %T cell	Wald ratio	1	0.81	1.00E-02	1.59E-06	8.18E-01	0.05	37.96	TRUE	2.82692E-08
HVEM on effector memory CD8+ T cell	Wald ratio	1	0.83	4.03E-02	9.47E-06	6.96E-01	0.08	157.55	TRUE	3.68938E-12
HVEM on central memory CD4+ T cell	Wald ratio	1	0.83	4.25E-02	9.47E-06	6.96E-01	0.09	166.83	TRUE	8.65081E-13
HVEM on CD45RA- CD4+ T cell	Wald ratio	1	0.83	4.58E-02	9.47E-06	6.96E-01	0.09	180.31	TRUE	1.0565E-13
HVEM on T cell	Wald ratio	1	0.83	4.06E-02	9.47E-06	6.96E-01	0.08	158.78	TRUE	3.0409E-12
HVEM on CD4+ T cell	Wald ratio	1	0.83	4.44E-02	9.47E-06	6.96E-01	0.09	174.38	TRUE	2.66275E-13
HVEM on central memory CD8+ T cell	Wald ratio	1	0.84	4.93E-02	9.47E-06	6.96E-01	0.09	194.87	TRUE	1.09886E-14
CD4 regulatory T cell %CD4+ T cell	Inverse variance weighted	2	0.86	2.35E-02	2.17E-06	8.18E-01	0.05	45.13	TRUE	1.04689E-17
CD4 on HLA DR+ CD4+ T cell	Inverse variance weighted	2	2.34	2.47E-02	2.76E-04	6.96E-01	0.53	47.52	TRUE	5.01601E-14
Effector memory CD8+ T cell %T cell	Inverse variance weighted	2	1.05	2.65E-02	7.35E-06	8.18E-01	0.03	51.15	TRUE	1.54993E-19

on other immune cells (34). Two categories of BTLA are CTLA-4/CD28/CD80/CD86 (function at the early phase of T-cell activation) and PD-1/PD-L1/PD-L2 (control the effector phase of the immune response in peripheral tissues) (35). The former (CTLA-4) expression has been found lower in MG patients, and the latter (PD-1) has been linked with immune checkpoint inhibitor-related myasthenia gravis (36, 37). As an immune inhibiting ligand, higher HVEM expression on T cells may be a protective factor for MG. The other function of HVEM is that it mediates the entry of herpes simplex virus type 1 (HSV-1) and HSV-2 into cells, which do not include other subtypes such as Epstein–Barr (EB) virus and varicella zoster virus (VZV) (38). We think that this might explain why fewer HSV-infected MG patient cases were reported than those EB and VZV cases in clinical settings (39, 40). However, studies with larger sample size and stringent design are needed to validate this in future.

Noted that MG is a rare neuromuscular disease; the sample size derived from now available GWAS datasets is still not satisfactory for data-driven analysis. However, we attempted to replicate the findings in another two independent biobanks. In the replication process, only similar protective tendencies, albeit not significant, were found in these exposures, which is restrained by the small power due to paucity in patients. Given that our results can explain the potential biological mechanism underlying T cells in MG genesis, this MR analysis basically satisfied the required assumptions in MR studies (relevance, independence, and exclusion restriction) (25).

There are several limitations in this study: 1) The primary results were derived from AChR+ MG patients, and the secondary results derived from MG with unknown subtypes. Hence, caution is needed to interpret the results. 2) There is insufficient validation in large exposure and outcome datasets. 3) The participants of the FIN and UK biobanks were enrolled by self-reported results, which may introduce biases in the results. 4) Horizontal pleiotropy was found in selected SNPs with other autoimmune diseases, which may interfere with MG pathogenesis by other immunological

TABLE 4 Detailed MR result in the secondary analysis (UK biobank).

Exposure	Method	SNP N	OR	r2.exposure	r2.outcome	P value (adjusted)	Power	F statistic	Correct causal direction	Steiger pval
CD8 on terminally differentiated CD8+ T cell	Inverse variance weighted	2	0.61	4.21E-02	1.09E-05	7.52E-01	0.24	82.46	TRUE	3.72264E-28
HVEM on naive CD4+ T cell	Wald ratio	1	0.75	3.30E-02	1.52E-06	9.96E-01	0.10	128.21	TRUE	1.30105E-10
HVEM on terminally differentiated CD4+ T cell	Wald ratio	1	0.83	2.81E-02	5.54E-07	9.96E-01	0.06	108.40	TRUE	3.14977E-09
HVEM on CD8+ T cell	Wald ratio	1	0.84	3.41E-02	5.54E-07	9.96E-01	0.06	132.69	TRUE	5.56983E-11
HVEM on effector memory CD4+ T cell	Wald ratio	1	0.82	4.15E-02	9.42E-07	9.96E-01	0.07	162.47	TRUE	4.52695E-13
CD4 regulatory T cell %T cell	Wald ratio	1	1.05	1.00E-02	1.36E-08	9.96E-01	0.03	37.96	TRUE	4.94225E-09
HVEM on effector memory CD8+ T cell	Wald ratio	1	1.00	4.03E-02	5.05E-11	9.96E-01	0.03	157.55	TRUE	7.87879E-13
HVEM on central memory CD4+ T cell	Wald ratio	1	1.00	4.25E-02	5.05E-11	9.96E-01	0.03	166.83	TRUE	1.72983E-13
HVEM on CD45RA- CD4+ T cell	Wald ratio	1	1.00	4.58E-02	5.05E-11	9.96E-01	0.03	180.31	TRUE	1.92226E-14
HVEM on T cell	Wald ratio	1	1.00	4.06E-02	5.05E-11	9.96E-01	0.03	158.78	TRUE	6.43707E-13
HVEM on CD4+ T cell	Wald ratio	1	1.00	4.44E-02	5.05E-11	9.96E-01	0.03	174.38	TRUE	5.04944E-14
HVEM on central memory CD8+ T cell	Wald ratio	1	1.00	4.93E-02	5.05E-11	9.96E-01	0.03	194.87	TRUE	1.80808E-15
CD4 regulatory T cell %CD4+ T cell	Inverse variance weighted	2	0.77	2.35E-02	2.81E-06	9.96E-01	0.07	45.13	TRUE	4.98314E-19
CD4 on HLA DR+ CD4+ T cell	Inverse variance weighted	2	0.96	2.47E-02	1.11E-05	9.96E-01	0.03	47.52	TRUE	1.3121E-17
Effector memory CD8+ T cell %T cell	Inverse variance weighted	2	0.83	2.65E-02	9.55E-06	9.96E-01	0.06	51.15	TRUE	5.80203E-21

pathways, not only through T cells. 5) The ancestry of GWAS data used in this study is mainly of European origin, and further GWASs from other races are needed to validate the results.

Conclusions

In conclusion, we found three T-cell-related traits as potential protective factors for the risk of MG in the primary analysis: 1) CD8 on terminally differentiated $CD8^+$ T cells, 2) $CD4^+$ regulatory T cell% T cells, and 3) HVEM on overall T cells. In the future, these factors may serve as biomarkers for forecasting MG development and provide new insights into the underlying mechanism.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the ethical committees of the original GWAS studies analyzed in this study. All original studies have obtained ethical approval and informed consent from the participants. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

SL and CZ conceived the presented idea. HZ, KJ, and XH performed the computations and manuscript writing. MS, XZ, and ZZ were involved in interpretation of data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.931821/full#supplementary-material

SUPPLEMENTARY FILE 1
The detailed instrumental SNPs information of primary and secondary analysis.

SUPPLEMENTARY FILE 2
The detailed MR results of all exposures in primary and secondary analysis.

SUPPLEMENTARY FILE 3
Pleiotropy analysis results for those significant SNPs displayed in primary and secondary analysis.

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Nature vs. nurture: FOXP3, genetics, and tissue environment shape Treg function

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The importance of regulatory T cells (Tregs) in preventing autoimmunity has been well established; however, the precise alterations in Treg function in autoimmune individuals and how underlying genetic associations impact the development and function of Tregs is still not well understood. Polygenetic susceptibly is a key driving factor in the development of autoimmunity, and many of the pathways implicated in genetic association studies point to a potential alteration or defect in regulatory T cell function. In this review transcriptomic control of Treg development and function is highlighted with a focus on how these pathways are altered during autoimmunity. In combination, observations from autoimmune mouse models and human patients now provide insights into epigenetic control of Treg function and stability. How tissue microenvironment influences Treg function, lineage stability, and functional plasticity is also explored. In conclusion, the current efficacy and future direction of Treg-based therapies for Type 1 Diabetes and other autoimmune diseases is discussed. In total, this review examines Treg function with focuses on genetic, epigenetic, and environmental mechanisms and how Treg functions are altered within the context of autoimmunity.

KEYWORDS

Treg - regulatory T cell, T cell, autoimmunity, type 1 diabetes, genetic, FOXP3

Introduction

At the crossroads of autoimmunity and health are regulatory T cells (Tregs) - a crucial immune cell involved in tolerance towards self and suppression of auto-antigen specific T cells. Tregs were first identified as a subpopulation of CD4 T cells that expressed the high affinity IL-2 receptor chain CD25 (1). However, it took several more years to identify the lineage specific transcription factor, Forkhead Box Protein 3 (FOXP3), that is a core regulator of suppressive Treg function (2–4), and acts as both

a positive and negative regulator of gene expression (2–4). For example, FOXP3 directly upregulates CD25 expression, but suppresses IL-2 production (5). With the knowledge of how to identify Tregs and a basic understanding of their function, the field was propelled towards key findings regarding their developmental source, suppressive mechanisms, and therapeutic potential (6–8).

While the transcription factor FOXP3 was initially considered the "master regulator" of CD4 Treg development and function (7, 8) we now understand that a more complex system is at work. Rather than a single element, the Treg suppressive program is regulated by a combination of transcription factors, genetic and epigenetic elements, as well as tissue-microenvironment cues. Due to the complexity that underlies the Treg suppressive phenotype, it has become apparent that loss of Treg lineage commitment can occur through either loss of FOXP3 or through a number of alternative genetic and/or transcriptional dysregulations. However, the precise alterations that occur in autoimmune individuals that affect Treg-mediated tolerance, and how underlying genetic variations impact the development and function of Tregs during autoimmunity are only partially elucidated. Polygenetic susceptibly is a key driving factor of many autoimmune diseases. However, while genome wide association studies (GWAS) alone were suggestive, they were not sufficient to formally link Treg dysfunction to disease. Integration of GWAS studies with functional and other omics-data now implicate alterations or defects in regulatory T cell function in autoimmune pathogenesis (9–11).

In this review we consider the function and regulation of FOXP3 both during homeostasis and autoimmunity, as well as how FOXP3 and mutations in key Treg genes influence Treg function and stability. In addition, we examine epigenetic modifications that regulate FOXP3 activity and how inflammation in the surrounding tissue environment impacts Tregs. Finally, we feature how Treg based therapies for autoimmunity have changed since their inception as well as factors that need to be improved in order to make these therapies efficacious as treatments for autoimmunity.

Mutations in the FOXP3 gene

Immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome is a rare disorder that often results from mutations within the *FOXP3* gene (2, 12, 13). However, in a cohort of 173 patients with IPEX syndrome symptoms, only 50.9% had direct mutations in *FOXP3*, underscoring the fragility of Treg function and its sensitivity to modulation of alternative pathways (14). Of the 85 patients that had no discernable *FOXP3* mutation, 25% had mutations in key Treg genes such as *LRBA, STAT1, STAT3, CTLA4, IL2RA, STAT5B*, and *DOCK8* which are responsible for various aspects of Treg differentiation and function. This suggests that although FOXP3 is critical for Treg mediated tolerance, other factors also participate in maintaining a functional Treg population (Figure 1A). For example, mice lacking the inhibitory molecule CTLA4 develop severe lymphoproliferative disease reminiscent of *Foxp3* mutant mice (37, 38). Furthermore, another study of 15 IPEX patients bearing *FOXP3* mutations revealed that Treg signature genes were still expressed, although with variable expression levels, indicating that Tregs can still maintain partial lineage characteristics after loss of FOXP3 expression (28). A transcriptomic disease signature was observed across both Tregs and conventional CD4 cells and was likely induced by global immune dysregulation. To put it differently, transcriptomic changes occur as a result of both cell-intrinsic and cell-extrinsic mechanisms, where Tregs first have dysregulated core genes involved in Treg stability and suppressive function *(i.e. Il2ra, Tnfrsf4, Tnfrsf9, Tnfrsf18, Capg, Ikzf2, and Ctla4)*, which in turn alter the tissue environment, ultimately leading to enhanced broad transcriptomic changes affecting all T cells (28). In the absence of cell-extrinsic inflammatory signals in heterozygous mothers of IPEX patients, patient *FOXP3* mutations impacted only a narrow set of genes directly under FOXP3 control. In combination, these observations point to limited direct impacts of *FOXP3* mutations and an increased role for activation of inflammatory feedback loops leading to cumulative dysregulation of both regulatory and effector T cells. This further underscores the importance of Tregs' ability to integrate information from their environment and alter their subsequent functions.

FOXP3 has four structural domains that are used to interact with diverse binding partners to exert transcriptional regulation. The examination of *FOXP3* mutations in IPEX patients and in mouse models has provided important insights into the function of the specific domains within FOXP3. Mutations in *FOXP3* identified in IPEX patients have been localized to all four structural domains of the transcription factor, although to some level they are concentrated in the DNA-binding FKH domain (29). For example, identification of a patient with a mutation within the dimerization motif in the FKH domain of *FOXP3* showed that FOXP3's domain swap interface is crucial for restricting Th2 immune responses in Tregs. When the domain swap interface is mutated, FOXP3 interacts with Th2 specific loci inducing expression of Th2 signature cytokines that are normally repressed in Tregs (29).

While most mutations within *FOXP3* result in systemic immune dysregulation and global autoimmune manifestations, partial disruption of interactions between FOXP3 and its binding partners can have understated effects on FOXP3-driven gene activity. Foxp3-GFP reporter mice that express GFP fused to Foxp3 at its N-terminus provided a system to observe how subtle changes can have disease-specific impacts (30, 31). The Foxp3-GFP reporter mouse shows no abnormal

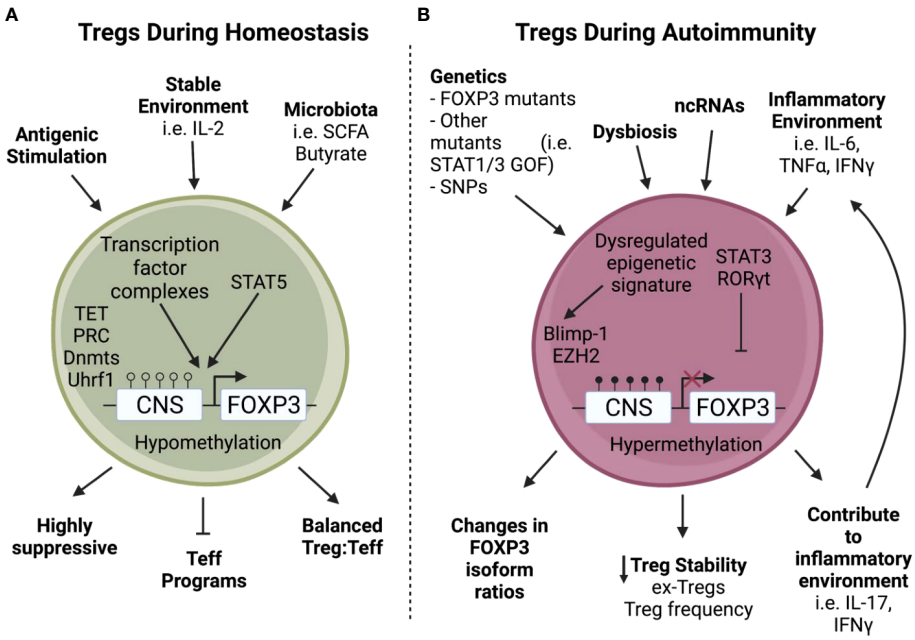

FIGURE 1
Tregs encounter increased stressors during autoimmunity. **A)** During homeostasis, Tregs are stimulated through TCR activation and proliferate using IL-2 from the surrounding environment (1, 5), allowing downstream transcription factor complexes to bind to a hypomethylated Foxp3 and enact critical Treg functions such as suppressive capabilities and repression of Teff programming (15–18). **B)** In contrast, Tregs found during autoimmunity often have intrinsic defects in addition to environmental stressors (2, 12–14, 19–23). For example, non-coding RNAs can be dysregulated during autoimmunity (24–26) leading to a dysregulated epigenetic signature with increased methylation of the Foxp3 Treg Specific Demethylated Region (TSDR) which can cause a loss of Foxp3 (27). In addition, a tissue environment rich in inflammatory cytokines can convert Tregs into Th17-like cells leading to the creation of ex-Tregs and decreased Treg stability (32–34). Furthermore, these stressors encountered during autoimmunity can also lead to perturbations in FOXP3 isoform ratios (35, 36) and expression of inflammatory cytokines (32, 33) which in turn leads to decreased Treg stability.

Treg function on the C57BL/6 genetic background; however, when backcrossed to the NOD autoimmune-susceptible strain it resulted in rapidly accelerated autoimmune diabetes development (30, 31). Foxp3-GFP showed reduced interaction with several binding partners involved in *Foxp3* gene regulation, suggesting Foxp3 instability and loss of Treg function under increased inflammatory stress (30). Interestingly, the GFP-modified Foxp3 was protective in a model of arthritis due to disruption in HIF1α binding and increasing Foxp3 interactions with Interferon Regulatory Factor 4 (IRF4) leading to improved Treg control of Th2 and Th17 responses (31). Perturbations in Treg function were observed in autoimmune prone, but not autoimmune resistant mice suggesting that the genetic or inflammatory environment has a direct influence on the ultimate functionality of Tregs. The loss of Treg stability under inflammatory conditions has been a concern in situations of chronic autoimmunity and has been directly observed in mouse models of autoimmune diabetes, multiple sclerosis, and rheumatoid arthritis (32–34). Loss of Foxp3 expression in these situations resulted in the formation of 'ex-Tregs' that acquired an effector pro-inflammatory phenotype (Figure 1B). However, such ex-Tregs have not been directly observed in human autoimmune conditions, and if they exist are more likely to be localized directly in inflammatory tissues.

Polygenetic susceptibilities and Treg function

Autoimmune manifestations that result from direct mutations of the *FOXP3* gene and related Treg functional genes, such as *CTLA4*, can be traced to loss of Treg numbers and/or function (38–40). However, it has been more challenging to infer the target immune cell population in polygenetic autoimmune susceptibilities. HLA alleles associated with autoimmunity contribute the largest risk for development of autoimmunity, including type 1 diabetes (9). For some HLA alleles, such as DQ8 and DQ2, loss of self-tolerance is thought to be prompted by the structure of the peptide binding grooves, which lead to increased selection or peripheral activation of autoimmune T cells (41). Many other T1D associated SNPs are located in close proximity to immune genes, such as *CTLA4*, and components of the IL-2 and TCR signaling pathways among others. The cytokine IL-2 binds to CD25 (*IL2RA*) and signals

through STAT5 to regulate FOXP3 expression in Tregs (Figure 1A) (42). Complete *IL2RA* deficiency can lead to severe autoimmunity with IPEX like symptoms (43), and *IL2RA* variants have been associated with reduced Treg numbers, suboptimal Treg function, and an increased risk for development of T1D (44). Since HLA alleles, *CTLA4*, and *IL2RA* among others are implicated in both T effector (Teff) and Treg function, the ultimate impact on either population is difficult to determine. Nevertheless, several T1D related SNPs have been connected to Treg function (45–47) and the Treg to T effector cell ratio (48). Additionally, evidence suggests that Tregs from T1D patients may not be as suppressive and may have a more inflammatory phenotype (49). Therefore, there is a growing consensus that Treg function is altered in T1D, and Treg dysregulation might be in part due to genetics.

In many other autoimmune and inflammatory disorders it is not so clear whether there is an underlying defect in regulatory T cells. Many polymorphic variants are shared between several autoimmune diseases, including PTPN22 (TCR signaling), TKY2 (cytokine signaling), and TNFAIP3 (TNF signaling) among others (50, 51). These variants point to genes besides FOXP3 that could influence T cell and Treg function during autoimmunity. For example, in the context of rheumatoid arthritis there is still an ongoing debate regarding Treg dysfunction. There are a number of conflicting observations on whether Treg frequency decreases or remains stable (52–55), whether there are changes in Treg suppressive capability, or the relative expression of Treg associated regulatory molecules, such as CTLA-4 (52, 56, 57). The markers used to define Tregs as well as disease severity should be carefully considered in these studies, and could potentially explain some of the discrepancies in observations. Nevertheless, the lack of clear loss in Treg number or function in RA supports the idea that Treg dysfunction is disease specific.

The majority of disease-associated genetic variants defined by GWAS studies are found in non-coding areas of the genome, which presents a challenge in determining the ultimate relationship between SNPs, gene expression, and downstream effects on cellular function. Importantly, many disease associated SNPs are mapped to regulated chromatin regions and enhancers, i.e. epigenetically regulated transcription factor binding sites (58–60). Several mechanisms for non-coding regions' impact on immune genes have been described. These range from direct disruption of transcription factor binding at SNPs located within enhancer regions (61) to distal effects mediated by genomic misfolding and interconnection of enhancers in 3D chromatin organization (62). Recent studies have coupled epigenetic profile analyses of isolated T cell populations to determine the effects of particular SNPs on chromatin accessibility in the context of T cell populations. Interestingly, the chromatin accessibility at these loci is preferentially associated with naive and activated Tregs, rather than conventional T cells (10, 63, 64). These observations imply that genetic susceptibility disproportionally effects Treg function compared to effector T cells in the context of autoimmunity. Based on cumulative genetic studies we can infer that genetic polymorphisms have connections to FOPX3+ Treg function and predisposition to autoimmunity (9, 44–48). Therefore, it is critical to examine the transcriptional regulation of the Treg lineage and the factors that impinge on Treg stability.

Genetic regulation of the FOXP3 locus

Genetic control and regulation of *FOXP3* plays a major role in Treg development and function during both homeostasis and disease. While several FOXP3 isoforms have been identified in humans, there are two distinct isoforms that are necessary for optimal Treg function; the full length FOXP3 isoform and the alternatively spliced FOXP3 isoform which lacks exon 2 (FOXP3Δ2) (65). The full length FOXP3 isoform has recently been identified as a critical component of regulating FOXP3 activity and maintaining Treg stability (66). FOXP3Δ2 on the other hand, has been shown to be upregulated during Treg activation, and is linked to transcription of the transmembrane protein, Glycoprotein A Repetitions Predominant (GARP), which tethers TGFβ to the cell membrane and potentiates cell-contact dependent TGFβ function (67, 68). While both isoforms are necessary for optimal Treg function (65), regulation of FOXP3 isoform ratios appears to alter the disease course in some autoimmune diseases (Figure 1B) (35, 36).

Regulation of the *FOXP3* locus is multifaceted and involves several key enhancer regions that recruit a number of regulators that control Treg development and stabilize the Treg lineage (Figure 1A). The *FOXP3* locus has four enhancer regions known as conserved non-coding sequences (CNS; CNS0, CNS1, CNS2, and CNS3) that work in tandem to drive *FOXP3* transcription and downstream gene expression necessary for Treg stability (15–17, 69, 70). These enhancer regions are embedded throughout upstream-promoter and intronic regions of *FOXP3* (71, 72) and alter *FOXP3* transcription and activity by controlling methylation status, chromatin accessibility, and act as docking sites for unique sets of binding partner complexes (15–17, 73, 74). For example, the transcription factor SATB1 binds CNS0 (18) which along with the transcription factor HIVEP2 co-regulates pathways involved in Treg immunosuppression (75). SATB1 is an important transcription factor in regulating T cell differentiation (76); however, it is repressed by FOXP3 in Tregs to balance Treg proliferation and function. Loss of SATB1 increases Treg frequency but diminishes Treg suppressive function (77, 78). In Tregs, *SATB1* is epigenetically regulated through histone trimethylation and acetylation changes, as well as by microRNAs such as mir-155, mir-21a, mir-7, mir-34a, and mir-18a (79). During development, IL-2 signaling directs the pioneer factor SATB1 to bind nucleosome dense regions in Tregs leading to chromatin remodeling and accessibility of critical Treg

signature genes (77). This is aided by the transcription factor Foxp1 which enhances IL-2 signaling and Foxp3 expression (78), making IL-2 signaling a critical step in differentiating Tregs from CD25+Foxp3- Treg precursors in the thymus (15).

CNS1 is primarily associated with peripheral induction of Tregs and is bound by several transcription factors including AP-1, NFAT, Foxo1, Hhex, Batf3, and importantly Smad3 induced by TGFβ signaling. Batf3 represses FOXP3 expression and downregulates the differentiation of naïve CD4 T cells into Tregs (80). In addition, Hhex (Hematopoietically expressed homeobox) is a transcription factor that binds to CNS1/CNS2 and represses FOXP3 expression; particularly under inflammatory conditions (81). CNS2 is a critical response element during thymic Treg development, and is bound by Ets-1, CREB, Stat5, NFAT, c-Rel, Runx, Foxp3, and AP-1. Importantly, CNS2 contains the Regulatory T cell Specific Demethylated Region (TSDR) (82), which maintains FOXP3 expression in Tregs and allows FOXP3 to positively regulate its own transcription even in the absence of TCR signaling (18). Lastly, CNS3 is another region important for the development of thymic Tregs and can bind Foxo and c-Rel (17, 83–85). These transcription factor binding complexes can alter FOXP3 activity, downstream targets of FOXP3, and additional pathways involved in Treg function (75). In addition, CNS regions CNS0 and CNS3, were recently determined to be sites that help initiate Treg development when bound by transcription factor complexes that allow chromatin remodeling and drive FOXP3 transcription (16). Beyond FOXP3 enhancer regions, transcription of Treg signature genes is also regulated by cooperation of Foxp3 and one of the five transcription factors Eos, IRF4, GATA-1, Lef1, and Satb1. These cofactors, referred to as the "quintet", enhance Foxp3 activity by 'locking in' and stabilizing Foxp3 to its binding sites (18).

Furthermore, demethylation status of the *FOXP3* TSDR was determined to be key for maintaining FOXP3 expression and stabilizing Treg identity. However, while demethylation of the TSDR is enough to stabilize FOXP3 expression in Tregs, it is not enough to confer suppressive function (86). This suggests that Treg suppressive function is not solely linked to FOXP3 expression, and that additional transcription factors are required. As an example, the transcription factor Helios is expressed in approximately 70% of Tregs and helps to maintain Treg stability by controlling certain aspects of Treg function, differentiation, and survival (87). However, mice lacking Helios are still able to convert naïve T cells into functional Tregs; indicating a level of redundancy in transcriptional regulation of Treg function (88).

Heterogeneity within the Treg population

The FOXP3+ Treg population exhibits phenotypic and functional complexity driven by tissue and context specific transcription factors. Similar to conventional T cells (Tconv), the majority of lymphoid derived thymic Tregs maintain a non-activated phenotype, characterized by expression of CD62L, CCR7 and TCF1 (a transcription factor associated with stemness) (89). However, Tregs can also be derived from naïve CD4 T cells in the periphery through TGFβ signaling (90, 91) (Figure 2). TGFβ signal can be provided in the form of latent TGFβ on the cell surface of tTregs, which leads to induction of additional Foxp3+ T cells (pTregs), in a process that is described as "infectious tolerance" (Figure 2) (98). Upon differentiation from naïve T cells, *in vivo* induced pTregs repress CD4 effector T cell programming, stabilize expression of FOXP3, and maintain a fully demethylated TSDR, similar to tTregs (92–94). In addition, recent work suggests that type 1 interferons can stabilize expression of STAT3, STAT5, and FOXP3 in peripheral CD4 T cells allowing their differentiation into pTregs (Figure 2) (99). However, Type 1 interferons have been shown to have opposing effects on Tregs depending on the timing of exposure. In the short-term, Type 1 interferons lead to decreased Treg frequency and function; however, in the long run they can stabilize expression of FOXP3 and promote Treg expansion (100). Nevertheless, since no definitive markers of pTregs have been identified, the functional importance of pTregs during autoimmunity is still heavily debated (101–103).

The widely accepted approach to induce Treg differentiation *in vitro* relies on a combination of TCR ligation in the context of TGFβ and high concentrations of IL-2 (Figure 2) (104). While studies show that iTregs have suppressive function both *in vitro* and *in vivo*, their long-term stability is more controversial (93, 105). Stability is measured by quantification of methylation at the TSDR region, and TCR and IL-2 stimulation can promote demethylation of TSDR in iTregs, thus stabilizing the lineage (106, 107). However, iTregs that have a hypermethylated TSDR can still be functional (108, 109).

As Tregs migrate from lymphoid organs to peripheral tissues they accumulate a common tissue-resident signature and are further differentiated into unique phenotypes dependent on tissue-specific signals. These tissue-resident Tregs (tissue Tregs) have the potential to be derived from both tTregs and pTregs, with the change from a lymphoid-resident phenotype to a tissue-resident phenotype, a process that is mediated by a combination of transcriptional regulators (Figure 3) (111, 112). In the spleen and lymph nodes, the transcription factor BATF drives the stepwise progression of tissue Treg precursors into tissue Tregs by increasing chromatin accessibility of tissue specific Treg genes (113). Repression of BATF impairs tissue Treg function and contributes to induction of autoimmunity (120). In addition, tissue Tregs often exhibit specialized functions associated with upregulation of tissue specific transcription factors, such as PPARγ in visceral fat tissue and Eos in the skin (89, 110). Although, more recently PPARγ has been linked to skin and liver Tregs as well (121, 122). Interestingly, upregulation of IL-33R (ST2) and its

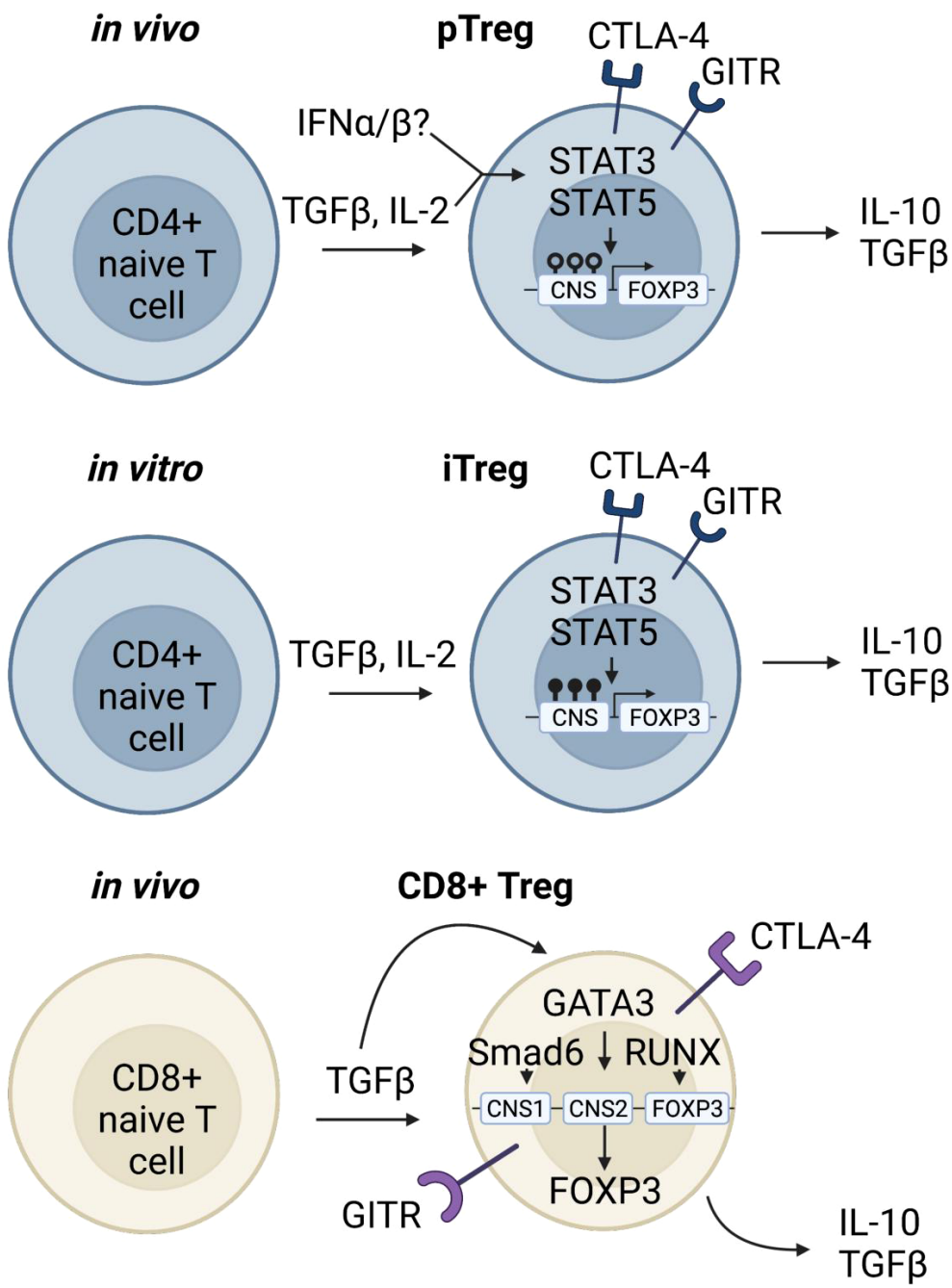

FIGURE 2
Induced regulatory T cells. In the periphery, T lymphocytes can encounter stimuli that turn on downstream signaling leading to genetic reprogramming and a regulatory phenotype (90, 91). pTregs which have the capacity to be immunosuppressive and traffic to inflamed tissue sites are differentiated from CD4 naïve T cells under inflammatory conditions (92–94). A rare and unique subpopulation of Tregs is the CD8+Foxp3+ Treg. In the periphery, when naïve CD8 Tconv cells encounter TGFβ, pSmad3 binds to CNS1 of *Foxp3*, and along with transcription factors Runx3 and Gata3 promote expression of Foxp3 (95–97). CD8+Foxp3+ Tregs express similar markers as CD4 Tregs and have immunosuppressive functions.

downstream target cytokine, amphiregulin, is a trait shared among many Tregs that are transitioning towards tissue phenotype; indicative of an acquired ability to participate in tissue repair in response to inflammation or injury (Figure 3) (89, 123–125). The growth factor amphiregulin is expressed by tissue Tregs in response to alarmin cytokines released by injured tissue cells, including IL-33 (114–116). The ramifications of this discovery show that Tregs upregulate receptors necessary to

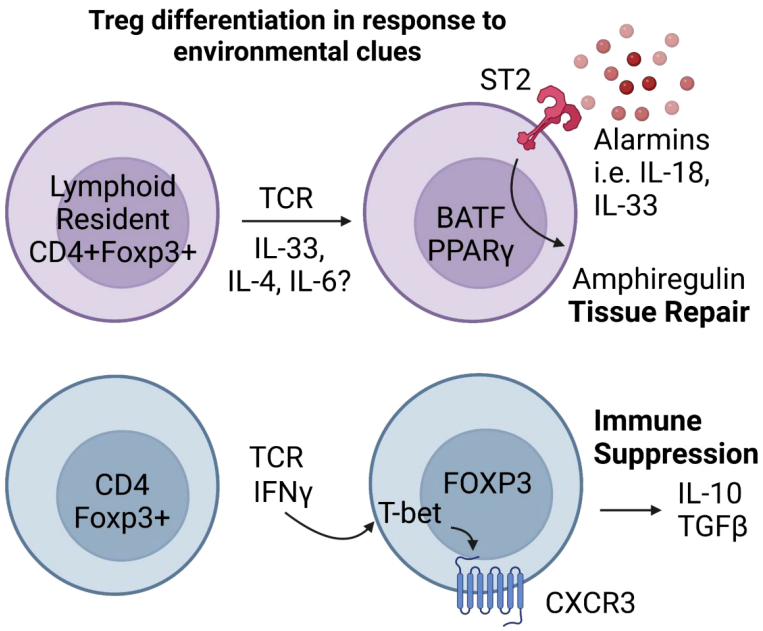

FIGURE 3
Inflammatory and tissue specific signals shape Treg responses. Tissue Tregs are poised to respond to inflammatory tissue environments. In the presence of alarmin cytokines, tissue Tregs expressing the transcriptional regulators Batf and PPARγ (89, 110–113) secrete the wound repair factor Amphiregulin (114–116). Inflammatory cytokines that normally drive T-helper lineage specific factors like T-bet can similarly induce T-helper transcription factor expression in Tregs. IFNγ and TCR stimulation induce T-bet expression in Foxp3+ Tregs, which provides them with increased ability to suppress Th1 effector T cells (117–119).

sense the tissue microenvironment in order to rapidly respond to environmental changes.

In addition, CD4 T-helper lineage defining transcription factors can shape Treg responses during inflammation. A prime example of this is T-bet, which in addition to being the major Th1 lineage-defining transcription factor, provides Tregs with increased ability to suppress Th1 effectors (117). T-bet is upregulated in Tregs in response to IFNγ and TCR ligation and is directly responsible for the upregulation of chemokine receptor CXCR3, allowing Tregs to traffic to sites of inflammation (Figure 3) (118, 119).

Furthermore, CD8+FOXP3+ regulatory T cells constitute a smaller proportion of the Treg compartment but are still functional contributors to the regulatory arm of the immune system. They are transcriptionally similar to CD4+FOXP3+ T cells; and although they seem to be less potent than CD4+FOXP3+ Tregs they have been shown to be effective in models of GVHD and lupus (126, 127). In contrast to CD4+FOXP3+ T cells, CD8 Treg suppressor programs are controlled by the transcription factors RUNX3 and GATA3 (Figure 2). In naïve CD8 T cells, GATA3 binds to the CNS1 region of *FOXP3* to inhibit FOXP3 expression, however in CD8 Tregs, GATA3 binds to the CNS2 region to maintain FOXP3 expression (95). Furthermore, RUNX3 binds to the promoter region of *FOXP3* to initiate transcription, and under conditions with high levels of TGFβ, Smad3 is phosphorylated and binds to CNS1 inducing FOXP3 expression (Figure 2). nCD8+CD25+ Tregs are also somewhat functionally similar to CD4+FOXP3+ Tregs as they express suppressive markers such as GITR and CTLA4 (96), as well as cytokines such as IL-10 and TGFβ (Figure 2) (95, 97).

Epigenetic regulation of Treg lineage

Epigenetic regulation of gene expression can have major consequences for cells. During development, thymocytes that are fated to become Tregs undergo a series of epigenetic modifications to CNS regions of *FOXP3* to activate transcription of *FOXP3* and downstream Treg signature genes. At the same time, T cell effector cellular differentiation programs are repressed (Figure 1A) (128, 129). Stepwise histone tail acetylation at the *FOXP3* promoter initiates chromatin remodeling and *FOXP3* transcription (130). *FOXP3* histone tail acetylation allows ten-eleven translocation (TET)-mediated DNA demethylation to occur in the CNS2 region of the *FOXP3*

locus and maintains *FOXP3* transcription by increasing chromatin accessibility; thus, removing the need for further histone acetylation (130). Positive regulation of Treg lineage is also accomplished by repression of alternative T-helper lineage programs. Polycomb-repressive complexes (PRC) are multi-protein enzymes that transcriptionally silence genes through histone H2A ubiquitylation and H3K27 methylation (131). PRCs silence Th17 related genes and enhance the Wnt signaling pathway to favor Treg development and stability (132, 133).

Once chromatin remodeling and access to core Treg genes is achieved, additional epigenetic changes occur that maintain stable chromatin accessibility. For example, the protein ubiquitin like with PHD and ring finger domains 1 (Uhrf1) is an epigenetic regulator that recruits DNA methyltransferases (Dnmt) such as Dnmt1, Dnmt3a, and Dnmt3b to stabilize methylation patterns (134–137) during Treg development (Figure 1A), as well as following TCR engagement in the periphery (138). Similarly, ablation of Dnmt1 in Tregs severely impairs their function through global changes in methylation (139).

However, many of these normal epigenetic modifications fail to function and/or maintain Treg stability during autoimmunity, as Tregs derived from autoimmune patients often have epigenetic and transcriptomic changes. For example, effector Tregs derived from juvenile idiopathic arthritis patients present with consistent changes that include methylation changes in enhancer regions, as well as upregulation of functional and core Treg genes (140). The upstream regulatory elements that are dysregulated can be numerous due to the complexity of epigenetic mechanisms that control Treg lineage. For instance, in a model of multiple sclerosis, methylation of CNS2 normally repressed by Dnmt3a and controlled by Blimp1 is disrupted and leads to loss of Treg identity (141). There are indications that similar disruptions occur in human autoimmunity. The chromatin-modifying enzyme Ezh2 maintains Treg identity after activation, and its reduction is observed in RA patients (Figure 1B) (142, 143). Moreover, tissue antigens themselves can produce variable epigenetic responses in antigen-specific Tregs. For example, Tregs expanded *in vitro* using APCs expressing insulin B:9-23 peptide were found to have transcriptomic and epigenetic signatures representative of highly suppressive Tregs compared to Tregs expanded using whole insulin peptide (144). This provides evidence for the importance of T cell receptor signaling and antigen specificity in the development of optimally functional and stable Tregs.

Understanding the epigenetic changes that Tregs undergo during chronic inflammation is important for gaining new targeting strategies for autoimmune therapies. Tregs function differently during homeostasis and acute infection compared to chronic inflammatory conditions, implying context and inflammation specific Treg functional programs potentially regulated at the epigenetic level (19–21). In addition, Treg frequency and core signature gene expression mainly associated with DNA accessibility, transcription, translation, signal transduction, and cytokine receptors are prone to changing throughout the span of autoimmune disease pathology (145).

Microbiota have also been shown to influence Treg function and stability. Interestingly, some microbial-derived signals directly engage with Treg epigenetic elements (146). While still a new field of study, there is increasing evidence that short chain fatty acids (SCFA), such as butyrate, can be produced by commensal bacteria and positively regulate Treg differentiation (147). This appears to be CNS1 dependent, and is mediated by enhanced acetylation at the *FOXP3* locus (148, 149). However, it is still unclear whether SCFAs are the key signal for pTreg induction in the mesenteric lymph nodes (150). Importantly, gut dysbiosis is a feature of several autoimmune diseases such as IBD, SLE, RA, Graves' Disease and T1D, and it might contribute to disbalance of immune homeostasis (Figure 1B) (151–156).Thus, it is relevant to ask if inflammation or other microenvironmental cues at tissue sites can play a direct role in changing Treg function through epigenetic and/or transcriptomic changes.

Shifting the Treg/Teff equilibrium

A major question that remains regarding Tregs in autoimmunity is how they inevitably fail throughout the course of disease. One hypothesis is a decrease in the ratio of Tregs : Teffs, which can be seen in several autoimmune diseases (157–160). The shifts seen in this equilibrium could be the result of direct mutations in *FOXP3* such as in IPEX syndrome, other polymorphisms that affect Treg function or stability, or could occur due to the influence of the surrounding tissue environment, since normal cellular mechanisms of differentiation and function that work to maintain the Treg : Teff balance are often dysregulated during autoimmunity (Figure 1).

For example, a major pathway that diverts CD4 T cells away from Treg differentiation and towards a Th17 program is the IL-6/STAT3 pathway commonly associated with inflammation. Dysregulation of the IL-6/STAT3 pathway seen in patients with gain of function mutations in STAT3 is correlated with increased susceptibility to T1D; most likely related to the Treg : Th17 imbalance seen in these patients (22). Inflammatory environments high in IL-6 have been shown to increase the Th17 transcription factor RORγt in both tTregs and pTregs (23), and lead to the creation of ex-Tregs that are capable of secreting inflammatory cytokines (32, 33). These ex-Treg cells lose FOXP3 expression and convert into pathogenic Th17 cells capable of producing IFNγ and destabilizing Tregs in the surrounding environment (33, 161–163). Formation of ex-Tregs promotes a

shift in the Treg : Teff ratio skewed towards destructive Teff cells. Treg-derived IFNγ can also act as a negative feedback regulator of Treg stability and lead to further loss of suppressive function, indicating an important role for the environment in continually shaping and sometimes destabilizing Treg responses (164). Additionally, antigen exposure and/or scarcity can impact the balance between Th17 and Treg differentiation (165). Recent evidence suggests that T cells can trogocytose MHCII molecules from APCs displaying specific antigens, and subsequently display the MHCII to other antigen-specific T cells. When differentiation is favored towards Tregs there is a high APC:T cell ratio, however, when the reverse occurs (high T cell:APC ratio) differentiation is skewed towards Th17 cells (166).

These inflammatory pathways implicated in Treg lineage destabilization can be effectively targeted for therapeutic purposes. Small molecule targeted inhibition of IL-6 or STAT3 promotes Treg development and leads to the establishment of homeostasis between Treg and Th17 cells in a model of multiple sclerosis (MS) (167, 168). MS patients often exhibit dysregulated cytokine levels - including an increase in IL-6 in their cerebral spinal fluid, which could be targeted with the goal of shifting the balance between anti- and pro-Treg micro-environment cues (169). However, blocking the IL-6R in early onset T1D patients with a mAb did not prevent or delay beta cell loss (170), illuminating the limitations of therapies that target a single inflammatory pathway.

Non-coding RNAs during autoimmunity

Evidence shows dysregulated microRNA (miRNA) and long non-coding RNA (lncRNA) expression is also associated with many autoimmune diseases (24, 25). miRNAs are small non-coding RNAs that regulate proteins largely by binding to the 3' UTR of mRNA and preventing translation, or by targeting the mRNA for degradation. Similarly, lncRNAs modulate chromatin architecture and mRNA stability (171). Both miRNA and lncRNA can impact Treg genetic regulation by altering expression of epigenetic regulators, directly targeting *FOXP3*, and by altering the signaling pathways that allow Tregs to respond to the surrounding microenvironment. Through these mechanisms, miRNAs influence Treg frequency and modify Treg functional capabilities.

Further, miRNAs can play an important role in regulating Treg epigenetics. For example, miR-142-3p, which is upregulated during T1D in humans and mice, can bind to lysine demethylase 6A (KDM6A) and demethylate H3K27me3 in Tregs leading to increased autophagy, decreased apoptosis, and increased Treg function (172). While two different Treg-specific miR-142 deficient mouse models showed impaired Treg function, whether or not Treg frequency is altered in these mice remains unclear since the two studies showed conflicting results (173, 174). Furthermore, miR-142-3p function in Tregs may operate through multiple pathways as miRNA142-3p also destabilizes Tregs by interacting with TET2 to alter Treg methylation in both humans and mice (27).

In addition to modifying Treg epigenetic signatures, non-coding RNAs can target *FOXP3* and other Treg signature genes. In humans, several miRNAs including, mi-R206, miR-133a, miR-133b, and miR-31 have been identified that directly target the 3' UTR of *FOXP3* mRNA leading to FOXP3 translational downregulation (175, 176). miR-31 is among the better studied miRNAs that target *FOXP3* and has been implicated in numerous autoimmune diseases. In murine models of autoimmunity, mi-R31 is upregulated upon TCR stimulation, but is inhibited by TGFβ/NF-κB signaling (177). miR-31 functions by directly targeting *FOXP3*, and also acts indirectly by promoting HIF1α and downregulating Nrp1 and retinoic acid-inducible protein 3 (Gprc5a) (178). miR-31 also inhibits carcinoembryonic antigen related cell adhesion molecule 1 (CEACAM1)-S, which represses Treg development in a model of murine liver autoimmunity but promotes Treg development in peripheral blood mononuclear cells (PBMCs) isolated from systemic lupus erythematosus (SLE) patients (179). The ultimate effect of miR-31 on Treg development and frequency depends on the balance between its inhibitory and enhancer functions. However, the factors that determine this require further investigation.

In addition, miRNAs can also influence FOXP3 by targeting pathways that regulate its expression. miR-21, which is among the best studied miRNAs that regulate Tregs in this manner, is dysregulated in several autoimmune disorders in both humans and mice. miR-21 acts indirectly to positively regulate Foxp3 expression (180); however, in autoimmunity, reduced miR-21 expression is correlated with increased STAT3 and reduced STAT5 and Foxp3 expression (26, 181–183). miR-21 directly targets STAT3 resulting in its downregulation and subsequently reduces effector molecules IL-17 and IL-22 (182, 184). Maresin 1 (MaR1) and the EGF/c-Jun pathway have both been shown to induce miR-21, restore Treg : Teff ratios through FOXP3 induction, and reduce autoimmunity (183, 185).

While some studies show that transfection of naïve human CD4 T cells with miR-21 is sufficient to induce Treg development by increasing Foxp3, TGFβ, and IL-10, another study found that miR-21 promotes RORγt and suppresses Foxp3 and IL-10 (180, 186, 187). Indeed, Treg specific depletion of miR-21 in mice induced the expression of both IL-17 and IL-10 indicating that miR-21 may play a role in opposing pathways (184). In line with these opposing observations, increased miR-21 expression inhibited FOXP3+ Tregs in human gastric cancer (188) whereas it induced FOXP3 in human and mouse autoimmunity (182, 183). Interestingly, LPS stimulation of PBMCs from RA patients down-regulated miR-21; however, PBMCs from healthy controls responded to LPS in the opposite

fashion by up-regulating miR-21 (175). The opposite regulation and effects of miR-21 in autoimmune patients compared to healthy controls and in cancer settings suggests that a complex network of factors determines whether miR-21 promotes or inhibits Treg stability and function.

Furthermore, lncRNAs can also modulate Treg epigenetics. For example, FOXP3 long intergenic noncoding RNA, *Flicr*, reduces chromatin accessibility to the CNS3/Accessible Region 5 in mature Tregs and represses FOXP3 expression in both humans and mice. Knockout of *Flicr* on the NOD mouse background results in stabilized Foxp3 expression with a reduction in diabetes incidence (25). Additionally, in both humans and mice the lncRNA lnc-Smad3 interacts with the histone deacetylase HDAC1 to silence SMAD3 transcription. Upon TGFβ stimulation SMAD3 inhibits lnc-Smad3, thus allowing for greater SMAD3 transcription (189).

lncRNAs are also integral in regulating key Treg transcription factors. For example, Homeobox D gene cluster antisense growth-associated long noncoding RNA (HAGLR) is another lncRNA involved in autoimmunity. In human Tregs, HAGLR suppresses RUNX3 expression resulting in reduced Treg frequency (190). Additionally, lncRNA DQ786243 induces FOXP3 expression in human Tregs and promotes Treg suppressive function (191).

Noncoding RNAs are also important participants in regulating and responding to environmental cues. In inflammatory environments rich in IL-6 and TNFα, NF-κb upregulates the expression of miR-34a in humans and mice (192), which attenuates FOXP3 expression and can result in a shift of the Treg : Teff ratio. miR-124, which is dysregulated in numerous autoimmune diseases (24, 26), inhibits IL-6/STAT3 signaling and promotes Treg development (193). Similarly, miR-146a normally targets *STAT5b* to enhance Treg function and differentiation, but loss of miR-146a during inflammatory conditions leads to reduced FOXP3 expression and reduced Treg frequency. IL-2 represses *Flicr* thus removing *Flicr's* inhibition of FOXP3 expression, while TGFβ inhibits the Foxp3-repressive noncoding RNAs miR-31 and lnc-Smad3 (194). The anti-inflammatory molecules MaR1 and EGF promote miR-21 (183, 185). The field of noncoding RNAs and their role in Treg development and function is growing, but additional studies are still required to reveal the full extent they may have in autoimmunity.

Treg-based therapies

With the central role for regulatory T cells in autoimmune diseases, it is unsurprising that investigation is underway as to how Tregs can be used therapeutically (195). One example is the use of Tregs as a treatment for T1D. The current standard of care for T1D patients is exogenous replacement of insulin. When managed well, the administration of synthetic insulin results in more stable blood glucose levels but does not entirely negate the risk of comorbidities (196). Thus, having an immunomodulatory therapy that prevents, attenuates, or reverses the course of pancreatic islet destruction is crucial.

Altering Treg to Teff ratio using immunomodulation

Due to potential imbalance in the Treg : Teff homeostasis seen during T1D, much attention has been focused on changing the ratio either by depleting effector T cells or expanding the Treg population. One of the earliest immunomodulatory therapies attempted in T1D patients was the use of anti-CD3 antibodies (197–199). Even a single dose of anti-CD3 lessened T1D progression and allowed reduction or complete withdrawal from exogenous insulin replacement therapy in some patients (197). Following initial positive observations in early diagnosed patients, anti-CD3 mAb therapy was used in a clinical trial of relatives of T1D patients who had at least two diabetes related auto-antibodies and confirmed dysglycemia prior to the start of the trial (200). A subgroup of participants in the treatment arm of the trial displayed delayed onset of T1D compared to controls, showing that modulation of T cell function after loss of tolerance but prior to overt disease can influence disease outcomes. Anti-CD3 antibodies appear to function by altering the ratio of Tregs : Teffs, as Teffs are susceptible to depletion by anti-CD3, whereas Tregs are more resistant (201). Additionally, following anti-CD3 mAb therapy a temporary increase in PD1+FOXP3+ Tregs was seen that paralleled a rise in anergic/exhausted CD4 and CD8 Teff cells (202). While early versions of anti-CD3 mAbs resulted in significant side effects that limited their use, genetic engineering and proteolytical removal of Fc domains alleviated many of the side effects (203, 204). The recent successes obtained with the anti-CD3 mAb therapy in T1D allow us to conclude that (1) immunotherapeutic interventions can be successful in T1D, (2) timing of immunotherapy is important, but success can be achieved even after anti-beta cell responses are detected, and (3) shifting the balance between inflammatory and regulatory pathways might be sufficient to acquire long-term tolerance. Although anti-CD3 mAb therapy is highly promising, it is not effective for ~25% of T1D patients and its positive effects can be temporary, which necessitates further investigation of the mechanisms underlying persistence of autoimmune T cells and their resistance to anti-CD3 therapy in certain individuals (197, 198).

Direct expansion of Tregs

Another avenue to address Treg frequency is by isolating and expanding endogenous Tregs from T1D patients directly *in vitro* followed by adoptive transfer back into the patient (Figure 4) (196). One way to expand Tregs utilizes the IL-2

pathway. For example, several studies have used low-dose IL-2 as a way to expand Tregs *in vivo* and increase their suppressive function (223, 224). Careful dosing of IL-2 in this approach is critical since high-dose IL-2 also expands effector T cells and other immune cell populations. Recent studies have addressed this dosing issue and improved upon this approach by modifying the IL-2 cytokine so that it selectively binds to Tregs (225–227). Targeting the IL-2 pathway is logical, as the decrease in Tregs seen during NOD diabetes progression is thought to be due to dysregulated IL-2 production within the pancreatic islets leading to loss in Treg function and survival (158), and IL-2R dysfunction is implicated in development of T1D (9). However, combining IL-2 therapy with autologous polyclonal expanded Treg infusion can have the potential to induce more harm than good. When IL-2 and Tregs are concomitantly administered to T1D recipients, IL-2 induces the proliferation not only of Tregs, but also of potentially cytotoxic cells, highlighting the need for Treg specific IL-2 (228). Although, low-dose IL-2 was well tolerated and specifically expanded Tregs in individuals of other autoimmune diseases (229).

An additional caveat to Treg therapy is how Tregs may change during the manufacturing process, i.e., expansion of Tregs *ex vivo*. While *ex vivo*-expanded Tregs maintain suppressive capacity (205), they can also upregulate inflammatory effector T cell-associated cytokines, such as IFNγ, which can lead to loss of Treg stability (Figure 4) (207). Genome wide DNA methylation sequencing on Tregs undergoing *in vitro* expansion show increased methylation in enhancer and promoter regions of genes associated with T cell activation and function, as well as hypomethylation of genes associated with T cell exhaustion. These results are donor independent and are consistent throughout manufacturing runs (206), raising the question of whether Tregs expanded under current *in vitro* protocols are poised for long term function *in vivo*, regardless of their transcriptomic landscape or suppressive capacities at the end of expansion. Findings such as this could elucidate why current Treg therapies often fail to suppress disease long term. Fortunately, recent experiments have shown that the Cas9/CRISPR system can be used for targeted TET-mediated demethylation of the Treg TSDR (220); potentially providing a solution for Treg manufacturing

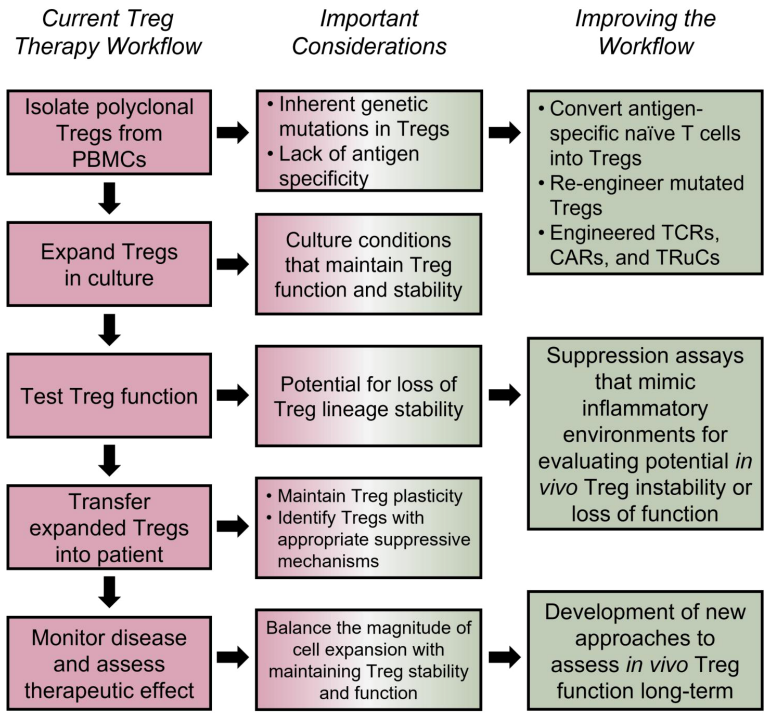

FIGURE 4
Tailoring Treg therapies for improved efficacy. Using human autologous Tregs is a promising approach for treatment of autoimmune and inflammatory disorders (196); however, the efficacy of such approaches depends on several factors. Loss of Treg suppressive capacity, stability, or stemness could be a side effect of *in vitro* expansion protocols (205–207). The potential inherent defects in Tregs, lack of antigen specificity (208–211), TSDR methylation status post expansion (206), and long-term functionality must also be considered. Potential solutions include engineering antigen specific TCRs (212), TRuCs (213–215), and CARs (216–219), utilizing Cas9/CRISPR technology for targeted demethylation of the TSDR (220), and using cytokine cocktails to optimize Treg expansion and functionality long-term (221, 222). Furthermore, clinical studies should be focused on accurately assessing the long-term *in vivo* Treg lineage stability, survival and disease-specific Treg suppressive mechanisms.

complications. Indeed, simply using a chemical inducer of TSDR demethylation was shown to decrease NOD diabetes disease (230).

Another standard approach for *in vitro* expansion of T cells, including Tregs, is based on anti-CD3/CD28 crosslinking that leads to engagement of TCR and co-stimulatory pathways. However, strong and continuous TCR stimulation might result in loss of Treg stability or lead to Treg exhaustion. As an alternative to using anti-CD3/CD28, a combination of cytokines and CD28 superagonist antibodies (CD28SA) can induce robust Treg expansion while maintaining superior Treg stability (221, 222). Collectively, these findings suggest that a more tailored approach is necessary to create Treg-based treatments, and that increased Treg frequency, while helpful, needs to be accompanied by a high suppressive capacity in order to fully curtail disease.

Antigen specificity in Treg therapy

Another important consideration for effective Treg therapy is their tissue antigen specificity, which was shown to be necessary for optimal Treg function in mouse models of T1D (209, 211). Indeed, islet auto-antigen specific, but not polyclonal Tregs transferred into NOD mice are capable of engrafting and expanding following anti-CD3 Ab treatment (208). This may be due to antigen specific Tregs' ability to traffic to the site of autoimmune inflammation more efficiently than polyclonal Tregs. For example, a clinical trial that recently concluded in MS patients saw that *ex vivo* expanded polyclonal CD4 +CD25^{high}CD127-FoxP3+ Tregs injected intrathecally, but not intravenously, had the ability to reduce disease severity, suggesting that inflammatory signals alone are not sufficient for recruitment of Tregs to the autoimmune tissue (210). Various approaches have been in development to increase antigen specific Tregs. One approach involved expansion of antigen specific Tregs *in vitro* using CD8+ splenic dendritic cells presenting islet antigens. Islet-antigen specific Tregs generated using this method had the ability to suppress diabetogenic T cells (231). Antigen specific Tregs can also be induced directly *in vivo*, as was observed in a recent clinical trial that utilized the *in vivo* delivery of beta cell peptide antigens (232). One potential problem that exists with this approach, however, is that some patients have inherent defects in their Treg populations, and thus it may be difficult to increase the number of functional Tregs. To address a potential lack of Treg precursors, one approach is to insert an enhancer before the *FOXP3* coding region in bulk CD4 T cells (233). This approach overcomes epigenetic repression of the *FOXP3* gene and can be used on antigen-specific CD4 conventional T cells (Figure 4). In addition, these edited Tregs express Treg signature genes and have a similar suppressive potential as naturally derived tTregs (234).

Understanding and identifying various subpopulations of Tregs is an important step to improving Treg-based therapies for autoimmune diseases, as the ability to isolate highly functional Tregs would be beneficial in enriching potentially more efficacious Tregs. As an example, TIGIT+ human Tregs positively correlate with stable FOXP3 expression (demethylated TSDR) while CD226+ Tregs are associated with effector cytokine expression and increased TSDR methylation (235). Furthermore, additional Treg subpopulations have been identified, that may increase our understanding of Treg biology and function (236).

Another approach to conferring antigen specificity to Tregs is with engineered TCRs, TCR-fusion constructs (TRuCs), or chimeric antigen receptors (CARs) (Figure 4). As the name suggests, engineered TCR Tregs are Tregs transfected with an antigen-specific TCR, however this approach may not create TCRs with a high enough affinity to be effective in resolving autoimmunity (212). Alternatively, CD4+FOXP3+ T cells can be transduced with a high affinity CAR specific for an autoimmune antigen (216, 217). Current results suggest that CAR Tregs specific for autoimmune antigens can traffic to the correct tissue site and maintain suppressive function (218, 219). TRuCs on the other hand, are tissue-protein specific antibody fragments fused to TCR, allowing for antigen recognition to be combined with natural TCR signaling (214). This approach may be superior to CAR Tregs when there is low density of the antigen available at the tissue site (213, 215).

Discussion

Understanding the genetic elements that lead to loss of regulatory T cell function in autoimmunity requires a foundational understanding of Treg function in a homeostatic environment. Control of Treg lineage and stability often revolve around the transcription factor FOXP3, although FOXP3 activity only accounts for a part of all Treg signature gene expression. Recent evidence has shown that FOXP3 expression and activity is tightly controlled through many different cis- and trans-regulatory factors including enhancer regions, transcription factor complexes, and epigenetic modifications. In turn, these regulatory factors can be influenced by the surrounding tissue environment, allowing for tight control of tolerance in healthy individuals. Thus, ultimate Treg function is a matter of both nature and nurture.

Genetic mutations leading to IPEX syndrome and polygenetic autoimmune susceptibilities revealed through GWAS analyses (9–11) converge on several pathways crucial to Treg stability and function and imply their dysregulation during autoimmunity. The dysregulation can be caused by mutations in *FOXP3* itself, mutations in Treg functional genes, or SNPs that affect regulatory elements such as enhancer regions or genes critical for proper Treg function. In addition, transcription factor complexes that

associate with CNS regions of *FOXP3*, are another component that give Tregs a 'manual' for how they should function in maintaining immune tolerance. However, this so-called manual often becomes distorted or destroyed during pathological autoimmunity, which might be attributed to chronic inflammation present in the tissue environment.

We know that Tregs are poised to interact with their environment and to make functional changes in response to seemingly minute alterations; especially compared to their effector T cell counterparts. The ability for a lymphoid resident Treg to undergo transcriptional reprogramming in order to become a tissue Treg is only one example of such functional changes. Additional evidence can be found in the sensitivity Tregs have to IL-2 in their surrounding environment, and the ability of Tregs to utilize unique metabolites (237–239). The idea that Tregs are influenced by their environment is not novel; however, there is growing appreciation that the environment or so-called 'nurture' can impose permanent changes in Treg nature.

GWAS and other -omics studies point to Treg defects as a partial contribution to autoimmune susceptibility. However, the ultimate trigger that destabilizes the immune system and leads to autoimmunity is hard to define. Do Tregs become dysfunctional due to the tissue environment created during inflammation or autoimmune attack, or are they dysfunctional prior to the initial triggering event? Perhaps, Tregs in autoimmune patients may be poised for dysregulation, but are only partially impaired and progressively lose function in response to specific environmental changes. Perturbations in the environment might provoke a series of downstream events related to epigenetic and transcriptomic changes of Tregs; ultimately leading to a loss of function and self-tolerance.

Author contributions

AR, DA, and MB conceptualized and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Polygenic autoimmune disease risk alleles impacting B cell tolerance act in concert across shared molecular networks in mouse and in humans

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Most B cells produced in the bone marrow have some level of autoreactivity. Despite efforts of central tolerance to eliminate these cells, many escape to periphery, where in healthy individuals, they are rendered functionally non-responsive to restimulation through their antigen receptor *via* a process termed anergy. Broad repertoire autoreactivity may reflect the chances of generating autoreactivity by stochastic use of germline immunoglobulin gene segments or active mechanisms may select autoreactive cells during egress to the naïve peripheral B cell pool. Likewise, it is unclear why in some individuals autoreactive B cell clones become activated and drive pathophysiologic changes in autoimmune diseases. Both of these remain central questions in the study of the immune system(s). In most individuals, autoimmune diseases arise from complex interplay of genetic risk factors and environmental influences. Advances in genome sequencing and increased statistical power from large autoimmune disease cohorts has led to identification of more than 200 autoimmune disease risk loci. It has been observed that autoantibodies are detectable in the serum years to decades prior to the diagnosis of autoimmune disease. Thus, current models hold that genetic defects in the pathways that control autoreactive B cell tolerance set genetic liability thresholds across multiple autoimmune diseases. Despite the fact these seminal concepts were developed in animal (especially murine) models of autoimmune disease, some perceive a disconnect between human risk alleles and those identified in murine models of autoimmune disease. Here, we synthesize the current state of the art in our understanding of human risk alleles in two prototypical autoimmune diseases – systemic lupus erythematosus (SLE) and type 1 diabetes (T1D) along with spontaneous murine disease models. We compare these risk networks to those reported in murine models of these diseases, focusing on pathways relevant to anergy and central tolerance. We highlight some differences between murine and human environmental and genetic

factors that may impact autoimmune disease development and expression and may, in turn, explain some of this discrepancy. Finally, we show that there is substantial overlap between the molecular networks that define these disease states across species. Our synthesis and analysis of the current state of the field are consistent with the idea that the same molecular networks are perturbed in murine and human autoimmune disease. Based on these analyses, we anticipate that murine autoimmune disease models will continue to yield novel insights into how best to diagnose, prognose, prevent and treat human autoimmune diseases.

KEYWORDS

systemic lupus erythematosus (SLE), autoimmune type 1 diabetes mellitus (T1D), polygenic, monogenic, genome-wide association study (GWAS), autoimmune disease mouse model, central and peripheral tolerance (anergy), B cell receptor (BCR) signaling pathway

Introduction: B cell development, autoimmunity and autoimmune pathology

Upwards of 75% of bone marrow produced B cells express B cell antigen receptors (BCRs) that bind self-antigen (1–8). Several mechanisms conspire to remove these autoreactive BCRs from the diverse repertoire needed to provide effective protective humoral immunity without autoimmunity. These mechanisms act both centrally by receptor editing and clonal deletion and peripherally by anergy (7). Central tolerance mechanisms typically remove clones from the wild type repertoire with the most avid interaction with autoantigens. However, peripheral tolerance or anergy is the operative mechanism that silences most autoreactive B cells (3–6). Anergy arises as a consequence of chronic antigen receptor stimulation in the absence of second signals (4, 7, 8). It is defined by non-responsiveness to re-stimulation through the BCR. Importantly, in several B-cell dependent human autoimmune diseases, most individuals with clinically apparent autoimmune disease develop serologically detectable autoantibodies prior to clinical diagnosis (9–13). While we would define B cell dependence as the ability of a B cell depleting therapy to prevent or treat human disease, the inclusion of type 1 diabetes and multiple sclerosis as a B-cell dependent diseases is not universally accepted. However, paired with the clinical efficacy of B-cell targeted therapies either in prevention or treatment of diverse autoimmune pathologies (11, 13–29) these observations implicate dysregulation of central tolerance mechanisms, peripheral tolerance mechanisms or both in the etiopathogenesis of these diseases. Evidence supporting regulatory defects in both central (30–33) and peripheral (31, 32) tolerance mechanisms have been described in numerous human autoimmune pathologies. Central B cell tolerance defects have been described in human SLE (34–36), T1D (37), RA (38, 39) and Sjogren's Syndrome (40). Peripheral B cell tolerance defects have been described in T1D (41), Autoimmune Thyroid Disease (AITD) (42), SLE (43–46), RA (47–49) and anti-neutrophil cytoplasmic antibody (ANCA)–associated vascluitis (AAV) (50). Current immunologic paradigms hold that immune systems have been selected to balance response to pathogens with damage to self (51–53). If this dominant theoretical framework of immunology is correct, the observation that such high levels of autoreactivity are the norm in some ways challenges our teleology of (auto-) immunity. Indeed, this apparent paradox is perhaps not surprising, as our aim is to reduce a complex system that has evolved to specifically, efficiently and flexibly respond to a universe of molecules with a range of approximately quintillion possibilities (54) to a simple and understandable set of rules.

There are obvious (and non-obvious) differences and drawbacks inherent in extrapolating principles to human pathologies from animal model systems (55). Nevertheless, our understanding of the mechanisms that regulate both central (33) and peripheral B cell tolerance (3, 56, 57)as well as the development of autoreactive B-cell dependent autoimmune pathologies (58–61) has been informed by frameworks developed in murine animal models. Indeed, our current models of the etiopathogenesis of human autoimmune pathology largely consist of a consilience of inductions from both observation and experimentation on living humans, model systems comprised of human tissues/cells and study of

murine model systems. However, several have challenged the use of animal models to understand autoimmune pathologies (55). One reason cited for this challenge is that advanced tools for studying human immune responses (62–66) (i.e. CyToF, single cell RNA-sequencing, spectral flow cytometry) now allow more precise definition of human immune responses. Another reason cited for this challenge are high-profile failures in translating findings from animal model of autoimmune disease to humans (67, 68) (some oft cited failures in translation include: oral tolerance with insulin in type 1 diabetes prevention (69), subcutaneous administration of partial agonists to induce antigen-specific T cell tolerance in multiple sclerosis (70–72), the use of interferon gamma (73) and inhibition of TNF-alpha (74, 75) in multiple sclerosis). Importantly, the most often cited high-profile failures in translation have arisen from observations in the EAE (Experimental Autoimmune/Allergic Encephalitis) murine model of multiple sclerosis. Notwithstanding the difference between mice and human beings, challenges in translation are perhaps not surprising, given that clinically defined human phenotypes may well represent congeries of etiopathogenic and pathogenetic mechanisms (76–78). That is, in these diseases each individual actually takes a single path to disease development out of many possible routes. Likewise, each murine model system of autoimmune pathology may well represent a single pathogenetic route to disease development.

Here we synthesize the recent advances in our understanding of the complex genetic basis of two paradigmatic human B-cell dependent autoimmune diseases: Systemic Lupus Erythematosus (SLE) and Type 1 Diabetes Mellitus (T1D). SLE is the prototypical protean multi-system autoimmune disease, whereas type 1 diabetes is the prototypical organ-specific autoimmune disease invariably leading to pancreatic beta-cell destruction. Importantly, both of these disease states have long been modeled with mouse strains that spontaneously develop disease features that closely resemble several of the key phenotypes and pathophysiologies of the human diseases being modeled. Because of the long history of investigation of the cellular and molecular mechanisms of these models, we expect that models of these two diseases are likely to have a more complete list of the genetic contributors and understanding of the relevant cellular and molecular mechanisms leading to murine autoimmune disease.

To address this overlap, we also synthesize what is known regarding the function of putative causal genes across murine models of both systemic autoimmune pathologies (SLE and T1D) and autoreactive B cell tolerance. We discuss several plausible potential explanations for the non-monotonic relationship between currently known human and murine autoimmune risk alleles. Through this analysis, we show that the molecular networks comprised of putative human and murine risk alleles for B-cell dependent autoimmunity and autoimmune pathology substantially overlap. Finally, we propose a framework for steps toward more successful translation of findings from murine model systems to clinical application in humans.

SLE and T1D: Heritability and epidemiology

In humans both SLE and T1D have heritable component with sibling recurrence risk ratios (lambda S) indicating a substantive genetic contribution (Lambda S SLE = 20, Lambda S T1D = 15) (79). Both are incompletely penetrant, with the monozygotic twin concordance rate estimated to be *at most* 40-50% but likely substantially lower for both diseases (79). Thus, for both of these autoimmune pathologies, non-heritable factors also impact disease development. These non-heritable risk factors are often assumed to represent exposure to one or more environmental triggers. Other stochastic events, such as somatic mutation or particular antigen receptor rearrangement towards a pathologic autoantigen could also plausibly contribute. In SLE the non-heritable component has been estimated to account for ~56% of disease risk (80) and in T1D, this has been estimated at ~34% (81).

In terms of epidemiology, SLE is both more prevalent and more severe in several populations of predominately non-European ancestry than in populations with European ancestry (82). A recent cause of death analysis puts these differences in stark contrast (83). Whereas SLE is the 10th leading cause of death in all female persons aged 15-24 in the US, it is the 5th leading cause of death in African American and Hispanic female persons. Similarly, a recent population-based registry reported approximately 30% mortality within 10 years of diagnosis in Black SLE patients, whereas white SLE patients from the same population exhibited approximately 10% mortality. These differences are likely due to a complex mixture of factors. Potential contributions to these disparities likely include systematic population level differences in access to healthcare and possibly also genetic variants that are exclusive to a particular ancestral group (84, 85). However, population level genetic differences explain only 16% of genetic variability in human populations (86). Therefore, systemic population level differences in access to care may have a greater impact on outcome differences in SLE. A recent report estimates that SLE occurs in US male persons at a rate of 8 to 53 per 100 000 and US female persons at a rate of 84 to 270 per 100 000, depending on the population (87). Importantly, SLE exhibits sexual dimorphism, occurring more commonly in female persons at rate of 9:1 (87). A caveat to the studies referenced above is that they rely on medical record abstraction and administrative data analysis methods that by their nature preclude obtaining sex, gender, race and ethnicity self-identification.

In terms of epidemiology, T1D is reported to be more prevalent in persons who self-identify as non-Hispanic white, followed by non-Hispanic black, Hispanic and other racial/

ethnic identities (0.35 to 2.55 per 1 000) with approximately equal prevalence in boys and girls in the US (1.93 per 1 000) (88). T1D incidence increases with age, peaking between 10-14 years of age. Notably, cases with onset < six months of age are not entirely uncommon (89). However, for reasons that remain incompletely clear, the overall incidence of T1D is increasing according to several studies performed in the US (90–92). As a result, based on anticipated demographic shifts, the prevalence is projected to increase from 2.13 per 1 000 in 2010 to 5.20 per 1 000 by 2050 (88). Increasing incidence in recent decades is not unique to type 1 diabetes amongst other autoimmune diseases (93).

When taken together with the observations that different geographies have different rates of autoimmune diseases (94) and autoimmunity (at least the rate of antinuclear antibody seropositivity) has also increased over the same time course (95), these data have been interpreted to strongly imply a changing autoimmunity/autoimmune disease risk environmental exposure has change in recent decades, as the kinetics seem too fast for a genetic explanation.

Several environmental factors have been associated with SLE, including smoking, silica exposure, exogenous sex hormones and infection, especially prior Epstein-Barr virus infection (96, 97). Similarly, in T1D, microbiome, micronutrient, diet, early life metabolism and immune stimuli (infection and vaccination) have been implicated with risk for incident disease (98).

In sum, both SLE and T1D in humans are complex diseases where both genetic and environmental factors contribute both to disease development and disease manifestations.

Nosology and classification – *Autoimmune* T1D and the heterogeneity of SLE

Both SLE and autoimmune type 1 diabetes pose practical challenges in disease definition, diagnosis and classification that should be considered when evaluating the utility and applicability of any disease model. One cannot evaluate whether a model recapitulates human disease pathogenesis if the definition of disease is unclear.

The particular nomenclature of autoimmune type 1 diabetes may strike the reader as oddly redundant, but it makes the point that type 1 diabetes is a clinical diagnosis. This diagnosis is made in part through typical seropositive autoimmunity to several pancreatic islet expressed proteins (insulin, ZnT8, IA-2, GAD65) (9) in the setting of insulin deficiency. This clinical scenario has been alternately referred to as type 1a diabetes or as immune-mediated type 1 diabetes (99–101). However, a small proportion of individuals clinically diagnosed with type 1 diabetes in large cohort studies have been found to have an alternative etiology for their disease that is non-autoimmune. These individuals commonly have either childhood onset monogenic type 2 diabetes (102) or fulminant onset diabetes with non-autoimmune beta-cell destruction. This latter category of disease has been alternatively referred to as type 1b diabetes, idiopathic type 1 diabetes or nonautoimmune diabetes plus IS (Insulin Sensitivity) (99–101). In some type 1 diabetes cohorts this proportion may be as high as 10% (103). Prior decades of careful phenotyping and molecular characterization has led to description of several subphenotypes of what would have previously considered either type 1 diabetes (young onset, insulin sensitive and autoimmune) or type 2 diabetes (later onset, insulin resistant non-autoimmune). These include latent autoimmune diabetes of adults (LADA), type 1.5 diabetes, ketosis-prone type 2 diabetes and maturity-onset diabetes of the young. See (104) for an excellent review of the nosological challenges of clinical diabetes classification. Our distinction in nomenclature seeks to differentiate monogenic causes of clinical type 1 diabetes with pathologic autoimmunity from monogenic causes of diabetes that clinically resemble autoimmune type 1 diabetes, but arise from non-autoimmune causes. This distinction is clinically important, as management is substantially different (insulin replacement vs. sulfonylureas and other therapies) (105). Indeed, cohorts clinically diagnosed and treated as type 1 diabetics with potential alternative etiologic explanations have been described (106). There is a growing body of literature that using polygenic risk scores (106) and/or sequencing panels of non-autoimmune monogenic risk alleles can help distinguish these two phenotypes. This approach may even be cost effective in select situations (107). Further highlighting the potential for case misclassification in type 1 diabetes cohorts, several recent studies applied type 1 diabetes polygenic risk scores (PRS) to define individuals with clinical type 1 diabetes with low genetic risk (108–110). As expected, these analyses identified rare T1D risk variants in or near genes with well-known effects on immune responses. In addition, these studies identified several rare risk variants in genes with metabolic function or impacts on obesity and no known function in immune responses. Taken together, they suggest that many of the type 1 diabetes cohorts used for GWAS studies likely include a mixture of individuals with autoimmune type 1 diabetes (T1aD) and individuals with non-autoimmune type 1 diabetes (T1bD).

By the same token, SLE is a clinical diagnosis. In order to develop homogeneous patient populations for clinical studies, several iterations of classification criteria have been developed (111–115). The most recent revision was published in 2019 (115). However, most studies of SLE in the past two decades defined SLE cases according to the 1997 revised classification criteria (113). It has been observed that the 1997 criteria lead to 330 possible combinations of clinical manifestations that could satisfy SLE classification (76). Thus, despite being unified by anti-nucleic acid/anti-nucleoprotein autoimmunity (116), human SLE remains a clinically heterogenous disease state.

Since particular patients differ in which features of SLE they manifest, attention must be paid to which features of human SLE a particular murine model recapitulates.

Genetic structure: The usual structure of human autoimmune diseases is polygenic

It is becoming increasingly clear that in most humans who develop autoimmune disease, disease most commonly arises from a complex interplay between many polygenic risk factors and one or more environmental triggers (79). Decreased cost of genotyping and the increasing size of autoimmune disease genetic cohorts has led to a seemingly ever-increasing list of disease risk loci. Indeed, for several common autoimmune diseases, the number of risk genetic loci across the genome now exceeds 200 (117). Each of these loci makes at most a modest contribution to relative risk of disease (odd ratio < 1.2) (117) and most are favored to act by regulating target causal genes (118–120). Together these risk alleles are thought to set a liability threshold that allows the development of autoimmune pathology in certain circumstances. These rules for human autoimmune pathologies appear to generally apply in the case of SLE and T1D with some subtle differences (caveats)?. One notable difference is that of association genetic association with the Major Histocompatibility Complex (MHC)/Human Leukocyte Antigen(HLA) Locus. In T1D, specific HLA alleles are associated with disease. Together, three amino acid variants account for nearly 30% of the phenotypic variance in T1D in European ancestry populations (121). This is similar to the case in RA, where specific HLA alleles have been shown to facilitate binding and presentation of the classic RA autoantigen, citrullinated peptides (122). In SLE, on the other hand, the major contribution to genetic association with the MHC/HLA locus has been mapped to Complement component 4 (*C4A* & *C4B*) gene copy number (123). Both *C4A* and *C4B* are genes that lie within the SLE association interval within the MHC/HLA locus. It has been shown that, in contrast to RA and T1D, the contribution of amino acid sequence variants to the SLE association at the MHC/HLA locus is minimal. HLA is not uninvolved in SLE etiopathogenesis, as there are additional contributions to SLE risk at this complex genetic locus that are attributable to regulation of MHC class II expression (123). However, the bulk of the risk from HLA in SLE arises from regulation of the complement system and not specific MHC alleles (123).

In terms of genetic structure, SLE is most commonly polygenic (117), but numerous monogenic forms of SLE have been described, 51 of which we are aware (124–196). Monogenic SLE presents more commonly with childhood onset and a severe disease phenotype (117, 124–126). It appears that in addition a minority of childhood onset cases, currently estimated at approximately 15% exhibit a probable mix of monogenic and polygenic genetic etiologies (197, 198). Ongoing studies suggest that rare or private mutations also partially contribute to risk in multipatient SLE pedigrees. However, the extent to which such mutations contribute to SLE risk is still being defined (199). To synthesize what is known about polygenic causes of SLE, we applied a previously described approach to published SLE risk variants in the NHGRI-EBI GWAS catalog (117). First, we grouped SLE risk variants listed in the GWAS catalog (200) into loci/regions, then integrated published results the from Open Targets Genetics (201) Locus to Gene (L2G) (202) algorithm. L2G is a machine learning pipeline that predicts a causal gene by integrating several sources of evidence. These sources include distance from causal credible set variants to gene, molecular QTL co-localisation, chromatin interaction data and where applicable variant pathogenicity prediction from the variant effect predictor algorithm. This evidence is then weighted by gold-standard functionally demonstrated causal variants from different GWAS studies. For loci where L2G was able to be confidently annotate a likely causal gene, that gene was included in the molecular network. This list is not comprehensive. Our approach to region definition obscures several known regions with multiple independent genetic effects. Despite this, we find 182 polygenic human SLE risk loci. By applying the L2G automated machine learning pipeline and manual annotation our final list includes 109 loci with assignable putative causal genes within these loci (Supplementary Table 2A).

In contradistinction to SLE, only very few (8 of which we are aware – Supplementary Table 1B) monogenic causes of autoimmune type 1 diabetes have been described (203–213). Monogenic autoimmune T1D arises in genetic syndromes of polyendocrinopathy. These autoimmune diseases are characterized by autoimmunity that adversely impacts multiple endocrine organs, not merely the pancreas. Only eight monogenic routes to autoimmune diabetes have been described provides a contrast to SLE. This may be in part due to the diffuse, systemic nature of SLE versus the more narrow target organ range of T1D. While SLE exhibits considerable clinical and phenotypic heterogeneity (214) that is unified around anti-nucleic acid/anti-nucleoprotein autoimmunity (116), type 1 diabetes leads to autoimmune pancreatic beta cell destruction. So, it may merely be that in this case there are more opportunities to develop an immune dysregulation syndrome resembling one or more features of SLE, as the manifestations of SLE are both numerous and diverse.

In individuals with T1D, the disease more commonly arises from the aggregate effects of polygenic risk alleles, just as with SLE. Indeed, in the comprehensive review of monogenic autoimmune type 1 diabetes to date reflects the experience of approximately 500 individuals worldwide (203). Thus, monogenic genetic effects or rare genetic effects of large effect

size do not likely explain a significant proportion of type 1 diabetes patients and this also appears to be the case in several autoimmune diseases (215). To explore this risk gene network we applied the same approach to define a high confidence causal polygenic risk gene network in human type 1 diabetes. This analysis of type 1 diabetes risk loci from the GWAS catalog yields a list of 131 polygenic human T1D risk loci. The L2G algorithm was able to confidently identify 63 putative causal genes within these loci (Supplementary Table 2B). Again, our approach likely obscures the presence of multiple independent signals in a particular region. A recent GWAS meta-analysis of T1D reported that 33% of the independent association signals occurred in loci with multiple independent association signals within the same locus. These independent signals within the same locus might exert their biological effects on disease risk through the same gene. Alternately, these multiple independent signals might exert their biological effects on disease risk through multiple independent genes.

IL2RA stands out as an algorithmically defined putative causal genes that is also present in the list of monogenic autoimmune type 1 diabetes genes (Supplementary Table 1B) as has been observed by others (216). Like SLE (Figure 1), the monogenic and polygenic type 1 diabetes risk networks overlap at this hub node (Figure 2). This suggests that these hub nodes may be particularly attractive as targets that span disease states based on their central location in both monogenic and polygenic disease molecular networks. In sum, the overlap between polygenic and monogenic disease genetic networks in both human autoimmune Type 1 Diabetes and SLE indicates that the monogenic forms of these diseases perturb the same diseases networks as polygenic disease.

Beyond polygenic genetic structure: Human autoimmune disease and the omnigenic model

A few general points concerning polygenic genetic structure should be considered. One objection that has been raised to polygenic structure in complex human disease is that sporadic cases are common. Sporadic refers to cases without a known family history of disease. However, statistical genetic models predict that sporadic cases of complex genetic disease will commonly occur even in disease with a polygenic genetic structure (217). Second, the bulk of polygenic risk alleles reported to date in common autoimmune disease only have small effects. In human SLE, as an example, only a handful of common genetic risk factors (four that we know of) impact disease *relative* risk from 2-10-fold (117). Applying knowledge of population prevalence, the genetic factor with the largest effect would change the absolute risk of SLE from approximately 0.1% to 0.4% (117). This kind of polygenic genetic architecture is present in many human phenotypes. This observation prompted the proposal of the Omnigenic model of complex traits (218). In this model, larger effect size variants (>1.1-fold increase in relative risk) operate within core disease pathways. However, thousands of loci with infinitesimally small effect size spanning the entire genome change absolute genetic liability (218). In this

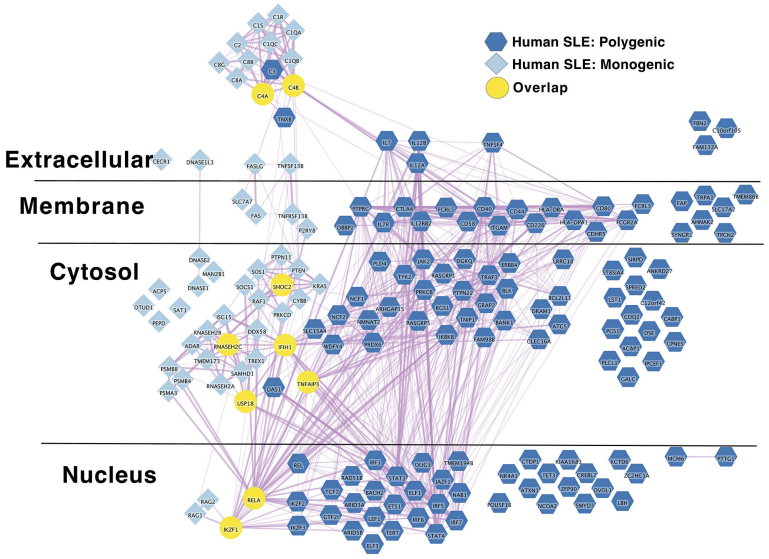

FIGURE 1
Monogenic and Polygenic human SLE risk gene networks overlap at hub genes. Light blue diamond – Monogenic human SLE genes; dark blue hexagon – Polygenic human SLE genes; Yellow circles – overlapping genes. Downloadable/Interactive network diagram can be found at: https://doi.org/10.18119/N9231T.

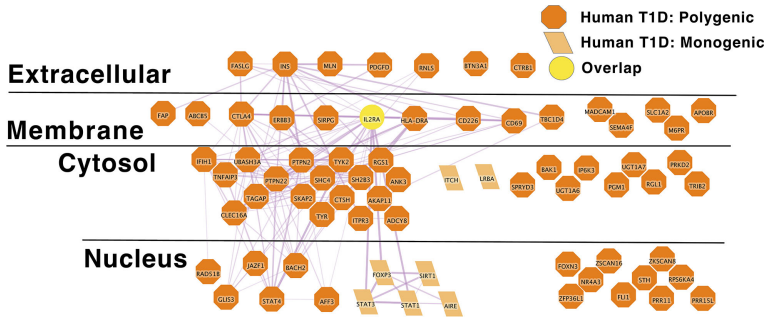

FIGURE 2
IL2RA is the link between Monogenic and Polygenic human type 1 diabetes risk gene networks; light orange parallelogram– human monogenic autoimmune type 1 diabetes gene; dark orange octagon– human polygenic autoimmune type 1 diabetes gene; Yellow circles – overlapping genes.Downloadable/Interactive network diagram can be found at: https://doi.org/10.18119/N94W34.

model, the entire genome is ultimately involved in disease risk, with each variation outside of the core disease pathway adding a very tiny amount of residual risk. In simple terms, it seems perhaps tautological to state that the whole genome is involved in any given trait, even if only slightly changing the trait. It is worth noting that predictions of this model appear to hold in other complex human genetic traits, such as height (219).

As an aside, the omnigenic model provides a potential explanation for why autoimmune disease genes have not been eliminated *via* natural selection. If most of the hundreds of core risk alleles are inherited independently (low correlation or linkage disequilibrium) and they each have a small effect, then selective pressure would not be expected to be strong in individuals with polygenic autoimmune disease. By way of analogy, being related to someone who wins the lottery does not make winning the lottery more likely for you, unless you buy more lottery tickets. On the other hand, many monogenic disease genes represent either *de novo* mutations or recent founder effects. Therefore, monogenic mutations have not had a very long to be subject to natural selection. These observations when combined with theoretical frameworks describing the balance between host collateral damage from immune responses and microbe clearance (51–53) may also explain the retention of these alleles in the wider gene pool. That is, there are several ways in which immune responses can be balanced to avoid damage to host. Genetic variation that modulates an immune response that is too weak or too strong for one context, may, in another context or in another generation better strike that balance.

If the omnigenic model is correct and thousands of risk loci are involved in determination of common polygenic traits, then sample sizes of > 1 000 000 affected individuals may be needed to develop risk scores that capture enough variants to explain the majority of variation in genetic risk (220). For most autoimmune diseases, these samples exceed the total number of affected individuals living on entire continents. If true, it would make systematically dissecting genetic network interaction with environmental disease triggers so complicated as to be potentially intractable. Our aim is to deconstruct disease processes, in order to improve our ability to diagnose, prognose, prevent and treat autoimmune diseases. Therefore, we must reduce the complexity of the systems we aim to deconstruct. In this way, we can build conceptual models of autoimmune disease development and maintenance that we can actually comprehend.

One approach is murine models. Such models may strike an appropriate balance between over-simplification and a sufficient degree of biological complexity such that core disease relevant cellular and molecular networks are conserved. Thus, findings can be expected to translate to humans. When proper controls and careful attention to potential confounders is observed, mouse models of disease have been very powerful in advancing our understanding of autoimmune pathologies (59).

Even the lousiest models of autoimmune disease would predict success if considered in context

Having an intermediate model of sufficient biological complexity is likely necessary for many types of causal evidence that allow inference regarding mechanism in cellular and molecular disease networks. In many cases this kind of inference cannot be achieved for either ethical or technical reasons in humans and are inadequately modeled *in vitro*. Many therapies that are promising *in vitro* do not stand up to testing in the more complex biological system that a whole organism *in vivo* represents. One recent example of relevance to autoimmune disease is that of hydroxychloroquine (a mainstay of SLE and Rheumatoid Arthritis therapy (221)) in the treatment of COVID-19. Indeed, hydroxychloroquine robustly inhibited SARS-CoV-2 (and other coronaviruses) *in vitro* (222), but was

shown to be ineffective in prevention of SARS-CoV-2 infection and treatment of COVID-19 in randomized controlled trials in humans (223–226). While it is a moot point now that the high-quality human data exist, an intermediate *in vivo* model system may have been able to predict and understand this therapeutic failure and thereby reprioritized COVID-19 patients for more suitable trials.

Several criticisms of mouse models of human autoimmune pathologies specifically and human disease writ large (with the use of SOD1-deficient mice in Amyotrophic Lateral Sclerosis representing a high-profile model with several issues of phenotypic non-correspondence) have been raised [notably (55, 67, 227, 228)]. See section 5 for our attempt at a comprehensive list of some key variables to consider in modeling human autoimmune disease in mice.

One major criticism that has been raised for why mouse models of human autoimmune disease are 'lousy' is failures in translation from experimental autoimmune/allergic encephalomyelitis into successful therapy for multiple sclerosis. However, we would submit that careful attention to both the details of the murine and human pathology and careful reexamination of models in light of the clinical, phenotypic, cellular and molecular features of the human diseases we seek to model would have predicted successful therapeutic targets even in this 'lousiest' of autoimmune disease models.

Failed trials of TNF-alpha inhibitors as well as oral and IV tolerance autoantigen-specific tolerance protocols that succeeded in mice, but failed in MS patients are often cited. Incidentally, TNF-alpha inhibition did not merely fail, but was subsequently discovered to be a risk factor for incident demyelination, just as it is a cause of drug-induced lupus. It is worth noting that despite many high-profile therapeutic failures, reassessment of successes, failures and refinement of models have led to several successful novel therapeutic approaches for MS treatment in the interim (68). Subsequently, phenomenally successful trials of B cell-depleting monoclonal antibodies directed against CD20 were performed in MS. In fact, B cells are so important in this autoimmune disease, that B cell depletion using anti-CD20 monoclonal antibodies is now the mainstay of therapy. This is not necessarily a conclusion that would have been reached by solely relying on data from the EAE model (229–234), even though careful experimentation ultimately revealed an important contributory role for B cells once early studies demonstrated the efficacy of anti-CD20 therapies in human MS (235). Subsequent work by many groups has demonstrated that antigen presenting B cells play a central role in the pathogenesis of human MS (236). Building on the principle of the oral tolerance studies in MS, re-enforcing tolerance in formerly anergic B cells remains an active area of investigation (237). More recent data has further advanced our understanding of the role of B cells in MS, as prior Epstein-Barr virus infection (but not other common latent viral infections) was shown to be an independent risk factor for MS development (238), leading commenters to infer that "These findings provide compelling data that implicate EBV as the trigger for the development of MS" (239). These data led to pan-proteome analysis of the auto-specificities of the pathognomonic oligoclonal bands found in the CSF of MS patients. Crossreactivity was shown between a human CNS autoantigen, GlialCAM and the EB viral latency transcription factor EBNA-1 (240). Indeed, as a final attempt to prove etiopathogenesis of EBV in MS – using a modified version of Koch's postulates, the authors of the latter paper immunized EAE mice and concluded that "EBNA1 immunization aggravates EAE". In doing so, they have nominated yet another potential therapeutic approach for MS that relies, in part, on the EAE model, the prevention of EB virus infection. In retrospect, the story of the EAE model seems to us more like the typical pattern of advances in science where models are challenged by data and refined so that the model predictions better fit the observed data. Indeed, it now appears that the use of proper controls, challenging murine models with ideas from human data and *vice versa* has an aggregate effect of reducing the influence of potential confounders. In so doing this approach would be expected to lead to a more accurate model autoimmune etiopathogeneis than either approach would have been able to do on its own (60). (many important potential variables are detailed in section 5.)

Thus, despite oft being cited as a model of autoimmune disease with high profile failures in translation, careful attention to the human processes being modeled by the EAE model continues to yield insight into MS pathology. In a similar manner, we expect that careful attention to potential confounders of lupus and T1D models, the use of multiple models and iterative comparison to intermediate human disease phenotypes would be expected to yield important insight into these human autoimmune pathologies.

Gene networks for murine autoimmune type 1 diabetes, lupus, central and peripheral B cell tolerance overlap

To better understand the relationship between human autoimmune pathology and murine models of autoimmune disease, we compared their respective gene networks. We have focused on making our comparison in long-standing murine disease models of two human autoimmune diseases that are fairly-well characterized in terms of correspondence across spontaneous disease models. For models of both diseases, excellent reviews of the convergent and divergent immunopathogenic bases for disease development between mice and humans have been written and we refer the interested reader to read them: [murine lupus (60, 61, 241, 242): murine type 1 diabetes (243, 244)].

The prevailing model of autoimmune disease risk is that the genetic networks regulating lymphocyte tolerance are core to autoimmune disease and span multiple autoimmunities (56, 57, 245, 246). That is, human genetic risk alleles shared across multiple autoimmune diseases perturb the normal function of lymphocyte self-tolerance networks. To begin both to evaluate this model more systematically and to more fully understand the differences between the murine and human autoimmune disease genetic risk networks, we reviewed the literature and collected lists of putative causal genes in murine models of SLE and type 1 diabetes, as well as genes whose disruption lead to B cell central or peripheral tolerance defects (247–450). Together, each of these sets of genes comprise a molecular network and many of the genes in each network overlap with those in the other networks (Figure 3). Taken together, these data point towards an important role of B cell central and peripheral tolerance regulatory networks in murine models of type 1 diabetes and SLE.

Risk gene networks for murine autoimmune type 1 diabetes, lupus, central and peripheral B cell tolerance overlap with risk gene networks for human SLE and autoimmune type 1 diabetes

To understand how autoimmune disease gene networks overlap, we merged the murine and human risk gene networks for SLE and T1D in several ways. Our goal was to evaluate whether the published studies support the prevailing model – that the genes regulating tolerance induction and escape of autoreactive B cells are central to the risk gene network of these seropositive autoimmune diseases. First, we combined risk genes from monogenic human SLE (Supplemental Table 1A), polygenic human SLE (Supplemental Table 2A) and murine Lupus genes (Supplemental Table 3) into a single network (Figure 4). Second, we combined gene from monogenic human T1D (Supplemental Table 1B), polygenic human T1D (Supplemental Table 2B) and murine autoimmune diabetes (Supplemental Table 3) genes into a single network (Figure 5). Finally, we combined both of the disease-specific networks (from Figures 4, 5) along with both B cell central (Supplemental Table 3) and peripheral tolerance (Supplemental Table 3) gene networks into a single network (Figure 6). Strikingly each of these gene sets formed a distinct protein-protein interaction network with greater overlap than expected by chance (Table 1). Further, the human monogenic and polygenic and murine genetic networks overlap 16-fold to 63-fold more than would be expected by chance (Table 2). Likewise, these networks overlap with one another or the overall B cell tolerance and murine disease networks between 15-fold and 86-fold more often than expected by chance (Table 3).

Overall, this analysis reveals a densely interconnected core autoimmunity gene network centered around genes that regulate B cell peripheral tolerance. This observation provides some degree of support for the prevailing model in the field, that the genes regulating tolerance induction and escape of autoreactive B cells are central to the risk gene network of these seropositive autoimmune diseases. Intermixed within this core are the murine type 1 diabetes and lupus gene networks. While this approach has utility in providing a high-level overview of autoimmune disease risk regulatory networks, it does have

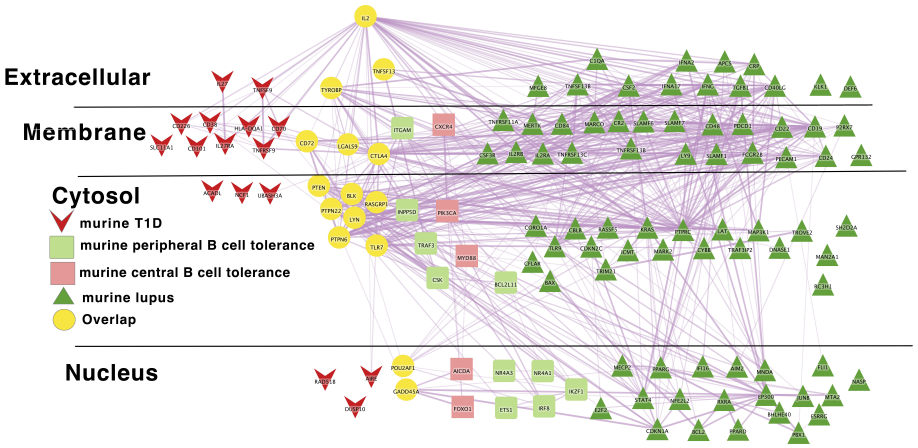

FIGURE 3
Murine autoimmune diabetes and lupus networks are densely connected to peripheral autoreactive B cell tolerance networks; dark green triangle – murine lupus gene; light green rounded rectangle – murine peripheral B cell tolerance gene; Yellow circles – overlapping genes. Downloadable/Interactive network diagram can be found at: https://doi.org/10.18119/N9161J.

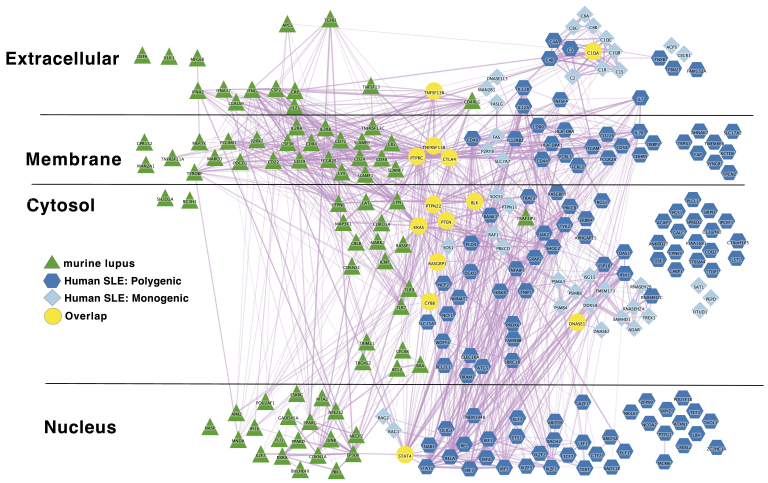

FIGURE 4
Murine Lupus risk genes connect to Polygenic Human SLE risk genes at the periphery of the core network in a manner similar to the monogenic risk SLE network; Light blue diamond – Monogenic human SLE gene; dark blue hexagon – Polygenic human SLE gene; dark green triangle – murine lupus gene; yellow circles – Overlapping genes. Downloadable/Interactive network diagram can be found at: https://doi.org/10.18119/N9WC8P.

some drawbacks. In each particular network, there are several putative causal genes that are not well connected to the central network. Certainly, it is possible that these genes have yet to be discovered function in the genesis of autoimmunity. However, there are other potential explanations for lack of connection to this central network. In some cases, these may represent misattribution of causality. For example, while the L2G algorithm nominated *PTTG1* as a putative causal gene for SLE, we have previously shown that altered function of the microRNA, *MIR146A*, likely better explains the observed association with SLE at this locus (454). Alternately, these genes may impact lupus function in a way that has not yet been represented in the molecular networks of the STRING database. For example, recent work has established *DNASE1L3*

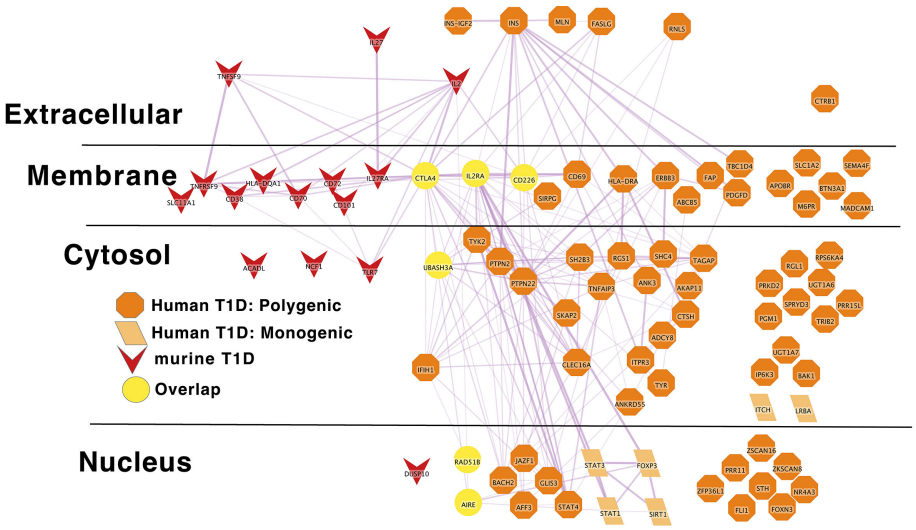

FIGURE 5
Murine autoimmune diabetes risk genes connect to Polygenic Human T1D risk genes at the periphery of the core network in a manner similar to the monogenic risk T1D network dark red inverted triangle – murine autoimmune type 1 diabetes gene; light orange parallelogram– human monogenic autoimmune type 1 diabetes gene; dark orange octagon– human polygenic autoimmune type 1 diabetes gene. Downloadable/Interactive network diagram can be found at: https://doi.org/10.18119/N9RP6S.

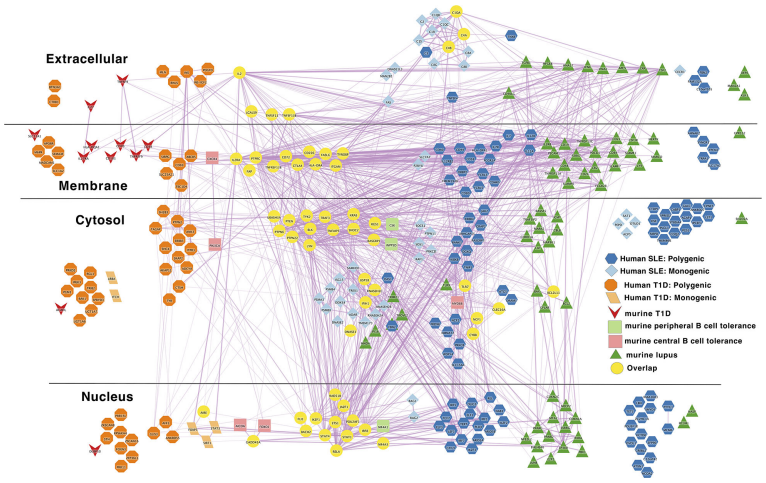

FIGURE 6
Murine autoimmune disease model genes center around the autoreactive B cell peripheral tolerance network in the middle of combined human autoimmune disease polygenic risk networks. Light blue diamond – Monogenic human SLE gene; dark blue hexagon – Polygenic human SLE gene; dark green triangle – murine lupus gene; light green rounded rectangle – murine peripheral B cell tolerance gene; light red rectangle – murine central B cell tolerance gene; dark red inverted triangle – murine autoimmune type 1 diabetes gene; light orange parallelogram– human monogenic autoimmune type 1 diabetes gene; dark orange octagon– human polygenic autoimmune type 1 diabetes gene; yellow circles – Overlapping genes. Downloadable/Interactive network diagram can be found at: https://doi.org/10.18119/N9MW3G.

as casual for SLE. First, non-synonymous coding changes in *DNASE1L3* explain the bulk of the genetic association with SLE near the *PXK* locus (455). Second, germline mutations in this gene have been described as a monogenic route to lupus (198, 456–458). Third, titers of autoantibodies against this enzyme correlate with disease flare in patients with lupus nephritis (459). Fourth, functional studies implicate the function of this secreted, extracellular DNAse in digesting the nucleic acids present in autoantigenic debris from dying cells (460–462). Thus, while the role of *DNASE1L3* in SLE risk is becoming abundantly clear, the STRING database (451) has not yet codified this new understanding. At the same time, there may be other information missing from the gene network as we have defined it. At this same locus, *DNASE1L3-PXK*, an additional contribution to genetic association with SLE is seen (455). This additional association is due to variation near *PXK*, a phox-homology kinase implicated in B-cell receptor endocytosis (463). There is evidence for a potential role of PXK in modulating B-cell receptor signaling and generating autoreactivity. However, the automated algorithmic approach that we used did not place

TABLE 1 Network characteristics.

Network	#nodes[a]	#edges[b]	degree[c]	clustering[d]	exp. Edges[e]	P[f]
Monogenic SLE	54	169	6	0.65	33	1.0E-16
Polygenic SLE	127	497	8	0.44	107	1.0E-16
Monogenic T1D	8	12	3	0.64	3	2.8E-05
Polygenic T1D	70	140	4	0.37	22	1.0E-16
murine lupus	92	523	11	0.58	111	1.0E-16
murine T1D	20	31	3	0.58	3	1.0E-16
peripheral tolerance[g]	22	63	6	0.58	8	1.0E-16
central tolerance[g]	7	7	2	0.24	1	6.7E-04

Network characteristics for each string protein-protein interaction network reveals a highly connected disease network in each gene list.
[a]#nodes indicates the number of genes in the network. [b]#edges indicates the number of pairwise predicted protein-protein interactions according to the default settings in the string database (http://www.string-db.org) (451). [c]Degree indicates average node degree. Per the string database manual: "The average node degree is a number of how many interactions (at the score threshold) that a protein have on the average in the network". [d]Clustering indicates the average clustering coefficient. Per the string database manual: "The clustering coefficient is a measure of how connected the nodes in the network are. Highly connected networks have high values". [e]Exp. Edges indicates "The expected number of edges gives how many edges is to be expected if the nodes were to be selected at random.". [f]P indicates the P value for enrichment of this protein-protein interaction network. "A small PPI enrichment p-value indicate that the nodes are not random and that the observed number of edges is significant." Note: the minimum enrichment p-value reported by string is 1E-16.[g]peripheral tolerance and central tolerance indicate networks of genes implicated in peripheral and central B cell tolerance.

TABLE 2 Disease Network Overlap.

	Disease	Exp. Overlaps[a]	Fold O-R[b]	P[c]
Human Polygenic: Monogenic Overlap	SLE	0.35	26	6.8E-11
	T1D	0.03	35	2.8E-02
Combined Human: Murine Overlap	SLE	0.82	16	1.6E-12
	T1D	0.08	63	1.2E-08

Overlap of disease networks supporting Figures 1, 2 (Human Polygenic: Monogenic Overlap) and Figures 4, 5 (Combined Human: Murine Overlap). [a]Exp. Overlaps indicate the number of expected overlapping nodes. Assuming similar length lists were randomly selected from the genome (unassociated). [b]Fold O-R indicates the fold over-representation compared to expectation. [c]P indicates p-value for hypergeometric distribution assuming independence of the two networks.

PXK within the polygenic SLE risk network. While this approach provides a useful overview of the interrelationships between gene networks, by its nature, it also provides an incomplete picture of disease risk due to incomplete information.

On a more granular level, these analyses revealed overlapping networks between monogenic and polygenic SLE. This overlap was between complement, cytosolic nucleic acid sensors, Ikaros and NF-kB pathways (Figure 1). In terms of monogenic and polygenic autoimmune type 1 diabetes, not surprisingly, there is limited overlap (Figure 2). However, there is still more than expected by chance. This includes a preponderance of key transcriptional regulators (*STAT1*, *STAT3*, *FOXP3*, *AIRE*) that are central regulators of T lymphocyte development in monogenic T1D. Close inspection of these networks shows that they do not overlap at *AIRE*. This lack of overlap highlights one of the drawbacks of the automated, algorithmic approach to putative causal gene definition. A rare variation in *AIRE*, rs74203920, was recently reported in a large GWAS of human autoimmune type 1 diabetes (464). This non-synonymous variation results in an amino acid change that is predicted to be deleterious. It has a minor allele frequency of ~2% in individuals with European continental ancestry in the 1000 Genomes project. Further, using Bayesian statistical approaches, the authors report a posterior probability of association > 99% (464). There are examples of non-synonymous coding changes in GWAS genes whose biological effects on disease risk may be through modulation of gene expression (465). However, it seems most parsimonious to conclude that *AIRE* is, in fact, the likely causal gene at this T1D risk locus. That our approach using L2G did not identify this particular variant and it therefore did not overlap with the monogenic T1D risk network highlights one of the drawbacks of this approach in terms of misattrubtion. It further suggests that our overlaps are more likely to represent a lower bound on the overlap between the true disease risk networks than an upper bound.

Turning to the network that combnines murine lupus, murine T1D and murine B cell tolerance gene networks, we

TABLE 3 Overlaps of disease networks supporting Figures 3 (Murine T1D, Lupus, Peripheral and Central tolerance) and Figure 6 (all 8 networks combined).

Network	Overlaps in Figure 3			Overlaps in Figure 6		
	Exp. Overlaps[a]	Fold O-R[b]	P[c]	Exp. Overlaps[a]	Fold O-R[b]	P[c]
Monogenic SLE	X	X	X	0.92	18	1.5E-17
Polygenic SLE	X	X	X	2.17	15	1.5E-28
Monogenic T1D	X	X	X	0.14	22	2.6E-04
Polygenic T1D	X	X	X	1.19	16	4.3E-18
murine lupus	0.58	26	1.9E-17	1.57	16	1.9E-23
murine T1D	0.13	32	6.8E-06	0.34	26	1.6E-11
peripheral[d]	0.14	86	1.5E-21	0.38	51	2.2E-31
central[d]	0.04	45	8.2E-04	0.12	17	5.8E-03

Overlap of disease networks supporting Figures 3 (Murine T1D, Lupus, Peripheral and Central tolerance) and Figure 6 (all 8 networks combined). [a]Exp. Overlaps indicate the number of expected overlapping nodes. Assuming similar length lists were randomly selected from the genome (unassociated). [b]Fold O-R indicates the fold over-representation compared to expectation. [c]P indicates p-value for hypergeometric distribution assuming independence of the two networks. [d]peripheral and central indicate networks of genes implicated in peripheral and central B cell tolerance. As a negative control, comparison was made to the L2G predicted causal genes in a large GWAS of osteoarthritis (452) and type 2 diabetes (453). In both cases, overlap was substantially less than in the table above. A single putative causal gene out of 19 for osteoarthritis overlapped with the network in Figure 6. This corresponds to 3-fold overrepresentation with P-value of 0.27. 17 putative causal gene out of 343 for type 2 diabetes overlapped with the network in Figure 6. This corresponds to 2.9-fold overrepresentation with P-value of 9E-5. Of note, the overlapping genes were enriched for genes within apoptosis and cellular proliferation pathways. As these core cellular processes impact both the genesis of autoimmune pathology and insulin resistance, this degree of overlap is perhaps not surprising.
OA network: https://version-11-5.string-db.org/cgi/network?networkId=bWV0Pd2gEYYx.
DM2 network: https://version-11-5.string-db.org/cgi/network?networkId=boNoFGYSyFUn.

also find substantial overlap. This overlap occurs within several pathways: IL2 (IL2), BCR signaling (BLK, Lyn etc.), tolerance response to nucleic acid (CD72, TLR7), tolerance to self-nucleic acid and control of viral infection. These overlaps serve as unifying pathways in these models of autoimmune pathology (Figure 3). Overlap of murine and human lupus occurs at B-cell signaling hubs involving BAFF, APRIL and B cell antigen receptor signaling. Of note, despite its central importance in SLE etiopathogenesis (117), TLR7 is absent from the human disease networks, though its signaling intermediates remain. Likewise, LYN is absent from the human disease networks despite its identification as a likely causal gene for SLE in GWAS follow-up studies. (Figure 4) Thus, our analysis likely underestimates the true extent of overlap between these various gene networks. Similar to Lupus, type 1 diabetes in mouse and humans is unified by T-cell tolerance regulators (CTLA4, IL2RA, CD226, AIRE, etc.) (Figure 5). Finally, peripheral B cell tolerance is the most over-represented compared to no association when looking at the unified network of all these states of pathologic autoimmunity (Figure 6). The substantial overlap between these different networks is consistent with a prominent role of particular environmental drivers in specifying the target organ focus of autoimmunity.

One question that arises is whether these associations represent an increase over what would be expected by chance. Indeed, overlap between the gene networks in type 2 diabetes (453) and osteoarthritis (452) are much less with these non-autoimmune traits than any of the autoimmune pathology networks (Table 3). Another question is how to address cell type specificity of these networks. One might assume that these gene networks only operate in concert within specific cell types. PTPN22 may serve as a counterexample to this – a recent review highlighted evidence for six independent mechanisms of the PTPN22R620W variant each operating in different cellular lineages (466). It may be that some autoimmune disease risk alleles do act in a cell type and cellular context-specific way. However, for many complex human traits, the genetic structure predicted by the omnigenic model appears to be the case. That is, hundreds to thousands of genetic variants of (mostly) very small effect size act in aggregate to set a genetic liability threshold. The central nodes in these disease gene networks have the largest effect size and therefore likely a lower statistical power requirement to demonstrate association. Thus, like many pharmacotherapies (467), it may well be that these core disease genes have multiple mechanisms through which they modulate disease risk. Hence, they are centrally located and have outsized effect sizes. Certainly, BLK, Lyn and the BAFF family genes in these networks could be argued to have effects selective to the B cell lineage. However, both BAFF (468) and Lyn (469) have well described actions outside of B cells. Likewise, BLK exhibits high expression in human plasmacytoid dendritic cells (470, 471) and the most strongly associated eQTL variants are within human fibroblasts. Both of these cell lineages are independent from B cells and have direct relevance to SLE etiopathogenesis. A role for these three genes acting to increase SLE risk within B cells is certainly more parsimonious. Alternately, it has been argued that several of the polygenic risk variants for human type 1 diabetes exhibit opposite action in effector and regulatory T cells (472). That is, several risk variants increase the likelihood of activation in effector T cells and simultaneously increase the likelihood of inhibition in regulatory T cells. Thus, even with specific cellular mechanisms, the risk alleles of the strongest effect size may be the most likely to have multiple mechanisms whereby they alter disease risk. Cogent arguments can be made for the cellular specificity of gene networks acting within a disease state. However, much work remains to be done to convincingly demonstrate cell-type specificity of genetic effects, over against disease risk networks that span and exert their effects within multiple cellular lineages.

Potential explanations for gaps in translation

What are the explanations for challenges in translatability of autoimmune disease mouse models?

We have discussed spontaneous, induced and humanized murine autoimmune disease models above in general terms. Here we focus on key potential differences that in our estimation are likely to affect several spontaneous models of lupus, such as those derived from the NZB/NZW F1 (BW) mice and the NOD mouse model of type 1 diabetes.

Recombinant inbred mice/Polygenic disease in Humans vs. Monogenic disease in mice

The use of recombinant inbred mice more closely resembles consanguinity that is seen more commonly the parents of individuals with childhood onset autosomal recessive disease. In this way, these murine models may offer more opportunities to develop monogenic mutations and sub-strain differences can profoundly alter physiology (473). One example sticks out in particular. The most commonly used lab mouse strain, C57BL6/J, developed a loss of function mutation in *Nnt*, the gene encoding for the nicotinamide nucleotide transhydrogenase (473). This mutant *Nnt* diverges from another commonly used lab mouse strain C57BL6/NJ. Unfortunately, *Nnt* mutation inadvertently serves as a model of familial glucocorticoid deficiency, which has been described in mice and humans who have mutant NNT (474). This could conceivably confound interpretation of results obtained using models that have not controlled for this mutation in lupus in particular, where glucocorticoids are a mainstay of therapy. As another example,

a body of literature describing functions previously attributed to caspase-1 are in fact due caspase-11 deficiency due to inadvertent gene-targeting leading to generation of caspase-1/caspase-11 double knockout mice (475).

Genetic & evolutionary divergence of both host and microbiota

Sixty-five million years of evolutionary history seems like a long time. Certainly, it is long enough to develop changes in how genes respond to the environment. As a stark example, Gout is a disease of higher primates. It is one of the most common forms of inflammatory arthritis and is estimated to affect 1 in 200 people worldwide. Gout occurs when uric acid levels are too high and uric acid crystals precipitate out of the serum, driving acute and chronic inflammation. Gout is thought to have arisen ~ twenty-two million years ago when one of a series of loss of function mutations in uricase (which converts uric acid to the much more water-soluble allantoin) and URAT1 and important renal uric acid transporter. As this system non-redundantly regulates blood pressure, it stands to reason that changes across similarly complex immune networks could have also developed differences in some critical regulatory genes. Indeed, many immune phenotypes that diverge between mice and humans have been described (476). Two select examples of gene to phenotype non-correspondence include MyD88 and STAT5B. MyD88 deficiency leads to early life susceptibility to only pyogenic infections in humans whereas it leads to long lasting susceptibility to a broad array of infections in mice (477). STAT5B deficiency leads to different phenotypes in terms of Treg generation, IL2R signaling and *in vivo* T cell effector function in mice as compared with humans (478).

Environmental enrichment

While humans are housed in varied circumstances, housing of mice is somewhat uniform. Environmental enrichment (EE) makes mouse housing more "fun" and leads to reductions in a variety of depressive/anxious behaviors and indicators of stress response in mice (479). At the same time, there is evidence that EE substantively impacts the antitumor response of NK cells and immunotherapy treated anti-cancer T cells (480). Thus, differences in the monotony and variety of environment may be a factor that alters immune system responses and could impact autoimmune disease pathways.

Thermoneutral housing

When given the option, mice, like humans tend to inhabit places with comfortable ambient temperature or change their environment to maintain their own core temperature in the thermoneutral zone. Humans do this by wearing clothes, whereas mice tend to fill their burrows with bedding and insulation. Observation of mice in the wild indicates that during their light cycle, mice tend to maintain a thermoneutral zone of 30-32 degrees Celsius. For historical reasons and for the comfort of clothed humans, most mouse facilities house mice at room temperature 19-25 degrees Celsius. Thus, mice are subjected to chronic "cold stress" which carries with it attendant increased sympathetic nervous system/beta-adrenergic tone and changes in whole organism metabolism and physiology (481). Removal of this cold stress through thermoneutral housing has been demonstrated to impact several immune phenotypes, including notably, induction of oral tolerance (482–485). Further there is growing evidence that the parasympathetic nervous system impacts autoimmune disease. For example, vagal nerve (parasympathetic) (486) stimulation has led to improvement of systemic inflammatory parameters in short-term trials (487, 488).

Circadian rhythms

Mice are typically handled in the vivarium during daylight hours, a period during which they commonly sleep in the wild. Several autoimmune diseases are associated with sleep disturbance (489) due to incompletely clear mechanisms. Indeed, less than 7 hours of sleep is associated with the onset of human SLE in longitudinal cohort studies (490). Further, several reports indicate that systematically sleep deprived NZB/NZWF (1) mice develop increased lupus activity (491, 492). Thus, differences in circadian cycles may be an additional factor to consider when modeling human autoimmune pathologies in mice.

Microbiota/pet store mice

Our immune system gene networks have subject to selective pressure for the sixty-five million years since divergence from mice. At the same time, the mutualistic relationship with our microbiota has been under pressure from our immune system and *vice versa*. This may be another important meta-genomic divergence that leads to non-correspondence of murine models of human disease (59). Following our reductionist tendencies, the character and make up of mouse microbiota is being intensively defined and simplified as specific-pathogen-free facilities are increasingly used (493, 494). Normalizing the microbiome to one that more closely resembles wild mice leads to several substantial changes in immune response (495–498). Thus, colonization with comparatively non-immunogenic

microbiota may be yet another factor that needs to be accounted for when modeling human autoimmune disease in mice.

Humans (usually) already have disease: Early disease therapy vs. established disease therapy

Most therapies given to people with autoimmune disorders are usually administered to counter a matured, often chronic disease. While prevention trials are underway in several human autoimmune diseases (221), many therapies employed in mouse models are preventive in nature. That is, intervention occurs prior to the onset of disease.

Mice are not free to eat what they want (but they can usually eat as much as they want)

Many lab rodent diets contain substantial proportions of alfalfa meal (499, 500). Alfalfa sprout consumption was long ago associated with incident lupus-like disease in higher primates and attributed to the presence of canavanine, a non-canonical arginine-related amino acid (501). Subsequent studies have also found epidemiological evidence of association with lupus (502), to the point that a commonly used Lupus patient education website recommends avoidance of alfalfa sprouts (503). Curiously, anti-cyclic citrullinated peptide antibodies (against peptides with the non-canonical arginine related amino acid citrulline) are commonly seen in individuals with rheumatoid arthritis as well as those with clinical features of both SLE and RA (504). Recent work has also implicated peptide processing that leads to hybrid-insulin peptide formation, generating a neoepitope as etiologic in type 1 Diabetes (505). Protein dietary and metabolic changes could theoretically alter the generation of neoepitopes in alfalfa fed mice and more broadly appear to have an important role in the genesis of several autoimmune pathologies.

Humans are free

Established disease in humans almost always means confounders – behavior, medications, adherence, understanding, communication, health literacy, numerical literacy, risk perception and risk calculus [COVID-19 pandemic as a global example (506)], to name a few. There is a situation when established disease in humans tends to go along with fewer confounders – early life. However, ethical and practical issues usually prevent trials in children for diseases that also develop in adults. Maybe it isn't that mice are simple, but that humans are just too complicated?

Mice are not free and cannot access sunlight

Most research animal facilities, have strict policies against taking mice out of the viviarium for a walk in the sun. This likely lowers the risk for the skin manifestations of lupus, which are importantly mediated by UV. While the artificial environment of the vivarium can be addressed artificially with transient UV exposure (507), vitamin D is also an independent protective factor for lupus flares and the development of several autoimmune disease (508–511).

Mice have fur

The absence of extensive hair follicles, dermal and epidermal layers that are twice as thick and the absence of a specialized muscle layer (*Panniculus carnosus*) all distinguish human from murine skin (512–514). If histological differences do not pose a sufficient challenge in modeling human skin pathologies in mice, it has been observed that only ~30% of the top skin-expressed genes overlap between mouse and human skin (515). Taken together, these differences pose several problems in modeling SLE, as autoimmune response in the skin is the first disease manifestation in many affected humans.

Mice are not naturally susceptible to infection by EBV

In addition to implication in MS (discussed above), EBV infection in humans is associated with SLE. There are mechanistic links implicating molecular mimicry by EBNA-1 (516) and substantial enrichment of EBNA-2, the latency transcription factor, at GWAS loci for SLE and other autoimmune diseases (516). There are also examples of allele specific binding of EB viral transcription factors to causal risk alleles. How might this confound translatability of murine model data? The closest gammaherpes virus to EBV that infects mice is murine gamma-herpesvirus 68. While murine gamma-herpesvirus 68 does infect mice, it lacks several features of EBV (517). If one of those divergent features omits a critical step in the EBV-dependent development of autoimmune disease, then this divergence would impact our ability to model autoimmune disease development in a way that parallels what is suspected to occur in humans.

In this section we point out some differences to consider when interpreting murine model data in light of human autoimmune pathology. There are several features of humans

that make modeling an inherently error-prone process. These complicating features are in addition to the potential intractability of understanding gene X environment interactions, if the omnigenic model proves true. Despite these drawbacks, murine models of autoimmune diseases have advanced our understanding of the gene networks that regulate autoimmune pathologies. At the same time, efforts at translation require both careful attention to potential confounders and continual reexamination of our models in light of the clinical, phenotypic, cellular and molecular features of the human diseases we seek to model.

Implications and a potential path towards translation

Simply put, the need for improved understanding and more diverse and less toxic therapeutic options for SLE and Type 1 diabetes is dire. The discrepant severity of SLE outcomes between populations simply cannot be accepted in a just society. To the extent that our lack of understanding contributes to this discrepancy, it needs to be corrected. In a similar manner, Type 1 diabetes disproportionately afflicts some of the most vulnerable members of our society with a burden of chronic disease and a concomitant burden of co-morbidity and mortality. Despite life-saving advances in therapy in the prior decades, the incidence of this disease is rising. So, we must better understand its genesis in order to more effectively intervene.

We need to understand disease mechanisms and define causal genetic immunophenotypes in humans. For this understanding to be certain regarding causal relationships, parallel understanding of mechanism in model systems is required for effective trial design. Mice have proven to be excellent sacrificial companions on our collective journey of disease deconstruction for both SLE and T1D. They have facilitated perturbations of genes and environmental triggers, allowing assessment of the impacts on murine intermediate immune cellular and molecular phenotypes and correlates of pathology. It continues to be prudent to advance therapies that can prove efficacy in these model systems along the path toward clinical application. However, careful attention to the details of both the model system and the disease processes being modeled is necessary to fully evaluate both therapeutic candidate successes and failures. Nearly 90% of trialed pharmaco-therapeutic candidates do not advance to the FDA approval (518). These rates are better for biologics than for small molecules at each stage of drug development, possibly due to the more specifically targeted nature of biologic therapies versus small molecules (519, 520). This failure is despite the best efforts of many who are employed by pharmaceutical companies. Our ability to fully understand these incredibly complex biological systems remains incomplete. Thus, it is perhaps not surprising that there have been several high-profile failures to develop autoimmune disease therapy.

How best to evaluate therapeutic leads for autoimmune diseases? Our proposed approach follows. Cellular/molecular phenotypes and pathological correlates of disease would need to be ameliorated by candidate therapeutic leads in murine systems to a reasonable degree of certainty in terms of causality. At the same time parallel approaches could be validated in human *in vitro* (cell lines), *ex vivo* (primary cells) or *in vivo* (hu-mice) reductionist model systems and shown to return the cellular/molecular phenotypes and pathologic correlates move to a healthier status with any therapeutic lead. Therapies that pass this bar could be trialed in first in human trials after primate evaluation or if repurposing (if already FDA approved), moved directly to phase 3 trials. Human trials based on the cellular, molecular and pathologic frameworks derived from model systems would need to include assessment of correlates of the postulated mechanism. Additionally, evaluation of any competing mechanisms would assist post-hoc evaluation of whether a given trial represented a true trial of therapy. Indeed, two recent (the first two since the 1950s) FDA-approved therapies for SLE, belimumab (anti-BAFF) and anifrolumab (anti-IFNAR1), both took approaches similar to the approach that we lay out. Following identification of antigen-presentation by B cells (521–528) as key in the genesis of murine autoimmune type 1 diabetes there is now a focus on B cell tolerance pathways in human T1D (41, 529–533). Further characterization of the role of B cell tolerance (534) and efforts to manipulate pathogenic autoantigen-reactive B cells in type 1 diabetes promise (530) to bring therapeutic successes in this disease, where T cells have long been the subject of focus. Our analysis highlights a potential role for autoreactive B cell tolerance in the development of multiple autoimmune pathologies. In doing so, it adds to a growing body of work that supports viewing seropositive autoimmunity as an endophenotype of multiple autoimmune diseases (535–541). As our efforts to more broadly understand autoimmune disease polygenic genetic risk network impacts on B cell function advance, we anticipate that murine disease models will continue to be critically important to furthering understanding of autoimmune diseases and advancing the goal of improved outcomes for patients.

Author contributions

IH conceived of the article, carried out the analyses, drafted and revised the manuscript. KA contributed to data analysis and interpretation and critically revised the manuscript. RS

contributed to data analysis, interpretation and critically revised the manuscript All authors contributed to the article and approved the submitted version.

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Conflict of interest

IH is partly funded by the Pfizer Global Grants Foundation Rheumatology program #51849703, but those funds did not support his work on this project.

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.953439/full#supplementary-material

SUPPLEMENTARY TABLE 1A
Monogenic Routes to Human Lupus – Gene(s) refers to the gene or genes that when mutated has been reported to lead to lupus or a lupus-like phenotype. Gene name refers to the HGNC (HUGO Gene Nomenclature Committee [https://www.genenames.org/]) official full name for that gene. Locus refers to chromosome and cytoband for that gene. Protein refers to the common protein name for a particular gene. Inheritance indicates the mode of inheritance (if reported) according to the abbreviations at the end of the table. Pathway refers to the reported pathway disrupted by the mutation. Phenotype refers to the phenotype observed, whether part of another defined genetic syndrome, such as Noonan syndrome or whether part of bona fide SLE or another lupus-like phenotype. Reference refers to numbered reference in the bibliography of this publication. PMID or link refers to the pubmed.gov identifier (PMID) for the publication or publications establishing the gene as a monogenic route to lupus or a review of several publications.

SUPPLEMENTARY TABLE 1B
Monogenic Routes to Human autoimmune Type 1 Diabetes – Gene(s) refers to the gene or genes that when mutated has been reported to lead to lupus or a lupus-like phenotype. Gene name refers to the HGNC (HUGO Gene Nomenclature Committee [https://www.genenames.org/]) official full name for that gene. Locus refers to chromosome and cytoband for that gene. Protein refers to the common protein name for a particular gene. Inheritance indicates the mode of inheritance (if reported) according to the abbreviations at the end of the table. Pathway refers to the reported pathway disrupted by the mutation. Phenotype refers to the phenotype observed according to online mendelian mutation in man (OMIM) [https://omim.org/]. Reference refers to numbered reference in the bibliography of this publication. PMID or link refers to the pubmed.gov identifier (PMID) for the publication or publications establishing the gene as a monogenic route to type 1 diabetes or a review of several publications.

SUPPLEMENTARY TABLE 2A
Human SLE Polygenic risk loci from GWAS catalog and putative causal gene(s) as identified by OpenTargetsGenetics L2G pipeline – Variant & Risk allele: the genetic variant with smallest reported P-value for association with SLE by GWAS and the corresponding risk allele as reported by the EBI/NHGRI GWAS catalog [https://www.ebi.ac.uk/gwas/]. P-value: the p-value reported for that variant. P-value annotation: commentary on the P-value reported for that variant as reported in the EBI GWAS catalog (i.e. association in a specific population, conditional logistic regression based on covariates, etc.) RAF – risk allele frequency, if reported in the EBI GWAS catalog. OR – reported odds ratio for the risk allele as reported in the EBI GWAS catalog. Beta – effect size or natural logarithm of the odds ratio. CI – 95% confidence interval of the estimated odds ratio (or beta where reported). Mapped gene – contiguous or adjacent gene mapped to the location of the lead genetic variant. Reported Trait – trait for the GWAS that reported the lead marker from the EBI GWAS catalog. Only "Systemic lupus erythematosus" is included in this table over against, i.e. "lupus nephritis". Trait(s) – trait or subphenotype. Only "systemic lupus erythematosus" is included in this table over against, i.e. "neonatal lupus". Background trait – indicator of background trait (i.e. in the case of lupus nephritis in a cohort of SLE patients, SLE would represent the

background trait), if present. Study accession – GWAS catalog study identifier. PubMed ID – pubmed ID of the study reporting association. First Author (First author of the study in question). Location – chromosome:position of the lead variant on human genome build 38 (hg38). P – P value converted from format in EBI GWAS catalog to scientific notation. Chromosome – chromosome of lead variant. Position (hg38) position in base pairs of the variant of the lead variant on human genome build 38 (hg38). Region – numerical value of this GWAS region as associated with SLE. Putative Causal gene as predicted by open targets genetics L2G algorithm. Opentargets – link to opentargets genetics prediction and evidence supporting this prediction for that region. "NR" or "'-" indicates value not reported in the EBI GWAS catalog.

SUPPLEMENTARY TABLE 2B

Human T1D Polygenic risk variants from GWAS catalog and putative causal gene(s) as identified by OpenTargetsGenetics L2G pipeline – columns are identical to Supplementary Table 2A, except that they apply to type 1 diabetes and not Systemic lupus erythematosus.

SUPPLEMENTARY TABLE 3

Genes involved in lupus, type 1 diabetes, peripheral and central B cell tolerance from mouse models – Murine locus – genetic locus, gene name or common protein name of the gene. Gene location – murine chromosome and cytoband of the gene in question. GL Murine – gene location on chromosome in centimorgans. Human orthologue – where identifiable, the human orthlogue to the murine gene in question. Gene location (human) chromosome and cytoband of that human gene. Gene name – HGNC gene name of the gene where applicable. (HGNC = HUGO Gene Nomenclature Committee [https://www.genenames.org/]). Protein – abbreviation or common name for the protein encoded by the gene. Pathway – pathway implicated in the function of this gene in the corresponding class. Model – murine model where gene was implicated. Phenotype – phenotype resulting from gene alteration. Class – murine disease/model state implicated: either lupus, T1D, peripheral tolerance, central tolerance or some combination. PMID – PubMed ID of the publications supporting the link of the gene with a particular class.

SUPPLEMENTARY TABLE 4

hyperlinks to Networks. This table consists of references to networks in this paper along with links to permanent versions of the networks and analyses that were used to develop the conclusions of this paper. Disease network – disease network as referred to in this paper. Color – color encoding of the disease network in question in –. Shape – shape encoding of the disease network in question in –. URL (NDEX) – universal resource locator for network @ http://www.ndexbio.org URL(string-db.org) – universal resource locator for network @ https://www.string-db.org/URL (Enrichr) – universal resource locator for network @ Enrichr pathway and geneset enrichment analysis: https://maayanlab.cloud/Enrichr/.

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Natural history of type 1 diabetes on an immunodysregulatory background with genetic alteration in B-cell activating factor receptor: A case report

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The immunological events leading to type 1 diabetes (T1D) are complex and heterogeneous, underscoring the necessity to study rare cases to improve our understanding. Here, we report the case of a 16-year-old patient who showed glycosuria during a regular checkup. Upon further evaluation, stage 2 T1D, autoimmune thrombocytopenic purpura (AITP), and common variable immunodeficiency (CVID) were diagnosed. The patient underwent low carb diet, losing > 8 kg, and was placed on Ig replacement therapy. Anti-CD20 monoclonal antibody (Rituximab, RTX) was administered 2 years after diagnosis to treat peripheral polyneuropathy, whereas an atypical mycobacteriosis

manifested 4 years after diagnosis and was managed with prolonged antibiotic treatment. In the fifth year of monitoring, the patient progressed to insulin dependency despite ZnT8A autoantibody resolution and IA-2A and GADA autoantibody decline. The patient had low T1D genetic risk score (GRS = 0.22817) and absence of human leukocyte antigen (HLA) DR3/DR4-DQ8. Genetic analysis identified the monoallelic mutation H159Y in *TNFRSF13C*, a gene encoding B-cell activating factor receptor (BAFFR). Significant reduced blood B-cell numbers and BAFFR levels were observed in line with a dysregulation in BAFF–BAFFR signaling. The elevated frequency of PD-1$^+$ dysfunctional Tfh cells composed predominantly by Th1 phenotype was observed at disease onset and during follow-up. This case report describes a patient progressing to T1D on a BAFFR-mediated immunodysregulatory background, suggesting a role of BAFF–BAFFR signaling in islet-specific tolerance and T1D progression.

KEYWORDS

type 1 diabetes (T1D), common variable immunodeficiency (CVID), BAFFR mutation, islet autoimmunity, circulating T follicular helper cells (cTfh)

Introduction

Type 1 diabetes (T1D) is a disease of multifactorial origin caused by the autoimmune destruction of insulin-producing pancreatic β cells. Several immune players have been identified as contributors to the disease immunopathogenesis, involving both the innate and adaptive arms of the immune system (1–3). T cells seem to play a dominant role during the disease pathogenesis and are directly involved in the pancreatic β-cell killing. The possible role of B cells and autoantibodies (AAbs) in T1D remains elusive, which are thought to act mainly as antigen-presenting cells. Islet-specific AAbs—such as glutamic acid decarboxylase 65 (GAD65), insulin, the tyrosine phosphatase–like autoantigen IA-2, or the ZnT8—are the most reliable biomarkers for disease diagnosis and prediction (4, 5). Today, T1D patients can be subdivided into three stages based on the presence of islet-specific AAbs and impaired glucose tolerance: stage 1 T1D, with individuals positive for at least two islet-specific AAbs and no metabolic dysregulation; stage 2 T1D, with individuals who developed impaired glucose tolerance; and stage 3 T1D, with individuals with multiple AAb-positive and fasting hyperglycemia (clinical diabetes) (6, 7)

A poorly defined interaction between genetic and environmental factors underlies T1D pathogenesis. HLA accounts for the majority of T1D genetic risk, whereas single-nucleotide polymorphisms (SNPs) in non-HLA genes, such as *INS*, *PTPN22*, *IL2RA*, *IFIH1*, and *CTLA4*, are considered additional contributing genetic factors (8, 9). Recently, several T1D genetic risk scores (GRSs) have been developed based on HLA and non-HLA T1D-risk genes (30-97 SNPs). These scores can discriminate T1D from type 2 diabetes (T2D), monogenic diabetes from T1D, and monogenic autoimmunity from early onset T1D associated with poly-autoimmunity (10, 11).

Common variable immunodeficiency (CVID) is a heterogenous disease classified as predominantly antibody deficiency (12), with a broad variety of clinical spectrum, characterized by low levels of immunoglobulins (Ig) and failure to produce antigen-specific antibodies with a normal or low levels of B cells and different involvement of cellular immunity. Reduced B-cell counts, isotype-switched B cells (13, 14) and plasmablasts (15) have been described in individuals affected by CVID. In addition, several T-cell defects have been described that often account for the failed B-cell helper support occurring in germinal centers (GCs) (16–19). Patients with CVID often present autoimmune manifestations, mainly autoimmune cytopenia and inflammatory bowel disease (20). T1D in CVID has been

Abbreviations: AAb, Autoantibody; AITP, Autoimmune thrombocytopenic purpura; BAFFR, B-cell activating factor receptor; cTfh, Circulating T follicular helper cell; cTfr, Circulating T follicular regulatory cell; cTreg, Circulating T regulatory cell; CVID, Common variable immunodeficiency; FACS, Fluorescence activated cell sorter; FC, Flow cytometry; FMO, Fluorescence minus one; FPG, Fasting plasma glucose; FU, Follow-up, GADA, Glutamic acid decarboxylase antibodies; GRS, Genetic risk score; HC, Healthy control; HLA, Human leukocyte antigen; I-A2, Insulinoma-associated antigen 2; IAA, Insulin autoantibodies; IQR, Interquartile range; ITP, Immune thrombocytopenia; MFI, Median fluorescence intensity; PBMCs, Peripheral blood mononucleated cells, SD, Standard deviation; SNP, Single-nucleotide polymorphism; T1D, Type 1 diabetes; WES, Whole exome sequencing, ZnT8, Zinc transporter 8.

described in a handful of reports, but the underlying mechanism and genetic causes remain unknown (21). In this study, we report a patient who at 16 years of age was diagnosed with stage 2 T1D and CVID. Genetic analyses identified a monoallelic mutation in the B-cell activating factor receptor (BAFFR). T1D GRS analysis showed a reduced risk for T1D, suggesting that the identified BAFFR mutation together with other factors, genetic, and environmental determined the progression to T1D.

Case description

A healthy 16-year-old man with a Caucasian ethnic background underwent a medical visit for a pre-participation sport evaluation. As part of the checkup, urinalysis was performed, resulting positive for glycosuria (99 mg/dl) but negative for ketones. Biochemical analysis revealed the presence of prediabetes (FPG 120 mg/dl, HbA1c 42 mmol/mol) associated with mild thrombocytopenia (89,000/µl) and microcytemia (MCV 78 fl) that was treated with iron supplementation for 1.5 months. Of note, glycosuria (252 mg/dl), not further addressed, and a platelet count at the lower limit of normal (166,000/µl) were present at the age of 12 years, according to his medical records. Stage 2 T1D was diagnosed by the presence of three islet AAbs (IA-2, GADA, and ZnT8A), dysglycemia (FPG 101 mg/dl, HbA1c 40 mmol/mol), glucose intolerance (FPG 309 mg/dl at 2-h 75-g Oral Glucose Tolerance Test (OGTT)), and a partially impaired insulin secretion (fasting insulin and C-peptide: 15.45 mU/L and 1.85 ng/ml; 2-h 75-g Oral Glucose Tolerance Test insulin and C-peptide: 47.52 mU/L and 3.46 ng/ml). Family history included autoimmune Hashimoto's thyroiditis (treated with levothyroxine) (father), anti-thyroid peroxidase antibodies (younger brother), and T2DM (maternal grandmother). No signs of celiac disease, atrophic gastritis, or autoimmune thyroid disease were found in the patient. A low-carb diet was recommended with a consequent decline in weight (> 8 kg in a 3-month period) and blood glucose normalization. Concomitant to stage 2 T1D, immune thrombocytopenia (ITP) (PLT 47,000/µl, anti-PLT antibodies positive), and hypogammaglobulinemia (IgG: 323 mg/dl; IgM: 21 mg/dl; IgA: 48 mg/dl) were diagnosed (22). Bone marrow biopsy excluded any lymphoproliferative diseases confirming the ITP diagnosis. Microbiological analysis and EBV serology were negative, except for low copies of HHV6 and Parvovirus B19 in the bone marrow. Two months later, the patient was hospitalized for severe immune thrombocytopenia (platelets: 20,000/µl), which was treated with high-dose intravenous immunoglobulin (IVIg) with a good response. During hospitalization, hypogammaglobulinemia was confirmed (IgG: 344 mg/dl; IgM: 33.10 mg/dl; IgA: 6.92 mg/dl). Immunological investigations showed mild lymphopenia with an increase in memory T-cell subsets and alteration in B-cell maturation, with low memory B-cell frequencies, absent switched memory B cells, and low/absent antigen-specific T-cell responses. In the same year, the patient had experienced recurrent tonsillitis, but his past medical history was negative for severe or recurrent infections, with the exception of laryngospasm episodes in pre-scholar age. Consequently, after excluding other secondary causes and considering the persistence of hypogammaglobulinemia, a clinical diagnosis of CVID was made and he started IVIg replacement therapy.

During a 5-year follow-up, he did not experience any ITP relapses and his platelet count remained stable between 100,000 and 150,000/µl.

Two years after CVID and T1D stage 2 diagnosis, the patient was admitted to the hospital for asymmetric axonal sensitive polyneuropathy, probably triggered by CMV infection, which was managed with high-dose IVIg, RTX, Pregabalin, and Duloxetin. Steroids were not considered due to his comorbidities (pre-clinical diabetes and hypertension). Neurological improvement occurred with a mild persistence of sensitive alterations.

Two years later, an atypical mycobacterial pulmonary infection associated with generalized lymphadenopathy and worsening splenomegaly was discovered and treated with long-time pluri-antibiotic therapy.

The patient remained insulin free for 4 years after the initial prediabetes diagnosis when the dysglycemia evolved into stage 3 T1D (at 21 years of age) marking the start of insulin therapy.

Despite receiving three doses of the anti–SARS-CoV-2 vaccine (the last dose in December 2021) and showing a good humoral and cellular response (23), the patient was infected by SARS-CoV-2 virus in April 2022 and experienced a paucisymptomatic clinical course without the necessity of additional therapies and viral clearance in 15 days. Currently, the patient is on subcutaneous Ig replacement therapy (20 gr/28 days) and insulin Glargine 20 UI/day.

Timeline

The complete timeline from the time of diagnosis (07/2016) to now is shown in Figure 1.

Genetic assessment

The index patient underwent genetic screening by whole exome sequencing (WES). A monoallelic mutation in *BAFFR* (H159Y) was identified and confirmed by Sanger sequencing. The mother carried the wild-type allele, whereas the father carried the same mutation. Additionally, T1D GRS was calculated by typing 30 common HLA and non-HLA genetic variants associated with T1D, as previously described (10). The index patient did not have a T1D-risk HLA (X/X for DR3/DR4-DQ8) and his T1D GRS score was 0.22817 (Figure 2A).

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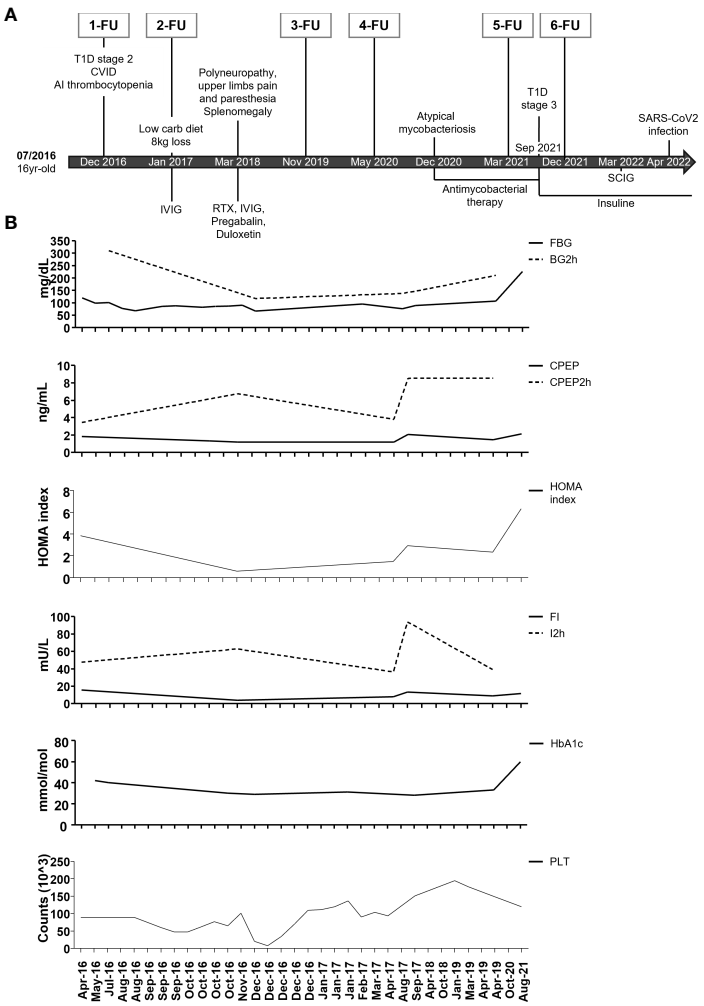

FIGURE 1
Timeline of clinical events, therapeutic interventions, and diagnostic procedures. **(A)** Summary of major clinical manifestations and therapeutic interventions. IVIG: intravenous immunoglobulins; SCIG: subcutaneous immunoglobulins; RTX: Rituximab. At each follow-up, an extensive immune cell phenotyping was conducted. **(B)** Timeline of fasting blood glucose (FBG), 2-h blood glucose (BG2h), fasting C-peptide (CPEP), and 2-h C-peptide (CPEP2h), HOMA index, fasting insulin (FI) and 2-h insulin (I2h), glycohemoglobin (HbA1c), and platelet count (PLT) from April 2016 to August 2021.

Moreover, the monoallelic mutation in BAFFR was associated with reduced gMFI BAFFR expression on the B cell, Tfh, and T regulatory cell (T_{reg}) surface as compared with HC. BAFFR decrease was more pronounced in B cells (MFI reduction 82.1%) than in T cells (reduction 15.4%, 18.9%, and 18.5% in Tfh, T_{reg}, and Tfr, respectively) (Figure 2B). Similar to the index patient, the father expressed reduced levels of BAFFR on the surface of his circulating B cells (Figure S1).

Immunological assessment

The diagnosis of CVID was confirmed by the patient's immunological profile. The patient showed mild lymphopenia with a global decrease and altered distribution of the B- and T-cell compartment already at disease onset and during follow-up as compared with age- and gender-matched healthy donors (HC) (Table 1).

By assessing the expression of CXCR5 and FoxP3 among $CD3^+CD4^+$ cells, the frequency of Circulating T follicular helper cell (cTfh) ($CXCR5^+FoxP3^-$), Circulating T follicular regulatory cell (cTfr) ($CXCR5^+FoxP3^+$), and cT_{reg} ($CXCR5^-FoxP3^+$) cells was determined. While cTfr cell frequencies in the patient were within the normal range, cT_{reg} cell frequencies were within the lower range at first but returned to average normal values in subsequent FUs (CVID cTfr, 1-FU = 3.13%; 2-FU = 3.76%; 3-FU = 4.24%; 4-FU = 2.66%; 5-FU = 2.44%; 6-FU = 0.63% vs. HC median, IQR = 1.62, 0.97–2.18, n = 80) (CVID cT_{reg}, 1-FU = 2.67%; 2-FU =

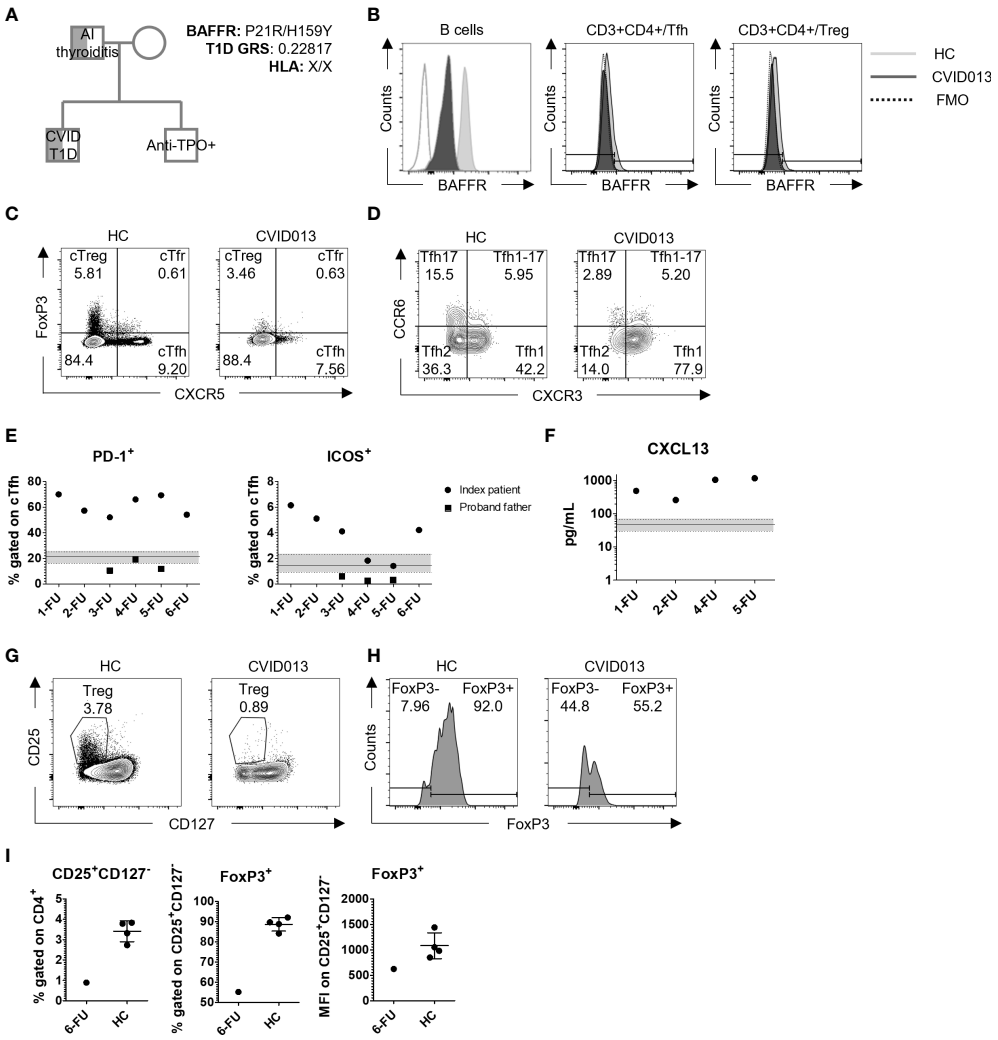

FIGURE 2
Genetic and immunological characteristics of a patient with CVID and stage 2 T1D. **(A)** Genetic testing identified low T1D GRS (0.22817, HLA: X/X), and H159Y mutation in *BAFFR* inherited in a patrilineal fashion. The father was diagnosed with autoimmune thyroiditis, and the brother was positive for anti-TPO autoantibody production. **(B)** Representative gating strategy to evaluate BAFFR distribution on B cells, Tfh, and T_{reg}. White, dark, and light gray slopes for control, HC, and CVID013, respectively. **(C)** FoxP3 and CXCR5 staining on $CD3^+CD4^+$ lymphocytes identifies Tfh ($CXCR5^+FoxP3^-$), Tfr ($CXCR5^+FoxP3^+$), and T_{reg} ($CXCR5^-FoxP3^+$) cells, and **(D)** CXCR3 and CCR6 staining on $CD4^+CXCR5^+CD45RA^-$. The following subsets were identified: cTfh1 ($CXCR3^+CCR6^-$), cTfh2 ($CXCR3^-CCR6^-$), and cTfh17 ($CXCR3^-CCR6^+$). **(E)** $PD\text{-}1^+$ and $ICOS^+$ expressing cells among cTfh are increased over time compared to the HC group ($PD\text{-}1^+$ median, IQR = 21.3%, 16.4–25.3, *n* = 65; $ICOS^+$ median, IQR = 1.45, 0.91–2.32, *n* = 65). Solid dots and squares represent the index patient and the proband father, respectively. **(F)** CXCL13 was evaluated in plasma by ELISA assay. Stable higher levels of this chemoattractant were detected over time in CVID patient when compared with the HC (median, IQR = 47.68 pg/ml, 29.52–68.24; *n* = 65), represented by the continuous line within the light gray area. (**G, H**) T_{reg} gating strategy based on CD25 and CD127 expression, and FoxP3 expressing cells among $CD25^+CD127^-$ T_{reg}. **(I)** $CD25^+CD127^-$ T_{reg} were reduced in the index patient at the first follow-up (HC mean ± *SD* = 3.42% ± 0.51) and expressed lower levels of FoxP3 (mean HC FoxP3 ± *SD* = 88.7% ± 3.3; mean HC FoxP3 MFI ± *SD* = 1080 ± 254.2).

2.43%; 3-FU = 5.22%; 4-FU = 4.26%; 5-FU = 3.39%; 6-FU = 3.46% vs. HC median, IQR = 4.4, 3.12–5.68) (Figures 2C, S2A, Table 1). T_{reg} cells ($CD25^+CD127^-$/loFoxP3$^+$) and FoxP3 levels (γMFI) in the proband were reduced at disease (Figures 2G–I).

cTfh cells, on the other hand, were elevated at the onset but declined in year 5 of FU (Table 1). Further analyses on cTfh cell subset distribution and activation status identified a remarkable shift toward Tfh1 ($CXCR3^+CCR6^-$) cells at the expense of the Tfh2 and Tfh17 subsets ($CXCR3^+CCR6^-$ and $CXCR3^-CCR6^+$, respectively) (Figure 2D) that was maintained throughout the 5-year FU (Figure S2B, Table 1). Moreover, the frequency of $PD\text{-}1^+$ cTfh cells was substantially higher and remained elevated over time in comparison with HC (median, IQR = 21.30, 16.40–25.30), whereas $ICOS^+$ cTfh cell frequency remained higher

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TABLE 1 Immunological phenotyping of B and T cells, autoantibodies titres, and analysis of cytokine production by FC.

		1-FU	2-FU	3-FU	4-FU	5-FU	6-FU	Father	HC group
%					*B-cell phenotyping*				
B cells ($CD19^+$)		2.00	–	1.19	1.77	0.50	2.1^+	5.40 (1.15)	10.28 (3.74)
B naïve ($CD19^+CD27^-$)		94.00	–	90.20	93.60	81.94	91.6^+	58.31 (6.76)	82.1 (73.0-87.3)
B memory ($CD19^+CD27^+$)		5.96	–	6.99	5.98	16.10	8.4^+	35.80 (8.0)	17.0 (12.6-25.2)
Class-switched memory B cells ($CD27^+$ IgM^- IgD^-)			–	7.69	2.70	–	1.3^+	73 (5.09)	46.46 (7.07)
IgM-memory B cells ($CD27^+$ IgM^+)			–	7.89	7.21	–	7.1^+	11.15 (1.48)	20.16 (10.21)
$CD38^{low}CD21^{low}$		11.10	–	18.30	38.00	30.50	26.3^+	9.84 (3.47)	2.42 (1.30-4.58)
Transitional ($CD24^+CD38^+$)		23.80	–	16.60	8.54	–	32.2^+	2.08 (1.92)	7.64 (4.08-10.7)
Breg ($CD27^+CD24^+$)		–	–	–	6.0	5.4	–	40.2 (6.79)	36.3 (12.30)
%					*Autoantibodies*				
IAA		0.06	0	0*	0*	0	0	0.00 (0.00)	0-0.2336
GADA		14.78	2.61	2.66*	3.62*	3.23	1.23	0.02 (0.005)	0-0.8761
IA-2A		48.30	55.87	31.72*	18.89*	18.93	11.72	0.06 (0.015)	0-0.9793
ZnT8A		379.49	26.71	12.52*	3.57*	1.32	2.49	0.42 (0.25)	0-2.5091
%					*T-cell phenotyping*				
$CD3^+$		79.2	75.4	81.3	47.1	79.7	81.6	70.35 (5.06)	39.2 (8.3)
$CD3^+CD4^+$		38.1	38.7	38.6	47.8	46.2	57.1	40.9 (5.45)	75.9 (11.8)
cTfh ($CXCR5^+FoxP3^-$)		39.20	34.20	18.60	30.40	34.40	7.56	6.4 (5.87)	10.85 (8.35-12.60)
cTfr ($CXCR5^+FoxP3^+$)		3.13	3.76	4.24	2.66	2.44	0.63	0.69 (0.63)	1.62 (0.97-2.18)
cTreg ($CXCR5^-FoxP3^+$)		2.67	2.43	5.22	4.26	3.39	3.46	7.65 (5.78)	4.4 (3.12-5.68)
Tfh1 ($CXCR3^+CCR6^-$)		63.90	53.90	58.60	62.10	75.40	52.00	33.83 (7.63)	26.75 (5.90)
Tfh2 ($CXCR3^-CCR6^-$)		19.40	32.40	34.10	26.70	17.10	25.80	28.93 (8.31)	36.47 (8.04)
Tfh17 ($CXCR3^-CCR6^+$)		8.29	7.83	4.64	5.05	2.65	8.22	27.33 (9.95)	26.06 (5.04)
PD1 ($CD4^+CXCR5^+$)		70.00	57.30	52.10	66.10	69.30	54.10	13.87 (4.75)	21.30 (16.40-25.30)
ICOS ($CD4^+CXCR5^+$)		6.15	5.12	4.11	1.83	1.41	4.22	0.39 (0.18)	1.45 (0.91-2.32)
$CXCR3^+PD1^-$ ($CD4^+CXCR5^+$)		22.40	23.80	7.01	12.70	13.20	–	12.31 (13.22)	8.17 (5.1)
%					*Cytokine production FC-analysis*				
$CXCR5^+$	$IFN\text{-}\gamma^+$	–	–	–	2.21	1.99	2.72	3.22 (1.21)	9.51 (10.88)
	$IL\text{-}17^+$	–	–	–	2.09	0.51	0.80	1.26 (1.04)	5.06 (6.18)
	$IL\text{-}21^+$	–	–	–	3.49	3.43	5.53	2.13 (0.87)	8.98 (12.07)
$CXCR5^-$	$IFN\text{-}\gamma^+$	–	–	–	10.20	13.50	25.80	11.01 (5.34)	3.69 (2.86)
	$IL\text{-}17^+$	–	–	–	0.92	0.21	1.34	1.32 (1.57)	1.14 (0.37)
	$IL\text{-}21^+$	–	–	–	6.17	15.10	20.07	2.8 (1.61)	3.32 (2.06)
ng/ml					*IgM and IgG production assay*				
CVID BM + CVID cTfh	IgM	7.78	8.35	–	–	–	–	–	1.37 (0.84)
	IgG	0.3	ND	–	–	–	–	–	9.77 (3.76)
CVID BN + CVID cTfh	IgM	1.1	0.81	–	–	ND	–	–	1.22 (1.05)
	IgG	ND	ND	–	–	ND	–	–	5.52 (3.68)

Available measurements for the index patient, for the father and for the HC pool (B and T cell phenotyping, HC n = 85; cytokine production FC-analysis, HC n = 65; IgM and IgG production assay, HC n = 16; autoantibodies, HC = internal laboratory reference) are included in the table as mean (SD) or median (IQR). The detection of autoantibodies was performed as previously described (24, 25). $^+$Values were determined in June 2022; *titres have been determined in serum samples; ND = undetermined.

since disease onset (~4 times higher than the control, 6.15% vs. HC median, IQR = 1.45%, 0.91–2.32) (Figure 2E, Table 1). Additionally, higher levels of plasma CXCL13, a GC blood biomarker, were observed during the 5-year FU (Table 1, Figure 2F). The father had normal frequencies and subset distribution of follicular T cells (Table 1).

The percentage of total $CD19^+$ B cells was low during the 5-year follow-up (FU) (CVID B cells, 1-FU = 2%; 3-FU = 1.19%; 4-FU = 1.77%; 5-FU = 0.5%; 6-FU = 2.1% vs. HC mean ± *SD* = 10.28 ± 3.74, *n* = 90). The frequency of B memory cells ($CD19^+CD27^+$) was lower than HC (median, IQR = 17.0, 12.6–25.2). Potentially autoreactive B cells defined as $CD19^+CD21^{low}CD38^{low}$ B cells (Figure 3A) were present at higher frequency in the index patient at diagnosis as compared with HC (CVID013 = 11.1% vs. HC median, IQR = 2.42%, 1.30–4.58) and increased over time (3-FU = 18.30%;

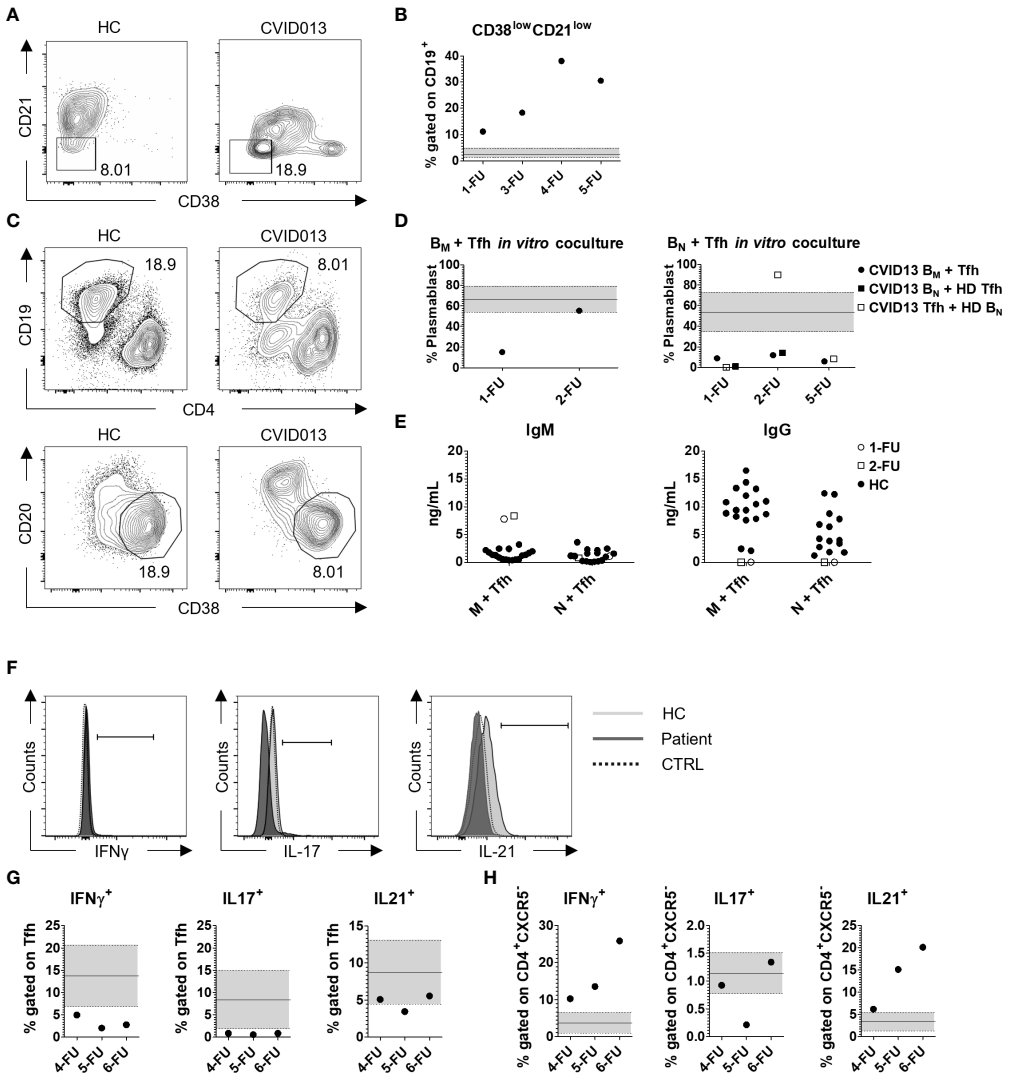

FIGURE 3
Functional analysis of B- and T-cell subsets. **(A)** Representative gating strategy for $CD38^{low}CD21^{low}$ autoreactive B cells, gated on $CD19^{+}$ cells and **(B)** their frequency over time. $CD38^{low}CD21^{low}$ cell percentage was higher compared with the HC median, IQR (2.42%, 1.3–4.58; *n* = 85), increasing from 11.10% at the first follow-up up to 38.00% in 2020, and decreasing to 30.50% in the last monitoring. **(C, D)** Functional analysis of IgM and IgG production. Sorted B memory or B naïve cells were co-cultured with Tfh cells (1:1 ratio) in autologous (solid dot) or heterologous settings (CVID B cells with HC Tfh, solid square, or HC B cells with CVID Tfh, clear square), and the percentage of $CD38^{+}CD20^{-}$ was analyzed within $CD19^{+}CD4^{-}$ cells after 1 week. The black continuous line is representative for the mean HC percentage value ± *SD* (66.32% ± 12.46, *n* = 16) represented by the light gray area within the two dashed lines. The production of IgM and IgG was evaluated in the supernatant **(E,** Table 1**)**. The white dots and squares are representative for the 1-FU and 2-FU, respectively, whereas the black dots represent the HC. **(F, G)** Evaluation of IFN-γ, IL-17, and IL-21 production in $CD4^{+}CXCR5^{+}$ cells after 2-h stimulation with PMA/Ionomycin. The HC and patient slopes are identified with the light and dark gray, respectively, whereas the unstimulated control is represented by the dashed line. IFN-γ and IL-17 production was lower compared with the HC (IFN-γ mean ± *SD* = 16.66% ± 6.84; IL-17 mean ± *SD* = 8.35% ± 6.63; *n* = 65), whereas IL-21 production was lower than HC mean and comprised within the *SD* (IL-21 mean ± *SD* = 8.74% ± 4.30; *n* = 65). **(H)** IFN-γ, IL-17, and IL-21 production in $CD4^{+}CXCR5^{-}$ cells after 2-h stimulation with PMA/Ionomycin. IFN-γ and IL-21 production was higher compared with the HC (IFN-γ mean ± *SD* = 3.69% ± 2.86; IL-21 mean ± *SD* = 3.32% ± 2.06; *n* = 65), whereas IL-17 production was comparable with HC (IL-17 mean ± *SD* = 1.14% ± 0.37; *n* = 65). .

4-FU = 38.00%; 5-FU = 30.50%; 6-FU = 26.30%) (Figure 3B, Table 1). In contrast to the index patient, circulating B cell frequency and subset distribution in the father were normal (Table 1).

To assess the functionality of B and Tfh cells, we performed *in vitro* B-cell helper assay. FACS-sorted memory and naïve B cells were co-cultured with cTfh cells in autologous (CVID B cells with CVID Tfh cells) and heterologous settings (CVID B cells with HC

Tfh cells or *vice versa*) and the percentage of $CD20^{-}CD38^{+}$ plasmablasts together with IgM and IgG levels were evaluated after a week (Figures 3C–E). Due to technical constraints, we were able to perform the assay at three FU. The percentage of plasmablast differentiating from memory B cells in the presence of autologous Tfh cells was lower with respect to HC at diagnosis but improved in 2-FU (Figure 3D, left panel). Patient Tfh cells were able to induce the production of IgM by autologous B memory cells at levels that were higher than the control co-cultures, (1-FU = 7.78ng/ml; 2-FU = 8.35ng/ml vs. HC mean ± *SD* = 1.37ng/ml ± 0.84, *n* = 18). On the other hand, IgM production by B naïve cells was similar to HC (1-FU = 1.1ng/ml; 2-FU = 0.80ng/ml vs. HC mean ± *SD* = 1.22 ng/ml ± 1.05, *n* = 16). Tfh cells co-cultured either with autologous or heterologous B naïve cells were unable to induce class switching and IgG production *in vitro* (Figures 3D, E).

Tfh ($CD4^{+}CXCR5^{+}$) and non-Tfh ($CD4^{+}CXCR5^{-}$) cell functional status was also evaluated *in vitro* by intracellular cytokine profile. Total PBMCs were activated with PMA/Ionomycin and the expression of interferon-γ (IFN-γ), interleukin-17 (IL-17), and IL-21 was evaluated by flow cytometry (FC) (Figures 3F, G). Within the $CXCR5^{+}$ compartment, IFN-γ and IL-17 producing cells were fewer compared with HC (Table 1). Also, IL-21 production was lower than HC (CVID013 IL-21 range = 3.43% - 5.53% vs. HC IL-21 mean ± *SD* = 8.74% ± 4.30; *n* = 65). On the contrary, higher frequencies of IFN-γ and IL-21 producing cells were observed within the $CXCR5^{-}$ compartment (Table 1, Figure 3H).

Discussion

This case report describes a patient diagnosed with ITP, CVID, and T1D with a monoallelic mutation in *BAFFR* (H159Y) inherited from the father. Two years after CVID and T1D stage 2 diagnosis, RTX was administered to treat peripheral polyneuropathy with a potentially positive impact on diabetes progression. Additional diet adjustment (hypoglycemic/ketogenic) led to an 8-kg weight loss that possibly impacted the disease course. Eventually, the patient progressed to insulin dependency, despite a decline in islet AAbs levels. The patient displayed the typical immunological signs of CVID, that is, reduction in circulating B cells, switched memory B cells, and an increase in autoreactive $CD21^{low}CD38^{low}$ B cells. B cell counts remained low during a 5-year FU. The patient was positive for SARS-CoV-2 antibodies prior to infection and vaccination, probably secondary to the presence of these antibodies in IVIg. After receiving three vaccine doses and natural SARS-CoV-2 infection, his anti–SARS-CoV-2 antibodies remained detectable. Generally, CVID patients, especially those with autoimmunity, have variable alterations in humoral responses against vaccines, including against SARS-CoV-2, that could account for a low specific response to some infections and vaccination (23). Interestingly, B naïve and memory subset frequencies increased over time but remained reduced and even declined in absolute numbers. When cultured *in vitro* with autologous and heterologous Tfh cells derived from HC, memory B cells were able to produce IgM, whereas IgG production was compromised, suggesting dysfunctional B and/or Tfh cells.

cTfh cells were present at elevated frequencies during the first 4 years of FU and produced reduced amounts of IFN-γ and IL-21 when challenged *in vitro*. cTfh cells showed a shift toward a Tfh1 phenotype accompanied by an increase in activation markers PD-1 and ICOS. cTfh cell activation status was reflected in the blood where elevated plasmatic concentrations of CXCL13 were found (26). Interestingly, IL-21 production by $CXCR5^{-}CD4^{+}$ cells was highly elevated when compared with HC. Given the connection between IL-21 production and T1D (27), elevated IL-21 production by $CXCR5^{-}CD4^{+}$ T cells could have influenced T1D development in the index patient.

BAFFR is essential for B-cell development, and reduced BAFFR expression or signaling, as in BAFFR deficiency, leads to decreased B cell survival and hypogammaglobulinemia (28). BAFFR can be expressed on the surface of activated T cells including T_{regs} albeit at low levels (29–32). By re-analyzing our previously published RNA-seq data in sorted Tfh cells from the index patient (CVID013) (19), BAFFR mRNA levels were elevated as compared with controls (Figure S3). However, at a protein level, Tfh cells expressed slightly reduced BAFFR levels on their cell surface. The BAFFR H159Y mutation identified in the patient has been previously associated not only with autoimmune diseases, such as systemic lupus erythematosus, multiple sclerosis, and Sjogren's syndrome, but also in non-Hodgkin's lymphoma (33). It is currently unknown how this variant affects protein trafficking, signaling, and degradation. Previous studies have shown that it increases TRAF2, TRAF3, and TRAF6 recruitment to BAFFR, potentiating NF-κB1 and NF-κB2 activity and immunoglobulin production in B cells (28, 33–38). According to our RNA-seq data, BAFFR-mediated dysregulation affected Tfh cell cycle, T-cell activation, and proliferation pathways, and altered the expression of genes involved in signal transduction, apoptosis, and Tfh identity (i.e., BCL-6) (Figures S4–S6). On the other hand, the UV response pathway was down-regulated including pathways involved in apoptosis, cell cycle, proliferation, and immune functions (promoting proliferation) (Figures S4–S7) (19). Further analyses are required to determine the functional role of H159Y in human Tfh cells and B cells and their contribution to CVID and T1D development.

The H159Y variant has been previously described in association with another polymorphism, P21R, which has been described in some patients with CVID (37). These patients displayed lower B cell numbers due to reduced BAFFR expression levels. Possibly, other genetic variants in BAFFR or in other genes related to this pathway are present and contributed to the clinical course of CVID and T1D in the index patient. Of note, the patient's father is affected by autoimmune thyroiditis and has no T1D nor CVID despite having the same BAFFR mutation and reduced surface BAFFR levels on his B cells. Thus, incomplete

disease penetrance might underlie the discrepancies between father and son, similarly to previous CVID reports where family members carry the same heterozygous mutation (34).

Given the absence of a T1D-HLA risk, alterations in BAFFR and humoral dysregulation might have led to T1D. In contrast to other autoimmune diseases, for example, SLE, where BAFF–BAFFR signalling has been extensively studied, limited studies have been conducted in T1D. In one of such studies, reduced BAFFR levels on circulating B cells were observed in patients with longstanding T1D (39). Given the 6-year time window from the time of stage 2 T1D to stage 2 T1D diagnosis, we speculate that BAFFR humoral dysregulation contributed to T1D with slow kinetics or, perhaps, RTX and IVIg therapy delayed the disease onset.

The effect of IVIg therapy in B cells seems to be rather complex and not well understood (40), and there is not enough evidence supporting a beneficial role of IVIg in T1D progression. In the index patient, the treatment did not alter circulating B-cell frequency over the 6 years follow-up and did not affect B-cell ability to stimulate IgM production *in vitro*. It is possible that the alterations in B-cell subset composition were partly mediated by IVIg and could have affected T1D progression, possibly by AAb dilution or by affecting autoreactive B-cell frequency (41). Tfh were able to stimulate the production of IgM but no IgG in B-cell co-cultures *in vitro*; however, we did not explore the possibility that the patient had less class-switched IgG^+ memory B cells explaining our *in vitro* B cell help findings. Additional experiments with sorted IgM^+ vs. IgM^- memory B cells will be necessary to clarify this point.

Belimumab, the human monoclonal antibody that blocks BAFF, is currently employed for the treatment of persistently active systemic lupus erythematosus (33) BAFFR blockade in murine models of T1D was also shown to protect from disease development, a mechanism that involved Breg induction (42). RTX depletes B cells and was shown to preserve C-peptide levels in patients with new-onset T1D (43). The index patient received RTX treatment 2 years after stage 2 T1D diagnosis and 3 years later; after partial B-cell reconstitution, he progressed to insulin-dependent T1D. In the NOD model of T1D, no synergy between RTX and anti-BAFFR mAb treatment was seen as RTX eliminated anti-BAFFR–induced Bregs (42). It remains unknown the effect of RTX on Bregs in the index patient, but possibly RTX did not aggravate disease progression but was rather beneficial.

Despite several weaknesses emanating from the study of a single case and the lack of studies of BAFFR signaling, our data suggest a possible involvement of the BAFFR H159Y variant in T1D pathogenesis and suggest that the BAFF/BAFFR axis might be a target of interest for the pharmacological modulation of T1D.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Ethical Committee of HSR (Tiget06, Tiget09 and DRI004 protocols). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

BL and LPac contributed equally. Conception and design: BL, LPac, CCa, and GF. Development of methodology: BL, LPac, and GM. Acquisition of data: BL, LP, GM, TJ, SC, JG, IMa, IMe, MB, EZ, BR, CCi, FB, CG, PZ, NR, AD, GA, LPas, PC, PP, AF, VL, PC, and RS. Analysis and interpretation of data: BL, LPac, SG, MPC, GDM, AA, LPi, CCa, and GF. Writing, review, and revision of the manuscript: BL, LPas, SG, GA, LPa, MPC, GDM, AA, LPi, CCa, and GF. Study supervision: GF. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.952715/full#supplementary-material

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