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CD4 T cell differentiation in type 1 diabetes

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Summary

Susceptibility to type 1 diabetes is associated strongly with human leucocyte antigen (HLA) genes, implicating T cells in disease pathogenesis. In humans, CD8 T cells predominantly infiltrate the islets, yet their activation and propagation probably requires CD4 T cell help. CD4 T cells can select from several differentiation fates following activation, and this choice has profound consequences for their subsequent cytokine production and migratory potential. In turn, these features dictate which other immune cell types T cells interact with and influence, thereby determining downstream effector functions. Obtaining an accurate picture of the type of CD4 T cell differentiation associated with a particular immune-mediated disease therefore constitutes an important clue when planning intervention strategies. Early models of T cell differentiation focused on the dichotomy between T helper type 1 (Th1) and Th2 responses, with type 1 diabetes (T1D) being viewed mainly as a Th1-mediated pathology. However, several additional fate choices have emerged in recent years, including Th17 cells and follicular helper T cells. Here we revisit the issue of T cell differentiation in autoimmune diabetes, highlighting new evidence from both mouse models and patient samples. We assess the strengths and the weaknesses of the Th1 paradigm, review the data on interleukin (IL)-17 production in type 1 diabetes and discuss emerging evidence for the roles of IL-21 and follicular helper T cells in this disease setting. A better understanding of the phenotype of CD4 T cells in T1D will undoubtedly inform biomarker development, improve patient stratification and potentially reveal new targets for therapeutic intervention.

Keywords: cytokine differentiation, diabetes, T cells

Introduction

Multiple lines of evidence support key roles for both CD4 and CD8 T cells in the immune response that drives type 1 diabetes (T1D). The primary human leucocyte antigen (HLA) associations with T1D are with the class II genes [1], the function of which is to activate CD4 T cells, and CD4 T cells are responsible for 'licencing' CD8 T cell activation [2], making an understanding of the CD4 T cell compartment particularly relevant. It has been shown recently that single nucleotide polymorphisms (SNPs) associated with T1D and other autoimmune diseases are enriched preferentially within CD4 T cell super-enhancers [3], a subset of transcriptional enhancers important for cell identity [4]. Intriguingly, super-enhancer-associated genes show a striking enrichment for cytokines, cytokine receptors and factors that regulate T cell differentiation [3], suggesting that control of T cell cytokine identity may be an important component of the genetic contribution to disease susceptibility. Understanding CD4 T cell differentiation may therefore hold the key to understanding T1D disease mechanisms and ultimately developing new therapeutic interventions.

The Th1 paradigm in T1D

Evidence in favour of the T helper type 1 (Th1) paradigm

Early models of CD4 T cell differentiation were based on a simple dichotomy between interferon (IFN)-γ-dominated

Th1 responses and interleukin (IL)-4-dominated Th2 responses. Th1 cells can be induced by IL-12 and are important for macrophage activation and clearance of intracellular pathogens, while Th2 cells provide defence against helminth infection, and are associated with allergic disorders (e.g. asthma, rhinitis, eczema) involving immunoglobulin (Ig)E, mast cells and eosinophils. Viewed through this lens, autoimmune diabetes appeared to fall firmly into the Th1 camp, with a seminal paper by Katz *et al.* demonstrating that T cells expressing a diabetogenic T cell receptor (TCR) elicited diabetes in neonatal NOD mice when differentiated to a Th1, but not a Th2, phenotype [5] (Fig. 1). Consistent with this idea, increasing levels of IFN-γ were shown to correlate with progression to diabetes in non-obese diabetic (NOD) mice [6], and IFN-γ was shown to be required for diabetes in a virus-induced model [7].

A cornerstone of the Th1/Th2 dichotomy is the capacity of the products of one T cell subset to reciprocally inhibit the development of the other [8]. In this respect, exogenous provision of IL-4 was shown to inhibit diabetes in NOD mice [9], and transgenic expression of IL-4 in the islets under the control of the insulin promoter completely prevented the development of diabetes [10]. In addition, helminth infection, a strong driver of the Th2 response, was shown to protect from diabetes in animal models [11].

A number of studies have pointed to a direct role for the Th1 cell signature cytokine, IFN-γ, in driving the disease process. Expression of IFN-γ under the control of the human insulin promoter was shown to be sufficient to cause the development of diabetes in mice [12] and conversely blockade of IFN-γ in NOD mice could prevent diabetes [13,14]. There are a number of ways in which IFN-γ could be envisaged to contribute to the disease process including by up-regulating expression of major histocompatibility complex (MHC) classes I and II, facilitating macrophage activation, and increasing leucocyte extravasation by inducing adhesion molecules and chemokines (reviewed in [15]). Indeed IFN-γ has been implicated in promoting the homing of diabetogenic T cells to the pancreatic islets in the NOD mouse [16]. There is also a substantial literature directly implicating the IFN-γ signalling pathway in beta cell death, the critical destructive event at the heart of autoimmune diabetes. IFN-γ drives a persistent signal in pancreatic beta cells that can be inhibited by overexpression of suppressor of cytokine signalling-1 (SOCS1) [17], and islet expression of SOCS1 was found to be protective in the rat insulin promoter-lymphocytic choriomeningitis virus (RIP-LCMV) mouse model of diabetes [18]. Both IFN-$\gamma^{-/-}$ [16] and IFN-$\gamma R^{-/-}$ [19] islets are killed less effectively *in vitro* by CD8 T cells [16] and cytokines [19]. It is particularly striking that beta cells lacking IFN-γR show reduced sensitivity not just to IFN-γ induced death, but also to TNF-α- and IL-1β-induced death [19], highlighting the capacity of IFN-γ to sensitize beta cells to multiple potential death triggers.

The balance between Th1 and Th2 responses has also been studied intensively in humans with T1D. Analysis of peripheral blood T cells from newly diagnosed adults (average age ∼29 years, average disease duration ∼5 weeks) provided support for an IFN-γ-dominated response to islet autoantigens, revealing that the balance between IFN-γ and IL-10 differed between patients and healthy controls. Individuals with T1D were more likely to have autoantigen-specific T cells producing IFN-γ alone, or to a lesser extent a mixed IFN-γ and IL-10 response, whereas non-diabetic subjects showed a clear bias towards production of IL-10 alone [20]. Analogous results were obtained in a separate patient cohort with a similar demographic (average age 28·5 years, average diabetes duration 7 months): interestingly, first-degree relatives also showed autoantigen-specific responses that were characterized by more IFN-γ and less IL-10 than healthy controls, although the ratios were not as skewed as in T1D patients [21]. A study assessing mRNA expression in whole blood revealed that levels of IFN-γ mRNA were significantly higher in new-onset T1D patients (average age ∼15 years, average diabetes duration 80 days) compared with an age-matched at-risk cohort

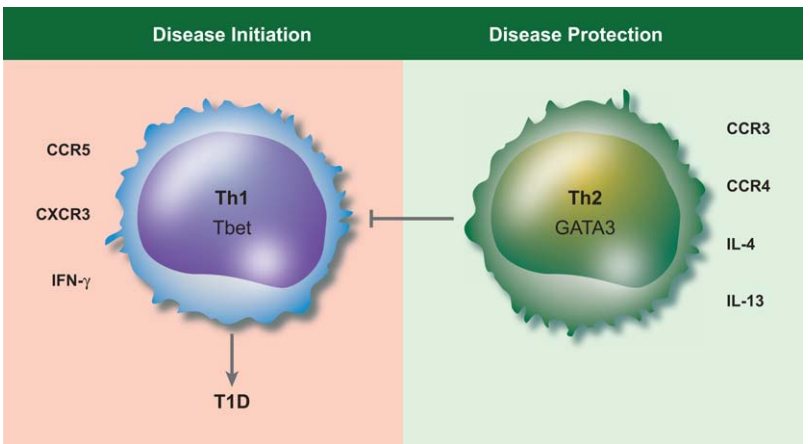

Fig. 1. The original T helper type 1 (Th1)/Th2 paradigm in type 1 diabetes. Type 1 diabetes has been viewed traditionally as a Th1-mediated pathology, with Th2 cells playing a protective role. The characteristic transcription factors and a selection of surface markers associated with Th1 cells or Th2 cells is shown.

[22]. This could potentially reflect a heightening of the Th1 response during conversion to overt disease. Thus, a considerable body of evidence supported the concept that an IFN-γ-producing T cell could be responsible for the pathogenic process in T1D (Fig. 2a).

Evidence against the Th1 paradigm

Although numerous studies support a Th1 bias in T1D, not all evidence is consistent with this conclusion. Some studies using the NOD mouse concluded that beta cell destruction was a Th2- rather than a Th1-mediated event [23], while others concluded that both types of response were involved [24]. At odds with data from short-term Th2 clones [5], long-term cultured Th2 clones derived from the same TCR transgenic animals have the capacity to induce diabetes, and could even enhance the ability of Th1 cells to cause disease [25]. The effect of helminth products on the immune response was also shown to be more complex than anticipated originally, with effects on regulatory T cells and innate lymphoid cells [11], and it is now clear that helminth infection can protect from diabetes without necessarily invoking Th2 differentiation [26,27].

The finding that NOD mice deficient in IFN-γRα exhibited striking resistance to diabetes [28] appeared to provide strong support for the Th1 paradigm; however, protection was subsequently attributed to a closely linked gene on chromosome 10 that was carried over from the 129 background [29,30]. In fact, deficiency in IFN-γ [31] or the β chain of its receptor [30] surprisingly leads to only a mild delay in diabetes development. Deficiency of IL-4 failed to exacerbate disease in NOD mice [32], while injection of recombinant IFN-γ did not accelerate diabetes [33] and, indeed, could even inhibit it [34]. In certain experimental settings, the regulatory T cells protecting from diabetes actually required IFN-γ [35]. Perhaps most surprising was the revelation that the ability of Complete Freund's adjuvant (CFA) to protect NOD mice from diabetes, traditionally assumed to reflect IL-4 or IL-10 production, was largely dependent upon IFN-γ [36]. Collectively, these data questioned the traditional view that diabetes was caused by Th1 cells making IFN-γ, and suggested that the situation might be rather more complex.

Data deriving from analysis of patient samples is also not clear-cut. Insulin-reactive T cells cloned from the pancreatic lymph nodes of individuals with long-standing diabetes expressed IL-13 but not IFN-γ in response to stimulation [37]. In individuals newly diagnosed with T1D, some reports suggested IFN-γ production to be lower [38,39], while others suggested an initial increase in IFN-γ in the early weeks following diagnosis followed by a subsequent decrease [22,40]. In an analysis of serum cytokine levels in 44 newly diabetic children compared with 22 age-matched controls, although Th1-associated products such as regulated upon activation normal T cell expressed and secreted (RANTES) and macrophage inflammatory protein (MIP)-1α were elevated, so too were factors considered indicative of a Th2 response such as IL-4, IL-5 and IL-10 [41]. Similarly, the increase in IFN-γ mRNA detected in whole blood from newly diabetic individuals compared with at-risk individuals was mimicked by a similar increase in IL-4 and IL-10 [22]. A separate analysis concluded that T cells from people with T1D produced equivalent amounts of IFN-γ and IL-13 in response to autoantigens as T cells from control subjects [42]. Regarding the prediabetic period, the Th1 response to autoantigen has been reported to be increased in one study [43] and decreased in another [44] in at-risk individuals. One must be mindful when assaying peripheral blood that a lower response could potentially signify the migration of disease-relevant T cells to the pancreas. Notwithstanding this consideration, it is clear that not all data from mouse models and patients provide consistent support for the dominance of a Th1 response in T1D.

The Th17 revision

Th17 cells in mouse models of autoimmune diabetes

The emergence of Th17 cells [45,46] provided a major revision of the Th1/Th2 paradigm [47] and raised the possibility that tissue-specific autoimmunity might be driven by IL-17-producing T cells rather than Th1 cells. However, the role of Th17 cells in diabetes remains far from clear. In mice, early work implicated IL-17 in the pathogenic

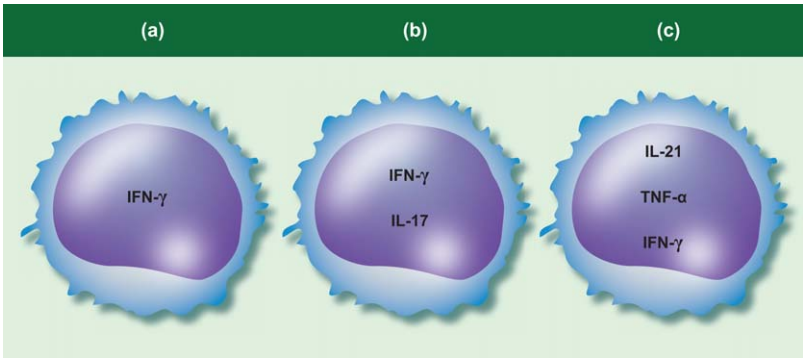

Fig. 2. T cell cytokine production in type 1 diabetes (T1D). (a) Many studies have assessed interferon (IFN)-γ in isolation as a measure of the T helper type 1 (Th1) response. (b) Some studies suggest T cells co-expressing IFN- γ and interleukin (IL)-17 may be expanded in people with type 1 diabetes [60,71]. (c) IL-21-producing T cells in the pancreas in mouse models of diabetes have been shown to co-express tumour necrosis factor (TNF)-α and IFN-γ [106]. IL-21-producing T cells are elevated in T1D patients [67,106], and can co-express TNF-α and IFN-γ [106].

process [48,49]; however, it was shown subsequently that silencing IL-17 expression did not protect NOD mice from diabetes [50]. Furthermore, there were even suggestions that IL-17 could protect from diabetes. Kriegel *et al.* [51] took advantage of the fact that colonization of the gastrointestinal tract with segmented filamentous bacteria (SFB) is known to cause Th17 induction [52], and asked whether SFB-colonized NOD mice developed diabetes with different kinetics to their SFB-negative counterparts. Strikingly, the presence of SFB appeared to delay diabetes, with only 16% of SFB^+ mice developing diabetes by 30 weeks compared with 91% of SFB^- animals. As expected, Th17 signature genes were up-regulated strongly in SFB^+ animals, whereas transcripts associated with Th1, Th2 and regulatory T cells (T_{reg}) were unchanged [51]. These data are clearly more consistent with a role for IL-17, or other Th17 cell products, in delaying rather than promoting diabetes development. A similar conclusion emerged from studies in the diabetes-prone Bio-Breeding (BB) rat, in which oral transfer of a particular *Lactobacillus* strain promoted Th17 differentation and protected from diabetes [53]. Furthermore, injection of Th17 polarized cells from CFA-injected NOD mice delayed diabetes in NOD/severe combined immunodeficiency (SCID) recipients in a manner that depended, at least in part, on IL-17 [54].

The role of Th17 cells in diabetes has also been addressed using adoptive transfer of TCR transgenic T cells specific for pancreatic antigen. Highly purified Th17-polarized BDC2·5 T cells were capable of inducing diabetes, but appeared to achieve this by differentiating further to a Th1 phenotype [55,56]. Indeed, the ensuing disease could be inhibited by antibodies to IFN-γ but not IL-17 [55,56]. Conversely, two reports documented the ability of IFN-$\gamma^{-/-}$ Th17 cells to transfer diabetes successfully [57,58], arguing against a requirement for a Th1 transition. Interestingly, if T cells expressing a different pancreatic antigen-specific TCR (BDC6·9) are used, far less Th17 to Th1 conversion is observed, yet diabetes is still induced [58]. Thus, individual T cell clones may differ in the cytokines they use to elicit disease, perhaps depending upon the affinity of their TCR–antigen interactions.

Taken together, the murine studies to date suggest that although IL-17 is up-regulated in the early stages of diabetes development [58,59], it does not necessarily follow that this cytokine, or indeed the Th17 subset, is necessary for disease.

Th17 cells in humans with type 1 diabetes

Several studies have indicated an increase in T cell IL-17 production in humans with T1D, especially in the very early stages of disease. Children with new-onset and long-standing T1D (mean age 8·7 years) were shown to have more IL-17-positive T cells compared with age-matched non-diabetic controls [60]. In a separate study, children within 6 months of T1D diagnosis (mean age 9·6 years) were shown to exhibit increased IL-17 secretion from both CD4 and CD8 T cells [61]. Both IL-6 and IL-1β can promote Th17 development [62,63], so the demonstration that monocytes from T1D patients expressed elevated levels of mRNA for IL-6 and IL-1β provided a potential explanation for increased IL-17 production [64]. However, this has not been observed universally [60], and may depend upon the demographic of the cohort. A separate analysis of first-degree relatives of T1D patients showed that monocytes from those who were autoantibody-positive produced more IL-1β and less IL-6 in response to TLR ligation compared with those from seronegative individuals. IL-1β plays multiple roles in autoimmune islet infiltration, and in addition to promoting IL-17 production can also directly modify beta cell survival and function [65].

A key challenge associated with studying T cell differentiation in diabetes patients is the limitation of focusing only on peripheral blood samples. In an impressive attempt to circumvent this problem, Ferraro and colleagues studied T cells isolated from the pancreatic lymph nodes of T1D patients undergoing pancreas or pancreas/kidney transplant. These were compared with pancreatic lymph nodes from non-diabetic donors. Careful analysis of T cell cytokine production and chemokine receptor profiles established that the pancreatic lymph nodes of type 1 diabetic subjects had a higher frequency of Th17 cells [66]. More recently, an increase in the frequency of $IL\text{-}17^+$ cells was found in the peripheral blood of adult T1D patients when gating on $CD45RA^-CCR6^+$ population of CD4 T cells [67]. Thus, several lines of evidence point to an increase in IL-17 production in the T1D setting.

Possible role for Th1/17 cells in type 1 diabetes?

One area worthy of note in considering the contribution of Th17 cells to T1D is the role of cells with a propensity to make both IL-17 and IFN-γ (sometimes called Th1/17 cells). Cells co-producing IL-17 and IFN-γ were identified originally in the gut of patients with Crohn's disease [68] and were shown subsequently to be present in the mouse colon in an adoptive transfer model of intestinal inflammation [69]. Fate mapping experiments in mice established that in autoimmune settings, IL-17-positive T cells can initiate IFN-γ production leading to the presence of a substantial number of T cells co-expressing both cytokines [70]. A closer analysis of the literature reveals hints that cells co-producing IFN-γ and IL-17 may also be present in the T1D setting. By measuring IFN-γ transcripts within sorted IL-17-producing cells, Reinert-Hartwall *et al.* [71] found an increased propensity of IL-17+ cells to make IFN-γ in children with T1D (mean 8·3 years) compared with healthy controls. Interestingly, this phenomenon was even more striking in children who had not developed diabetes but exhibited advanced beta cell autoimmunity and

impaired glucose tolerance (mean age 7·7 years) [71]. Consistent with this theme, a separate study of IL-17 production in the T1D setting found that T cells co-producing IL-17 and IFN-γ were present in four of 11 of the diabetic children examined [60]. In addition, when T cells from human pancreatic lymph nodes were examined, there was a suggestion that IFN-γ as well as IL-17 expression was up-regulated in those deriving from T1D patients, although the results did not reach statistical significance [66]. Finally, both IL-17 and IFN-γ were up-regulated at the mRNA level within the pancreatic islets of an individual who died within 5 days of T1D diagnosis [72]. Thus, the presence of T cells that co-produce IL-17 and IFN-γ could potentially be a feature of T1D, and this area may warrant further investigation (Fig. 2b).

IL-21 production in type 1 diabetes

IL-21 is required for autoimmune diabetes in mice

IL-21 is familiar to the diabetes community as a candidate gene at the diabetes susceptibility locus Idd3 [73–75], and levels of IL-21 mRNA have been shown to increase during diabetes development in mice [76–78] (Table 1). IL-21 is a member of the common-γ chain receptor family of cytokines that includes IL-2, IL-4, IL-7, IL-9 and IL-15. Its receptor is a heterodimer, comprising the common γ-chain and an IL-21Rα subunit, which is broadly expressed on a wide range of haematopoietic cell types. It came as something of a surprise when two groups reported that IL-21 signalling was required critically for diabetes in NOD mice [78,79]. In one report, fewer than 10% of IL-21R$^{-/-}$ NOD had developed diabetes by 35 weeks [79], while in the other none of the IL-21R$^{-/-}$ NOD animals were diabetic even at 60 weeks of age, a time-point at which > 90% of IL-21R-sufficient animals had developed disease [78].

The timing of these reports coincided with the discovery that IL-21 can enhance Th17 differentiation and can itself be produced by Th17 cells to exert feedback in an autocrine fashion [80–82]. This prompted the question of whether a defect in Th17 differentiation might underlie the lack of diabetes in IL-21R$^{-/-}$ mice. Support for such a notion came from the observation that decreased numbers of IL-17-producing T cells were detected in IL-21R $^{-/-}$ mice in one study [79]. However, in the other study [78], IL-17-producing T cells were slightly increased, and the amount of IL-17 following *in-vitro* restimulation was actually slightly higher in IL-21R$^{-/-}$ mice, arguing against a reduction in IL-17 production being responsible for disease protection. Therefore, the role of IL-21 in the development of diabetes appeared to be more than just an effect on Th17 differentiation.

Possible roles for IL-21 in autoimmune diabetes

If IL-21 did not exert its pro-diabetogenic effects by IL-17 up-regulation, how else could its ability to promote disease be explained? The answer to this critical question is not yet elucidated fully. It seems unlikely that IL-21 acts directly on pancreatic beta cells, as they appear to lack expression of the IL-21 receptor [78]. However, the presence of IL-21 local to beta cells is sufficient to trigger the cascade necessary for diabetes induction, even in non-autoimmune prone C57BL/6 mice [78]. Accordingly, expression of IL-21 under the human insulin promoter elicited spontaneous diabetes in ∼80% of mice, with substantial islet infiltration by CD4 T cells and macrophages as well as dendritic cells (DC) and B cells [78]. Forced expression of IL-21 was therefore sufficient to trigger islet infiltration – but is IL-21 required for spontaneous islet infiltration occurring in the absence of transgenic over-expression? The answer appears to be yes, as mice deficient in IL-21 signalling were virtually devoid of inflammatory infiltration in the islets [78,79]. Furthermore, short-term blockade of the IL-21 pathway appeared to reverse established insulitis in NOD mice, resulting in a significantly reduced number of lymphocytes with the islet lesion [83].

Which cell is the key target for the pro-diabetogenic effects of IL-21? IL-21 is an extraordinarily pleiotropic

Table 1. Interleukin (IL)-21 production at the site of the autoimmune attack in mouse models of type 1 diabetes. Table shows data relating to IL-21 expression in mouse models of diabetes.

Publication	Findings	Mouse model
Clough *et al.* [77]	Increased IL-21 mRNA in pancreatic lymph nodes of diabetic compared with non-diabetic mice	DO11 × rip-OVA
Sutherland *et al.* [78]	Increased IL-21 mRNA were in the pancreas of diabetic compared with non-diabetic mice	NOD
McGuire *et al.* [84]	Enrichment of IL-21-producing T cells within the pancreas compared with peripheral lymphoid organs. Fewer IL-21^{+} T cells were observed in the pancreas of NOD mice bearing the B6 Idd3 region	NOD
Kenefeck *et al.* [106]	Enrichment of IL-21-producing T cells within the pancreas compared with peripheral lymphoid organs. The IL-21-producing T cells co-express TNF-α and IFN-γ but not IL-17	DO11 × rip-OVA

NOD = non-obese diabetic; OVA = ovalbumin; IFN = interferon; TNF = tumour necrosis factor.

cytokine with the capacity to act on a broad array of cell types, including CD4 and CD8 T cells, natural killer (NK) cells, B cells, macrophages and DC (Fig. 3). In this respect, McGuire *et al.* [84] found that when diabetes was induced in NOD/SCID mice by adoptive transfer of CD4 and CD8 T cells, it was the CD8 T cells that required IL-21R in order for diabetes to develop. Loss of IL-21 sensitivity in the CD4 compartment led to only a partial reduction in diabetes incidence. In contrast, a similar experiment by Van Belle *et al.* [85] concluded that CD4 T cells were the obligate targets of IL-21, with IL-21 responsiveness in CD8 T cells having only a partial affect. While, at first sight, these findings are hard to reconcile, it is probably reasonable to conclude that IL-21 can act on both CD4 and CD8 T cells to promote diabetes, with the relative importance of each axis being dependent upon the precise experimental context.

The effects of IL-21 on the CD4 compartment include the promotion of cell survival, as illustrated by the increased sensitivity of IL-21R$^{-/-}$ T cells to activation-induced cell death [85]. In addition, IL-21 may act on macrophages, increasing their capacity to stimulate CD4 T cell proliferation [86]. A further manner in which IL-21 may contribute to autoimmunity is by imparting resistance to T_{reg} suppression. Several investigators have shown that IL-21 is able to counteract the suppressive function of T_{reg} [77,87] and that this requires the IL-21 to act on conventional CD4 T cells [85,88], rather than the T_{reg} themselves. Interestingly, resistance to T_{reg} suppression has been reported in both mice [77] and humans with T1D [89,90], although many other factors in addition to IL-21 are likely to contribute to this effect [91].

IL-21 is also known to play key roles in orchestrating T cell : B cell interactions, and this may be relevant in the light of the possible contribution of B cells to diabetes pathogenesis [92–94]. IL-21 can promote the formation of antibody-producing plasma cells [95,96], and can also instruct germinal centre B cell development by up-regulating expression of the transcription factor B cell lymphoma 6 protein (Bcl6) [97,98]. It was shown recently that IL-21 can up-regulate B cell CD86 expression [99] which, in turn, can influence the homeostasis of follicular helper T cells (Tfh) [100] (see later) and facilitate further B cell stimulation. IL-21 could therefore conceivably contribute to diabetes development by acting on the B cell compartment. Interestingly, recent data suggest that an alteration at the IL-2/IL-21 locus that confers increased risk for T1D is associated with decreased production of IL-10 in memory B cells [101]. Thus, there are likely to be additional IL-21-dependent control points relevant to diabetes pathogenesis that are not yet elucidated fully.

In an interesting development, it has also been shown that IL-21 can act on DCs to influence their maturation

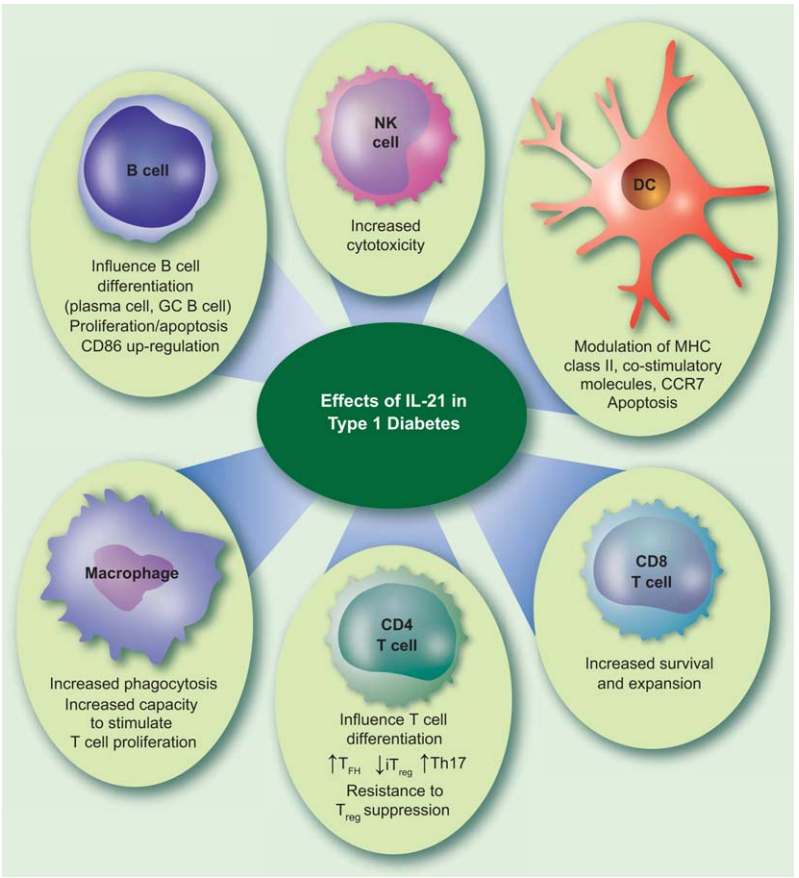

Fig. 3. Potential effects of interleukin (IL)-21 on immune cells in type 1 diabetes. IL-21 is a highly pleiotropic cytokine and could potentially act on several different cell types in the context of type 1 diabetes development (see text for references).

and migration. In a virus-induced diabetes model, pancreatic DCs required IL-21R signals to acquire CCR7 and MHC class II and migrate to the draining lymph node [85]. Indeed, disease resistance associated with IL-21R deficiency could be overcome by the adoptive transfer of IL-21R-sufficient DC [85]. This contrasts with the capacity of IL-21 to inhibit DC maturation *in vitro* [102], and suggests that its effects may be highly context-dependent. In this regard, it has emerged that IL-21 is a potent inducer of DC apoptosis, an effect that is reversed in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF) [103]. This is reminiscent of the situation in B cells, where IL-21 can induce either activation or apoptosis depending on the context [104,105], and suggests that the capacity of IL-21 to influence DC biology may be far greater that appreciated previously.

IL-21 in human T1D

It has been found recently that CD4 T cells from T1D patients produce higher levels of IL-21 in response to *in-vitro* restimulation than T cells from non-diabetic individuals [67,106]. Work by Kenefeck *et al.* [106] focused on adult patients, with a mean age of 37, while analysis by Ferreira and colleagues [67] also included children (median age 14, range 6–42 years). Importantly, both studies normalized their analysis to the memory cell population to avoid variation in the frequency of memory T cells affecting their results. Interestingly, the IL-21-producing T cells in T1D patients co-expressed high levels of IFN-γ and TNF-α [106] (Fig. 2c). T cells co-expressing IL-21, IFN-γ and TNF-α were also detected infiltrating the pancreas in a TCR transgenic mouse model of diabetes [106].

In a separate study, mRNA levels of IL-21 were found to be higher in total CD4 T cells from T1D patients [107], although Kenefeck *et al.* found no significant difference in IL-21 mRNA levels, even when homing in on the memory T cell population [106]. It is possible that IL-21 production is more pronounced at the earlier disease stages, as the former study [107] examined individuals within 2 years of onset while most participants in the latter study [106] were long-standing diabetes patients. In all three studies, the increase in IL-21 was observed in bulk CD4 T cells and the authors did not attempt to identify autoantigen-specific cells. The changes in IL-17 expression in T1D discussed earlier [60,66,71] were also detected in polyclonal T cell populations. Conceivably, the factors that influence the propensity of T cells to produce a particular cytokine may act on all CD4 T cells, rather than solely those responding to islet antigens; however, this issue warrants further investigation.

Taken together, analysis of mouse models has clearly demonstrated that the loss of IL-21 signalling inhibits diabetes development while, conversely, its local production is sufficient to initiate immune-mediated islet destruction. The over-production of IL-21 in T1D patients highlights this cytokine as worthy of further investigation in respect of disease pathogenesis in humans.

Follicular helper T cells in T1D

Expansion of Tfh in mice and humans with autoimmune diabetes

Given the link between IL-21 production and diabetes pathogenesis, a key question becomes what is the identity of the IL-21-producing cell? IL-21 is the signature cytokine for Tfh, the subset that specializes in providing help for B cell antibody production [108,109]. In this regard, a recent unbiased microarray analysis of T cells responding to islet antigen in the pancreatic lymph node of mice revealed a striking signature of Tfh differentiation [106]. The top 20 most significantly up-regulated genes in T cells responding to islet antigen included four archetypal Tfh genes (CXCR5, PD-1, IL-21, Bcl6), and flow cytometry analysis demonstrated that T cells with a Tfh phenotype were over-represented in the pancreatic lymph nodes [106]. Tfh are so-named due to their capacity to enter the B cell follicles of secondary lymphoid tissues, where they initiate the formation of germinal centres. These are specialized structures where B cells mutate their immunoglobulin molecules, so that those with higher affinity for antigen can be selected to enter the long-lived plasma cell and memory B cell pools. The capacity of B cells to solicit help from Tfh within the germinal centre is a key factor in the selection procedure. Importantly, germinal centres could be demonstrated in the pancreatic lymph nodes (LN) of diabetic mice by confocal microscopy [106], consistent with the presence of a functional Tfh population.

The finding that diabetes was associated with Tfh differentiation in mice prompted an analysis of Tfh cells in the peripheral blood of humans with T1D. Within the memory pool, the % $CXCR5^+$ and $CXCR5^+$inducible T cell co-stimulator $(ICOS)^+$ T cells was found to be significantly higher in individuals with T1D [106]. Consistent with this, an increase in peripheral blood T cells with a Tfh phenotype, has also been reported in two independent cohorts of T1D patients, one of which comprised exclusively new onset patients (within 2 years' diagnosis, mean age 23) [107], while the other included individuals with disease duration ranging from 2 to 20 years (median age 32) [67]. Thus, in both mouse models and in humans, an expansion of cells with a Tfh phenotype appears to be a feature of autoimmune diabetes (Table 2). Regarding the issue of whether Tfh might represent the source of IL-21 in T1D, both Kenefeck *et al.* and Ferreira *et al.* demonstrated a highly significant correlation between the frequency of Tfh and the frequency of $IL\text{-}21^+$ T cells [67,106], providing strong support for such a notion. However, as IL-21 can also be produced by other T cells, including Th17 cells

Table 2. Link between follicular helper T cells (Tfh) differentiation and autoimmune diabetes. Table collating some of the evidence that suggests Tfh differentiation may be a feature of autoimmune diabetes in mice and humans.

Mouse	Human
Increase in Tfh genes in microarray of T cells responding to islet antigen in the pancreatic LN [106]	Increased production of IL-21 after *ex-vivo* stimulation of memory T cells from T1D patients compared with matched controls [67,106]
Increased Tfh differentiation due to roquin mutation exacerbates diabetes [137]	Increased IL-21 mRNA in memory T cells from new onset T1D patients compared with matched controls [107]
CXCR5-enriched T cells preferentially induce diabetes upon adoptive transfer [106]	Increased numbers of cells with a Tfh phenotype in blood of T1D patients compared with controls [67,106,107]. Correlation between frequency of Tfh and IL-21$^+$ T cells [67,106]

IL = interleukin; T1D = type 1 diabetes; LN = lymph node.

[81,82], and immunosuppressive Tr1 cells [110], the contribution of non-Tfh populations cannot be excluded.

The precise relationship between CXCR5$^+$ T cells in the blood and bona fide Tfh cells remains controversial [111]. There is now good evidence that Tfh can become circulating memory cells [112–115], but in so doing they down-regulate many of their characteristic Tfh markers, although these can be regained upon antigen re-encounter [116]. Interestingly, one study showed that Tfh that lose their phenotype after antigen deprivation retain intermediate levels of CXCR5 [117], suggesting that this marker may offer the best chance to track such cells. The presence of blood-borne Tfh in SAP-deficient mice and humans suggests that they arise prior to intimate T cell : B cell interactions within the Germinal Center [118], and while it is technically possible for bona fide Tfh to exit the GC to enter the circulation, this appears to be a rare event [119]. Thus, the CXCR5$^+$ cells in the circulation of T1D patients may derive from pre-Tfh cells that have bifurcated from those entering the GC, choosing instead to commit to a memory pathway. The generation of blood-borne Tfh-phenotype cells prior to the development of GC [118] is consistent with recent data highlighting that the homeostasis of this population in LN and blood can be strikingly different [120]. Despite the many ongoing controversies, it is clear that the blood-borne CXCR5$^+$ fraction, while heterogeneous, contains circulating Tfh memory cells that can traffic to B cell follicles of secondary lymphoid tissues and contribute to GC reactions [120].

Tfh and autoantibody status

The presence of autoantibodies is a key predictor of diabetes development in at-risk individuals, with the number of antibodies and the timing of their appearance being particularly telling [121,122]. The key role of Tfh in class-switching and affinity maturation is consistent with a role for these cells in autoantibody production. Intriguingly, one study focusing on newly diagnosed patients identified a small difference in the percentage of Tfh cells between individuals that were either positive or negative for certain autoantibodies. Tfh numbers appeared to be independent of glutamate decarboxylase (GAD) autoantibody status, but were higher in those deemed positive for ZnT8 or IA-2 autoantibodies compared with autoantibody-negative individuals [107]. The relationship between circulating cells with a Tfh phenotype and the emergence of autoantibodies will be important to elucidate fully in future studies. Given the role of Tfh in honing the quality of the B cell response, it is noteworthy that autoantibodies to islet antigens frequently exhibit affinity maturation [123] and, indeed, the presence of high-affinity autoantibodies may help to identify those individuals most likely to progress from at-risk status to overt diabetes [124–126]. It is possible that measuring peripheral blood Tfh, as well as autoantibodies, could be useful in at-risk individuals and might provide additional power to predict progression to overt disease.

IL-2 signalling impairs Tfh differentiation

One notable aspect of Tfh biology is that IL-2 signalling is known to impair Tfh differentiation. Accordingly, signals generated by the IL-2 receptor during early T cell activation can influence the balance between Tfh differentiation and other effector T cell fates [127,128] via signal transducer and activator of transcription (STAT)-5-dependent skewing of the Bcl6/PR domain zinc finger protein 1 (BLIMP1) ratio [129]. Elegant experiments have used MHC class II tetramers to home in on antigen-specific T cells following influenza infection in mice and have shown that IL-2 administration selectively decreases the number of Tfh cells (CXCR5$^+$PD1$^+$), but not other effector T cells (CXCR5$^-$PD1$^-$) [130]. Conversely, it has been shown that under conditions of IL-2 deprivation, Th1 cells can acquire a Tfh phenotype [131]. Thus, the availability of IL-2 in the local environment has significant consequences for the development and homeostasis of the Tfh population. These findings may be relevant to the observed increase in Tfh in type 1 diabetes, as multiple defects in the IL-2 signalling pathway have been associated with this disease setting [132–136]. Interestingly Kenefeck *et al.* found an inverse relationship between the ability of T cells from type 1 diabetes patients to respond to IL-2 and propensity to acquire a Tfh phenotype *in vitro* [106]. It is therefore possible that suboptimal IL-2 signalling could contribute to increased Tfh differentiation in T1D.

Could Tfh be responsible for driving disease?

Whether Tfh cells are directly responsible for autoimmune pathology is hard to assess in patients; however, data from mouse models provides support for such a notion. In a TCR transgenic diabetes model, a mutation in *Roquin* leading to dysregulated Tfh generation dramatically accelerated diabetes development [137]. In a second TCR transgenic model, based on a different pancreatic antigen and different transgenic T cells, enriching for T cells with a Tfh phenotype increased their capacity to cause diabetes upon adoptive transfer [106]. Anecdotal evidence also links the Tfh response with diabetes in NOD mice: the spontaneous formation of germinal centres in NOD spleen has been documented [138], and germinal centres have even been detected within the lymphoid mass infiltrating the pancreas itself [139,140].

Although IL-21 is the characteristic cytokine associated with Tfh cells, they are also known to be capable of producing other cytokines, including IFN-γ, which could explain the association of this cytokine with T1D [112,141]. Indeed, human Tfh isolated from lymph nodes of chronically HIV-infected subjects were shown to be capable of substantial co-production of cytokines, including IL-21, TNF-α and IFN-γ [142]. Several studies have implicated persistent antigen presentation in Tfh differentiation [117,143], a notion that might fit with the inability of self-antigens to be cleared in autoimmune settings. Interestingly, Tfh differentiation is subject to regulation by a number of pathways that are linked genetically to autoimmunity, including the CD28/cytotoxic T lymphocyte antigen (CTLA)-4 axis [144–150], IL-2 [127–130] and the lymphoid-specific tyrosine phosphatase (LYP) encoded by protein tyrosine phosphatase, non-receptor type 22 (PTPN22) [151]. The link between these loci and disease initiation is complex, but modulation of Tfh differentiation adds an additional consideration to the other known roles of the candidate genes in these locations.

Concluding comments

The complexities of CD4 T cell differentiation are still emerging, but we have clearly moved beyond the era of a simple Th1/Th2 dichotomy. Recent data suggest that early T cell differentiation is likely to be even more diverse than was appreciated previously [152], with a broad selection of functional phenotypes being generated, the most useful of which are then expanded selectively. Moreover, intermediate differentiation states exist, including a Tfh-like transitional stage shared by Tfh and Th1 cells [153], and phenotypical conversions are possible, such as the switch from Th1 to Tfh under conditions of limiting IL-2 [131]. These developments argue for a more nuanced view of T cell differentiation in type 1 diabetes that does not focus solely on Th1 cells, but also encompasses the possible involvement of IL-21-producing T cells such as Tfh, as well as T cells co-producing IFN-γ and IL-17.

The demonstration of an augmented Tfh population in type 1 diabetes [67,106,107] is in line with a growing appreciation that Tfh differentiation is a feature of several autoimmune diseases ([154] and reviewed in [155]). Increasingly refined genetic analysis suggests that T1D may be more similar to other autoantibody-positive diseases, such as juvenile idiopathic arthritis and rheumatoid arthritis, than to conditions lacking characteristic autoantibodies, such as ulcerative colitis and Crohn's disease [156]. This is clearly consistent with the potential involvement of Tfh cells in the underlying immunological processes.

Tfh cell numbers in the peripheral blood are being linked increasingly with protective anti-viral immunity [157–159]. This is interesting, given the long-standing debate regarding the putative contribution of viral infection to diabetes initiation [160,161]. One could envisage that evolutionary selection for characteristics that confer an advantage in infectious settings might have also influenced susceptibility to autoimmunity in parallel.

One potential benefit of broadening our perception of T cell differentiation in diabetes beyond the simple Th1 paradigm is the prospect of identifying new disease biomarkers (Fig. 4). Exploration of CXCR5, or other markers of circulating Tfh-like cells, may present new opportunities for assessing diabetes risk, tracking disease progression or gauging response to therapeutic interventions. We envisage that an increasingly refined understanding of the CD4 T cell population in type 1 diabetes will help us to monitor

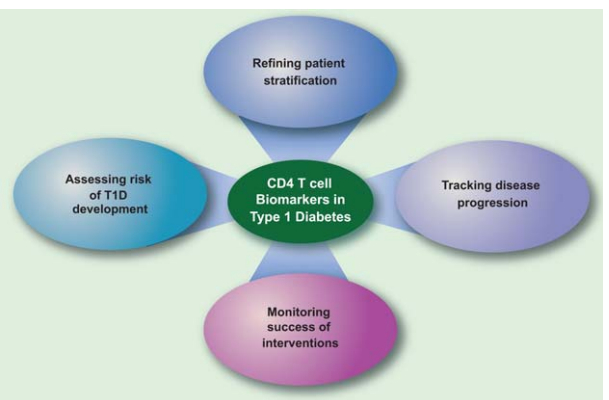

Fig. 4. Potential utility of biomarkers arising from a better understanding of the T cell phenotype in type 1 diabetes (T1D). Surface markers and/or secreted products from CD4 T cells can potentially be used to gauge the risk of T1D development, in conjunction with established risk indicators. They may also be used to refine patient stratification, perhaps selecting groups that might be predicted to benefit from a particular immune intervention. Longitudinal studies may reveal whether particular markers can be used to stage the disease process. Phenotypical markers may also be of utility in assessing the efficacy of therapeutic interventions, perhaps in combination with tetramer technology.

the autoimmune response and ultimately deploy effective immunomodulatory strategies in this disease setting.

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Disclosure

L.S.K.W. declares no conflicts of interest. M.v.H. declares a commercial interest in developing IL-21 blockade reagents at NovoNordisk.

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