CD4 T cell differentiation in Type 1 Diabetes

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Abstract

Susceptibility to type 1 diabetes is strongly associated with HLA genes, implicating T cells in disease pathogenesis. In humans, CD8 T cells predominantly infiltrate the islets, yet their activation and propagation likely 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 1 (Th1) and T-helper 2 (Th2) responses, with type 1 diabetes being mainly viewed 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 IL-17 production in type 1 diabetes and discuss emerging evidence for 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.

Multiple lines of evidence support key roles for both CD4 and CD8 T cells in the immune response that drives T1D. The primary 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 recently been shown that SNPs associated with type 1 diabetes and other autoimmune diseases are preferentially enriched 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 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 Th1 paradigm

Early models of CD4 T cell differentiation were based on a simple dichotomy between IFN-γ-dominated Th1 responses and 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 defense against helminth infection, and are associated with allergic disorders (e.g. asthma, rhinitis, eczema) involving IgE, 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 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 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 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 upregulating expression of MHC class I and II, facilitating macrophage activation, and increasing leukocyte 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 signaling 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 signaling-1 (SOCS1)[17] and islet expression of SOCS1 was found to be protective in the RIP-LCMV mouse model of diabetes [18].

Both IFN-γ-/- [16] and IFN-γR-/- [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 intensively studied in humans with T1D. Analysis of peripheral blood T cells from newly diagnosed adults (av. age ~29yr, av. disease duration ~5wk) 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 repsonse, whereas nondiabetic 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 28.5yr, average diabetes duration 7 months): interestingly first degree relatives also showed autoantigen specific responses that were characterised 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 levels of IFN-γ mRNA were significantly higher in new onset T1D patients (av. age ~15yr, av. diabetes duration 80 days) compared with an age-matched at risk cohort [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-mediated rather than 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 did 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 originally anticipated, 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 CFA to protect NOD mice from diabetes, traditionally assumed to reflect IL-4 or IL-10 production, was largely dependent on IFN-γ [36]. Collectively these data questioned the

traditional view that diabetes was caused by Th1 cells making IFN-γ and suggested 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 other 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 RANTES and MIP-1α were elevated, so too were factors considered indicative of a Th2 response such as IL-4, IL-5 and IL-10 [41]. Likewise, 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 type 1 diabetes 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 type 1 diabetes.

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 process [48, 49] however it was subsequently shown 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-colonised 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 strongly upregulated in SFB+ animals whereas transcripts associated with Th1, Th2 and Treg cells were unchanged [51]. These data are clearly more consistent with a role for IL-17, or other Th17 cell products, in delaying rather that 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 polarised cells from CFA-injected NOD mice delayed diabetes in NOD/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-polarised BDC2.5 T cells were capable of inducing diabetes but appeared to achieve this by further

differentiating to a Th1 phenotype [55, 56]. Indeed the ensuing disease could be inhibited by antibodies to IFN-γ but not IL-17 [55, 56]. On the other hand, two reports documented the ability of IFN-γ-/- Th17 cells to successfully transfer diabetes [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 on the affinity of their TCR-antigen interactions.

Taken together, the murine studies to date suggest that although IL-17 is upregulated 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 longstanding 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 universally observed [60] and may depend on the demographic of the cohort. A separate analysis of first-degree relatives of T1D patients showed that monocytes from those that 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 nondiabetic 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-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 originally identified in the gut of patients with Crohn's Disease [68] and were subsequently shown 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 be present in the T1D setting too. 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 age 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 4 out 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 upregulated in those deriving from T1D patients, although the results did not reach statistical significance [66]. Finally, both IL-17 and IFN-γ were upregulated 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 signaling was critically required for diabetes in NOD mice [78, 79]. In one report less 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 timepoint 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

So if IL-21 did not exert its pro-diabetogenic effects by IL-17 upregulation, how else could its ability to promote disease be explained? The answer to this critical question is not yet fully elucidated. It seems unlikely that IL-21 acts directly on pancreatic beta cells since 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 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 overexpression? The answer appears to be yes since mice deficient in IL-21 signaling 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 numbers 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 cytokine with the capacity to act on a broad array of cell types including CD4 and CD8 T cells, 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 on the precise experimental context.

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 Treg suppression. Several investigators have shown that IL-21 is able to counteract the suppressive function of Treg [77, 87] and that this requires the IL-21 to act on conventional CD4 T cells [85, 88] rather than the Treg themselves. Interestingly resistance to Treg suppression has been reported in both mice [77] and humans with type 1 diabetes [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 center B cell development by upregulating expression of the transcription factor Bcl6 [97, 98]. It was recently shown that IL-21 can upregulate B cell CD86 expression [99] which in turn can influence the homeostasis of follicular helper T cells (T_{FH}) [100] (see below) 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 fully elucidated.

In an interesting development, it has also been shown that IL-21 can act on dendritic cells to influence their maturation 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 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 previously appreciated.

IL-21 in human T1D

It has recently been found 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 6-42yr). 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 since 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 3 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 signaling inhibits diabetes development while conversely its local production is sufficient to initiate immune-mediated islet destruction. The overproduction 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 Type 1 Diabetes

Expansion of T_{FH} 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 follicular helper T cells (T_{FH}), 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 T_{FH} differentiation [106]. The top 20 most significantly upregulated

genes in T cells responding to islet antigen included 4 archetypal T_{FH} genes (CXCR5, PD-1, IL-21, Bcl6) and flow cytometry analysis demonstrated that T cells with a T_{FH} phenotype were overrepresented in the pancreatic lymph nodes [106]. Follicular helper T cells are so-named due to their capacity to enter the B cell follicles of secondary lymphoid tissues where they initiate the formation of germinal centers. 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 T_{FH} within the germinal center is a key factor in the selection procedure. Importantly germinal centers could be demonstrated in the pancreatic LN of diabetic mice by confocal microscopy [106], consistent with the presence of a functional T_{FH} population.

The finding that diabetes was associated with T_{FH} differentiation in mice prompted an analysis of T_{FH} cells in the peripheral blood of humans with T1D. Within the memory pool, the % CXCR5+ and CXCR5+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 T_{FH} phenotype has also been reported in 2 independent cohorts of T1D patients, one of which comprised exclusively new onset patients (within 2yr diagnosis, mean age 23) [107] while the other included individuals with disease duration ranging from 2-20yr (median age 32) [67]. Thus in both mouse models and in humans, an expansion of cells with a T_{FH} phenotype appears to be a feature of autoimmune diabetes (**Table 2**). Regarding the issue of whether T_{FH} 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 T_{FH} and the frequency of IL-21+ T cells [67, 106] providing strong support for such a notion. However since IL-21 can also be produced by other T cells,

including Th17 cells [81, 82], and immunosuppressive Tr1 cells[110], the contribution of non-T_{FH} populations cannot be excluded.

The precise relationship between CXCR5+ T cells in the blood and bona fide T_{FH} cells remains controversial [111]. There is now good evidence that T_{FH} can become circulating memory cells [112-115], but in doing so they downregulate many of their characteristic T_{FH} markers, although these can be re-gained upon antigen re-encounter [116]. Interestingly, one study showed that T_{FH} that lose their phenotype after antigen deprivation retain intermediate levels of CXCR5 [117], suggesting this marker may offer the best chance to track such cells. The presence of blood-borne T_{FH} in SAP-deficient mice and humans suggests that they arise prior to intimate T cell:B cell interactions within the GC [118] and while it is technically possible for *bona fide* T_{FH} 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-T_{FH} cells that have bifurcated from those entering the GC, choosing instead to commit to a memory pathway. The generation of blood borne T_{FH}-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, whilst heterogeneous, does contain circulating T_{FH} memory cells that can traffic to B cell follicles of secondary lymphoid tissues and contribute to GC reactions [120].

T_{FH} 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 T_{FH} 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 T_{FH} cells between individuals that were either positive or negative for certain autoantibodies. T_{FH} numbers appeared to be independent of 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 T_{FH} phenotype and the emergence of autoantibodies will be important to fully elucidate in future studies. Given the role of T_{FH} 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 T_{FH}, as well as autoantibodies, could be useful in at risk individuals and might provide additional power to predict progression to overt disease.

IL-2 signaling impairs T_{FH} differentiation

One notable aspect of T_{FH} biology is that IL-2 signaling is known to impair T_{FH} differentiation. Accordingly, signals generated by the IL-2 receptor during early T cell activation can influence the balance between T_{FH} differentiation and other effector T cell fates [127, 128] *via* STAT5-dependent skewing of the BCL6/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 shown that IL-2 administration selectively decreases the number of T_{FH} 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 T_{FH} phenotype [131]. Thus, the availability of IL-2 in the local environment has significant consequences for the development and homeostasis of the T_{FH} population. These findings may be relevant to the observed increase in T_{FH} in type 1 diabetes, since multiple defects in the IL-2 signaling 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 T_{FH} phenotype in vitro [106]. It is therefore possible that suboptimal IL-2 signaling could contribute to increased T_{FH} differentiation in T1D.

Could T_{FH} be responsible for driving disease?

Whether T_{FH} cells are directly responsible for autoimmune pathology is hard to assess in patients, however data from mouse models does provide support for such a notion. In a TCR transgenic diabetes model, a mutation in *Roquin* leading to dysregulated T_{FH} 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 T_{FH} phenotype increased their capacity to cause diabetes upon adoptive transfer [106]. Anecdotal evidence also links the T_{FH} response with diabetes in NOD mice: the spontaneous formation of germinal centers in NOD spleen has been documented [138] and germinal centers have even been detected within the lymphoid mass infiltrating the pancreas itself [139, 140].

Although IL-21 is the characteristic cytokine associated with T_{FH} 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 T_{FH} 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 T_{FH} differentiation [117, 143], a notion that might fit with the inability of self antigens to be cleared in autoimmune settings. Interestingly, T_{FH} differentiation is subject to regulation by a number of pathways that are genetically linked to autoimmunity, including the CD28/CTLA-4 axis [144-150], IL-2 [127-130] and the LYP tyrosine phosphatase encoded by PTPN22 [151]. The link between these loci and disease initiation is complex, but modulation of T_{FH} 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 previously appreciated [152], with a broad selection of functional phenotypes being generated, the most useful of which are then selectively expanded. Moreover, intermediate differentiation states exist, including a T_{FH}-like transitional stage shared by T_{FH} and Th1 cells [153], and phenotypic conversions are possible, such as the switch from Th1 to T_{FH} 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 T_{FH}, as well as T cells co-producing IFN-γ and IL-17.

The demonstration of an augmented T_{FH} population in type 1 diabetes [67, 106, 107] is in line with a growing appreciation that T_{FH} 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 potential involvement of T_{FH} cells in the underlying immunological processes.

T_{FH} cell numbers in the peripheral blood are increasingly being linked with protective antiviral immunity [157-159]. This is interesting given the longstanding 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 T_{FH}-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 the autoimmune response and ultimately deploy effective immunomodulatory strategies in this disease setting.

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Conflict of Interest Statements

LSKW declares no conflicts of interest. MvH declares a commercial interest in developing IL-21 blockade reagents at NovoNordisk.

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Figure Legends

Fig. 1. The original Th1 / Th2 paradigm in Type 1 Diabetes. Type 1 diabetes has traditionally been viewed 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.

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

Fig. 3. Potential effects of 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 manuscript text for references).

Fig. 4. Potential utility of biomarkers arising from a better understanding of the T cell phenotype in 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. Phenotypic markers may also be of utility in assessing the efficacy of therapeutic interventions, perhaps in combination with tetramer technology.

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

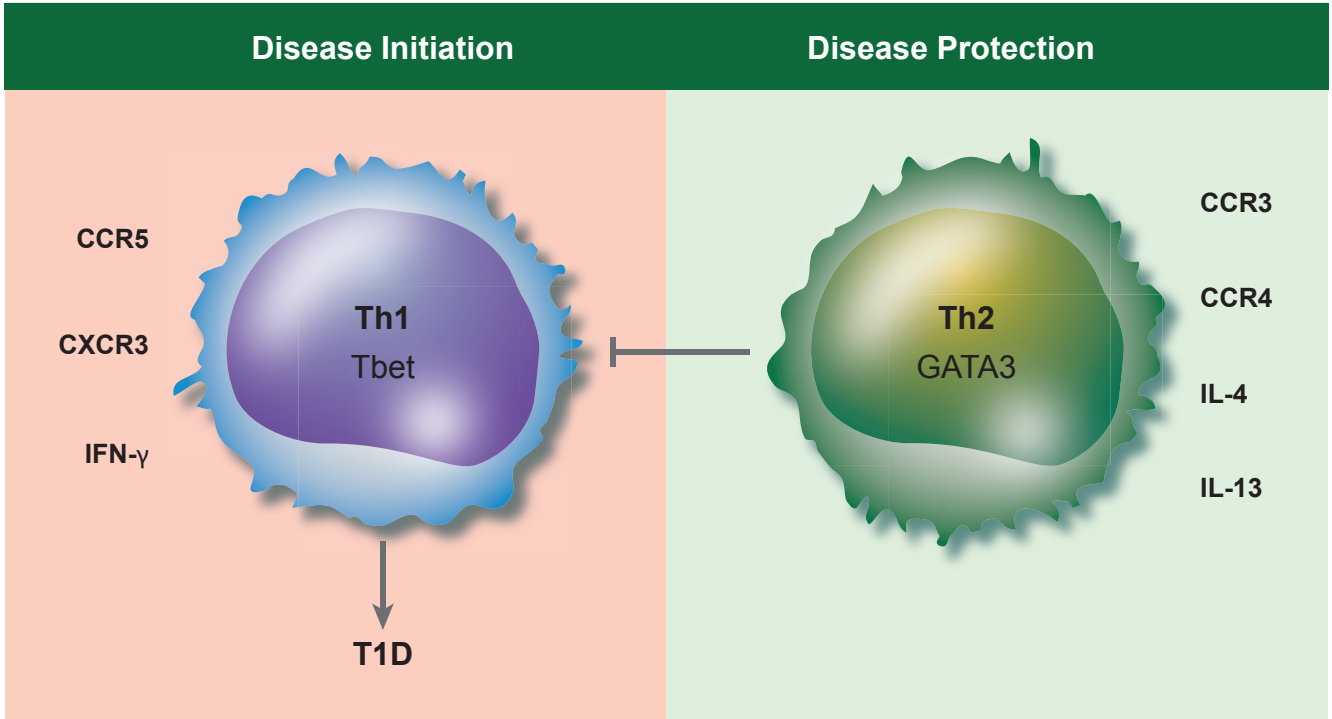
Table 2. Link between T_{FH} differentiation and autoimmune diabetes. Table collating some of the evidence that suggests T_{FH} differentiation may be a feature of autoimmune diabetes in mice and humans.

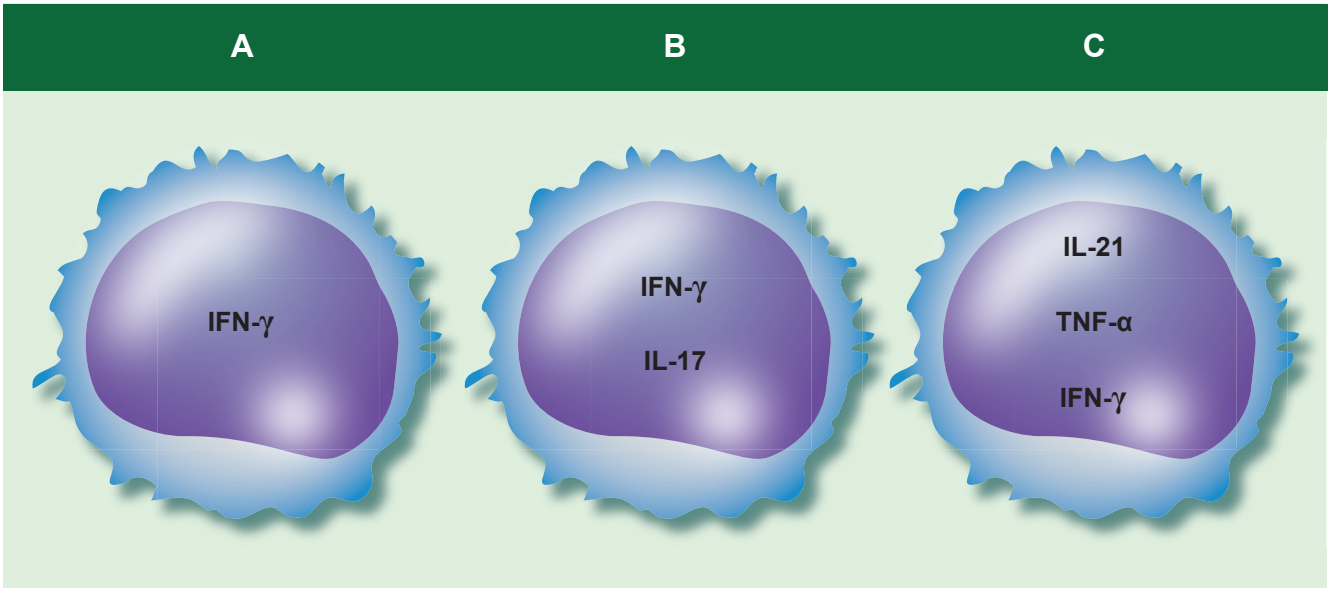
Publication	Findings	Mouse Model
Clough et al. (ref 77)	Increased IL-21 mRNA in pancreatic lymph nodes of diabetic compared with non-diabetic mice.	DO11 x rip-OVA
Sutherland et al. (ref 78)	Increased IL-21 mRNA were in the pancreas of diabetic compared with non-diabetic mice.	NOD
McGuire et al. (ref 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. (ref 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 x rip-OVA

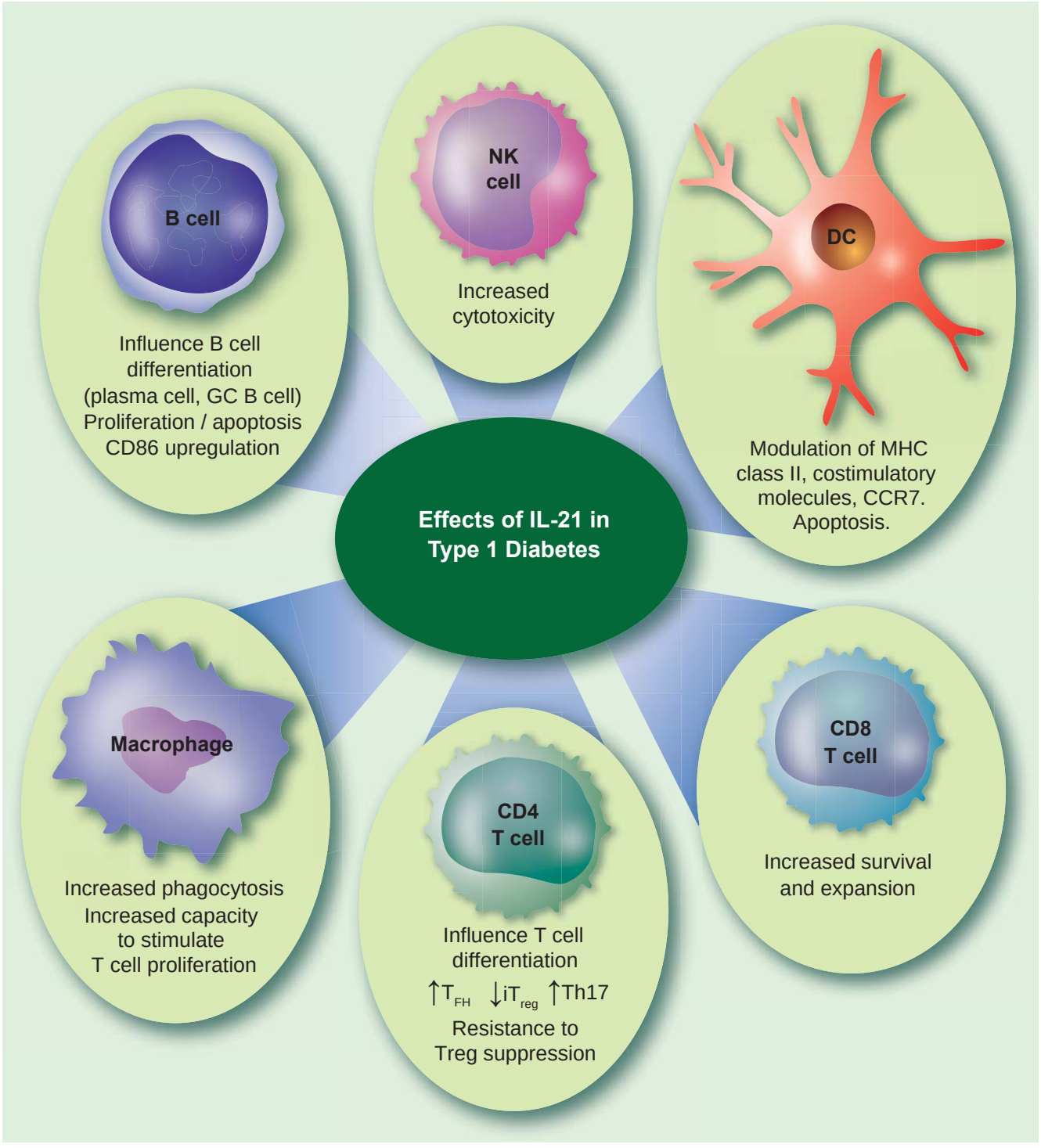
Table 1. IL-21 production at the site of the autoimmune attack in mouse models of type 1 diabetes.

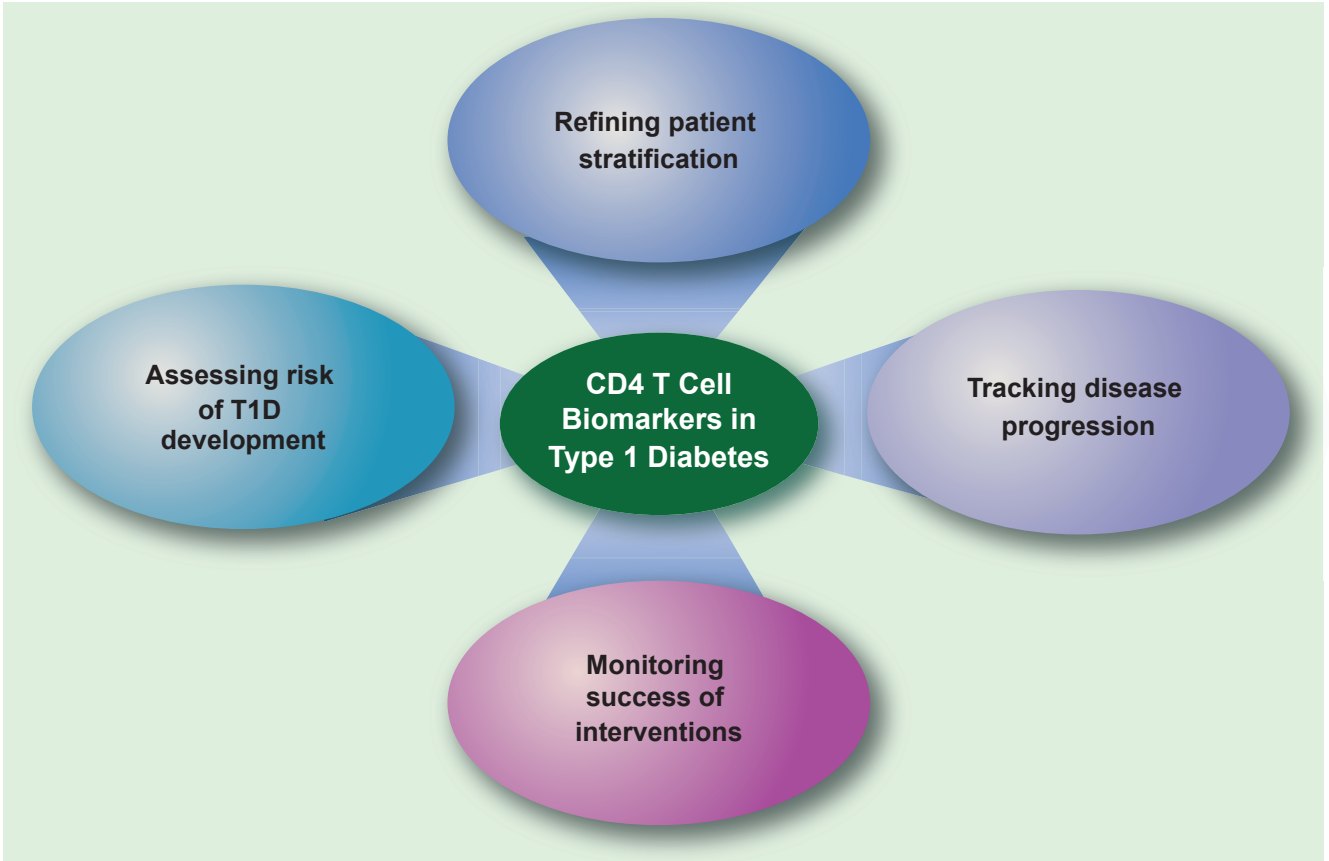
Mouse	Human
Increase in T_{FH} genes in microarray of T cells responding to islet antigen in the pancreatic LN (ref 106)	Increased production of IL-21 after *ex vivo* stimulation of memory T cells from T1D patients compared with matched controls (ref 106, 67)
Increased T_{FH} differentiation due to roquin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

Table 2. Link between T_{FH} differentiation and autoimmune diabetes.


Disease Initiation
Disease Protection
CCR5
CXCR3
IFN-γ
Th1
Tbet
T1D
Th2
GATA3
CCR3
CCR4
IL-4
IL-13


A
B
C
IFN-γ
IFN-γ
IL-17
IL-21
TNF-α
IFN-γ


Effects of IL-21 in Type 1 Diabetes
B cell
Influence B cell differentiation (plasma cell, GC B cell)
Proliferation / apoptosis
CD86 upregulation
NK cell
Increased cytotoxicity
DC
Modulation of MHC class II, costimulatory molecules, CCR7.
Apoptosis.
Macrophage
Increased phagocytosis
Increased capacity to stimulate T cell proliferation
CD4 T cell
Influence T cell differentiation
↑T_{FH} ↓iT_{reg} ↑Th17
Resistance to Treg suppression
CD8 T cell
Increased survival and expansion


Refining patient stratification
Assessing risk of T1D development
CD4 T Cell Biomarkers in Type 1 Diabetes
Tracking disease progression
Monitoring success of interventions