

# New insight into type 1 diabetes development: resolving early diabetogenic CD4<sup>+</sup> T cell responses that precede seroconversion

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Although it can arise at any age, type 1 diabetes (T1D) represents one of the most prevalent and severe chronic diseases in childhood and adolescence that results from the autoimmune-mediated destruction of insulin-producing  $\beta$  cells in the pancreatic islets of Langerhans causing insulin deficiency and hyperglycemia (1). There is currently no cure or prevention for T1D, leading to lifelong dependence on exogenous insulin therapy, which is essential in managing metabolic symptoms, but does not interfere with the underlying autoimmune process and is frequently associated with serious secondary complications such as retinal, renal, and neurological disease (2).

T1D represents a multifactorial disease caused by a combination of events, including inherited genetic susceptibility, as well as environmental and immunological factors (3). The strong genetic component of T1D is reflected by a major contribution of specific alleles and haplotypes of human leukocyte antigen (HLA) class II genes. Polymorphisms in multiple non-HLA genes (such as insulin, *PTPN22*, *CTLA4*, and *IL2RA*) some of which exert important functions in immune regulation, also contribute to T1D risk. In genetically susceptible individuals, manifestation of clinical T1D is a multi-step process that is precipitated by ill-defined environmental

factors (viruses, allergens, toxins, etc.) in conjunction with conditions of immune dysregulation [e.g., impaired function of Foxp3<sup>+</sup> regulatory T (Treg) cells], leading to the generation of pathogenic CD4<sup>+</sup> and CD8<sup>+</sup> T effector cells that mediate progressive  $\beta$  cell destruction. Notably, loss of self-tolerance and initiation of an autoimmune response to  $\beta$  cell antigen occurs well before clinical onset of T1D: the T cell-mediated selective loss of pancreatic  $\beta$  cell mass is preceded by an asymptomatic pre-clinical period of variable duration, which is characterized by the gradual emergence of a number of autoantibodies (AABs) with high affinity to  $\beta$  cell-associated self-antigens, such as insulin and 65 kDa glutamic acid decarboxylase (GAD65). AABs with  $\beta$  cell reactivity are thought to be dispensable for T1D pathogenesis, but represent valuable serum biomarkers for  $\beta$  cell autoimmunity prior to manifestation of clinical T1D and evaluation of disease risk in individuals with an inherited HLA predisposition (4,5). Importantly, naïve CD4<sup>+</sup>CD45RA<sup>+</sup> T cells with  $\beta$  cell reactivity (insulin, GAD65) can already be detected in cord blood of healthy neonates at frequencies that positively correlate with HLA-dependent genetic T1D susceptibility (6). However, the mechanisms underlying their inappropriate activation and differentiation into diabetogenic CD4<sup>+</sup> T effector cells, as

well as their relationship to  $\beta$  cell-specific B cells secreting high affinity AAbs has remained largely elusive. As the human pancreas (and thus pancreas-infiltrating immune cells) is not readily accessible, the study of immune events that precede the generation of CD4<sup>+</sup> T effector cells and AAbs reactive to  $\beta$  cell antigen is inevitably dependent on peripheral blood mononuclear cells (PBMC). Major challenges include the limited availability of PBMC samples from cohorts of individuals with high genetic T1D risk and exceedingly low frequencies of  $\beta$  cell-specific T cells among PBMCs.

Now, a recent study by Bonifacio and colleagues (7) has shed new light on early immunological events in T1D, suggesting that a unique subset of naïve CD4<sup>+</sup>CD45RA<sup>+</sup> T cells with  $\beta$  cell reactivity and an unusual proinflammatory T helper (T<sub>H</sub>)-like phenotype marks a latent phase of autoimmune susceptibility, which might be of diagnostic value well before the first appearance of memory CD4<sup>+</sup>CD45RO<sup>+</sup> T cells and seroconversion to multiple AAbs.

### Longitudinal tracking of $\beta$ cell-responsive CD4<sup>+</sup> T cells in at-risk children

Taking advantage of a collection of PBMC samples, which were regularly obtained from infancy through early childhood of at-risk individuals (family history and HLA susceptibility) and made available through the BABYDIET study (8), the authors had the rare opportunity to correlate quantitative and qualitative differences in  $\beta$  cell-specific CD4<sup>+</sup> T cell responses with the progression to  $\beta$  cell autoimmunity and overt T1D (i.e., before, at and after appearance of AAbs). Overall, the study encompassed longitudinal samples from 16 at-risk children who developed  $\beta$  cell-reactive AAbs during the observation period. For comparison, samples of 12 genetically susceptible children who remained free of any signs for  $\beta$  cell autoimmunity were also included.

Low frequencies of  $\beta$  cell-reactive CD4<sup>+</sup> T cells were identified among total PBMCs based on their proliferative capacity (dilution of a fluorescent dye) and acquisition of the early T cell activation marker CD25, upon *in vitro* re-stimulation with whole protein antigen (proinsulin, GAD65). This highly specific approach further allowed discriminating  $\beta$  cell-specific CD4<sup>+</sup> T cells with a naïve or memory phenotype based on differential surface expression of CD45RA and CD45RO, respectively. At 6 months of age, none of the 28 at-risk children initially included in

the study exhibited detectable memory CD4<sup>+</sup>CD45RO<sup>+</sup> T cells or AAbs to  $\beta$  cell antigen. However, in the cohort of children who developed  $\beta$  cell autoimmunity at later time points, the first appearance of  $\beta$  cell-specific AAbs closely correlated with the detection of  $\beta$  cell-reactive CD4<sup>+</sup> T cells that exhibited a CD45RO<sup>+</sup> memory phenotype. On the basis of their frequency and proliferation profile after *in vitro* re-stimulation with cognate self-antigen, the *in vivo* abundance of  $\beta$  cell-reactive CD4<sup>+</sup>RO<sup>+</sup> T cells was estimated to represent less than 0.1% of PBMC-derived CD4<sup>+</sup> T cells, underscoring the high sensitivity of this assay. In striking contrast, CD4<sup>+</sup>CD45RO<sup>+</sup> memory T cells and AAbs to  $\beta$  cell antigen remained below the detection limit in the control cohort. Hence, reminiscent of adoptive immune responses to foreign (i.e., non-self) antigens,  $\beta$  cell antigens appear to synchronously drive activation and differentiation of both CD4<sup>+</sup> T cells and AAb-producing B cells.

Consistent with the previous observation of  $\beta$  cell-specific, naïve CD4<sup>+</sup>CD45RA<sup>+</sup> T cells in healthy neonates (6), both AAb-positive and -negative cohorts of at-risk children were found to be largely comparable with regard to frequencies of  $\beta$  cell-specific CD4<sup>+</sup> T cells with a naïve CD45RA<sup>+</sup> phenotype, as judged by the up-regulation of CD25 expression and low, but clearly detectable proliferative responsiveness *in vitro* to proinsulin or GAD65 protein. Surprisingly, subsequent gene expression profiling revealed that many children who later on developed  $\beta$  cell autoimmunity exhibit a strong bias towards a rather unusual naïve  $\beta$  cell-reactive CD4<sup>+</sup> T cell phenotype with proinflammatory features well before the appearance of memory CD4<sup>+</sup> T cells and AAbs reactive to  $\beta$  cell antigen.

### Transcriptional signatures of diabetogenic CD4<sup>+</sup>T cell subphenotypes

Gene expression profiling at the single-cell level offers the opportunity to unravel inter-cellular transcriptomic variations within a defined cell population and to identify cell subsets involved in disease pathogenesis, which are otherwise obscured by the bulk analysis of those populations. Yet, despite recent technological advances, single cell transcriptomic has remained challenging, in particular, with small cell populations and rare clinical samples. To address these issues, Heninger *et al.* took advantage of a robust quantitative RT-PCR-based approach, employing fluorescence activated cell sorting-based purification of single  $\beta$  cell-responsive CD4<sup>+</sup> T cells in conjunction with multiplex primers, interrogating

the expression of a selected set of 19 mRNA transcripts encoding proteins (transcription factors, cytokines, surface markers, chemokine receptors) with well-defined roles in CD4<sup>+</sup> T cell-mediated effector function (e.g., IFN- $\gamma$ , TNF, IL-21) and immune regulation (e.g., IL-10, FOXP3). Despite the observed quantitative similarities in their overall responsiveness to proinsulin and GAD65, children at 6 months of age exhibited a remarkable transcriptional heterogeneity among the  $\beta$  cell-reactive naïve CD4<sup>+</sup> T cell population, which could be attributed to the presence of distinct subphenotypes with predominantly immune regulatory (e.g., FOXP3, TGF- $\beta$ , and CTLA-4, in the absence of proinflammatory cytokines) or proinflammatory features. The latter included a naïve CD4<sup>+</sup>CD45RA<sup>+</sup> T cell subset, which could be defined by consistently increased expression of a unique set of genes (*CCR6*, *IL21*, *TBX21*, *TNF*, *RORC*, *EGR2*, *TGFB1*, and *ICOS*) that play essential roles in different aspects of CD4<sup>+</sup> T<sub>H</sub> responses, such as naïve T-cell activation/proliferation, differentiation, and acquisition of T<sub>H</sub> effector functions. This proinflammatory phenotype appears rather unusual, as it combines genes whose protein products are involved in the overall control of T<sub>H</sub> differentiation (e.g., EGR2, TGF- $\beta$ 1) with individual facets of functionally distinct CD4<sup>+</sup> T<sub>H</sub> lineages (T<sub>H</sub>1: T-bet, TNF; T<sub>H</sub>17: ROR- $\gamma$ T, CCR6; follicular T<sub>H</sub> (T<sub>FH</sub>): IL-21, ICOS), whereas the expression of the Treg cell lineage specification factor Foxp3 and the T<sub>H</sub>17 signature cytokine IL-17 were found to be underrepresented.

Strikingly, in 3 of the 6 children included in the gene expression survey, the T<sub>H</sub>-like phenotype was shared among the vast majority of naïve CD4<sup>+</sup>CD45RA<sup>+</sup> T cells with both proinsulin- and GAD65-responsiveness, preceding subsequent development of memory CD4<sup>+</sup> T cells and AAbs reactive to  $\beta$  cell antigen. Importantly, this particular proinflammatory subphenotype was essentially absent in all tested at-risk children who remained free of any signs for  $\beta$  cell autoimmunity, and was clearly discernable from the transcriptional profile of naïve CD4<sup>+</sup>CD45RA<sup>+</sup> T cells responsive to the non- $\beta$  cell antigen tetanus toxoid, formally excluding the possibility that genetically susceptible children with  $\beta$  cell autoimmunity exhibit an overall bias in their antigen-specific CD4<sup>+</sup> T cell responses. A predictive algorithm for progression to  $\beta$  cell autoimmunity based on the proinflammatory gene signature of naïve CD4<sup>+</sup>CD45RA<sup>+</sup> T cells with  $\beta$  cell responsiveness further supported the relevance of these findings.

### Transition to memory CD4<sup>+</sup> T cell responses and overt $\beta$ cell autoimmunity

Their independent identification in several at-risk children as early as 6 months of age and selective predominance in those who progressed to  $\beta$  cell autoimmunity already indicated that the mixed 'T<sub>H</sub>1/T<sub>H</sub>17/TF<sub>H</sub>'-like phenotype of naïve  $\beta$  cell-responsive CD4<sup>+</sup> T cells may represent a transitional but rather long-lasting stage of partial differentiation with the potential to give rise to fully differentiated, diabetogenic CD4<sup>+</sup> T effector cells. Consistently, comparative gene expression profiling of single  $\beta$  cell-responsive CD4<sup>+</sup> T cells before and after seroconversion provided direct evidence that the progression towards  $\beta$  cell autoimmunity is indeed accompanied by the transition to a more homogeneous T<sub>H</sub>1-type response characterized by the expression of *IFNG*, *TNF* and *CCR7*, with low frequencies of cells expressing *IL17*, *IL4*, *IL10* or *FOXP3* mRNA. However, the replacement of semi-committed 'T<sub>H</sub>1/T<sub>H</sub>17/TF<sub>H</sub>'-like T cells was not dominantly driven by proliferative clonal expansion, as the paired analysis of TCR/ $\alpha/\beta$  chains at single-cell resolution revealed a largely diverse TCR repertoire expressed by  $\beta$  cell-responsive memory CD4<sup>+</sup> T cells and provided no evidence for the selection of dominant or public clones.

### Naïve T<sub>H</sub>-like CD4<sup>+</sup> T cells with $\beta$ cell reactivity: marker for early T1D diagnosis and target for autoimmune intervention?

In addition to providing novel insight into the immune pathogenesis of T1D, the present study is likely to have important implications for disease diagnosis and prevention of  $\beta$  cell autoimmunity. So far,  $\beta$  cell-specific AAbs have been most commonly used as immune biomarker with predictive value for progression of genetically susceptible individuals to clinical T1D. A T-cell-readout based on the naïve CD4<sup>+</sup> T<sub>H</sub>-like phenotype reported by Heninger *et al.* may improve early detection of  $\beta$  cell autoimmunity prior to the establishment of  $\beta$  cell-specific memory CD4<sup>+</sup> T cell responses and seroconversion, thereby further strengthening diagnosis and prevention of metabolic complications often associated with clinical T1D onset. The mixed proinflammatory transcriptional signature was detected in naïve  $\beta$  cell-responsive CD4<sup>+</sup> T cells of some (50%) but not all at-risk children who progressed to  $\beta$

cell autoimmunity, perhaps due to the modest cohort size or technical peculiarities inherent to the RT-PCR-based analysis of single cells. However, it appears important to emphasize that the environmental factor(s) driving the initial activation of  $\beta$  cell-responsive CD4<sup>+</sup> T cells have yet to be determined. Indeed, it might well be that quantitatively and qualitatively different environmental cues, which impinge early on non-committed, truly naïve CD4<sup>+</sup> T cells during initial priming with  $\beta$  cell antigen, result in imprinting of distinct proinflammatory CD4<sup>+</sup> T cell subphenotypes. Provided that the entire spectrum of pancreas-infiltrating T cell subsets is reflected in the blood, genome-wide single-cell transcriptomic of PBMC-derived  $\beta$  cell-responsive T cells may provide first hints towards the identity of the involved environmental stimuli. Clearly, further studies are warranted to further corroborate the predictive value and diabetogenic potential of the newly identified naïve CD4<sup>+</sup> T cell subset.

With regard to autoimmune intervention, the development of immune-modifying approaches to halt the underlying autoimmune response targeting  $\beta$  cell antigen has been a long-standing goal in T1D, but encouraging observations in animal models of T1D have not yet demonstrated comparable efficacy in humans. Such efforts have now gained considerable momentum through the identification of antigen-specific CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells as key mediators of dominant tolerance and immune homeostasis. The central role of Foxp3<sup>+</sup> Treg cells in autoimmune protection of pancreatic  $\beta$  cells is probably best exemplified by the observation that T1D represents a hallmark of the IPEX (immune dysfunction, polyendocrinopathy, enteropathy, X-linked) syndrome in male patients lacking functional Foxp3<sup>+</sup> Treg cells due to mutations in the *FOXP3* gene (9). Conversely, Foxp3<sup>+</sup> Treg cell-based therapy for prevention and even interference with established  $\beta$  cell autoimmunity has shown promise in preclinical animal models of T1D and other autoimmune diseases (10,11). This includes the adoptive transfer of  $\beta$  cell-specific Foxp3<sup>+</sup> Treg cells and tolerogenic administration of  $\beta$  cell antigen (such as proinsulin) to promote both recessive (deletion and anergy of diabetogenic T effector cells) and dominant (enhancement of Treg cell activity) mechanisms of immunological tolerance. While additional treatment options without pharmacological immunosuppression such as immunoprotective artificial pancreas systems (12) are also emerging, the transplantation of pancreatic islets to restore endogenous insulin secretion is still restricted to a small subset of T1D patients with high metabolic lability, due to

the long-term need for systemic immunosuppression and shortage of donor organs. Thus, it appears highly desirable to preserve endogenous  $\beta$  cell mass and insulin production by interference with autoimmune responses prior to the destruction of the majority of insulin-producing  $\beta$  cells. However, the successful development of efficacious T1D prevention strategies will require detection of the earliest events in the autoimmune process. In this context, it appears tempting to speculate that  $\beta$  cell-responsive naïve CD4<sup>+</sup> T cells with a semi-committed T<sub>H</sub>-like phenotype may represent a suitable target for the assessment of early T cell responses to immune-modulating T1D prevention strategies, in particular tolerogenic vaccination with  $\beta$  cell antigen.

Several clinical Foxp3<sup>+</sup> Treg cell-based trials that have been recently conducted in patients at different time points after T1D onset. This includes cell-based immunotherapy by intravenous infusion of children (13,14) and adults (15) with *ex vivo*-expanded autologous polyclonal FOXP3<sup>+</sup> Treg cells, as well as antigen-based immunotherapy of adult patients using repeated intradermal injections of an immunodominant proinsulin peptide antigen to promote Foxp3<sup>+</sup> Treg cell function (16). Both treatment strategies were well tolerated and no severe adverse effects were observed, strongly encouraging further clinical trials to provide more detailed insight into the potential of Treg cell-based therapy to halt cell autoimmunity in patients with established T1D. Notably, results of the Pre-POINT clinical trial provided first evidence that self-antigen-based therapy might indeed be suitable to prevent progression to overt  $\beta$  cell autoimmunity: oral administration of insulin protein to genetically susceptible children prior to seroconversion (i.e.,  $\beta$  cell AAb-negative children) elicited an antigen-specific immune response, which, among other things, was characterized by insulin-responsive CD4<sup>+</sup> T cells with Treg cell-like properties, while hyperglycemia or other severe side effects were not observed (17). In light of the present study by Heninger *et al.*, it will be of considerable interest to assess in future clinical studies whether early tolerogenic vaccination of at-risk children can interfere with the establishment of the unique proinflammatory phenotype of the naïve  $\beta$  cell-responsive CD4<sup>+</sup> T cells, preventing their developmental progression to memory CD4<sup>+</sup> T cells, production of  $\beta$  cell-reactive AAbs and clinical T1D.

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## Footnote

**Conflicts of Interest:** The authors have no conflicts of interests to declare.

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