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Evaluating T cell responses prior to the onset of type 1 diabetes

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Abstract

Aims: In the current study we aimed to evaluat T cell phenotypes and metabolic profiles in high-risk individuals who progressed to type 1 diabetes compared to those remaining disease free.

Methods: A Fluorspot assay was used to examine T cell responses to a panel of islet autoantigen peptides in samples obtained 6- and 30-months preceding disease onset and at the same timepoints in non-progressors.

Results: We noted a significant increase in the magnitude of the proinflammatory interferon-γ response to proinsulin and insulin peptides in individuals who progressed to type 1 diabetes. In contrast, in the non-progressors, we observed an increase in the regulatory IL-10 response to proinsulin peptides. Furthermore, the T cell responses to the islet peptide panel predisposed towards a proinflammatory interferon-γ bias in the progressors.

Conclusions: Collectively, these data suggest that a proinflammatory T cell response is prevalent in high-risk individuals who progress to type 1 diabetes and can be detected up to 6 months prior to onset of disease. This observation, albeit in a small cohort, can potentially be harnessed in disease staging, particularly in identifying autoantibody-positive individuals transitioning from stage 2 (dysglycemia present and pre-symptomatic) to stage 3 (dysglycemia present and symptomatic). The detection of these different T cell phenotypes in progressors and non-progressors suggests the presence of disease endotypes.

KEYWORDS

antibody, cytokines, interferon-γ, proinsulin, T cells, type 1 diabetes

1 | INTRODUCTION

Type 1 diabetes results from a sustained immune-mediated attack on insulin-producing β cells culminating in insulin deficiency and the requirement for exogenous insulin.[1]

The disparity in several factors including genetic susceptibility, variable prodrome periods, type and number of autoantibodies and inconsistent responses to immuno-intervention illustrates the heterogeneous features of the disease.[2] Studies into the nosology of type 1 diabetes have prompted a scrutiny

of this heterogeneity which may be explained by the presence of disease endotypes. The existence of type 1 diabetes endotypes has major implications for therapeutic intervention and highlights the need for patient stratification during every stage of the natural history of the disease.

By far, the most robust biomarkers of impending type 1 diabetes risk and progression are autoantibodies to islet targets such as insulin, insulinoma antigen-2 (IA-2), glutamic acid decarboxylase (GAD) and Zinc transporter 8 (ZnT8)[3] and indeed, multiple-autoantibody positive individuals can be stratified for risk of disease development based on the characteristics or combinations of autoantibodies.[4,5] However, the order of appearance and titres of autoantibodies are highly variable and, this coupled with inconsistent prodrome periods from seroconversion calls for additional biomarkers which may be able to distinguish the rate of progression.

There is an abundance of evidence underscoring the role of T cells in the pathogenesis of type 1 diabetes.[6,7] Despite the technical challenges in evaluating T cell responses, they have been used as potential biomarkers in addressing both responsiveness to therapeutics and stratifying patients.[8] Indeed, the cytokine secretion profile of CD4+ T cells has been used to distinguish subjects with type 1 diabetes from healthy controls[9]; furthermore, quantitative T cell responses differ between children and adults with type 1 diabetes thus highlighting the age-related heterogeneity observed in the disease.[10] Finally, distinct T cell phenotypes have been described in at-risk individuals.[11]

The issue of heterogeneity can be further addressed using metabolic parameters such as C-peptide and HbA_{1c}. C-peptide levels vary according to age[12,13] and type 1 diabetes duration, with levels decreasing with increased duration of disease.[14] HbA_{1c} is a metabolic measure used to determine long-term glycaemic control.

We set out to examine the heterogeneity in type 1 diabetes by exploring specific immune and metabolic profiles in high-risk subjects to determine if this impacted disease progression. We wanted to evaluate the antigen-specific T cell responses and metabolic parameters such as C-peptide and HbA_{1c} levels in individuals who developed disease compared to those remaining diabetes free over a 30-month period. Finally, we also wanted to assess how these responses differed in individuals just prior to disease onset compared to several months earlier.

What's new?

- T cells have been demonstrated in preclinical type 1 diabetes where they illustrate the immunological heterogeneity of the disorder by exhibiting distinct phenotypes.
- The current study expands on this and shows that there is a proinsulin-specific pro-inflammatory T cell phenotype present specifically in a subgroup of high-risk individuals who progress to type 1 diabetes and this can be detected within 6 months prior to onset.
- This proinsulin-specific response may be potentially useful indisease staging.

2 | METHODS

2.1 | Participants

Fresh heparinised blood samples were obtained from 42 children, all first-degree relatives of individuals with type 1 diabetes (21 males; median age 12 years 8 months (age range: 3 years 8 months to 19 years 8 months); of these, 41 were autoantibody positive (GADA): 86%; IA2-A: 64%; IAA: 40%; ZnT8-A: 69%; ICA: 67%). Participants were recruited via the Type 1 Diabetes TrialNet Pathway to Prevention study.[13]

Individuals were genotyped for HLA-DR3/DQ HLA-DR4/DQ8 haplotypes and 35/41 (85%) were positive for one or more of these haplotypes. Where possible longitudinal blood samples were collected every 6 months for 2.5 years (visits 1–5) or until the participant developed type 1 diabetes. Over the course of the study, 14 participants progressed to type 1 diabetes. At the final visit, samples were available on eight participants who developed type 1 diabetes and 16 individuals who did not. Demographic details of the participants are shown in Table 1. These studies were carried out with the approval of the U.K. National Research Ethics Service, and for blood studies, informed consent was obtained from all participants or their parents/guardians.

2.2 | Autoantibodies

IAA, GADA, IA-2A, ZnT8-A and ICA were measured according to previous TrialNet studies.[15–17]

2.3 | HLA typing

DNA-based HLA typing using oligonucleotide probes at full resolution was used to type individuals for HLA class II alleles (HLA DRB1, HLA DQB1) as previously reported in the TrialNet Pathway to Prevention Study.[18]

TABLE 1 Summary of characteristics of participants of the study

	Progressors ($n = 14$)	Non-progressors ($n = 28$)
HLA DR3/DR4 (%)	93	79
Males (%)	64	46
Median age (years)	13.11	12.5
Mean follow-up (months)	16.5 (SD = 6.9)	13.8 (SD = 3.6)
Autoantibodies		
GADA (%)	93	82
IA−2 (%)	79	57
IAA (%)	71	25
ZnT8-A (%)	79	64
ICA (%)	79	61

2.4 | Detection of β-cell-specific cytokine-secreting CD4+ T cells

Peptides based on sequences of naturally processed and presented proinsulin (C13-32; C19-A3; C22-A5), IA-2 (752–775; 853–872), GAD65 (335–352; 555–567) epitopes, and overlapping regions of insulin B chain (B1-20; B6-25) were synthesised and purified by high-performance liquid chromatography (Thermo Hybaid) and used at 10 μg/ml. Recombinant human proinsulin (Biomm) and recombinant GAD (Diamyd) were both used at 10 μg/ml. Pediacel, a penta-vaccine (Sanofi Pasteur Ltd.), was used at 1 μl/ml to examine anamnestic responses induced by vaccination or infection as previously described.[10]

Cytokine responses were measured using combined interferon-γ/IL-10 indirect FluoroSpot assays (U-Cytech) to examine cytokine secretion and co-secretion by individual cells. Briefly, cells prestimulated with peptides described above, proinsulin, GAD or the positive control (Pediacel) were transferred to wells of a pretreated FluoroSpot plate coated with high affinity anti-IL-10 and anti-interferon-γ antibodies.

After 24 h, the cells were removed by washing, and antibody-bound cytokine identified using a mixture of anti-IL-10 and anti-interferon-γ detection antibodies followed by two fluorescent conjugates containing Alexa 488-labelled anti-FITC antibodies and R-phycoerythrin (R-PE)-labelled streptavidin made up according to manufacturer's instructions. Green, fluorescent spots represent interferon-γ-producing cells, red spots, IL-10 and yellow spots signify cells that release both interferon-γ and IL-10 (Figure S1).

2.5 | Metabolic measurements

Participants undertook an oral glucose tolerance test (OGTT) after fasting overnight. C-peptide (nmol/L) and glucose (mmol/L) measurements were performed as recently described in other TrialNet studies.[13] HbA_{1c} was measured as previously described in TrialNet studies.[19]

2.6 | Data analysis

FluoroSpot data were expressed as the mean number of spots per triplicate compared with the mean spot number in the presence of diluent alone (stimulation index [SI]) and an SI≥3 was considered positive for both interferon-γ and IL-10.

The proportion of participants responding to individual T cell epitopes was compared using Fisher's exact test. Responses to peptides at the first and final visits were compared using Wilcoxon and Mann–Whitney tests (data were tested for normality using Shapiro–Wilk) and were analysed with GraphPad Prism 9 software. $p < 0.05$ was considered statistically significant.

3 | RESULTS

During the study, 14 individuals developed type 1 diabetes, and thus, we were able to compare immunological and metabolic measurements in these individuals with those in individuals who did not progress to disease ($n = 28$) (Table S1).

Within the progressors, the genotypes associated with diabetes susceptibility, *HLA-DRB1*0301* and/or **0401* genotypes were present in 13/14 individuals (93%) compared to 22/27 (81%) of individuals who did not progress to type 1 diabetes.

Multiple autoantibodies were present in 93% of progressors and 81% of non-progressors.

3.1 | Immune responses just prior to the onset of type 1 diabetes

Of the individuals who developed type 1 diabetes, longitudinal samples were available on eight participants with the last sample analysed within 6 months of diabetes onset. We examined immunological parameters at this final visit and compared the data to the first visit (obtained 12–30 months earlier, median 12 months duration of follow-up) and to individuals who remained diabetes free on whom samples were acquired at identical time points ($n = 16$).

3.2 | T cell responses prior to onset of type 1 diabetes

We have previously shown that high-risk multi-autoantibody-positive individuals present as two immunological phenotypes, one characterised by pro-inflammatory T cell responses and the other a partially regulated IL10 response indicating different immune phenotypes.[11] Based on this, we determined interferon-γ and IL-10 T cell responses in the current cohort.

The prevalence of interferon-γ and IL-10 T cell responses did not differ amongst the two groups at the first visit (positive responses identified as interferon-γ responses ≥SI = 3): 0%–29% in progressors and 4%–29% in non-progressors; IL-10 responses (≥SI = 3): 0%–29% in progressors and 11%–39% in non-progressors (Figure S2). At the final visit, the T cell response was shown to be higher in the progressors, and this was particularly notable for the interferon-γ response to proinsulin and insulin peptides (25%–63%).

The magnitude of interferon-γ and IL-10 T cell responses was measured as the agglomerated stimulation index (SI) across all the peptides for each islet autoantigen to provide an indication of the size of the T cell immune response.

In progressors, interferon-γ T cell responses were higher in the visit preceding the onset of disease compared to the first visit for proinsulin and insulin peptides ($p = 0.0011$ and $p = 0.0157$) respectively (Wilcoxon test). In contrast, interferon-γ responses to IA-2 and GAD peptides were similar at both visits $p = 0.89$ and $p = 0.81$ respectively (Figure 1a).

In the non-progressors, there was no significant difference in interferon-γ T cell responses between both visits for any islet peptide. There were a few non-progressors who did, however, have an increased IFN-γ T cell (SI>3) responses to proinsulin and insulin peptides at the final visit. These individuals had a higher prevalence of HLA DR4 haplotypes (5/6 [63%]) compared to those in whom IFN-γ T cell responses remained stable or decreased (3/8 [38%]).

Although the numbers were small, we noted that progressors with interferon-γ T cell responses to proinsulin and insulin peptides were slightly younger than non-progressors (median age: 9.2 years vs. 11.1 years).

IL-10 T cell responses were similar at both visits in the progressors for all the islet peptides except for GAD peptides where IL-10 responses were significantly lower at the final visit ($p = 0.04$); this was at a similar frequency for both GAD peptides (335–352 and 555–567 (Figure 1b). Similarly, in the non-progressors, IL-10 responses were similar at both visits except for responses to proinsulin peptides which were higher at the last visit ($p = 0.0135$, (Wilcoxon test) (Figure 1b))).

In summary, progressors present with a proinsulin/insulin-targeted pro-inflammatory T cell response and proinsulin peptides elicit a regulatory IL-10 response in non-progressors.

3.3 | Dual interferon-γ/IL-10-producing T cell responses prior to onset of type 1 diabetes

Whereas it is difficult to definitively infer the pathogenic potential of T cells purely based on cytokine secretion, studies have suggested that cells with a regulatory phenotype are characterised by IL10 with co-secretion of interferon-γ.[20] This includes cells with a Tr1-like phenotype known to secrete both interferon-γ and IL-10, hence are dual cytokine-producing cells.

The prevalence of dual (interferon-γ + IL-10) cytokine-producing cells was measured in both progressors and non-progressors. GAD and GAD peptide 335–352 each elicited dual cytokines in one patient (12.5%) prior to progression to type 1 diabetes at the final visit, and in each case, only one cell produced both cytokines.

In contrast, in the non-progressors, dual cytokine-producing cells were observed in 9/16 (56%) of individuals at the last visit. Dual cytokine-producing cells were elicited by GAD ($n = 5$ individuals), GAD peptides: 335–352 ($n = 5$); 555–567 ($n = 2$), insulin peptides: B1-20 ($n = 2$); B6-25 ($n = 2$), IA-2 peptide: 853–872 ($n = 3$), proinsulin peptides: C13-32 ($n = 1$), C19-A3 ($n = 2$), C22-A5 ($n = 3$) and proinsulin ($n = 2$) (Figure S3).

In summary, the higher prevalence of dual cytokine-producing cells in non-progressors suggests the presence of cells with a regulatory phenotype.

3.4 | Ratio of interferon-γ and IL-10 responses

We examined the ratio of interferon-γ to IL-10 responses in individuals preceding type 1 diabetes development to determine if responses were polarised towards an inflammatory or regulatory phenotype. In subjects who progressed to diabetes, we observed a predominant interferon-γ bias with only 3/11 islet autoantigens showing an IL-10 bias; this pattern persisted at the final visit just before disease onset (Figure 2a,b). In contrast, although responses initially were biased towards interferon-γ, by the final visit, responses in non-progressors were biased towards IL-10—this was particularly prominent for the proinsulin peptides (Figure 2c,d).

These data further indicate the presence of different immune phenotypes in progressors and non-progressors.

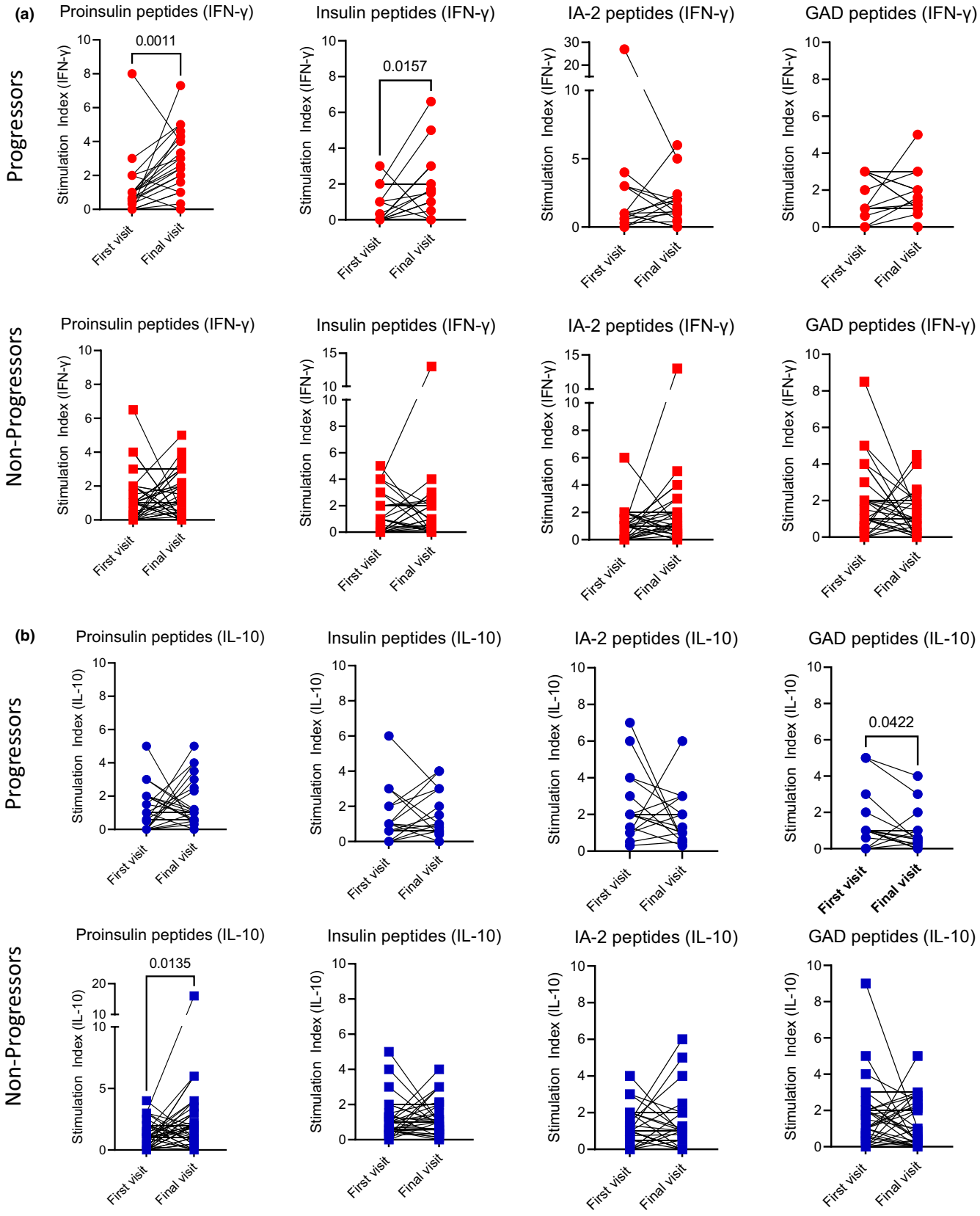

FIGURE 1 Magnitude of T cell responses. Stimulation indices for (a) interferon-γ (red) and (b) IL-10 (blue) responses in progressors ($n = 8$) (circles) and non-progressors ($n = 16$) (squares) at the first and final visits preceding type 1 diabetes in progressors and at the same time points in non-progressors. p values (Wilcoxon test) where significant are shown

3.5 | Prevalence of autoantibodies preceding type 1 diabetes

We determined autoantibody prevalence at both visits to determine if this were related to disease progression. At the start of the study (visit 1) overall, the prevalence of autoantibodies tended to be higher in the progressors compared to non-progressors for GADA, IA2A and IAA (insulin autoantibodies) (Table 1). At the final visit, the prevalence of autoantibodies was also higher in the progressors ($n = 8$) for IAA (50% vs. 12.5%) albeit not significantly, this was likely to be due to the small number of participants. Interestingly, every individual positive for IAA except one had an interferon-γ T cell response to proinsulin and/or insulin peptides.

3.6 | Metabolic measurements prior to the onset of type 1 diabetes

In addition to the immunological data, we also assessed metabolic measurements, specifically HbA_{1c} and C-peptide to see if these were related to disease progression. At the final visit before type 1 diabetes onset, HbA_{1c} levels were greater in progressors (ranging from 4.4 to 6.3% (25–45 mmol/mol)) compared to non-progressors (ranging from 4.5% to 5.6% (26–38 mmol/mol)) ($p = 0.047$, Mann–Whitney test) (Figure S4a).

We examined interferon-γ responses to proinsulin/insulin peptides and insulin autoantibodies in this group as the data above show that these are the most discriminatory parameters in progressors versus non-progressors. In the five individuals with the HbA_{1c} values >5.45% (>36 mmol/mol) (as determined by the 75th percentile value) at the final visit, 4/5 (80%) showed an interferon-γ response to proinsulin/insulin peptides with proinsulin peptide C22-A5 eliciting a response in all four individuals.

In the non-progressor cohort, one individual had a HbA_{1c} value >5.45% at the final visit and no interferon-γ response to proinsulin/insulin peptides was observed ($p = 0.02$) (Fisher's exact test).

C-peptide values were lower in most individuals who developed type 1 diabetes in samples just prior to diabetes onset compared to those who did not develop disease (Figure S4b) albeit not significantly. The 25th percentile of C-peptide values was determined to be 488 so this was set as a threshold. Five individuals (63%) in the progressor group had C-peptide values <488 AUC just prior to the onset of type 1 diabetes compared to two subjects (14%) in the non-progressors group ($p = 0.05$) (Figure S4b). All five individuals (100%) had an interferon-γ response to proinsulin/insulin peptides and three (60%) were positive for insulin autoantibodies. In the non-progressors, interferon-γ response to proinsulin/insulin peptides was noted in both subjects and neither had insulin autoantibodies.

In contrast, 38% of progressors and 21% of non-progressors had C-peptides values >488 AUC (p = ns); 33% of progressors had an interferon-γ T cell response to proinsulin/insulin peptides and insulin autoantibodies; and 21% of non-progressors showed an interferon-γ T cell response to these peptides and 7% were positive for insulin autoantibodies.

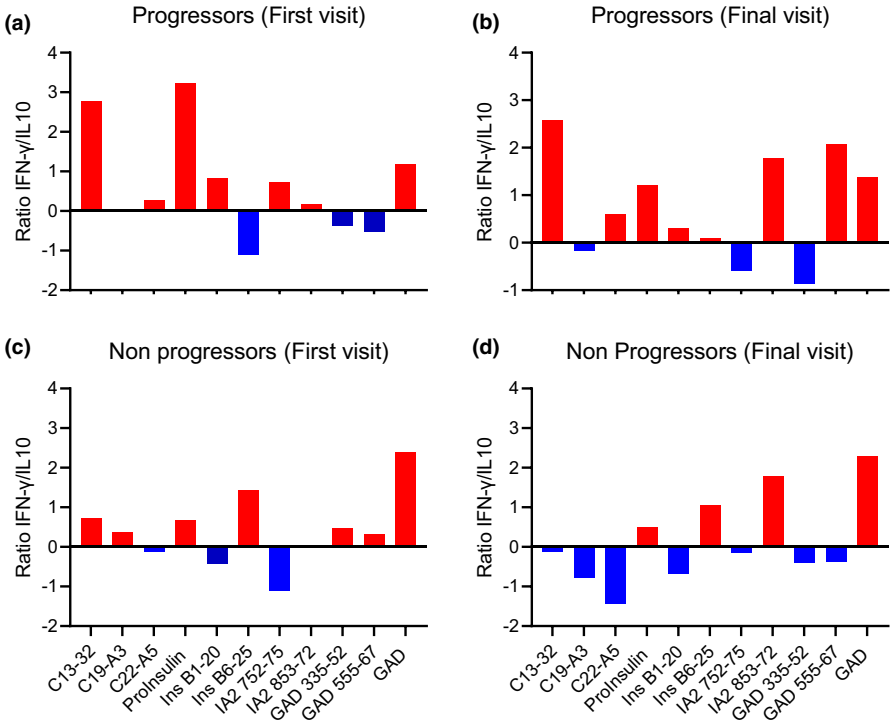

FIGURE 2 Ratio of IFN-γ (red) and IL-10 (blue) T cell responses to peptides of proinsulin, whole proinsulin, peptides of insulin, IA-2, GAD and whole GAD for progressors ($n = 8$) (top panel (a) and (b)) and non-progressors ($n = 16$) (bottom panel (c) and (d)) at the first (left) (a) and (c) and final (right) (b) and (d) visits (p = ns for progressors and non-progressors at both visits). The total positive responses for IFN-γ responses were divided by the total positive responses for IL-10 to calculate ratios which are shown as Ln values on the y-axis

These data suggest that there are differences in metabolic parameters in subjects who progressed to type 1 diabetes.

4 | DISCUSSION

In the present study, we detected antigen-specific T cells in individuals at high risk for type 1 diabetes consistent with our previous findings.[11] We show a significant increase in antigen-specific interferon-γ T cell responses specifically in individuals who progress to type 1 diabetes in samples obtained up to 6 months prior to disease onset compared to those collected several months previously. Also, prior to disease onset, the T cell response is preferentially biased towards an interferon-γ for most of the islet autoantigenic peptides tested. Collectively, these data suggest that a pro-inflammatory, interferon-γ T cell response particularly to proinsulin and insulin peptides is significantly prevalent in high-risk individuals who progress to type 1 diabetes. This observation is in a small group of patients and needs to be confirmed in larger cohorts, if corroborated it can potentially be harnessed in disease staging.

We have previously shown that proinsulin and insulin peptides are preferentially targeted in children with type 1 diabetes[10]; the fact that proinsulin and insulin peptides elicit the strongest interferon-γ T cell responses in the current study further highlights the key role of these peptides in high-risk children progressing to type 1 diabetes. Finally, although the numbers of participants were very small, the children with interferon-γ T cell responses to these peptides who progressed to type 1 diabetes were younger than those who remained disease free.

It is intriguing that in individuals who progressed to type 1 diabetes, just preceding disease onset, the proinsulin peptide, C13-32, specifically elicited only pro-inflammatory interferon-γ responses and no IL-10. Furthermore, the interferon-γ bias observed in progressors was highest for peptide C13-32 amongst all the peptides tested. These observations together with our previous studies in preclinical individuals,[11,21] and a recent study of at-risk individuals reporting proliferative reactivity to proinsulin C16-C30 which is encompassed in C13-32,[22] highlight a possible key role for C13-32 in type 1 diabetes pathology.

We also examined individuals who did not go on to develop type 1 diabetes at the same time points and noted a significant increase in proinsulin peptide-specific IL-10 responses, suggesting possible immune regulation at play in a subgroup of high-risk individuals consistent with our previous reports.[11,21] This is further supported by the data on dual (interferon-γ + IL-10) cytokine-producing cells which are found predominantly in the individuals who did not progress to type 1 diabetes and suggest that in dual cytokine-producing cells, IL-10 may be regulating pro-inflammatory responses in non-progressors. This group is high risk, and the individuals will also develop type 1 diabetes albeit in a possible protracted manner as the participants did not develop disease within the same timeframe as the progressors. The slower disease progression in this group further highlights the heterogeneity of type 1 diabetes.

For both progressors and non-progressors, the prodrome period is not known and it is possible that the time since the appearance of the first autoantibodies may differ in the two groups. With this study design, it is not possible to assess whether thedifferent T cell responses elicited in progressors and non-progressors may beconfounded by differences in the time since the appearance of the firstautoantibody. It is possible that there are differences in disease progression and the prodrome periods may vary in the two groups.

Using a combination of proinsulin/insulin peptide-specific T cell responses and insulin autoantibodies just prior to onset of disease, we were able to identify all but one subject who progressed to type 1 diabetes. These data illustrate the biomarker potential of this combined panel not only in predicting disease but also narrowing the prodrome window to within less than 6 months. Naturally, applying a combination of proinsulin/insulin peptide-specific T cell responses and insulin autoantibodies as a biomarker panel needs to be validated extensively in a clinical setting to see if imminent disease can be predicted. A few non-progressors also had responses to proinsulin/insulin peptides; hence, a large cohort needs to be tested preferably with prior knowledge of the length of the prodrome period, this will determine if this panel can be used as biomarker.

The number of individuals with high HbA_{1c} and low C-peptide was small; hence, the data need to be interpreted with a degree of caution; however, it is interesting that in progressors with higher HbA_{1c} levels, all but one of the individuals had an interferon-γ T cell response to proinsulin peptide C22-A5 that was rarely observed in the non-progressors. A relationship between islet antigen peptide-specific T cell responses and HbA_{1c} has been described previously in preclinical individuals, but these were mainly detected in autoantibody negative individuals[23] and the individuals were not followed up for disease onset.

The small number of participants in this study is a limitation of the study and the likelihood of disease progression in subjects with proinsulin/insulin peptide-specific interferon-γ T cell responses needs to be addressed in larger cohorts of high-risk insulin autoantibody-positive individuals.

To our knowledge, this is the first study that examines antigen-specific T cells in the prodrome period. The phenotype of the response in progressors is pro-inflammatory and directed specifically at proinsulin and insulin peptides; conversely in non-progressors, there is a significant regulatory IL-10 response to proinsulin peptides; these distinct immune phenotypes in high-risk individuals warrant further investigation.

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CONFLICT OF INTEREST

None declared.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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