The β-cell specific T-lymphocyte response has a distinct inflammatory phenotype in juvenile compared to adult type 1 diabetes

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Novelty statement
- T1DM development in children appears more rapid and severe compared to adults. This paper shows that immune responses against β-cells are more common and target more autoantigens in children compared to adults. In addition, the immune response in children is particularly focused on proinsulin and insulin as the main drivers of the autoimmune response.
- These findings suggest age-related immunological heterogeneity in T1DM. This may be important in developing age-appropriate immune-intervention strategies.
Aims/Hypothesis
We examined the hypothesis that the quality, magnitude and breadth of helper T lymphocyte responses to β-cells differs in T1DM according to diagnosis in childhood or adulthood.

Methods
We studied helper T-lymphocyte reactivity against β-cell autoantigens by measuring production of the pro-inflammatory cytokine: interferon-γ (IFN-γ) and the anti-inflammatory cytokine: IL-10, using Elispot assays in 61 people with T1DM (within 3 months of diagnosis, positive for HLA DRB1*0301 and/or *0401) of whom 33 were children/adolescents, and 91 unaffected siblings.

Results
IFN-γ responses were significantly more frequent in children with T1DM compared to adults (85% vs. 61% p=0.04). Insulin and proinsulin peptides were preferentially targeted in children (p=0.0001-0.02) and the breadth of the IFN-γ response was also greater with 70% of children having an IFN-γ response to three or more peptides compared to 14% of adults (p<0.0001).

β-cellantigen-specific IL-10 responses were comparable in children and adults in terms of frequency, breadth and magnitude, with the exception of responses to GAD65 which were significantly less frequent in adults.

Conclusion/Interpretation
At diagnosis of T1DM, pro-inflammatory autoreactivity is significantly more prevalent, focuses on a wider range of targets, and is more focused on insulin/proinsulin in children than adults. We interpret this as indicating a more aggressive immunological response in the younger age group that is especially characterised by loss of tolerance to proinsulin. These findings highlight the existence of age-related heterogeneity in T1DM pathogenesis that could have relevance to development of immune-based therapies.
The incidence of type 1 diabetes (T1DM) has increased worldwide during the last decade; especially in children (1) who typically develop disease with more severe, and rapid onset of symptoms than adults (2). This clinical observation might have several different explanations or be attributable to a combination of effects. The possibility that it reflects a different disease tempo in children compared with adults, resulting in turn from differences in the autoimmune response, is an attractive and important notion, since it would potentially influence strategies for the deployment of immunological interventions. For example, children might be treated at an earlier stage in the disease process, more aggressively or with a different set of therapeutic agents if it transpired that their autoimmune response has a different quality or magnitude compared to adults.

Comparative studies on β-cell specific autoimmunity around the time of diagnosis in adults and children are scant, but studies conducted in the setting of childhood onset T1DM indicate that there may be within-disease, age-related effects on some aspects of autoimmunity, notably autoantibodies. For example, the appearance of autoantibodies within the first 2 years of life is usually accompanied by the development of multiple specificities (3) and rapid progression to T1DM; in contrast, children who develop autoantibodies later have a slower progression to multiple autoantibodies and disease (4). However, the relationship of these age-related differences in autoantibodies to the tempo of T1DM development is difficult to gauge, since antibodies are not considered to be directly responsible for β-cell damage. Rather, it is generally proposed that CD4 and CD8 T-lymphocytes act in concert to destroy β-cells, through a combination of inflammatory mediators and direct cytotoxicity, with β-cell-specific CD4 T-lymphocytes (also known as helper T-lymphocytes) as the main orchestrators of the process (5) (6). Thus the current lack
of comparative data on autoreactive helper T-lymphocytes in children and adults developing T1D represents a significant knowledge gap that potentially impacts upon the translation of new intervention strategies into paediatric clinics.

We have previously shown the existence of disease endotypes in T1DM (7) based on heterogeneity in both the adaptive immune response and islet pathology; and have now sought to extend these findings by examining the frequency, magnitude, breadth or quality of the β-cell-specific helper T-lymphocyte responses that prevail at diagnosis of T1DM in children and adults.
Materials and Methods

Subjects and autoantibodies
Between 2009-2012 fresh heparinized blood was obtained from 61 people with newly-diagnosed T1DM (duration ≤ 12 weeks; 33 children/adolescents (defined as aged 16 years or under); 28 adults) and from 91 of their autoantibody-negative siblings without T1DM (48 children; 43 adults) (Table 1). Children and adults with T1DM did not differ significantly for disease duration, gender distribution, frequency of HLA genes, HbA1c or autoantibody prevalence (Table 1). Studies were approved by the National Research Ethics Service and informed consent was obtained from all participants/parents/guardians. Participants were enrolled if they possessed one or both of HLA-DRB1*0301 and HLA-DRB1*0401 (Table 1). Autoantibodies to GAD65, intra-cytoplasmic (606–979) IA-2 and ZnT8 were measured by radioimmunoassay (8, 9). Insulin autoantibodies (IAA) were not tested. The present work is an extension to our previously study showing the existence of disease endotypes in T1DM (7).

Measuring β-cell-specific cytokine secreting CD4+ T cells
Peptides representing naturally processed and presented IA-2, proinsulin and GAD-65 epitopes, and overlapping regions of the insulin B and A chain, were used as stimuli at a concentration of 10μg/ml to stimulate 2 x 10^6 cells (7). Pediacel pentavaccine (Sanofi Pasteur Ltd Berkshire, UK) was used at 1μl/ml to examine anamnestic responses induced by vaccination or infection. IFN-γ and IL-10 production by CD4+ T-cells was detected by enzyme-linked immunospot (ELISPOT) assay, performed as described in the TrialNet T cell Validation blinded workshop, in triplicate for each peptide and data were expressed as SI (stimulation index); a response was considered positive when SI is ≥3 (7).
Inter-assay coefficient of variation (CV) was evaluated by repeated measurement of spot numbers to recall antigens using the same donor over a 12 month period. The CVs for the spot number for both the IFN-$\gamma$ and IL-10 assays were 12.3% and 10.7%, respectively.

**Statistical analysis**

Positive responses were compared using Fisher’s exact test. T cell response data were aggregated for an autoantigen (proinsulin, insulin, GAD65, IA-2) and if any of the derivative peptides elicited a response, this autoantigen was considered positive (7).

**Results**

*IFN-$\gamma$ responses in children and adults with newly diagnosed T1DM*

A higher frequency of children with T1DM (28/33; 85%) mounted an IFN-$\gamma$ response to one or more of the islet-autoantigen peptides compared with adults (17/28 61%; p=0.04, Figure 1a). Two findings suggest that this difference does not simply reflect a difference in age. First, we found that children who are autoantibody-negative siblings without T1DM, had significantly lower IFN-$\gamma$ responses (12/48 (25%)) compared with children (p=0.0001) and adults (p=0.003) with T1DM; autoantibody-negative adult siblings also had significantly lower IFN-$\gamma$ responses (13/43 (30%) compared to adults (p=0.015) and children (p<0.0001) with T1DM. Second, the prevalence of anamnestic IFN-$\gamma$ responses to pentavalent vaccine was similar in children and adults (96% and 94% respectively) (Table 1). There were no gender biases in the detected responses.
β-cell specific CD4 responses in juveniles and adults

IFN-γ response specificity also differed between children and adults notably to insulin and proinsulin peptides. Responses to insulin A1-21 (33% in children vs. 7% in adults, p=0.025), insulin B1-20 (42% vs. 11%, p=0.009), proinsulin peptides: C13-32, (55% vs. 11%, p=0.0004), C19-A3 (42% vs.14%, p=0.02) and C22-A5 (27% vs. 4% p=0.0001) and GAD 555-67 (30% vs. 4%, p=0.007) were all significantly higher in children (Figure 1b). In contrast, responses to individual peptides were detected in 2-8% of the younger siblings and 2-11% of the adult autoantibody-negative siblings without type 1 diabetes.

The breadth of the IFN-γ response, as measured by the number of peptides an individual responded positively against, differed significantly between children and adults with T1DM: only 4/28 (14%) adults showed a response to 3 or more islet peptides whereas this was observed in 23/33 (70%) children (p<0.0001). The median number of peptides eliciting an IFN-γ response in children was higher: 4 in children vs 1 in adults (p<0.0001) (Figure 1c). Thus, at diagnosis of T1DM children have IFN-γ responses to multiple islet peptides more frequently than adults.

The magnitude of the autoreactive response was assessed in subjects who made positive peptide-specific IFN-γ responses. The magnitude of the response to each peptide was similar in children and adults. However, differences were observed for insulin peptide B1-20 and IA-2 752-775 (mean SI higher in children; p=0.0009 and p=0.01, respectively) (Figure 1d). The magnitude of the response to pentavalent vaccine was not significantly different between across the study groups (Table 1).

**IL-10 responses in children and adults with newly diagnosed T1DM**
In contrast to IFN-γ responses, the frequency of people making an IL-10 response to islet-autoantigen peptides was similar in children (19/33 (58%) and adults 13/28 (46%) p=0.4) with T1DM (Figure 1a) and unaffected siblings (31/48 (65%) children; 30/43 (70%) adults) without T1DM. Again, there was no gender bias and the prevalence of IL-10 responses to pentavalent vaccine was similar in children and adults (96% and 97%, respectively).

IL-10 response specificity to individual peptides was varied and showed no distinct pattern (Figure 1e). The breadth of the IL-10 response, was not significantly different between children and adults with T1DM; 5/28 (18%) adults showed a response to three or more islet peptides compared to 11/33 (33%) children (p=0.2). The median number of peptides eliciting an IL-10 response in children was 1 and 0 in adults (Figure 1c). The magnitude (SI) was not significantly different between children and adults (Figure 1f) and pentavalent vaccine responses were similar across groups. The IL-10 response to pentavalent vaccine was similar in all groups (Table 1).

Overall, the responses in children with T1DM show an IFN-γ predominance (Figure 2a): 13/33 children (39%) show an exclusive IFN-γ response compared to 4/33 (12%) with IL-10 only (p=0.02). Such skewing is not observed in adults (9/28, 32% for IFN-γ and 6/28, 21% for IL-10; p=0.5). When examining only insulin and proinsulin specific responses which are far more prevalent in children, this pro-inflammatory polarisation is much more apparent: 81% (27/33) of children make an IFN-γ response compared to 39% (11/28) adults (p=0.0012). IL10 responses to proinsulin and insulin peptides are similar in children (55% (18/33) and adults (46% (13/28) (p=0.6)
None of the autoimmune phenotypes were influenced by the presence of HLA-
*DRB1*0301 and *0401 genotypes in either children or adults with T1DM (data not shown).

**Autoimmune phenotypes in children and adults with T1DM**

We agglomerated peptide-specific responses into their parent antigens and analysed these alongside autoantibodies (Figure 2b). The prevalence of autoantibodies in children and adults was similar. However, IFN-γ responses against proinsulin and insulin were significantly more frequent in children (70% and 63% compared to adults; 18% and 36% respectively, p<0.0001 and p=0.04 respectively). Also notable was a significantly lower GAD65-specific IL-10 response in adults compared to children (12.5% vs. 36% in children, p=0.03).

**Autoantibody and T-lymphocyte responses**

Of the people positive for GAD autoantibodies, a corresponding T-lymphocyte inflammatory (IFN-γ) response was seen in more children than in adults (17/20, 85% versus 11/21, 52%; p=0.04). For IA-2 autoantibody positivity, a corresponding T-lymphocyte inflammatory response was seen in more children than in adults (23/25, 92% versus 8/16, 50%; p=0.007). For ZnT8 autoantibody positivity, there was a trend for corresponding T-lymphocyte inflammatory response to be greater children than in adults but this was not statistically significant (19/22, 86% versus 9/16, 56%; p=NS). In contrast there was no relationship between any autoantibody positivity and IL-10 responses. Overall, the stronger relationship between autoantibodies and
β-cell specific CD4 responses in juveniles and adults

inflammatory T-lymphocyte responses in children emphasises the stronger pro-inflammatory bias in the young.
**Discussion**

This study compares islet antigen-specific cellular immune responses in recent-onset T1D arising in childhood and adult life; and has led to a novel observation: near to diagnosis of T1D, pro-inflammatory autoreactivity is significantly more prevalent, and targets a wider range of islet peptides in children than adults, which is consistent with a broader and thus more aggressive autoimmune response in the younger age group. This finding is consistent with the proposition that islet autoreactivity is broader and more aggressive (or less well regulated) in the younger age group. Younger participants also preferentially target epitopes of proinsulin and insulin, suggesting that the antigenic driver(s) of disease also differ with age. We speculate that these findings are linked to, and provide a mechanistic explanation for the known tendency of C-peptide reserve to decline at a faster rate in younger people after T1DM diagnosis (10).

The increased frequency of IFN-γ responses in children could be attributable to several different influences, which will need to be examined in future studies; control of autoreactivity by naturally arising CD4+CD25\textsuperscript{hi}FoxP3+CD127\textsuperscript{lo} regulatory T cells (nTregs) could differ between adults and children; indeed lower numbers of T regs have been reported in children with T1DM (11, 12). Furthermore, it has been previously demonstrated that there is a correlation between increasing age and frequency of nTregs in T1D (13) and it is conceivable that as a consequence adaptive immune regulation is stronger in adulthood, leading to a more limited autoreactive T cell response. A further possibility is that the reduced observed autoreactivity in adults reflects a relatively reduced genetic load of T1DM predisposing genotypes (14), some of which are likely to influence disease susceptibility via effects on immune regulation of effector pathways. In a recent study
of people aged 17 years and over at diagnosis, the slower progression toward autoimmune insulin deficiency was ascribed to a lower T1DM-predisposing genetic load (14). Also the same authors noted that non-HLA genes conferring susceptibility were associated with a lower age of diagnosis (15).

Interestingly, although the frequency and breadth of the pro-inflammatory autoimmune response was greater in children in our study, the magnitude of the response as measured by stimulation index was generally similar in children and adults. The stimulation index acts as a surrogate for the number of responder cells, suggesting that what marks out children developing T1DM is a poly-specific response that targets more autoantigens and/or more epitopes, in keeping with the notion that determinant spreading is a key immunological driver (16).

We further explored this response to proinsulin and insulin peptides and demonstrated significantly higher IFN-γ responses in children compared to adults; IL10 responses did not differ amongst the two groups. This is an interesting finding and suggests a polarisation towards a pro-inflammatory response against peptides of proinsulin and insulin specifically in children which has major implications for choice of immuno-therapy in this population.

Responses characterised by release of the natural immune suppressive cytokine IL-10 did not differ between children and adults, apart from with respect to GAD65, which was significantly less frequent in adults. It is tempting to speculate that this relatively poor GAD65-specific immune regulation is related to the greater propensity for adult T1DM to focus on GAD65 as a major autoantigen for autoantibody responses (17).
There are some caveats and limitations in our study. For example, future studies in cohorts followed longitudinally will be needed to address whether age-related differences in T cell responses are persistent and have the same behaviour for other autoantigens, and whether they are influenced by high-risk HLA alleles of $DQA1$ and $DQB1$ genes and the extent to which they relate directly to rate of disease progression and loss of C-peptide. Larger numbers of subjects could also explore how responses to other autoantigens differ, especially in those people with T1DM and an additional autoimmune disease. Although not significant, we observed a trend for vaccine-specific IL-10 and IFN-$\gamma$ responses to be lower in adults. We speculate that this reflects the distance in years that adults are from exposure to these recall antigens in vaccines or wild-type infections, compared with the children who would have been actively immunized more recently, and this should be explored in future studies.

It would also be of interest to see whether these distinct phenotypes are present during pre-clinical disease as such studies have not been conducted.

The present study provides evidence to substantiate the hypothesis that the autoimmune response in children developing T1DM is more pro-inflammatory/less regulated than in adults, further highlighting recent reports of heterogeneity in disease pathogenesis (7). Viewed from the perspective of designing intervention trials and selecting therapeutic agents, our findings suggest that these may require greater attention to age/inflammatory set-point than has been the case hitherto.
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Contribution Statement

SA designed and performed experiments, analysed data and wrote the manuscript; SA, and MP conceived ideas and oversaw the research. VG and VN performed experiments. CD, JP, CG, DBD, PJB and JAT recruited and characterized participants. AL performed all the statistical analyses. All authors participated in the interpretation and analysis of the data, the critical revision and final approval of the manuscript. MP is the guarantor of this work and, as such, had full access to all of
the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Legends to figures

Figure 1: Frequency and magnitude of IFN-γ and IL-10 responses in T1DM according to age at disease diagnosis.

a) The frequency of IFN-γ and IL-10 responses in children (open bars) and adults (shaded bars) with T1DM. Bars represent means of percent responders to any peptide (*p=0.04).

b) Stacked bars showing the prevalence of IFN-γ responses to each islet autoantigenic peptide in children (open bars) and adults (shaded) with T1DM. The frequency of responses to each peptide has been compared by Fishers exact test and p values of <0.05* and p<0.0005** are shown.

c) Scatter plot represent the number of peptides eliciting IFN-γ and IL-10 responses in children (open squares) and adults (black circles) with T1DM; the median response is represented by the black horizontal line (**p<0.001).

d) The mean Stimulation Index (SI) for each peptide response in children (open squares) and adults (black circles) with T1DM for IFN-γ responses. The frequency of responses to each peptide has been compared by an unpaired t-test and p values of <0.05* and p<0.005**** are shown.

e) Stacked bars showing the prevalence of IL-10 responses to each islet autoantigenic peptide in children (open bars) and adults (shaded) with T1DM.

f) The mean Stimulation Index (SI) for each peptide response in children (open squares) and adults (black circles) with T1DM for IL-10 responses.
Figure 2: The autoimmune response is skewed towards a pro-inflammatory phenotype in children and peptides of proinsulin and insulin are preferentially targeted by this pro-inflammatory immune response.

(a) Autoreactive T cell responses to β-cell peptides in children (open red triangles) and adults (open blue circles). Positive peptide responses (SI>3 for interferon-γ and/or IL10) have been plotted for each cytokine; the numbers in each quadrant represent number of positive responses.

(b) CD4 T cell responses to islet target peptides agglomerated according to parent antigen. Graph shows frequency of response to islet autoantigens in children (x-axis) and adult (y-axis). Red circles denote IFN-γ responses; blue: IL-10 responses, and green: autoantibody responses. Filled circles indicate a statistically significant difference ($P<0.05^*$) in the frequency of responses between the two groups. Grey lines are 95% CIs. Ins, insulin; PI, proinsulin.
Table 1: Demographic data on people with T1DM and unaffected siblings

N/A: not applicable; Abbreviations; GADA, glutamic acid decarboxylase antibodies; IA2-A, insulinoma-associated antigen 2 antibodies; ZnT8-A, zinc transporter 8 antibodies

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<th>Children (33)</th>
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References


Figure 1

**c**

Number of peptides eliciting a response

**d**

Mean stimulation index

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Figure 1

(f) Mean stimulation index

(e) Frequency of subjects making a response (%)
Figure 2

(a) Interferon-γ stimulation index vs. IL-10 stimulation index for Children and Adults. The numbers indicate the count of observations.

(b) Scatter plot showing the correlation between Children (n=33) and Adults (n=28) with specific markers indicating different conditions. The green line represents the linear relationship.