Blood and islet phenotypes indicate immunological heterogeneity in type-1 diabetes

Running title: Immunological heterogeneity in type 1 diabetes

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Abstract

Studies in type 1 diabetes indicate potential disease heterogeneity, notably in the rate of β-cell loss, responsiveness to immunotherapies and, in limited studies, islet pathology. We sought evidence for different immunological phenotypes using two approaches. First, we defined blood autoimmune response phenotypes by combinatorial, multi-parameter analysis of autoantibodies and autoreactive T-cell responses in 33 children/adolescents with newly-diagnosed disease. Multi-dimensional cluster analysis showed two equal-sized patient agglomerations, characterized by pro-inflammatory (IFN-γ+, multi-autoantibody-positive) and partially-regulated (IL-10+, pauci-autoantibody-positive) responses. Multi-autoantibody-positive non-diabetic siblings at high-risk of disease progression showed similar clustering. Second, pancreas samples obtained *post mortem* from a separate cohort of 21 children/adolescents with recently-diagnosed type 1 diabetes were examined immunohistologically. This revealed two distinct types of insulitic lesion, distinguishable by degree of cellular infiltrate and presence of B-lymphocytes, that we term "hyper-immune CD20Hi" and "pauci-immune CD20Lo". Notably, subjects had only one infiltration phenotype and were partitioned by this into two equal-size groups that differed significantly by age of diabetes-onset, hyper-immune CD20Hi subjects being 5 years younger. These data indicate potentially related islet and blood autoimmune response phenotypes that coincide with, and precede disease. We conclude that different immunopathological processes (endotypes) may underlie type 1 diabetes, carrying important implications for treatment/prevention strategies.

Type 1 diabetes is a chronic inflammatory disease resulting from selective immune-mediated destruction of insulin-producing β-cells in the islets of Langerhans (1). Numerous features of the syndrome are constant, including absolute requirement for exogenous insulin administration, and in the narrower definition of type 1A diabetes, evidence of ongoing autoimmunity directed against the islets (2). The latter is usually manifest via circulating islet antigen-specific autoantibodies (islet AAbs), but there is also now considerable evidence that circulating autoreactive CD4 and CD8 T cells are detectable (3-7). These insights have been critical in the translational course towards the development and implementation of immune-based therapies, aiming to subvert or incapacitate immune pathways that lead to β-cell death.

In most intervention studies to date, the major inclusion criterion is positivity for a single islet AAb, of any specificity. In light of recent suggestions that type 1 diabetes is heterogeneous in nature, this aspect of clinical trial design is worth closer inspection. There is evidence, for example, that patients vary in their presenting level of functional β-cell reserve and its rate of loss (8), as well as the number and type of AAbs (9). There is also an emerging impression that islet pathology, in terms of extent of β-cell damage and immune cell infiltration, may have a broad range of presentations, although data in this regard are currently very limited (10). If these observations of clinico-pathological heterogeneity reflect diverse immunological pathways to disease, then this could have important implications for the selection and testing of agents designed to halt ongoing immune-mediated damage.

We previously described circulating CD4 T cells specific for islet autoantigens and secreting inflammatory cytokines (IFN-γ, IL-17) as a characteristic of type 1 diabetes (4; 6). Those studies provided proof-of-concept that autoreactive T cells are present at disease diagnosis, but there have been limited attempts to date to link this finding to the presence of islet AAbs and gain a more holistic view of the adaptive immune response. Since patients with type 1 diabetes may have absent, single or multiple islet specific autoantibodies at diagnosis, this broader view of the autoimmune response may offer insights into the nature of this heterogeneity.

From these considerations, we identified two important, and potentially linked knowledge gaps. The first is whether there are distinct patterns of the adaptive autoimmune response detectable in the blood that can be revealed in combinatorial approaches; and the second is whether there is evidence of heterogeneity of islet pathology. To address these, we combined measurements of autoimmunity (functional phenotype of autoreactive CD4 T cells and islet AAbs) with multi-dimensional data analysis to explore whether distinct autoimmune response phenotypes are present in children with type 1 diabetes and how they relate to disease risk in siblings. In addition, we profiled the islet immune cell infiltration in a cohort of children who died close to type 1 diabetes onset. Our data provide evidence for distinct immunopathological processes in type 1 diabetes development, which may have important implications for intervention and prevention strategies.

Materials and Methods

Autoantibody assays

Autoantibodies to insulin (IAA), glutamic acid decarboxylase (GAD65Ab), islet antigen (IA-2Ab), and zinc transporter 8 (ZnT8Ab) were measured by radioimmunoassay as previously described (11; 12). IAA, and ZnT8Ab levels were expressed in arbitrary units and GADAb and IA-2Ab in DK units/mL (12).

Studies on peripheral blood

Fresh heparinized blood samples were obtained from 33 children with type 1 diabetes (median age 11 years, range 5-16 years; duration of type 1 diabetes ≤ 12 weeks: median 8 weeks; 60% males). Positivity for AAbs was as follows: GADAb, 54%, IA-2Ab, 73%; and ZnT8Ab, 65%. Insulin autoantibody measurements were not conducted on patients with type 1 diabetes. In addition, blood samples were obtained from unaffected siblings of patients with type 1 diabetes within a similar age range (n=72; median age 13 years; range 6-16 years). Forty-four were negative for all AAbs; 10 had a single AAb; 5 were positive for 2 AAbs; 7 were positive for 3 AAbs; 3 had all 4 AAbs. A further 4 subjects were recruited via the Type 1 Diabetes TrialNet Pathway to Prevention study (TN-01) (median age of 16 years; range 12-18 years; 1 subject with a single AAb; 3 positive for 2 AAbs). To avoid biases in CD4 T cell responses due to varied possession of HLA class II molecules, only participants with one or both of the *HLA-DRB1*0301* and **0401* genotypes were enrolled. In our previous studies the frequency of responses to IA-2, proinsulin and GAD65 peptides has not been significantly different between *HLA-DRB1*0301* and **0401* subjects (6; 13). Of subjects available for study, 12 children/adolescents with type 1 diabetes and 33 unaffected siblings were excluded on the basis of non-*HLA-DRB1*0301 or *0401*genotype. These studies were carried out with the approval of the UK National Research Ethics Service, and for blood studies informed consent was obtained from all participants or their parents/guardians.

Detection of β-cell specific cytokine secreting CD4$^+$ T cells

Peptides based on sequences of naturally processed and presented IA-2, proinsulin and GAD-65 epitopes and overlapping regions of insulin B and A chains were synthesised and purified by HPLC (Thermohybaid, Germany). Pediacel, a penta-vaccine, was obtained from Sanofi Pasteur Ltd (Berkshire, UK) and used at 1µl/ml to examine anamnestic responses induced by vaccination or infection as previously described (4). Detection of interferon (IFN)-γ and IL-10 production by CD4$^+$ T cells was carried out using an enzyme-linked immunospot (ELISPOT) as previously described (6; 13) and performed blinded to the clinical status of the donor (type 1 diabetes or sibling; AAbs or not). Data are expressed as the mean number of spots per triplicate and compared with the mean spot number in the presence of diluent alone (stimulation index; SI) and a response is considered positive when the SI is ≥3 (6). IFN-γ responses to pediacel were similar in patients with type 1 diabetes and unaffected siblings (mean SI 79.7 versus 78.8, respectively) as were IL-10 responses (mean SI 20.5 versus 26.7, respectively).

Studies on islet-infiltrating leukocytes

Formalin fixed paraffin embedded pancreas samples from 21 patients with type 1 diabetes from a previously described cohort (14) were included in the present study on the basis of confirmed insulitis, as defined in a recent position statement (10). These cases had a median age of 12 years (range 1-23 years) at disease onset and median disease duration of 1 week (1 day-6 months). Sections of pancreas (4µm) were mounted on glass slides previously coated with (3-aminopropyl)-triethoxysilane (Sigma, Dorset, UK) then de-waxed and rehydrated in alcohol. Antigens were visualised by standard horseradish peroxidase staining and, with the exception of insulin, were unmasked using heat induced epitope retrieval (HIER). For CD45 and CD20, HIER was achieved in 10mmol/l citrate buffer, while CD4, CD8 and CD68 were retrieved with 10mmol/l Tris, 1mmol/l EDTA, pH9.0. Primary antibodies where applied for 1 hour at room temperature, with exception of CD4 which required overnight incubation at 4°C. Antigens were visualised using the Dako REAL^{TM} Detection System and analysed by light microscopy. Ten insulin-containing islet sections were

selected at random in each case and the individual immune cell numbers per islet section were monitored by visual inspection of immunostained serial sections and recorded manually. Two additional cases were selected for study from within the Juvenile Diabetes Research Foundation network of Pancreatic Organ Donors (nPOD) on the basis of being the only samples in the bioresource from patients with recent onset diabetes (≤12 months). These cases (6113 and 6052) were examined by a combination of immunofluorescence, horseradish peroxidase and alkaline phosphatase staining for at least 2 antigens per section to minimise sample usage. Antigens were unmasked by HIER in 10 mmol/L citrate buffer, pH6. Combined staining was achieved by employing guinea pig anti-insulin (Dako, #A056401) in sequence with mouse anti-CD45 (Abcam, #M0701); rabbit anti-glucagon (Dako, #A0565) after mouse anti-CD20 (Dako, #M0755); and mouse anti-CD68 (Dako, #M0876) with rabbit anti-CD8 (Abcam, #GR404-4). Secondary antibodies were labelled with Dylight 405 (anti-guinea-pig 405, Stratech Scientific Ltd, Suffolk, UK; #106-475-003-JIR) Alexafluor anti-mouse 488 (#A11029) or anti-rabbit 568 (#A1136; Invitrogen, Paisley, UK). Alkaline phosphatase was detected using a Vector Red Substrate kit (Vector Labs, UK; #AK-5100 and #SK-5100) and cell nuclei were stained with TOPRO 3 Iodide (red; Invitrogen; #T3605) or DAPI (blue). IF and brightfield images were captured on a Nikon 80i and Nikon 50i Eclipse Microscope, respectively.

Statistical analysis

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. If responses to derivative(s) were positive, but any relevant test condition (peptide, IFN-γ, IL-10) for the same antigen was missing, the autoantigen test value was considered positive and the subject was included in the analysis. If responses to derivatives were negative, but any relevant test condition for the same antigen was missing, the autoantigen test value was considered missing and the subject was excluded from analysis. A total of 33 children/adolescents with type 1 diabetes out of 55 tested and 72 unaffected siblings out of 97 tested were included in the analyses. The prevalence of positive

responses was established with confidence intervals of 95% and compared using Fisher's exact test. Clustering was analysed by agglomerative hierarchical clustering with Ward's method, based on squared Euclidean distance between tests/patients. Support for clusters was estimated by multi-scale bootstrap re-sampling with 1000 replications, implemented in the R package pvclust (15). Principal component analysis (PCA) was performed using unscaled binary data with standard R functions on healthy siblings with at least two AAbs positive and rotation values from this dataset were applied to samples from children/adolescents with type 1 diabetes. Differences in the immune cell infiltration into islet sections were assessed by Mann Whitney U test. Data were analysed using GraphPad Prism 5 software or in statistical software environment R. A p value <0.05 was considered statistically significant.

Results

Autoimmune inflammatory phenotypes in children with new-onset type 1 diabetes

Blood samples from 33 ~~consecutively~~ newly-diagnosed children with type 1 diabetes were analyzed for the quality (IFN-γ, IL-10, AAbs) and specificity (proinsulin, insulin, IA-2, GAD65 and ZnT8) of autoimmune responses in a combinatorial approach. This analysis generated results for 27 analytes (3 AAbs, 12 islet peptides each tested for two cytokines) which, in the case of the T cell responses, were combined to show responsiveness to the parent antigens (**Table 1**). Analysis of these data by agglomerative hierarchical clustering showed the formation of two highly stable autoimmune response clusters (bootstrap support for the main nodes ≥95%; **Figure 1A**) representing (i) a combination of islet AAbs and IFN-γ responses to all antigens, as distinct from (ii) IL-10 responses to all antigens. In the same analysis, patients with type 1 diabetes formed two distinct clusters (bootstrap support for the main nodes >97%; **Figure 1B**) of approximately equal size.

Combined analysis of the patient and autoimmune response clusters using unbiased hierarchical clustering in a heatmap illustrates that the major discriminating factor between the two patient agglomerations is the presence of an IL-10 response (cluster-1, bottom left, **Figure 1C**). We applied additional analytical methods to support this conclusion. Cluster-1 patients have a significantly higher frequency of IL-10 response to GAD, insulin, proinsulin (all $p<0.0001$), IA-2 ($p<0.02$) than are seen in cluster-2. Importantly, there are also differences in the frequency of islet AAbs between clusters. AAbs against IA-2 ($p<0.002$) and ZnT8 ($p<0.05$) are significantly less frequent in the IL-10-dominated cluster-1. Moreover, within cluster-1, 2 children had no islet AAbs present at diagnosis, 5 had only a single autoantibody and 8 had ≥2 AAbs. The frequency of multiple AAbs was significantly higher in cluster-2, in which all 18 children had ≥2 ($p=0.0015$).

We did not observe significant biases in the distribution of the main diabetes-associated HLA alleles in the two agglomerations (*DRB1*0401/X* and *DRB1*0301/X* genotypes both present in 6/15 (40%) of subjects in cluster-1, the remaining 3 subjects being heterozygous *DRB1*0401/*0301*; and

*DRB1*0401/X* present in 11/18 (61%) and *DRB1*0301/X* in 3/18 (17%) of subjects in cluster-2, the 4 remaining subjects (22%) being heterozygous). In contrast to the stable clustering we observed for aggregated analytes (**Figures 1A-B**), when single analytes are used the main clusters are less stable (bootstrap scores for the two main nodes 81% and 85%; data not shown), supporting an approach in which epitope-specific responses are aggregated. Principal component analysis (PCA) was explored to assist in subdivision of subjects or enable assay refinement by exclusion of redundant analytes. As shown in **Figure 1D,** although clustering patterns were largely reproduced, we did not find single prevalent sources of variation using this approach.

To summarise, these data show that combining autoantibody and the quality of CD4 T cell responses to specific islet autoantigens partitions children with newly diagnosed type 1 diabetes into two distinct autoimmune response phenotypes, characterised by islet AAbs plus IFN-γ or a pauci-AAb, IL-10 dominated response. These data reveal a hitherto unidentified association between IL-10 responses and regulated B lymphocyte responses *in vivo*.

Autoimmune inflammatory phenotypes in non-diabetic siblings of children with type 1 diabetes

We next ~~further~~ explored the disease relationship of the autoimmune response phenotypes we had defined by studying non-diabetic siblings of type 1 diabetes. First, we studied a group of siblings of type 1 diabetes patients. As expected, when comparing siblings with newly diagnosed children, islet AAbs, and especially IA-2Ab and ZnT8Ab were strongly associated with disease (**Figure 2**). However, IFN-γ CD4 T cell responses to derivative peptides of specific antigens, notably insulin and proinsulin, were also strongly and significantly diabetes-associated (**Figure 2A-B**). Indeed, when T cell responses were combined into a single analyte (ie the presence of an IFN-γ CD4 T cell response to any autoantigen), disease-association is comparable to detecting any islet AAb in terms of its ability to discriminate disease and health with equal sensitivity and specificity (**Figure 2C**).

In contrast, IL-10 CD4 T cell responses show a similar prevalence in siblings and children with diabetes (**Figure 2**). However, when the clustering of aggregated analytes from the type 1 diabetes samples (**Figure 1**) was superimposed on the non-diabetic sibling population it is notable that there is

a large group of healthy islet AAb-negative children (low risk for type 1 diabetes) who have IL-10 responses, but not IFN-γ responses to islet antigens (cluster top left, **Figure 3A**). Unsupervised clustering of aggregated analytes in non-diabetic siblings shows similar clustering (bootstrap scores for main clusters >93%; not shown).

Autoimmune inflammatory phenotypes in multiple-autoantibody-positive non-diabetic siblings of children with type 1 diabetes

To examine the relevance of autoimmune inflammatory phenotypes to the natural history of progression to type 1 diabetes, we identified a group of subjects with multiple (≥2) islet AAb positivity, a status known to confer high risk of progression to disease. In these subjects (n=14), analysis using the clustering model superimposed from the type 1 diabetes samples (**Figure 1**) shows a pattern of responses comparable to those seen in type 1 diabetes. Non-diabetic subjects with ≥2 islet AAbs partition into two main clusters characterised by (i) the presence of IL-10 with sparse IFN-γ, and (ii) the presence of IFN-γ with sparse IL-10 (**Figure 3B**). Unsupervised analysis did not generate robust evidence of clustering, presumably reflecting the small numbers of high risk subjects studied. We also examined whether PCA could have potential as a tool for identifying outcomes in the same high-risk subjects with multiple (≥2) islet AAbs. In the analysis superimposed on type 1 diabetes patients, we identified two potential clusters by arbitrary means (labelled A and B, **Figure 1D**). These are dominated by IFN-γ and IL-10, respectively, tending to reinforce the findings from cluster analysis. Applying the same analysis in high-risk siblings (**Figure 3C**) indicates subjects within the same potential clusters. Since our studies were initiated, 3 of the high-risk subjects developed diabetes. The subject in the "IL-10" dominated cluster was aged 18 years at diagnosis, whilst those in the "IFN-γ" cluster were aged 6 and 10 years.

Heterogeneity of islet infiltration in recent onset type 1 diabetes

We considered the possibility that findings in the blood might have correlates in the pancreata of patients with type 1 diabetes (not available from the same cohort) and therefore examined insulitic profiles in a large historical tissue collection from children (n=21) who had died close to diagnosis of

diabetes (14). This revealed considerable variation in the absolute numbers of immune cells per islet identified by staining for CD45, CD20, CD4, CD8 and CD68. We noted a particularly high coefficient of variation for CD20 staining as an indication of dispersion of the frequency distribution (**Table 2**). This was consistent with our subjective analysis by microscopy in which we observed two distinct patterns of infiltration: one characterised by relatively large numbers of infiltrating immune cells, but especially CD20+, and the other by a relative paucity of immune cells with very low numbers being CD20+. Of note, similar patterns were seen in an additional two patients obtained via the nPOD collection, ascertained within 12 months of diagnosis (**Figure 4A-F**).

To simplify the analysis, we ranked the 21 subjects in our collection by CD20 staining and identified an inflection, the mid-point of which (mean of 3.7 CD20+ cells per islet section; **Supplementary Figure 1**) was used to define "CD20Hi" and "CD20Lo" staining patterns. This divided the cohort into two approximately equal-sized groups (n=9 and n=12, respectively). These two groups significantly differed in the number of total (CD45+) immune cells (mean (SEM) of 29.9 (4.3) versus 13.2 (0.8) cells per islet section for the CD20Hi and CD20Lo groups, respectively, $p<0.01$) and in cell numbers of the constituent subsets such as CD20, CD8 (**Figure 4G-H**; $p<0.01$ for both) and CD4 ($p=0.03$), leading us to term these "hyper-immune CD20Hi" and "pauci-immune CD20Lo" patterns. CD68+ cells were not significantly different between the groups ($p>0.05$). Differences between the two groups could not be ascribed to differences in the β-cell status of the islets studied, since we examined the immune cell infiltration only in those islets with residual immune-staining for insulin (ie islets with remaining β-cells). These data indicate that patterns of infiltration may represent functionally important pathological phenotypes, a contention borne out by two further observations. First, we found that hyper-immune CD20Hi subjects had significantly fewer insulin-containing islets as a proportion of all islets identified than pauci-immune CD20Lo subjects (mean (SEM) 15.5% (4.8) versus 38.3% (6.9); $p=0.02$). Second, we noted that the mean age of subjects with hyper-immune CD20Hi infiltration was significantly lower than that of pauci-immune CD20Lo subjects among this cohort (mean (SEM) 7.8 (1.7) years versus 13.0 (1.5) years; $p=0.03$).

Taken together, these data suggest that children and adolescents studied close to diagnosis of type 1 diabetes may be distinguished by their tissue pathology into two age-related and residual insulin-related patterns, characterised by high and low degrees of cellularity and B lymphocyte infiltration.

Discussion

In the present study we use two complementary analyses, in tissue and blood, to identify disease-related sub-phenotypes in type 1 diabetes. Our blood-based study shows that approximately half of patients analyzed close to diagnosis have T cell responses characterised by IFN-γ, whilst half are distinguished by having IL-10 responses along with significantly fewer autoantibodies. In pancreas tissue obtained close to disease diagnosis, approximately half of the patients studied show an inflammatory islet infiltration distinguished by high numbers of CD20+ B lymphocytes, whilst half of the patients have sparse infiltration and significantly fewer B lymphocytes. We speculate that these blood and tissue phenotypes are related, provide a potential pathophysiological basis for disease heterogeneity of relevance to stratification for therapeutic trials.

β-cell specific autoreactivity in the blood is the hallmark of type 1 diabetes (2). The present study is the first, to our knowledge, to simultaneously assess multiple parameters of autoimmunity that span CD4 T cell and B lymphocyte responses using integrated, multi-dimensional clustering analysis. We show that in children/adolescents who develop type 1 diabetes, it is likely that one or other autoimmune response phenotype, definable as [AAb++ and IFN-γ>>IL-10] and [AAb± and IFN-γ<<IL-10] is dominant at the late stage of disease. In addition, we find the same autoimmune response phenotypes in non-diabetic individuals with multiple (≥2) AAbs and high-risk of progression to type 1 diabetes. Successive studies will require longitudinal follow-up of the high risk group to establish whether the autoimmune response phenotypes are stable over time and indicate a fixed pathway of disease progression, which would be important for deployment of such analyses as stratification tools in prevention studies. Further development and refinement of the multi-parameter model could be achieved through inclusion of additional measurements of autoreactivity, such as circulating CD8 T cells specific for β-cell epitopes (3; 16). This approach may prove of clinical value but will require greater numbers to be studied and a longer period of follow-up.

In addition to these studies in the blood, we returned to a collection of samples obtained *post mortem*, close to diagnosis of type 1 diabetes, on which we have previously published our findings on the immune cell infiltrate (10; 14; 17; 18). Using a new consensus classification of insulitis (10) and focusing on defining *patient-specific* immune cell infiltration, we made the novel observation that patients can be categorized according to hyper-immune CD20Hi and pauci-immune CD20Lo insulitis phenotypes. When considered in the light of our observations in the blood, an obvious conclusion is that hyper-immune CD20Hi subjects have the [AAb++ and IFN-γ>>IL-10] autoimmune response phenotype, whilst pauci-immune CD20Lo subjects demonstrate [AAb± and IFN-γ<<IL-10] reactivity. This speculation derives some support from the fact that both sub-phenotypes (immunohistological and blood-derived) divided the subjects studied into roughly equal-sized halves. In addition, clear mechanistic links can be drawn between having pauci-AAb status in the blood (presumably as a result of antigen-specific immune regulation by islet-specific IL-10+ CD4 T cells (19)) and low or absent levels of B lymphocyte infiltration in the islets. Future studies will be required to focus on the mechanisms of this effect, and whether it involves additional cell types such as the recently described T follicular helper and T follicular regulatory cells (20; 21). The linking of these blood and islet findings represents a vital conjecture, which will require additional studies of both the T cells and histology from the same patient, for example via the Juvenile Diabetes Research Foundation network of Pancreatic Organ Donors (nPOD) collection. This inability to link blood and islet phenotypes in the same individual represents an important limitation of our study, as does the relatively brief follow-up and lack of prospective studies, but these issues can be addressed in future cohorts.

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The observations in relation to age and type of autoimmune response in this and our previous study (6) raise the question as to the underlying basis for the different sub-phenotypes we have observed. It could be that disease endotypes exist that reflect different immunological pathways to disease, as has been proposed in asthma (22; 23). An alternative possibility is that the underlying effector pathways

are not intrinsically different, but that the degree of immune regulation is a dominant discriminative feature. In this scheme, the pauci-immune CD20Lo and [AAb± and IFN-γ<<IL-10] immune phenotypes could be considered to indicate "partially regulated" effector responses which may be more likely to be present in older subjects (24). We have previously shown that IL-10-secreting β-cell specific CD4 T cells have potent regulatory properties (19) and are present in healthy subjects and relatively enriched in older adults developing type 1 diabetes (19) . In the present study we reinforce this concept by reproducing a previous finding that IL-10-mediated autoreactivity is frequently detected in siblings of type 1 diabetes patients who are AAb-negative and have very low disease risk (25). We also extend the concept by showing evidence of operational autoreactive B lymphocyte non-responsiveness (tolerance) *in vivo* when IL-10 responses are present. Taken together these observations emphasise the importance of autoreactive CD4 T cells that secrete IL-10 in influencing disease pathogenesis.

Most relevant to current translational objectives in type 1 diabetes perhaps, are the potential implications of our study for therapeutic strategies. The question of whether type 1 diabetes is a heterogeneous disease has taken on considerable importance in recent years as successful immune-based therapies begin to be tested in Phase II-III clinical trials (26-30). Heterogeneity, or the existence of endotypes defined by discrete pathophysiological mechanisms with the potential to explain distinct clinical features and response to treatment, could open up the prospect of stratification and personalised medicine, both of which are emerging aspirations for the management of complex autoimmune and inflammatory diseases in modern healthcare systems (31). As one obvious example of the "translatability" of our findings, it is likely that B lymphocyte depletion using rituximab would have a different therapeutic impact, depending on whether CD20+ cells are a dominant feature of insulitis and actively engaged in the inflammatory process as indicated by biomarkers such as multiple circulating AAbs and IFN-γ production, or not, as evidenced by few or no AAbs and high IL-10 responses. Indeed, the original report of rituximab intervention therapy in

type 1 diabetes shows trends for greater treatment efficacy (preservation of stimulated C-peptide) in younger *versus* older patients and those with greater than or equal to two AAbs as opposed to those with a single specificity (30). Thus it seems probable that future studies of this and other therapeutics for prevention of β-cell destruction are likely to benefit from a greater emphasis on prospective and *post hoc* analyses that make use of multi-parameter measurements of T and B lymphocyte autoreactivity such as those defined in our study.

SA designed and performed experiments, analyzed data and wrote the manuscript; SA, MP, JT, AKF and NGM conceived ideas and oversaw the research. VN, KM, ME, NMN, PL, AW and SJR performed experiments. CG, DD, PB and JP recruited and characterized patients. DKV, ER and AL performed all the statistical analyses. 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.

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Table 1. Autoantigens and derivative peptide epitopes tested in T cell assays

Parent antigen	Region	Sequence	Relevant T cell studies	Designation in analyses
Insulin (A + B chains)	A1-21	GIVEQCCTSICSLYQLENYCNK	N/A	Ins (1)
	B1-20	FVNQHLCGSHLVEALYLVCGK	N/A	Ins (2)
	B6-25	LCGSHLVEALYLVCGERGFFK	N/A	Ins (3)
	B11-30	LVEALYLVCGERGFFYTPKTK	N/A	Ins (4)
Proinsulin	C13-32	GGGPGAGSLQPLALEGSLQK	cited(6)	PI (5)
	C19-A3	GSLQPLALEGSLQKRGIV	cited(4; 6; 13)	PI (6)
	C22-A5	QPLALEGSLQKRGIVEQ	cited(6)	PI (7)
IA-2	709-736	LAKEWQALCAYQAEPNTCATAQGEGNIK	cited(4; 6; 13)	IA-2 (8)
	752-775	KLKVESSPSRSDYINASPIIEHDP	cited(4; 6; 13)	IA-2 (9)
	853-872	SFYLKNVQTQETRTLTQFHF	cited(4; 6; 13)	IA-2 (10)
GAD65	335-352	TAGTTVYGAFDPLLAVAD	cited(4; 13)	GAD (11)
	555-567	NFIRMVISNPAAT	cited(4)	GAD (12)

Table 2. Immunohistological findings in *post mortem* analysis of pancreas from 21 Type 1 diabetes patients

	Immune cell markers			
	CD8	**CD68**	**CD20**	**CD4**
Mean (cells stained per islet)	11.2	7.0	5.5	3.7
Std. Deviation	9.4	6.1	8.0	3.1
Lower 95% CI of mean	6.9	4.2	1.8	2.3
Upper 95% CI of mean	15.4	9.7	9.1	5.1
Number of subjects	21	21	21	21
Coefficient of variation (%)	83.9	87.1	147.0	83.9

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Figure 1: Cluster analysis of autoreactive T cell and autoantibody responses in the blood close to diagnosis of type 1 diabetes. Blood samples from 33 ~~consecutively~~ newly-diagnosed children with type 1 diabetes were analyzed for the quality (IFN-γ, IL-10, AAbs) and specificity (proinsulin, insulin, IA-2, GAD65 and ZnT8) of autoimmune responses, generating results for 27 analytes summed to show responsiveness to the parent antigens. (A) Dendrogram shows agglomerative hierarchical clustering analysis which reveals two highly stable autoimmune response clusters (bootstrap support for the main nodes ≥95%) a combination of islet AAbs and IFN-γ responses to all antigens tested (right cluster), as distinct from IL-10 responses to all antigens tested (left cluster). (B) Dendrogram shows agglomerative hierarchical clustering analysis in which patients with type 1 diabetes form two distinct clusters (bootstrap support for the main nodes ≥97%) of approximately equal size. (C) Combined analysis of the patient and autoimmune response clusters using unbiased hierarchical clustering in a heatmap illustrates that the major discriminating factor between the two patient agglomerations is the presence of an IL-10 response (cluster-1 bottom left). Yellow indicates positive response to an analyte; blue is negative. CD4 T cell peptide-specific responses are summarised into proteins (IA-2, GAD, PI and Ins). Panel (D) shows plots of the two principal components of autoreactive T cell responses in patients with type 1 diabetes, each identified as circles. The colour of the symbol indicates AAb positivity (open = no AAb; yellow = 1 AAb+; orange = 2 AAb+; red = 3 AAb+). Numbered, arrowed blue lines represent vectors indicating the influence of individual analyte responses on PC1 and PC2, in which the length of the arrow reflects the strength of the effect; continuous blue lines are IFN-γ and broken lines IL-10 responses, respectively and numbers 1-12 are the peptide identifiers (Table 1). The dashed oval outlines indicate putative patient clusters, with Cluster A predominantly reflecting IFN- γ and Cluster B IL-10 responses, respectively.

Figure 2: Autoimmune inflammatory phenotypes in non-diabetic siblings of children with type 1 diabetes. Graphs compared frequency of response to islet autoantigens in siblings of type 1

diabetes patients collected in a cross-sectional study (y-axis) with that in type 1 diabetes patients matched for age and studied close to diagnosis (x-axis). Blue circles denote IL-10 responses; red circles IFN-γ responses; and green circles AAb responses. Filled circles indicate a statistically significant difference ($p<0.05$) in the frequency of responses between the two groups. Grey lines are 95% confidence intervals. Numbered symbols in panel A indicate individual autoantigen peptides (see Table 1). (A) Comparison of response frequency against single analytes. Islet AAbs, and especially IA-2Ab and ZnT8Ab are strongly diabetes-associated measurements. In (B), single T cell analytes have been summed to indicate a response to a single autoantigen. IFN-γ CD4 T cell responses to specific antigens, notably insulin and proinsulin peptides, are strong and significant. In (C) T cell analytes have been summed to show a positive response to any autoantigen and AAb responses summed to show positivity to any autoantigen; disease-discrimination is comparable between AAbs and T cell responses.

Figure 3: Clustering analysis of autoimmune inflammatory phenotypes in siblings. Unbiased hierarchical clustering analysis of autoimmune CD4 T cell and AAb responses represented as heatmaps. (A) Analysis of unaffected siblings collected in an unbiased, cross-sectional study. (B) Analysis of high-risk, unaffected siblings with multiple (≥2) islet AAbs positivity showing two main clusters characterised by the presence of IL-10 with sparse IFN-γ (lower left) and the presence of IFN-γ with sparse IL-10 (upper right). Yellow indicates positive response to an analyte; blue is negative. CD4 T cell peptide-specific responses are summarised into proteins (IA-2, GAD, PI and Ins). Panel (C) shows plots of the two principal components for siblings with ≥2 islet AAbs and at high-risk of progression to disease; each subject is identified by a diamond. The colour of the symbol indicates AAb positivity (open = no AAb; yellow = 1 AAb+; orange = 2 AAb+; red = 3 AAb+; brown = 4 AAb+). For explanation of blue lines see Figure 1D legend. The same dotted-line ovals as in Figure 1D are overlaid and the corresponding Clusters A (IFN-γ dominated) and B (IL-10 dominated) are identifiable in high-risk subjects. The subjects identified by arrows (a) and (b) and

present in Cluster A developed type 1 diabetes (ages 6 and 10 years respectively at diagnosis); the subject identified by arrow (c) in Cluster B developed type 1 diabetes aged 18 years.

Figure 4. Immunohistological analysis of pancreas from patients with type 1 diabetes reveals heterogeneity of insulitis. Panels show staining for CD20+ cells in 3 representative islets from a single patient with abundant positivity (nPOD6052; panels A-C) and a single patient with absence of positivity (nPOD6070; panels D-F). Staining patterns correspond to "hyper-immune CD20Hi" and "pauci-immune CD20Lo", respectively. CD20 staining was used to subdivide the 21 patients into CD20Hi and CD20Lo categories using mean CD20+ cell counts above and below 3.7 cells/islet.