# Detecting autoreactive B cells in the peripheral blood of people with type 1 diabetes using ELISpot

[1*]Powell WE, [1*]Hanna SJ, [1]Hocter CN, [1]Robinson E, [1]Lewis M, [2]Dunseath G, [2]Luzio S, [1]Howell A, [1]Dayan CM, [1]Wong FS

[1]Division of Infection and Immunity, Cardiff University School of Medicine, Cardiff CF14 4XN, UK

[2]Diabetes Research Unit Cymru, Grove Building, Swansea University, Swansea SA2 8PP, UK

Correspondence to F. S. Wong, email: wongfs@cardiff.ac.uk

*These authors contributed equally to this work

**Abstract**

Type 1 diabetes mellitus (T1D) is an autoimmune disorder where T lymphocytes damage the islet beta cells but B lymphocytes also play an important role. Although changes in peripheral B cell phenotype have been observed, little is known about the B cells that secrete the autoantibodies.

We developed a sensitive B cell enzyme-linked immunospot assay (ELISpot assay) to detect individual B cell antibody responses to glutamic acid decarboxylase (GAD) and islet antigen-2 (IA-2).

We found that even healthy donors have B cells that secrete antibodies in response to GAD and IA-2 in the ELISpot. There was increased B cell reactivity to autoantigens in the peripheral blood of individuals with newly-diagnosed, but not long-standing, type 1 diabetes. However, no correlation with serum autoantibody levels was found, indicating that additional factors such as antigen affinity or exposure to antigens *in vivo* are required for antibody secretion, and that even healthy donors have potentially autoreactive B cells.

*Keywords:* B cells; type 1 diabetes; ELISpot; autoantibodies.

*Abbreviations:* BAFF-R, B cell activating factor receptor; CD, cluster of determination; CXCR, chemokine receptor; EDTA, ethylenediaminetetraacetic acid; ELISpot, enzyme-linked immunospot assay; GAD, glutamic acid decarboxylase; IA-2, islet antigen 2; LS, long-standing; ND, newly diagnosed; PBMC, peripheral blood mononuclear cell; TACI, transmembrane activator and CAML interactor; T1D, type 1 diabetes; ZnT8, zinc transporter 8.

## 1. Introduction

Type 1 diabetes (TID) is an immune-mediated disease that occurs in genetically susceptible individuals, with risk modified by the environment. Although T cells play a central role in islet beta cell damage, B cells clearly are also important in pathogenic processes. Autoantibodies secreted by B cells provide an early indication of autoimmunity. The risk of developing type 1 diabetes is indicated by the presence of autoantibodies against islet antigens such as insulin, glutamic acid decarboxylase (GAD), islet antigen-2 (IA-2) and the zinc transporter (ZnT8) [1]. High serum antibody titres of one or more autoantibodies are often present upon initial diagnosis of T1D. Healthy individuals can have one or two autoantibodies present at a lower titre and may not proceed to develop T1D. Anti-GAD antibodies can remain detectable for many years whereas anti-IA-2 or ZnT8 antibodies tend to decrease rapidly after diagnosis [2]. Although these serum autoantibody-titres are useful markers of disease onset, they cannot be used as markers of ongoing autoimmunity after diagnosis and do not provide information regarding the cells that produce them.

B cells are found in the pancreas of individuals with type 1 diabetes [3, 4], and B cell depletion with rituximab can slow the loss of insulin production [5]. We, and others, have demonstrated that changes in the phenotype of peripheral B cells are also observed in people with type 1 diabetes. These changes included a decrease in CXCR3, B220, CD24, CD95, BAFFR and TACI expression [6-8]. In addition, decreased percentages of $CD24^{++}CD38^{++}$ B cells (thought to be regulatory B cells) [9], increased plasmablasts [6], increased frequencies of marginal zone $CD19^{+}CD21^{+}CD23^{-}$ B cells and decreased frequencies of

regulatory $CD1d^{+}CD5^{+}CD19^{+}$ and follicular $CD19^{+}CD21^{-}CD23^{+}$ B cells [10] have all been observed in individuals with type 1 diabetes.

In terms of antigen specificity, “insulin binding” B cells have been investigated for phenotype and frequency in the peripheral blood. These were found to be predominantly naïve anergic cells in healthy donors, whereas in people with newly-diagnosed diabetes they were lost, suggesting that they had trafficked to the pancreas [11]. Long-term antibody titres are maintained by long-lived antibody-secreting plasma B cells, and memory B cells can also differentiate into plasma cells to replenish the pool. Memory B cells are unique in function and phenotype compared to other types of B cells. They remain quiescent for a long duration but rapidly respond on re-exposure to antigen and can quickly differentiate into plasma cells to produce high-affinity antibodies to eliminate pathogens not removed by existing circulating antibodies [12-15]. In type 1 diabetes, it is not known whether there are autoreactive memory B cells in the circulation, which could contribute to the replenishment of the autoantibody-producing plasma cell pool.

To investigate whether circulating B cells recognise autoantigens and produce IgG auto-antibodies, we used a polyclonal activation protocol to stimulate memory B cells from the peripheral blood, before determining their ability to secrete GAD- and IA-2-targeting IgG antibodies using ELISpot analysis.

## 2. Methods

### 2.1 Study subjects

PBMCs were isolated from the peripheral blood of individuals with newly diagnosed, ND (<1 year, n=20, female=7, mean age: 30 years (range: 18-47 years), mean disease duration 0.4 years (range 0.1-1.0 years)), and long-standing, LS (>3 years) type 1 diabetes (n=20, female= 11, mean age: 34 years (range 18-48 years), mean disease duration: 17.7 years (range: 3-36 years )), as well as age- and sex-matched healthy donors, HD (n=35, female= 12, mean age: 33 years (range 18-48 years)). Type 1 diabetes was diagnosed according to criteria established by the American Diabetes Association [10], and insulin treatment was commenced within one month of diagnosis. The details of individuals studied are shown in Supplementary Table A1, A2 and A3.

This study was approved by the South East Wales Research Ethics Committee and conducted in accordance with the principles of Good Clinical Practice established by the International Council for Harmonization/World Health Organization. All subjects provided written informed consent prior to enrolment as mandated by the Declaration of Helsinki.

### 2.2 HLA class II genotyping

Genomic DNA was extracted from whole blood samples collected in ethylenediaminetetraacetic acid (EDTA). HLA-DRB alleles were resolved using polymerase chain reactions with sequence-specific primers [16] and are listed in Supplementary Table A2 and A3.

**2.3 Blood samples**

Serum samples were separated on the day of collection and stored in 1.5 ml Eppendorf tubes at -20$^{o}$C before levels of serum antibodies were analysed in batches. Serum autoantibodies specific for GAD and IA-2 were quantified by ELISA (RSR Limited, Cardiff, UK). Positive thresholds were set at 5 U/mL and 7.5 U/mL respectively. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized samples of whole blood via density gradient centrifugation over Lymphoprep (Stem Cell Technologies).

**2.4 Cell culture**

Freshly isolated PBMCs were cultured for 5-7 days, 37$^{o}$C at a cell concentration of 0.5x10$^{6}$ /ml in RPMI/10% heat inactivated human AB serum/penicillin/streptomycin/ L-glutamine, 0.001% v/v pokeweed mitogen extract (gift from Prof. Shane Crotty, La Jolla Institute for Allergy & Immunology), 0.1µg/ml (2.4nM) protein A soluble from Staphylococcus aureus Cowan strain (SAC) (Sigma) and 4µg/ml (0.52µM) CpG-ODN 2006 (Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven).

**2.5 ELISpot**

Multiscreen-IP 96 separation system, 0.45µm Immobilin-P PVDF membrane (Merck Millipore) plates were pre-wetted with 70% molecular grade ethanol (Sigma), then washed and coated with either PBS, 0.5µg/ml goat antihuman IgG (Newmarket scientific) (as a positive control, data not shown), or recombinant human antigens (RSR Limited, Cardiff, UK) overnight at 4˚C. Antigen concentrations were as follows: rhGAD$_{65}$ used at 0.4µg/ml

(6.2nM), 0.8 µg/ml (12.3nM) or 1.6µg/ml (24.6nM); rhIA-2 intracellular fragment (aa 604-979) (RSR Limited, Cardiff UK) used at 0.4µg/ml (8.7nM), 0.8 µg/ml (17.4 nM) or 1.6µg/ml (34.8nM). Plates were then washed and blocked with RPMI 10% heat inactivated foetal bovine serum (HI-FBS), overnight at 4˚C.

The following day, blocking solution was removed and cells were washed in RPMI 10% HI-FBS, resuspended at $4x10^6$ cells/ml and 100µl was placed in each well ($4x10^5$/well). For the IgG positive control cells were resuspended at $5x10^4$/ml and 100µl was placed in each well ($5x10^3$/well). Plates were incubated for 5 hours at 37˚C, then washed with PBS/0.05% Tween 20, then incubated overnight at 4˚C with goat-anti-human IgG Fc biotin antibody (eBioscience) at 0.5µg/ml in PBS/0.05% Tween 20/5% HI-FBS.

The following day, the plates were washed and developed with ExtrAvidin (Sigma) diluted 1/5000 in PBS/0.05% Tween 20/ 5% HI-FBS, followed by BCIP/NBT substrate (Sigma). Plates were then washed and dried overnight before being read on a BIO-SYS GmbH Bioreader 4000 using a minimum spot size of 30µm. All bioreader images were checked manually to ensure spots were correctly identified. The background number of spots, produced in wells coated with PBS, was subtracted from the counts for each antigen-coated well. A minimum of 6 wells were counted and averaged for each condition. All individuals responded to the positive control stimulation.

**2.6 Statistics**

Data were analysed using Graphpad Prism 5 and R v3.4.1 for the plot matrix (ggplot2 and GGally packages, ggpairs plot). ELISpots were analysed using a Mann Whitney test to compare between two groups and a Kruskal-Wallis test, followed by Dunn's multiple

comparison for comparison between three groups, as the data were not normally distributed.

**3. Results**

We used a range of antigen concentrations to coat the ELISpot plates. Healthy donors (HD) exhibited a response to both GAD and IA-2 above the background level of spots. A dose titration was observed, with the effect dependent on the coating concentration for both antigens (Fig 1A, D). After the background levels of spots were subtracted, the median number of spots (from 400,000 cells) was 5.5 at 0.4μg/ml (6.15nM) GAD and 15.9 at 0.8μg/ml (12.3nM) GAD ($p<0.005$). For IA-2, the median number of spots (from 400,000 cells) was 4.2 at 0.4μg/ml (8.7nM) IA-2 and 10.5 at 0.8μg/ml (17.4nM) IA-2 ($p<0.05$). Compared to healthy donors (HD), the newly diagnosed (ND) cohort had a significantly higher response to GAD at both 0.4μg/ml (6.2nM) (HD 5.5 spots, ND 16.8 spots, $p<0.01$), and 0.8μg/ml (12.3nM) (HD 15.9 spots, ND 36 spots $p<0.05$), (Fig 1B, C). The long-standing (LS) cohort was not significantly different to the healthy donors (median 14.8 spots at GAD 0.4μg/ml (6.15nM) and 33.2 at GAD 0.8μg/ml (12.3nM)). The spot counts were not normally distributed and appeared to have a bimodal distribution.

In response to IA-2, the newly diagnosed (ND) cohort had increased spots compared to the healthy donors (HD) at 0.8μg/ml (17.4nM) (HD 10.5 spots, ND 28 spots, $p<0.05$), but not 0.4μg/ml (8.7nM) (HD 3.7 spots, ND 10.4 spots), (Fig 1E, F). Again, long-standing (LS) donors were not significantly different at either concentration of antigen (median 6.9 spots at IA-2 0.4μg/ml (8.7nM) and 19.6 at IA-2 0.8μg/ml (17.4nM)).

We compared spots in the GAD and IA-2 0.8μg/ml (12.3nM and 17.4nM respectively) ELISpots, with GAD and IA-2 antibody titres. The concentration of antigen was chosen

because there was a significant difference in the number of spots seen between healthy donors and newly diagnosed donors at this coating concentration. In the newly diagnosed and long-standing cohorts, serum titres of GAD or IA-2 autoantibodies were not correlated with spots produced in the respective GAD or IA-2 0.8ug/ml ELISpot assay, with high ELISpot counts occurring even when antibody titres were below the cut-off for a positive antibody result (GAD 5 U/ml, IA-2 7.5 U/ml) [17]. Indeed for both, there was a non-significant negative correlation between GAD antibody and GAD ELISpot (LS r= -0.191, ND r =-0.422) and for the newly diagnosed cohort between IA-2 antibody titre and IA-2 ELISpot (LS r= 0.221, ND r =-0.435) (Fig 2). However, GAD and IA-2 antibody titres were weakly correlated for both of these cohorts (LS r= 0.164, ND r =0.271) consonant with findings of others [18]. There was no correlation or differences found related to HLA type (supplementary table A3).

**4. Discussion**

Here we have demonstrated that ELISpots can be used to detect B cell responses to autoantigens in T1D. Moreover, we showed that whilst B cells from healthy donors and people with long-standing diabetes could secrete antibody in response to diabetic autoantigens, the increase in the number of autoreactive B cells was only significant in the PBMCs of people with newly-diagnosed diabetes.

B cell responses in type 1 diabetes are normally assessed by quantification of serum antibodies, produced by plasma cells. However, this does not provide a measurement of the memory B cells that may be circulating but not secreting antibodies [19, 20]. Whilst B cell ELISpots have been widely used to detect vaccine and infectious disease responses, the few reported incidences of their use in autoimmune disease relate to multiple sclerosis [21], SLE

[22] and pemphigus vulgaris [23], showing that autoreactive B cells are detectable by ELISpot.

We used a method of stimulating memory B cells using Pokeweed mitogen (PWM) combined with *S. aureus* (Cowan strain, SAC) and a CpG oligonucleotide (ODN-2006), adapted from the method used by Crotty *et al* [20]. This pre-stimulation induces memory B cells to differentiate into antibody secreting cells [24]. Crotty and colleagues [20] used this method to identify memory B cells with a response to vaccines, and demonstrated that the responding cells in their assay were CD27+.

It has been found that many autoreactive B cells in healthy donors are anergic with a mature naïve ($CD19^{+}$ $CD27^{-}$IgD+) phenotype and low affinity for autoantigens. At the onset of autoimmune diseases including T1D, SLE and autoimmune thyroid disease [25] [26], these anergic, naïve B cells are transiently lost from the peripheral blood suggesting that they may have become activated and travelled to the site of autoimmunity. However, it has also been demonstrated that a substantial minority of insulin-binding cells in the peripheral blood of both healthy donors and people with diabetes are CD27+ memory B cells [11]. Indeed insulin-binding cells are found in all major B cell compartments, although their affinity for insulin may be very low, with high-affinity insulin binding cells found only in the anergic naïve subset [11, 26].

Our data demonstrated that even healthy donors had B cells that could produce antibodies in response to GAD and IA-2 in a concentration-dependent manner. The polyclonal pre-stimulation may overcome anergy present in these B cells, and the ELISpot will potentially detect B cells with receptors that have a range of affinity of antigen binding. We therefore hypothesise that the antigen-specific antibody secreting cells from the healthy donors may

be either anergic *in vivo* and/or have very low affinity for the autoantigens, such that at physiological antigen concentrations they are not induced to secrete antibodies. However, it is likely that they exist in the peripheral blood as CD27+ memory cells, as these are the cells that are activated by the pre-stimulation, rather than mature naïve B cells.

The fact that people with long-standing type 1 diabetes did not have significantly more antigen-specific antibody secreting cells than healthy donors was perhaps not surprising as it is known that levels of serum autoantibodies decline in people with long-standing diabetes [27]. T cell responses to auto-antigenic peptide epitopes also decline in people with long-standing diabetes to levels observed in people with no type 1 diabetes [28].

We observed that people with newly diagnosed type 1 diabetes had significantly more antigen-specific antibody secreting cells than healthy donors. This novel finding suggests that there are more circulating B cells in the peripheral blood capable of producing autoantibodies, and potentially that there are stimuli present that induce these to produce the autoantibodies.

In summary, we found that B cells that secrete autoantibodies to specific-antigens are more abundant in people newly diagnosed with type 1 diabetes, and that those B cells can remain for many years in people considered long-standing. More interestingly, they are also present in people with no history of type 1 diabetes. Further experiments will be required to elucidate which B cell subsets produce these antibodies and whether the antibodies from healthy donors and people with diabetes have equivalent affinities for autoantigens.

Figure 1. Healthy donors and people with type 1 diabetes have B cells in the peripheral blood that can produce autoantibodies to GAD and IA-2. A. Titration of GAD coating concentration: Spot-forming response of healthy donor PBMCs to indicated GAD coating concentration (with the background response to PBS coating subtracted). B. Spot forming response of healthy donors and people with ND and LS type 1 diabetes to 0.4μg/ml (6.15nM) GAD coating solution (with the background response to PBS coating subtracted). C Spot forming response of healthy donors and people with ND and LS type 1 diabetes to 0.8μg/ml (12.3nM) GAD coating solution (with the background response to PBS coating subtracted). D. Titration of IA-2 coating concentration: Spot-forming response of healthy donor PBMCs to indicated IA-2 coating concentration (with the background response to PBS coating subtracted). E. Spot forming response of HDs and people with ND and LS type 1 diabetes to 0.4μg/ml (8.7nM) IA-2 coating solution (with the background response to PBS coating subtracted). F. Spot forming response of HDs and people with ND and LS type 1 diabetes to 0.8μg/ml (17.4nM) IA-2 coating solution (with the background response to PBS coating subtracted). * $p<0.05$, ** $p<0.01$, *** $p<0.005$ (A, D; Mann Whitney test. B, C, E, F; Kruskal-Wallis test, followed by Dunn's multiple comparison). Lines represent the median.

Figure 2. The number of antigen-specific antibody secreting cells is not correlated to serum antibody titres. Plot matrix of serum antibody concentrations (GAD, IA-2, log values) and PBMC responses in ELISpot to GAD 0.8 μg/ml (12.3nM) (PBS background subtracted) and IA-2 0.8 μg/ml (17.4nM) (PBS background subtracted). On the lower left side: scatterplots of the variables plotted against each other as indicated on the axes. Correlation (r value) for the variables as indicated on the axes are to the right of each graph. Diagonally: Histograms showing the distribution of the variables. Pink- healthy donor. Green- LS type 1 diabetes. Blue- ND type 1 diabetes. Black (r value only) - all samples.

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Duality of Interest: The authors declare that there is no duality of interest associated with this manuscript.

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Figure 1

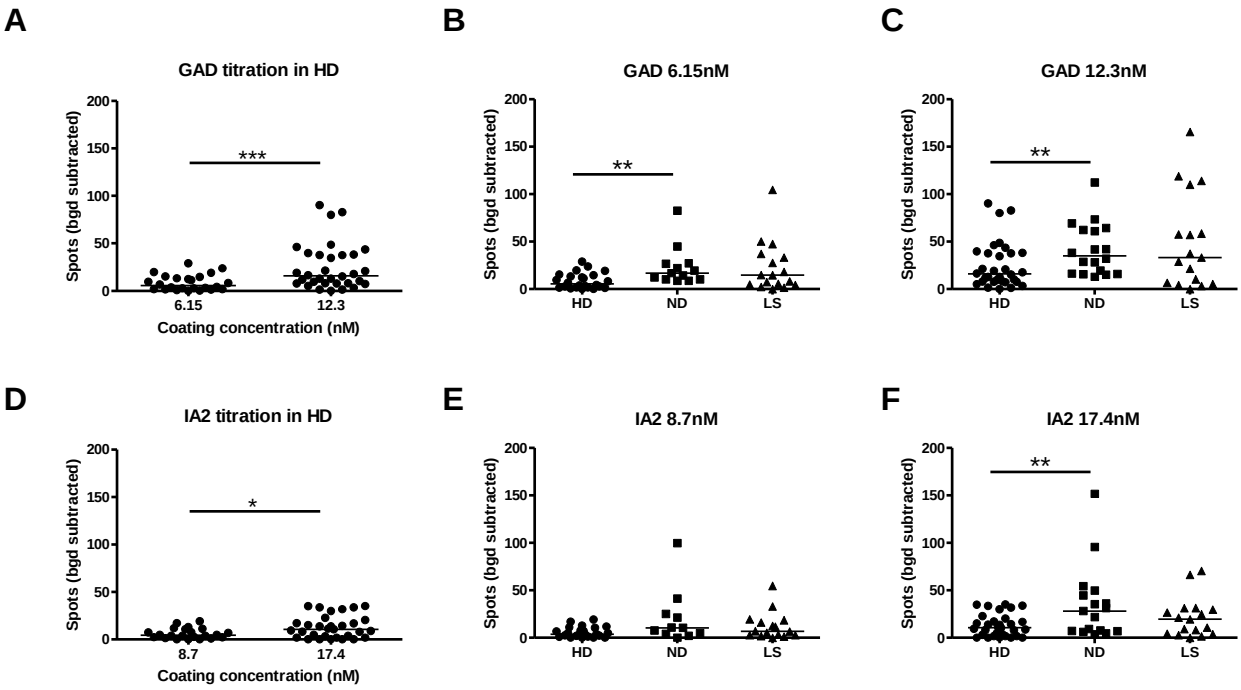
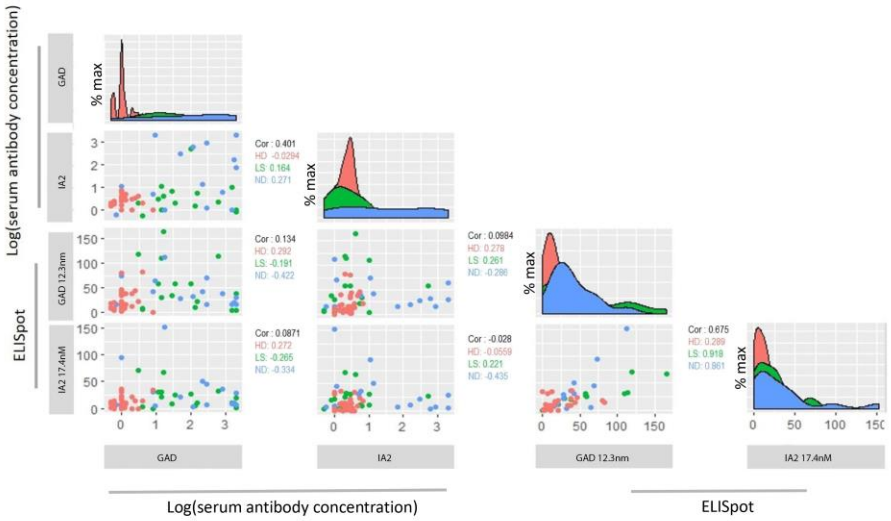

A
GAD titration in HD
Spots (bgd subtracted)
200
150
100
50
0
***
6.15
12.3
Coating concentration (nM)
B
GAD 6.15nM
Spots (bgd subtracted)
**
HD
ND
LS
C
GAD 12.3nM
Spots (bgd subtracted)
**
HD
ND
LS
D
IA2 titration in HD
Spots (bgd subtracted)
*
8.7
17.4
Coating concentration (nM)
E
IA2 8.7nM
Spots (bgd subtracted)
HD
ND
LS
F
IA2 17.4nM
Spots (bgd subtracted)
**
HD
ND
LS

Figure 2


Log(serum antibody concentration)
ELISpot
GAD
IA2
GAD 12.3nm
IA2 17.4nM
% max
Cor : 0.401
HD: -0.0294
LS: 0.164
ND: 0.271
Cor : 0.134
HD: 0.292
LS: -0.191
ND: -0.422
Cor : 0.0871
HD: 0.272
LS: -0.265
ND: -0.334
Cor : 0.0984
HD: 0.278
LS: 0.261
ND: -0.286
Cor : -0.028
HD: -0.0559
LS: 0.221
ND: -0.435
Cor : 0.675
HD: 0.289
LS: 0.918
ND: 0.861
Log(serum antibody concentration)
ELISpot