**Ex-vivo** whole blood secretion of interferon (IFN)-γ and IFN-γ-inducible protein-10 measured by enzyme-linked immunosorbent assay are as sensitive as IFN-γ enzyme-linked immunospot for the detection of gluten-reactive T cells in human leucocyte antigen (HLA)-DQ2·5+-associated coeliac disease

N. Ontiveros,*† J. A. Tye-Din,*†‡ M. Y. Hardy*† and R. P. Anderson*†§

*The Walter and Eliza Hall Institute of Medical Research, †Department of Medical Biology, The University of Melbourne, ‡Department of Gastroenterology, The Royal Melbourne Hospital, Parkville, Vic., Australia, and §ImmusanT, Inc., Cambridge, MA, USA

**Summary**

T cell cytokine release assays are used to diagnose infectious diseases, but not autoimmune or allergic disease. Coeliac disease (CD) is a common T cell-mediated disease diagnosed by the presence of gluten-dependent intestinal inflammation and serology. Many patients cannot be diagnosed with CD because they reduce dietary gluten before medical workup. Oral gluten challenge in CD patients treated with gluten-free diet (GFD) mobilizes gluten-reactive T cells measurable by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) or major histocompatibility complex (MHC) class II tetramers. Immunodominant peptides are quite consistent in the 90% of patients who possess HLA-DQ2·5. We aimed to develop whole blood assays to detect gluten-specific T cells. Blood was collected before and after gluten challenge from GFD donors confirmed to have CD (n = 27, all HLA-DQ2·5+), GFD donors confirmed not to have CD (n = 6 HLA-DQ2·5+, 11 HLA-DQ2·5−) and donors with CD not following GFD (n = 4, all HLA-DQ2·5+).

Plasma IFN-γ and IFN-γ-inducible protein-10 (IP-10) were measured by enzyme-linked immunosorbent assay (ELISA) after whole blood incubation with peptides or gliadin, and correlated with IFN-γ ELISPOT. No T cell assay could distinguish between CD patients and controls prior to gluten challenge, but after gluten challenge the whole blood IFN-γ ELISA and the ELISPOT were both 85% sensitive and 100% specific for HLA-DQ2·5+ CD patients; the whole blood IP-10 ELISA was 94% sensitive and 100% specific. We conclude that whole blood cytokine release assays are sensitive and specific for detection of gluten-reactive T cells in CD; further clinical studies addressing the utility of these tests in patients with an uncertain diagnosis of CD is warranted.

**Keywords:** coeliac disease, diagnostics, gluten challenge

**Introduction**

Coeliac disease (CD) is a T cell-mediated autoimmune-like disease triggered by the ingestion of gluten from wheat, rye, barley and sometimes oats in genetically susceptible individuals. The only medical treatment for CD is a strict lifelong gluten-free diet (GFD). The community prevalence of CD in the United States is 0·7%, but 83% of cases go unrecognized [1]. Addressing this unmet medical need by diagnosing patients accurately is compromised by self-diagnosis based on symptomatic response to GFD [2], and also by inconsistent adherence to clinical guidelines which recommend confirmatory small bowel biopsy while gluten is included in the diet [3,4]. Indeed, false-positive CD-specific serology is common even in individuals who are genetically susceptible to CD because they carry major histocompatibility complex (MHC) class II genes for human leucocyte antigen (HLA)-DQ2·5, HLA-DQ2·2 and/or HLA-DQ8 [5]. Consequently, there may be many patients strictly avoiding gluten who would be better suited to other treatments [6]. Meanwhile, complications of CD such as osteoporosis may not be addressed in other patients who truly do have CD but are following GFD and not confirmed by small bowel histology. In either case, patients on...
GFD are often reluctant to reintroduce gluten for the weeks or months required to cause serological and histological relapse in order to confirm CD [7].

Prior to our observation that 3-day oral gluten challenge mobilizes gluten-reactive T cells, it was thought that T cells circulating in blood did not reflect the specificities of gluten-reactive T cells in the gut [8–10]. Subsequently, overnight interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays and MHC-peptide multimers using freshly isolated peripheral blood mononuclear cells (PBMC) have provided detailed understanding of epitope hierarchies and the phenotypes of CD4 T cells circulating in CD patients 6 days after commencing oral gluten challenge [10–12].

Detection of T cells in blood specific for immunodominant gluten-derived epitopes has been proposed as a diagnostic tool for CD [13,14]. Indeed, several assay platforms to measure antigen-specific T cells have entered clinical practice. For example, enzyme-linked immunosorbent assay (ELISA) and ELISPOT to detect IFN-γ or monokine (IFN-γ-inducible protein-10, IP-10) release in whole blood or PBMC incubated with antigenic peptides can be used to diagnose Mycobacterium tuberculosis infection [15–19].

As proof of concept, we sought to test whether plasma levels of IFN-γ or IP-10 in whole blood collected after oral gluten challenge and then incubated with immunodominant peptides or gliadin could differentiate patients with CD from other patients who have adopted GFD.

### Materials and methods

#### Participants and clinical procedures

Informed consent was obtained from all study participants. The study was approved by the Human Research Ethics Committees of The Royal Melbourne Hospital (2003·009) and The Walter and Eliza Hall Institute of Medical Research (03/04). CD subjects were recruited by advertisement in the Coeliac Victoria and Tasmania newsletter. Subjects who had excluded gluten from their diet because they attributed various digestive and systemic symptoms to gluten, but did not have CD (non-CD control subjects), were referred by Dr Jessica Biesiekierski, Department of Gastroenterology, Monash University, Box Hill. Enrolment was open from July 2009 to September 2010, and from April to October 2011. Volunteers fulfilling entry criteria were enrolled consecutively. Recruitment and clinical aspects were performed in a clinical research unit attached to The Royal Melbourne Hospital. Subjects enrolled were all Caucasian and are described in Table 1. All enrolled subjects completed the study.

Inclusion in the study required that subjects had been diagnosed with CD and claimed to have followed strict GFD for at least 6 months, or had been diagnosed recently with CD but did not exclude gluten from their diet, or did not have CD but reported adverse symptoms after gluten ingestion and had followed a strict GFD for 6 months or more. CD subjects were included only if they had...
biopsy-proven CD conforming to ESPGHAN (European Society of Paediatric Gastroenterology, Hepatology and Nutrition) criteria [20]. Control subjects following GFD were included only if they had been evaluated previously for CD and had normal small bowel histology while following a ‘normal’ diet or they did not possess HLA-DQ2·5, DQ2·2 and DQ8 genotypes. Use of immunosuppressants was an exclusion.

Blood for serology and cytokine release assays was drawn in the morning immediately before (d0) and on day 6 (d6) after commencing gluten challenge, or prior to commencing GFD in untreated CD patients. Blood was transported at ambient temperature to the laboratory, where it was received within 1 h of collection. Oral gluten challenge was as described originally by Anderson et al. [10], and occurred from the morning of day 1 (d1) to day 3 (d3). A symptom diary was completed daily from d1 to d6, with subjects recording symptoms and grading them on a three-point Likert scale (mild, moderate or severe). All symptomatic subjects were interviewed by telephone 4–6 weeks following gluten challenge.

Serology and HLA typing

Serum transglutaminase (tTG-IgA), deamidated gliadin peptide (DGP)-immunoglobulin (Ig)A and DGP-IgG were evaluated with commercial kits (INV 708760, 704525 and 704520; INOVA Diagnostics, San Diego, CA, USA) by a clinical diagnostic laboratory (Gribbles-Healthscope, Clayton, Australia). The presence of alleles encoding HLA-DQ2·5, DQ2·2 and DQ8 was determined in leucocyte-derived DNA using one of two previously reported methods [21,22].

Antigens

Two synthetic peptides that each encompassed two overlapping epitopes (DQ2·5-glia-α1a/α2 and DQ2·5-glia-α01/α02) were custom-synthesized to at least 95% purity confirmed by high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (Pepscan, Lelystad, the Netherlands) [23]. Peptides were first dissolved in dimethylsulphoxide (DMSO) (Sigma D2650) (Sigma, St Louis, MO, USA) to 100 mg/ml then diluted to 5 mg/ml in phosphate-buffered saline (PBS). The maximum concentration of DMSO in incubations with blood or PBMCs was 0·1% v/v. Gliadin (#101778; ICN Biomedicals, Aurora, OH, USA) was incubated in 10-fold excess with chymotrypsin (Sigma #C3142) in ammonium bicarbonate (pH 8) for 4 h at 37°C and was then boiled for 15 min. Protein concentration of the gliadin hydrolysate was determined using the BioRad Protein Assay Dye Reagent #500-0006 method (BioRad, Hercules, CA, USA). Deamidation of the gliadin hydrolysate with guinea pig liver transglutaminase (Sigma T5398) was performed as described previously [10,13].

Whole blood incubation and IFN-γ and IP-10 ELISA

Cytokine release assays were performed in a dedicated academic immunology laboratory at the Walter and Eliza Hall Institute by a single, trained postgraduate research student (N. O.), who was not blinded to the diagnosis of subjects. Fresh heparinized whole blood (1 ml) was added to cryotubes (1 ml NUNC Cryotube, #377224; Roskilde, Denmark) containing peptides, deamidated chymotrypsin-digested gliadin (‘gliadin’, 100 μg/ml), or tetanus toxoid (10 LFU/ml; CSL, Melbourne, Australia). Cryotubes were inverted vigorously 10 times to ensure mixing of blood with antigens. After incubation at 37°C for 24 h, samples were centrifuged at 1000 g for 10 min at room temperature (RT). Plasma was collected and stored at −80°C until analysis.

Plasma IFN-γ levels on d0 and d6 were measured in triplicate by ELISA; 96-well flat-bottomed polystyrene plates (NUNC Maxisorb #442404) were coated with 100 μl capture antibody at 2 μg/ml (1-D1K, diluted 1:500 with PBS; Mabtech, Nacka Strand, Sweden) and incubated overnight at 4°C, then washed twice with PBS and incubated for 1 h at RT with 200 μl of blocking solution [PBS/0·05% Tween 20/1% bovine serum albumin (BSA)]. The blocking solution was discarded and 100 μl of Mabtech recombinant human IFN-γ or 100 μl test sample diluted 1:2 in ELISA diluent (Mabtech #3652-D2), were added to individual wells. After overnight incubation at 4°C, wells were washed five times with PBS/0·05% Tween 20. 100 μl of the biotinylated detection antibody at 1 μg/ml (Mabtech 7-B6-1-biotin diluted 1:1000 with PBS/0·05% Tween 20/0·1% BSA) was added to each well and incubated for 1 h at RT. Wells were washed as before, then 100 μl of streptavidin–horseradish peroxidase (Mabtech diluted 1:1000 with PBS/0·05% Tween 20/0·1% BSA) was added to each well and incubated for 1 h at RT. Wells were washed as before, then 100 μl tetramethyl benzidine (TMB) substrate for 10 min at RT. H2SO4 2 M (50 μl) was used as stop solution. An automated ELISA reader (Multiskan Ascent, Thermo Labsystems, Finland) was used to measure absorbance at 450 nm, and levels of IFN-γ were determined using standard curves.

Plasma IP-10 levels on d6 were measured in triplicate by ELISA (BD OptEIA 550926; BD Biosciences, San Jose, CA, USA) with minor modifications to the manufacturer’s protocol; 96-well plates (NUNC Maxisorb 442404) were coated with 100 μl of capture antibody (51-27321E) diluted 1:500 with PBS and incubated overnight at 4°C. Plates were washed three times with PBS containing 0·05% Tween 20, then blocked with PBS/10% fetal calf serum (FCS) for 1 h at RT. The blocking solution was discarded and then 100 μl standards or samples diluted 1:20 or 1:10 in PBS/10% FCS were added to individual wells. After overnight incubation at 4°C, wells were washed five times as above and 100 μl of the detector solution was added to each well and incubated for 1 h at RT. The detector solution consisted of both the
detection antibody (51–27322-E diluted 1:1000) and streptavidin–horseradish peroxidase conjugate (51–27326-E diluted 1:250) in PBS/10% FCS. Wells were washed seven times as above and 100 μl TMB substrate solution was added for 30 min in the dark at RT. H2SO4 2 M (50 μl) was used as stop solution. Absorbance was measured as described previously.

IFN-γ ELISPOT assay

PBMC were separated from heparinized whole blood collected on d6 using Ficoll-Paque Plus (GE Healthcare) and density-gradient centrifugation in 50 ml Leucosep™ tubes (Griener Labotechnik, Kresmuster, Austria). After being washed three times in PBS, PBMC were resuspended in complete RPMI supplemented with 10% heat-inactivated pooled human serum (Australian Red Cross Blood Service, Melbourne, Australia), 2 mM GlutaMAX™ (Gibco, Invitrogen, Carlsbad, CA, USA), 100 μM modified Eagle’s medium (MEM) non-essential amino acids (Gibco, Invitrogen), and 50 μM 2-mercaptoethanol (Sigma). Overnight IFN-γ ELISPOT assays (Mabtech) using 96-well plates (MSIP-S45-10; Millipore, Bedford, MA, USA) were performed as described previously [10,13]. Briefly, 0.5 × 10⁶ PBMCs per well were plated for each condition and the spots were developed after incubation at 37°C for 24 h. The capture and detection anti-IFN-γ antibodies (1-D1K and 7-B6-1, respectively) were the same as those used to detect IFN-γ by ELISA in plasma from whole blood. Phytohaemagglutinin-P (PHA; Sigma) was used as the positive control at a final concentration of 2·5 μg/ml, as it proved more consistent than tetanus toxoid in the IFN-γ ELISPOT assay. Each antigen was tested in triplicate. Spot-forming units (SFU) in individual wells were counted using an automated ELISPOT reader (AID ELISPOT Reader System; AID Autoimmun Diagnostika GmbH, Strassberg, Germany) and results expressed as SFU per 10⁶ PBMC.

Statistical analysis

Data were analysed using GraphPad Prism version 5·0 (GraphPad Software, San Diego, CA, USA). The Kruskal–Wallis test was used to calculate the differences between IFN-γ ELISPOT, IFN-γ ELISA and IP-10 ELISA responses between groups. Cytokine assay responses were correlated using Spearman’s rank test. Fisher’s exact test was used to compare the proportion of symptomatic subjects and symptoms between treated CD and controls. Significance was taken to be P < 0·05.

The diagnostic performance of the assays was compared using receiver operating characteristic (ROC) curve analysis and area under the curve (AUC) analysis utilizing MedCalc software (http://www.medcalc.org), according to the method of Hanley and McNeil [24]. Cut-offs for antigen-dependent IFN-γ and IP-10 were estimated using MedCalc

and at the ideal false-positive rate (0%). Results were graded ‘positive’ if the responses to the equimolar peptide cocktail of DQ2.5-glia-α1a/α2 with DQ2.5-glia-α1/α2 (‘peptide mix’) or gliadin was above the selected cut-off, irrespective of the positive control response; ‘negative’ if the responses to peptide mix or gliadin was below the selected cut-off and not indeterminate; and ‘indeterminate’ if the responses to peptide mix or gliadin was negative and the positive control response was also negative.

Responses to peptides, gliadin and the positive control were assessed following subtraction of background responses (Nil/no antigen). Test samples with IFN-γ and IP-10 levels at or above the upper limit of quantification were assigned a value equal to the maximum standard. Final results presented are the mean of ELISA and ELISPOT values from three replicate plasma or PBMC samples. For whole blood assays, quality control was determined by interassay coefficient of variation (CV) using the formula CV = [standard deviation (s.d.)/mean] × 100. Interassay variability was not assessed for the ELISPOT assay, because this would have required the use of frozen PBMCs, and provided information regarding the variability of both the assay and also cryopreservation and thawing of cells.

Results

Clinical response and tolerability of the 3-day gluten challenge

All subjects were able to complete the 3-day challenge protocol, and none required any medical intervention (Table 2). One non-CD subject and two CD subjects reported nausea and vomiting on the first day of challenge.

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Treated CD n = 27 (%)</th>
<th>Non-CD control subjects n = 17 (%)</th>
<th>P-value (Fisher’s exact test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic</td>
<td>6 (22·2)</td>
<td>2 (11·7)</td>
<td>0·688</td>
</tr>
<tr>
<td>Nausea</td>
<td>4 (14·8)</td>
<td>3 (17·6)</td>
<td>1·0</td>
</tr>
<tr>
<td>Bloating</td>
<td>5 (18·5)</td>
<td>7 (41·1)</td>
<td>0·278</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>7 (25·9)</td>
<td>5 (29·4)</td>
<td>1·0</td>
</tr>
<tr>
<td>Lethargy</td>
<td>6 (22·2)</td>
<td>7 (41·1)</td>
<td>0·178</td>
</tr>
<tr>
<td>Vomiting</td>
<td>2 (7·4)</td>
<td>1 (5·8)</td>
<td>1·0</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>7 (25·9)</td>
<td>6 (35·2)</td>
<td>0·737</td>
</tr>
<tr>
<td>Constipation</td>
<td>2 (7·4)</td>
<td>4 (23·5)</td>
<td>0·174</td>
</tr>
<tr>
<td>Headache</td>
<td>2 (7·4)</td>
<td>1 (5·8)</td>
<td>1·0</td>
</tr>
<tr>
<td>Irritability</td>
<td>0</td>
<td>3 (17·6)</td>
<td>0·045</td>
</tr>
<tr>
<td>Flatulence</td>
<td>2 (7·4)</td>
<td>1 (5·8)</td>
<td>1·0</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>0</td>
<td>2 (11·7)</td>
<td>0·133</td>
</tr>
<tr>
<td>Insomnia</td>
<td>0</td>
<td>1 (5·8)</td>
<td>0·372</td>
</tr>
<tr>
<td>Mouth ulcers</td>
<td>0</td>
<td>2 (11·7)</td>
<td>0·133</td>
</tr>
<tr>
<td>‘Swollen glands’ in neck</td>
<td>0</td>
<td>1 (5·8)</td>
<td>0·372</td>
</tr>
</tbody>
</table>

CD: coeliac disease.
There was no significant difference between the numbers of CD (21 of 27) and non-CD subjects (15 of 17) reporting symptoms during gluten challenge. Symptoms provoked by the challenge had resolved fully by d6 in 12 of 21 symptomatic CD subjects and in one of 15 symptomatic non-CD control subjects. Follow-up of all symptomatic participants 4–6 weeks after completion of gluten challenge confirmed resolution of challenge-associated symptoms.

Serology is stable during 3-day gluten challenge

Serology findings did not change between d0 and d6 in any subjects. All non-CD control subjects were seronegative for tTG-IgA, DGP-IgA and DGP-IgG (Table 1), but eight treated CD subjects showed mild or moderate elevations in at least one CD-specific serology test. Borderline elevations in serology on d0 were found in five treated CD subjects in one test (tTG-IgA 22 units in one, DGP-IgA 20–30 units in three and DGP-IgG 36 units in one; normal range 0–20 units), one CD subject had moderately elevated tTG-IgA at 82 units, and another showed elevations in both DGP-IgA (62 units) and DGP-IgG (32 units). One other subject had borderline to moderately elevated levels in all three serology tests, but claimed to be following strict GFD (tTG-IgA 62, DGP-IgA 27 and DGP-IgG 20 units). In contrast, all four untreated HLA-DQ2·5+ CD subjects were seropositive for tTG-IgA while three had elevated DGP-IgA, and three had abnormal DGP-IgG consistent with ongoing gluten exposure.

Antigen optimization for maximal ex-vivo IFN-γ responses

While we and others have shown that IFN-γ ELISPOT is an effective tool to assess gluten-specific T cell responses [12,25], we sought to assess and compare the performance of an ELISA-based format using whole blood which could be implemented in clinical practice, as it has in the diagnosis of M. tuberculosis infection [26]. The incubation conditions selected for the cytokine release assays were adapted from those used for the whole blood cytokine release test approved for the diagnosis of M. tuberculosis infection (Quantiferon-TB Gold; Cellestis, Chadstone, Australia), which utilizes three blood collection tubes each with 1 ml anti-coagulated blood incubated without additives as the negative control, or with PHA as the positive control, or with test peptides. In this study, the IFN-γ ELISPOT assay was considered the reference standard. In our hands, the IFN-γ ELISPOT assay using a 17mer peptide encompassing DQ2-5-glia-α1α2 epitopes is qualitatively reproducible using the same donor’s PBMC collected on days 6 and 7 after commencing oral gluten challenge, and also when the same donor is rechallenged 6–12 months later [13].

The IFN-γ ELISA and ELISPOT assays utilized the same antibody pair [10,11,13]. The magnitude of responses in the IFN-γ ELISPOT and whole blood ELISA IFN-γ release assays stimulated by either the DQ2-5-glia-α1α2 or DQ2-5-glia-α1α2 peptide were similar in six treated HLA-DQ2·5+ CD subjects on d6 (Fig. 1). There was a trend for an equimolar mixture of the DQ2-5-glia-α1α2 or DQ2-5-glia-α1α2 peptides (peptide mix) to elicit stronger IFN-γ responses than either peptide alone. The magnitude of responses stimulated by peptide mix was similar to chymotrypsin-digested, deamidated gliadin (‘gliadin’) in both the IFN-γ ELISPOT and ELISA, but in three of six cases responses to gliadin were also detected before gluten challenge. In blood collected on d6 from HLA-DQ2·5+ CD subjects, incubation with peptide mix stimulated plasma IFN-γ levels that were correlated closely with SFU in the IFN-γ ELISPOT (r = 0.839, P < 0.0001) (Fig. 2a).

Gluten peptide-stimulated production of IFN-γ and IP-10 is specific for treated CD following gluten challenge

There were no indeterminate or missing results in any of the 27 treated CD and 17 non-CD control subjects following GFD. In the treated CD group, the IFN-γ whole blood ELISA response to peptide mix was significantly greater on d6 following gluten challenge than on d0, and when compared to no antigen (Fig. 3a). Responses to gliadin by
treated CD patients on d6 were significantly higher than the no antigen control, but the difference between d0 and d6 did not reach statistical significance (Fig. 3a). IFN-γ responses to gluten-derived peptides above the negative control were not detected in untreated CD patients (data not shown). In non-CD subjects there was no significant difference between responses to the peptide mix or gliadin on d0 and d6 (Fig. 3b). In addition, subgroup analysis indicated IFN-γ levels in plasma after whole blood was incubated with peptide mix or gliadin were no different between HLA-DQ2-5+ and HLA-DQ2-5+ non-CD control subjects on d0 or d6. Supporting these findings, similar results were observed in d6 samples when IP-10 levels were measured alongside the IFN-γ in plasma from whole blood (Fig 3c,d). IFN-γ and IP-10 levels in plasma after whole blood incubation with antigens were also related closely to IFN-γ ELISPOT responses using PBMC from the same blood sample (Fig 3e,f). In line with the IFN-γ whole blood ELISA, there was a close correlation between IP-10 whole blood ELISA and IFN-γ ELISPOT measured following gluten challenge and stimulation with peptide mix (Fig. 2b) (n = 25 treated CD; r = 0.822, P < 0.0001). Correlations

Fig. 2. Correlation between interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) and whole blood IFN-γ and interferon-gamma inducible protein-10 (IP-10) ELISA responses to a two-peptide mixture after gluten challenge. (a) Peptide mix responses in the IFN-γ ELISPOT versus IFN-γ ELISA (n = 27 treated celiac disease (CD) (r = 0.839, P < 0.0001)). (b) Peptide mix responses in the IFN-γ ELISPOT versus IP-10 ELISA (n = 25 treated CD (r = 0.822 P < 0.0001)). The horizontal and vertical dotted lines represent the ELISA and ELISPOT cut-off determined by receiver operating characteristic (ROC) curve analysis. For the analysis, responses to no antigen (Nil) were subtracted. Untreated CD (n = 4) and non-CD control subjects (n = 17) are also shown.

Fig. 3. Interferon (IFN)-γ and IFN-γ-inducible protein-10 (IP-10) enzyme-linked immunosassay (ELISA) and IFN-γ enzyme-linked immunospot (ELISPOT) responses in coeliac disease (CD) patients and non-CD control subjects. Responses are shown after incubation of whole blood or peripheral blood mononuclear cells (PBMC) with no antigen (Nil), two-peptide mix (peptide mix), gliadin and tetanus toxoid (Ttox) or phytohaemagglutinin (PHA). (a,c,e) IFN-γ and IP-10 ELISA and IFN-γ ELISPOT responses in treated CD after gluten challenge (d6 closed circles, n = 27 or 25); (a only) treated CD patients before gluten challenge (d0 open circles, n = 27). (b,d,f) IFN-γ and IP-10 ELISA and IFN-γ ELISPOT responses in non-CD control subjects after gluten challenge (d6 closed circles, n = 17); (b only) non-CD control subjects before gluten challenge (d0 open circles, n = 16). Median indicated by horizontal lines. *Significant difference P < 0.05 by Kruskal–Wallis test.
In order to determine the diagnostic performance of the IFN-γ and IP-10 release assays in this exploratory study, ROC curve analysis was employed after subtraction of the no antigen responses (Fig. 4). For the analysis, CD subjects following GFD were considered 'disease' cases, and HLA-DQ2-5+ and HLA-DQ2-5− non-CD control subjects following GFD were regarded as 'non-disease' cases. There were no statistically significant differences between the AUC of the cytokine release assays using peptide mix or gliadin as antigen. However, the AUC of IFN-γ whole blood ELISA using gliadin as antigen [0·79, 95% CI: 0·63–0·90] (P < 0·05) was significantly lower than the corresponding assay using peptide mix (0·96, 95% CI: 0·85–0·99) (P < 0·05). Similar findings were obtained comparing the AUC of the IP-10 release assay using gliadin as antigen (0·79, 95% CI: 0·63–0·90) and peptide mix (0·99, 95% CI: 0·90–1·0) (P < 0·05). In contrast, there was no statistically significant difference between the AUC of IFN-γ ELISPOT using gliadin (0·81, 95% CI: 0·66–0·92) or peptide mix (0·93, 95% CI: 0·81–0·99) (P > 0·05).

The cut-offs for a positive test were estimated using ROC curve analysis on the antigen-dependent values and at ideal false positive cut-offs for both peptide mix and gliadin as antigen. Sensitivities and specificities using these cut-offs are shown in Table 3.

Ideal false-positive cut-offs were also evaluated. Notably, four of six ideal false-positive cut-offs were the same as those calculated by MedCalc, indicating that the cut-off for these assays were already optimized at a 0% false-positive rate. Whole blood IFN-γ and IP-10 ELISAs and IFN-γ ELISPOT with fresh PBMC using peptide mix yielded sensitivities of 26, 48 and 19%, respectively, that were higher than when gliadin was utilized. For IP-10 whole blood ELISA using peptide mix, the ideal false-positive cut-off was higher with a lower detection rate (92%, 95% CI: 74·0–99·0) for IFN-γ whole blood ELISA using gliadin as antigen, the ideal false-positive cut-off was higher, with a substantially lower detection rate (11%, 95% CI: 2·4–29).

Table 3. Diagnostic accuracy of cytokine release assays with optimized cut-offs.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Peptide mix</th>
<th>Gliadin</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>85·1 (66·3–95·8)</td>
<td>59·2 (38·8–77·6)</td>
</tr>
<tr>
<td>IFN-γ ELISA</td>
<td>85·1 (66·3–95·8)</td>
<td>52·0 (31·3–72·2)</td>
</tr>
<tr>
<td>IFN-γ ELISPOT</td>
<td>94·1 (69·8–99·8)</td>
<td>88·2 (61·7–98·4)</td>
</tr>
<tr>
<td>IP-10 ELISA</td>
<td>100 (86·3–100)</td>
<td>100 (79·4–100)</td>
</tr>
<tr>
<td>IP-10 ELISPOT</td>
<td>100 (86·3–100)</td>
<td>100 (79·4–100)</td>
</tr>
</tbody>
</table>

IFN: interferon; IP-10: IFN-γ-inducible protein-10; ELISPOT: enzyme-linked immunospot.
The sensitivity and specificity for IFN-γ ELISA using peptide mix was not affected significantly when ROC curve analysis was undertaken with 'non-disease' cases including only the six HLA-DQ2·5+ non-CD control subjects instead of all 17 non-CD subjects. As ongoing gluten consumption maintains disease activity and is associated with elevated CD-specific serology, further exploratory subgroup analysis compared non-CD and only those CD subjects who were seronegative for tTG-IgA, DGP-IgA and DGP-IgG (n = 19 CD and n = 16 non-CD control subjects). In this scenario, the IFN-γ and IP-10 whole blood ELISA and ELISPOT on d6 with peptide mix yielded diagnostic sensitivities of 89% (95% CI: 66·8–98·7), 100% (95% CI: 81·5–100) and 95% (95% CI: 73·9–99·8), respectively; specificities were 100% (95% CI: 79·4–100) for the IFN-γ ELISA, and 94% (95% CI: 69·7–99·8) for both the IP-10 ELISA and ELISPOT. Sensitivities of cytokine release assays using gliadin as antigen were consistently less than 69% and specificities were 88% (95% CI: 69·7–99·8) for the IFN-γ ELISA and 100% (95% CI: 79·4–100) for both the IP-10 ELISA and ELISPOT. Taken together, these findings support the high sensitivity and specificity of cytokine release assays using peptide mix incubated with blood collected from treated CD patients after gluten challenge.

Finally, ROC curve analysis was performed in 24 CD and 16 non-CD control subjects to evaluate if the incremental response to peptide mix between d0 and d6 in the IFN-γ whole blood ELISA was a better predictor of CD than responses on d6 alone. Sensitivity was 83% (95% CI: 62·6–95·2) and specificity was 94% (95% CI: 69·7–99·8) for the incremental response between d0 and d6, slightly lower than for d6 responses alone (Table 3). Together, these results indicated that assessment of plasma IFN-γ in whole blood incubated with peptide mix was sufficient to distinguish between HLA-DQ2·5+ CD patients following GFD and non-CD subjects following GFD, whether or not they carried HLA-DQ2·5.

Reproducibility of the whole blood assay and IFN-γ and IP-10 ELISA assays is high

Because elevated responses to gluten-derived peptides in the whole blood ELISA were found only on d6 after commencing gluten challenge and the assay requires fresh blood, it was not possible to assess samples from the same patient on different days to determine interassay variation. Therefore, we determined the reproducibility of the whole blood assay with a single blood sample from six CD donors on d6 that was divided between three independent whole blood experiments (total of 18 whole blood assays). After stimulation with peptide mix or gliadin, plasma IFN-γ concentrations were measured by ELISA in duplicate, and the CV was calculated (Table 4). The interassay variation of the whole blood experiments was 12·8% when using peptide mix and 16·9% for gliadin.

To assess the IFN-γ and IP-10 ELISA interassay variability, plasma samples with levels outside the limits of quantification were assigned the lower or upper limit of quantification (for example 4000 for some IP-10 results). As this generates a CV of 0, they were not included. Therefore, not all CD and non-CD control subjects enrolled into the study were included in each analysis (right column, Table 4). The CVs were less than 12% for the treated CD subjects and non-CD control subjects when calculated by taking the mean of individual CVs using each of the three replicates on each subject (Table 4).

Discussion

Reliable detection of rare antigen-specific T cells is critical for translation of the field of T cell immunology to diagnostics, therapeutics and preventative vaccines. Although antigen-specific T cell assays have been used to monitor novel vaccines and immunotherapies, clinical diagnostic tests measuring antigen-specific T cells have been limited to
chronic infections, such as *M. tuberculosis* and cytomegalovirus infection [27]. In principle, T cell diagnostics might be relevant to T cell-mediated autoimmune diseases, but the frequencies of pathogenic T cells in blood are at or below levels of detection for even the most sensitive assays.

Our original observation, that oral gluten challenge mobilizes gluten-reactive T cells to frequencies that are readily detected by IFN-γ ELISPOT, has been replicated by several groups [10–12,13,25]. This ‘real life’ antigen challenge to reactivate memory T cell responses has provided the first definitive T cell epitope hierarchy for a human T cell-mediated disease [11]. In the current study we used blood collected before and after gluten challenge, tested responses to antigenic proteins and peptides and compared three assay designs to permit efficient detection of gluten-reactive T cells that are specific for HLA-DQ2·5+ CD patients.

In this exploratory study, the only patients to consistently show detectable T cell responses to gluten peptides after oral gluten challenge were HLA-DQ2·5+ CD subjects following GFD. Prior to gluten challenge, these patients were indistinguishable from both non-CD subjects following GFD and untreated CD subjects. IFN-γ and IP-10 release assays using ELISPOT and/or ELISA formats with blood collected after gluten challenge performed equally well for the detection of gluten-reactive T cells. In these assays, a mixture of two synthetic peptides containing immunodominant HLA-DQ2·5-restricted gluten-derived epitopes provided sufficient antigenic stimulation to achieve high sensitivity and specificity for HLA-DQ2·5+ CD.

The current proof of concept study was limited to HLA-DQ2·5+ CD patients because this genotype is found in 90% of affected patients and the T cell epitope hierarchy recognized by gluten-reactive T cells in HLA-DQ2·5+ patients is conserved and well understood [11,28]. The identity and relative importance of gluten-derived peptides recognized by T cells isolated from CD patients who do not carry HLA-DQ2·5 is not as well defined as in HLA-DQ2·5+ CD.

It is not clear that gluten itself provokes gastrointestinal symptoms in patients who do not have CD but choose to avoid gluten-containing food [6], but oral gluten challenge for 3 days was tolerated equally well by patients with confirmed CD and control patients who excluded dietary gluten because they associated it with various digestive and systemic symptoms. However, if tested in large prospectively recruited patient cohorts, it is possible that oral gluten challenge even for 3 days may not be as well tolerated as observed in this current group of volunteers who willingly agreed to eat gluten. Fortunately, oral gluten challenge for 3 days does not cause villous atrophy or significant architectural disruption to the small intestinal mucosa in CD patients in histological remission [14].

Brottveit *et al.* recently assessed the potential of a fluorescence-activated cell sorter (FACS)-based assay utilizing MHC class II-peptide tetramers detecting DQ2·5-glia-α1a and DQ2·5-glia-α2 epitope-specific T cells in blood after 3-day gluten challenge for the diagnosis of CD in patients following GFD [14]. The cytokine release assays described in the present study performed as well as the tetramer assay, which was 85% sensitive and 100% specific for HLA-DQ2·5+ CD [14]. Recently, these findings using MHC tetramers have also been replicated in CD patients from the United States [29].

The current study highlights that whole blood-based IFN-γ and IP-10 release assays are as effective as ELISPOT for the detection of rare gluten-specific T cells. IFN-γ ELISPOT is a robust and sensitive assay for the detection of rare antigen-specific T cells and has been utilized in clinical trials, but requires that blood be transported to a central facility with specialist technical capability [30–32]. Transporting fresh blood can result in reduced T cell functionality and introduces a risk of tests being false negative [33]. When translated to the diagnosis of *M. tuberculosis* infection, on-site incubation of whole blood drawn directly into dedicated *M. tuberculosis* antigen-stimulation tubes followed by measurement of plasma IFN-γ by ELISA has proved to be practical and is now approved for the diagnosis of *M. tuberculosis* infection [15–17].

As a potential diagnostic test for HLA-DQ2·5+ CD patients following GFD, whole blood cytokine release assays appear to be sensitive and specific. As an added benefit over current diagnostic tests being performed on patients already following GFD, the mobilization of gluten-reactive T cells specific for CD into the bloodstream requires oral gluten challenge for only 3 days instead of the weeks or months required for diagnosis based on abnormal small bowel histology.

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**Author contributions**

N. O.: main investigator, acquisition, analysis and interpretation of the data, statistical analysis, manuscript.
preparation; J. T.-D.: study concept and design, analysis and interpretation of the data, statistical analysis, manuscript preparation; M. H.: study design, analysis and interpretation of the data, revision of the manuscript; R. P. A.: study concept and design, supervision of the study, statistical analysis, revision of the manuscript. All authors approved the final version of the manuscript. Guarantor of the article: Dr Robert Anderson.

Disclosures

J. T.-D. and R. P. A. are co-inventors of patents pertaining to the use gluten peptides in therapeutics, diagnostics and non-toxic gluten; both are shareholders of Nexpep Pty Ltd and R. P. A. also of ImmusanT, Inc. (USA). R. P. A. is Chief Scientific Officer and J. T.-D. is a consultant to ImmusanT, Inc. Full disclosure was provided to all study participants.

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