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Blood-Based T-Cell Diagnosis of Celiac Disease

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BACKGROUND & AIMS: Current diagnosis of celiac disease (CeD) is inaccurate in patients consuming a gluten-free diet (GFD). Blood-based diagnostics targeting gluten-specific T cells, such as tetramer assays, are highly sensitive and specific but are impractical for clinical use. We evaluated the potential of a simple, whole-blood assay measuring interleukin 2 (IL2) release (WBAIL-2) for detecting gluten-specific T cells to aid in CeD diagnosis. **METHODS:** WBAIL-2 was assessed in 181 adults; 88 with CeD (75 consuming a GFD, 13 consuming gluten) and 93 controls (32 consuming a GFD with nonceliac gluten sensitivity, 61 healthy). In vitro IL2 release in whole blood after gluten peptide stimulation was measured. The assay's performance was compared with tetramer-based methods, and serum IL2 levels were monitored before and after a single-dose gluten challenge. Correlations between IL2 levels, tetramer-positive T-cell frequencies, and symptoms were examined. **RESULTS:** The WBAIL-2 assay demonstrates high accuracy for CeD diagnosis, even in patients consuming a strict GFD. Optimized dual cutoffs in HLA-DQ2.5$^+$ patients showed high sensitivity (90%) and specificity (95%), with lower sensitivity (56%) in HLA-DQ8$^+$ CeD. WBAIL-2 correlated strongly with the frequency of tetramer-positive gluten-specific cluster of differentiation (CD) 4$^+$ T cells and serum IL2 levels after a gluten challenge. Elevated WBAIL-2 levels predicted gluten-induced symptom severity, such as vomiting. The assay required only small blood volumes and performed comparably with tetramer-based methods. **CONCLUSIONS:** Gluten-stimulated IL2 secretion indicates the presence of pathogenic gluten-specific CD4$^+$ T cells and is a useful diagnostic for CeD. WBAIL-2 and serum IL2 after gluten could be complementary and allow biopsy-free CeD diagnosis. WBAIL-2 may help diagnose and monitor other CD4$^+$ T cell-driven diseases.

Keywords: Celiac Disease; T cells; Interleukin 2; Diagnostics.

Celiac disease (CeD) is caused by an adaptive immune response to dietary gluten mediated by cluster of differentiation (CD) 4$^+$ gluten-specific T cells that results in chronic small intestinal injury and diverse chronic morbidities.[1] The only available treatment is a strict, life-long, gluten-free diet (GFD). Expeditious diagnosis is important to minimize long-term complications, but global prevalence is high (1%–2%), with 50% to 80% of cases undiagnosed or diagnosed late.[2–4]

Reliable diagnosis of CeD requires patients to consume gluten, because first-line serology tests and confirmatory endoscopic duodenal biopsy specimens depend on the detection of gluten-induced injury, which normalizes on a GFD. This requirement poses challenges for patients already avoiding gluten, and with the anticipated approval of non-dietary therapies, accurate tools for confirming diagnosis and monitoring disease severity will become increasingly important.[5]

The diagnosis and therapeutic monitoring of CeD and other chronic immune diseases, as well as infectious diseases and cancers, could be significantly advanced by measuring disease-relevant antigen-specific effector CD4$^+$ T cells. However, this approach has seen limited clinical application outside of academic studies, vaccine development, and pharmaceutical research. Tuberculosis is one of the few conditions diagnosed by measuring antigen-specific T cells. Circulating mycobacteria-specific T cells are relatively abundant in latent tuberculosis and can be detected by enzyme-linked immunospot (ELISpot) or whole-blood cytokine release assay.[6,7]

Unfortunately, the frequency of antigen-specific T cells in blood without antigen rechallenge is below the detection limits of these platforms for most diseases, and in many cases, the identity of the triggering antigenic peptides is poorly defined. Although flow cytometry with peptide-major histocompatibility complex multimers has the sensitivity to detect these rare cells, it requires knowledge of the relevant T-cell epitopes and is restricted to highly specialized laboratories.

Abbreviations used in this paper: **AUC, area under the curve; CeD, celiac disease; CD, cluster of differentiation; CI, confidence interval; CLIP, class II-associated invariant chain peptide; ELISpot, enzyme-linked immunospot; CV, coefficient of variation; GCIL-2, grass carp interleukin 2; GFD, gluten-free diet; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; T_{EM}, T-effector memory; TNF, tumor necrosis factor; WBA, whole-blood assay; WBAIL-2, whole-blood assay measuring interleukin 2 release.**

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WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Celiac disease (CeD) diagnosis is challenging, particularly for patients on gluten-free diets. A whole blood interleukin (IL) 2 release assay (WBAIL-2) may simplify detection of gluten-specific T cells and be a straightforward diagnostic tool for CeD.

NEW FINDINGS

WBAIL-2 is a simple and accurate CeD diagnostic even in patients consuming a gluten-free diet. IL2 production reflects the activation of gluten-specific T cells, correlates with their frequency, and stratifies symptom severity to gluten.

LIMITATIONS

This was a single-center study with relatively small subgroup sizes and untested reproducibility across laboratories. Pediatric populations and patients on immunosuppressants were not assessed, and prospective validation is required.

CLINICAL RESEARCH RELEVANCE

WBAIL-2 is an accurate diagnostic tool for CeD, even in patients consuming a gluten-free diet. It matches the sensitivity of major histocompatibility complex-tetramer detection but requires significantly less blood and is easier to perform. It has the potential to replace gastroscopy and small intestinal biopsies. WBAIL-2 could aid in diagnosing and monitoring other diseases driven by cluster of differentiation 4+ T cells.

BASIC RESEARCH RELEVANCE

This study confirms IL2 as a sensitive and specific biomarker of gluten-specific T-cell activation, capable of detecting a single gluten-specific T cell in 4 mL of blood. WBAIL-2's practical advantages, including minimal blood requirements and no need for specialized infrastructure, make it a valuable tool for studying immune responses in CeD and other T cell–driven disorders.

CeD is an appealing candidate for a T-cell diagnostic due to the central role of gluten-specific $CD4^+$ T cells in its pathogenesis and the clear identification of immunodominant gluten epitopes driving these cells.[8] Gluten-specific $CD4^+$ T cells are specific to CeD; however, they circulate in low numbers in the blood and can only be detected by peptide-major histocompatibility complex tetramers[9,10] or by functional T-cell assays, such as ELISpot, which require a preceding oral gluten challenge.[11,12]

Recent advances suggest that ultrasensitive assays detecting interleukin 2 (IL2) release after gluten ingestion could complement serology and enable non-biopsy specimen–diagnosis of CeD.[13–17] IL2 release has demonstrated high sensitivity and specificity in distinguishing CeD patients from healthy controls and nonceliac gluten sensitivity.[18,19] A whole-blood assay for IL2 (WBAIL-2) was developed, in which gluten peptides stimulate blood in vitro, and IL2 release is measured.[20]

This study assessed the feasibility and performance of these novel, but technically straightforward, in vivo and in vitro gluten-stimulated IL2 release assays in CeD. The findings provide proof of concept for these ultrasensitive assays being comparable to HLA-DQ:gluten peptide tetramer assays in detecting rare antigen-specific $CD4^+$ T cells and having clinical utility in the diagnosis of CeD.

Materials and Methods

Study Design and Participants

This was a single-center, investigator-led study performed at the Walter and Eliza Hall Institute, with recruitment via the Royal Melbourne Hospital. Adult participants aged 18 to 75 years with medically diagnosed CeD (treated and active cohorts), nonceliac gluten sensitivity, and healthy controls were recruited. All participants provided written informed consent. The study was approved by the Human Research Ethics Committees of the Royal Melbourne Hospital (2021.210) and the Walter and Eliza Hall Institute (21/18) and was conducted in accordance with the ethical principles in the Declaration of Helsinki.

Inclusion criteria for CeD participants was documentation confirming a past CeD diagnosis based on duodenal villous atrophy (Marsh 3), positive celiac serology, and supportive clinical criteria. The treated cohort of CeD participants were consuming a GFD for at least 12 months. The active CeD cohort were recruited at diagnosis before starting a GFD and had positive transglutaminase-IgA or deamidated gliadin peptide-IgG and duodenal histology that subsequently confirmed CeD. Inclusion criteria for nonceliac gluten-sensitivity participants were self-report of gluten sensitivity, adherence to a GFD, documentation of prior exclusion of CeD based on negative CeD serology or small intestinal histology, or both, while eating gluten, or the presence of an HLA genotype not consistent with CeD. Inclusion criteria for nonceliac, non-gluten–sensitive controls were the absence of self-reported gluten sensitivity, regular gluten consumption, and documentation of prior exclusion of CeD confirmed by negative CeD serology or duodenal histology, or both, while eating gluten. Participants with autoimmune disease, such as type 1 diabetes mellitus and autoimmune thyroid disease, were diagnosed based on accepted clinical criteria. Exclusion criteria were the use of systemic immunosuppressant medication, pregnancy, or the presence of refractory CeD.

Procedures

Eligible participants attended a single visit where blood was collected for CeD serology (transglutaminase-IgA and deamidated gliadin peptide-IgG, Melbourne Health) and HLA-DQ2/DQ8 genotyping via single-nucleotide polymorphism tagging[21] or polymerase chain reaction (Melbourne Pathology). Medical history and medications were recorded. A subset of participants with treated CeD, nonceliac gluten sensitivity, and healthy controls on a GFD for at least 4 weeks underwent a single-dose open-label gluten challenge (10 g vital wheat gluten) for additional immune studies or to follow-up WBAIL-2 results discordant with their diagnosis. Serum for IL2 assessment was collected at baseline and 4 hours after a gluten challenge (IL2 found in grass carp [GCIL-2]), while whole blood was collected at baseline (8 mL) and day 6 (8 mL) for WBAIL-2. In some cases, additional blood (50–300 mL) was collected at

baseline and day 6 for tetramer analysis. A subset of treated CeD participants undertook a 3-day oral gluten challenge (10 g/d) to enable cytokine capture assays on peripheral blood mononuclear cells (PBMCs) collected on day 6. Participants undergoing gluten challenges completed a diary (modified CeD patient-reported outcome)[22] to record symptoms before, during, and after the challenge.

Cytokine Assays

The WBAIL-2 assay was adapted from a previously reported 96-well plate approach[20] to an individual tube format. The WBAIL-2 assay used in this study is a prototype version of the Novoleukin-C.G8 whole-blood assay, which is a proprietary commercial product developed and distributed by Novoviah Pharmaceuticals (Brisbane, Queensland, Australia). The stimulation tube contains a mixture of 8 synthetic immunoreactive gluten T-cell stimulatory peptides, including the well-characterized, overlapping DQ2.5-glia-α1b, DQ2.5-glia-α2, DQ2.5-glia-ω1, and DQ2.5-glia-ω2 epitopes included in the tetramers used in this study, as well as selected additional HLA-DQ2.5, HLA-DQ2.2, and HLA-DQ8–restricted epitopes derived from wheat α-, γ-, and ω-gliadins, and low-molecular-weight glutenin.[8] The scrambled peptide negative control condition matched the amount and amino acid composition of the peptide mix in the stimulation tube. Synthetic peptides were >95% pure by high-performance liquid chromatography, and identity was confirmed by liquid chromatography-mass spectrometry (Mimotopes, Clayton, Victoria, Australia).

Whole-blood was collected in 4 mL heparinized tubes, and within 30 minutes, the peptide cocktail or a negative control (scrambled peptide) was added. Samples were incubated at 37°C in 5% CO_2 for 24 hours ± 20 minutes. Plasma was then collected and stored at −80°C. IL2 levels were measured using an electrochemiluminescence IL2 S-PLEX kit (Meso Scale Diagnostics [MSD], Rockville, MD) following the manufacturer's instructions. A subset of samples was also assessed for interferon-γ (IFN-γ), IL17A, IL6, tumor necrosis factor-α, and IL10 using Proinflammatory and Cytokine V-PLEX assays. Plates were run on an SQ 120MM instrument (MSD) , and mean cytokine levels from duplicate wells were analyzed using MSD Discovery Workbench software. The fold-change (peptide/control) and IL2 concentration (peptide minus control) were calculated. The optimal diagnostic cutoff was determined post hoc using receiver operating characteristic curve analysis. Interassay and intra-assay variability were calculated (Supplementary Figure 1).

Tetramer Generation

The extracellular domain of HLA-DQ2.5 (HLA-DQA1*5:01 and HLA-DQB1*02:01) with the DQ2.5-hor-3a epitope or the DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1, DQ2.5-glia-ω2 epitopes, or a class II-associated invariant chain peptide (CLIP; ATPLLMQALPMGA) control covalently linked to the N-terminus of the HLA-DQ2.5 β-chain via a flexible linker (GSGGSIEGRGGSG) was produced and purified essentially as described.[23] Purified HLA-DQ2.5-peptide monomers were biotinylated on the C-terminal *Escherichia coli* BirA ligase recognition sequence (GLNDIFEAQKIEWHE) of the HLA-DQ2.5 β-chain using BirA ligase buffer exchanged on a HiTrap Desalting column (Cytiva) to remove excess biotin, and complexed with streptavidin-phycoerythrin (PE) (BD Pharmingen) to form tetramers.

Peripheral Blood Mononuclear Cell Studies

Immune studies on PBMCs used tetramers and cytokine capture assays. PBMCs were isolated from whole-blood samples by density gradient centrifugation (Leucosep, Interpath Services) and cryopreserved. PBMCs were incubated with 50 nmol/L dasatinib at 37°C for 30 minutes (Cell Signaling Technology), followed by an incubation with Fc receptor blocker (Miltenyi Biotec or Stem Cell Technologies). PBMCs were stained with HLA-DQ2.5-CLIP tetramer or HLA-DQ2.5:gluten tetramers conjugated to PE, representing the epitopes DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1, DQ2.5-glia-ω2, and DQ2.5-hor3 (10 μg/mL of each tetramer). Tetramer-stained PBMCs underwent tetramer-enrichment using the EasySep Release Human PE positive selection kit (Stem Cell Technologies) following the manufacturer's instructions (not the CLIP control). The CLIP negative control was used to establish a pre-enrichment number of $CD4^+$ T cells. Cells were stained with Zombie UV live/dead marker (BioLegend) for 30 minutes at room temperature, followed by an antibody mix comprising anti-human CD3-BUV395 (clone: UCHT1; BD), CD4-BV480 (clone: RPA-T4; BD), CD8-BV750 (clone: SK1; BioLegend), CD62L-fluorescein isothiocyanate (clone: DREG-56; BioLegend), CD14-APC-H7 (clone: MDP9; BD Pharmingen), CD45RA-PerCP-Cy5.5 (HI100; BD), Integrin β7-BV421 (clone: FIB504; BD), and CD38-APC/Fire810 (clone: HB-7; BioLegend), prepared in brilliant stain buffer (BD), for 30 minutes at 4°C. Cells were analyzed on a Cytek Aurora cytometer, and flow cytometry data were analyzed by FlowJo 10 software (BD Biosciences). $CD4^+$ T cells were analyzed by flow cytometry for $CD3^+CD14^-CD4^+$ HLA-DQ2.5:gluten $tetramer^+CD62L^-CD45RA^-$Integrin $\beta7^+$, hereafter termed $tetramer^+\beta7^+$ T-effector memory (T_{EM}) cells. Expression of $CD38^+$ $tetramer^+\beta7^+$ T_{EM} cells was assessed to determine activation status. The gating strategy is shown in Supplementary Figure 2. The number of $tetramer^+\beta7^+$ T_{EM} cells was normalized to 10^6 $CD4^+$ cells estimated from a pre-enriched sample.

Interleukin 2 Interferon-γ Capture Assay

Cytokine capture assay was performed on fresh or frozen PBMCs acquired from treated CeD patients 6 days after a 3-day oral gluten challenge (10 g vital wheat gluten daily). Briefly, as large numbers of PBMC were required, $CD4^+$ T cells were isolated using EasySep Human $CD4^+$ T Cell Isolation Kit (Stem Cell Technologies) then stained with HLA-DQ2.5-gluten tetramers and magnetically enriched, as above. Tetramer-enriched samples were incubated with 900,000 autologous PBMCs and glia-α1/α2–, glia-ω1/ω2– and hor-3a–containing 14 to 16 mer peptides (GL Biochem) at 15 μg/mL equimolar in T-cell media (Iscove's Modified Dulbecco's Medium supplemented with 5% heat-inactivated pooled human serum [Australian Lifeblood], 1x GlutaMAX [Gibco], 1x nonessential amino acids [Gibco], and 50 μmol/L 2-mercaptoethanol [Sigma-Aldrich]) for 4 hours at 37°C in 5% CO_2. A CLIP condition and unstimulated condition were included. Cytokine-secreting cells were identified using the IL2-allophycocyanin and IFN-γ–fluorescein isothiocyanate secretion assay kits (Miltenyi Biotec), following the manufacturer's instructions, with a shortened secretion period of 30

minutes. Cells were stained with Zombie UV Live/Dead marker (BioLegend) for 30 minutes at 4°C, followed by an antibody mix comprising anti-human CD3-Brilliant UltraViolet (BUV) 395 (clone: UCHT1; BD), CD4- Brilliant Violet (BV) 480 (clone: RPA-T4; BD), CD8-BV750 (clone: SK1; BioLegend), CD62L-BV510 (clone: DREG-56; BD), CD14-BUV661 (clone: M5E2), CD11c-BUV661 (clone: B-ly6; BD), CD19-BUV661 (clone: HIB19), CD56-BUV661 (clone: MY31; BD), CD45RA-peridinin-chlorophyll protein-Cy5.5 (HI100, BD), integrin-β7–BV421 (clone: FIB504; BD), CD38-allophycocyanin/Fire810 (clone: HB-7; BioLegend), and CD60-BUV737 (clone: FN50; BD), prepared in Brilliant Stain Buffer (BD), for 30 minutes at 4°C. Cells were analyzed on a Cytek Aurora cytometer, and flow cytometry data were analyzed by FlowJo 10 software.

Interassay and Intra-assay Variability

The interassay and intra-assay variability of the WBAIL-2 and plasma IL2 assessment (MSD S-PLEX) were evaluated (Supplementary Figure 1*A*). The interassay variability of the IL2 whole-blood assay (WBA) was determined by testing samples on 2 occasions 1 to 2 weeks apart. The mean IL2 fold change and pg/mL from triplicate WBA tests was used to compare across the 2 time points. The intra-assay variability was determined by triplicate IL2 WBA tests performed from the same blood draw. Additionally, a different operator performed an additional triplicate set of IL2 WBA tests to determine interoperator variability. The coefficient of variation (CV) was calculated as the ratio of the standard deviation to the mean, expressed as a percentage (%CV = standard deviation/mean × 100).

Statistical Analyses

Data were analyzed using GraphPad Prism 5 software (GraphPad Prism Software). Nonparametric tests were applied: the Mann-Whitney *U* test for unpaired data and Wilcoxon's signed rank test for paired data, with corrections for multiple comparisons.

Results

Participant Demographics

Between August 23, 2022, and December 18, 2024, 220 adults were screened for inclusion. Of these, 39 were excluded, and 181 were enrolled, comprising individuals with treated CeD (n = 75), active CeD (n = 13), nonceliac gluten sensitivity (n = 32), and healthy controls (n = 61) (Table 1 and Supplementary Table 1). Women comprised 72% to 85% of the cohorts. Among the CeD participants, 85% (treated) and 92% (active) carried the celiac-susceptibility genotype HLA-DQ2.5 compared with 39% of healthy controls and 31% of nonceliac gluten-sensitive participants.

The Whole-Blood Assay Measuring Interleukin 2 Release Is Sensitive and Specific for Celiac Disease

The diagnostic utility of WBAIL-2 was evaluated in a cohort of 88 adults with CeD (75 treated, 13 active) and 93 controls (32 nonceliac gluten sensitivity, 61 healthy). IL2 fold change and concentration were significantly higher in treated CeD (median, 7.77–3.195 pg/mL) and active CeD (median, 3.73; 1.162 pg/mL) compared with healthy (median, 0.79; −0.0078 pg/mL) and nonceliac gluten-sensitive controls (median, 1.08; 0.001 pg/mL) (Figure 1*A* and *B*).

All CeD individuals were compared with all non-CeD individuals and an optimal cutoff of 1.99-fold change yielded 88% sensitivity (77 of 88) and 89% specificity (83 of 93; area under the curve [AUC], 0.92; 95% confidence interval [CI], 0.87–0.95; $P < .0001$) (Figure 1*C*; green). A secondary cutoff of 0.271 pg/mL achieved 90% sensitivity (79 of 88) and 83% specificity (77 of 93; AUC, 0.91; 95% CI, 0.86–0.96; $P < .0001$) (Figure 1*D*; green). Combining both cutoffs improved specificity to 95% (88 of 93) with 86% sensitivity (76 of 88) (Figures 1*E* and *F*). Treated CeD was also compared with nonceliac gluten sensitivity to determine specificity in these 2 cohorts consuming a GFD. The 1.99-fold change cutoff yielded 81% specificity (26 of 32; AUC, 0.89; 95% CI, 0.82-0.95; $P < .0001$) (Figure 1*C*; blue), and the 0.271 pg/mL cutoff yielded 84% specificity (27 of 32; AUC, 0.90; 95% CI, 0.83-0.96; $P < .0001$) (Figure 1*D*; blue). The combined cutoff yielded 91% specificity (29 of 32) in participants consuming a GFD.

No significant differences in WBAIL-2 were observed across HLA types or zygosity (Figure 1*G*). However, sensitivity was lower (56%) in participants with the less common HLA-DQ8 genotype. Excluding this genotype from the analyses increased WBAIL-2 sensitivity to 90% (71 of 79). Using dual cutoff criteria in HLA-DQ2.5$^+$ CeD participants, WBAIL-2 achieved 90% sensitivity (71 of 79) and 95% specificity (88 of 93).

Of 5 non-CeD participants with positive WBAIL-2 responses, 4 were first-degree relatives of a person with CeD (Table 2). First-degree relatives had a higher IL2 fold change (median, 1.35) than nonrelatives (median, 0.74; $P = .0146$) (Figure 1*H*). Of these 5, 2 were healthy, and 3 reported nonceliac gluten sensitivity. Three participants (1 healthy, 2 nonceliac gluten sensitive) agreed to a repeat WBAIL-2 assessment and underwent a single-dose gluten challenge after a 4-week GFD. The healthy participant and 1 nonceliac gluten-sensitive participant again showed positive WBAIL-2 but had a negative GCIL-2. The other nonceliac gluten-sensitive participant had a negative WBAIL-2 on retesting, suggesting an initial false positive.

Negative WBAIL-2 responses occurred in 12 CeD participants (n = 10 treated, n = 2 active) (Table 2). Among them were 4 HLA-DQ8$^+$ individuals: 1 had no follow-up tests, 1 had prior positive IFN-γ ELISpot T-cell responses to rye and barley but not wheat, and 2 had high IL2 concentrations in the WBA control tube. This pattern was also observed in 2 HLA-DQ2.5$^+$ treated CeD nonresponders, who initially had substantially higher IL2 concentrations in the control tube of their WBAIL-2 assays, but upon retesting, they had lower control tube IL2 levels and their WBAIL-2 results were positive.

WBAIL-2 responses were initially negative in 2 treated CeD nonresponders, but these became positive on day 6 after a gluten challenge. One treated CeD participant had consistently negative WBAIL-2 results, including repeat WBAIL-2, postchallenge WBAIL-2, and GCIL-2. Both active CeD participants, after starting a GFD, showed positive WBAIL-2 responses when the test was repeated.

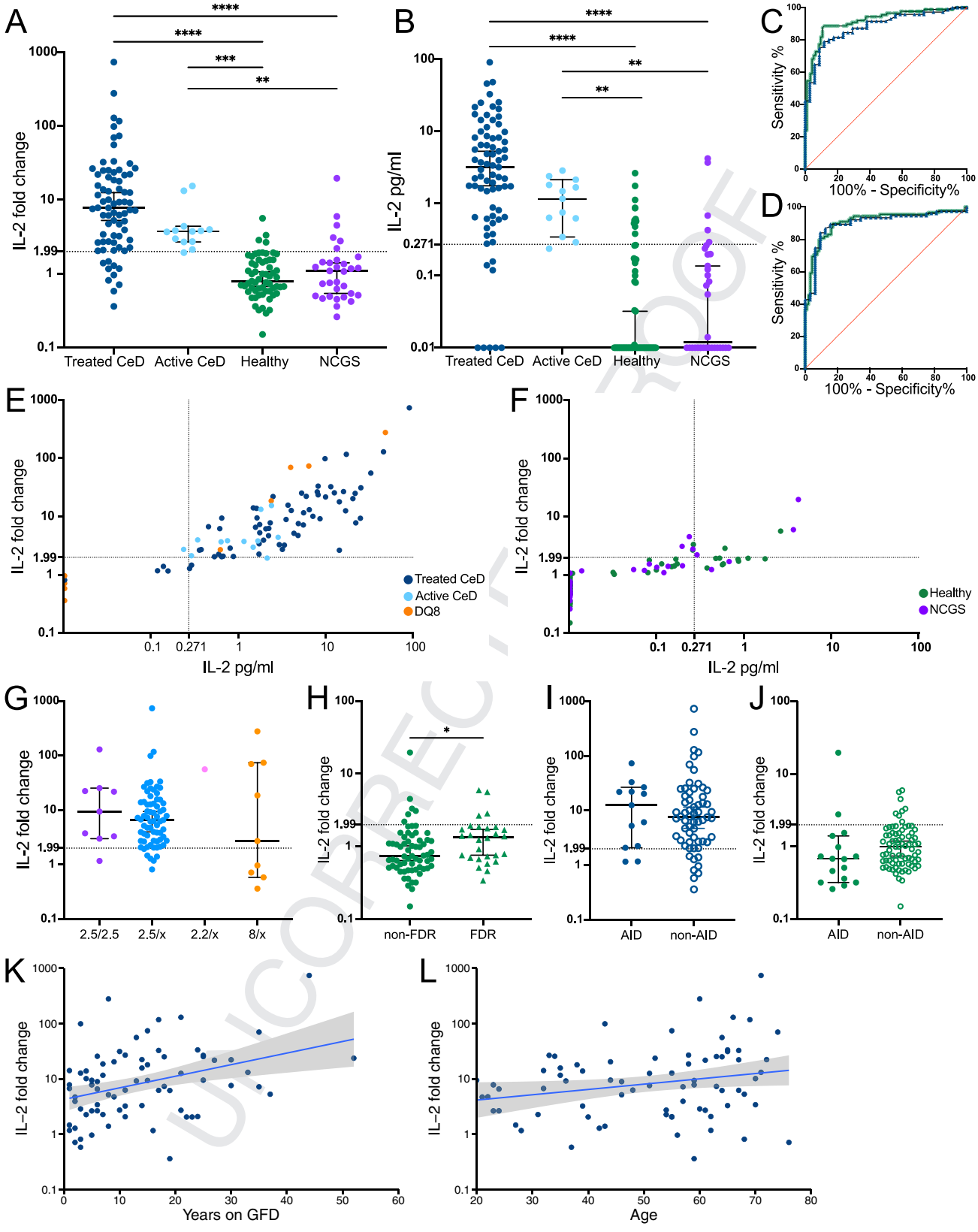
Table 1. Demographics and Clinical Characteristics of Participants

Variable	Treated CeD (n = 75)	Active CeD (n = 13)	Healthy (n = 61)	NCGS (n = 32)
Female participants	64 (85)	10 (77)	44 (72)	27 (84)
Age, *y*	55 (20–76)	28 (18–52)	44 (19–71)	47 (24–75)
HLA-DQ				
2.5/2.5	7 (9)	2 (15)	3 (5)	0 (0)
2.5/x	35 (37)	7 (54)	14 (23)	9 (29)
2.5/2.2	14 (19)	2 (15)	2 (3)	0 (0)
2.5/7	3 (4)	0 (0)	1 (2)	0 (0)
2.5/8	7 (9)	1 (8)	4 (7)	1 (3)
2.2/2.2	0 (0)	0 (0)	7 (12)	0 (0)
2.2/x	1 (1)	0 (0)	1 (2)	6 (19)
2.2/8	0 (0)	0 (0)	0 (0)	1 (3)
8/8	2 (3)	0 (0)	0 (0)	0 (0)
8/7	1 (1)	1 (8)	0 (0)	1 (3)
8/x	5 (7)	0 (0)	9 (15)	3 (10)
7/x	0 (0)	0 (0)	6 (10)	7 (23)
x/x	0 (0)	0 (0)	13 (22)	3 (10)
Positive TTG and DGP serology	4 (5)	10 (77)	1 (2)	0 (0)
Positive TTG serology only	8 (11)	3 (23)	2 (3)	0 (0)
Positive DGP serology only	2 (3)	0 (0)	2 (3)	1 (3)
Years on a GFD, *y*	11 (1–52)	0	NA	7 (0.5–32)
Autoimmune disease other than CeD	14 (19)	0 (0)	10 (16)	6 (19)
Hashimoto's thyroiditis	5 (7)	0 (0)	3 (5)	4 (13)
Grave's disease	2 (3)	0 (0)	0 (0)	0 (0)
Rheumatoid arthritis	3 (4)	0 (0)	0 (0)	1 (3)
Type 1 diabetes mellitus	2 (3)	0 (0)	7 (11)	1 (3)
Dermatitis herpetiformis	1 (1)	0 (0)	0 (0)	0 (0)
Scleroderma	1 (1)	0 (0)	0 (0)	0 (0)
Addison's disease	1 (1)	0 (0)	0 (0)	0 (0)
Autoimmune hepatitis	1 (1)	0 (0)	0 (0)	0 (0)
Sjogren's syndrome	0 (0)	0 (0)	0 (0)	1 (3)
Lupus	0 (0)	0 (0)	0 (0)	1 (3)
GCIL-2, n	35	0	5	16
Tetramer analysis, n	10	0	0	4
Baseline WBAIL-2, n	75	13	61	32
Day 1 pregluten challenge WBAIL-2, n	31	0	1	12
Day 6 postgluten challenge WBAIL-2, n	31	0	1	11

NOTE. Data are presented as n (%), or median (range).
DGP, deamidated gliadin peptide antibody; NA, not applicable; NCGS, nonceliac gluten sensitivity; TTG, tissue transglutaminase antibody.

To evaluate the disease specificity of WBAIL-2, the CeD cohort was stratified by the presence (n = 14) or absence (n = 61) of concurrent autoimmunity. IL2 fold change did not differ significantly between CeD participants with autoimmunity (median, 12.52) and those without (median, 7.66) (Figure 1*I*). Similarly, in the nonceliac cohort (n = 93), there was no significant difference between participants with (n = 16; median, 0.68) and without autoimmune disease (n = 77; median, 0.99) (Figure 1*J*). WBAIL-2 was positively correlated with years on a GFD ($r = 0.35$; 95% CI. 0.13–0.54; $P = .002$) (Figure 1*K*) and participant age ($r = 0.49$; 95% CI, 0.01–0.45; $P = .034$) (Figure 1*L*).

To determine whether the diagnostic utility of the WBAIL-2 could be improved, additional cytokines reported to be increased by acute gluten challenge (IFN-γ, IL17A, IL6, tumor necrosis factor-α, and IL10)[14,24] were measured in a subset of CeD (n = 43) and nonceliac (n = 26) participants. Samples were selected based on WBAIL-2 results either concordant (n = 58) or discordant (n = 11) with the diagnosis. Across cohorts, IL2 correlated most strongly with



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IFN-γ ($r = 0.632$, $P = 2.2^{-07}$) and IL-17A ($r = 0.595$, $P = 1.6^{-07}$) (Supplementary Table 2). After IL2, IFN-γ and IL17A showed the highest diagnostic performance (AUC, 0.69 and 0.64; $P = .018$ and $P = .091$, respectively). In participants with WBAIL-2, results consistent with their diagnosis, IFN-γ and IL17A had lower diagnostic accuracy than IL2 (AUC, 0.84 and 0.75; $P = .0002$ and $P = .005$, respectively), and neither cytokine was positive in CeD participants with negative WBAIL-2 results. These findings confirm IL2 as the optimal cytokine for CeD diagnostic assays. High IFN-γ responses in nonceliac participants with positive WBAIL-2 suggest a true biological response rather than technical issues or nonspecific IL2 production.

The variability of WBAIL-2 and the IL2 S-PLEX was assessed in 3 treated CeD participants and 3 nonceliac controls (Supplementary Figure 1*A*). For WBAIL-2, the mean CV was 24.1% to 29.7% for interassay variability, 28.9% to 46.3% for intra-assay variability, and 17.8% to 19.4% for interoperator variability (Supplementary Figure 1*B–E*). In the IL2 S-PLEX, replicate wells had a mean CV of 2.5%, and interassay variability had a mean CV of 7.6% (Supplementary Figure 1*F*).

The Whole-Blood Assay Measuring Interleukin 2 Release Response Correlates With Serum Interleukin 2 After Oral Gluten Challenge

Serum IL2 production after the oral gluten challenge (GCIL-2) was assessed to determine its diagnostic accuracy. GCIL-2 levels were significantly higher in CeD participants (n = 35; median, 16.1 pg/mL) compared with nonceliac participants (n = 21; median, 1.0 pg/mL; $P < .0001$) (Figure 2*A*). The optimal diagnostic cutoff for GCIL-2 was identified as a 2-fold increase from baseline to 4 hours postchallenge, achieving 97% sensitivity and 100% specificity for detecting CeD (AUC, 0.96; 95% CI, 0.9–1.0; $P < .0001$) (Figure 2*B*). Additionally, GCIL-2 levels were positively correlated with WBAIL-2 results ($r = 0.72$, 95% CI 0.56–0.83; $P < .0001$) (Figure 2*C*).

The Whole-Blood Assay Measuring Interleukin 2 Release Response Correlates With the Frequency of Activated Gluten-Specific Cluster of Differentiation 4$^+$ T Cells

The WBAIL-2 and GCIL-2 were compared with matched HLA-DQ2.5:gluten tetramer analysis. Higher tetramer$^+\beta7^+$ T_{EM} cell frequencies in CeD participants (n = 10; median, 2.15) than in nonceliac participants (n = 4; median, 0.19; $P = .024$) (Figure 2*D*) were shown. A cutoff of 0.59 cells per million CD4$^+$ T cells achieved 100% sensitivity and 75% specificity for CeD (AUC, 0.90; 95% CI, 0.7–1.0; $P = .024$) (Figure 2*E*). Tetramer$^+\beta7^+T_{EM}$ cell frequency correlated with GCIL-2 ($r = 0.74$, $P = .0035$) (Figure 2*F*) and with WBAIL-2 both with ($r = 0.79$; 95% CI, 0.58–0.90; $P < .0001$) (Figure 2*G*) and without ($r = 0.73$; 95% CI, 0.48–0.87; $P < .0001$) the gluten challenge (Figure 2*H*). A positive WBAIL-2 ($\geq$2-fold change over control) was detected with as few as 1 tetramer$^+\beta7^+T_{EM}$ cell per million CD4$^+$ T cells, equivalent to 1 cell in 4 mL of blood. WBAIL-2 also correlated with CD38$^+$ tetramer$^+\beta7^+T_{EM}$ cells ($r = 0.62$, $P = .0005$) (Figure 2*I*), representing activated gluten-specific T cells.

In Vivo Gluten Challenge Increases the Frequency of Tetramer$^+\beta7^+$T-Effector Memory Cells and the Whole-Blood Assay Measuring Interleukin 2 Release Response

WBAIL-2 and tetramer frequency were assessed at baseline (day 1) and day 6 after an oral gluten challenge in treated CeD. The gluten challenge expands gut-homing gluten-specific T cells in the blood by day 6.[11,12,16,25,26] The WBAIL-2 increased by a median of 3-fold on day 6 compared with baseline, with IL2 fold changes rising from a median of 7.8 on day 1 to 34.1 on day 6 ($P = .0013$) (Figure 3*A*). Two CeD participants with negative WBAIL-2 results on day 1 had positive responses on day 6, indicating gluten-induced expansion of gluten-specific T cells enhances in vitro IL2 production. The oral gluten challenge increased absolute IL2 concentrations in vitro by a median of 2.5-fold, from 1.5 pg/mL on day 1 to 7.4 pg/mL on day 6 ($P < .0001$) (Figure 3*B*). Similarly, the frequency of tetramer$^+\beta7^+T_{EM}$ cells increased by a median of 2.4-fold, from 2.2 on day 1 to 8.7 on day 6 ($P = .0098$) (Figure 3*C*). CD38$^+$tetramer$^+\beta7^+T_{EM}$ cells, indicative of recent gluten-induced activation,[16] rose from 0.12 on day 1 to 6.8 on day 6 ($P = .004$) (Figure 3*D*). One participant showed a decrease in tetramer$^+\beta7^+T_{EM}$ cells on day 6, consistent with their WBAIL-2 response. The percentage of tetramer$^+\beta7^+T_{EM}$ cells expressing CD38 increased from 3.6% on day 1 to 76.6% on day 6 ($P = .0098$) (Figure 3*E*). These findings demonstrate that the WBAIL-2 reflects the expansion of circulating gluten-specific T cells after a gluten challenge.

Figure 1. WBAIL-2 diagnostic performance. (*A*) IL2 fold change and (*B*) IL2 concentration in treated CeD (n = 75), active CeD (n = 13), healthy controls (n = 61) and nonceliac gluten sensitivity (NCGS; n = 32). (*C*) Receiver operating characteristic curves are shown for IL2 fold change and (*D*) concentration for treated CeD compared with NCGS and healthy (*green*) or compared with NCGS alone (*blue*). Performance of combined cutoffs using both IL2 fold change and concentration in (*E*) CeD and (*F*) control cohorts. (*G*) IL2 fold change in treated CeD based on HLA type and zygosity. (*H*) IL2 fold change in non-CeD controls stratified by presence of a first-degree relative (FDR) with CeD. IL2 fold change in (*I*) CeD and (*J*) non-CeD cohorts stratified by the presence or absence of autoimmune diseases (AID) other than CeD. IL2 fold change correlation with (*K*) years consuming a GFD and (*L*) age. The cutoffs determined by the receiver operating characteristic curves are shown by the *dashed lines*. Negative IL2 concentrations were set at 0.01 to allow graphing on a $\log_{10}$ scale. Median and 95% CIs (*shaded area*) are shown. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

Table 2. Participants With Discordant Whole-Blood Assay Measuring Interleukin 2 Release Responses

Participant	Baseline WBA	Repeat baseline WBA	Day 6 after challenge WBA	GCIL-2	HLA	Notes
Active CeD						
1	Negative	Positive[a]			2.5/8	Baseline WBA close to cutoff (1.93-fold change, 2.14 pg/mL)
2	Negative	Positive[a]			2.5/x	Baseline WBA close to cutoff (2.72-fold change, 0.24 pg/mL)
Treated CeD						
1	Negative				8/x	No follow-up tests due to death
2	Negative				8/x	Previously responded to rye and barley on ELISpot but not wheat
3	Negative	Positive	Positive	Positive	8/8	Baseline WBA high control IL2 (0.75 pg/mL) compared with positive repeat WBA (0.22 pg/mL)
4	Negative	Negative			8/x	High control IL2 in baseline (7.9 pg/mL) and repeat WBA (35.4 pg/mL)
5	Negative	Negative	Positive	Positive	2.5/x	
6	Negative	Negative	Positive	Positive	2.5/x	
7	Negative	Positive	Positive	Positive	2.5/x	Baseline WBA high control IL2 (2.4 pg/mL) compared with positive repeat WBA (0.16 pg/mL)
8	Negative	Positive	Positive	Positive	2.5/2.2	Baseline WBA high control IL2 (0.35 pg/mL) compared with positive repeat WBA (0.08 pg/mL)
9	Negative	Negative	Negative	Negative	2.5/2.5	In clinical trial on α-melanocyte–stimulating hormone for type 1 diabetes
10	Negative	Negative				No gluten challenge as pregnant
Healthy						
1	Positive	Positive	Positive	Negative	2.2/x	FDR, negative serology while eating gluten (2024)
2	Positive				2.5/x	FDR, negative serology and histology while eating gluten (2024)
NCGS						
1	Positive	Positive		Negative	7/x	FDR, refused gluten challenge
2	Positive	Negative	Negative	Negative	8/x	FDR
3	Positive	Positive		Negative	7/x	Refused gluten challenge

FDR, first degree relative; NCGS, nonceliac gluten sensitive.
[a]Repeat baseline WBAIL-2 done after 1 week on GFD for active CeD.

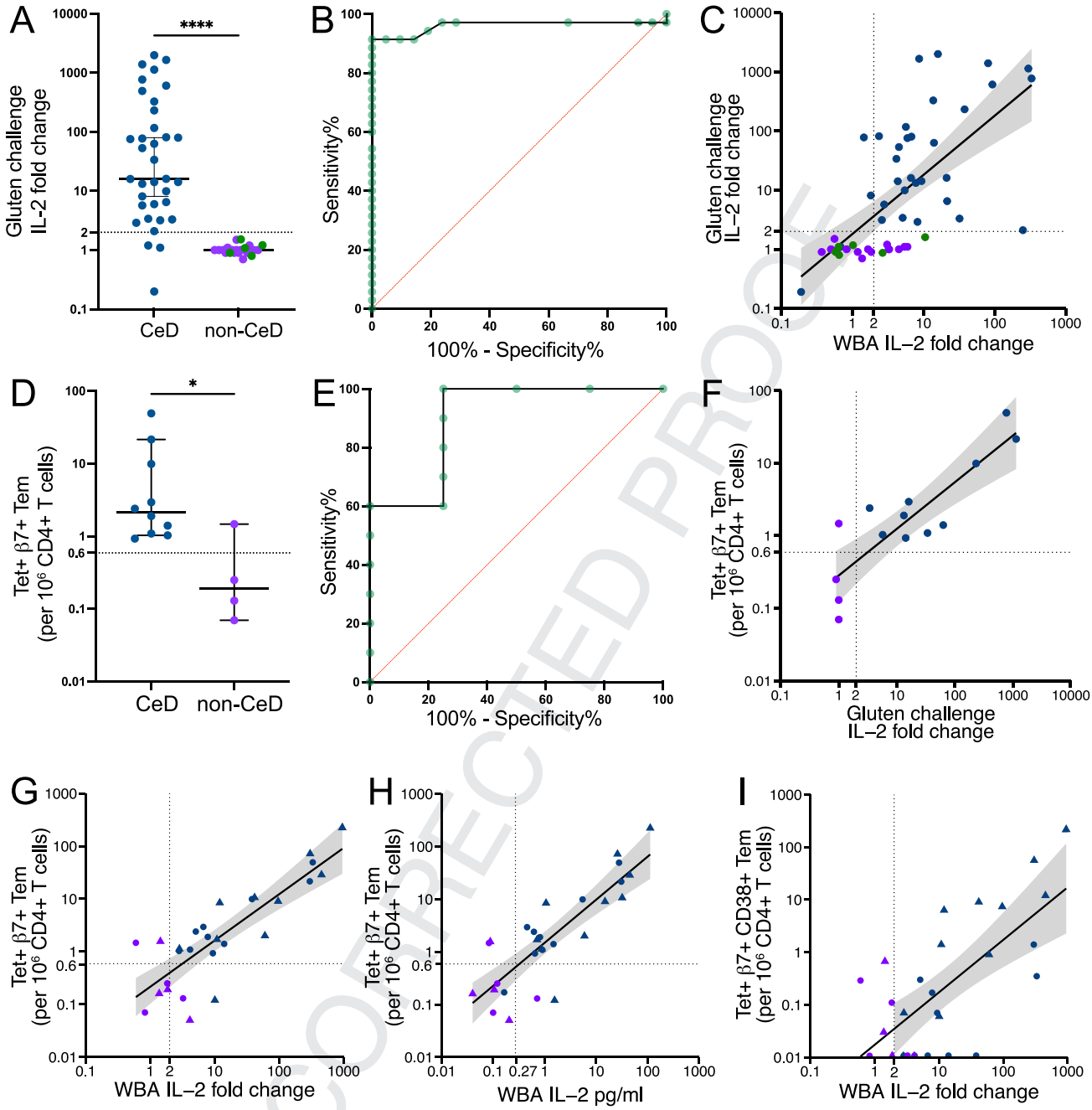

Figure 2. Comparison of WBAIL-2 with GCIL-2 and tetramer frequency. (*A*) GCIL-2 calculated as the fold change in IL2 between baseline and 4 hours after the gluten challenge is shown for CeD (n = 35) and non-CeD cohorts (n = 21). (*B*) Receiver operating characteristic curve is shown for the GCIL-2. (*C*) GCIL-2 responses are plotted against IL-2 WBA responses (calculated as fold change between peptide and control tube). The cutoffs for a positive GCIL-2 and WBA (2-fold change) are indicated by the *dotted lines*. (*D*) The number of tetramer$^+\beta7^+T_{EM}$ T cells per million CD4$^+$ T cells are shown for CeD (n = 10) and non-CeD cohorts (n = 4). (*E*) Receiver operating characteristic curve is shown for the frequency of tetramer$^+\beta7^+T_{EM}$ T cells per million CD4$^+$ T cells. The frequency of tetramer$^+\beta7^+T_{EM}$ T cells per million CD4$^+$ T cells is plotted against (*F*) GCIL-2 fold change, (*G*) WBAIL-2 fold change, and (*H*) WBAIL-2 concentration (pg/mL). (*I*) Frequency of CD38$^+$ tetramer$^+\beta7^+T_{EM}$ T cells per million CD4$^+$ T cells is plotted against the WBAIL-2 fold change. Treated CeD are shown in *blue*, NCGS in *purple*, and healthy participants in *green*, and baseline frequencies are indicated by *circles*, and frequencies on day 6 after the gluten challenge are indicated by *triangles*. Median and 95% CIs (*shaded area*) are shown. *$P < .05$, ****$P < .0001$.

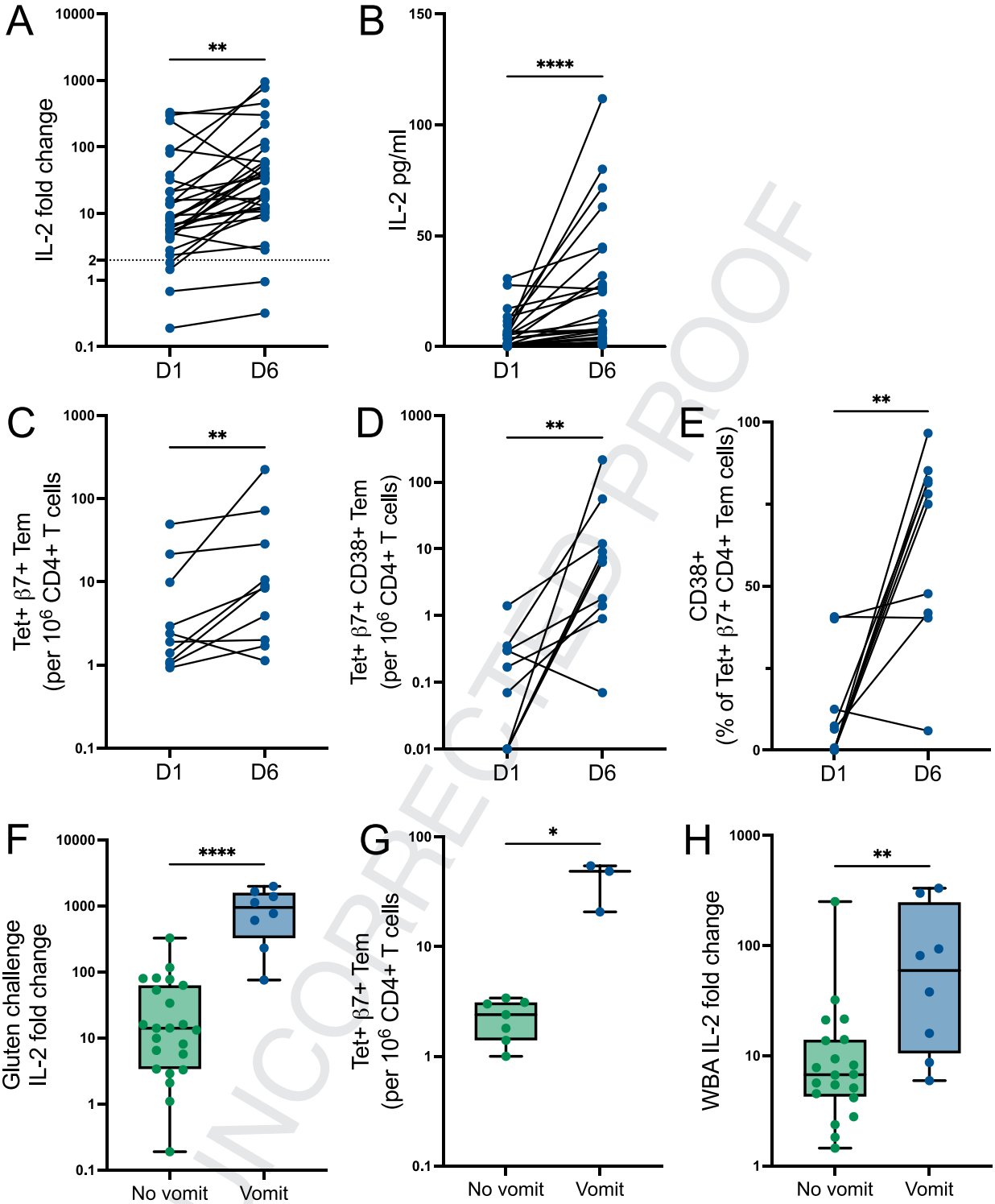

Figure 3. WBAIL-2 responses and tetramer frequencies reflect gluten challenge–induced T-cell expansion and symptoms in treated celiac disease. (*A*) WBAIL-2 fold change, (*B*) WBAIL-2 concentration (pg/mL), (*C*) frequency of tetramer$^+\beta7^+T_{EM}$ T cells per million CD4$^+$ T cells, (*D*) frequency of CD38$^+$ tetramer$^+\beta7^+T_{EM}$ T cells per million CD4$^+$ T cells, and (*E*) percentage of tetramer$^+\beta7^+T_{EM}$ T cells expressing CD38 are shown before (day 1; D1) and after (day 6; D6) the gluten challenge in treated CeD participants (n = 31 for WBAIL-2 and n = 10 for tetramer). Treated CeD participants were stratified based on whether or not they vomited after the oral gluten challenge. (*F*) GCIL-2 is shown, and both (*G*) frequency of tetramer$^+\beta7^+T_{EM}$ T cells per million CD4$^+$ T cells, and (*H*) IL2 WBA performed before the gluten challenge are shown. Box and whisker plot: The *boxes* indicate the 25th percentile (*bottom border*), median (*center line*), and 75th percentile (*top border*), the *whiskers* show the maximum and minimum ranges, and the *circles* indicate outliers. *$P < .05$, **$P < .01$, ****$P < .0001$.

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In Vivo and In Vitro Interleukin 2 Responses and Tetramer Frequency Correlate With Patient Symptoms

The relationship between gluten-induced symptoms and gluten-specific T-cell responses was assessed in 31 treated CeD participants. The most common symptoms after gluten exposure were tiredness (55%; mean severity, 4.5 of 10), nausea (55%; mean severity, 5.1 of 10), and headache (52%; mean severity, 3.7 of 10), with peak symptoms most commonly occurring at 3 to 4 hours (symptom heat map, Supplementary Figure 3). Severe symptoms (≥7 of 10) were reported by 9 participants (29%), 7 of whom experienced vomiting. Vomiting participants had significantly higher gluten-specific T-cell responses: GCIL-2 was 60-fold higher, with a median 949-fold change compared with 14-fold in nonvomiting participants ($P < .0001$) (Figure 3*F*). Tetramer$^+\beta7^+T_{EM}$ cell frequency was 20-fold higher, with a median of 48.5 compared with 2.4 ($P = .017$) (Figure 3*G*), and WBAIL-2 was 10-fold higher, with a median 60-fold change compared with 6.7-fold ($P = .004$) (Figure 3*H*). No consistent relationship was observed between other symptoms and T-cell responses, although notably, 1 nonvomiting participant with a >100-fold WBAIL-2 increase reported severe tiredness (Figure 3*H*). These findings suggest that the magnitude of WBAIL-2 responses at baseline can identify treated CeD participants likely to experience severe symptoms after gluten exposure.

Gluten-Specific Cluster of Differentiation 4$^+$ T Cells Are Responsible for Gluten-Induced Interleukin 2 Production

To confirm acute IL2 production is a biologically relevant marker of pathogenic gluten-specific T cells, a cytokine capture assay was performed using PBMCs from treated CeD participants after the gluten challenge (Figure 4*A*). From 125 to 205 million starting PBMCs, a median of 323 tetramer$^+\beta7^+T_{EM}$ T cells was detected, with 64.7% (median, 209 cells) expressing CD69 (Figure 4*B*). The cytokine profile of CD69$^+$tetramer$^+\beta7^+T_{EM}$ T cells varied: 1 participant had more IL2$^+$IFN-γ^+ cells (38.9%) than IL2$^-$IFN-γ^+ cells (29.1%), and the other 2 had predominantly IL2$^-$IFN-γ^+ cells (61.7% and 28.7%) compared with IL2$^+$IFN-γ^+ cells (12.4% and 16.6%, respectively) (Figure 4*C*). All participants had few IL2$^+$IFN-γ^- cells (median 4.97%). Higher frequencies of IL2$^+$ cells correlated with higher WBA IL2 fold changes (Figure 4*D*). A similar trend was observed for IFN-γ, although 1 participant had a high frequency of IFN-γ^+ cells but low IFN-γ fold change, likely due to using fresh PBMCs instead of frozen, which may affect cytokine secretion. Shorter secretion periods favor IL2 capture, whereas longer periods favor IFN-γ. No IL2 was detected from naïve, central memory, or CD8$^+$tetramer$^+\beta7^+$ T cells (data not shown). These findings confirm that activated gluten-specific CD4$^+$ T cells drive gluten-induced IL2 production.

Discussion

Accurate blood-based T-cell diagnostics to support clinical practice are highly attractive, but none are used routinely outside of the QuantiFERON-Gold (Labcorp) assay for tuberculosis. CeD is an ideal candidate for T-cell diagnostics due to the central role of gluten-specific CD4$^+$ T cells in its pathogenesis and the well-defined immunodominant gluten epitopes driving them.[8] The field is increasingly shifting toward an immune-based diagnosis of CeD[5,27,28] with increased acceptance of an immune (serology) non–biopsy specimen–based diagnosis when transglutaminase-IgA is 10 times the upper limit of normal in both children[29,30] and, increasingly, adults.[31] However, serology has limited positive-predictive value in average-risk patients and is uninformative for those avoiding gluten.[3]

Our findings show that the WBAIL-2 provides a direct measure of pathogenic gluten-specific T cells and offers high accuracy for diagnosing CeD, even in patients adhering to a strict GFD. The assay performed optimally in patients with the common HLA-DQ2.5 genotype, present in >85% of CeD cases.[32] We confirm that IL2 production in vitro correlates with serum IL2 and the circulating frequency of gluten-specific T cells before and after gluten exposure. Strong WBAIL-2 responses predicted severe symptoms, such as vomiting, even before gluten ingestion. Importantly, we show that IL2 is produced by tetramer$^+$ gluten-specific T cells, highlighting the biological relevance of this diagnostic approach.

Detection of gluten-specific T cells is challenging due to their low frequency (∼1 per 100,000 CD4$^+$ T cells in treated CeD and negligible in individuals without CeD).[10,16] To date, this has only been possible using HLA-DQ-gluten tetramer–based assays[10] or prior expansion using a 3-day gluten challenge coupled with T-cell ELISpot or enzyme-linked immunosorbent assays.[11,33] Although the tetramer-based approach incorporating 5 T-cell epitopes (HLA-DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1, DQ2.5-glia-ω2, and DQ2.5-hor-3) has excellent sensitivity (97%) and specificity (95%) for CeD, the requirement for large blood volumes (30 to ≥50 mL), knowledge of patient HLA genotype, technical expertise, and access to flow cytometry, makes this approach impractical for clinical translation.[10,16] Further, approaches using a gluten challenge are less appealing to patients. The WBAIL-2 has similar sensitivity and specificity to these approaches but avoids the gluten challenge, is simple to perform, and requires limited blood (4 mL per tube).

In our community study, the WBAIL-2 demonstrated high sensitivity and specificity for the detection of CeD. The findings confirm the accuracy of the WBAIL-2 in a simple tube-based format. Variability (mean CV, 18%–46%) was similar to the QuantiFERON-Gold assay (mean CV, 13%–30%),[34] and likely reflects the challenges of detecting gluten-specific T cells that are circulating in low frequencies. Although GCIL-2 offered optimal diagnostic performance, the WBAIL-2 required only 8 mL of blood and no gluten challenge. We showed that priming with oral gluten

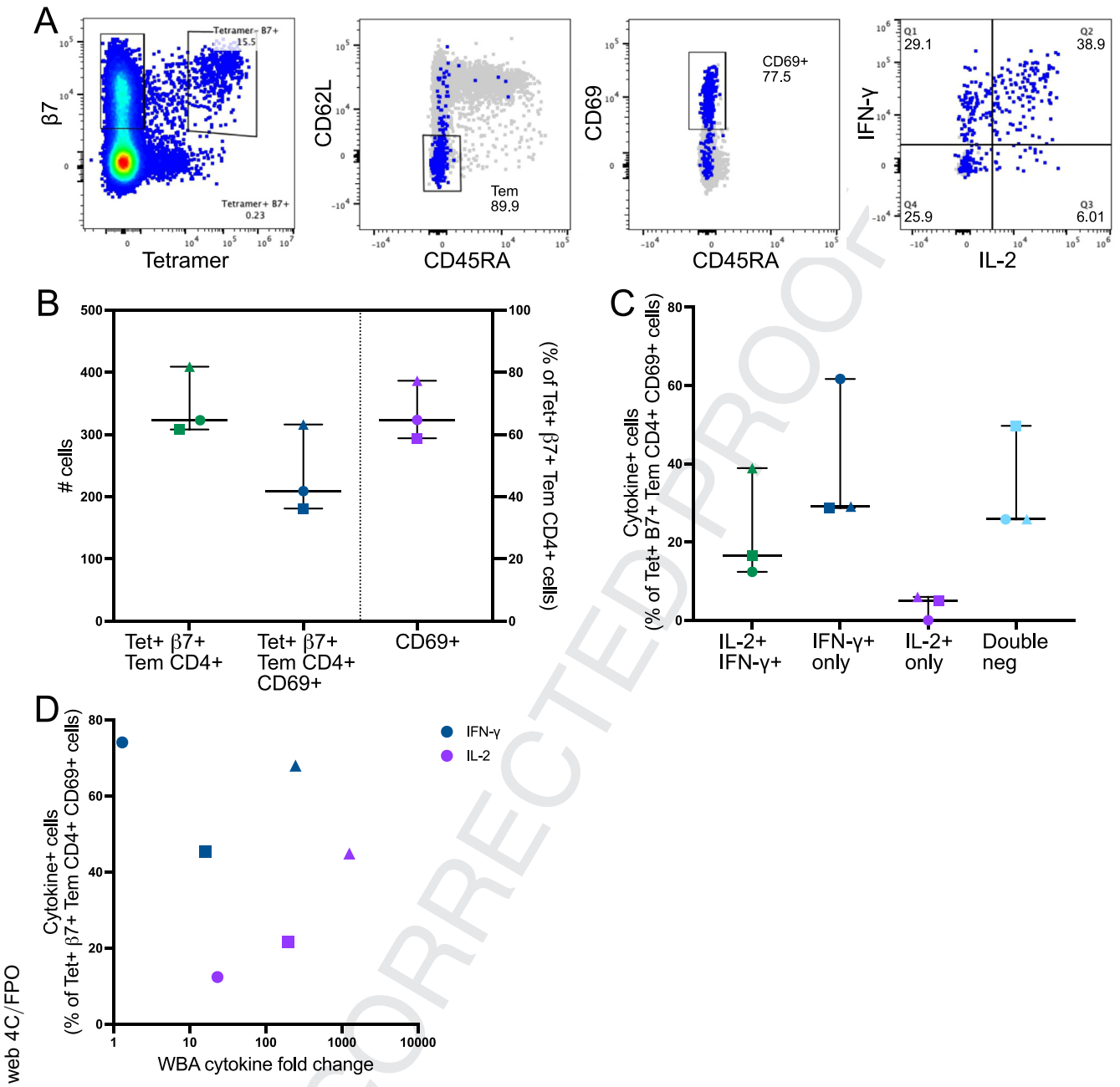

Figure 4. IL2 and IFN-γ production by gluten-specific $CD4^+$ T cells. (*A*) Gating strategy for IL2- and IFN-γ–producing cells gated on $tetramer^+\beta7^+$ cells in *blue* and $tetramer^-\beta7^+$ in grey, $CD45RA^-CD62L^-T_{EM}$, and activated $CD69^+$ cells, 1 representative of 3 similar experiments. (*B*) The number of $tetramer^+\beta7^+T_{EM}$ cells and $CD69^+tetramer^+\beta7^+T_{EM}$ cells (*left y-axis*) and the frequency of $tetramer^+\beta7^+T_{EM}$ T cells that express CD69 (*right y-axis*). (*C*) IL2 and IFN-γ double positives, single positives, and double negatives as a frequency of $CD69^+$ $tetramer^+\beta7^+T_{EM}$ cells. (*D*) Frequency of $CD69^+$ $tetramer^+\beta7^+T_{EM}$ cells that express IFN-γ (*blue*) or IL2 (*purple*) are plotted against the fold change in the respective cytokine from a WBA. Median and 95% CIs are shown. Each shape represents a different patient. Q20

enhances the sensitivity of the WBAIL-2 by expanding the gluten-specific T-cell pool.

The lower sensitivity of the WBAIL-2 for $HLA\text{-}DQ8^+$ CeD may have been impacted by the small sample size for this less common genotype, and another possibility is that HLA-DQ8–restricted responses in some CeD patients were below the assay's detection limit. Indeed, we previously showed that serum IL2 responses after an oral gluten challenge in $HLA\text{-}DQ8^+$ CeD is lower than $HLA\text{-}DQ2.5^+$ CeD, suggesting that HLA type can impact the T-cell response.[18] Further, there may be other HLA-DQ8–restricted peptides that could enhance WBAIL-2 sensitivity, although detailed characterization of HLA-DQ8–restricted immunodominant epitopes is considerably more limited than in $HLA\text{-}DQ2.5^+$ CeD.[8]

Prospective studies in larger populations are needed, both to better understand WBAIL-2 performance in HLA-

DQ8$^+$ CeD and also inform the optimal testing algorithm for this assay. The WBAIL-2 could potentially be used as a confirmatory diagnostic in lieu of gastroscopy or as a first-line test in people consuming a GFD. The role of a single-dose gluten challenge to measure serum IL2 or boost WBAIL-2 responses to clarify "equivocal" WBAIL-2 results also warrants investigation.

Confirming published work, we show a single-dose gluten challenge triggers a rise in serum IL2 within hours, with high sensitivity (97%) and specificity (100%) for CeD, and an expansion of circulating CD38$^+$ activated gluten-specific CD4+ T cells within days.[14,16] Although WBAIL-2 closely correlates with GCIL-2 responses, some discordant results occur, with positive GCIL-2 but negative WBAIL-2, or vice versa. This may reflect differences in the assays: GCIL-2 measures systemic IL2 responses to ingested wheat gluten, where a broad range of polyclonal gluten-specific T cells have been activated, whereas WBAIL-2 uses in vitro stimulation with a small number of deamidated gluten peptides, targeting a narrower T-cell repertoire in a low blood volume that may only contain a few gluten-specific T cells.

This study is the first to demonstrate that the gluten-specific T cell is responsible for gluten-induced IL2 production, and it does this generally alongside IFN-γ production. These T cells fall into 3 functional subsets: IL2$^+$IFN-γ^-, IFN-γ^+IL2$^-$, and IFN-γ^+IL2$^+$, with the IFN-γ^+IL2$^-$ subset being most common in 2 of 3 participants. However, IL2$^+$IFN-γ^- cells may be underestimated due to rapid IL2 internalization,[35] and whether cytokine levels reflect a few highly active T cells or many less active ones remains unclear. Future work should examine how gluten-specific T cells shift between functional states over time and in relation to acute gluten exposure. These data confirm that gluten-induced IL2 in the WBAIL-2 and GCIL-2 directly reflects the activation of pathogenic gluten-specific CD4$^+$ T cells, confirming its biological relevance as a diagnostic biomarker in CeD.

We confirmed that higher GCIL-2 levels correlate with more severe symptoms[14,36] and extended this to show that prechallenge WBAIL-2 responses and tetramer$^+$ T-cell frequency predict gluten-induced vomiting. This suggests WBAIL-2 could predict symptom severity without an oral gluten challenge, aiding in selecting symptomatic patients for therapy trials where symptoms are key end points. Another advantage of the WBAIL-2 is monitoring the functional status of gluten-specific T cells without the need for a gluten challenge, which can be counterproductive and undesirable in the trial setting.

This study has several limitations. First, the single-center design and rigorous inclusion criteria limit the generalizability of the findings to diverse real-world patient populations, such as those taking systemic immunosuppression.

Second, the sample size for certain subgroups, particularly patients with less common non–HLA-DQ2.5 HLA genotypes, was small, reducing the robustness of the conclusions drawn for these populations.

Third, although the WBAIL-2 demonstrated promising results, its reproducibility across different laboratories was not assessed, which is critical for its adoption in routine clinical practice.

Fourth, the study also does not address the cost-effectiveness of implementing the WBAIL-2 compared with current diagnostic methods, an important consideration for real-world applicability. Studies assessing IL2-based T-cell diagnostics in children are also needed, especially as we showed WBAIL-2 responses correlated positively with older participant age and previously showed serum IL2 responses to oral gluten may be lower in younger patients with CeD.[18] Future prospective validation studies with multiple centers and a larger patient numbers, including those with non–HLA-DQ2.5 genotypes, will provide important data to validate the diagnostic performance of the WBAIL-2 in different clinical settings.

Using the WBAIL-2 approach, our findings highlight the potential for practical, blood-based T-cell diagnostics to extend beyond infectious diseases into autoimmunity. The ultrasensitive electrochemiluminescent platform is well-suited to clinical laboratories due to its compact design, minimal setup requirements, and automation that reduces the need for extensive technical training. Its high sensitivity, broad dynamic range, and capability to analyze various sample types, including serum and plasma, make it a practical choice for diagnostic purposes. Such a platform could be adapted for a variety of T cell–driven diseases and conditions, including type 1 diabetes, malignancies, transplant immunology, and other infectious diseases, whenever circulating antigen-specific T cells are likely to be present, even at low frequencies. Future studies should explore how whole-blood cytokine release assays, combined with ultrasensitive cytokine detection, can further advance T-cell diagnostics in these contexts, improving early detection, disease monitoring, and therapeutic stratification.

Conclusion

Our study indicates that gluten-stimulated IL2 release assays are practical and correlate with the presence of a pathogenic gluten-specific CD4$^+$ T-cell response in CeD. Availability of complementary in vivo and in vitro IL2 release assays in CeD could address the diagnostic needs of clinicians managing patients with an uncertain diagnosis while avoiding a prolonged gluten rechallenge, children and adults unsuitable or unwilling to undergo endoscopy for histologic diagnosis, and provides a biomarker for stratifying disease severity by the "strength" of antigluten immunity. In immune conditions with T cells specific for well-defined antigens, in-tube WBAIL-2 release assays could be feasible and facilitate personalized diagnosis and therapy previously not possible in clinical care.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://dx.doi.org/10.1053/j.gastro.2025.05.022

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Conflicts of interest
These authors disclose the following: Melinda Y. Hardy is a consultant for Takeda. Robert P. Anderson is an advisor to Immunic, Takeda Pharmaceutical, AMYRA Biotech, DBV Technologies, Barinthus Biotherapeutics, EVOQ Therapeutics, Pecigen ActoBio, Allero Therapeutics, Kanyos Bio, GSK, Treg Therapeutics, Bioniz Therapeutics, AdAlta, Universal Cells, and Bristol-Myers Squibb Australia; is a director and shareholder of Novoviah Pharmaceuticals; and is inventor of patents relating to diagnosis and treatment of celiac disease. Jason Allan Tye-Din has privately or via his institution been a consultant or advisory board member for Anatara, Anokion, Barinthus Biotherapeutics, Chugai Pharmaceuticals, DBV Technologies, Dr. Falk, EVOQ Therapeutics, Equillium, Forte Biosciences, IM Therapeutics, Janssen, Kallyope, Mozart Therapeutics, Takeda, TEVA, and Topas, has received research funding from Barinthus Biotherapeutics, Chugai Pharmaceuticals, Codexis, DBV Technologies, Kallyope, Novoviah Pharmaceuticals, Topas, and Tillotts Pharmaceuticals, and is an inventor on patents relating to the use of gluten peptides in celiac disease diagnosis and treatment. The remaining authors disclose no conflicts.

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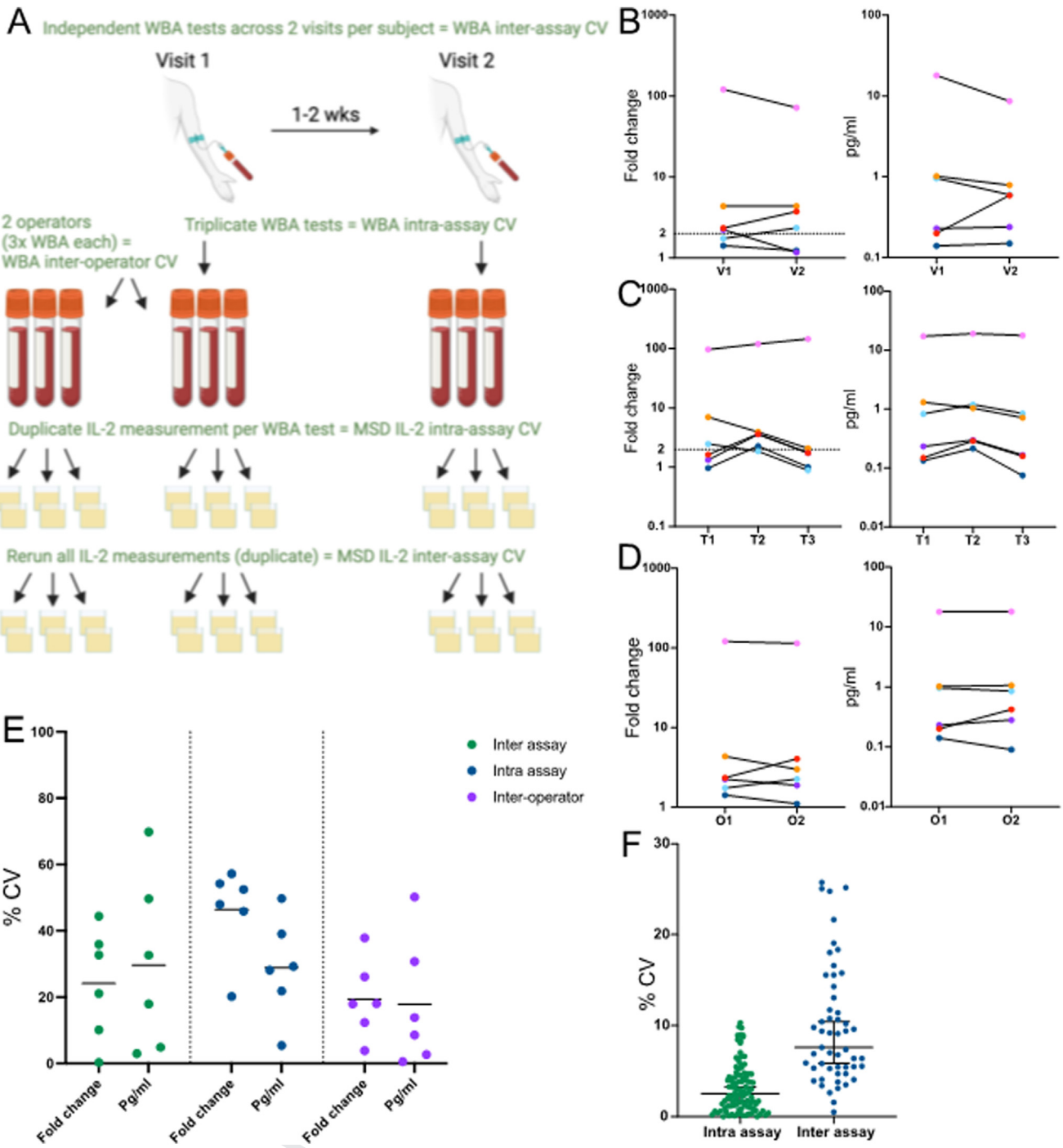
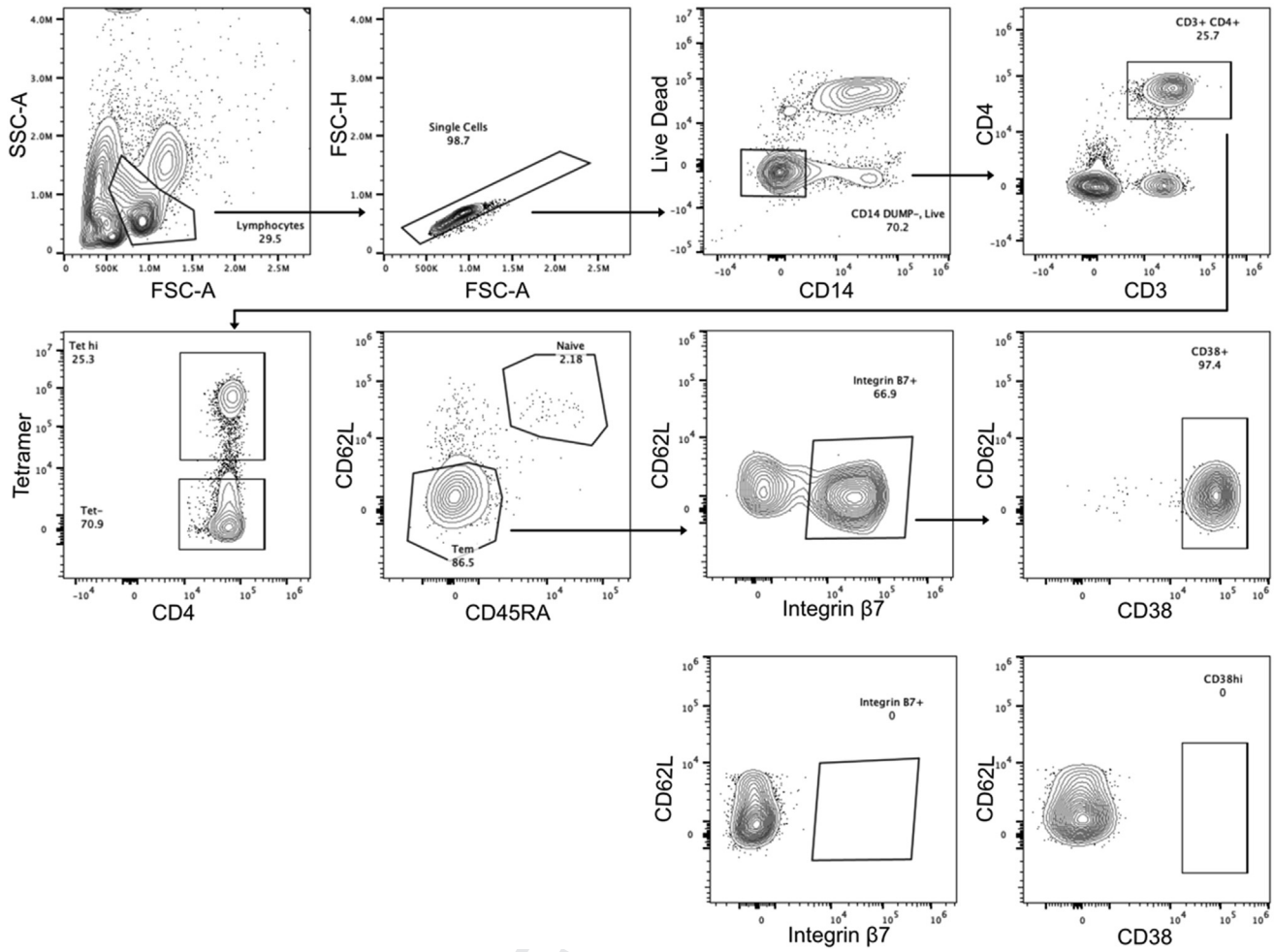
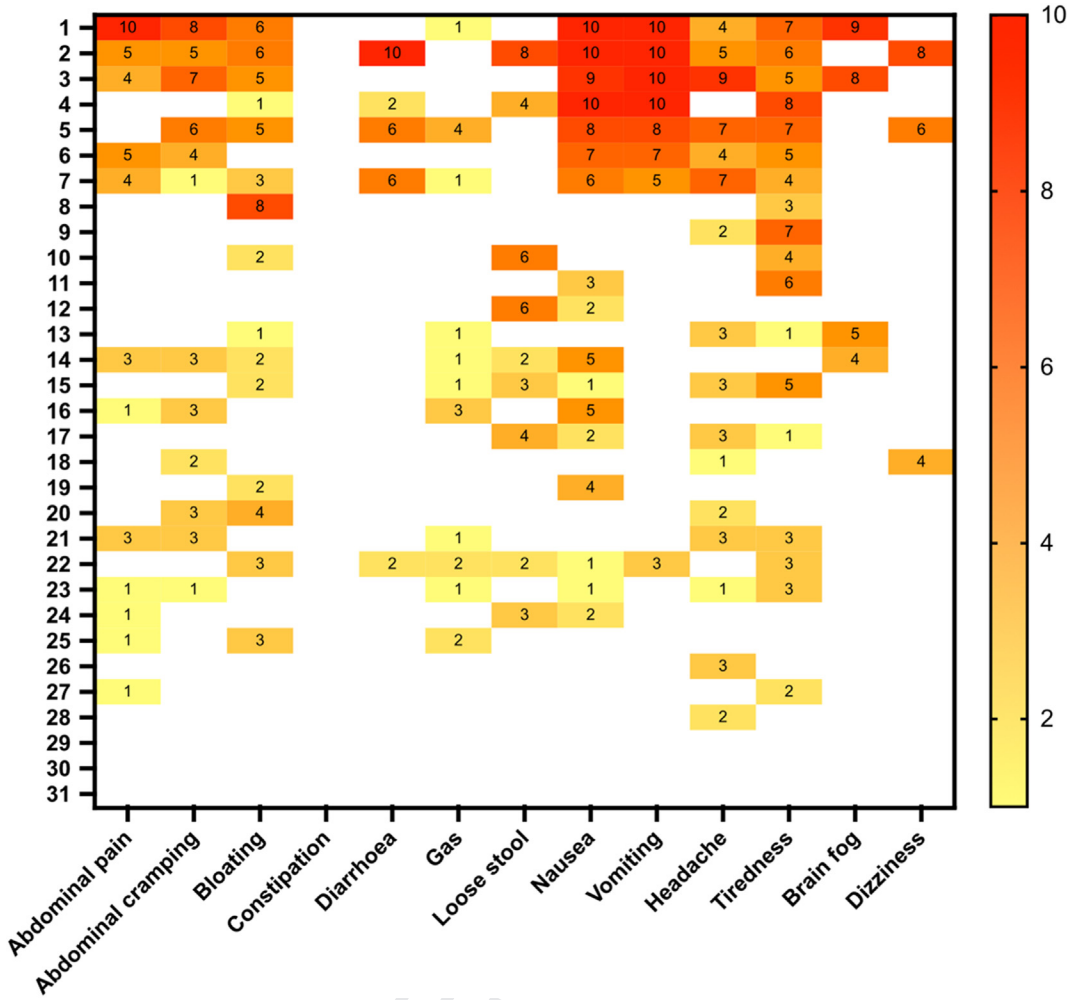
Data Availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.



Supplementary Figure 1. Evaluation of interassay and intra-assay variability of the WBAIL-2 and the MSD IL2 S-PLEX. (*A*) Experimental design for assessment of interassay and intra-assay and interoperator variability. IL2 fold change (*left*) and concentration (pg/mL; *right*) are shown for (*B*) interassay replicates at visit 1 and 2, (*C*) intra-assay replicates for tests 1, 2 and 3, and (*D*) interoperator replicates for operators 1 and 2 for the IL2 WBA. (*E*) Percentage CVs are shown for fold change and pg/mL for interassay, intra-assay, and interoperator variability of the IL2 WBA. (*F*) Percentage CVs are shown for intra-assay and interassay variability of the MSD IL2 S-PLEX.



Supplementary Figure 2. Tetramer gating strategy. Example gating strategy of the tetramer-enriched sample. Shown is 1 representative treated CeD patient on day 6 after the gluten challenge. Firstly, cleanup gates were applied to gate lymphocytes, single cells, live $CD14^-$ cells and $CD3^+CD4^+$ cells (*top row*). FSC-A, forward side scatter area; SSC-A, side scatter area. Next, $tetramer^+$ cells were gated on, followed by effector memory cells identified as $CD45RA^-CD62L^-$, then integrin $\beta7^+$ cells, and lastly, CD38hi cells (*middle row*). Integrin $\beta7^+$ and $CD38^+$ gates are shown for respective fluorescence minus one samples (*bottom row*).



Supplementary Figure 3. Heat map of gluten-induced symptoms. Heat map of patient-reported symptom scores after gluten challenge in treated CeD participants (n = 31). Each *row* represents 1 participant. Maximum reported severity scores are shown, ranging from 0 (no symptoms; *white*) to 10 (severe symptoms; *red*).

Supplementary Table 1. Participant Screening, Inclusion and Exclusion Numbers

Variable	Treated CeD (n)	Active CeD (n)	Healthy (n)	NCGS (n)	Total (n)
Screened	96	13	66	45	220
Included	75	13	61	32	181
Excluded	21	0	5	13	39
Reason for exclusion					
Use of immunosuppressants	5	0	2	1	8
Uncertain diagnosis[a]	12	0	1	8	21
Laboratory technical issues	1	0	2	1	4
Nonadherent with GFD	3	0	0	3	6

NCGS, nonceliac gluten sensitivity.
[a]Uncertain diagnosis includes insufficient documentation to confirm the diagnosis, diagnosis on biopsy or serology alone and serology/biopsy specimen testing performed while the patient was consuming a GFD.

Supplementary Table 2. Additional Cytokine Analysis Performed on the Whole-Blood Analysis Plasma

	Correlation with IL2				Diagnostic performance					
Variable	pg/mL		Fold change		All participants (N = 69)			Participants with IL-2 WBA consistent with diagnosis (n = 58)		
Cytokine	Spearman's *r*	*P* value	Spearman's *r*	*P* value	AUC	95% CI	*P* value	AUC	95% CI	*P* value
TNF-α	0.257	.033	0.348	.003	0.58	0.44-0.71	.287	0.64	0.50-0.79	.066
IL6	0.293	.015	0.261	.030	0.62	0.49-0.76	.090	0.66	0.52-0.80	.041
IL10	0.218	.072	0.225	.063	0.55	0.41-0.69	.492	0.61	0.46-0.76	.157
IFN-γ	0.684	9.66^{-11}	0.706	1.27^{-11}	0.75	064-0.86	.0005	0.87	0.77-0.96	<.0001
IL17A	0.683	1.02^{-10}	0.595	6.80^{-08}	0.73	0.61-0.85	.0016	0.82	0.71-0.93	<.0001