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SERUM CYTOKINES ELEVATED DURING GLUTEN-MEDIATED CYTOKINE RELEASE IN COELIAC DISEASE

Short Title: Gluten-mediated cytokine release

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Key words: Coeliac disease, gluten, cytokines, cytokine release syndrome, IL-2, IL-17

Abbreviations: Celiac Disease Patient Reported Outcome tool (CeD PRO), human leukocyte antigen-DQ (HLA-DQ), immunoglobulin (Ig), interleukin (IL), interferon (IFN), patient reported outcome (PRO), tumor necrosis factor (TNF), helper T cell (Th).

ABSTRACT

Background: Cytokines have been extensively studied in coeliac disease, but cytokine release related to exposure to gluten and associated symptoms has only recently been described. Prominent, early elevations in serum IL-2 after gluten support a central role for T-cell activation in the clinical reactions to gluten in coeliac disease.

Aims: Establish a quantitative hierarchy of serum cytokines and their relation to symptoms in patients with coeliac disease during gluten-mediated cytokine release reactions

Methods: Sera were analyzed from coeliac disease patients on gluten free diet (n=25), and from a parallel cohort of healthy volunteers (n=25) who underwent an unmasked gluten challenge. Sera were collected at baseline, and 2, 4, and 6 hours after consuming ten grams vital wheat gluten flour. 187 cytokines were assessed. Confirmatory analyses were performed by high sensitivity electrochemiluminescence immunoassay. Cytokine elevations were correlated with symptoms.

Results: Cytokine release following gluten challenge in coeliac disease patients included significant elevations of IL-2, CCL20, IL-6, CXCL9, CXCL8, IFN-γ, IL-10, IL-22, IL-17A, TNF-α, CCL2, and amphiregulin. IL-2 and IL-17A were earliest to rise. Peak levels of cytokines were generally at four hours. IL-2 increased most (median 57-fold), then CCL20 (median 10-fold). Cytokine changes were strongly correlated with one another, and the most severely symptomatic patients had highest elevations.

Conclusions: Early elevations of IL-2, IL-17A, IL-22 and IFN-γ after gluten in patients with coeliac disease implicates rapidly activated T cells as their likely source. Cytokine release after gluten could aid in monitoring experimental treatments and support diagnosis.

INTRODUCTION

Circulating levels of IL-2 are elevated as early as two hours after gluten food challenge in patients with coeliac disease on gluten free diet, and are closely linked to the onset of gastrointestinal symptoms (1). A closely related systemic cytokine release phenomenon also occurs when patients with coeliac disease receive an intradermal injection of short,

deamidated gluten peptides corresponding to immunodominant epitopes for gluten-specific CD4+ T cells (1). Gluten food challenge also elevates the frequencies of gut-homing gluten-specific CD4+ T cells in blood six days later that can be detected by using overnight IFN-γ ELISpot assay(2), HLA-DQ2.5-peptide tetramers (3), and by measuring CXCL10 (IFN-γ induced protein-10), IL-2, or IFN-γ in fresh blood incubated with peptides recognized by gluten-specific CD4+ T cells (1, 4). Collectively, these observations link reactivation of gluten immunity to acute symptoms, cytokine release, and subsequent expansion of gluten-reactive CD4+ T cells specifically in patients affected by coeliac disease.

Characterizing the sequence and magnitude of cytokine elevations, and how they relate to clinical manifestations can indicate which parts of and in what order the immune system is engaged. These insights could guide the development of diagnostics combining gluten food challenge with cytokine assessments and aid in monitoring experimental treatments to ameliorate symptoms or modify immunopathology.

In coeliac disease, peptides derived from gluten are known to specifically activate intestinal and peripheral blood CD4+ T cells (2, 5), bind to Ig-A and Ig-G (6), B cells and plasma cells (7), and also activate innate immune cells in vitro (8, 9). Initial studies of cytokines elevated by gluten food challenge in patients with coeliac disease indicated that only IL-2, CXCL8, and IL-10 were elevated out of a panel of 19 cytokines: IFN-γ, IL-1β, IL-2, IL-4, IL-6, CXCL8, IL-10, IL-12p70, IL-13, TNF-α, CCL11 (eotaxin), CCL26 (eotaxin-3), CXCL10, CCL2 (monocyte chemoattractant protein-1), CCL13 (monocyte chemoattractant protein-4), CCL22 (macrophage-derived chemokine), CCL3 (macrophage inflammatory protein-1α), CCL4 (macrophage inflammatory protein-1β), and CCL17 (thymus and activation regulated chemokine) (1). The absence of elevation in IFN-γ was unexpected as most gluten-specific CD4+ T cells in vitro show a pro-inflammatory T helper cell (Th)1 phenotype and secrete IFN-γ (10). Furthermore, no evidence of early innate immune activation preceding T cell activation was observed after gluten food challenge.

Profiling cytokines in cytokine release syndromes has generally relied on conventional ELISAs and bead assays that have generally been insufficiently sensitive to quantify levels of IL-2 and several other T cell-derived cytokines in unstimulated serum and plasma (11-13). The range of cytokines assessed has also been relatively limited. Recent improvements in cytokine assays allow for substantially expanded multiplex screening and significantly improved sensitivity. The present study aimed to provide a detailed, quantitative hierarchy of serum cytokines and their relation to symptoms in patients with coeliac disease when they experienced gluten-mediated cytokine release.

MATERIALS AND METHODS

Participants and study design

The study design is outlined in Figure 1. Sera and clinical data collected in volunteers with coeliac disease during an unmasked gluten food challenge on the first day of screening for the "Nexvax2-1005" study (NCT03543540) were re-assessed along with matching sera and data from an identical unmasked gluten food challenge performed in a parallel study in healthy adults. Truitt et al reported the Nexvax2-1005 study in detail elsewhere, and Tye-Din et al reported a detailed account of symptoms associated with the unmasked food challenge, which included pre-specified IL-2 assessments during gluten challenge that were performed separately from the present study (14, 15). Briefly, the first cohort consisted of all 25 patients diagnosed with coeliac disease who enrolled and completed screening procedures at their first visit. The study was approved by Bellberry Human Research Ethics Committee (Application No: 2017-12-962-A-2). All patients gave written, informed consent prior to undergoing any trial-related procedures. The control cohort consisted of 25 healthy adults aged 18 to 70 years who did not restrict their dietary intake of gluten and were recruited at a separate Australian site according to a protocol that was aligned with the first screening day of the Nexvax2-1005 study. The study was approved by the Human Research Ethics Committee at the Walter and Eliza Hall Institute and Melbourne Health (identifiers 03/4 and 2003.009 respectively). Participants were required to be between 18 and 70 years. Participants in the coeliac disease cohort needed to have a history of medically diagnosed coeliac disease that included assessment of duodenal biopsies, and to

attest to being adherent to a gluten free diet for at least one year. Participants in the control cohort were excluded if their screening IgG specific for deamidated gliadin peptide or IgA specific for transglutaminase 2 was above the normal range, if they had any history of coeliac disease or symptoms attributed to gluten intolerance, or if they avoided food because it contained gluten, wheat, barley, or rye. Volunteers were excluded if they had any medical condition that in the opinion of the investigator would impact the immune response (other than coeliac disease), confound interpretation of study results, pose an increased risk to the patient, or interfere with study conduct. Specific exclusions for enrollment were lactation, pregnancy, refractory coeliac disease, inflammatory bowel disease and/or microscopic colitis, immunomodulatory or immune-suppressing medical treatment within six months, oral or parenteral immunomodulatory corticosteroids within six weeks, or past participation in a clinical study with the experimental immunotherapy Nexvax2.

Gluten challenge

Participants consumed approximately 10 g of vital wheat gluten flour (Manildra Group, Auburn, New South Wales, Australia) mixed thoroughly with 100 ml water. The food challenge material was prepared in individually sealed packets by Rutgers Food Innovation Center (Rutgers University, Bridgeton, New Jersey, USA), and food safety testing confirmed the material was free of enteropathogens (Eurofins Microbiology Laboratories Inc., Lancaster, Pennsylvania, USA). The protein content of the vital wheat flour was 77-79% protein and 5.5-6.4% water. Neogen Corp. (Lansing, Michigan, United States), and according to the Osborne method indicated that approximately 6 g gluten protein would be consumed. The presence of gluten was confirmed by a positive Veratox Gliadin R5-ELISA result, and analysis by Monash University (Melbourne, Victoria, Australia) indicated that exposure to fermentable oligo-, di-, monosaccharides, and polyols was “low”. After the gluten challenge, participants had nothing to eat or drink for 15 minutes, but later could consume gluten-free foods and drinks ad libitum. During the subsequent six-hour observation period patients completed a modified version of the Celiac Disease Patient Reported Outcomes tool (CeD PRO®), and a single-item patient global impression of gastrointestinal symptom severity. Patients responded to the prompt, “Thinking about your worst experience in the past 1 hour, how severe was each of the following symptoms?” A

whole number rating on a 0-to 10 scale was recorded individually for abdominal cramping, bloating, gas, pain, nausea, diarrhea, loose stool, headache and tiredness (0 is absent, and 10 is worst possible). The single-item patient global impression of gastrointestinal symptom severity was recorded on the same 0 to 10 numeric scale as the modified CeD PRO, and in parallel a severity descriptor was selected from one of six options: "A. no symptoms", "B. Very mild symptoms", "C. Mild symptoms", "D. Moderate symptoms", "E. Severe symptoms", or "F. very severe symptoms". Overall severity of gluten reactions was assigned according to the worst severity descriptor rating during the six-hour observation period.

Blood collection and laboratory assessments

Coeliac serology tests (QUANTA Lite® R h-tTG IgA and Gliadin IgA II, INOVA Diagnostics, San Diego, California, USA) were performed by Dorevitch Pathology (Footscray, Victoria, Australia). *HLA-DQA* and *HLA-DQB* alleles were determined using leukocyte-derived DNA from acid citrate dextrose (ACD) whole blood tube. *HLA-DQA* and *HLA-DQB* alleles were determined using leukocyte-derived DNA with a panel of sequence-specific primers (Australian Red Cross, Victorian Transplantation and Immunogenetics Service, Parkville, Victoria, Australia). Blood samples for serum cytokine assessments were collected within one hour before gluten challenge, and at two, four, and six hours afterwards. At each time point, blood was drawn into one 8.5 mL Vacutainer plus plastic Serum Separator Tube (BD #367988, Becton Dickinson, Franklin Lakes, New Jersey, USA) via a 21G butterfly needle or cannula with a tourniquet lightly applied. After collection blood tubes were gently inverted five times, and then set aside upright for 30 minutes at room temperature. Subsequently, samples were centrifuged at 2000 x g for 20 mins, and 1 ml aliquots of serum were withdrawn and frozen within three hours of blood collection. Sera were stored at -60 to -80^{o}C. Olink Proteomics (Uppsala, Sweden) performed proximity extension assays with the 92-plex Proseek® Multiplex Inflammation I panel and 92-plex Proseek® Multiplex Immune Response (v.302) panel using sera collected at baseline and at four hours from all 25 coeliac disease patients and 13 healthy volunteers, including all eight positive for HLA-DQ2.5. Time-to-peak cytokine levels were determined by also assessing two-, and six-hour sera for ten patients in coeliac disease

cohort who experienced more severe clinical reactions (vomiting or severe nausea). Subsequently, the laboratory at ImmusanT, Inc performed high sensitivity electrochemiluminescence immunoassay assays using kits from Meso Scale Discovery (Rockville, Maryland, USA) to confirm the top hits, and to screen biomarkers considered strong candidates but not included (e.g. IL-15) or were negative (e.g. IFN-γ) in proximity extension assays. The assays utilized the following kits: V-PLEX IL-15, R-PLEX CXCL9, V-PLEX Proinflammatory panel (IFN-γ, IL-1β, IL-2, IL-4, IL-6, CXCL8, IL-10, IL-12p70, IL-13, and TNF-α) and V-PLEX Th17 panel (IL-17A Gen. B, IL-21, IL-22, IL-23, IL-27, IL-31, and CCL20) and were performed at ImmusanT, Inc. according to the manufacturer's instructions.

Statistics

The sample size was empirical for this exploratory study. For screening, serum cytokine levels at four hours and baseline for 180 analytes measured by proximity extension assay were compared by Wilcoxon signed-rank test with false discovery rate adjustment by Benjamini-Hochberg method. Confirmatory serum cytokine tests by electrochemiluminescence immunoassay compared serum cytokine levels at their peak time point (previously defined during screening) with baseline by Wilcoxon signed-rank test without correction for multiple comparisons. Cytokine levels were compared as their measured concentration, and as fold-change relative to baseline. Peak serum cytokine levels were compared with each other, and with symptom scores assessed by patient reported outcome surveys were compared by Wilcoxon signed-rank test with false discovery rate adjustment by Benjamini-Hochberg method. (Cytokine) responders were defined as participants with a fold-change in cytokine level >1.2, and >3 standard deviations above mean in controls. There was no missing data.

RESULTS

Participant characteristics

For the 25 participants with coeliac disease, the mean age was 39 years (standard deviation: 16), 88% were women, all were positive for HLA-DQ2.5, and 20% had mild elevations of one of the two coeliac serology tests performed, which is compatible with coeliac disease

and occasional exposure to dietary gluten. The median years since diagnosis of coeliac disease was 4 (1-25), and duration on gluten free diet was 4 (1-20). For the control cohort of 25 volunteers, the mean age was 47 years (standard deviation: 11), 68% were women, and 32% were positive for HLA-DQ2.5. The 13 healthy volunteers, including all eight positive for HLA-DQ2.5, whose sera were used for exploratory analyses had a mean age of 42 years (standard deviation: 11), and 10 (77%) were women. Sera from all 25 patients with coeliac disease, and all 25 healthy controls were used for confirmatory high sensitivity cytokine testing.

Exploratory multiplex cytokine screen

In our previous studies, the most common time for peak levels of cytokines was at four hours after gluten ingestion (1), and this was also true for IL-2 assayed separately in the present study (14). Levels of 180 unique immune and inflammatory biomarkers measured by multiplex proximity extension assays in sera at four hours compared with their baseline levels identified nine that were significantly elevated after correction for multiple comparisons and also had a responder rate over 40% in the coeliac disease participants (Table 1). No more than one control participant was considered a responder to any of these nine biomarkers. The group of nine gluten-responsive biomarkers included four chemokines (CCL20, CXCL8, CCL2, and CXCL9), four cytokines (IL-6, IL-17A, IL-10, and IL-2), and amphiregulin, a member of the epidermal growth factor family with potent intestinal epithelial mitogenic effects that is also implicated in immune tolerance and resistance to infection (16). The time course for elevations in these nine biomarkers was evaluated in ten coeliac disease participants who experienced more severe symptomatic responses to gluten. Peak levels were at four hours except for IL-17A, which was at two hours, and for CXCL9, which delayed until six hours.

Confirmatory high sensitivity cytokine testing

Because baseline and four-hour serum levels of several cytokines implicated in coeliac disease were at or below the lower level of detection for the proximity extension assay, a high sensitivity electrochemiluminescence immunoassay was used to more accurately assess seven biomarkers elevated at screening, and also several chemokines and cytokines associated with Th1, Th2, Th17, and Th22 responses. IL-15 was also assessed because it is

a cytokine with innate immune functions that is thought to play a central role in gluten-induced mucosal immunopathogy (17), and is under investigation as a therapeutic target in coeliac disease (18).

Sera from all time-points for each of the 25 coeliac disease and control participants were assessed by electrochemiluminescence immunoassay (Table 2). At their peak, levels of all seven of the tested biomarkers that had been identified during screening were significantly elevated after gluten challenge in coeliac disease participants. The electrochemiluminescence immunoassay resolved the hierarchy of biomarkers according to relative change from baseline because it was better able to quantify serum levels of low-abundance biomarkers including IL-2. One consequence of this improved assay performance was to clarify that IL-2 was the most dynamic of the biomarkers tested. Median fold-changes from baseline for IL-2 were substantially greater than the next most prominent biomarkers at two hours (21 versus 1.6 for IL-17A and IFN-γ, which are both relatively specific for activated T cell or NK cell), at four hours (57 versus 10 for CCL20, which is a Th17-associated chemokine), and also at six hours (22 versus 6.6 for IL-6, which is a ubiquitous proinflammatory cytokine secreted by T cells, B cells and innate immune cells). Several of the candidate cytokines that had not been detected or not assessed by proximity extension assay also showed significant elevations. Peak median fold-changes were 2.6 at four hours for IFN-γ, 1.6 at six hours for IL-22, and 1.2 at each time point for TNF-α. Over 80% of participants in the coeliac disease cohort were responders for IL-2, CCL20, CXCL9 (a monocyte-derived chemokine induced by IFN-γ), or CXCL8 (a ubiquitous pro-inflammatory chemokine) alone. Notably, no change from baseline was evident for IL-15, or for IL-21.

Timing and coordination of cytokine release

Individuals with greater immune activation measured by relative elevations in IL-2 from baseline typically showed elevations in a more diverse range of cytokines than those with lower IL-2 elevations (Figure 2). Cytokine elevations after gluten challenge were closely linked; peak relative elevations for each of the serum cytokines were significantly correlated except with IL-6 (Table 3). In general, peak levels of cytokines relatively

specific for activated T cells (IL-2, IFN-γ, IL-17A, IL-22) were more strongly correlated with select chemokines that peaked later than with any other T cell-associated cytokine. Among the cytokines closely linked to T cell activation, peak IFN-γ levels (at four hours) were correlated most closely with peak CXCL9 levels (at six hours), and IL-17A levels (at two hours) were correlated most closely with peak CCL20 levels (at four hours), and IL-2 levels (at four hours) were correlated most closely with peak levels of TNF-α (at six hours), CXCL8 (at four hours), and CXCL9 (at six hours).

Relationship between symptoms and cytokine release

Peak scores for nine self-rated symptom severity assessments, and for the global digestive symptom score were compared with peak serum levels of ten cytokines. For the full cohort of 25 coeliac disease patients, statistically significant correlations after correction for multiple comparisons were found for nausea and IL-2 (Spearman's correlation coefficient, $r_s = 0.65$, $p = 0.020$), and for global digestive symptom score with CCL20 ($r_s = 0.66$, $p=0.020$), CXCL9 ($r_s = 0.61$, $p = 0.030$), CXCL8 ($r_s = 0.61$, $p = 0.030$) and TNF-α ($r_s = 0.58$, $p = 0.048$). Clinical reactions to gluten in the coeliac disease cohort were categorized as severe (n=4, all with vomiting), moderate (n=7, including two who had vomiting), mild (n=8), or very mild (n=6) according to their worst global digestive symptom descriptor during the six hours after gluten challenge. None of the coeliac disease patients were categorized as asymptomatic. There was a consistent trend for the severe group (n = 4) to have highest levels of each cytokine at both two and four hours, and usually also at six hours (Figure 3). Serum cytokines followed a similar profile in patients with moderate symptoms (n = 7), but levels were lower than in the severe group. Cytokine elevations were lower and usually later in patients with mild (n = 8) or very mild symptoms (n = 6), but were clearly distinguished from the control cohort (n = 25) showing no change in any cytokine throughout the observation period. No demonstrable elevation in IL-2 was observed in three coeliac disease patients, two had “mild” and one had “very mild” symptoms after gluten challenge, but elevations in IL-6 or CCL20 were observed in these three patients (Figure 2).

DISCUSSION

We profiled gluten-stimulated systemic cytokine release in patients with coeliac disease and a control cohort using two complimentary multiplex cytokine assays to assess serum collected over six hours following gluten food challenge. The cytokine profiles yielded an unambiguous, personalized assessment of immune activation that was correlated with patient reported outcome data. The present study complements two companion reports that describe assessments limited to only IL-2 in the same set of sera assessed in this study using a formally qualified electrochemiluminescence assay performed by a third party vendor under good laboratory conditions. IL-2 levels were always below the lower limit of quantitation (0.5 pg/ml) at all time points in the healthy volunteers and also at baseline in the coeliac disease patients, but 23 of the 25 coeliac disease patients had elevated IL-2 levels four hours after gluten (14, 15). Unlike ex vivo or in vitro assays with gluten-stimulated fresh blood cells, intestinal tissue, or especially T cell lines and clones, cytokine assessments in serum are unlikely to be prone to significant laboratory artifact, and at least for IL-2, IL-8 and IL-10, appear to be consistent findings after injection of deamidated gluten peptides, and for food challenges with 3- or 6-grams of gluten protein, and gluten ingested in a variety of formats such as bread, or as vital gluten added cooked in muesli bars uncooked in the format used in the present study (1). Early elevations of IL-2, IL-17A, IL-22 and IFN-γ after gluten in patients with coeliac disease implicates rapidly activated T cells as their likely source. A conserved set of chemokines were elevated later and was consistent with T-cell activation being followed by coordinated, downstream activation of effector cells in the innate immune system. Elevations in serum IL-10 and amphiregulin suggest that a regulatory, anti-inflammatory arm of the immune response was also induced after gluten exposure. As with cytokine release syndromes, elevations in serum cytokines after gluten exposure appeared to be clinically important as they were correlated with severity of acute digestive symptoms. Cytokine elevations after gluten were sensitive and specific for coeliac disease in these relatively small cohorts and could ultimately yield a new diagnostic approach avoiding the need for prolonged gluten challenge and endoscopic biopsy in patients already avoiding dietary gluten. Direct measurement of cytokines in serum four hours after gluten exposure is technically less demanding and scalable than

proposals to measure gluten-specific CD4+ T cells by flow cytometry, or ex vivo cytokine release assays with blood collected six days after three-day gluten challenge (4, 19).

The term "cytokine release syndrome" has been applied in the setting of immune effector cell therapies or following administration of biologics that target lymphocytes, and is regarded as a manifestation of supraphysiologic immune activation causing systemic cytokine elevation (20). For the purpose of adverse event reporting, cytokine release syndrome is currently defined as "a disorder characterized by fever, tachypnea, headache, tachycardia, hypotension, rash, and/or hypoxia caused by the release of cytokines", and graded from 1 (mild) to 5 (death) (21). Accordingly, the acute symptom complex after gluten food challenge would not meet this definition of cytokine release syndrome. Our findings suggest that a formal clinical definition and severity grading system could be established for acute clinical reactions to gluten mediated by cytokine release. This would advance clinical research and facilitate improved endpoint definitions in therapeutics development for coeliac disease (22). In the setting of efficacy assessments or comparing potency of gluten preparations, laboratory assessments of serum cytokines elevated by gluten exposure, such as IL-2 alone or in parallel with the cytokines defined in the current study, provides an objective measure that could overcome the subjectivity of patient reported outcomes (symptoms), and address the concerns of nocebo effects.

A limitation of the present study was that the gluten challenge was unmasked and restricted to one format at a single "dose" level. These features of the food challenge do not control for the possibility that patients with coeliac disease anticipate adverse symptoms after knowingly consuming gluten ("nocebo" effect) (23). However, nocebo effect is not a plausible explanation for the consistent pattern and timing of serum cytokine elevations, or their correlation with severity of symptoms. Demonstrating an association between symptoms and cytokines after gluten ingestion in the present study may have been aided by the gluten challenge format being minimized to just ten grams of vital wheat gluten flour. This design allowed approximately six grams of gluten protein to be delivered without symptoms being caused by co-administering substantial amounts of carbohydrate including

FODMAPs present in standard wheat flour. The dose level of six grams of gluten protein was selected because this is similar to many other gluten challenge studies (24), and equates to about half the daily consumption of gluten typical of an adult on an unrestricted diet in the United States and Europe (25, 26). In previous gluten challenge studies calculated to deliver approximately six grams of gluten protein in muesli bars, and also administering three grams of gluten protein in vital wheat gluten flour, we have shown consistent elevations of IL-2 with less prominent rises of CXCL8 and IL-10 in patients with coeliac disease adhering to a gluten free diet (1). Ingestion of six grams of gluten protein would, however, be a substantial indiscretion for a patient with coeliac disease normally adhering to a gluten free diet. Relating the dose of gluten protein, and the food matrix to symptoms, and qualitative and quantitative changes in serum cytokines and symptoms will be important when considering the direct immunological effects of gluten, rather than the consequences of other matrix constituents such as FODMAPs.

A weakness of many clinical studies utilizing multiplex immunoassay or gene expression platforms to profile cytokine levels has been their inability to quantify low abundance cytokines present at concentrations below the threshold for quantitation. Clearly, if physiologic serum levels of an important cytokine such as IL-2 cannot be measured before and often after gluten exposure, as was the case with the proximity extension assay in the present study, little can be concluded regarding its relative importance compared to a high abundance analyte such as CCL20. Furthermore, modest, statistically significant elevations of both IFN-γ and TNF-α after gluten challenge were not apparent by multiplex proximity extension assay but were revealed by the more sensitive electrochemiluminescence immunoassay. Therefore, our strategy to screen using the multiplex proximity extension assay may have overlooked elevations in some low abundance cytokines not subsequently measured by electrochemiluminescence immunoassay. Gluten-specific CD4+ T cells are the likely source of cytokines such as IL-2, IL-17 and IFN-γ elevated early after gluten food challenge. Previously, we showed that elevation of IL-2 after gluten ingestion was correlated with the frequency of gluten-specific CD4+ T cells in blood (1). Here we show that the cytokine release profile in serum evoked by gluten challenge is strikingly similar to

that four hours after patients with coeliac disease receive antigenic gluten peptides (150 μg) by intradermal injection. These short antigenic gluten peptides corresponding to epitopes commonly recognized by gluten-specific CD4+ T cells show systemic bioavailability (14), and also cause gastrointestinal symptoms mimicking those observed after gluten food challenge here (27). Further, prominent elevation of IL-2 is a consistent observation among the cytokines circulating after administering the first dose of biologics directed against and activating T cells (11, 28, 29), which is absent after administering biologics targeting B cells that elevate circulating levels of TNF-α and IL-6 (13). Interestingly, IL-2, CCL20, CXCL8, IL-10, IL-22, TNF-α, IFN-γ, and amphiregulin were all recently reported to arise from a gluten-specific CD4+ T-cell clone activated using plate-bound CD3/CD28 (30). Indeed, IL-2 from antigen-specific CD4+ T cells activated by dietary ovalbumin in vivo, or gluten in vitro, synergises with mucosal IL-15, or TNF-α respectively, to expand and activate intra-epithelial CD8+ T cells (30, 31). Evidence for secretion of IL-17A, IL-21 and IL-22 by gluten-reactive T cells has been contradictory (32-35), but mucosal gene expression studies have reported elevated IL-17A levels in coeliac disease (36, 37) and we also observed elevated plasma IL-17A after administering T cell stimulatory gluten peptides to coeliac disease patients by intradermal injection (1). CXCL9 is mainly secreted by monocytes, endothelial cells, and fibroblasts following exposure to IFN-γ and TNF-α, but has not previously been implicated in coeliac disease despite efforts to identify it in intestinal mucosa from affected patients (38). The absence of cytokines such as IL-15 that are increased in the inflamed gut mucosa in coeliac disease may be because they remain tissue or cell-bound (39). Future studies are now needed to understand the cells producing these cytokines/chemokines shortly after patients consume gluten, and to ascertain whether T cells or other immune cells in the proximal small intestine are the primary source. Patient-based research utilizing gluten challenge combined with analyses of fresh tissue samples or blood appears to be essential to provide unambiguous findings regarding cytokine production induced by gluten.

Whether gluten exposure induces the same range of cytokines in patients regularly exposed to gluten with an unrestricted diet is unclear and should also be addressed in future

research. The relative absence of gastrointestinal symptoms in many untreated patients recently diagnosed with coeliac disease suggests that gluten-mediated cytokine release in this setting is quantitatively less, or qualitatively different from that in treated patients. This paucity of symptoms in coeliac disease patients regularly exposed to gluten may be a natural consequence of gluten-specific CD4+ T cell becoming less responsive to antigenic stimulation. We have shown that coeliac disease patients have marked digestive symptoms after the first, but not later intradermal administrations of T cell-stimulatory gluten peptides (1). Potentially, this scenario may be akin to reduced allergen-induced symptoms following allergen-specific immunotherapy or regular, natural exposure to allergen (40).

The cytokine profile we have described using a functional, proteomic approach could also point towards therapeutic targets, and compliment extrapolations from genome-wide association studies. A recent study concluded that pleiotropic genetic variants implicated in coeliac disease could potentially regulate gene expression in different subsets of T cells, mostly Th17 and regulatory (41). Indeed, the previously highlighted role for IL-21 had been supported by a strong, reproducible association of coeliac disease with a locus at 4q27 that encodes both IL-2 and IL-21. Our finding that IL-2, but not IL-21 is elevated by gluten exposure suggests that IL-2 may account for the functional importance of this genetic association at 4q27 (42). Similarly, CCR2, which is a receptor for CCL2, is among the cluster of chemokine receptor genes encoded on 3p21, and now may be functionally implicated as the causally important gene within this linkage region (42).

Potential therapeutic targets implicated by our study analysing cytokines released after gluten and by genome-wide association studies include the regulatory and pro-inflammatory Th1 and Th17 gluten-specific CD4+ T cells, IFN-γ, CCL2, IL-6, TNF-α, and IL-2 (41, 42). Various repurposed or developmental compounds might be considered for specific unmet clinical needs, or as short-term adjuncts with antigen-specific immunotherapy, for example, antagonists of IFN-γ such as salicylates (43), IL-6 (44), TNF-α antagonists, IL-17 (45), or CCL2/CCR2 blockade by natural compounds (46). At present, IL-15 is the only target tested as anti-cytokine therapy in coeliac disease (18), but our

findings do not support a quantitatively important role for IL-15 during acute, systemic gluten-mediated cytokine release.

Elevation of IL-10 after gluten challenge, and after injecting gluten peptides as a potential therapeutic vaccine, may reflect the involvement of gluten-specific CD4+ T cells with a regulatory phenotype. Tolerance induction by peptide-based therapeutic vaccines in murine models of autoimmunity and in patients with allergy appears to be, in part, mediated by regulatory CD4+ T cells secreting IL-10 (47, 48). IL-10 is known to suppress gluten-mediated activation of patient-derived gluten-specific CD4+ T cells in vitro (49). Gluten-specific regulatory CD4+ T cells secreting IL-10 have also been isolated from patients with coeliac disease (50), and in murine models of coeliac disease gluten-specific CD4+ T cells secreting IL-10 mediate gluten-specific immuno-modulation (51).

The present study establishes that gluten-mediated cytokine release is tightly coordinated and comprises elevations of a highly conserved set of cytokines linked directly to T cell activation or to subsequent downstream activation of innate immune cells. Consistent with our previous studies, changes in serum levels of IL-2 are the most prominent and among the earliest of any cytokine elevated by gluten challenge. The tissue source of circulating cytokines elevated by gluten is not directly identified, but the early prominence of upper gastrointestinal symptoms and presence of gluten-specific CD4+ T cells in the duodenum suggests the upper gastrointestinal tract makes a substantial contribution (52). Our findings are consistent with gluten-specific CD4+ T cells as the primary cause of acute gluten-mediated symptoms and highlight the potential of a diagnostic strategy measuring serum cytokines to support the diagnosis of coeliac disease in patients already established on a gluten-restricted diet. Further studies are warranted to evaluate the diagnostic value of cytokine assessments after gluten challenge in patients with suspected coeliac disease.

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provided data integration and analysis; GG and RPA wrote the manuscript and prepared the Tables and figures. All authors reviewed and approved the manuscript, tables and figures. The authors made the decision to submit the manuscript for publication and vouch for the accuracy of the data and analyses and for the fidelity of this report to the trial protocol. RPA had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Conflict of Interest: ImmusanT, Inc., Cambridge, Massachusetts USA provided funding for the study. HE has no conflicts to disclose. GG, SW, ES, LJW, JLD, KMN, KET and RPA are employees of ImmusanT, Inc. JAT-D and AJMD have served as advisors to ImmusanT, Inc. RPA is inventor of Patents, owned or licensed by ImmusanT, Inc, relating to the diagnostic application of gluten challenge. HCE has no conflicts to disclose.

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645

646 **Figure Legends**

647

648 **Figure 1.** Study design

649

650 **Figure 2.** Heatmap of serum cytokine elevations after gluten challenge. Peak fold change in
651 cytokine levels were normalized by dividing by the sum of the mean plus 3x(standard
652 deviation) of fold change observed in unaffected cohort.

653

654 **Figure 3. Serum cytokine profiles after gluten challenge and self-rated severity assessments.**
655 Coeliac disease participants (n=25) were grouped according to their overall severity of digestive
656 symptoms, and compared to healthy volunteers (n=25).

657

Table 1. Changes in serum cytokines after gluten by proximity extension multiplex assays

Cytokine	Concentration † Median (25-75th %)		P value††	Fold change at 4 h Median (25-75th %)		% Responders‡	
	Baseline	4 h	BSL vs 4 h	Coeliac	Controls	Coeliac	Controls
CCL20	38 (19-62)	314 (62-1235)	0.0002	5.99 (1.47-36.8)	0.79 (0.65-1.08)	72	0

IL-6	11 (8-17)	49 (31-89)	2.74E-05	4.50 (2.32-9.11)	1.15 (0.90-1.38)	56	8
IL-17A	5 (3-6)	12 (6-32)	0.0145	2.45 (0.95-4.50)	1.06 (0.94-1.14)	64	0
CXCL8	162 (118-194)	426 (243-1352)	0.0014	2.93 (1.09-7.37)	0.93 (0.84-1.02)	60	0
CCL2	6847 (5229-8445)	11468 (7050-17622)	0.0043	1.65 (1.12-2.64)	0.97 (0.84-1.02)	52	0
CXCL9	301 (262-559)	539 (401-1089)	0.0079	1.61 (0.97-2.95)	0.88 (0.83-0.94)	48	8
Amphiregulin	11 (8-15)	15 (11-27)	0.0043	1.43 (1.11-1.83)	0.99 (0.90-1.14)	52	0
IL-10	25 (20-36)	38 (27-67)	0.0121	1.38 (1.01-2.93)	1.07 (0.77-1.16)	44	0
IL-2	2 (2-3)	3 (3-5)	0.0043	1.35 (1.06-2.18)	0.94 (0.85-1.07)	40	0

† Data presented as normalized protein expression (NPX) in proximity extension assays, Olink Proteomics' arbitrary unit on log2 scale.

†† Wilcoxon signed-rank test with false discovery rate adjustment by Benjamini-Hochberg method for 180 comparisons

‡ % of coeliac disease (n=25) or control (n=13) participants with a fold-change in cytokine level >1.2, and >3 standard deviations above mean in controls.

Table 2. Serum cytokine concentrations and fold change from baseline after gluten

	Coeliac disease patients, median (25th - 75th%)				**Peak elevation ††**		**% Responders ‡**	
	Baseline (pg/ml)	2 h fold-change	4 h fold-change	6 h fold-change	Hour	P-value	Coeliac	Controls
IL-2	0.03 (0.03-0.04)	21 (2.4-103)	57 (13-151)	22 (3.5-43)	4	3.66E-10	88	4
CCL20	0.89 (0.64-1.5)	1.1 (0.9-1.8)	10 (5.0-83)	4.2 (1.8-20)	4	9.73E-11	88	0
IL-6	0.14 (0.11-0.22)	1.9 (1.3-3.6)	6.4 (2.6-8.7)	6.6 (3.6-13)	4	9.73E-11	76	0
CXCL9	66 (53-113)	0.9 (0.9-1.0)	1.4 (1.1-3.1)	3.1 (2.2-6.5)	6	9.73E-11	92	0
CXCL8	4.9 (3.6-6.1)	1.3 (1.1-2.3)	3.0 (1.6-7.5)	2.2 (1.4-3.7)	4	5.51E-08	84	0
IFN-γ	0.9 (0.5-2.0)	1.6 (1.1-3.1)	2.6 (1.7-5.3)	2.2 (1.4-4.0)	4	2.64E-09	64	0
IL-10	0.08 (0.06-0.11)	1.3 (1.0-1.9)	2.7 (1.7-9.0)	2.5 (1.6-4.0)	4	9.73E-11	60	0
IL-22	0.3 (0.1-0.4)	1.0 (0.8-1.1)	1.4 (0.9-2.0)	1.6 (1.2-2.7)	6	8.85E-07	48	0
IL-17A	0.3 (0.3-0.5)	1.6 (1.0-6.8)	1.3 (1.0-3.9)	1.0 (1.0-2.3)	2	0.001	52	0
TNF-α	1.4 (1.2-1.8)	1.2 (1.0-1.9)	1.2 (1.1-1.7)	1.2 (1.1-1.3)	6	0.0001	52	0

† Measured by electrochemiluminescence immunoassay. †† Peak elevation versus baseline by Wilcoxon signed-rank test

‡ Coeliac disease patients (n=25) versus controls (n=25) at their peak in serum previously defined by proximity extension assay

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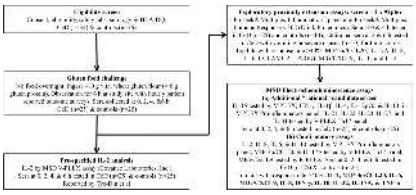
Table 3. Correlation between fold-change elevations in serum cytokines after gluten challenge

		Spearman's correlation coefficient (r_s) for peak serum levels in coeliac disease subjects (n=25)									
		IL-2	CCL20	IL-17A	CXCL9	CXCL8	IL-10	IL-22	TNF-α	IFN-γ	IL-6
	IL-2	-	0.76	0.44	0.83	0.82	0.61	0.51	0.86	0.64	n.s.
	CCL20	*7.3E-05*	-	0.65	0.81	0.83	0.53	0.64	0.8	0.66	n.s.
	IL-17A	*0.035*	*0.0012*	-	0.5	0.59	0.53	0.58	0.56	0.5	n.s.
	CXCL9	*9.7E-06*	*9.8E-06*	*0.015*	-	0.7	0.61	0.69	0.74	0.82	n.s.
P-value	*CXCL8*	*9.7E-06*	*9.7E-06*	*0.0033*	*0.0004*	-	0.64	0.53	0.83	0.46	n.s.
	IL-10	*0.0029*	*0.0096*	*0.0093*	*0.0029*	*0.0017*	-	0.44	0.62	0.61	n.s.
	IL-22	*0.013*	*0.0015*	*0.0038*	*0.0004*	*0.0094*	*0.035*	-	0.56	0.58	n.s.
	TNF-α	*5.3E-07*	*9.7E-06*	*0.0059*	*0.0001*	*2.3E-06*	*0.0021*	*0.0057*	-	0.61	n.s.
	IFN-γ	*0.0018*	*0.0013*	*0.016*	*9.7E-06*	*0.027*	*0.0029*	*0.0037*	*0.0028*	-	n.s.
	IL-6	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	-

n.s. not significant

664



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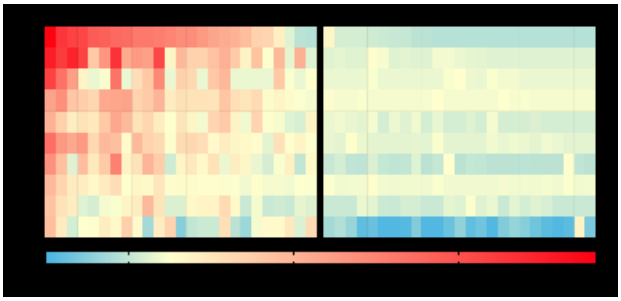

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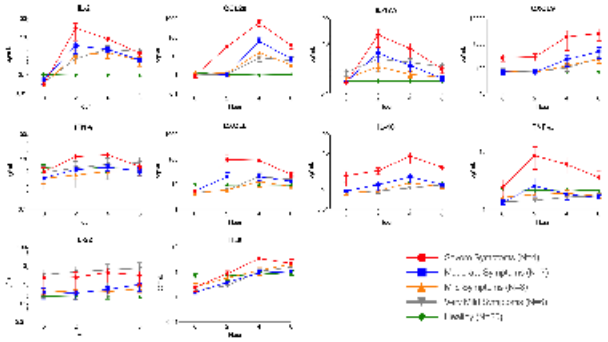

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