LESSONS ON T-CELLS AND IMMUNE-TARGETING THERAPEUTICS IN COELIAC DISEASE

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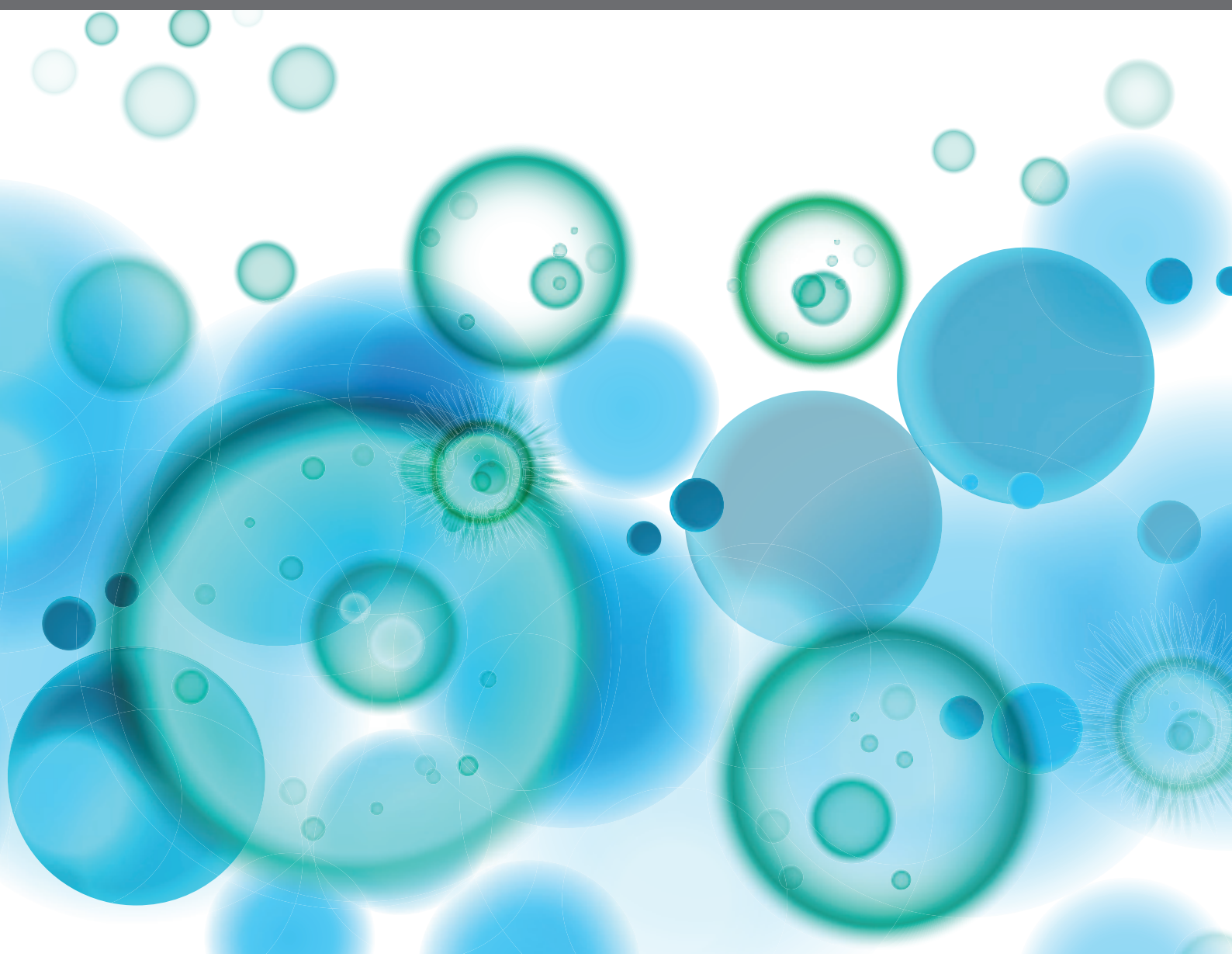

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LESSONS ON T-CELLS AND IMMUNE-TARGETING THERAPEUTICS IN COELIAC DISEASE

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Table of Contents

04 ***Editorial: Lessons on T-Cells and Immune-Targeting Therapeutics in Coeliac Disease***
Melinda Y. Hardy, Daniel Agardh and Robert P. Anderson

07 ***Celiac Disease Causes Epithelial Disruption and Regulatory T Cell Recruitment in the Oral Mucosa***
Javier Sanchez-Solares, Luis Sanchez, Carmela Pablo-Torres, Celso Diaz-Fernandez, Poul Sørensen, Domingo Barber and Cristina Gomez-Casado

19 ***Frequency of Gluten-Reactive T Cells in Active Celiac Lesions Estimated by Direct Cell Cloning***
Shuo-Wang Qiao, Shiva Dahal-Koirala, Linn M. Eggesbø, Knut E. A. Lundin and Ludvig M. Sollid

27 ***Missing Insight Into T and B Cell Responses in Dermatitis Herpetiformis***
Esko Kemppainen, Teea Salmi and Katri Lindfors

34 ***The Evolving Landscape of Biomarkers in Celiac Disease: Leading the Way to Clinical Development***
Glennda Smithson, Jenifer Siegelman, Toshihiko Oki, Joseph R. Maxwell and Daniel A. Leffler

41 ***Comprehensive Analysis of CDR3 Sequences in Gluten-Specific T-Cell Receptors Reveals a Dominant R-Motif and Several New Minor Motifs***
Shiva Dahal-Koirala, Louise Fremgaard Risnes, Ralf Stefan Neumann, Asbjørn Christophersen, Knut E. A. Lundin, Geir Kjetil Sandve, Shuo-Wang Qiao and Ludvig M. Sollid

54 ***Can Celiac Disease Be Prevented?***
Renata Auricchio and Riccardo Troncone

60 ***A Sensitive Whole Blood Assay Detects Antigen-Stimulated Cytokine Release From CD4+ T Cells and Facilitates Immunomonitoring in a Phase 2 Clinical Trial of Nexvax2 in Coeliac Disease***
Melinda Y. Hardy, Gautam Goel, Amy K. Russell, Swee Lin G. Chen Yi Mei, Gregor J. E. Brown, Suyue Wang, Evan Szymczak, Ruan Zhang, Kaela E. Goldstein, Kristin M. Neff, Leslie J. Williams, Kenneth E. Truitt, John L. Dzuris, Jason A. Tye-Din and Robert P. Anderson

75 ***Interplay Between Gluten, HLA, Innate and Adaptive Immunity Orchestrates the Development of Coeliac Disease***
Jordan Voisine and Valérie Abadie

88 ***Apolipoprotein A4 Defines the Villus-Crypt Border in Duodenal Specimens for Celiac Disease Morphometry***
Juha Taavela, Keijo Viiri, Anna Välimäki, Jani Sarin, Kristiina Salonoja, Markku Mäki and Jorma Isola

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Editorial: Lessons on T-Cells and Immune-Targeting Therapeutics in Coeliac Disease

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Keywords: autoimmunity, coeliac disease, dermatitis herpetiformis, prevention, T cells, transglutaminase, treatment

Editorial on the Research Topic

Lessons on T-Cells and Immune-Targeting Therapeutics in Coeliac Disease

Moving forward from sole reliance on gluten-free diet to treat coeliac disease (CeD) requires consensus on the mechanism of gluten toxicity relevant to CeD. A more precise understanding of the role of gluten-reactive CD4+ T cells underpins innovative efforts to design targeted approaches to diagnose, treat, or prevent CeD. The field will also benefit from confirmatory studies that may provide useful road-maps for monitoring disease modulation during clinical trials. This Research Topic describes the current knowledge and highlights possible targets for new CeD treatment strategies.

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INSIGHTS INTO CeD IMMUNE PATHOGENESIS

Memory CD4+ T cells orchestrating and maintaining an adaptive immune response directed against a conserved set of selectively deamidated gluten peptides is broadly accepted as the primary cause for CeD. Dietary gluten provides the antigenic substrate for the immune response in CeD, but only after selective deamidation by host transglutaminases do CD4+ T cells efficiently recognise gluten. CD4+ T cells specific for gluten also support B cells and plasma cells specific for deamidated gluten peptides. While some highlight autoimmune aspects of CeD, these are limited to a humoral response to transglutaminase 2 associated with enteropathy, and also transglutaminase 3 and 6 in patients with skin and neurological complications, respectively. Humoral autoimmunity without an accompanying autoreactive CD4+ T-cell response has generally been explained by extracellular transglutaminases in host tissues serving as the carrier in classical "hapten-carrier" complexes, and immunogenic gluten peptides being the "hapten". By virtue of their catalytic activity mediating transamidation, transglutaminases crosslink gluten peptides onto themselves. This allows transglutaminase-specific B cells to take up transglutaminase-gluten complexes, present antigenic gluten peptides, and receive help from gluten-specific CD4+ T cells. In clinical practice, exclusion of dietary gluten slowly reduces intestinal injury and is paralleled by falling levels and eventually disappearance of transglutaminase autoantibodies. CeD is driven by an environmental rather than self-antigen. Gluten and transglutaminase-gluten complexes being extracellular antigens is also the likely explanation for the preponderance of the CD4 subtype of T cells responding to gluten,

and very few if any gluten-specific T cells of the CD8 subtype. Whether direct effects of gluten on innate immunity, non-immune cells, or structures such as tight junctions actually contribute to pathology in patients consuming gluten is an open question awaiting convincing, reproducible studies in patients to confirm *in vitro* findings. To date, therapeutics targeting innate immunity such as IL-15 and leaky tight junctions are yet to show clear benefit in CeD patients. In summary, the T cell-mediated adaptive immunity model serves well to explain the natural history, genetics, and reductionist immunological observations associated with CeD, and predicts that a variety of immune strategies may be effective for diagnosis, monitoring, treatment, and prevention of disease.

Voisine and Abadie provide a thorough review on the interplay between innate and adaptive immune compartments, gluten, tissue transglutaminase 2, and HLA in the development of CeD. They highlight that no separate element is sufficient to induce intestinal damage typical of CeD. They discuss use of their transgenic mouse model and highlight the importance of cytokines in these processes. This paper nicely ties together many elements of CeD pathogenesis and the topics published in this e-book.

The difficulty in isolating gluten-specific CD4+ T cells from small amounts of intestinal tissue means that the true proportion of CeD-specific pathogenic T cells in the gut is unclear. Qiao et al. report generating T cells clones by limiting dilution from duodenal tissue from six CeD patients with active disease (one refractory) and 3 controls. From 1652 T cell clones they observed gluten-reactivity in 24 (1.5%). A large proportion of T cell clones responded to whole protein but not to peptides containing previously described T cell epitopes (1), suggesting that undescribed epitopes still exist within gluten. However, due to pre-screening with wheat gluten in this study, the proportion of T cells reactive to peptides derived from barley, rye, or oats, or to additional sub-dominant gluten peptides recognised by circulating CD4+ T cells will need further investigation (2, 3).

CeD can present with a number of extra-intestinal manifestations, including skin and oral manifestations. Kemppainen et al. highlight the key differences in pathogenesis between CeD and the cutaneous form of CeD, dermatitis herpetiformis. They also discuss the gaps that remain in our understanding of immune cell subset involvement in the characteristic skin lesions. Sanchez-Solares et al. describe an increase in FoxP3+ cells within the oral mucosa of active and treated CeD patients. They suggest a role in tissue repair due to a correlation with altered epithelial integrity in CeD patients and increases in peripheral amphiregulin mRNA expression. These findings open the possibility for oral mucosal targeting during tolerogenic immunotherapy. Further studies are required to understand the immune milieu and functional capacity of cells within this tissue site in CeD.

BIOMARKERS TO PREDICT DEVELOPMENT, DIAGNOSE, AND MONITOR CeD

The advancement of new therapies for CeD is dependent on sensitive methods to detect changes in gluten-specific immune responses, as well as clinical responses. Smithson et al. describe potential biomarkers for CeD and refractory CeD in their review, and the utility of such biomarkers in measuring therapeutic intervention in CeD trials. They describe a number of different immune markers that should be considered.

Further to this, Hardy et al. describe the utility of a simple and highly sensitive whole blood cytokine release assay to monitor changes to the gluten-induced CD4+ T-cell response in CeD patients, which was then utilised for monitoring responses during the RESET CeD Phase 2 clinical trial testing a gluten-peptide immunotherapy (Nexvax2). The assay was effective on samples from patients without the need of a gluten challenge. They describe the optimal conditions for blood collection and highlight the promise of the assay in detecting attenuated gluten-peptide induced IL-2 and IFN-γ release following Nexvax2 treatment. An assay that does not require gluten challenge provides a useful tool for future CeD trials and following validation, could be used as a less invasive T cell-based CeD diagnostic.

Taavela et al. describe another useful aid in clinical trial monitoring and possibly in CeD diagnosis. They utilised APOA4 staining (a lipid-binding glycoprotein) on histological sections in CeD patients and controls, and showed a clear distinction of the villus-crypt border. Use of this marker increased the reliability and reproducibility of morphometrical villus height:crypt depth readings, in particular for hard to interpret samples where intestinal damage was present.

Rare gluten-specific T cells can be detected and enriched for using MHC class II tetramers (4), and may be useful to predict for CeD (5). Using tetramer-based cell sorting Dahal-Koirala et al. isolated CD4+ T cells specific for immunodominant gluten epitopes and performed a comprehensive analysis of the T cell receptor (TCR) sequences expressed in CeD patients. This is the largest TCR database of gluten-specific CD4+ T cells studied so far consisting of TCRs expressed by 3122 clonotypes from 63 CeD patients. They showed a large proportion expressed TRAV26-1:TRBV7-2 chains and the dominant CDR3 R-motif. Importantly, a consistent proportion of public TCRs were observed within different individuals. This points to the possibility of utilising TCR sequencing to support CeD diagnosis. Further validation studies are warranted.

Lastly, Auricchio et al. discuss biomarkers predictive for CeD development (e.g. microRNA's and lipidomic modifications), and the possibility of disease prevention in at-risk individuals. They review prevention trials conducted to date and studies that target risk factors, such as childhood infections, changes in the microbiota, effects of vaccinations, gluten infant feeding and use of probiotics. They argue that successful prevention may require a combination in approaches.

CONCLUSIONS

Together these articles highlight how understanding the contributions of CD4+ T cells in CeD pathogenesis is enabling clinical programs seeking to improve CeD clinical management or even prevent CeD altogether. Additional tools to detect disease

processes are discussed, which will greatly benefit the field. As the adaptive immune paradigm strengthens, there could still be long-held beliefs about gluten toxicity in patients that are substantially revised. There are also a number of important unanswered questions, such as the exact triggers of CeD, the separation of the symptomatic response, gluten immunity, and the damaged intestine, the drivers of extra-intestinal manifestations, and if it is possible to prevent CeD in genetically at-risk populations. The next decade of research is set to provide new insights into CeD pathogenic processes, less invasive diagnostic tests, and better treatment options for CeD and refractory CeD patients with greater focus on targeting immune processes, and in particular halting the gluten-specific CD4+ T-cell response. Moreover, longitudinal follow-up of several ongoing primary and secondary prevention trials conducted in at-risk birth cohorts will be completed. Such insights will benefit researchers in the entire field of T cell-mediated disease.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Conflict of Interest: RA has served as consultant for Takeda, GSK, Anokion, Allero Therapeutics, Treg Therapeutics, EVOQ Therapeutics, and Bristol-Myers Squibb. He is also founder and shareholder of Novoviah Pharmaceuticals and is the inventor of patents relating to the diagnosis and treatment of coeliac disease.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Celiac Disease Causes Epithelial Disruption and Regulatory T Cell Recruitment in the Oral Mucosa

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Celiac disease (CD) is a chronic autoimmune disease characterized by an immune-triggered enteropathy upon gluten intake. The only current treatment available is lifelong Gluten Free Diet (GFD). Several extraintestinal manifestations have been described in CD, some affecting the oral mucosa. Thus, we hypothesized that oral mucosa could potentially be a target for novel biomarkers and an administration route for CD treatment. Six *de novo* diagnosed and seven CD patients under GFD for at least 1 year were recruited. Non-celiac subjects (n = 8) were recruited as control group. Two biopsies of the cheek lining were taken from each subject for mRNA analysis and immunohistochemical characterization. We observed a significant decrease in the expression of epithelial junction proteins in all CD patients, indicating that oral mucosa barrier integrity is compromised. FoxP3+ population was greatly increased in CD patients, suggesting that Tregs are recruited to the damaged mucosa, even after avoidance of gluten. Amphiregulin mRNA levels from Peripheral Blood Mononuclear Cells (PBMCs) and epithelial damage in the oral mucosa correlated with Treg infiltration in all the experimental groups, suggesting that recruited Tregs might display a "repair" phenotype. Based on these results, we propose that oral mucosa is altered in CD and, as such, might have diagnostic potential. Furthermore, due to its tolerogenic nature, it could be an important target for oral immunotherapy.

Keywords: celiac disease, oral mucosa, remodeling, regulatory T cells, immunotherapy, tolerance induction, immune mediated disorders, autoimmune disease

INTRODUCTION

Celiac disease (CD) is characterized by an immune-mediated chronic enteropathy of the small intestine, triggered by the ingestion of gluten in genetically predisposed individuals. The prevalence of CD is 1–3% worldwide. Although this prevalence varies from country to country, i.e. 1% in Western countries, due to environmental, autoimmune, and genetic factors. CD is regarded as one of the most common genetic disorders, as virtually all the patients are HLA-DQ2+ and/or HLA-DQ8+ (1–4).

It is characterized by the atrophy of the villi of the small intestinal mucosa, which entails a syndrome of nutrient malabsorption (1). CD diagnosis is currently based on several features that include, besides the HLA haplotypes, serological markers (IgA anti-endomysial and/or IgA anti-tissue transglutaminase -tTG2-) and gluten-induced intestinal morphological changes. These changes have led to the Marsh classification score based on a) lymphocyte infiltrates at the intraepithelial compartment; b) crypt hyperplasia; and c) villous atrophy (5). Therefore, CD diagnosis requires a biopsy from the small intestine, a sampling technique that is invasive and entails possible complications for the patients.

The typical form of the disease is a malabsorption syndrome with chronic diarrhea, abdominal pain, distention, and weight loss (6). However, due to the deficiencies of the nutrients absorbed in the small intestine such as iron, folic acid, or vitamin B12, atypical forms include extra-intestinal manifestations as iron-deficiency anemia, dermatitis herpetiformis, osteoporosis, and osteopenia. Lesions in the oral and gingival mucosa, tongue, palate, tooth, and enamel are also frequently associated to CD. In fact, recurrent aphthous stomatitis and atrophic glossitis are present in more than 20% of CD patients (6–8).

Currently, the only available treatment for CD is the adherence to a lifelong GFD. After gluten removal, an improvement in clinical symptoms and intestinal histological findings is observed. However, some patients find this diet cumbersome, as it is more expensive and socially restrictive than ordinary diets (9, 10). In this regard, several adverse effects and negative psychosocial implications have been reported (11). As a result, an increasing number of trials have recently begun to explore alternative therapeutic strategies, such as enzymes designed to digest gluten, the use of inhibitors of paracellular permeability to decrease the migration of gluten peptides into the lamina propria, binding of gluten by polymers, the use of tissue transglutaminase (tTG2) inhibitors, or the modulation of cytokine production. Interestingly, gluten tolerization by antigen specific immunotherapy is also being recently pursued (12). The latter approach is based on allergy immunotherapy (AIT), which is a well described tolerance-inducing and disease modifying treatment for allergic diseases that acts through several mechanisms, including the generation of B and T regulatory responses (13, 14). Among AIT strategies, sublingual immunotherapy (SLIT) is both a safe and effective treatment for allergic rhinitis and asthma (15–17). Recent accumulating evidence suggests that a possible "mouth-gut axis" may exist in the pathogenesis of gastrointestinal diseases (18), and as such, oral mucosa may be a key target organ for the development of CD-specific immunotherapy.

The oral mucosa lines the inside of the mouth and consists of primarily non-keratinized stratified squamous epithelia, and an underlying connective tissue, the lamina propria, which is highly vascularized (19). Epithelial integrity is maintained by junction protein complexes, such as adherens junctions (AJs) and tight junctions (TJs) (20). Besides being a physical barrier, the oral mucosa contains immune cells that maintain mucosal homeostasis. In fact, the mucosal epithelium plays a key role in the immune regulatory system of the oral mucosa, whose function is primarily tolerogenic (19, 21, 22). When the epithelium is disrupted, epithelium-derived cytokines, such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33, are released. Another cytokine recently associated with epithelial tissue damage is periostin, which provides signals for tissue development and remodeling (23–25). Factors such as platelet-activating factor (PAF) can contribute to barrier remodeling by activating epithelial cells to release IL-33 (26). IL-33, in turn, signals for epithelial remodeling associated inflammation and the recruitment of Tregs (27). The recruited Tregs contribute to mucosal homeostasis by promoting wound healing and repair processes. Recruited "repair" Tregs express and/or produce repair factors such as amphiregulin and keratinocyte growth factor (28).

Although the immune processes taking place in the oral mucosa are still poorly understood, CD pathogenesis in the gut is very well characterized. IL-15, a cytokine structurally related to IL-2 that it is widely distributed, has been found to be upregulated in enterocytes in active CD. It drives epithelial damage by stimulating the production of Th1 cytokines, such as IFNγ, and the cytotoxicity of intestinal intraepithelial lymphocytes (IELs) (29–31). IL-15 contribution to oral manifestations in CD has not been addressed yet. Consistent with a gliadin-driven Th1 response, IELs in CD are enriched in cytolytic proteins, such as perforin, and produce large amounts of IFNγ; therefore, participating in the severe mucosal damage (32–34). Gluten-specific CD4+ T-cell lines and clones derived from CD patients were shown to produce interferon (IFN), in response to activated dendritic cells isolated from the mucosa of active CD (31, 35). There are a few studies describing oral intraepithelial dendritic cells (36, 37). These cells express langerin and are highly abundant in the oral mucosa (38). However, their role in CD has not been explored. Regarding the T-lymphocytic inflammatory infiltrate in the oral mucosa, it was found to be significantly increased in patients with active CD (6), in contrast to other studies that found no differences with healthy control (39, 40).

Previous studies by our group with allergic patients to grass pollen, olive pollen, or house dust mite (HDM) have demonstrated that they undergo oral epithelial remodeling characterized by reduced expression of claudin-1, occludin, and E-cadherin proteins (25, 41). In fact, severe grass pollen allergic patients also presented an increased number of CD11c+ and CD4+ infiltrates and increased gene expression of IL-33 (25). Moreover, these patients were characterized by unique transcriptomic and metabolomic fingerprints (42).

In view of these evidence, we formulated the hypothesis that oral mucosa remodeling could also be present in CD patients and would not only help us understand the extraintestinal manifestations associated to CD, but also provide a rationale for oral IT strategies in CD.

In this study, we have found that the oral epithelial barrier of CD patients is compromised, even when they adhere to a GFD. Moreover, increased Treg numbers in the oral mucosa are observed in CD patients. Our data suggest that the characterization of the oral mucosal barrier may be a potential

tool for advancing novel oral diagnostic markers and disease modifying and tolerization treatments for CD.

MATERIALS AND METHODS

Study Subjects

Twenty-one subjects were recruited: six CD patients *de novo* diagnosed, seven CD patients under GFD treatment for at least 1 year, and eight non-celiac subjects as a control group. *De novo* CD patients were included after confirmation of duodenal biopsy histological classification Marsh III (villi atrophy), positive Anti-Transglutaminase Antibodies (ATA), and DQ2+ allele of the Human Leukocyte Antigen (HLA). GFD treated group included patients with positive clinical response to diet and ATA loss. Medical history from all subjects was revised by the gastroenterologist in charge of patient enrolment. All subjects with a recent history of nasopharyngeal disease or severe respiratory allergic disease were excluded from the study. During sample collection, all patients were further investigated to ensure that no significant oral lesions were present. Complying with EU regulation 2016/679 from the European Parliament and the Council and Spanish Royal Decree-Law 5/2018, all subjects provided written informed consent and protocol approval was obtained from the Research and Ethics Committees of San Agustin Hospital of Aviles. Clinical features and detailed information of the studied population are shown in **Table 1**.

Duodenal Samples

Duodenal sampling was carried out under local anesthesia, obtaining a total of six biopsies from the second portion of the duodenum and the duodenal bulb. Endoscopy was carried out using a Fujifilm gastroscope series 200 or 500 with a biopsy channel of 2.8 mm. Biopsy specimens were fixed in 4% PFA, and later processed to paraffin blocks, sectioned at 4 μm and stained with H&E following manufacturer´s protocols. Duodenal damage was categorized according to Marsh classification (**Supplementary Figure 1**). Duodenal biopsies were performed at the moment of diagnosis, duodenal histopathological information for GFD treated patients was obtained from archived data.

Serum Antitransglutaminase 2 Determinations

Serum antitransglutaminase 2 antibodies (IgA class) were measured using Triturus automated ELISA analyzer (Grifols). Total IgA was measured by immunoturbidimetry using a Cobas© 8000 modular analyzer (Hoffman-La Roche).

Oral Mucosa Samples

Two biopsies were taken from the buccal mucosa of each study subject using a 3 mm surgery biopsy punch under local anesthesia. One biopsy was embedded in 4% paraformaldehyde (PFA) and processed to paraffin for immunohistochemical (IHC) studies; and the other was conserved in RNALater™ (ThemoFisher) for qPCR studies.

Blood Sampling

Twenty milliliters of heparinized blood were collected from each study subject. Ficoll-Paque (GE Healthcare™) density gradient centrifugation was performed to obtain both plasma and Peripheral Blood Mononuclear Cells (PBMCs). Plasma samples were frozen at −80°C for cytokine quantification by ELISA. PBMCs were further lysed in RLT buffer (Qiagen) and stored at −20°C for later RNA extraction.

Oral Mucosa Immunohistochemical Analyses

Paraffin blocks were cut into 1 μm-thick sections and fixed onto a poly-L-lysine treated glass slide and then used for histological

TABLE 1 | Detailed information on study celiac disease patients.

	Age	Sex	Serology		Time since GFD	Genotype	Clinical manifestations	Other diseases	Marsh classification	Response to GFD
			Total IgA (mg/dl)	*IgA Anti-tTG2 (kU/L)*		*DQ2*				
De novo	42	F	143	158	–	+	ID	V	3b	
	64	F	80	12	–	+	ID, DY	–	3b	
	64	F	253	18.3	–	+	WL, N, D	HP	3b	
	25	F	<5*	26.1	–	+	ABP, ID, HC	SA, RD	3a	
	62	M	370	45.7	–	+	AN, GIB	HTN	3b	
	49	F	149	657	–	+	DY, D/CON	–	3a	
GFD	48	F	133	>125/5	6 y 9 m	+	DY, ID, AN	–	3c	+
	67	F	133	152/<5	20 y	+	D, WL	HT, FM, DE	3a	+
	26	F	152	>300/<5	1 y 1 m	+	ABP, WL	–	3b	+
	38	F	277	41.2/<5	12 y 5 m	+	AN, D	–	3b	+
	54	F	222	1.2	2y	+	ID, B12	VE, DE, AG	3a	+
	54	F	284	>300/9.8	1y 6 m	+	VO, D/CON, ABP	–	3b	+
	53	M	266	93/<5	1y 2 m	+	ABP	–	3b	+

M/F, male/female; y/m, years/months; AG, autoimmune gastritis; ABP, abdominal pain; AN, anemia; B12, B12 deficiency; CON, constipation; DE, depression; D, diarrhea; DY, dyspepsia; FM, fibromyalgia; GIB, gastrointestinal bleeding; HC, hematochezia; HP, Helicobacter pylori; HT, hypothyroidism; HTN, hypertension; ID, iron deficiency; N, nausea; RD, rheumatic disease; SA, sulfonamide allergy; V, vitiligo; VE, vertigo; VO, vomit; WL, weight loss.

and immunohistochemical analyses. All sections were stained with H&E following manufacturer's protocol to achieve correct orientation of the biopsy. Immunohistochemical staining was performed using Bond Polymer refine Detection kit on a BOND-MAX Automated IHC/ISH Stainer (Leica Biosystems) following the manufacturer's protocol. The following antibodies were used: mouse monoclonal anti-human CD19 (NCL-L-CD19-163, Leica Biosystems), rat anti-human CD3 (MCA1477,Bio-Rad), mouse monoclonal anti-human CD4 (NCL-L-CD4-368, Leica Biosystems), mouse monoclonal anti-human CD8 (NCL-L-CD8-4B11, Leica Biosystems), mouse monoclonal anti-human Claudin-1 (ab56417, Abcam), mouse monoclonal anti-human E-Cadherin (36B5) (PA0387, Leica Biosystems), mouse monoclonal anti-human FoxP3 (ab22510, Abcam), mouse monoclonal anti-human γδ-TCR (sc-100289, Santa Cruz Biotechnology), mouse monoclonal anti-human Langerin (ab49730, Abcam), rabbit recombinant monoclonal [EPR20992] to Occludin (ab216327, Abcam), and rabbit polyclonal to Neutrophil Elastase (ab68672, Abcam). Positive and negative controls were included for each experiment.

Luna Staining

Sections were stained with a mixture of hematoxylin and Biebrich scarlet for 5 min. Subsequently, they were rinsed with 1% acid alcohol solution (hydrochloric acid), and water. Finally, samples were counterstained with 0.5% lithium carbonate solution and rinsed in running water for 2 min.

Oral Mucosa Image Analysis

Scanning of the samples was performed using pathology scanner Aperio Versa 8 (Leica Biosystems) at 40× magnification. A sufficient number of images covering the whole biopsy were captured at an appropriate magnification for quantification.

Epithelial cell junctions image analyses were performed using Image-Pro Plus v4.5.0.29 (Media Cybernetics). Briefly, an adequate threshold that best adjusted to the actual DAB staining was established for a selected area. After applying the same threshold to all the images taken from the same sample, the software calculated the stained area relative to the total selected area. The final value was calculated as the weighted average of all measurements obtained for each image in one sample. These analyses were done by at least two independent observers for each staining.

Inflammatory infiltrate analyses were performed by using the "counter tool" integrated in Aperio ImageScope v12.3.2.8013 (Leica Biosystems) covering either epithelium, connective tissue, or the whole sample, when appropriate. Both area and stained cells were determined by three independent observers.

RNA Isolation From Oral Biopsies and Peripheral Blood Mononuclear Cells for qPCR

Oral mucosa tissue was digested in TRIzol™ (ThermoFisher) manually using a scalpel and later homogenized using TissueLyser (Qiagen). After centrifugation, aqueous phase was purified using RNeasy Mini Kit (Qiagen) columns with DNase treatment, according to manufacturer's protocol, and later retrotranscribed into cDNA with the High Capacity RNA-to-cDNA kit (Applied Biosystems). RNA from lysed PBMCs was also extracted using RNeasy Mini Kit (Qiagen) columns. SYBR Green master mix (Takara) was used for quantitative RT-PCR in the equipment Real Time HT 7900 (Applied Biosystems).

Expression data were normalized to the average median of housekeeping genes β-actin and GAPDH and the results were analyzed using the 2 -ΔΔCT method (26). Oligonucleotides for selected genes were designed using Primer3 software, NIH PrimerBlast and Olygoanalizer tool (IDT).

Plasma Cytokine Quantification

ELISA kits for IL-33 (CSB-E13000h-96T), IL-25 (CSB-E11715h-96T), and TSLP (CSB-E03316h-96T) (Cusabio) were used to determined cytokine plasma levels following manufacturer's recommended protocols for every specific kit. To detect PAF in plasma, LabClinics ELISA kit (EH4331) was used. Optical density of the ELISAs was measured at 450 nm in a Varioskan plate reader. A five-parameter logistic fit curve was generated for each cytokine from the seven standards.

Statistics

GraphPad Prism v8.0.1 software was used for statistical analysis. Non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test or One-way ANOVA test followed by Tukey's multiple comparison test were used when appropriate to compare data among experimental groups. For correlation analysis, Spearman correlation was applied. A p-value <0.05 was considered significant for all the analyses. Descriptive statistics along the text are expressed as "mean (s = standard deviation)." Inferential statistics are expressed as "(mean difference ± SE of difference, p-value)" when a parametric test is used, and "(median, p-value)" when the test is non-parametric.

RESULTS

Clinical Features

Of the study subjects, 76.2% were female: 83.3% in the *de novo* group, 87.5% in the GFD group, and 62.5% in the control group. Average age was 44.69 years: 51 (s = 15.62 years) in the *de novo* group, 48.57 years (s = 13.16 years) in the GFD group, and 34.5 years (s = 10.81 years) in the control group. All included study subjects were Caucasian.

Among CD patients, average age of diagnosis was 46.6 years: 51 years (s = 15.62 years) for the *de novo* diagnosed and 42.21 years (s = 12.12 years) for the GFD group. The most frequent clinical manifestations at diagnosis were iron deficiency and diarrhea, found in 38.46% of the CD patients, being 23.08% diagnosed with anemia. Other frequent manifestations were abdominal pain (30.77%), weigh loss (23.08%), and dyspepsia (23.08%). Comorbidities were described in some patients and included other autoimmune diseases such as vitiligo or rheumatic disease. Of the patients in the GFD group, 25% showed signs of depression (**Table 1**).

According to the inclusion criteria, all newly diagnosed patients involved in the study tested positive for tissue transglutaminase 2 (tTG2) antibodies, genotype HLA-DQ2+ and reach at least III in Marsh scale for duodenal biopsy. All patients had normal IgA levels, except one that presented IgA deficiency, but clear anti-tTG2 antibodies. In the GFD group, average time under diet was 6.42 years (s = 6.76 years) and ranged between 1.17 and 12.42 years (**Table 1**).

Epithelial Integrity of the Oral Mucosa Is Compromised in Celiac Disease Patients

Protein expression levels of epithelial junctional proteins were studied by IHC staining in histological sections of the oral mucosa. Paraffin-embedded samples from all subjects included in the study were stained targeting occludin and claudin-1, members of the tight junctional complex, and E-cadherin, member of the adherens junctions. We found a significant decrease in E-cadherin expression in both groups of CD patients: *de novo* diagnosed (−20.34 ± 5.30%, $p < 0.01$) and GFD patients (−20.58 ± 5.08%, $p < 0.01$) compared with non-celiac controls (**Figures 1A, B**). Claudin-1 was also significantly decreased in both groups of CD patients when compared to control subjects (*de novo* diagnosed −12.97 ± 2.99%, $p < 0.01$ and GFD −11.81 ± 2.99%) (**Figures 1C, D**). For occludin, a non-significant trend was observed (**Figures 1E, F**).

We also determined mRNA expression of the epithelial junctional protein occludin, and periplakin (PPL), a member of desmosomes. We found increased mRNA expression in GFD patients for both occludin (+0.62 ± 0.20-fold change, $p < 0.05$) and PPL (+1.26 ± 0.41-fold change, $p < 0.05$) when compared to *de novo* patients (**Figures 1G, H**).

Celiac Disease Patients Present Higher Treg Infiltrate in the Oral Epithelium

Oral mucosa biopsies were stained for common T lymphocyte and antigen-presenting cell (APC) markers, such CD3, CD4, CD8, FoxP3, Langerin, and CD11c (**Figure 2** and **Supplementary Figure 2**). We found no significant differences in the absolute counts of $CD3^+$, $CD4^+$, or $CD8^+$ T cells among the experimental groups in either epithelium or the lamina propria (**Figures 2B, C**). However, when the preferential location of these cells was examined, significant differences were found among study groups. The percentage of $CD3^+$ cells located in the epithelium

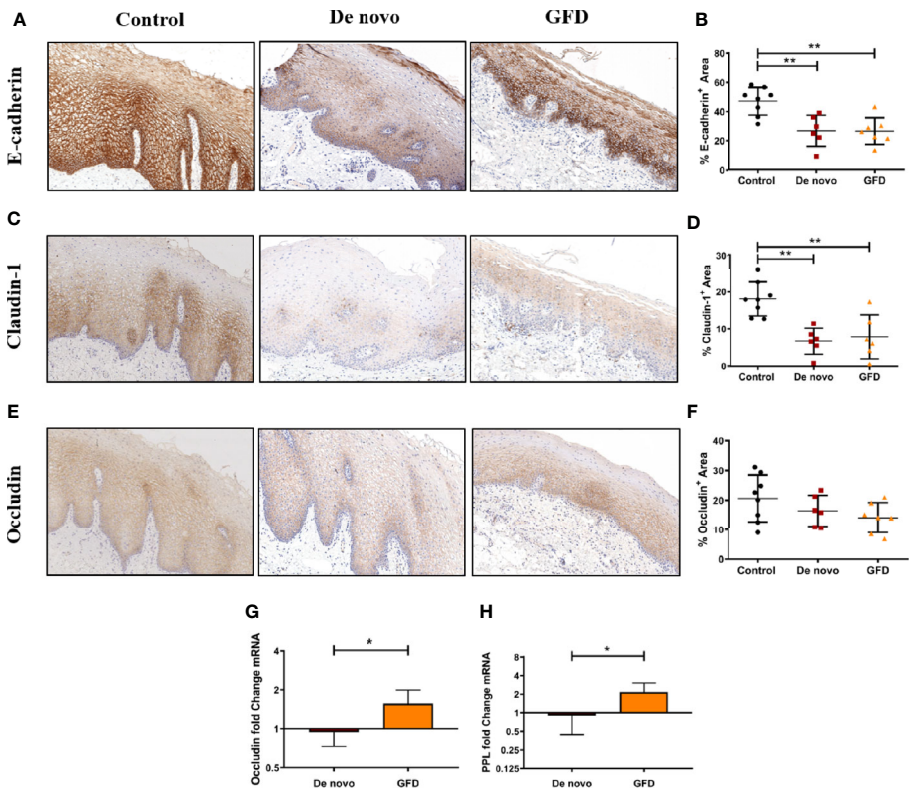

FIGURE 1 | Immunohistochemical staining of FFPE oral mucosal sections and quantification of protein expression from non-celiac subjects (control) (n = 8) and CD patients *de novo* diagnosed (*de novo*) (n = 6) and after Gluten Free Diet (GFD) (n = 7) for E-cadherin **(A, B)**, claudin-1 **(C, D)**, and occludin **(E, F)**. Images were captured at 8× magnification. Quantification is the percentage-stained area (mm^2) of the total epithelial area **(B, D, F)**. Scatter plots show mean ± SD *$p < 0.05$, **$p < 0.01$. Fold change expression of occludin **(G)** and periplakin (PPL) **(H)** in the oral mucosa for *de novo* diagnosed (n = 6) and GFD (n = 7) CD patients. Fold change is referred to non-celiac (control) samples using 2 -ΔΔCT method. Data were normalized using two housekeeping genes (GAPDH and β-actin). Bar plots show mean ± SD *$p < 0.05$.

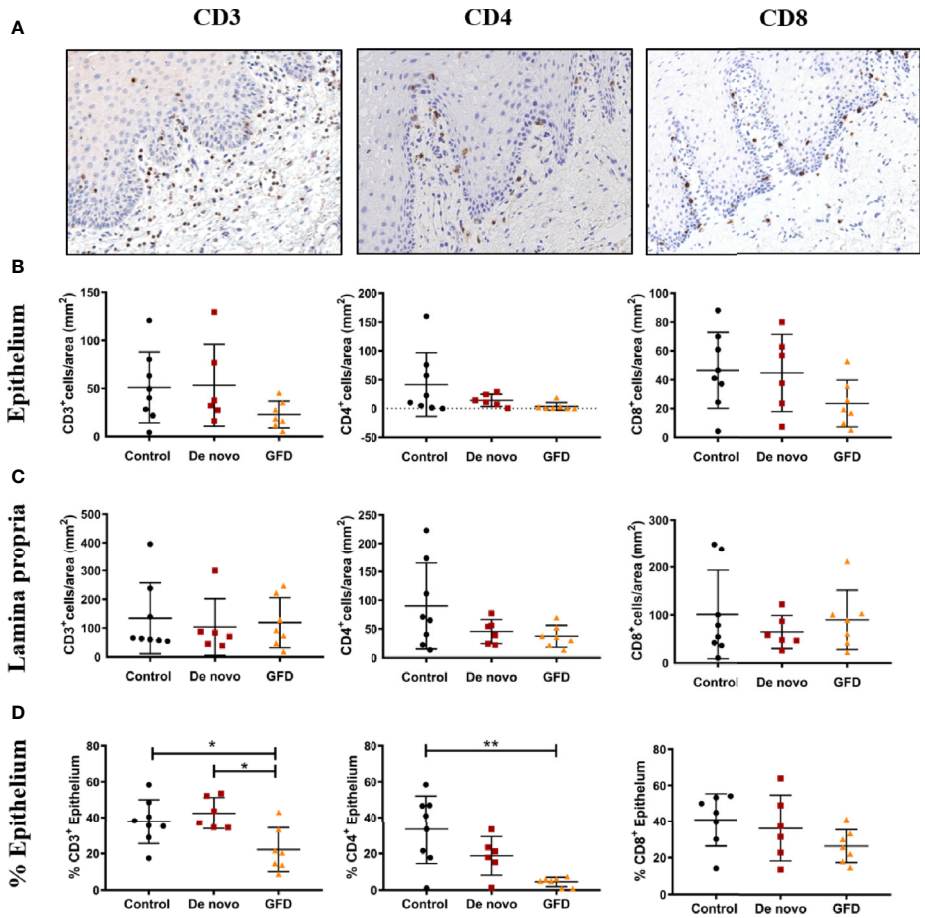

FIGURE 2 | Representative images of CD3, CD4, CD8 immunohistochemistry **(A)**. Images were captured at 15× magnification. Absolute counts in epithelium **(B)**, absolute counts in lamina propria **(C)**, and frequency of cells present in the epithelium (%) **(D)** for CD3+ (left), CD4+ (center), and CD8+ (right) from non-celiac subjects (control) (n = 8) and celiac disease patients *de novo* diagnosed (*de novo*) (n = 6) and after Gluten Free Diet (GFD) (n = 7). Scatter plots show mean ± SD *p < 0.05, **p < 0.01.

was significantly lower in GFD patients when compared to both control (−15.60 ± 5.86%, p < 0.05) and *de novo* subjects (−20.32 ± 6.30%, p < 0.05) (**Figure 2D**). $CD4^+$ presence in the epithelium was also significantly lower in GFD patients but only when compared to control subjects (−28.91 ± 7.27%, p < 0.01) (**Figure 2D**). $CD8^+$ cells showed no significant differences among experimental groups (**Figure 2D**). These lymphocytes lacked γδ T cell receptor expression (**Supplementary Figures 2A, E**).

Fox p 3+ cell numbers in the epithelium were negligible in most cases (data not shown). $FoxP3^+$ Treg counts in the whole mucosa were significantly higher in both *de novo* diagnosed (+11.54 cells/mm^2, p < 0.001) and GFD (+8.04 cells/mm^2, p < 0.05) CD patients as compared to the non-celiac (control) group (**Figures 3A, B**). When the population of Tregs among the total $CD3^+$ and $CD4^+$ cells was examined, similar results were observed. $FoxP3^+/CD4^+$ ratio was increased in *de novo* diagnosed (+11.00%, p < 0.01) and GFD (+9.00%, p < 0.05) groups. $FoxP3^+/CD3^+$ ratio followed the same pattern and was also increased in *de novo* (+27.11 ± 4.58%, p < 0.0001) and GFD (+16.01 ± 4.58%, p < 0.01) groups (**Figures 3C, D**).

We also stained for the APC markers langerin and CD11c to characterize whether there was a predominant APC population associated to CD. No significant differences were found in these two markers among the experimental groups (**Supplementary Figure 3**). Anti-CD19, anti-neutrophil elastase and Luna staining revealed absence of B cells, low presence of neutrophils, and absence of eosinophils, respectively, in buccal mucosa tissue (**Supplementary Figure 2**).

Recruitment of Tregs to the Damaged Oral Mucosa Is Associated to the Upregulation of Amphiregulin

Next, we wanted to determine whether the recruitment of Tregs to the oral mucosa presented characteristic features. First, we found a significant positive correlation between E-cadherin expression and langerin expression in the oral epithelium (r = 0.57, p < 0.01) for all the experimental groups (**Figure 4A**). In contrast, E-cadherin levels negatively correlated to $FoxP3^+$ Treg numbers (r = −0.65, p < 0.01), i.e. the higher the damage in the epithelium (the lower the E-cadherin expression), the higher the

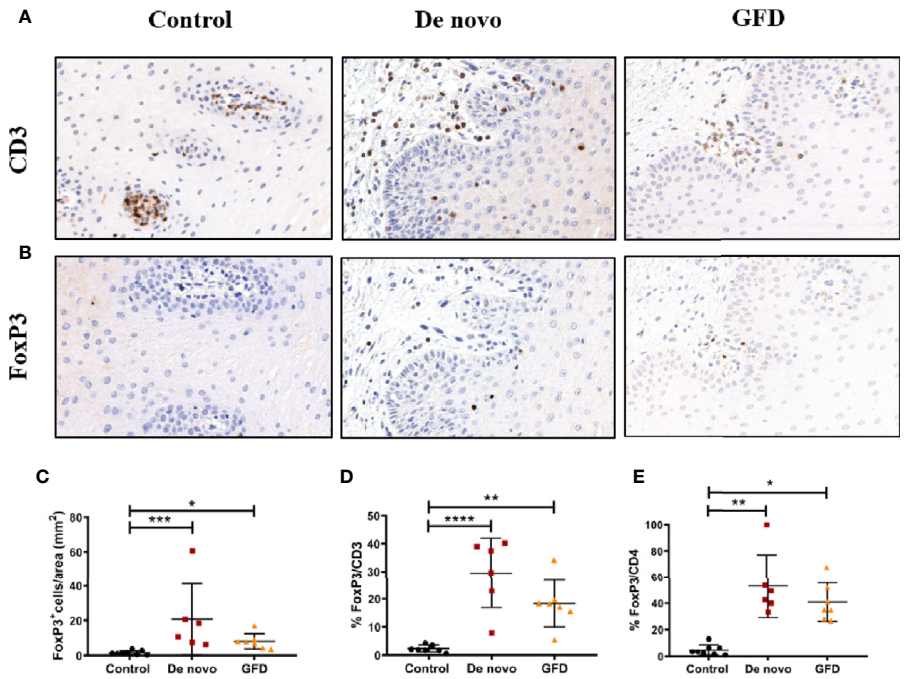

FIGURE 3 | Representative images of CD3 **(A)** and FoxP3 **(B)** staining of oral mucosal sections. Images were captured at 20× magnification. Quantification of FoxP3+ cells in the whole mucosa **(C)** for non-celiac subjects (control) (n = 8) and celiac disease patients *de novo* diagnosed (*de novo*) (n = 6) and after Gluten Free Diet (GFD) (n = 7). Quantification is expressed as counts per area (mm^2) of the total mucosa. Percentage of FoxP3+ cells in relation to CD3+ **(D)** or CD4+ **(E)** counting in the whole mucosa. Scatter plots show mean ± SD *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Treg infiltrate in the oral mucosa (**Figure 4B**). Moreover, Treg recruitment to the oral mucosa was found to be positively correlated with amphiregulin mRNA expression in PBMCs (r = 0.61, p < 0.05) (**Figure 4C**).

Markers Associated With Celiac Disease in the Intestinal Mucosa Are Not Increased in the Oral Mucosa

Next, we studied whether factors traditionally associated with intestinal IELs and inflammation in CD were also relevant in the oral mucosa of CD patients. For that, we determined IL15, IL15RA, IFNγ, and perforin mRNA levels in oral mucosa biopsies. Surprisingly, we found that all tended to decrease in CD patients, being this decrease significant for IL-15 in *de novo* diagnosed (0.55 ± 0.20 fold change, p < 0.05) and GFD (0.71 ± 0.19 fold change, p < 0.01) patients, and IFNγ expression in GFD patients (1.51 ± 0.55 fold change, p < 0.05) when compared to the control group (**Figure 5**).

IL33 Plasma Levels Are Increased in Celiac Disease Patients

Levels of the epithelial alarmins known to be released upon mucosal damage were determined in oral mucosa biopsies and in plasma samples of the study subjects. In oral mucosa biopsies, we did not find significant differences in mRNA expression for IL-33, TSLP, or periostin (*POSTN*) of CD patients (**Supplementary**

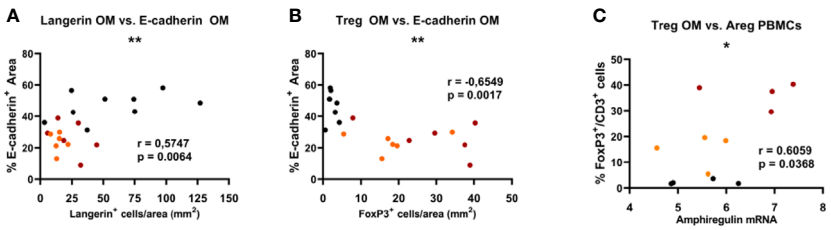

FIGURE 4 | Significant Spearman correlations found in the oral mucosa between the expression of epithelial E-cadherin and langerin **(A)**, and FoxP3 Treg frequency **(B)**. Significant correlations found in oral mucosa and PBMCs between the frequency of FoxP3 Treg cells in the oral mucosa and the expression of amphiregulin (Areg) **(C)**. Red dots represent *de novo* diagnosed patients, orange dots represent GFD, and black dots correspond to non-celiac (control) subjects. *p < 0.05, **p < 0.01.

Figure 4). Plasma levels of IL-33, IL-25, and TSLP were measured by ELISA. D*e novo* diagnosed (26.78 ± 10.21 pg/ml, $p < 0.05$) and GFD (27.53 ± 9.30 pg/ml, $p < 0.05$) CD patients presented significantly higher plasma levels of IL-33 as compared with non-celiac subjects (**Figure 6A**). There were no significant differences between the two groups of CD patients. We could not find any differences among the experimental groups for IL-25 or TSLP, although a trend of higher TSLP plasma levels was observed for both groups of CD patients when compared to controls ($p = 0.059$) (**Figures 6B, C**). We also determined PAF levels, as it is known to induce IL-33 release. We could not find any differences between non-celiac and either of the CD groups ($p = 0.09$). Nevertheless, PAF plasma levels tended to be higher in *de novo* diagnosed CD patients when compared to GFD ($p = 0.09$) (**Figure 6D**).

DISCUSSION

Celiac disease is a chronic autoimmune enteropathy triggered by gluten intake. As such, immune responses in the intestinal mucosa have been profoundly investigated (1, 43). However, it is well known that CD may present in atypical forms including extra-intestinal manifestations, some affecting the oral mucosa (6, 7). In fact, the oral mucosa of CD patients was shown to react to gliadin challenge with increasing numbers of lymphocytes (44). However, studies examining oral histopathological findings in CD are conflicting (8). In the present study, we examined buccal mucosa biopsies from CD patients both *de novo* diagnosed and under GFD, with the aim of determining its potential role as an immunomodulatory site for CD IT.

An intact functional oral mucosal barrier is crucial in the maintenance of homeostasis as it protects the mucosal immune system from the exposure to noxious environmental antigens (45). In our study, we have found that the expression of intercellular junctional proteins that are critical for epithelial integrity was altered in the buccal epithelium of CD patients even after avoidance of gluten for at least 1 year. In accordance, damage in the oral mucosa of GFD patients has been previously reported (6, 46). Unlike the previous studies that have focused on the characterization of the oral immune response, we have also examined the expression of intercellular junctional proteins to describe the remodeling of the oral mucosal barrier. This remodeling in the treated CD patients has been suggested not to result from poor dietary compliance, but rather as a late immune response reflecting chronic immune stimulation followed by regeneration of memory T cells (46). After gluten avoidance, mRNA levels of both occludin and PPL (a protein expressed in desmosomes and described to be a regulator of lung injury and repair) (47) are increased. These findings suggest that gluten depletion has a healing effect over the oral mucosa that is still not visible at the protein level. Therefore, an impaired epithelial barrier could account for the extraintestinal manifestations of CD observed in the oral cavity such as aphthous ulcers, even after the avoidance of gluten (48). In our study, all subjects were investigated by the gastroenterologist to ensure that no significant oral lesions were present in the buccal mucosa before enrolment. However, the overall health status of the oral cavity was not examined. Therefore, other alterations,

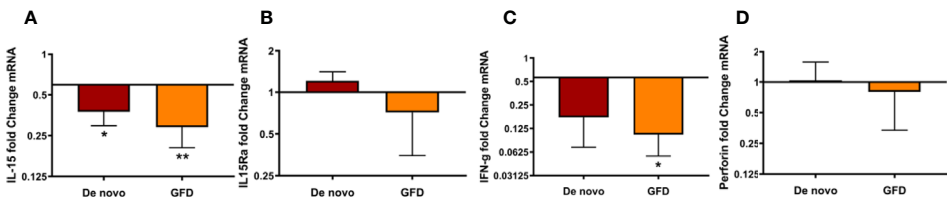

FIGURE 5 | mRNA expression of *de novo* diagnosed CD patients (*de novo*) (n = 6) and CD patients on GFD (GFD) (n = 7) for IL-15 **(A)**, IL15Ra **(B)**, IFNγ **(C),** and perforin **(D)**. Fold change is referred to non-celiac (control) samples using 2 -ΔΔCT method, data were normalized using two housekeeping genes (GAPDH and β-actin). Bar plots show mean ± SD *$p < 0.05$, **$p < 0.01$.

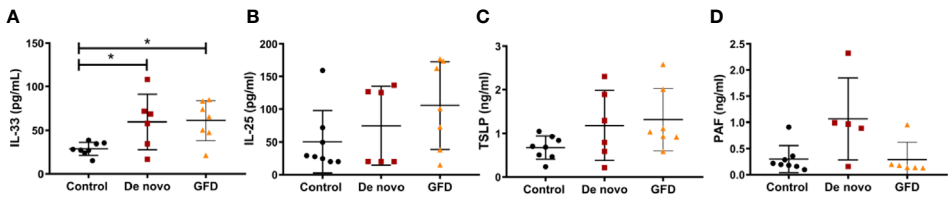

FIGURE 6 | Protein levels of IL-33 **(A),** IL-25 **(B),** TSLP **(C),** and PAF **(D)** in plasma samples of non-celiac subjects (control) (n = 8), *de novo* diagnosed CD patients (*de novo*) (n = 6), and CD patients on GFD (GFD) (n = 7). Scatter plots show mean ± SD. *p-value <0.05.

e.g. gingivitis or periodontitis, cannot be formally excluded, although none of the study subjects showed signs of oral disease at sampling.

Regarding the immune infiltrate in the oral mucosa of CD patients, there are not consensus studies (6, 8, 39, 40). In our study, we did not find significant differences in the global counts of langerin$^+$, CD11c$^+$, CD4$^+$, CD8$^+$, or CD3$^+$ cell populations. The study by Bardellini et al. describes an increased CD3 infiltrate in the stromal papillae that is decreased after avoidance of gluten (6). Although we could not see changes in absolute cell counts, we found that avoidance of gluten reduces the relative abundance of lymphocytes in the epithelium. Moreover, we also confirm that the vast majority of the IELs in the oral mucosa lack γδ-T cell receptor expression, in accordance with Lähteenoja et al. (46). These authors suggest that NK cells substantially contribute to lymphocyte recruitment; thus, NK cells may take part in the mucosa remodeling process. Although important in gut mucosa, we discard γδ-T cells as a biomarker in the epithelium of buccal mucosa of CD patients. In contrast, Krishnan S et al. found the presence of γδ+ cells at the gingiva in a mouse model of periodontitis. Moreover, these cells were producing amphiregulin for safeguarding the homeostasis of the oral mucosal barrier (49).

Interestingly, the population of Tregs was greatly increased in the oral mucosa of CD patients. However, previous research in circulating Tregs of CD patients suggests that they have an impaired suppressive function (50, 51). IL15, an important hallmark of CD, has been shown to perform a relevant role in Treg effect suppression (29, 52, 53). In the present study, we could not find an increase in IL-15 expression in the oral mucosa of CD patients. What we found is that Fox p3+ cell abundance inversely correlated with E-cadherin expression (i.e. the lower the E-cadherin expression, the more the Treg numbers in the buccal mucosa). Thus, we hypothesize that the Fox p3+ cells we observed in CD patients are recruited to protect against further tissue damage and maintain barrier integrity, as previously reported (28, 41). In fact, we found a positive correlation between Fox p3+ cell numbers in the oral mucosa and peripheral amphiregulin expression, which is a repair factor. Peripheral amphiregulin expression has been previously found associated to repair/remodeling features in other disease settings such as infant viral bronchiolitis (54). In this line, amphiregulin-producing pathogenic memory Th2 cells were found to control airway fibrosis resulting from chronic inflammatory stimulation (55). Besides, amphiregulin is described to restore integrity of damaged intestinal mucosa in murine models of acute graft-*versus*-host disease (aGVHD). In the study by Holtan S et al., high circulating amphiregulin levels reclassified patients into high risk subgroups helping further refine the aGVHD clinical risk score (56). Therefore, we propose that circulating amphiregulin could also be useful for the diagnosis of CD and helpful to establish an alternative classification score to the intestinal biopsy-based Marsh scoring system. Nevertheless, amphiregulin determination and Treg phenotypes in the oral mucosa deserve further study in the context of CD for solid conclusions.

We did not identify changes in langerin+ or CD11c+ cell counts in our study. However, langerin+ cells, described to have a tolerogenic role in the oral mucosa (57), correlated inversely with epithelial damage. This result supports the role of langerin+ cells as tolerance inductors.

Plasma cells have been recently identified as the most abundant gluten peptide MHC-expressing cells in the intestine of patients with active CD (58). Therefore, we investigated whether B cells were also more abundant in the oral mucosa of CD patients. Strikingly, our study revealed absence of B cells in the oral mucosa of CD patients. In addition and consistent with previous findings for allergic patients (25, 41), eosinophilic and neutrophilic infiltrates were negligible in the oral mucosa. According to Moutsopoulos et al., neutrophils are the gatekeepers of oral immunity. They can be found within the oral cavity, exhibiting varying levels of activation and functionality depending on the presence of oral inflammation. In patients with neutrophil defects, a dysregulated IL-17/Th17 response has been shown to drive immunopathology (19). Regarding eosinophils, there is limited evidence that they reside in the oral cavity, at least in the gingiva, in healthy individuals (19).

The immunomodulatory mechanisms taking place in the oral mucosa in CD support its potential role as a target for oral IT. The potential of the buccal mucosa as a drug delivery system has been previously reviewed (59) and trials have been made to develop an IT for CD (12, 60). Protein-based desensitization IT is widely used to treat allergic diseases (61). CD4+ Tregs are induced by peptide vaccination, which means that a sustainable induction of Tregs is responsible for the efficacy of this treatment (16, 62, 63). An important question we wanted to address was whether the inflammatory mechanisms taking place in the oral mucosa mirror those of the intestinal mucosa. In this regard, two studies have assessed the capacity of the oral mucosa of untreated CD patients to produce CD autoantibodies (64, 65). In our study, we assessed the expression of inflammatory factors such as IL-15, in the oral mucosa of CD patients. Surprisingly, IL-15 expression, along with that of IFNγ and perforin, was decreased. Therefore, the immunopathological mechanisms taking place in the oral mucosa of CD patients deserve further study.

In our study, the structural changes in the oral mucosa of CD patients take place with increased number of FoxP3 Tregs. Moreover, the inflammatory hallmark of CD in the gut (IL-15) seems to be absent in the oral mucosa. Furthermore, IL-33, TSLP, or POSTN mRNA levels are not elevated in buccal biopsies. Thus, we hypothesize that the oral mucosa remodeling observed may be a consequence of the systemic inflammation associated to CD. In fact, systemic immune deregulation is reflected in the function of the oral immune system (45). We, and others (66) have reported increased plasma levels of IL-33 in CD groups. Lopez-Casado et al. measured serum levels and determined intestinal expression of IL-33 and its receptor ST2 in patients with active CD, but not in patients following a GFD. They found that the higher levels of IL-33 and its receptor ST2 in the intestine and serum reflect an active inflammatory state. Thus, they suggest it may be a potential biomarker for CD. In this line,

Perez F et al. (67), found an increased expression of IL-33 in the duodenal mucosa of active CD patients. These findings highlight the potential contribution of IL-33 to exacerbate inflammation in CD pathology.

Based on our results, we conclude that oral mucosal integrity is compromised in CD patients, even after gluten avoidance. A remodeled epithelium may be key for the IT to gain access to both epithelial surfaces (apical and basolateral) and the local mucosa associated immune system, as previously suggested (25, 68, 69). Therefore, a disrupted epithelial barrier, together with the local recruitment of Tregs, make the oral mucosa a potential target for CD IT. These features could also help explain oral extraintestinal manifestations, and possibly assist CD diagnosis. Overall, our study highlights the relevance to characterize the specific immunopathological features of the oral mucosa in CD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research and Ethics Committees of San Agustin Hospital of Aviles. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LS, CDF, and JSS recruited the study subjects and conducted the biopsy and blood sampling. JSS, CPT, and CGC performed and analyzed the laboratory experiments. JSS, PS, DB, and CGC discussed the results and wrote the manuscript. All the authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.623805/full#supplementary-material

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Frequency of Gluten-Reactive T Cells in Active Celiac Lesions Estimated by Direct Cell Cloning

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Chronic inflammation of the small intestine in celiac disease is driven by activation of CD4+ T cells that recognize gluten peptides presented by disease-associated HLA-DQ molecules. We have performed direct cell cloning of duodenal biopsies from five untreated and one refractory celiac disease patients, and three non-celiac disease control subjects in order to assess, in an unbiased fashion, the frequency of gluten-reactive T cells in the disease-affected tissue as well as the antigen fine specificity of the responding T cells. From the biopsies of active disease lesions of five patients, 19 T-cell clones were found to be gluten-reactive out of total 1,379 clones tested. This gave an average of 1.4% (range 0.7% - 1.9%) of gluten-reactive T cells in lamina propria of active celiac lesions. Interestingly, also the patient with refractory celiac disease had gluten-reactive T cell clones in the lamina propria (5/273; 1.8%). In comparison, we found no gluten-reactive T cells in any of the total 984 T-cell clones screened from biopsies from three disease control donors. Around two thirds of the gluten-reactive clones were reactive to a panel of peptides representing known gluten T-cell epitopes, of which two thirds were reactive to the immunodominant DQ2.5-glia-α1/DQ2.5-glia-α2 and DQ2.5-glia-ω1/DQ2.5-glia-ω2 epitopes. This study shows that gluten-reactive T cells in the inflamed duodenal tissue are prevalent in the active disease lesion, and that many of these T cells are reactive to T-cell epitopes that are not yet characterized. Knowledge of the prevalence and epitope specificity of gluten-specific T cells is a prerequisite for therapeutic efforts that target disease-specific T cells in celiac disease.

Keywords: celiac disease, gluten, T cells, epitope, HLA, direct cloning

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INTRODUCTION

Celiac disease is a chronic inflammatory disease of the small intestine elicited by T-cell mediated immune response to dietary gluten. Almost all patients express the HLA-DQ2.5, HLA-DQ2.2 or HLA-DQ8 allotypes with HLA-DQ2.5 being expressed by more than 90% of the patients. The disease-associated HLA-molecules present gluten to CD4+ T cells. Gluten is a complex family of

Abbreviations: TCC, T-cell clone; SI, stimulatory index; TG2, transglutaminase 2; CPM, counts per minute.

proteins found in wheat. Currently, 27 distinct HLA-DQ2.5-restricted T-cell epitopes have been characterized from gluten and related proteins such as hordein (barley), secalin (rye) and avenin (oat) (1). CD4+ T cells reactive to these epitopes can only be found in the intestinal tissue of celiac disease patients, and not in controls including non-celiac gluten-intolerant patients (2, 3). All HLA-DQ2.5-restricted gluten T-cell epitopes contain one or several glutamic residues within the 9-mer core HLA-binding region. Native gluten has few glutamic acid residues, but contains over 35% glutamine residues (4). Certain glutamine residues in the native gluten can be post-translationally modified into glutamic acid residues by the action of transglutaminase 2 (TG2), in a reaction known as deamidation (5). The negatively charged glutamic acid residues increase the binding affinity of gluten peptides to HLA-DQ2.5 (6).

Among the multitude of HLA-DQ2.5-restricted gluten T-cell epitopes, only a few are known to be recognized by nearly all celiac disease patients. These immunodominant epitopes are the epitopes DQ2.5-glia-α1, DQ2.5-glia-α2, DQ2.5-glia-ω1, DQ2.5-glia-ω2, and DQ2.5-hor-3 (7, 8). T cells that recognize the other HLA-DQ2.5-restricted gluten epitopes are found occasionally in a minority of celiac disease patients. Despite decades of efforts in characterizing T-cell epitopes from gluten (reviewed in 1), there are still undiscovered T-cell epitopes in gluten, a fact manifested by T-cell clones that recognize deamidated peptic digest of gluten, but not any of the known epitope sequences. The proportion of gluten-reactive T cells that recognize hitherto uncharacterized gluten epitopes is largely unknown since few systematic efforts have been made in the past few years.

The fact that an unknown fraction of T cells from celiac disease patients recognize yet unknown gluten epitopes, impacts the estimation of the frequency of gluten-reactive T cells in the celiac intestinal tissue. Most studies use ELISPOT with overlapping peptides from a few well-characterized gluten proteins (8, 9) or tetramers presenting a limited number of the known gluten epitopes (10, 11) in their estimation of this frequency. Only one single study has reported the frequency of gluten-reactive T cells by direct cloning of T cells from the lamina propria of duodenal biopsies (10). This study, however, included only three treated patients, and two untreated patients. The average frequency of gluten-reactive T cells in this study was 1.0% (range 0.5% - 1.8%, four patients) in celiac lesion with Marsh 3, a histological score that indicates the most severe tissue damage. Yet other approaches such as cloning from polyclonal T-cell line can give some indication of the specificity distribution, including the proportion of cells that recognize unknown gluten epitopes. However, due to *in vitro* expansion of the polyclonal lines, this approach does not give the precursor frequency of gluten-reactive T cells in the celiac lesion.

In the current study, we have cloned T cells directly from the lamina propria of total nine subjects, of whom five were untreated patients with Marsh 3 lesion, one refractory celiac patient with Marsh 3, and three control subjects that do not have celiac disease. From the six celiac disease patients with Marsh 3 in the duodenum, 24 T-cell clones were found to be gluten-reactive of total 1,652 clones tested, giving an average frequency of 1.5% of gluten-reactive T cells. We also found that around one third of gluten-reactive T-cell clones from these patients only responded to deamidated gluten, but not to peptides containing any of the known HLA-DQ2.5-restricted gluten epitopes.

MATERIAL AND METHODS

Patients and Biopsies

Biopsies were taken as part of routine clinical follow-up or to investigate a suspected diagnosis of celiac disease. The regional committee for medical research ethics had approved the relevant protocols (REK 6544), and patients gave written consent before participating. One or two pieces of biopsies were taken from the descending duodenum for this study, and additional biopsies were taken from the duodenal bulb (bulbus duodeni) for diagnostic purposes. Patients received diagnosis based on the guidelines from European Society for the Study of Coeliac Disease (ESsCD) (12).

Duodenal biopsies were transported in sterile RPMI on ice. The epithelial layer was stripped off by two consecutive incubations with 5 ml PBS + 2% FCS + 2 mM EDTA, 10 min each in a rotating tube at 37°C. Single-cell suspension of the remaining lamina propria preparations was made by incubation with 10 ml PBS + 2% FCS + 1 mg/ml collagenase (Sigma) and 100 μg/ml DNase (Sigma), for 45 min at 37°C.

Cloning and Expansion

T cells were cloned and expanded in culture medium (RPMI (Gibco) + 10% human serum + 10 mM 2-ME (M-6250, Sigma) + penicillin/streptomycin) supplemented with 20 U/ml IL-2 (R&D systems), 1 ng/ml IL-15 (R&D Systems) and 1 μg/ml phytohemagglutinin (PHA, Remel), in the presence of 0.8 – 1 mill/ml irradiated (30 Gy) mixed allogeneic peripheral blood mononuclear (PBMC) feeder cells from 2-3 donors. For the initial cloning, single-cell suspension of duodenal lamina propria was washed, counted and re-suspended in the expansion medium containing the abovementioned ingredients. Cells in 20 μl were distributed into each well of the Terasaki plates (Nunc). Lamina propria cells were seeded at three different concentrations: 30, 10 and 5 cells per well. Growth was assessed microscopically after 10 days. Probability for clonal growth was calculated from the percentage of proliferating wells for each seeding concentration based on assumptions of Poisson distribution with the follow formula: $P_{clonal} = -\lambda(e^{\lambda}/(1-e^{\lambda}))$ where $\lambda = \ln(1-\text{observed frequency of growth})$. Proliferating T cells were transferred from Terasaki wells to flat-bottomed 96-wells containing 125 μl of the expansion medium containing freshly prepared PBMC feeder cells and cytokine as described above. Fresh culture medium containing IL-2 and IL-15 were added about every two days, and about half of the old medium was removed when necessary. Eight to 10 days after expansion in 96-wells, proliferating T cells were screened for reactivity to deamidated gluten antigen. T-cell clone (TCC) that showed proliferative response were further expanded in two 24-wells each, containing 1 ml expansion medium. These TCC were

further tested for gluten specificity and screened with a panel of peptides containing different HLA-DQ2.5-restricted gluten epitopes (**Supplementary Table 1**).

Screening by T-Cell Proliferation Assay

TCC were tested in proliferation assays where gluten or peptide antigens were presented by HLA-DQ2.5-expressing antigen-presenting cells. Gluten was prepared in-house from wheat flour and digested with chymotrypsin according to procedures described in (13). Gluten was subjected to TG2-mediated deamidation where 2 mg/ml gluten was incubated in 100 mM Tris + 2 mM Ca^{2+} with 50-100 µg/ml human recombinant TG2 for 2 h at 37°C. In each U-bottomed 96-well, 50,000 – 70,000 irradiated (75 Gy) HLA-DQ2.5 homozygous EBV-transformed B lymphoblastoid cells (CD114) were pre-incubated overnight at 37°C with native or deamidated wheat gluten (100 µg/ml unless otherwise stated) or synthetic peptides (10 µM unless otherwise stated) that contained HLA-DQ2.5-restricted gluten T-cell epitopes. On the following day, T cells were added and incubated for another 3 days. One µCi ^{3}H-thymidine (Hartman Analytics) was added 16-20 h before harvest and counting on a scintillation counter. Proliferative response was measured as stimulatory index (SI) defined as counts per minute (CPM) with antigen of interest divided by CPM with PBS only. For the initial screening of proliferating T cells from Terasaki wells, each TCC was tested in single well of either PBS or TG2-treated gluten (Gluten-TG2). TCC with SI above 2.5 from this single-well screening were selected for further expansion and testing. In subsequent assays, each antigenic condition was tested in either duplicates or triplicates. Only TCC that showed proliferative response (SI>2.5) to Gluten-TG2 or gluten peptide antigens in the subsequent re-testing was deemed gluten-reactive and included in the analysis.

Tetramer Staining and Flow Cytometry

Selected gluten-reactive TCCs were stained with FITC-CD8 (clone SK1, BD), PE-CD4 (clone SK3, BD), or corresponding isotype controls together with propidium iodide (BioLegend). TCC that were reactive to one of the four epitopes: DQ2.5-glia-α1, DQ2.5-glia-α2, DQ2.5-glia-ω1 or DQ2.5-glia-ω2, were stained with 10 ng/ml of PE- or APC- conjugated HLA-DQ2.5 tetramer presenting one of these four epitopes (11), for 40 min at room temperature. Samples were analyzed on a FACS Calibur II (BD) and data analyzed with FlowJo (BD).

RESULTS

T-Cell Clones Can Be Successfully Established by Direct Cloning of Lamina Propria Cells

In order to assess the precursor frequency of gluten-reactive CD4+ T cells in the active celiac disease lesion, we generated T-cell clones directly from unsorted lamina propria preparations of duodenal biopsies from nine individuals. These were subjects that were referred to gastroenteroscopy examination for suspicion of celiac disease. Five patients received the diagnosis of celiac disease based on Marsh 3 changes in histological examination; three subjects had none or minimal histological changes and did not receive the diagnosis. The last subject had refractory celiac disease, where gross morphological changes (Marsh 3C) persisted despite gluten-free diet (**Table 1**).

We successfully cultured T cells by directly seeding unsorted single-cell suspensions of lamina propria preparations from descending duodenal biopsies from all nine subjects, using an antigen-free cloning and expansion protocol. After 10 days *in vitro* culture in the presence of PHA, IL-2 and IL-15, we found wells containing growing T cells in frequencies that were directly correlated with the seeding concentration of unfractionated lamina propria cells (**Table 2**). In most cases, seeding 10 or 5 lamina propria cells per well resulted in the growth of T cells in less than 50% of the wells, which implied >70% likelihood that the T-cell populations in each well were clonal. For simplicity, we will refer to these cells as T-cell clones (TCC).

TABLE 1 | Patient characteristics and frequency of gluten-reactive T cells.

Patient	Gender	Age	HLA	Clinical status	IgA-TG2* (ref <3)	IgG-DGP* (ref <20)	Other clinical information	Histology*	Gluten-reactivity among T-cell clones (P_{clonal} > 70%)	
CD1334	F	62	DQ2.5	CONTROL	1.1	**26**		1	0/269¶	0.0%
CD1346	M	47	DQ2.5	CONTROL	**9**	10		1	0/370¶	0.0%
CD1350	F	56	DQ2.5	NCGS	0	0	2wks gluten challenge	0	0/345¶	0.0%
Total control subjects									0/984	0.0%
CD1329	F	25	DQ2.5	UCD	**5.8**	9		3B	5/286	1.7%
CD1335	F	54	DQ2.5	UCD	**23**	**57**		3B	2/271	0.7%
CD1336	F	49	DQ2.5	UCD	2.5	**41**	Graves' disease	3B	3/284	1.1%
CD1344	M	35	DQ2.5	UCD	**41**	12	1st relative with CD	3B	5/263	1.9%
CD1349	F	27	DQ2.5	UCD	<1	**100**	IgA deficiency	3C	4/275	1.5%
Total UCD									19/1379	1.4%
CD1348	F	56	DQ2.5	RCD	<1	10	CD diagnosis 2004	3C	5/273	1.8%

**Histology and serological results from the time point of sampling.*

¶All wells tested are included.

NCGS, non-celiac gluten sensitivity; UCD, untreated celiac disease; RCD, refractory celiac disease.

Serology results above cut-off values are denoted in bold.

TABLE 2 | Detailed cloning frequency.

	Terasaki seeding	Frequency of growth, cloning in Terasaki plates		P_{clonal}*	Frequency of growth, expansion in 96-wells		Frequency gluten-specific of tested T cells		Average frequency P_{clonal} > 70%		Average frequency all tested	
CD1334 (Marsh 1)	30/well	101/120	84%	35%	76/96	79%	0/45	0%			0/269	0%
	10/well	111/240	46%	**72%**	78/96	81%	0/78	0%	0/224	0%		
	5/well	211/1200	18%	**91%**	158/192	82%	0/146	0%				
CD1346 (Marsh 1)	30/well	98/120	82%	38%	92/96	96%	0/92	0%			0/370	0%
	10/well	98/210	47%	**72%**	92/96	96%	0/92	0%	0/278	0%		
	5/well	205/780	26%	**86%**	186/192	97%	0/186	0%				
CD1350 (Marsh 0)	30/well	117/180	65%	57%	99/103	96%	0/99	0%			0/345	0%
	10/well	99/300	33%	**81%**	84/96	88%	0/84	0%	0/246	0%		
	5/well	185/1200	15%	**92%**	162/185	88%	0/162	0%				
CD1329 (Marsh 3B)	30/well	202/240	84%	35%	95/96	99%	4/95	4.2%			9/381	2.4%
	10/well	143/300	48%	**71%**	95/96	99%	1/95	1.1%	5/286	1.7%		
	5/well	216/780	28%	**85%**	191/192	99%	4/191	2.1%				
CD1335 (Marsh 3B)	30/well	127/180	71%	51%	96/96	100%	2/96	2.1%			4/367	1.1%
	10/well	107/300	36%	**80%**	111/115	97%	1/111	0.9%	2/271	0.7%		
	5/well	176/1100	16%	**92%**	160/173	92%	1/160	0.6%				
CD1336 (Marsh 3B)	30/well	121/170	71%	50%	95/96	99%	4/95	4.2%			7/379	1.8%
	10/well	113/360	31%	**82%**	94/96	98%	1/94	1.1%	3/284	1.1%		
	5/well	195/1000	20%	**90%**	190/192	99%	2/190	1.1%				
CD1344 (Marsh 3B)	30/well	104/150	69%	52%	92/96	96%	0/92	0.0%			5/355	1.4%
	10/well	104/300	35%	**80%**	91/96	95%	0/91	0.0%	5/263	1.9%		
	5/well	198/1075	18%	**90%**	172/192	90%	5/172	2.9%				
CD1349 (Marsh 3C)	30/well	105/120	88%	30%	95/96	99%	2/95	2.1%			6/370	1.6%
	10/well	118/240	49%	**70%**	94/96	98%	2/94	2.1%	4/275	1.5%		
	5/well	227/840	27%	**85%**	181/192	94%	2/181	1.1%				
CD1348 (Marsh 3C)	30/well	107/180	59%	62%	94/96	98%	2/94	2.1%			7/367	1.9%
	10/well	105/360	29%	**84%**	95/96	99%	1/95	1.1%	5/273	1.8%		
	5/well	193/1192	16%	**91%**	178/192	93%	4/178	2.2%				

*When P_{clonal}, the probability that the growing wells is clonal, is larger than 70% (in bold), the wells are included in the calculations of frequency of gluten-specific T cells.

Gluten-Reactive T Cells Were Only Found in Duodenal Biopsies From Active Celiac Lesions

From each of the nine subjects, 269-381 TCC were expanded and screened for proliferative response to gluten. In the three subjects with little or no histological changes in the duodenum, we found no gluten-reactive T cells in any of the total 984 TCC screened. In comparison, from the six celiac disease patients with Marsh 3 changes, including one refractory CD patient, 38 TCC, or 1.7%, were gluten-reactive out of total 2,219 clones tested.

Since growth was detected frequently at the top seeding concentration of 30 lamina propria cells per Terasaki well, there is a high likelihood that many of these wells contained cells that originated from two or more seeded cells and thus were not truly clonal. As a consequence, the frequency of gluten-reactive T cells could thus be slightly over-estimated due to oligoclonal growth in some wells. To correct for this, we counted only proliferating T cells that were more than 70% likely to be clonal in the calculation of the frequency of gluten-reactive T cells. Among TCC with P_{clonal} > 70%, we found 19 TCC that were gluten-reactive out of total 1,379 clones tested from the five untreated celiac disease patients with Marsh 3 changes in the duodenum (**Table 1**). This gave an average of 1.4% (range 0.7% - 1.9%) of T cells in lamina propria of active celiac lesions that were gluten-reactive. In addition, in the refractory celiac disease patient who had Marsh 3C changes in the duodenum, we found five gluten-reactive TCC of 273 tested. Thus, despite gluten-free diet, the frequency of gluten-reactive T cells in this patient was found to be as high as that found in untreated celiac disease patients that were exposed to dietary gluten antigen. When data from the refractory patient was grouped with the five Marsh 3 samples from untreated celiac patients, 24 gluten-reactive TCC were found in total 1,652 P_{clonal} > 70% TCC tested, giving an average of 1.5% (range 0.7% - 1.9%).

Distribution of Gluten Epitope Specificity

All gluten-reactive T cells, including those generated with 30/well seeding in Terasaki wells, were screened against a panel of peptides that contained all known HLA-DQ2.5-restricted gluten epitopes (**Supplementary Table 1**), as well as 5 μg/ml recombinant TG2 in order to exclude any reactivity toward the TG2 component of TG2-treated gluten used in the initial screening. We found no TG2-reactivity in any of the T cells tested. Of the total 38 gluten-reactive TCC tested (**Table 3**), 12 TCC were reactive to deamidated gluten, but not to any of the peptides containing known gluten epitopes (**Figures 1A-C**). Of the 26 TCC where epitope specificity was ascertained, 17 were reactive to the immunodominant DQ2.5-glia-α1/DQ2.5-glia-α2 and DQ2.5-glia-ω1/DQ2.5-glia-ω2 epitopes (**Figure 2**) of which 15 were confirmed by specific staining with HLA-DQ2.5 tetramers presenting one of these four epitopes (**Supplementary Table 2** and **Supplementary Figure 2**), eight were reactive to various DQ2.5-glia-γ epitopes (**Figures 1D, E**)

TABLE 3 | Summary of epitope specificities of gluten-reactive TCC.

	G/TG only	α1 or α2	ω1 or ω2	α1 & ω1	γ3 or γ5	γ4	sec-3	TOTAL
CD1329	4	2	2		1			9
CD1335		1		2		1		4
CD1336	2	1	2	1			1	7
CD1344	2	2				1		5
CD1349	3	1			2			6
CD1348	1	1	1	1	1	2		7
TOTAL	12	8	5	4	4	4	1	38

G/TG, TG2-deamidated gluten digest; α1: DQ2.5-glia-α1; α2: DQ2.5-glia-α2; ω1: DQ2.5-glia-ω1; ω2: DQ2.5-glia-ω2; γ3: DQ2.5-glia-γ3; γ4: DQ2.5-glia-γ4; γ5: DQ2.5-glia-γ5; sec-3: DQ2.5-glia-sec-3.

and one was reactive to the DQ2.5-sec-3 epitope (**Figure 1F**). Of note, there was no significant difference between the magnitude of response in terms of SI values, between gluten only, α-gliadin and/or ω-gliadin, γ-gliadin or secalin specific T cells (**Supplementary Table 2**).

DISCUSSION

Gluten-reactive T cells are a hallmark of celiac disease. These cells recognize TG2-deamidated gluten presented on disease-associated HLA-DQ molecules and play pivotal roles in the pathogenesis of

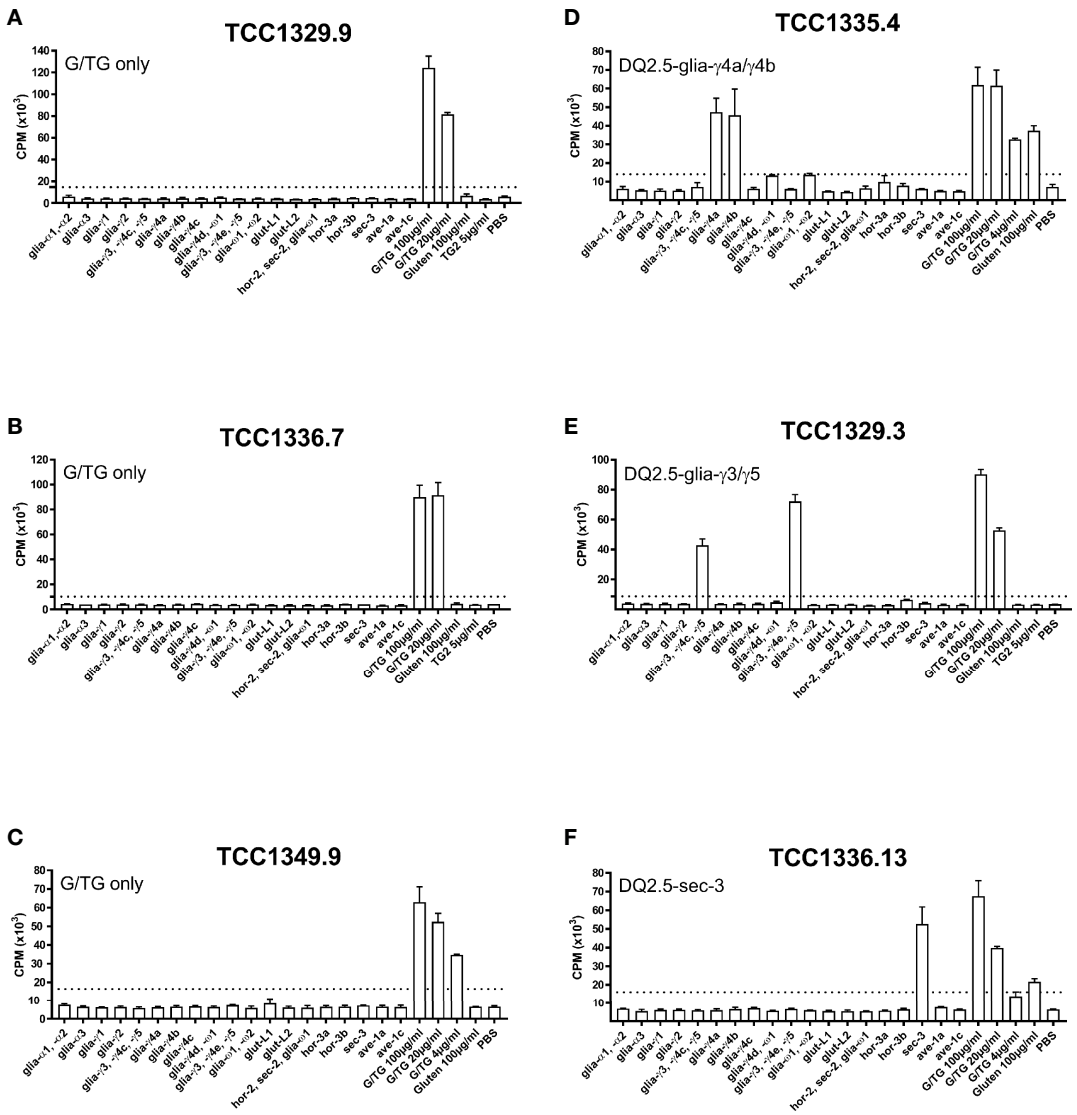

FIGURE 1 | Proliferation of T-cell clones that were specific to TG2-treated gluten only, various DQ2.5-glia-γ epitopes, or DQ2.5-sec-3. **(A-C)** Three T-cell clones from three different patients that responded to TG2-treated gluten digest only. **(D)** A DQ2.5-glia-γ4a and DQ2.5-glia-γ4b reactive T-cell clone. **(E)** A DQ2.5-glia-γ3 and DQ2.5-glia-γ5 reactive T-cell clone. **(F)** A DQ2.5-glia-sec-3 reactive T-cell clone. T-cell proliferation measured by ^{3}H-thymidine incorporation is visualized by CPM (counts per minute). Bars show average CPM response and standard error of the mean. Dotted line denotes the cut-off set at 2.5 times CPM with PBS. G/TG: TG2-treated gluten digest. All peptides were tested at 10 μM unless otherwise stated.

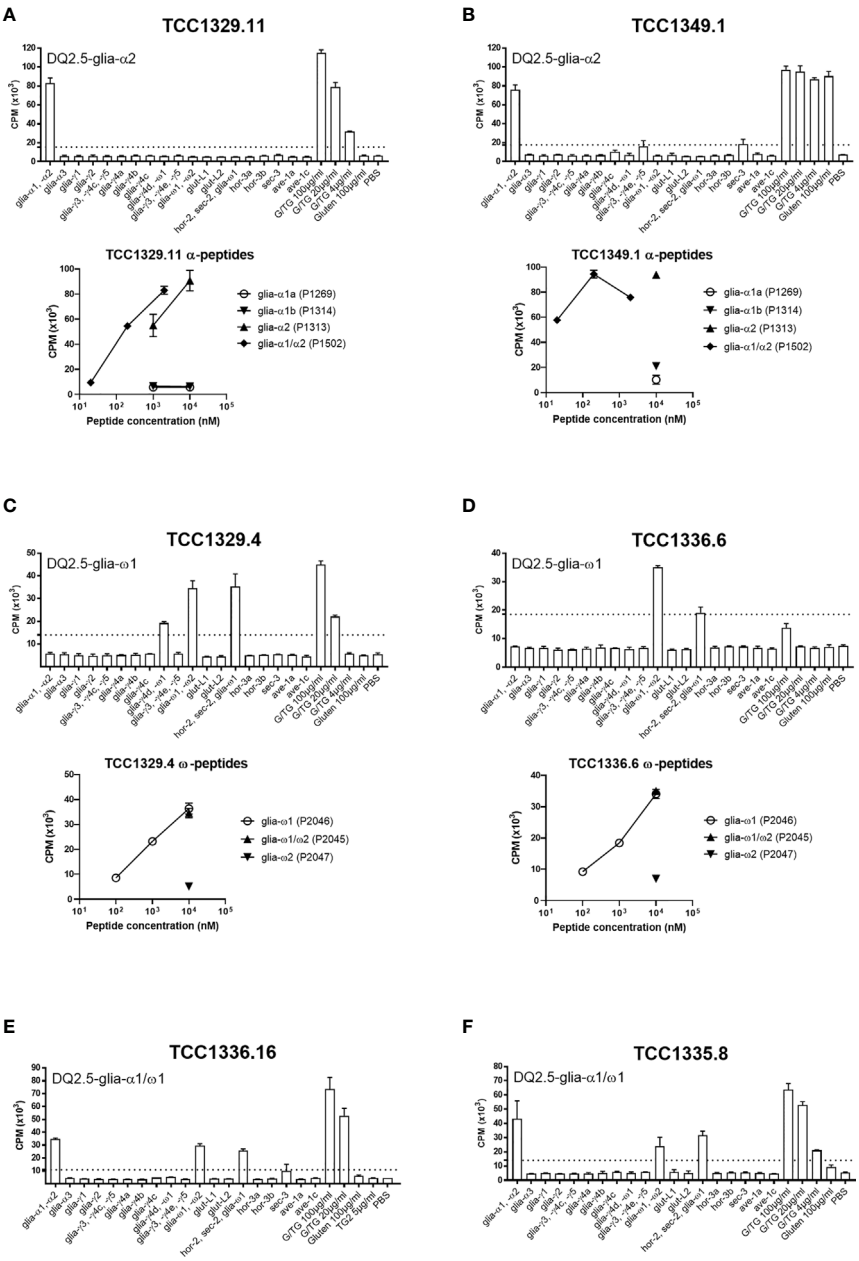

FIGURE 2 | Proliferation of T-cell clones that were specific to immunodominant DQ2.5-glia-α and DQ2.5-glia-ω epitopes. **(A, B)** Two T-cell clones reactive to DQ2.5-glia-α2. **(C, D)** Two T-cell clones reactive to DQ2.5-glia-ω1. **(E, F)** Two T-cell clones reactive to both DQ2.5-glia-α1 and DQ2.5-glia-ω1. T-cell proliferation measured by ^{3}H-thymidine incorporation is visualized by CPM (counts per minute). Bars show average CPM response and standard error of the mean. Dotted line denotes the cut-off set at 2.5 times CPM with PBS. G/TG: TG2-treated gluten digest. All peptides were tested at 10 μM unless otherwise stated.

celiac disease. In the current study, we have generated T-cell clones directly from lamina propria tissues and found that all six samples taken from tissues in active disease contained gluten-reactive T cells. In average, 1.4% (range 0.7% - 1.9%) of T cells from inflamed celiac tissue were gluten-reactive, compared to zero reactive clones of total 984 TCCs generated from non-celiac tissues. We found that almost half of the gluten-reactive TCC were specific to the four immunodominant DQ2.5-glia-α1/DQ2.5-glia-α2 and DQ2.5-glia-ω1/DQ2.5-glia-ω2 epitopes, whereas combined, only a quarter of the TCC were specific to any of the other 23 HLA-DQ2.5 restricted gluten epitopes. More surprisingly, we found that one third of the gluten-reactive TCC were specific to some not yet discovered epitopes in gluten.

To our knowledge, only one previously published study has estimated the frequency of gluten-reactive T cells by direct cloning (10). Of the four Marsh 3 patients investigated in this

study from 2013, the average frequency was 1.0% (0.5% - 1.8%) of all TCC tested. Our results are not statistically significantly different from this estimate. However, we could speculate that since the intestinal biopsies used in Bodd et al. (10) were mostly Marsh 3A, including from two subjects that were treated patients on gluten-free diet, less inflammation could be the cause of the somewhat lower frequency estimate found in that study.

Interestingly, a refractory celiac disease patient who had massive inflammation in the duodenum despite gluten-free diet, had gluten-reactive T cells with the same frequency as other untreated celiac disease patients with similar Marsh 3 changes. This finding is in accordance with Bodd et al. (10), where the frequency of gluten-reactive T cells estimated by direct cloning was correlated with the Marsh score, rather than with the gluten consumption status. Overall, our results further corroborate the notion that tissue injuries in celiac disease are driven by gluten-reactive T cells.

An important assumption for our estimate of gluten-reactive T cells based on *in vitro* cloning, is that all T cells have equal potential to grow under the *in vitro* culture conditions used. However, it is conceivable that recently *in vivo* activated gluten-reactive T cells could be less prone to proliferate *in vitro* due to activation-induced refractoriness. If that is the case, the frequency we have calculated would under-estimate the true frequency of gluten-reactive T cells. Similarly, our estimate would not include gluten-reactive cells that do not proliferate well under the *in vitro* culture and testing conditions used, including regulatory T cells. On the other hand, since 8%-29% of the TCC could in reality be oligoclonal as estimated by the growth in Terasaki wells, the frequency we have shown may slightly over-estimate the true frequency of gluten-reactive T cells. This last notion is supported by flow cytometry data where 7 of 37 gluten-specific TCC showed > 5% contamination with CD8+ T cells. Since the staining was performed after many rounds of expansion, the CD8+ T cells may have originated from unintended out-growth of feeder cells that is known to occur occasionally despite irradiation.

We have previously reported that gluten-reactive memory T cells persist for decades in celiac disease patient on gluten-free diet (11). A definite cure from the disease is therefore dependent on the eradication or 're-education' of existing gluten-reactive memory T cells that were primed and formed during the active disease phase. It is clearly a shortcoming of the current study that we did not assess the frequency of gluten-reactive memory T cells in treated celiac disease patients on gluten-free diet. As a method for assessing the frequency of gluten-reactive T cells, direct cloning is labor-intensive. Other methods such as tetramer staining is much less labor-intensive assuming the availability of the key reagents. The current study and Bodd et al. (10) have both found that around half of all gluten-reactive T cells are specific to one of the four immunodominant DQ2.5-glia-α or DQ2.5-glia-ω epitopes. By using this knowledge, the total frequency of gluten-reactive T cells can thus be extrapolated from tetramer-staining data.

It is a weakness of this study that TG2-deamidated gluten was used as the sole antigen during the initial screening. It is therefore likely that T cells that recognize hordein, secalin and avenin specific sequences did not pass this screening and were thus not included in the downstream analysis. This may explain the low prevalence of hordein, secalin and avenin specific T cells found in this study, where only one single DQ2.5-sec-3-specific TCC was found. T cells that recognize the DQ2.5-hor-3a epitope have been reported to be relatively prevalent in patients that consume barley (8). However, we did not find any DQ2.5-hor-3a TCC in our study possibly due to the limited variety of antigen that was used during the initial screening.

It was a surprise that up to one third of the gluten-reactive TCC apparently have unknown epitope specificity. In comparison, Bodd et al. found two TCC with unknown epitope specificity among 15 gluten-reactive in total (10). Decades of epitope hunting has resulted in an extensive list of total 27 HLA-DQ2.5-restricted gluten/secalin/hordein/avenin epitopes (1). Nevertheless, it is clear that there are still yet uncharacterized HLA-DQ2.5-restricted gluten epitopes. In our study, 'gluten-only' TCC was found in five of the six subjects, and was absent only in the subject that had the lowest number of gluten-reactive TCC (n=4). Future studies will show whether these TCC respond to a few commonly recognized novel epitopes, or a multitude of private epitopes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The regional committee for medical research ethics. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

S-WQ — Study design, data collection and analysis, and writing of paper. SD-K, LE — Data collection. KL — Procurement of materials, LS — Intellectual contribution to direction of study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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Missing Insight Into T and B Cell Responses in Dermatitis Herpetiformis

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Dermatitis herpetiformis is a cutaneous form of celiac disease manifesting as an itching rash typically on the elbows, knees and buttocks. It is driven by the ingestion of gluten-containing cereals and characterized by granular deposits of immunoglobulin A in the papillary dermis. These antibodies target transglutaminase (TG) 3 and in the majority of patients they are also found in circulation. The circulating antibodies disappear and skin symptoms resolve as a result of gluten-free diet but the cutaneous anti-TG3 IgA deposits may persist for several years. In dermatitis herpetiformis, plasma cells secreting antibodies against TG3 are located in the intestinal mucosa similarly to those producing TG2 antibodies characteristic for celiac disease. In fact, both TG2- and TG3-specific plasma cells and gluten responsive T cells are found in dermatitis herpetiformis patients but the interplay between these cell populations is unknown. The small bowel mucosal damage in celiac disease is believed to be mediated by co-operation of cytotoxic intraepithelial T cells and the inflammatory milieu contributed by gluten-reactive CD4+ T cells, whereas the skin lesions in dermatitis herpetiformis appear to be devoid of gluten reactive T cells. Thus, how celiac disease-type intestinal T and B cell responses develop into an autoimmune condition affecting the skin is still incompletely understood. Finally, the skin and small bowel lesions may reappear upon reintroduction of gluten in patients treated with gluten-free diet but virtually nothing is known about the long-lived B cell and memory T cell populations activating in response to dietary gluten in dermatitis herpetiformis.

Keywords: dermatitis herpetiformis, celiac disease, T cell, B cell, epitope spreading, transglutaminase

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INTRODUCTION

Dermatitis herpetiformis (DH) is a cutaneous form of celiac disease (CeD) usually presenting as a blistering, itching rash particularly on the elbows, knees and buttocks. Both manifestations are driven by the ingestion of dietary gluten in wheat, rye and barley, which induces an inflammatory response featured by B and T cell activation. DH is characterized by granular deposits of immunoglobulin A (IgA) in the papillary dermis, which is considered the primary diagnostic criterion for the disease (1). These antibodies target the human transglutaminase (TG) 3 and are also found in the circulation of the majority of DH patients (2, 3). The circulating antibodies disappear, and skin symptoms resolve on a gluten-free diet (GFD), the treatment of choice in DH,

while the anti-TG3 IgA deposits in the skin may persist for several years or even decades despite dietary adherence (2, 4, 5).

Regardless of the differing primary manifestations, DH and CeD share genetic susceptibility conferred by HLA-DQ2 or -DQ8 (6) and present often with partially overlapping features (**Table 1**). The great majority of untreated CeD patients are seropositive for gluten-dependent antibodies against gluten-derived gliadin peptides and autoantibodies against TG2 (anti-TG2 and endomysial antibodies), another member of the TG family of enzymes and the main autoantigen in CeD (7). Likewise, most DH patients are seropositive for TG2 autoantibodies (25). Moreover, approximately 30% of the CeD patients are also seropositive for TG3-autoantibodies while the corresponding number among DH patients has been shown to be considerably higher, ranging from 52-95% in reported studies (3, 16, 17). In addition, the vast majority of DH patients exhibit small-bowel mucosal tissue remodeling and damage, *i.e.* villous atrophy, crypt hyperplasia and inflammation, characteristic of CeD (26, 27). In this review, DH in patients either with or without villous atrophy was compared to CeD patients with no DH rash.

B CELL RESPONSES IN DH

Both DH and CeD are characterized by the occurrence of circulating TG2, gliadin and deamidated gliadin peptide antibodies (28). Despite this, the DH specific antibody response is considered to be targeted against the main autoantigen TG3 (2). However, circulating TG3-antibodies are found also in a subset of CeD patients without DH, but their significance in CeD is poorly understood. TG2, the main autoantigen in CeD, can both deamidate gluten peptides and form both iso-peptide linked and thioester-linked complexes with gluten, which are believed to drive TG2 autoantibody production in CeD (29). TG3 only forms enzyme-peptide complexes with lower affinity *via* thioester linkage (30). In addition, TG3 is also able to incorporate significantly fewer peptides per enzyme than TG2 (30). The differing end products resulting from TG2 or TG3-catalyzed reactions may explain the different dynamics of the autoantibody responses in CeD and DH. Furthermore, the complement of immunogenic gluten-derived peptides, which can act as TG3 substrates, the complement of T cell receptor subsets, and their impact on the development of B cell mediated immune response in DH have not been studied.

In CeD, TG2-antibody producing plasma cells are found in the small intestinal lamina propria (31, 32), although circulating antibodies may originate outside the intestine despite strong clonal relatedness between circulating and gut-derived autoantibodies (33). Intestinal plasma cells producing autoantibodies against TG2 have also been discovered in DH patients (14). Recently, the first studies on the occurrence of TG3 autoantibody producing cells in DH were published. *Ex vivo* cultures of duodenal biopsies as well as intestinal plasma cell stainings performed on DH patient tissue strongly suggest that TG3-antibody producing cells are present at least in the small intestine (14, 34). These cells seem to be highly DH-specific: despite the occasional occurrence of circulating TG3 antibodies, TG3 antibody producing plasma cells have only rarely been detected in CeD patients (14, 34). Furthermore, the TG3-specific plasma cells appear to be gluten-responsive as their frequency is increased during gluten challenge (14). However, according to current evidence, the presence of intestinal anti-TG3 plasma cells seems not to consistently correlate with the level of serum TG3 antibodies (14), raising the possibility that two or more subsets of autoantibodies with different plasma cell origins may exist in DH, as suggested for TG2 autoantibody producing plasma cells in CeD (33).

Supporting the hypothesis of a strictly DH-specific TG3 autoantibody plasma cell subpopulation, the number of intestinal TG3 autoantibody producing plasma cells detected in DH patients' gut biopsies using biotinylated TG3 to visualize TG3-specific antibody producing cells was not affected by preincubation with recombinantly produced TG2 (33). This suggests that these cells have a high specificity to TG3 alone. Likewise, CeD patients' recombinant monoclonal TG2 intestinal antibodies have been demonstrated to lack cross-reactivity with TG3 (35). Despite these findings implying very strict epitope specificities, it has been suggested that the multiple co-existing antibody populations would arise through epitope spreading, *i.e.* initial autoimmunity against TG2 would later expand to cover

TABLE 1 | Comparison of the features of dermatitis herpetiformis and celiac disease.

Feature	Dermatitis herpetiformis	Celiac disease	Reference
Primary autoantigen	Transglutaminase 3	Transglutaminase 2	(2, 7)
Dermal TG3-IgA deposits	100%	Not determined*	(8, 9)
Villous atrophy	Approx. 75%	Nearly 100%	(10)
CD3+/γδ+ IELs	70%/91% of patients	93%/93% of patients	(11, 12)
Intestinal TG2-IgA	79%	100%	(12, 13)
Intestinal plasma cells	Anti-TG2: detected Anti-TG3: detected	Anti-TG2: detected Anti-TG3: rarely detected	(14, 15)
Serum TG3-IgA	52-95% of patients	33-53% of patients	(3, 16–18)
Serum TG2-IgA	45-95% of patients	Nearly 100% of patients	(3, 17, 18)
Serum DGP-IgA/IgG	78%/78%	74%/65%	(19, 20)
HLA-DQ2/DQ8	86-100%/0-12%	88-95%/4-6%	(6, 21, 22)
T cell response	Not characterized	T_H1	(23, 24)

**IgA and TG3 deposits detected in some celiac disease patients (8).*

other closely related members of the same transglutaminase family (36). This hypothesis was supported by the pivotal work of Sárdy and colleagues (2) suggesting the possible existence of two distinct populations of TG3-antibodies: one highly specific to TG3 and present only in DH patients and the other recognizing both TG2 and TG3 and potentially present in both DH patients and CeD patients without DH. While the epitope spreading hypothesis remains plausible, testing it would first require identifying the specific TG3 epitopes recognized by the DH patient antibodies.

SYSTEMIC T CELL RESPONSES IN DH

The disease pathogenesis of both CeD and DH is considered to involve a major T cell component. In CeD, the gluten reactivity of intestinal T cells is strongly associated with the DQ2.5 molecule and the crosstalk between B cells and T cells reacting to covalently linked peptide-TG2 complexes is key in the generation of the TG2 autoantibody response. However, the actual intestinal epithelial cell destruction in CeD is mediated by intraepithelial cytotoxic CD8 T lymphocytes (23). The cascade of T and B cell driven events induced by the ingestion of gluten, is far less well characterized, however, in the case of DH although the early events occurring in the small intestine are thought to follow the same path in both manifestations (**Figure 1**.).

Only few studies have assessed the immune cell subsets and levels of T cell derived cytokines in DH. The peripheral T cell responses to gluten in GFD-treated DH and CeD patients following a gluten-challenge were assessed in a recent study (37). DH patient-derived peripheral blood mononuclear cells (PBMCs) responded to two CeD-associated gliadin peptides (DQ2-glia-α1a/α2 and DQ2-glia-ω1/ω2 peptides) in an interferon-γ ELISpot (enzyme-linked immunospot assay) qualitatively similarly to those obtained from CeD patients.

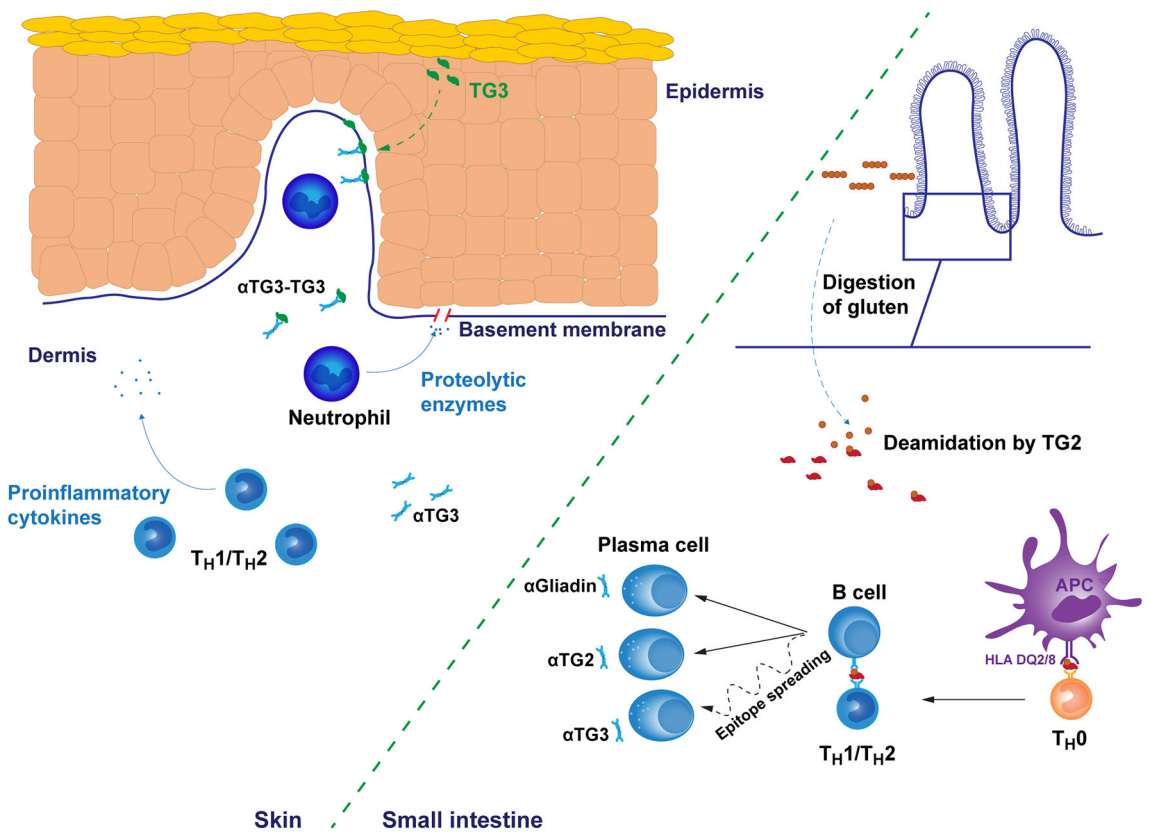

FIGURE 1 | Putative pathogenetic mechanisms of dermatitis herpetiformis. The early gluten-induced autoimmune response results in the production of autoantibodies targeting gluten-derived gliadin peptides and transglutaminase 2 (TG2) in the small bowel mucosa. Antibody specificity to transglutaminase 3 (TG3) may develop later by epitope spreading after initial autoreactivity against TG2. However, the dynamics of this processes are unknown. Immune responses are mediated by subtype 1 or 2 T helper cells. In the skin, IgA and TG3 form punctate structures, typically found in the dermal papillae. TG3 is endogenously produced in the upper layers of epidermis but may spontaneously diffuse towards dermis providing a possible explanation for the recruitment of autoantibodies into tissue bound immunocomplexes. Alternatively, IgA-TG3 immunocomplexes circulating free in dermatitis herpetiformis patient serum may bind to these sites. Tissue bound immunocomplexes attract neutrophil infiltration to dermal-epidermal junctions resulting in the cleavage of the basement membrane and finally in blister formation. The membrane cleavage is likely catalyzed by neutrophil secreted proteolytic enzymes targeting the fibrillar proteins of the basement membrane. αTG2/3, TG2/3 autoantibody, αGliadin, anti-gliadin antibody, APC, antigen presenting cell.

It is noteworthy, however, that while CeD is considered to be a strictly Th1-mediated disease (38), earlier studies suggest that this may not be the case with DH, *i.e.* the proportion of interferon-γ-secreting T cells among circulating immune cells might thus be low in DH patients. Th2-related cytokines in turn, such as interleukin (IL)-4 and IL-5 have been found to be overexpressed both in the skin and in the serum of patients with DH (24, 39). The exact nature of the gluten-induced T cell response thus remains to be ascertained.

SKIN LESIONS ARE DEVOID OF GLUTEN-REACTIVE T CELLS

The mechanisms underlying the skin lesions in DH are only superficially understood. The pathognomonic granular deposits of IgA co-localize with TG3 in the papillary dermis, and are located particularly in perilesional areas of the skin (2, 40, 41). TG3 is not endogenously produced by the cells lining the dermal-epidermal boundary and it is perplexing why TG3 and the autoantibodies precipitate to such persistent punctate structures at these loci. Hypothetically, the TG3-IgA immunocomplexes found in the circulation of DH patients (42) may simply adhere to structural proteins, for instance fibrinogen (40), potentially substrates for TG3, of the dermal papillae. Alternatively, TG3 may diffuse from the epidermis to the dermis, where it would be bound by circulating TG3 antibodies (43). Moreover, it is unclear why dermal TG3-IgA deposits may persist for years despite strict adherence to GFD and faster disappearance of detectable levels of circulating TG3-antibodies (1, 5). Prolonged clearance of the deposits is one possible explanation. However, the existence of small, persistent populations of antibody-producing cells in lymphatic tissue cannot be excluded since virtually nothing is known about the possible development of long-lived plasma cells or memory T cells in DH. For example, such long-lived TG2 antibody producing plasma cells populations discovered in CeD patients (44, 45) have not been investigated in DH.

It is noteworthy that the presence of the tissue bound TG3-IgA immunocomplexes alone appears not to be pathogenic, since these deposits are at times also detectable in skin areas of DH patients far away from the rash and also in the skin of DH patients in clinical remission (5, 46). Also, a few studies have presented granular IgA deposits-findings also in CeD patients without DH (8, 47, 48), but to our knowledge in only one study IgA was shown to co-localize with TG3 (8). Some of the early studies on the disease pathogenesis suggest that the formation of skin lesions in DH involves an influx of lymphocytes and macrophages (49, 50) but, contrary to the duodenum in CeD, the skin lesions appear to be devoid of gluten-reactive T cells (51). In a murine model of DH, the skin lesions develop virtually in complete absence of local CD4+ T cells, driven mainly by neutrophils and monocytes (52).

Indeed, the affected skin areas in DH have been shown to be infiltrated by neutrophils (53, 54), which have the ability to secrete proteolytic enzymes such as collagenases, elastases and granzyme B. These enzymes may be responsible for the disruption of connective tissue between the dermis and epidermis DH, resulting in blister formation (55). It is also noteworthy that dapsone, a drug that shows rapid clearance of DH rash (10) is a potent anti-neutrophilic agent (56, 57). Smith and colleagues showed that these dermal neutrophils have an increased ability to bind IgA *via* their Fc IgA receptors, indicative of prior priming (54). It is compelling to hypothesize that this neutrophil priming may occur in the inflamed intestine. The dermal immune infiltrate in DH also comprises at least αβ and γδ subtypes of T cells (58, 59). Increased numbers of intraepithelial αβ and γδ T cells in the small bowel mucosa is one of the hallmarks of both DH and CeD (11, 60) but whether the populations in skin and small bowel are linked remains an open and interesting question. The evidence at least for γδ T cells so far would suggest that this is not the case: Holtmeier and colleagues studied the TCR δ repertoires present in the inflamed duodenum, peripheral blood, involved and non-involved skin of DH patients and found that cutaneous TCR δ repertoires were oligoclonal, and that identical dominant γδ T cell clones were present throughout lesional and perilesional skin (61). Furthermore, the TCR δ repertoires of blood, the small intestine and skin were different and thus the cutaneous γδ T cells are not likely to originate from the inflamed duodenum.

The loss of tolerance to gluten and self-antigens may also be caused by impaired regulatory T (Treg) cell compartment. Loss of Treg suppressivity has been linked to CeD (62, 63) and the same impaired function could affect the cutaneous Treg population in DH. The potential role of T regulatory cells in DH pathology is supported by reduced levels of Foxp3+ Treg cells in DH patients' skin, as reported by Antiga and colleagues (64). This phenomenon has also been reported in other autoimmune disorders of the skin, such as systemic scleroderma (65) and bullous pemphigoid (66).

DISCUSSION

The understanding of DH pathogenesis has increased significantly in recent decades. The origins of the blistering skin condition are most likely in the inflamed small bowel, but it is unknown, how the autoimmunity progresses from the gut to the skin. Epitope spreading from initial immune response against TG2 to TG3 has been suggested as a possible mechanism (36). This is supported by the fact that DH generally tends to develop later than CeD and that CeD can progress into DH, particularly if dietary adherence is not optimal (67). In addition, CeD and DH patients have also been shown to develop antibodies against another closely related transglutaminase, TG6 (30, 36, 68). It is also noteworthy that such a pathogenic process may be exacerbated by a number of intrinsic and extrinsic factors. For example, ageing is considered detrimental to the functionality of T cell mediated immunity (69), but certain processes such as the impairment of the self/non-self-discrimination and subsequent accumulation of self-reactive memory B cells (70) may also contribute to the development of DH later in life. Furthermore, the impact of accumulating exposure to environmental stressors such as infections or environmental toxins has not been thoroughly studied in the case of DH, although it is known

that, for example iodine, exacerbates the skin lesions, potentially by causing aberrant activity of the skin immuno-complex associated TG3 (71). Finally, many of the potential environmental modulators are linked to the intestinal microbiota, and while gut dysbiosis has been tentatively linked to CeD (72), there have been no studies on how the maintenance of the microbial homeostasis throughout life might impact the development of DH.

The early T cell responses in DH in general are far less well understood than in CeD. The complement of gluten-derived immunogenic peptides, gluten-reactive T cell subsets and their receptor subsets in particular have not been thoroughly investigated. A plethora of cytokines, *e.g.* IL-8, IL-36 and IL-17 (73, 74) have been linked to DH, primarily by virtue of positive correlation between serum levels and disease status or gluten exposure. No conclusive evidence has so far been presented for their exact role in the disease pathophysiology, however. Neither have comprehensive systemic cytokine profiling studies been conducted on DH patients, such as that conducted by Goel and co-workers on CeD patients (75). Yet it is curious that CeD is considered a Th1-mediated autoimmune disorder, while Th2-linked cytokines dominate the molecular findings linked to DH. Whether such a profound difference between these two manifestations of the same disease truly exists, and the dynamics of a possible switch between effector cell subsets, could be an interesting novel avenue in DH research.

One distinguishing characteristic of DH is the pathognomonic granular IgA deposits in the dermal papillae of the skin. These IgA deposits are found particularly in non-lesional skin and thus the possibility of them being merely an epiphenomenon cannot be fully excluded, although their absence from the lesions could also be explained by phagocytic processes. With IgA being the predominant Ig class produced by the intestinal plasma cells, it is plausible that IgA antibodies in the dermal granular deposits originate from the gut. The cutaneous antibodies in DH appear to be dimeric (76) and predominantly of the IgA1 subclass (77, 78), the predominant subclass produced in the small intestine (79). Irrespective of their site of origin, the presence of the secretory component in the dermal immunocomplexes (76) implies that their transcytosis into circulation has been mediated by mucosal epithelial cells expressing the polymeric immunoglobulin receptor (80).

Two major questions thus remain unanswered as regards DH etiology: the origin of the cutaneous IgA deposits and their role in the development of the skin lesions. Very little is also known about the relationship between DH and other autoimmune bullous skin diseases. DH is often perceived primarily as an extraintestinal manifestation of CeD but understanding its immunology could be just as relevant for understanding similar autoimmune skin conditions and vice versa. For example, in a Finnish retrospective case-control study, patients with DH were found to have a 22-fold higher risk of developing bullous pemphigoid, another autoimmune blistering skin disease, compared to the only two-fold higher risk of subsequent bullous pemphigoid development among CeD patients (81).

Much of the work in understanding the cellular and molecular pathophysiology of DH dates back to the 1980s and 1990s and many of the questions could be and should be reassessed with modern methodology. A major hindrance in studying DH is recruitment of patients. Contrary to CeD, the incidence of DH is slowly declining with fewer than 10% of CeD patients developing DH (9). This phenomenon is interesting in its own right and most likely due to the increased awareness and improved diagnostics of CeD, resulting in fewer cases of untreated celiac disease developing into DH. Unfortunately, however, this otherwise positive trend also sets limitations for conducting clinical studies and limits access to patient material. Thus, long-term collaboration and careful coordination of research between clinics and research units is necessary. Furthermore, due to the multifaceted nature of DH, combining the expertise of clinicians from different fields of medicine with that of basic researchers is vital.

AUTHOR CONTRIBUTIONS

EK and KL conceptualized the manuscript. All authors contributed to the article and approved the submitted version.

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The Evolving Landscape of Biomarkers in Celiac Disease: Leading the Way to Clinical Development

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Celiac disease is a common immune-mediated disease characterized by abnormal T-cell responses to gluten. For many patients, symptoms and intestinal damage can be controlled by a gluten-free diet, but, for some, this approach is not enough, and celiac disease progresses, with serious medical consequences. Multiple therapies are now under development, increasing the need for biomarkers that allow identification of specific patient populations and monitoring of therapeutic activity and durability. The advantage of identifying biomarkers in celiac disease is that the underlying pathways driving disease are well characterized and the histological, cellular, and serological changes with gluten response have been defined in gluten challenge studies. However, there is room for improvement. Biomarkers that measure histological changes require duodenal biopsies and are invasive. Less invasive peripheral blood cell and cytokine biomarkers are transient and dependent upon gluten challenge. Here, we discuss established biomarkers and new approaches for biomarkers that may overcome current limitations.

Keywords: celiac disease, biomarkers, patient populations, diagnosis, disease monitoring, clinical development

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INTRODUCTION

Celiac disease (CeD) is a chronic disease mediated by a destructive immune response triggered by gliadin, a protein found in wheat, rye, and barley. The response to gliadin is characterized by activation of gliadin-specific T cells, anti-gliadin and tissue transglutaminase antibody response, and small intestine inflammation and damage to the epithelium resulting in a characteristic villous flattening (1). Biopsy-confirmed CeD currently has a worldwide prevalence of 0.7% and has been increasing in prevalence over the last 3 decades (2).

In most patients, eliminating gluten from the diet (gluten-free diet; GFD) reduces symptoms and recurring intestinal damage. However, for about 30% of patients, gluten restriction is not sufficient

Abbreviations: CeD, celiac disease; GFD, gluten-free diet; IEL, intraepithelial lymphocyte; γIFN, gamma interferon; IHC, immunohistochemistry; HLA, human leukocyte antigen; PBMC, peripheral blood mononuclear cell; PD, pharmacodynamic; RCD, refractory celiac disease; RCD1, RCD type 1; RCD2, RCD type 2; TCR, T-cell receptor; TTG-IgA, IgA antibodies against tissue transglutaminase; VCE, video capsule endoscopy; Vh:Cd, villous height:crypt depth.

to prevent symptoms or damage (3, 4). This lack of response is most commonly due to repeated inadvertent gluten exposure and/or high sensitivity to gluten at levels below what is considered 'gluten free' [20 ppm; (5)], but, in rare cases, may be related to refractory celiac disease (RCD). A diagnosis of RCD is based on continued intestinal damage and malabsorption after ≥ 12 months on a GFD. RCD1 is similar to active CeD with CD3+ polyclonal T cells comprising the majority of intraepithelial lymphocytes. Often, this disease type improves over time with strict adherence to a GFD. RCD2, in contrast, is characterized by the clonal expansion of aberrant intraepithelial lymphocytes that do not express surface CD3 or a T-cell receptor. These patients have a much poorer prognosis, with higher mortality and likelihood of progressing to enteropathy-associated T-cell lymphoma (3, 6–8).

There are currently no approved therapies for CeD; however, there are several therapies in development. Larazotide acetate (INN-202) is the most advanced program and is currently in phase III (ClinicalTrials.gov identifier: NCT03569007). Phase I/II programs include TAK-062 (ClinicalTrials.gov identifier: NCT03701555) and latiglutenase (IMGX003), which degrade ingested gliadin (9); PRV-015 (AMG 714), a monoclonal antibody that blocks IL-15, a cytokine associated with mucosal damage (10); and TAK-101, which elicits gliadin-specific immune tolerance. These therapies may target patients who are on a GFD but have ongoing symptoms and/or intestinal damage due to inadvertent gluten exposure. Currently, a diverse range of mechanisms is being investigated (11), and it is possible that selected therapeutics could be used for a broader segment of the patient population.

As the number of promising therapies for CeD grows, so does the need to measure therapeutic impact on clinically relevant endpoints and distinguish between different patient populations. This could be addressed, at least in part, by thoughtful biomarker selection. The Biomarkers, EndpointS and other Tools resource glossary (12) defines a biomarker as "*A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions. Molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers. A biomarker is not an assessment of how a patient feels, functions, or survives*". In this review, we describe the context of use and limitations of current biomarkers, and how new biomarkers under development may avoid these limitations and play an important part in the clinical development of new therapies.

PROGNOSTIC BIOMARKERS AND PREDICTING THE COURSE OF CeD

Prognostic biomarkers provide information about the likelihood of a clinical event, disease recurrence, or disease progression within a patient population (12). For example, a prognostic biomarker in CeD might predict if a patient had a greater likelihood of a negative response to gluten exposure, or was more likely to progress to a serious disease state such as RCD. Some, but not all, prognostic biomarkers are also predictive biomarkers, allowing the selection of patients who are more likely to have a favorable/unfavorable response to a specific therapy.

Human leukocyte antigen (HLA) class II has been proposed to be a prognostic genetic biomarker for CeD severity (**Table 1**) (9, 10, 13–30). Either HLA-DQ2.5 (encoded by the HLA-DQA allele, *HLA-DQA1*05* and HLA-DQB allele *HLA-DQB1*02*), HLA-DQ2.2 (*HLA-DQA1*02* and *HLA-DQB1*02* alleles), or HLA-DQ8 (*HLA-DQA1*03* and *HLA-DQB1*03:02* alleles) are present in almost all patients with CeD (31). HLA-DQ2 and/or HLA-DQ8, expressed on antigen-presenting cells, bind to immunogenic gliadin peptides and activate gliadin-specific CD4+ effector T cells (1). HLA-DQ2.5 binds and presents immunogenic gliadin peptides more effectively than HLA-DQ8 and HLA-DQ2.2 (32, 33). Homozygosity of the *HLA-DQB1*02* allele, which encodes the beta chain of HLA-DQ2, may impact the number of gliadin-specific T cells that are activated after gluten exposure. Patients with HLA-DQ2 who are homozygous for the *HLA-DQB1*02* allele appear more likely to respond to a gluten challenge with increased serum IL-2 and to have higher maximum serum concentrations of IL-2 than other genotypes (24, 34). In turn, this subset of patients may have a slower intestinal recovery rate after gluten challenge (14) and may be more likely to progress to RCD2 (35). These findings support *HLA DQB1*02* homozygosity as a determinant of gluten response and a potential prognostic biomarker for predicting the course of disease. Similar findings have not been associated with the gene for the alpha chains of HLA-DQ2.5, or HLA-DQ2.2, or the genes for the alpha or beta chains of HLA-DQ8.

The link between *HLA-DQB1*02* homozygosity and disease severity or progression to more complicated disease has not been seen in all populations (36, 37). Whether this discrepancy is a biologically relevant observation showing a lack of prognostic power in the number of *HLA-DQB1*02* alleles in these populations, or is a result of a small patient number, biased patient selection, or differences in study protocol, requires further investigation.

DIAGNOSTIC BIOMARKERS AND PATIENT SELECTION FOR CLINICAL TRIALS

In CeD, diagnostic biomarkers could be used to confirm that an individual has CeD and not another disease, such as irritable bowel syndrome, that clinically mimics symptoms of CeD (38, 39). They could also be used to distinguish between disease subtypes, for example patients with active CeD due to inadvertent gluten exposure versus patients with RCD1, just as current biomarkers allow for differentiation of RCD1 from RCD2 as described later (40). Distinguishing between patient subpopulations is important because some therapies, such as

TABLE 1 | Biomarkers used in celiac studies.

Biomarker	Biomarker type[a]	Context of use in clinical studies	Sample type (collected)	Assay[b]	Representative clinical studies[c]
HLA-DQ2 and/or HLA-DQ8	Prognostic Diagnostic	Confirm diagnosis	DNA (blood cells or cheek swab)	Molecular assay (13)	(10, 14)
Villous height:crypt depth ratio	Diagnostic Pharmacodynamic Monitoring	Confirm diagnosis Monitor response to therapy	Protein (mucosal biopsy)	IHC (15)	(9, 10)
IEL count	Diagnostic Pharmacodynamic Monitoring	Confirm diagnosis Monitor response to therapy	Protein (mucosal biopsy)	IHC (15)	(9, 10)
Celiac serology TTG-IgA level	Diagnostic Pharmacodynamic Monitoring	Confirm diagnosis Determine if gluten exposure has occurred	Protein (serum)	ELISA (16)	(9, 10, 17)
Number of HLA-DQ2 restricted gluten peptide binding CD4 T cells	Diagnostic Pharmacodynamic Monitoring	Gluten-specific T-cell response	Protein (blood cells)	HLA-tetramer binding measured by flow cytometry (18)	(19, 20)
Production of γIFN in response to *ex vivo* blood cell culture with gluten peptides (number of spot-forming units)	Pharmacodynamic Monitoring	Gluten-specific T-cell response	Protein (blood cells; PBMC)	γIFN ELISpot (21)	(20, 22)
Production of IP-10 in response to *ex vivo* culture with gluten peptides	Pharmacodynamic Monitoring	Gluten-specific T-cell response	Protein (blood cells; PBMC)	IP-10 ELISA (23)	(23)
Change in IL-2 with oral gluten challenge	Diagnostic Pharmacodynamic Monitoring	Gluten-induced immune response	Protein (serum or plasma)	Ultrasensitive ligand binding assays (24)	(20, 25)
Change in gut-homing γ δ T cells and CD8αβ T cells	Pharmacodynamic Monitoring	Gluten-induced immune response	Protein (blood cells; PBMC)	Mass cytometry or flow cytometry (26)	(20, 26)
RCD2 aberrant lymphocytes per total IELs	Diagnostic Pharmacodynamic	Confirm diagnosis Monitor response to therapy	Flow cytometry or IHC	Flow cytometry (27)	(28)
NKp46 positive IELs/100 epithelial cells Celiac minutes of enteropathy	Diagnostic Diagnostic Pharmacodynamic Monitoring	Confirm diagnosis Extent of villous damage	IHC Optical images	(29) VCE (30)	None (20)

IEL, intraepithelial lymphocyte; γ IFN, gamma interferon; IHC, immunohistochemistry; HLA, human leukocyte antigen; PBMC, peripheral blood mononuclear cell; RCD, refractory celiac disease; TTG-IgA, IgA antibodies against tissue transglutaminase; VCE, video capsule endoscopy.

[a]FDA-NIH Biomarker Working Group. BEST (Biomarkers, EndpointS, and other Tools) Resource [Internet]. Silver Spring (MD): Food and Drug Administration (US); 2016-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK326791/ Co-published by National Institutes of Health (US), Bethesda (MD).

[b]The listed assays are the most commonly used technologies, and the indicated references provide information on the technical development and/or an example of clinical validation.

[c]Representative clinical studies are studies where the indicated biomarker was included as part of the protocol and prospectively collected.

enzymes that degrade gluten, may be effective at blocking intestinal damage in a patient with disease driven by gluten exposure. However, a patient with RCD2, in whom intestinal damage occurs in the absence of gluten exposure, would have continuing pathological changes despite this type of therapy.

Currently, diagnosis of patients with CeD is based on serology, histology, and genetic biomarkers (e.g. HLA-DQ). In patients on a gluten-containing diet, detection of high titers of IgA antibodies against tissue transglutaminase (TTG-IgA) alone may be sufficient for a diagnosis of CeD (16, 41, 42). However, up to 5% of the Western population follow a GFD as a lifestyle choice (43), and, once a patient is on a GFD, antibodies to TTG and deamidated gliadin peptide subside and serology cannot be used for diagnosis. Confirmation of diagnosis in seropositive and seronegative patients is based on histology of duodenal biopsies and HLA typing (42). HLA-DQ2 or HLA-DQ8 expression alone is not sufficient for disease diagnosis because these are common HLA haplotypes, particularly in Western populations. Therefore, as diagnostic biomarkers, HLA-DQ2 or HLA-DQ8 alone have a negative predictive value of near 100%, but have a negligible positive predictive value (44, 45).

For clinical trial design, these biomarkers have been used in combination to help verify CeD diagnosis, for patient stratification and as part of assessment of disease activity (e.g. active disease, well-controlled disease). In the phase II studies for latiglutenase and larazotide, celiac serology was used to estimate gluten exposure and to identify patients likely to have active disease (9, 17). In addition to celiac serology, the latiglutenase and the AMG 714 phase IIa studies used duodenal histology to characterize epithelial damage, based on villous height:crypt depth ratio (Vh:Cd) and intestinal inflammation as assessed by intraepithelial lymphocyte (IEL) counts (10). In the latiglutenase study, patients were selected based on Vh:Cd of ≤ 2 and were stratified based on serology (9). The success or failure of these biomarkers to reduce variability and segregate patients into clinically meaningful and therapeutically important populations is difficult to assess because neither study met its primary endpoint. However, it is notable that in the latiglutenase study, a *post hoc* analysis found that seropositive patients preferentially showed symptomatic relief compared with seronegative patients (46).

The clinical manifestations of CeD vary widely between patients, as does the pathophysiologic response to gluten exposure. This variability presents a challenge to detecting therapeutic response in clinical studies. To directly reduce variability in patient response, gluten challenge has been incorporated into clinical trials (10, 47). By challenging patients with a specific gluten dose regimen, the temporal changes induced by gluten and the impact of therapy can be measured with a variety of disease-relevant biomarkers as described later. For this type of study, it is critical to know that a patient with CeD has the potential for a robust response to gluten. However, patients recruited into these studies are on a GFD for ≥ 6–12 months and are often intentionally selected based on negative celiac serology. Thus, only intestinal damage and HLA are currently widely available for use as indicators of disease status in these patients, and neither of these tests for a functional gliadin immune response.

One recent innovation, the HLA-DQ–gluten tetramer-based diagnostic assay, has been shown to differentiate patients with CeD from healthy controls, and to differentiate patients on a GFD from those who have recently ingested gluten. Overall, this assay is both sensitive and specific for identifying patients with CeD, regardless of diet. The HLA tetramers, major histocompatibility complex class II molecules loaded with immunogenic peptides, used in the assay have to be from the same HLA-DQ haplotype as those in the patient being tested. HLA-DQ2.5, HLA-DQ8, and HLA-DQ2.2 tetramers have been produced by academic groups, suggesting that the majority of patients with CeD could be tested (32, 48, 49). However, the assay takes a substantial amount of blood to perform, HLA-DQ tetramers are not commercially available and it is labor-intensive, suggesting it may not be feasible as a clinical diagnostic tool (50). A second approach exploits the fact that CD4 T-cell clonotypes are long-lived and persist for decades in patients with CeD (51). This assay sequences the rearranged T-cell receptor (TCR) β chain T cells and was shown to imply a diagnosis of CeD, based on TCR sequences common to patients with CeD (e.g. public sequences). The proof of principle was done using lamina propria T cells enriched with gluten-specific CD4 T cells; however, the ultimate goal of this approach will be to do the same assay in blood (52). The utility of these assays to confirm disease status as part of a clinical trial has yet to be tested.

Diagnosis of CeD subtype (uncontrolled CeD, RCD1, RCD2) is also key for the development of therapies, because the mechanisms driving each disease subtype may differ and, particularly for RCD2, which has a high mortality, the benefit–risk profile is quite different. RCD1 cannot be distinguished from gluten-induced active CeD *via* biomarkers. However, RCD2 can be identified based on biomarkers. The RCD2 IEL population is distinct from the polyclonal CD3+, CD8+ IELs associated with inflammation and damage after gluten challenge. RCD2 IELs are clonal TCR rearrangements and are positive for NKp46, tend to be CD8- and do not express surface CD3 or TCRs (29, 40). In a single phase IIa study, the safety and efficacy of anti-IL-15 (AMG 714) were tested in patients with RCD2. Patients were selected based on the percentage of aberrant IELs/100 total CD45+ IELs by flow cytometric analysis or > 50% aberrant IELs as measured by immunohistochemistry. This study did not meet the primary endpoint, reduction of aberrant intraepithelial lymphocytes from baseline measured at 12 weeks (28).

PHARMACODYNAMIC BIOMARKERS AND MEASURING THERAPEUTIC INTERVENTION IN CeD

Pharmacodynamic (PD) biomarkers measure the impact of therapeutic intervention on a biological process. In the development of therapies for CeD, PD biomarkers could be

used to evaluate therapeutic target engagement, gluten exposure or measure clinically meaningful endpoints, such as change in gluten-specific T cells and resolution of intestinal damage. Some PD markers can be tested serially to monitor drug-mediated changes over time and durability of therapeutic-induced responses to help build a rationale for a dosing regimen. A caveat for these PD biomarkers is that serial collection should have minimal impact on patient comfort or safety, thus, less invasive blood-based or imaging biomarkers are favored over duodenal biopsies.

PD biomarkers used in previous CeD clinical trials quantitatively measured changes in small intestine epithelial damage and inflammation by histology and evaluated immune response to gluten exposure by serology (9, 10). As a PD marker, histology has the advantage of measuring changes that are directly related to CeD processes, the influx of T cells into the epithelium, and the subsequent destruction of mucosal epithelium. Moving from the use of a subjective scoring system (such as Marsh–Oberhuber grade) to a quantitative evaluation of intestinal changes (e.g. measuring Vh:Cd ratio and IEL numbers), provides the sensitivity to detect relatively small, but clinically significant damage (15, 53). Vh:Cd is currently the standard for mucosal assessment in CeD clinical trials and is more reliable and responsive than traditional subjective histological measures. However, reliance on Vh:Cd has several limitations, including: mucosal biopsies are invasive and unsuited for serial testing, duodenal biopsy provides only a small representation of the entire disease area, expertise and significant time is needed to properly orient tissue sections, and Vh:Cd does not include a measure of lymphocytosis (34). Video capsule endoscopy (VCE) avoids these issues. It is less invasive than a duodenal biopsy and can be used as a method to monitor therapeutic impact while evaluating a much larger portion of the small intestine. VCE is unable to directly measure cellular changes, but rather records macroscopic changes in tissue (20, 30).

Anti-TTG antibodies have been used as a PD biomarker to understand immune response to gluten challenge (19, 20, 54) and have been incorporated into clinical trials for this purpose (9, 10). These antibodies are not considered by most to be pathogenic in the intestinal damage seen in CeD, but may be a contributor to extraintestinal manifestations, such as dermatitis herpetiformis or central nervous system lesions (55–57). Antibody response requires repeated gluten exposure, takes at least 2 weeks to appear after initial gluten challenge, and is still high 3–4 weeks after the last gluten exposure (20, 54). Although anti-TTG antibody measurement is useful for CeD diagnosis, it is certainly not a dynamic biomarker. In comparison, newer cytokine and cellular PD biomarkers are more responsive to gluten, with changes seen in days or hours after gluten challenge, quickly dropping to pre-gluten challenge levels. After a single gluten dose in patients on a GFD, levels of several inflammatory cytokines increase (58). IL-2 is one of the most consistently upregulated cytokines in patients and peaks 4 hours after gluten challenge, becoming undetectable in most patients by 6 days after initial gluten exposure (20). The presence of IL-2 in patients correlated with CeD symptoms, and no changes in IL-2 were seen in healthy participants with gluten challenge (58, 59).

Gluten-specific CD4 T cells are released into the blood 6 days after the start of a gluten challenge (20, 22). Gluten-specific T cells can be induced by *ex vivo* antigen challenge with gluten peptides and quantified by γIFN ELISpot, IP-10 ELISA or visualized by flow cytometry using HLA-DQ2 tetramers in combination with CD38 expression (60). The results of these assays correlate well with each other (22, 23, 50). As biomarkers for use in clinical trials, they have some pragmatic challenges: they require viable blood cells, reagents that are not commercially available, and large volumes of blood and CD4+ T-cell enrichment (tetramer assay only). However, the role of these gluten-specific CD4 T cells in CeD is clear, and a reduction in these cells would be highly suggestive of a disease-modifying effect.

Along with gluten-specific T cells that arise after gluten challenge, gut-homing CD8αβ T cells and γδ T cells that co-express CD103 and the activation antigen, CD38, also increase 6 days after the start of a gluten challenge. Although the role of these cells in CeD is less well understood, the gut-homing CD8 T cells are phenotypically similar to IELs found in patients with active CeD (26). The advantage of tracking these cells as a PD biomarker of active disease is that they are more plentiful in the blood and do not require pre-enrichment or cell culture prior to staining.

DISCUSSION

Translational medicine and biomarkers are becoming integral components of clinical development, contributing to trials by: 1) supporting dose and dose regimen selections for new therapeutic modalities that preclude traditional pharmacokinetic measures; 2) confirming unique therapeutic mechanisms of action; 3) providing proof of concept earlier in development; and 4) showing therapeutic efficacy in trials that require fewer patients. Because the etiology of CeD is better understood than that of most chronic inflammatory diseases, it has been possible to design biomarker assays that allow quantification of the earliest changes induced by gluten ingestion, tracking of the adaptive immune response, and evaluation of tissue inflammation and damage. However, biomarkers have some limitations in a real-world setting, and are only one approach to understanding disease progression and therapeutic efficacy (61). With advances in technology and the discovery of new biomarkers, it is possible that, in future studies, patient selection can be based on specific disease subtypes, or on prognosis, identifying the patient subpopulation most appropriate for the benefit–risk profile of a given therapy. From early clinical studies, pharmacokinetic data and data from PD markers can be combined to model the therapeutic dose response and gain a deeper understanding of the therapeutic mechanism of action.

AUTHOR CONTRIBUTIONS

GS, JM, and DL were responsible for the conceptualization of the manuscript. GS wrote the original draft. All authors reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Comprehensive Analysis of CDR3 Sequences in Gluten-Specific T-Cell Receptors Reveals a Dominant R-Motif and Several New Minor Motifs

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Gluten-specific CD4+ T cells are drivers of celiac disease (CeD). Previous studies of gluten-specific T-cell receptor (TCR) repertoires have found public TCRs shared across multiple individuals, biased usage of particular V-genes and conserved CDR3 motifs. The CDR3 motifs within the gluten-specific TCR repertoire, however, have not been systematically investigated. In the current study, we analyzed the largest TCR database of gluten-specific CD4+ T cells studied so far consisting of TCRs of 3122 clonotypes from 63 CeD patients. We established a TCR database from CD4+ T cells isolated with a mix of HLA-DQ2.5:gluten tetramers representing four immunodominant gluten epitopes. In an unbiased fashion we searched by hierarchical clustering for common CDR3 motifs among 2764 clonotypes. We identified multiple CDR3α, CDR3β, and paired CDR3α:CDR3β motif candidates. Among these, a previously known conserved CDR3β R-motif used by TRAV26-1/TRBV7-2 TCRs specific for the DQ2.5-glia-α2 epitope was the most prominent motif. Furthermore, we identified the epitope specificity of altogether 16 new CDR3α:CDR3β motifs by comparing with TCR sequences of 231 T-cell clones with known specificity and TCR sequences of cells sorted with single HLA-DQ2.5:gluten tetramers. We identified 325 public TCRα and TCRβ sequences of which 145, 102 and 78 belonged to TCRα, TCRβ and paired TCRαβ sequences, respectively. While the number of public sequences was depended on the number of clonotypes in each patient, we found that the proportion of public clonotypes from the gluten-specific TCR repertoire of given CeD patients appeared to be stable (median 37%). Taken together, we here demonstrate that the TCR repertoire of CD4+ T cells specific to immunodominant gluten epitopes in CeD is diverse, yet there is clearly biased V-gene usage, presence of public TCRs and existence of conserved motifs of which R-motif is the most prominent.

Keywords: celiac disease, T-cell receptors, gluten-specific T-cell receptors, CDR3 motifs, public T-cell receptors, R-motif

INTRODUCTION

Celiac disease (CeD) is a prevalent and autoimmune like disorder caused by a maladapted immune response to dietary cereal gluten in genetically predisposed individuals (1, 2). The majority of the patients (~90%) express the HLA-DQ allotype HLA-DQ2.5 (HLA-DQA1*05/HLA-DQB1*02), while the remaining express HLA-DQ2.2 (HLA-DQA1*02/HLA-DQB1*02) or HLA-DQ8 (3). These HLA-DQ molecules present deamidated gluten peptides to CD4+ T cells. On recognition of defined epitopes by their T-cell receptors (TCRs), the T cells become activated, and they then drive a pathogenic immune response by providing help to B cells to differentiate into antibody-producing plasma cells and by communicating with intraepithelial lymphocytes that kill enterocytes (4). Analysis of the TCR repertoires of T cells specific to given immunodominant gluten epitope has revealed V-gene bias and some examples of preferred usage of CDR3 motifs (5–15). The most striking feature has been observed in DQ2.5-glia-α2-specific T cells where dominant usage of TRAV26-1/TRBV7-2 and where many of these TCRs use a CDR3β motif with a conserved non-germline encoded arginine residue (ASSxRxTDTQY, so-called R-motif) and a CDR3α motif (NDYKLS) (5, 7, 11). So far, no comprehensive and comparative analysis of TCR repertoires and CDR3 motif usage by T cells specific for different immunodominant gluten epitopes have not been undertaken.

Here we have performed a systematic search for TCR CDR3 motifs which are prevalent and/or public (i.e. motifs shared by two or more individuals) by analyzing gluten-specific T cells that were isolated using a pool of HLA-DQ2.5:gluten tetramers representing the epitopes (DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1 and DQ2.5-glia-ω2) from peripheral blood or gut of a collection of HLA-DQ2.5+ CeD patients (11, 12, 16). From sequence analysis of more than 3000 gluten-specific T-cell clonotypes, we are able to describe TRAV and TRBV usage and the global picture of CDR3 motifs carried by T cells specific for these four immunodominant epitopes. The work is important as it lays the foundation for the potential use of gluten-specific TCRs as diagnostic markers of celiac disease.

MATERIAL AND METHODS

Generation and Analysis of Single-Cell TCR Sequences From Gluten-Specific CD4+ T Cells

We obtained T cells from a total of 50 HLA-DQ2.5+ patients comprising of patients in active disease state, in remission and patients undergoing gluten challenge recruited to several published/unpublished studies (**Supplementary Table 1**). The studies were approved by Regional Committee for Medical and Health Research Ethics South-East Norway (REK no. 6544).

The gluten-specific T cells were selected using HLA-DQ2.5: gluten tetramers from peripheral blood mononuclear cells (PBMCs), from lamina propria T cells of gut biopsies, or from *in vitro* cultured T-cell lines (TCLs) as described in previous study (16). Most T cells included in the analysis (82%) were isolated with a cocktail of four immunodominant gluten epitopes DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1 and DQ2.5-glia-ω2 (**Supplementary Table 2**). Single cell suspension generated from lamina propria of gut biopsies were stained with PE-conjugated HLA-DQ2.5:gluten tetramer/s (10 μg/mL) for 30–45 min at room temperature before adding antibody mixtures reactive with cell surface markers. Live, single, CD3+, CD11c−, CD14−, CD15−, CD19−, CD56−, CD8−, CD4+, CD8- and HLA-DQ2.5:gluten tetramer+ cells were then isolated by FACS. PBMCs were stained with PE-conjugated HLA-DQ2.5:gluten tetramer/s (10 μg/mL) for 30–45 min at room temperature prior to magnetic bead enrichment of tetramer binding cells followed by antibody staining. Live, single, CD3+, CD11c−, CD14−, CD15−, CD19−, CD56−, CD4+, CD45RA−, CD62L−, integrin β7+, and HLA-DQ2.5:gluten tetramer+ were sorted for TCR sequencing. TCLs were stained with PE conjugated HLA-DQ2.5:gluten tetramer (10 μg/mL) for 2 h at 37°C prior to adding antibody cocktail. We sorted live, single, CD3+, CD8−, CD4+ and HLA-DQ2.5:gluten tetramer+ cells. The following antibodies were used in the study: CD14-Pacific Blue (Biolegend), CD15-Pacific Blue (Biolegend), CD19-Pacific Blue (Biolegend), CD56-Pacific Blue (Biolegend), CD3-FITC (Biolegend) or CD3-Superbright 600 (eBioscience) or CD3-Brilliant Violet 510 (Biolegend), CD11c-Horizon V450 (BD Biosciences), CD4-APC-H7 (BD Biosciences), CD62L-PerCP/Cy5.5 (BD Biosciences), CD45RA-PECy7 (eBioscience), integrin-β7-APC (Biolegend), and CD8-PerCP (eBioscience). To exclude dead cells we used LIVE/DEAD marker fixable violet stain (Thermo Fischer Invitrogen). The FACS plot showing gating strategy for the isolation of T cells from blood, gut and TCLs is shown in **Supplementary Figure 1**.

Single T cells were sorted on 96-well plates and rearranged TCRα genes and TCRβ genes were amplified by multiplex PCR with V-gene specific primers described in detail elsewhere (16). Sequencing was performed on the Illumina MiSeq platform (250 bp PE) at the Norwegian Sequencing Center (Oslo University Hospital). The single-cell TCR-αβ sequencing raw data generated are available in European Genome-phenome Archive (EGAS00001003245) and NCBI's Sequence Read Archive database (SRP102399 and SRP102402) (12, 16), or as new deposits (EGAS00001005047) (**Supplementary Table 1**). A compilation of TCR nucleotide and amino acid sequences of 2918 clonotypes are available from the authors on reasonable request.

Raw Illumina sequencing reads were assembled into rearranged TCR sequences using the MiXCR (17) "analyze amplicon" macro (default settings). Subsequently, for each cell the three highest scorings in terms of read support, TCRα and TCRβ MiXCR sequences were submitted to the IMGT HighV-QUEST web portal (18), resulting in TCR annotation output that is compatible with our in-house Immune Receptor Information System (IRIS). IRIS is a data repository as well as a data analysis software. We upload the IMGT output files into the IRIS database followed by connecting the data with the metadata by assigning different dimensions (age, patient ID, status, tissue, well

ID, plate ID, library ID, etc.) to each of the sequences. This allows the user to choose any specific dataset based on these dimensions. Further filtering steps were carried out in the IRIS program in order to remove low quality or ambiguous sequences. Sequences with a read support of <50 were discarded. Valid cells were defined as comprising of one or two TCRα and TCRβ sequences, maximum three sequences. Dual TCRα or TCRβ were accepted based on read support proportions. The number and frequency of dual productive TCRα (median:6.9%) or TCRβ (median:0.2%) and unproductive TCRα (median:16.1%) or TCRβ (median:3.5%) in each patient is shown in **Supplementary Table 1** and **Supplementary Figure 2**. After the initial filter has been set up, IRIS will perform downstream analysis on the data set requested by the user on only the sequences that pass through the filter. So, using IRIS, users can choose specific data based on the dimensions, filter the sequences and the sequences can then be browsed directly, or used as the input when creating a report on V-gene usage, pairing and other relevant TCR analysis. Previously, we used pRESTO to process the raw sequencing reads (16), but here we have used MiXCR as it improved the processing pipeline and also increased the number of valid cells by roughly 10%. Clonotypes were defined based on identical TCRβ sequences (identical V (gene level) - and J genes and identical CDR3 nucleotide sequences); subsequent inspection confirmed that more than 99% of clonotypes were characterized by unique TCRα:TCRβ combinations. In total, 6627 valid cells from 50 patients gave rise to a total of 2918 clonotypes, ranging from 201 to 1 clonotypes per patient (**Supplementary Table 1**). Only the 34 patients with at least 20 clonotypes (2764 clonotypes in total) were included in the clustering and motif discovery analyses (**Supplementary Table 1**). The remaining 16 patients with less than 20 clonotypes per patient were included in assessing sharing of TCR sequences across patients.

V-gene usage for the single-cell sequencing libraries was exported from IRIS; here, sequences with ambiguous V gene calls were removed and the distinct V gene were counted. For paired TCR V-gene usage, the combinations of TRAV and TRBV genes were counted. In case of dual TCRα or TCRβ, both TRAV and TRBV gene combinations were included and each was counted 50%.

Hierarchical Clustering

For the hierarchical clustering we removed from the CDR3 sequences two amino acids from the N terminal (IMGT positions 105 and 106) and one amino acid from the C terminal (IMGT position 117) following the recommendation of Glanville et al. These authors demonstrated based on assessment of 52 ternary TCR-peptide-MHC structures that these TCR residues do not contact peptide antigen thereby leading to the recommendation that clustering analysis for shared specificity should be performed without these residues (19). Additionally, we removed all TCR sequences where the length of the remaining CDR3 was less than 8 amino acids. For paired TCR sequences with dual TCRα or TCRβ, one of the dual sequences was selected at random to represent the TCR. These operations reduced the size of the dataset from 2764 clonotypes to 2750 clonotypes.

Hierarchical clustering was done using the python "scipy.cluster.hierarchy" package, selecting average (UPGMA) linkage. Pairwise distances between amino acid TCRα or TCRβ CDR3s were calculated using the Levenshtein editing distance, using a value of 0 for matching positions and 1 for mismatches and indels. Pairwise distances between paired TCRα:TCRβ receptors were calculated as the sum of the TCRα and TCRβ Levenshtein distances.

Motif Discovery

New motif candidates were retrieved by examining all nodes of the hierarchical clustering dendrogram in a recursive, root-to-leaves ordering. Nodes below a given Levenshtein cut-off distance were assessed in terms of the V genes utilized by their leaf clonotype sequences. If all leaf clonotypes of a given node were utilizing similar V genes, and at least five leaf clonotypes were present, a new motif candidate was assigned to this node. In this case, the recursive search would stop. The required similarity of V genes was either defined as identical V genes (disregarding the allele numbers), or alternatively identical V-gene subgroups (as used by IMGT). Resulting motifs matching any of the established CeD motifs (**Table 1**) were removed from the list. For each remaining motif, a multiple sequence alignment was created using ClustalW (20); corresponding sequence logos were created using the WebLogo tool (21). In case of identical characters for a given column, the column character was added to the regex. If several distinct characters belonging to the same amino acid group; charged (K, R, E, D), polar (Q, N, H, S, T, Y, C), amphipathic (W, Y, M), hydrophobic (A, I, L, M, F, V, P, G) were present, a character group containing all amino acid residues for that group were added to the regex. If more than one amino acid group was represented, the position was added as an unrestricted wild-type character. If a column contains one or more gap characters, the amino acid character(s) for that position were marked as optional. Finally, all regex search terms were associated with the V gene used by the motif. Sequences constituting matches to a certain motif were required to match both the motif regex and express identical V gene. For paired TCR sequences, matches for both TCRα and TCRβ had to be found.

Linking Motifs to Epitope Specificity

In order to evaluate the epitope specificity of the new motifs obtained by hierarchical clustering, we looked into the TCRs of *in vitro* cultured gluten-specific T-cell clones (TCCs) with known epitope specificity. Over the years, we have generated TCCs reactive to different gluten epitopes from blood and gut samples obtained from 15 CeD patients of whom 13 are not included in the clustering analysis. The TCR sequences and relevant patient information for the TCCs used in this study is shown in **Supplementary Table 3**.The majority of the TCCs were generated from CD4 T cells sorted with HLA-DQ2.5:gluten tetramers by limited dilution cloning and antigen-free expansion. However, some of the TCCs were generated by cloning of T-cell lines generated from biopsies of CeD patients and the epitope specificity was confirmed by T-cell proliferation assays. Of the 328 gluten-specific TCCs, 231 TCCs expressed unique TCRs (the remaining 97 TCCs were sister clones) (**Supplementary Table 3**). We had 39, 111, 13 and 54 unique TCCs specific for DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1, and DQ2.5-glia-ω2, respectively. Further, we also had 10 and

TABLE 1 | CeD-relevant sequence motifs.

Motif name	Chain	Sequence	V gene	J gene	Pairs with	Frequency
R-motif	TCRβ	ASS.R.TDTQY	TRBV7-2 or TRBV7-3	TRBJ2-3	TRAV26-1 (87.6%) TRAV13-1 (1.3%) TRAV23/DV6 (1.3%) Others (9.8%)	291/2764 (10.5%)
Extended R-motif	TCRβ	ASS.R.*	TRBV7-2 or TRBV7-3	TRBJ2-3	TRAV26-1 (81.1%) TRAV14/DV4 (2.0%) TRAV13-1 (1.8%) TRAV23/DV6 (1.5%) TRAV4 (1.3%) Others (12.3%)	369/2764 (13.3%)
NDYKLS	TCRα	I.NDYKLS	TRAV26-1	TRAJ20	TRBV7-2 (88.6%)[$] TRBV20-1 (4.6%) TRBV11-2 (2.3%) TRBV5-1 (2.3%) TRBV7-3 (2.3%)	43/2764 (1.6%)
Paired R-motif	TCRαβ	ASS.R.TDTQY	TRBV7-2 or TRBV7-3/TRAV26-1	TRBJ2-3	N/A	

[$]*All of these TCRβ sequences contain the R-motif.*

The sequence patterns are displayed using regular expression syntax where "." Indicates any character and "." indicates any characters including no character.*

6 unique TCCs that were cross-reactive for DQ2.5-glia-α1a and DQ2.5-glia-ω1 epitopes, or DQ2.5-glia-α2 and DQ2.5-glia-ω2 epitopes, respectively.

Search of CDR3 Motifs in Public TCR Databases

We searched for all the CDR3 motifs in two different TCR databases, VDJdb repository (https://github.com/antigenomics/vdjdb-db/releases/tag/2020-01-20) and McPAS-TCR (22). These databases contain TCRs with specificity for known epitopes. TCR specific for CeD related epitopes were excluded prior to the search. Sequences matching a certain motif were required to match both the motif regex and possess identical V gene.

Immune-Receptor Generation Probability (IRGP) Calculations

The IGoR (23) and OLGA (24) programs were used for calculating IRGPs. For sequences with ambiguous V gene calls, one of the V gene possibilities were chosen at random to represent the sequence in question. Since sequencing reads were not long enough to allow confident allele identifications, the "*01" allele was assumed used for all V genes. IRGPs were calculated using a default IGOR installation, together with the default TCRα and TCRβ models included in (23). IRGPs for paired TCRαβ sequences (amino acid and nucleotide level) were calculated as the products of the TCRα and TCRβ IRGPs (25).

RESULTS

TRAV, TRBV and TRAV : TRBV Usage by Gluten-Specific TCRs in CeD

We analyzed the V genes used by gluten-specific T cells of 31 CeD patients. The T cells embodying altogether 2396 clonotypes were isolated using a cocktail of HLA-DQ2.5:gluten tetramers representing four immunodominant gluten epitopes (DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1 and DQ2.5-glia-ω2). For this analysis we excluded 20 patients where we isolated T cells using HLA-DQ2.5:gluten tetramers representing only one or two epitopes to avoid potential epitope-specific V-gene bias.

TRAV26-1, TRBV7-2 and TRAV26-1:TRBV7-2 were found to be the most prominently used V genes and V gene pair used by the gluten-specific T cells (**Figure 1**). The analysis revealed the biased expression of V genes that previously were described to be used by TCRs specific to one of these immunodominant epitopes such as TRAV26-1, TRAV4, TRAV35, TRAV12-2/3 for TRAV (**Figure 1A**), and TRBV7-2, TRBV29-1, TRBV20-1, TRBV5-1 and TRBV19 for TRBV (**Figure 1B**). Except for TRAV26-1:TRBV7-2 which was expressed in 12% of the clonotypes, the majority of the V gene pairs were expressed at low frequency (< 1%) indicating that the gluten-specific TCRs against these four immunodominant gluten epitopes express diverse TCRs with the exception of TRAV26-1:TRBV7-2 TCRs (**Figure 1C**). This suggested that the biased expression of TRAV26-1:TRBV7-2 is the most prominent feature of TCRs specific to HLA-DQ2.5-restricted immunodominant gluten epitopes in CeD.

Hierarchical Clustering on CDR3 Sequences Places the R Motif as the Most Prominent CeD Relevant Motif

In order to reveal TCR motifs associated with CeD, we performed hierarchical clustering on CDR3 amino acid sequences derived from 34 patients that had more than 20 clonotypes (2750 clonotypes in total). All clonotypes recognized one or more of the DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1 and DQ2.5-glia-ω2 epitopes presented on HLA-DQ2.5 (**Supplementary Table 2**). Clustering was done based both on the individual CDR3α and CDR3β sequences, and of paired TCRαβ receptors represented by their CDR3 sequences. Further, the pairwise distances between sequences were calculated using the Levenshtein editing distance.

The clustering dendrograms for CDR3β sequences revealed that the largest cluster (**Figure 2A**) observed at a level of average Levenshtein distance of ~1.85 contained R-motif (**Table 1**)

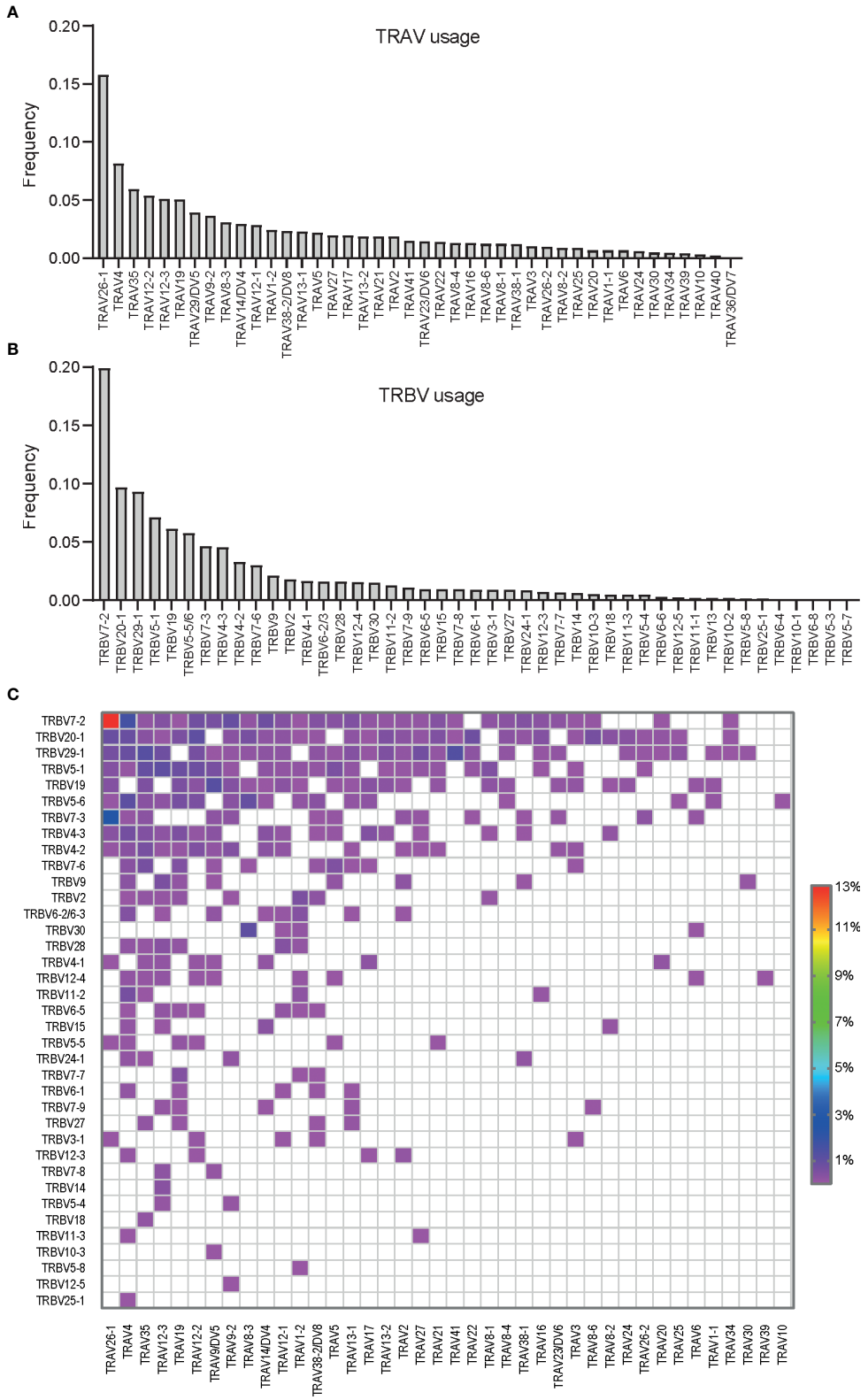

FIGURE 1 | The **(A)** TRAV, **(B)** TRBV and **(C)** paired TRAV : TRBV gene usage in T cells specific to immunodominant gluten epitopes isolated using a cocktail of HLA-DQ2.5:gluten tetramers representing four immunodominant gluten epitopes (DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1 and DQ2.5-glia-ω2). In total 2396 clonotypes from 30 CeD patients were analyzed where the V genes or V gene pair expressed in at least two clonotypes were included.

clonotypes in the dataset, and only 1.7% of the cluster clonotypes did not match this motif. Comparable clusters obtained by cutting the dendrogram tree at the same Levenshtein distance were considerably smaller, where the largest of which contained 27 clonotypes compared with 290 clonotype for the R-motif cluster.

Similarly, the largest cluster observed in clustering dendrograms for paired CDR3α:CDR3β sequences at a Levenshtein cut-off distance of 8.7 comprised mostly (85.3%) of paired R-motif (**Table 1**) and R-motif for TCRβs (91.1%) (**Figure 2B**). The relatively high Levenshtein editing distance of 8.7 compared to the TCRβ motif (**Figure 2A**) reflects the heterogeneity of the CDR3α sequences. However, if the dendrogram tree was cut at a distance of 2.4, the R-motif paired to the NDYKLS-motif was found to be the largest cluster (39 clonotypes) at that distance (**Supplementary Figure 3**).

Clustering the CDR3α sequences in the same manner as for CDR3β sequences above produced a dendrogram that did not contain any distinct clusters. However, at the cut-off distance of 1.85 (**Figure 2C**), we identified a cluster containing the NDYKLS-motif, with 56 sequences. This cluster was the largest CDR3α cluster at this cut-off distance as the other clusters contained 21 to 42 sequences.

Taken together, hierarchical clustering on CDR3 sequences revealed that the well-known R motif is by far the most prominent CeD relevant motif.

Hierarchical Clustering on CDR3 Sequences Reveals New Smaller Motifs

In order to identify potential new motifs, we looked for clusters of CDR3 sequences within a given Levenshtein distance with the additional requirement that all clonotypes within such a cluster should use the same V gene.

Performing a motif search using the CDR3α based clustering, 38 and 37 new CDR3α motif candidates for Levenshtein distance 1.0 (**Supplementary Figure 4A**) and 2.0 (**Supplementary Figure 4B, C, Supplementary Table 4**), were identified. For both distances, the largest new motif was about equal in size to the NDYKLS-motif.

Based on the CDR3β clustering, 25 and 32 new motif candidates were identified for Levenshtein distances of 1.0 (**Supplementary Figure 5**) and 2.0 (**Supplementary Figure 5B, C, Supplementary Table 5**). For both Levenshtein distances, the resulting new motif clusters were all substantially smaller than the R-motif cluster, which was represented with 290 sequences in the dataset.

Finally, we identified new motif candidates based on clustering of the CDR3α:CDRβ sequences, requiring identical V genes for both TCRα and TCRβ sequences. As we included both sequences, we doubled the allowed Levenshtein distances to 2.0 (**Supplementary Figure 6A**) and 4.0 (**Supplementary Figure 6B, C, Supplementary Table 6**). This yielded 18 and 27 new motif candidates, respectively. For a cut-off distance of 4.0, the largest new motif contained 16 sequences, substantially smaller than the number of receptors utilizing the paired R-motif (275). We also retrieved paired motifs based on identical V gene subgroups and increased the Levenshtein distance cut-off to 8.0 which resulted in 37 new motif candidates. Despite the increased Levenshtein distance, the largest new motif candidate still contained far less clonotypes than the paired R-motif cluster.

Taken together, we identified new CDR3 motif candidates used by the gluten-specific TCRs other that the R-motif. However, the occurrence of these novel motifs were much lower than the R-motif.

CDR3 Motifs Used by the Gluten-Specific TCRs in Public TCR Databases

As it would be of interest to learn whether the CDR3 motifs of the gluten-specific TCRs are unique to celiac disease, we searched the VDJdb and McPAS-TCR sequence repositories for presence of the motifs. Very few matches were found (**Table 2**). As expected, CDR3 motifs based on alignments with larger editing distances gave more matches. In addition, CDR3α motifs gave more matches than CDR3β motifs. No matches were obtained when searching with paired CDR3α:CDR3β motifs. Likewise, the R-motif and the NDYKLS-motif did not match any sequences. Of the gluten-specific TCRs, only 2.2%, 0.2% and 0% of the CDR3α, CDR3β and paired CDR3α:CDR3β motifs respectively matched sequences in the VDJdb repository. Similarly, the motifs that matched sequences in McPAS-TCR were 0.9%, 0.1% and 0%. These results may indicate that the novel CDR3 motifs, specially the CDR3β and the paired CDR3α:CDR3β motifs are fairly specific to CeD.

Linking Epitope Specificity to the Motifs Identified by Hierarchical Clustering

We wanted to pinpoint the epitope specificity of obtained novel motifs by comparing with the TCRs of the TCCs with known gluten epitope specificities (**Supplementary Table 3**) as well as single HLA-DQ2.5:gluten tetramers. We also looked for these motifs in the entire dataset comprising of single cell TCR sequencing data from 50 patients and TCR sequences from TCCs from 15 patients (**Supplementary Table 3**) and reported the prevalence in **Supplementary Tables S4-S6**. We found that 84% (31 of 37) CDR3α motifs (**Supplementary Table S4**) and 63% (20 of 32) CDR3β motifs (**Supplementary Table S5**) identified at Levenshtein distance of 4.0, could be linked to TCRs with known specificity. We found that out of 27 paired CDR3α:CDR3β motifs identified at a Levenshtein distance of 4.0, nine motifs were used by at least one gluten-specific TCC (**Supplementary Table 6**). Additional six motifs were linked to TCRs sequenced from T cells that had been isolated with single HLA-DQ2.5:gluten epitope tetramers. Further, three motifs were identified in TCRs of TCCs as well as T cells isolated with single HLA-DQ2.5:gluten epitope tetramers. As a result, we were able to identify 16 new paired CDR3α:CDR3β motifs for DQ2.5-glia-α1a (7), DQ2.5-glia-α2 (1), DQ2.5-glia-ω1 (1), DQ2.5-glia-ω2 (3) and DQ2.5-glia-α1a/DQ2.5-glia-ω1 (4).

Higher Number of Shared TCR Sequences Observed in Patients With Higher Number of Clonotypes

In order to identify TCR sequences that are shared across individuals (i.e. public sequences) among the gluten-specific T

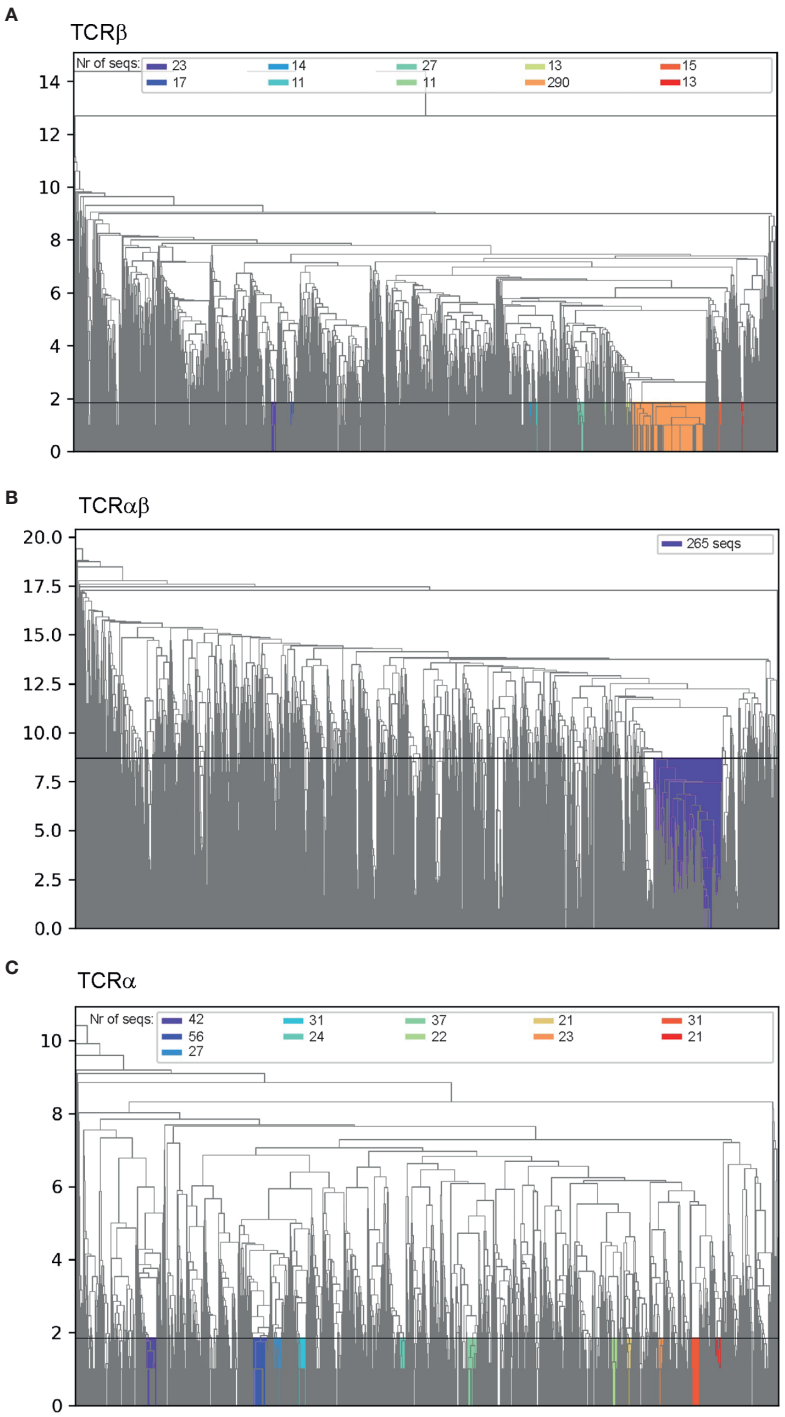

FIGURE 2 | Hierarchical clustering of **(A)** CDR3β sequences, **(B)** paired CDR3α:CDR3β sequences and **(C)** CDR3α sequences. The y-axis indicates the Levenshtein distance and the horizontal line shows distance that was used to cut the dendogram tree to generate the clusters. The clusters are colored and the respective number of sequences in each cluster are indicated in the legend.

cells, we analyzed TCR sequences derived from T cells of blood, gut biopsies, as well as *in vitro* cultured T-cell lines and T-cell clones obtained from 63 CeD patients. Collecting all sequences from these 63 CeD patients, we obtained 2996 unique paired TCRαβ sequences at amino acid level from a total of 3122 clonotypes (3109 were unique at the nucleotide level). The 2996 sequences were used to analyze for public TCRs. Shared TCRα and TCRβ sequences were found in 59 out of 63 patients.

TABLE 2 | The numbers of sequences from the VDJdb and McPAS-TCR that match the CDR3 motifs of gluten-specific TCRs.

	VDJdb (n=67689)			McPAS-TCR (n=34795)		
	CDR3α (n=28268)	CDR3β (n=39405)	CDR3α:CDR3β (n=22284)	CDR3α (n=11675)	CDR3β (n=13332)	CDR3α:CDR3β (n=7501)
R-motif	–	0	–	–	0	–
Paired R-motif	–	–	0	–	–	0
NDYKLS-motif	0	–	–	0	–	–
Motifs at distance=1	4[a]	1[c]	–	21[a]	3[h]	–
Motifs at distance=2	10[b]	4[d]	0[e]	21[g]	3[i]	0[e]
Motifs at distance=4	–	–	0[f]	–	–	0[f]

[a]*Sequence match with 2 out of 38 CDR3α motifs.*
[b]*Sequence match with 5 out of 37 CDR3α motifs.*
[c]*Sequence match with 1 out of 25 CDR3β motifs.*
[d]*Sequence match with 4 out of 32 CDR3β motifs.*
[e]*Sequence match with 0 out of 18 CDR3α:CDR3β motifs.*
[f]*Sequence match with 0 out of 27 CDR3α:CDR3β motifs.*
[g]*Sequence match with 2 out of 37 CDR3α motifs.*
[h]*Sequence match with 0 out of 25 CDR3β motifs.*
[i]*Sequence match with 0 out of 32 CDR3β motifs.*

The four remaining patients contained less than three TCRαβ clonotypes each making it less likely to detect public TCR sequences in these patients. We identified 325 TCRα or TCRβ sequences that were shared between 2 to 23 CeD patients (**Figure 3A**). Of the 325 public TCR sequences, 145, 102 and 78 were TCRα, TCRβ and paired TCRαβ sequences, respectively. Of the paired TCRαβ sequences, 42% were identical and 65% were highly similar to the TCRαβ clonotypes with known epitope specificity (**Figure 3B**, **Supplementary Table 7**).

We then assessed the contribution of the public TCRα and TCRβ sequences in the gluten-specific TCR repertoire of the individual patients. We found a higher number of shared TCRα or TCRα sequences in patients with higher number of TCRαβ clonotypes (**Figure 3C**). In order to investigate if the patients with higher number of TCRαβ clonotypes mostly contribute the public TCR sequences, we divided the patients into two groups where the first group contained patients with less than 100 clonotypes (1-100) and the second group contained those with more than 100 clonotypes (101-200). These two groups constituted almost equal number of clonotypes, but differed in number of patients which gave rise to two sets representing many patients-few clonotypes (47 patients, 1529 clonotypes) and few patients-many clonotypes (11 patients, 1585 clonotypes) (**Figure 3D**). Notably, we found that both groups contributed almost equally to the public TCR sequence pool. Further, we observed that the number of shared TCRα or TCRβ sequences per TCRαβ clonotypes in a given CeD patient appeared to be stable around 30-40% (median 37%) regardless of the number of gluten-specific clonotypes in the patient (**Figure 3E**).

R-Motif Dominates the Public T-Cell Response to Gluten

Upon analysis of the public paired TCRαβ sequences (n = 78), TRAV26-1:TRBV7-2 was the most dominant TRAV : TRBV pair (**Figure 4**). Further, the TCRβ sequences expressing the TRAV26-1:TRBV7-2/3 with R-motif were also the most prominent amongst the paired TCRαβ sequences (23%). When looking only into the collection of gluten-specific TCCs, 50% of the DQ2.5-glia-α2 specific TCCs (56/111) used TRBV7-2 and 30% also expressed the TRAV26-1 gene with the R-motif (33/111). Strikingly, we found T-cell clonotypes carrying the TRAV26-1:TRBV7-2 TCR with R-motif in all 41 patients where DQ2.5-glia-α2 tetramer was used. To sum up, the public T-cell response against wheat gluten in CeD is characterized by preferential expression of the TRAV26-1:TRBV7-2 and dominated by the use of the R-motif sequences.

Public T-Cell Receptor Sequences Exhibit Higher Generation Probabilities

In order to investigate what makes a TCR sequence public, we compared the TCR sequences shared in more than two individuals (public) with the TCR sequences observed only in one individual (private) in our dataset.

Assuming that the sharing of TCR sequences across patients is most likely influenced by a higher immune receptor generation probability (IRGP), we compared the mean IRGP values for private and public sequences. We observed that public TCR sequences indeed had significantly higher IRGPs than private ones (**Table 3**). This applied both to individual chains and paired receptors. Given the below-average CDR3 length of R-motif sequences (11 versus ~12.5 amino acids) and its prominent representation amongst public TCRβ sequences, we additionally created TCRβ datasets without R-motif sequences. As expected, the mean IRGPs of public TCRβs still remained significantly higher than private TCRβ sequences in the R-motif-free TCRβ dataset.

DISCUSSION

Unlike several other autoimmune disorders, the antigen (dietary gluten) driving the T-cell response is well established in CeD. Hence, lessons learned from CeD on features of the disease driving gluten-specific TCRs are likely applicable to other autoimmune disorders.

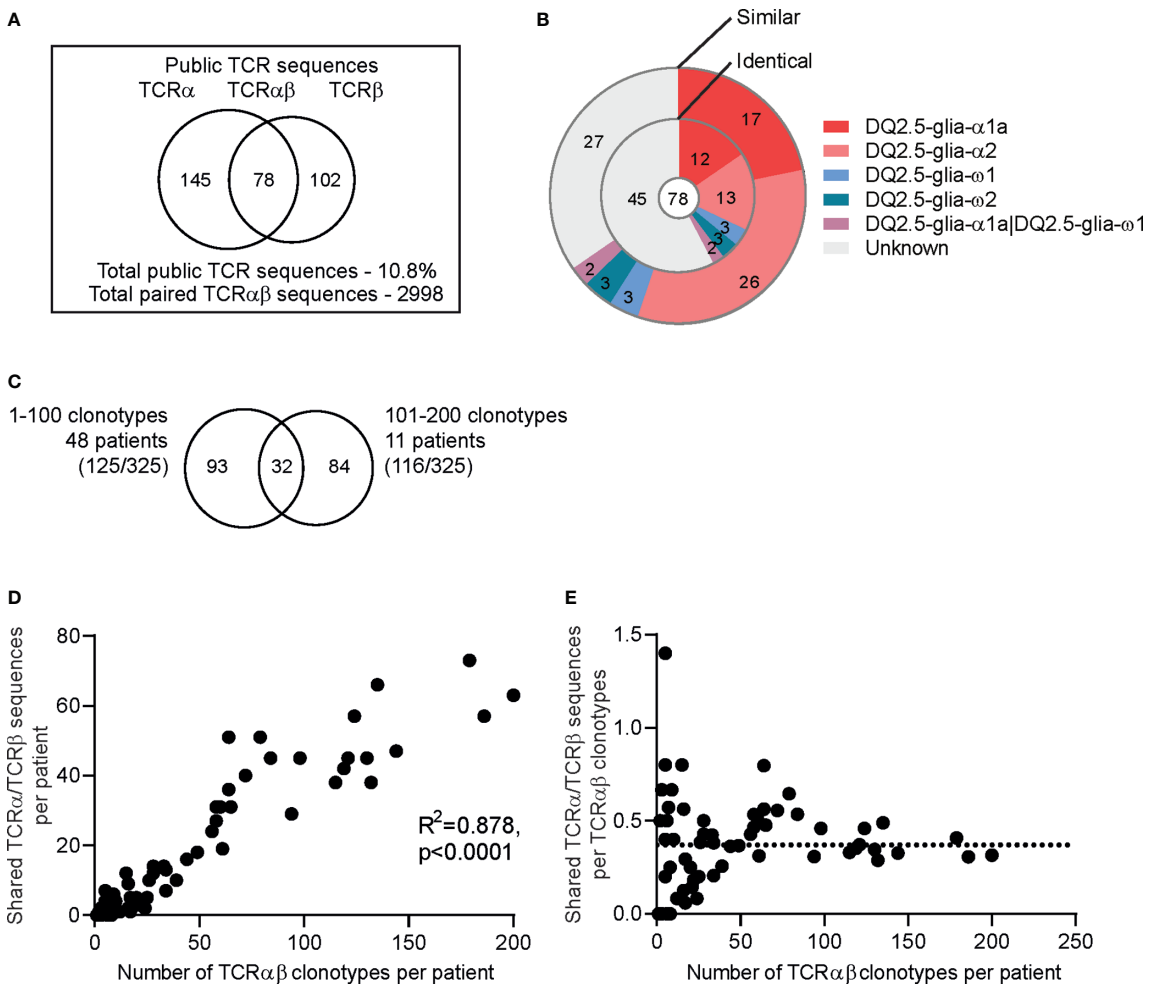

FIGURE 3 | Public TCR sequences among gluten-specific T cells. **(A)** The number of public TCRs defined as identical TCRα, TCRβ or paired TCRαβ amino acid sequences observed at least in two individuals in a dataset of total 2996 gluten-specific TCRαβ sequences from 63 patients. **(B)** Doughnut chart showing the epitope specificity of the 78 public paired TCRαβ sequences. The inner circle displays the paired TCRαβ sequences that are identical to the TCRαβ sequences of TCRs with known epitope specificity while the outer circle displays the paired TCRαβ sequences that have similar (1 amino acid difference) TCRβ or TCRαβ sequences. **(C)** Plot showing the number of shared TCRα/TCRβ sequences and the number of TCRαβ clonotypes in each patient. **(D)** Intra-comparison of the shared TCRα/TCRβ sequences between patients with 1 to 100 TCRαβ clonotypes (n=47) and 101-200 TCRαβ clonotypes (n=11). Total number of shared sequences within the groups are shown in parentheses. **(E)** The number of shared TCRα/TCRβ sequences per TCRαβ clonotypes in each patient. The stippled line indicates the median value.

The features of the TCR repertoire against the individual immunodominant HLA-DQ2.5 restricted gluten epitopes have been studied previously (5, 7, 9, 11, 12). However, no reports on public TCR motifs used by T cells specific for other immunodominant HLA-DQ2.5 restricted gluten epitopes exist apart from the CDR3β (R-motif) and CDR3α (NDYKLS-motif) used by DQ2.5-glia-α2-specific TCRs (5, 7, 9, 11). In order to comprehensively search for potential new motifs, we performed hierarchical clustering of a large dataset of CDR3α and CDR3β sequences obtained from 2750 gluten-specific TCRαβ clonotypes from 34 CeD patients. While the R-motif cluster stands out as the most dominant motif, several new CDR3α, CDR3β and CDR3α:CDR3β motifs were identified. Strikingly, more than half of the new paired CDR3α:CDR3β motifs could be linked to one or two of the four epitope specificities by searching among TCRαβ clonotypes of epitope-specific *in vitro* cultured TCCs and T cells isolated with single HLA-DQ2.5:gluten tetramers, resulting in novel TCR motifs specific to DQ2.5-glia-α1a, DQ2.5-glia-α2, DQ2.5-glia-ω1, DQ2.5-glia-ω2 and DQ2.5-glia-α1a/DQ2.5-glia-ω1. Although we cannot rule out that each such motif may encompass TCRs reactive to further epitopes among these four, the strictness of the motifs makes us believe that the motifs mostly capture specificities to a single of these four epitopes. Analysis of whether these motifs are unique to CeD patients and can serve as proxies for the disease will have to be tested in series of samples from CeD patients and non-affected subjects.

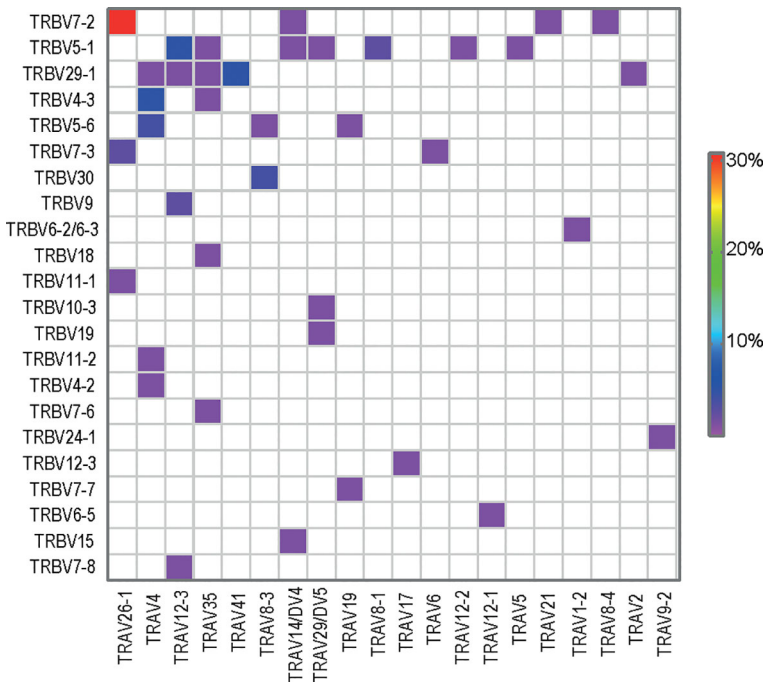

FIGURE 4 | TRAV : TRBV gene usage in public paired TCRαβ sequences (n=78).

TABLE 3 | The mean IRGP values for public and private sequences, with the number of sequences given in parentheses.

Sample	Amino acid sequences			Nucleotide sequences		
	private: mean (n)	public: mean (n)	p-value (U)	private: mean (n)	public: mean (n)	p-value (U)
TCRα	3.18e-08 (2230)	5.96e-08 (182)	9.77E-04 (174966)	3.99e-09 (2475)	3.61e-08 (123)	5.82E-06 (116660.5)
TCRβ	3.01e-08 (2182)	8.35e-08 (149)	3.15E-34 (66042.5)	1.04e-09 (2615)	6.94e-09 (73)	4.42E-27 (25379.5)
w/o R-motif	3.01e-08 (2182)	4.73e-08 (115)	1.38E-20 (61494.5)	1.04e-09 (2615)	2.44e-09 (29)	6.59E-06 (20103)
TCRα:TCRβ	1.10e-15 (2600)	8.35e-15 (72)	5.68E-08 (59372)	1.01e-17 (2758)	3.12e-16 (11)	1.73E-03 (7441)

For each sample, the p-value and the U statistic for the one-sided Mann Whitney U test comparing the samples is given.

Given the low frequency of gluten-specific T cells in blood and gut, we mostly used a pool of tetramers to maximize the yield of isolated gluten-specific T cells. While this approach had the limitation that it did not allow us to directly assign TCR usage with specificity, it was advantageous for the comparison of frequencies of TCR-motifs across epitopes.

The biased expression of TRAV26-1 and TRBV7-2 genes as well as the paired receptor resulting from these V genes emerged as the most prominent feature when analyzing the gene usage frequency of the gluten-specific T cells isolated using a cocktail of four immunodominant epitopes. V-gene bias has been proposed to be a consequence of several factors such as biases in somatic recombination, thymic selection, antigen-driven selection and structural features in TCR and peptide:MHC interaction (26–28). In line with this hypothesis, we speculate that the biased usage of TRAV26-1/TRBV7-2 TCR in T-cell response against gluten in CeD is the consequence of V-gene bias acting on numerous levels. The theory of antigen-driven selection bias suggests that the TCRs with a best fit for a peptide:MHC complex has an advantage over less-fit TCRs in the naive T-cell repertoire resulting in a biased repertoire (27). In this context, it is notable that the TCRs expressing TRAV26-1/TRBV7-2 with R-motif have shown higher affinity to the HLA-DQ2.5:DQ2.5-glia-α2 complex compared to the TRAV26-1/TRBV7-2 TCR with the extended R motif (8). Furthermore, the arginine that gives rise to the R-motif was shown to exert a key role in the interaction with HLA-DQ2.5:DQ2.5-glia-α2 complex (8). This indicates that the TRAV26-1/TRBV7-2 with R-motif due to its higher affinity to the peptide:MHC complex and crucial interactions might have an increased chances of selection during immune response resulting in increased prevalence in the subsequent effector pool.

The proportion of gluten-specific T-cell clonotypes that were public seemed to be around 30-40%, regardless of the number of gluten-specific TCRs we have acquired from each CeD patient. This indicates that these public T-cell receptors are central in the T-cell response against gluten. It has been proposed that the TCR

sequences that are closer to the germline sequences and that could be easily generated are potential candidates for such public TCRs (26, 28). In agreement with this hypothesis, we observed that the public gluten-specific TCRs in our dataset had fewer N insertions in the CDR3 and higher generation probabilities. On scrutiny of the CDR3β sequence with the R-motif, the CDR3β sequence is relatively short and closely matches the V, D and J gene germline sequences. This motif also contains a conserved non-germline arginine at the fifth position, which has crucial role in the interaction between TCR and the DQ2.5-glia-α2 presented by HLA-DQ2.5 (8). However, the arginine in the R-motifs observed between individuals and within the same individual has shown to be encoded by different recombination events (nucleotides) (5, 16). As arginine is encoded by six codons, this increased variety of ways in which the R-motif amino-acid sequence can be made, thus creating a higher chance for TCR sharing across individuals. Instances of this phenomenon known as convergent recombination contributing to public T-cell response are also found outside CeD (28). Therefore, conceivably TCRs with the R-motif become dominating public TCRs due to the cumulative effect of utilizing the preferentially expressed V gene pair with near-germline CDR3 regions and the convergent recombination giving conserved non-germline arginine residues.

There are several examples in the literature on universal public motifs in human disorders (29–32), but it is unclear how often one may find strong public TCR motifs for any given specificity. The fact that we did not observe any other prominent motifs apart from the R-motifs despite employing HLA-DQ2.5:gluten tetramers covering four immunodominant gluten epitopes, suggests that the formation of dominant public TCR motifs does not take place for all peptide-MHC specificities. It is notable that no dominant public TCR motifs was observed for the DQ2.5-glia-ω2 epitope, an epitope that is highly homologous to the DQ2.5-glia-α2 epitope. Future studies should address to which extent public TCRs constitute the response to various antigens.

Looking for the presence of the CDR3 motifs identified in our study in the VDJdb and McPAS-TCR repositories, we found no matches for paired CDR3α:CDR3β motifs and very few matches for the CDR3α and CDR3β motifs indicating disease specificity of these TCR motifs. Potentially such information can be utilized in a celiac disease diagnostic test. Our result suggests that combined TCRα and TCRβ information will provide a better prediction. This notion of importance of both TCR chains is supported by the functional data demonstrating that T-cell reactivity in DQ2.5-glia-α2 specific TCRs using TRBV7-2 is lost upon substitution of TRAV26-1 with TRAV26-2 (33). In a recent study, Yao et al. analyzed for presence of TCR sequences of gluten-specific T cells in gut biopsies of 7 CeD patients and 8 disease controls, and concluded that correct disease status could be assigned based on presence of TCRs sequences in 13 out of 15 donors (34). While these results are encouraging for the prospect of a diagnostic test, further analysis is required to conclude how frequent the identified TCR CDR3 motifs are in healthy subjects. As there is a non-negligible number (about 1%) of undiagnosed celiacs in the general population (35), in future studies it will be important to accurately ascertain disease status of the disease controls.

Taken together, in this study we have demonstrated that the gluten-specific T-cell response is composed of one very dominant CDR3 motif used by DQ2.5-glia-α2-specific TCRs and several less dominant motifs used by TCRs specific for other dominant gluten-derived T-cell epitopes. Together, these CDR3 motifs are part of a diverse TCR repertoire employed by gluten-specific CD4+ T cells in CeD. Therefore, these public TCR sequences and conserved CDR3 motifs can potentially be exploited as diagnostic markers of CeD.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Committees for Medical and Health Research Ethics, Norway. Written informed consent to participate in this study was provided by the participants or the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SD-K and LR isolated T cells and sequenced their receptors, performed data analysis and wrote the manuscript. RN performed data analysis and wrote the manuscript. AC isolated T cells. KL organized collection of CeD patient material. GS supervised the project and critically revised the manuscript. S-WQ isolated T cells, supervised the project and critically revised the manuscript. LS supervised the project, arranged the necessary funding and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.639672/full#supplementary-material

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Conflict of Interest: SD-K, LR, RN, AC, KL, S-WQ, GS and LS are holders of a patent application entitled "Method of diagnosing celiac disease" (US20210010077A1) on the use of the gluten-specific T cell receptor sequences described in the current work for diagnosis of celiac disease.

frontiers in Immunology

REVIEW
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Can Celiac Disease Be Prevented?

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Celiac disease (CD) is an autoimmune disorder triggered by gluten in genetically susceptible individuals characterized by a variable combination of gluten-dependent symptoms, presence of specific autoantibodies and enteropathy. The health burden of CD is considerable, as it reduces quality of life and, at a societal level, has extensive negative economic consequences. Prevention strategies are based on the identification of at-risk subjects and identification and elimination of risk factors. A number of prospective observational and interventional studies conducted on the general population, and more often in subjects at-risk, have given important information on the natural history of the disease. Both genetic and environmental factors have been identified with the former, in particular histocompatibility genes, playing a major role. Environmental factors, some operating already before birth, have been identified, with feeding pattern in the first year of life (breast feeding, amount and time of introduction of gluten) and infections being the most relevant. Prospective studies have also allowed the identification of biomarkers predictive of the disease which in perspective could better define the population on which to intervene. Interventions have been so far limited to modifications of feeding patterns. However, as also learnt from diseases that share with CD genetic risk factors and mechanisms of damage, such as type 1 diabetes (T1D), future strategies may be envisaged based on protection from infections, manipulation of microbiota, intervention on T cells.

Keywords: risk factors, biomarkers, celiac disease, children, gluten

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INTRODUCTION

Celiac disease (CD) is an immune mediated systemic disorder elicited by gluten and related prolamines in genetically susceptible individuals and characterized by a variable combination of gluten-dependent clinical manifestations, anti-tissue transglutaminase antibodies positivity and enteropathy (1). The disease results from a complex interplay between genetic, environmental and immune factors leading to an inappropriate mucosal T cell response to gluten and eventually to a remodeling of the small intestinal mucosa (villous atrophy) and its clinical consequences (2).

CD is a frequent disorder. A recent meta-analysis has indicated the pooled global prevalence of CD being 1.4%, based on positive results from tests for anti–tissue transglutaminase and/or anti-endomysial antibodies (called seroprevalence) and 0.7% that of biopsy-confirmed CD (3). The prevalence goes up to 10% in at risk groups, such as children and adolescents with first-degree relatives with CD, patients with autoimmune diseases (e.g. type 1 diabetes, T1D), IgA deficiency and

chromosomopathies. It is also worthy to note that the incidence has increased over the past several decades emphasizing the relevance of environmental factors (4).

The health burden of CD is considerable, as it reduces quality of life (5, 6) and in social life has extensive negative consequences. The costs of a particular disease in different Countries depends on the structure of a particular healthcare system. However, from all Countries there are reports of significant additional primary care costs associated with CD. When compared with other chronic illnesses, the costs of patients with CD were similar to those of patients with diabetes and hypertension (7). For these reasons primary prevention has become a priority in the research agenda of CD.

A strategy for primary prevention is based on the identification of at risk subjects primary target of the intervention and on the identification of environmental factors that favor disease development whose manipulation may decrease the risk. Recently a number of prospective studies have shed light on these aspects, in a first place helping to understand the natural history of the disease. Different phases have been identified, from the genetic predisposition with HLA alleles contributing to 30-50% of the genetic risk, to the pre-weaning phase where environmental factors may play a role even before birth, to the introduction of gluten in the diet, to the development of CD-specific autoantibodies, to the eventual development of small intestinal mucosal damage and the consequent clinical manifestations (**Figure 1**). At each of this stage it is possible to intervene to prevent the gluten-induced harm. Early diagnosis through screening policies based on the detection of CD-associated autoantibodies and efforts to assure compliance with the gluten free diet represent the basis for secondary and tertiary prevention of CD. However, this mini-review will mainly focus on primary prevention and on the possible strategies to halt the disease process before mucosal damage occurs.

The Target: Biomarkers to Identify at Risk Subjects

Those subjects who are at the highest risk to develop CD are the first target of intervention strategies. Much effort has been made in recent years trying to identify possible biomarkers that would allow clinicians to classify individuals based on their risk of developing CD before any clinical and serological signs of the disease.

More than ninety percent of CD patients have the HLA-DQ2.5 haplotypes (encoded by the DQA1*0501 and DQB1*0201 alleles) either in cis or trans position, the remaining showing the HLA-DQ8, DQ2.2 or DQ7 haplotypes (8). Furthermore, it is known that the risk among genetically predisposed first degree relatives increases up to 20% (9). However, not all DQ2 or DQ8 positive patients have the same probability to develop the disease, a gene dose effect due to the number of copies of DQB1*0201 having been reported (10). Studies in cohorts of children at risk for CD have prospectively confirmed these observations. In the European multicentre prospective study PREVENT-CD it was in fact shown that subjects homozygous for HLA DQ2 were those with the highest incidence of CD (11). Similar data were also produced by the Italian multicentre study, known as CELIPREV (12), and in the TEDDY study (13). Girls seem to be at higher risk to develop the disease. The contribution of non-HLA genes to the risk is less defined, but scores that take into account the contribution of single nucleotide polymorphisms related to CD have been developed (14) and could help in a more precise assessment of the genetic risk.

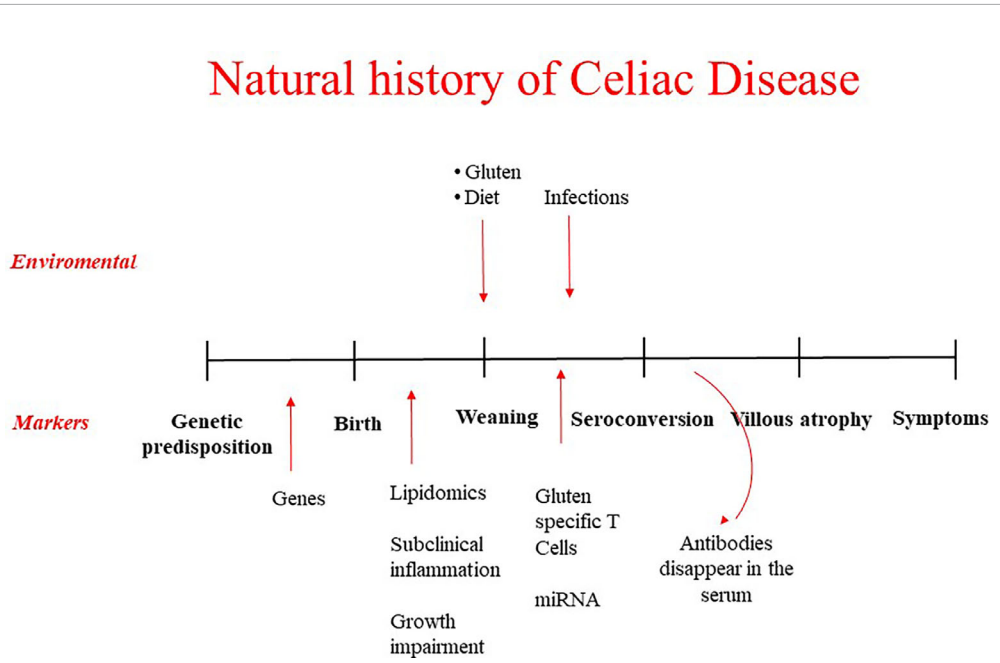

FIGURE 1 | Natural history of celiac disease: environmental factors possible target of prevention strategies and biomarkers to identify at risk children.

Prospective studies have helped to identify biomarkers predicting the development of the disease and then helping to identify subjects candidate to prevention strategies. Lipidomic modifications were considered as an early predictive marker for CD. In fact, it has been shown that since a very early age, even before the introduction of gluten, genetically predisposed children who will develop the disease during their life show a specific profile of phospholipids. A limited number of alkylacyl-phosphatidylcholine and lyso-phosphatidylcholine, together with the duration of breastfeeding, allows the discrimination of infants who will develop CD from those at a similar genetic risk who will not develop the disease (15). Interestingly similar observations have been made in T1D (16). These changes seem to be present already at 4 months of age, suggesting they are the result of very early phenomena, even in the gestational period, which may predispose to development of the disease in at risk subjects. In another study conducted in the DIPP cohort increased amounts of triglycerides of low carbon number and double bond count and decreased levels of phosphatydilcholine were noted before gluten introduction in subjects who later developed CD; in this case the changes were attributed to impaired lipid absorption (17).

Before seroconversion other features are reported in infants who will later develop the disease. In fact, Galatola et al. have shown that a small set of non-HLA genes is differently expressed in subjects at risk who then develop the disease already in the first year of life, long before the appearance of other clinical and serological signs of the disease (18).

Also microRNAs have been considered as possible biomarkers capable of predicting disease. In fact, some studies have shown that both at the tissue and blood level there are microRNA profiles able to distinguish celiac patients from controls (19).

The seroconversion i.e. the appearance in serum of anti-tissue transglutaminase antibodies and other autoantibodies related to CD is a major step of the natural history of the disease. However, they are not invariably associated to the mucosal damage; moreover, they do not automatically lead to evolution towards villous atrophy. On the contrary in percentages ranging from 30 to 90% antibodies can disappear from the serum (20, 21) indicating this condition of potential CD (normal mucosa but presence of autoantibodies) still represent a situation amenable to prevention, being possible to halt the progression to the full blown disease.

Identification of Risk Factors and Intervention on at Risk Infants: Feeding in the First Years of Life

Breastfeeding has for long time been considered the main protective factor for the development of CD (22). More recently, the evidence coming from most studies (23) including the two large interventional studies on children at risk for CD (11, 12), have concluded that exclusive or any breastfeeding, as well as breastfeeding at the time of gluten introduction, did not reduce the risk of developing celiac disease during childhood.

In recent past, studies had identified a "window of tolerance": gluten introduction between 4 and 6 months of life, was hypothesized to reduce the risk of developing CD (24). More recent studies on prospective cohorts at risk for CD such as PREVENT CD (11), CELIPREV (12) have instead shown that there is no substantial difference in the incidence of the disease whether gluten is introduced early (4 months) or later (after the 12th month of life). Other observational studies both in general population and in at risk groups for T1D also reached the same conclusions. Therefore, the current guidelines of the European Society for Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommend introducing gradually gluten between 4-12 months of life (25). Recently, several studies have been published describing the effect of challenging young babies with allergen containing foods at a very early age to induce lifelong tolerance. In this context data coming from the EAT study, an open labelled randomized clinical trial aimed to assess if early introduction of six allergenic foods can prevent food allergy (26), showed that introduction of gluten at 4 months was associated with reduction of CD prevalence. The small size and the criteria used for diagnosis suggest caution in the interpretation of these results.

Whether the amount of gluten at the time of weaning and in the first year of life can in any way influence the risk of CD has been the focus of different studies. In the PREVENT CD study it was shown that gluten consumption pattern, as well as the amount of gluten consumed in the first three years of life, do not influence the development of CD (27), although a trend to a positive effect was noted in the subgroup at lower genetic risk, suggesting that effect related to the gluten amount may become more visible in those with lower genetic risk. In fact, more recent data have been published on two cohorts at risk for T1D [TEDDY (28), DAISY (29)] and one on the Norwegian mother-child cohort (30), suggesting that gluten ingested (around 3-10 g/day) is associated with the development of autoimmunity to T1D and CD. Taken together all these studies, it could be concluded that one extra slice of bread (2 g/day of gluten) could cause 20-50% increase in the risk of CD. Interestingly, it is likely that the amount of gluten may concur with other risk factors (in particular infections in the first years of life) to the development of CD. This is suggested by a recent observation in the context of the TEDDY study of an additive effect of more than 10 g/day of gluten ingested and virus infection (31).

The idea that gluten is not the only nutritional factor for the development of the disease is gaining ground in the recent years, but rather it could be that specific dietary pattern could cause a basal inflammatory state increasing the susceptibility to chronic diseases, such as CD. Roager et al. have shown that a diet rich in whole grain is able to reduce the inflammatory state of adult obese patients (32). Likewise, the work of Barroso et al. showed that a "prudent" diet at one year of life, with more fruit, vegetables, vegetable oil and cereals and less snacks, confectionary and sugars, in other words more like the Mediterranean diet, is able to reduce celiac autoimmunity at 6 years (33).

Identification of Risk Factors and Intervention on at Risk Infants: Infections, Vaccines, Manipulation of Microbiota

Another important hypothesis is that the process of autoimmunity leading to CD is stimulated or switched on by the occurrence of

common infections during the period preceding its onset. The authors of several studies have suggested that early infections might contribute to the risk of developing CD. First of all, repeated infections by Rotavirus (34) and *Parechovirus* (35) were associated to the risk of CD. More recently, *Reovirus* infection was also indicated in association to CD (36). In the TEDDY study, gastrointestinal infections increased the risk of celiac autoimmunity by 33% in genetically predisposed children in the following 3 months of life (37). In the Norwegian Mother and Child Cohort Study, children with more than 10 infections before 18 months of age had a significantly higher risk of developing CD later in life than children with less than four infections (38). Subsequently, in the same cohort it was shown that the increased risk for CD was associated with gastrointestinal infection mainly caused by *Enteroviruses*, especially if infection was contracted before seroconversion (39). The increased risk associated with *Enterovirus* was recently confirmed in TEDDY study by Lindorfs et al. (31). Moreover, they hypothesized that there is a cumulative effect of enteroviral exposure and higher amount of gluten consumed in the first 2 years of life (31). Interestingly, also non-gastrointestinal infections were associated to the risk of CD in an Italian prospective cohort study: a higher frequency of respiratory tract infections among CD patients during the first 24 months of life significantly contributed to discrimination of case *versus* controls (40).

Given the important role of infections, vaccination has been indicated as a strategy for prevention. In fact, several studies have shown a reduced incidence of disease in subjects vaccinated for *Rotavirus* (37, 41). Furthermore, a trial is being conducted with the use of an anti-*Coxsackie* vaccination for the prevention of T1D and CD (Clinical Trial n° NCT04690426).

A number of studies have addressed the potential role of microbial composition in the evolution of CD. High risk children have been shown to possess a different microbiota in comparison to children with no or low genetic risk for CD (42). Contrasting data have been produced on the existence of an early microbial signature in infants from at risk groups who later progress to CD (43, 44). Manipulation of the microbiota has then become another possible strategy. Probiotics are candidate for their proven anti-inflammatory effects; but controversial results have been published about their ability to prevent CD (45). One ongoing clinical trial (NCT3562221) in this context addresses the effects of GFD and probiotics during the first 3 years of life on the development of celiac disease. It is clear that, also because the low risk of side effects, that will be one of the area of major development.

Finally, the identification of other risk factors could also be important in the design of prevention strategies based on multiple interventions. However, given also the relatively little impact of each of them, conflicting results have been so far obtained for example in relation to the use of antibiotics and the modalities of delivery (46).

Halting the Progression of CD: Analogies With Type 1 Diabetes

CD is now considered by most an autoimmune disease. It is often associated with other autoimmune diseases, first among all T1D. With T1D, CD shares genes conferring risk (both HLA and non HLA) (47) and immunological mechanisms inducing damage at the target tissue (intestinal mucosa or pancreatic islets). Lessons from what implemented for T1D prevention may be particularly useful for CD. Also for T1D the target population for primary prevention trials is individuals who carry high risk genotypes before the first appearance of islet autoantibodies and also for T1D these trials include mostly low risk dietary interventions such as the avoidance of cow's milk or gluten and the supplementation of n-3 fatty acids or vitamin D (47). The results of these trials have been so far quite disappointing. None of these specific dietary factors has been proved to be a definite risk factor inducing beta cell autoimmunity. More recently primary prevention has started focusing on the modulation of the immune system by antigen specific immunotherapy, such as oral insulin (48). Furthermore, the observation that as in CD certain viral infections e.g., *Coxsackie B* may promote autoimmune attack to pancreatic islets has prompted efforts to develop vaccines that are going to be tested in clinical trials.

Most of the efforts in T1D have been so far directed to slow or to halt the progressive beta cell destruction. Nicotinamide, antigen-specific immune therapy (oral and nasal insulin, GAD alum), monoclonal antibodies, immunosuppressive drugs, hydroycloroquine and anti-inflammatory agents have been tested (47). Particularly promising is the use of monoclonal antibody to CD3 (Tepluzimab) targeting CD8+ cells responsible of beta cell destruction in T1D (49). Here the analogies with potential CD (anti tissue transglutaminase antibodies, anti-TG2) present in the absence of mucosal damage) are very strong. Moreover, we know from clinical studies that the appearance of anti-TG2 antibodies in CD is still reversible, as shown by observations in infants born in celiac families and subjects with T1D (see above). That suggests that also in the presence of anti-TG2 antibodies the evolution to villous atrophy is not obligatory; in theory it may still be prevented as the experience with anti-CD3 in pre-diabetes seems to indicate (49). Other possibilities are based on the induction of specific tolerance to gliadin through "vaccination" with gliadin peptides (50) or use of nanoparticles containing gliadin peptides (51). Another attempt has been based on the use of probiotics. A randomized double blind placebo-controlled trial of *Lactobacillus plantarum* and *Lactobacillus paracasei* to suppress celiac autoimmunity in infants at risk has led to a decrease of anti-TG2 antibodies titres and to changes of the phenotype of PBMC (52). In general, one of the challenges of such trials is to balance the potential benefits against the risks, and this is particularly true when it comes to strategies which alter the immune response.

Another important indication that seems to come from studies on T1D is that further attempts should not concentrate on single hypothesis or intervention. It is very likely that a combination of different factors may be decisive, as suggested by the additive risk given by the amount of gluten and viral infections (31), and therefore a combination of different approaches could be the next strategy.

CONCLUSIONS

The pathogenesis of CD need to be clarified: environmental factors and genetic factors need to be better understood. Prospective studies have much improved our knowledge of the natural history and have provided biomarkers that help to define the different level to which intervene. Primary prevention remains the main goal to achieve, with interventions planned as early as possible even before birth. On the other hand, even if CD associated autoantibodies develop there is still potential therapeutic benefit from intervention to halt and possibly reverse the disease. We acknowledge that in the case of CD prevention is moving its first steps. Attempts based on the timing of gluten introduction in infants' diet are inconclusive. We are now waiting for the results of trials based on antiviral vaccinations. However, there is no doubt that studies aimed to find a prevention strategy for CD will represent in the next years one of the frontiers of the research in CD with important consequences also in the management of other autoimmune diseases.

AUTHOR CONTRIBUTIONS

RA and RT both contribute to select papers and write the review. All authors contributed to the article and approved the submitted version.

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A Sensitive Whole Blood Assay Detects Antigen-Stimulated Cytokine Release From CD4+ T Cells and Facilitates Immunomonitoring in a Phase 2 Clinical Trial of Nexvax2 in Coeliac Disease

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Improved blood tests assessing the functional status of rare gluten-specific CD4+ T cells are needed to effectively monitor experimental therapies for coeliac disease (CD). Our aim was to develop a simple, but highly sensitive cytokine release assay (CRA) for gluten-specific CD4+ T cells that did not require patients to undergo a prior gluten challenge, and would be practical in large, multi-centre clinical trials. We developed an enhanced CRA and used it in a phase 2 clinical trial ("RESET CeD") of Nexvax2, a peptide-based immunotherapy for CD. Two participants with treated CD were assessed in a pilot study prior to and six days after a 3-day gluten challenge. Dye-dilution proliferation in peripheral blood mononuclear cells (PBMC) was assessed, and IL-2, IFN-γ and IL-10 were measured by multiplex electrochemiluminescence immunoassay (ECL) after 24-hour gluten-peptide stimulation of whole blood or matched PBMC. Subsequently, gluten-specific CD4+ T cells in blood were assessed in a subgroup of the RESET CeD Study participants who received Nexvax2 (maintenance dose 900 μg, n = 12) or placebo (n = 9). The pilot study showed that gluten peptides induced IL-2, IFN-γ and IL-10 release from PBMCs attributable to CD4+ T cells, but the PBMC CRA was substantially less sensitive than whole blood CRA. Only modest gluten peptide-stimulated IL-2 release could be detected without prior gluten challenge using PBMC. In contrast, whole blood CRA enabled detection of IL-2 and IFN-γ before and after gluten challenge. IL-2 and IFN-γ release in whole blood required more than 6 hours incubation. Delay in whole blood incubation of more than three hours from collection substantially reduced antigen-stimulated IL-2 and IFN-γ secretion. Nexvax2, but not placebo treatment in the RESET CeD Study was associated with significant reductions in gluten peptide-stimulated whole

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blood IL-2 and IFN-γ release, and CD4+ T cell proliferation. We conclude that using fresh whole blood instead of PBMC substantially enhances cytokine secretion stimulated by gluten peptides, and enables assessment of rare gluten-specific CD4+ T cells without requiring CD patients to undertake a gluten challenge. Whole blood assessment coupled with ultra-sensitive cytokine detection shows promise in the monitoring of rare antigen-specific T cells in clinical studies.

Keywords: coeliac disease, T cells, cytokines, cytokine release assay, IL-2, diagnosis

INTRODUCTION

Antigen-specific CD4+ T cells are drivers of both inflammatory and tolerogenic immune responses. Functional blood-based biomarkers for antigen-specific CD4+ T cells play a central role in characterizing immune responses, and in monitoring vaccines and therapies intended to induce or suppress antigen-specific immunity, or restore immune tolerance (1). Autoimmune diseases including coeliac disease (CD) can often be associated with significant target organ injury, but in contrast to acute infectious diseases, conventional functional assays are often not sufficiently sensitive to detect relevant peripheral blood antigen-specific CD4+ T cells (2, 3).

Cytokine release assays (CRAs) employing fresh or cryopreserved peripheral blood mononuclear cells (PBMC) have been the mainstay for studying and monitoring T-cell immunity in patients (4). A practical challenge for immune monitoring in clinical trials is to preserve the functional properties of CD4+ T cells, which requires that PBMC should be separated from whole blood within 24 hours before cryopreservation or commencing an assay (5). CRAs using fresh, unseparated blood added directly to antigen soon after blood collection have previously been evaluated as research tools (6), and are now used for diagnosis of infectious diseases such as tuberculosis (7). Whole blood is typically "stimulated" by addition of an activator of innate or adaptive immunity, and maintained at 37°C in a sealed tube or dispensed into 96-well microplates and placed in an incubator. Plasma is separated after completing usually six to 48-hours incubation, frozen, and transported to a central laboratory for specialized biomarker assay. Newer versions of whole blood CRAs have been promoted for profiling cytokine release to various stimuli (8, 9), but in our past experience whole blood CRA has been lacking the sensitivity to detect cytokine release by rare gluten-specific CD4+ T cells in CD patients (10).

CD has many features in common with autoimmune disease (11). CD is strongly associated with HLA-DQ2.5, and CD4+ T cells specific for a well-documented hierarchy of gluten epitopes play a central role in intestinal injury, and are drivers of acute gluten-induced cytokine release and symptoms (12, 13). Gluten-specific CD4+ T cells isolated from intestinal tissue or in PBMC share many of the same surface markers suggesting blood is a valid source of gluten-specific CD4+ T cells to monitor gluten immunity (14).

Only two methods have consistently identified rare peripheral blood gluten-specific CD4+ T cells in CD patients. Blood is either collected 6 to 8 days after subjecting patients to a short gluten-food challenge to increase their frequencies allowing detection by conventional CRAs (10, 15–17), or PBMC are stained with customized MHC class II-gluten peptide tetramers, concentrated by immunomagnetic beads, and then enumerated by flow cytometry (18). These experimental approaches have shown that the frequency of peripheral blood gluten-specific CD4+ T cells is below ten per million CD4+ T cells in treated CD, but increases about ten-fold by six days after commencing gluten challenge (17, 19). Recently, in parallel to the current study, we first reported a simple whole blood CRA utilizing an electrochemiluminescence (ECL) immunoassay for interleukin-2 (IL-2) that appears to be as effective as MHC class II-gluten peptide tetramers for detection of these rare gluten-specific CD4+ T cells in CD, and can be combined with multiplex assessments of interferon-γ (IFN-γ) and interleukin-10 (IL-10) (3). Based on Poisson distribution analysis, we detected approximately 0.5 – 11 IL-2 secreting gluten-specific T cells per one mL of fresh whole blood collected from treated CD patients without gluten challenge.

In the present study, we utilize this novel CRA for rare gluten-specific CD4+ T cells with the aim of optimizing blood collection and processing to monitor the effects of an experimental peptide immunotherapy (Nexvax2) for CD during a phase 2 clinical trial.

MATERIALS AND METHODS

Study Design

Immune monitoring sub-studies supplementing the RESET CeD Study are outlined in **Figure 1**. Nexvax2 is an experimental antigen-specific immunotherapy intended to restore clinical and immunological tolerance to gluten in HLA-DQ2.5+ patients with CD. The "RESET CeD Study" was a double-blind, placebo-controlled phase 2 efficacy study of Nexvax2 conducted at 39 trial sites and randomized a total of 178 HLA-DQ2.5+ adults

Abbreviations: CRA, cytokine release assay; CD, coeliac disease; CTV, CellTrace™ Violet dye; DGP, deamidated gliadin peptide; DMSO, dimethyl sulphoxide; ECL, electrochemiluminescence; GC, gluten food challenge; ELISpot, enzyme-linked immune absorbent spot; GFD, gluten-free diet; HLA-DQ, Human Leukocyte Antigen-DQ; HLA-DR, Human Leukocyte Antigen-DR; Ig, immunoglobulin; IL-2, interleukin-2; IFN-γ, interferon-γ; LLOD, lower limit of detection; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; SFU, Spot-forming unit; TG2, transglutaminase 2; ULOD, upper limit of detection.

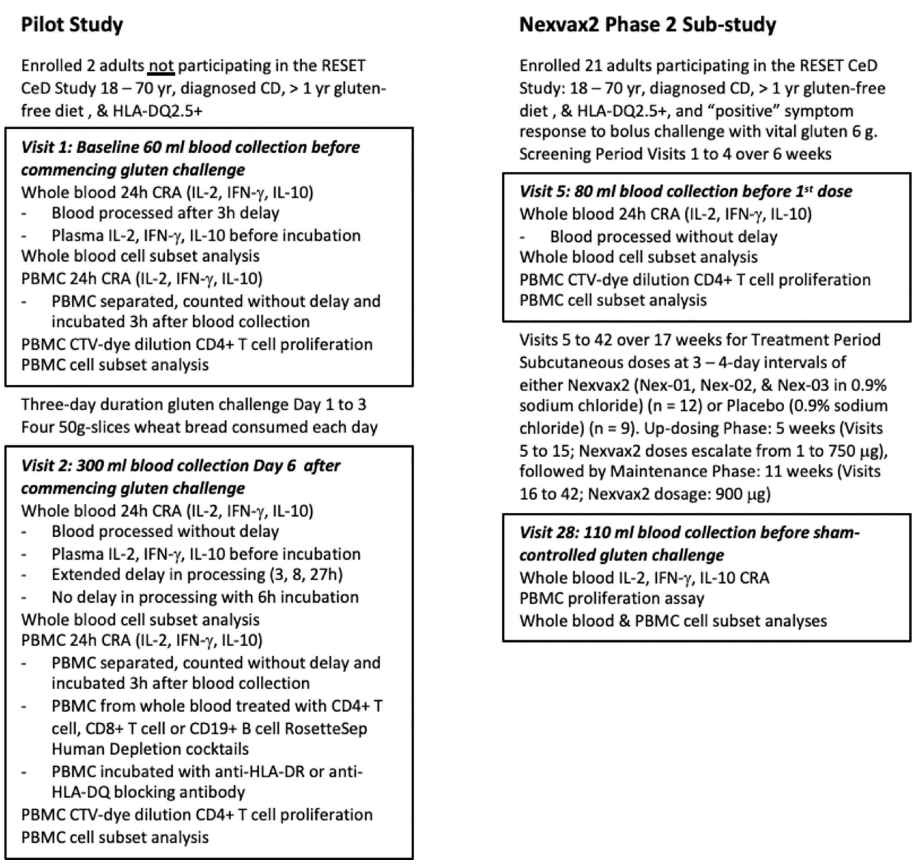

FIGURE 1 | Study overview. The pilot study included two participants prior to commencement of the Nexvax2 Phase 2 Sub-study.

with treated, medically confirmed CD (ClinicalTrials.gov identifier: NCT03644069) (20). The primary endpoint in the RESET CeD Study was self-reported gastrointestinal symptoms the day of a masked single bolus gluten challenge after 14-weeks treatment compared to a baseline period pre-treatment. Nexvax2 was administered in aqueous 0.9% sodium chloride twice weekly as a subcutaneous injection, which results in detectable plasma levels of each constituent peptide over about six hours (21). The bioactive component of Nexvax2 is limited to the three gluten-related peptides shown in **Table 1** (Nex-01, Nex-02, and Nex-03)

TABLE 1 | Peptides and their final concentrations in PBMC and whole blood incubations[†].

Peptide	Source	Length[‡]	Amino acid sequence (Z pyroglutamate)	Solvent	Control Pool	NX Pool	xNX Pool	NXxNX Pool	CEFT Pool
Nex-01	α-gliadin/Nexvax2	16	ZLQPFPQPELPYPQPQ-NH_2	PBS		5 μM		5 μM	
Nex-02	ω-gliadin/Nexvax2	15	ZQPFPQPEQPFPWQP-NH_2	PBS		5 μM		5 μM	
Nex-03	B-hordein/Nexvax2	16	ZPEQPIPEQPQPYPQQ-NH_2	PBS		5 μM		5 μM	
W04	ω-gliadin	16	ZPFPQPEQPIPVQPEQ-NH_2	PBS			5 μM	5 μM	
R03	ω-gliadin	16	ZPFPQPEQPTPIQPEQ-NH_2	PBS			5 μM	5 μM	
W14	ω-secalin	16	ZIQPEQPFPEQPEQIR-NH_2	PBS			5 μM	5 μM	
SCP1	Scramble	8	ZPFPLPQP-NH_2	PBS	15 μM	7.5 μM	7.5 μM		15 μM
SCP2	Scramble	8	ZPQYQPEQ-NH_2	PBS	15 μM	7.5 μM	7.5 μM		15 μM
SCP3	Scramble	8	ZPFEPQPL-NH_2	PBS	15 μM	7.5 μM	7.5 μM		15 μM
SCP4	Scramble	8	ZPQSYPEQ-NH_2	PBS	15 μM	7.5 μM	7.5 μM		15 μM
CEFT	CMV, influenza, EBV, & C. tetani	9 - 21	27 peptides with HLA class I or II restricted T-cell epitopes	DMSO					0.1 μg/ml

[†]*225 μl unseparated, heparinized blood was dispensed into wells containing 25 μl PBS with 10x final concentration of peptides;*
[‡]*Number of amino acids.*

that each includes immuno-dominant HLA-DQ2.5-restricted epitopes for gluten-specific CD4+ T cells (22).

A convenient blood test that did not require patients to be rechallenged with gluten was needed for serial assessments of rare CD4+ T cells specific for epitopes represented in Nex-01, Nex-02, or Nex-03, and for other gluten-specific CD4+ T cells that may be indirectly suppressed by Nexvax2 immunotherapy through induction of regulatory T cells (23, 24). Initially, in a pilot study separate from the RESET CeD Study, PBMC and whole blood were compared as sources of gluten-specific CD4+ T cells for the CRA. Subsequently, the CRA using whole blood was deployed to monitor Nexvax2 immunotherapy during the RESET CeD Study in a subgroup of CD patients enrolled at three sites in Melbourne, Australia. Additional blood was collected before treatment (Visit 5), during maintenance treatment (Visit 28), and at the end of treatment (Visit 42). However, after an interim analysis indicated the primary efficacy endpoint in the RESET CeD study would not be achieved, dosing was discontinued on 25 June 2019 and the supplementary sub-study was also halted. Consequently, sample analysis for the supplementary sub-study was limited to Visit 5 and 28.

Participants and Ethics Approvals

The pilot study was approved by the Human Research Ethics Committees at the Walter and Eliza Hall Institute and Melbourne Health (identifiers 03/4 and 2003.009 respectively), and the RESET CeD study was approved by Melbourne Health Human Research Ethics Committee (Study Number HREC/43048/MH-2018) and Bellberry Limited (Application Number 2018-07-562-A-13). Patients for the pilot study were recruited at The Royal Melbourne Hospital and the Walter and Eliza Hall Institute of Medical Research (Parkville, VIC). The two volunteers in the pilot study did not participate in the RESET CeD Study. Patients for the sub-study in the RESET CeD study were recruited from The Royal Melbourne Hospital/Walter and Eliza Hall Institute of Medical Research (n = 19), Box Hill Hospital (n = 2), and Alfred Hospital (n = 2). All patients enrolled at these sites were invited to volunteer and did enroll in the sub-study. All patients gave written, informed consent prior to undergoing any study procedures. Full eligibility criteria for the RESET CeD are published elsewhere (20). All participants in the RESET CeD Study were aged between 18 and 70 years, had a diagnosis of CD made on the basis of duodenal histology showing villous atrophy and supportive serology, had been on a gluten free diet (GFD) for at least one year, were HLA-DQ2.5 positive, and had worsening of gastrointestinal symptoms within six hours after consuming 10-grams vital wheat gluten as described elsewhere (20).

Clinical Procedures

Interventions for the pilot study included a 3-day gluten challenge and blood collections at baseline (60 ml) and one week later on Day-6 after commencing gluten challenge (300 ml). Blood was collected as previously described *via* 21G ¾" Surflo winged infusion set (Terumo, Shibuya City, Japan) into 10 ml lithium-heparin vacutainers (Becton Dickinson, Franklin Lakes, New Jersey, USA) (25). Gluten challenge for the pilot study comprised four 50-gram slices of gluten-containing wheat bread consumed daily for three consecutive days as previously described (15). For the supplementary sub-study of the RESET CeD Study, heparinised blood (80 ml) was collected at Visit 5 just before patients received their first dose of Nexvax2 or placebo, and again at Visit 28 (110 ml) after patients had received 12-weeks treatment with two-times weekly subcutaneous injections of study drug. The dosing schedule for Nexvax2 included eleven escalating doses from 1 μg to 750 μg described elsewhere (21), which was followed by maintenance dosing at 900 μg. Visit 28 marked the first of three masked single bolus food challenges that are described elsewhere and were gluten-free or contained 10-grams vital wheat gluten (20). Most patients in the supplementary sub-study had been discontinued from the RESET CeD Study before completing masked single bolus gluten challenge at Visit 34 or Visit 42 at the end-of-treatment. Consequently, immune monitoring was limited to blood collected at Visit 5 and Visit 28.

Peptides and Antigens

Table 1 shows the individual peptides and peptide pools used in the study. CS Bio (Menlo Park, CA, USA) synthesized each of the peptides except for CEFT (JPT Peptide Technologies, Berlin, Germany). Immunogenic gluten peptides sequences were selected from the hierarchy of gluten peptides determined by IFN-γ ELISpot using PBMC from blood collected from treated HLA-DQ2.5+ CD patients six days after they commenced gluten challenge (26). Peptide purity was > 95% by HPLC, and LC-MS confirmed each peptide's identity. As described elsewhere (3), peptide pool stock solutions were prepared as 10x final incubation concentration and were dispensed in 25 μl to individual wells in sterile 96-well U-bottom microwell plates (Thermo Fisher Scientific) that would later be used for blood incubations. Plates were sealed with sterile adhesive covers (Thermo Fisher Scientific), stored at -80°C, and shipped on dry ice to the study site. Immediately before use, to avoid condensation and seepage of peptide solutions between wells, microplates containing test solutions were thawed while being centrifuged at room temperature.

PBMC Separation

Heparinized blood was diluted with an equal volume of PBS/2% fetal bovine serum (FBS) (StemCell Technologies) before transfer to 50 ml SepMate™ tubes (StemCell Technologies) pre-filled with 15 ml Ficoll-Paque Plus (GE Healthcare). Blood was separated by centrifugation at 1200g for 10 min at room temperature. The majority of the plasma supernatant was removed and discarded. The remainder of the layer above the SepMate barrier was poured into a fresh tube and washed twice in PBS/2% FBS, centrifuge settings: 300g for 10 min at room temperature. PBMC were counted using the Scepter cytometer (Merck) with 40 μm sensors. PBMC were resuspended to 0.9-1.8 million per ml in RPMI 1640 media supplemented with 10% Human male AB serum (Sigma-Aldrich), 1x Glutamax (Gibco), 1x MEM non-essential amino acids (Gibco) and 50 μM 2-mercaptoethanol (Sigma-Aldrich).

Cytokine Release Assay

The CRA was performed as described elsewhere (3). Briefly, 225 µl aliquots of blood or PBMC in media were dispensed into wells pre-filled with 25 µl PBS containing "NX", "xNX", "NXxNX", "Control", or "CEFT" peptides (**Table 1**). Six replicate wells were assessed for each test condition. Incubation plates were immediately placed in a humidified incubator at 37°C in 5% CO_2. After the incubation period, plates were centrifuged at 500g for 10 min at room temperature. Plasma, 90 - 120 µl per well, was carefully collected to avoid blood cell contamination and transferred to a corresponding well in a "mirror image" sterile 96-well plate that was sealed with an adhesive cover, immediately frozen at -80°C, and later shipped to the ImmusanT laboratory for cytokine assessment. An ECL 3-plex IL-2, IFN-γ, and IL-10 immunoassay kit from Meso Scale Diagnostics LLC (Rockville, MD, USA) was used according to manufacturer's instructions. In the pilot study, one 25 µL plasma sample was assessed from each of six replicate incubations. In the RESET CeD Study, plasmas from two adjacent replicate wells were pooled making three measurements for each test condition. Cytokine concentration was determined using the MSD MESO™ Sector S600 plate reader and Discovery Workbench 4.0. The lower limit of detection (LLOD) was calculated for each cytokine on each assay plate.

HLA-DQ and HLA-DR Blocking

After resuspending PBMC to 0.9 - 1.8 million per ml in RPMI/10% Human AB serum, two 2 ml fractions were transferred to fresh tubes and incubated with anti-HLA-DQ antibody at 10 µg/ml (clone SPVL3; Beckman Coulter) or anti-HLA-DR antibody at 10 µg/ml (clone L243; BioLegend) for one hour at 37°C. Test conditions were assessed in duplicate wells.

Cell Depletions

Whole blood was depleted of either CD4+ T cells, CD8+ T cells or CD19+ B cells using RosetteSep Human Depletion cocktails and SepMate tubes (Stemcell Technologies), according to manufacturer's recommendations. Briefly, heparinized blood was divided between 3 tubes and incubated for 10 mins at room temperature with either CD4 Depletion Cocktail, CD8 Depletion Cocktail or a custom CD19 Depletion Cocktail (all at 1:20 dilution). The whole blood was then diluted and loaded into SepMate tubes and PBMC were isolated according to the procedure described above. The unwanted cells pellet with the red blood cells and therefore are depleted from the resulting PBMC. The depleted PBMC were resuspended at 0.9 - 1.8 million per ml in complete RPMI/10% Human AB serum, and then incubated with peptides in the cytokine release assay as described above. Test conditions were assessed with duplicate wells.

Peptide-Stimulated Proliferation of CD4+ T Cells

As described previously (3), proliferation assays consisted of three replicate wells per condition containing ~0.3 million in 225 µl per well of CTV-labelled PBMC and 25 µl test solution. After 8 days cells were stained with CD3-FITC (UCHT1), CD4-APC (SK3), and 7-Amino-Actinomycin D to discriminate dead cells (7-AAD; all from BD Biosciences). Cells were analysed on a BD FACSVerse cytometer and flow cytometry data was analysed by FlowJo software (version 10; FlowJo, LLC).

Lymphocyte Subset and Monocyte Frequencies in Fresh Blood and PBMC

Whole blood or PBMC were stained with antibody mix comprising anti-human CD3-Bv421, CD4-PE, CD8-APC, CD14-APCH7, CD19-Bv480, CD20-PECy7, and CD45-FITC (clones UCHT1, SK3, SK1, MphiP9, SJ25C1, L27, 2D1, respectively) and 7-AAD (all from BD Biosciences). For whole blood, BD Trucount™ tubes were used according to the manufacturer's instructions (**Figure S1**). Briefly, antibody mix was added followed by 50 µl whole blood by reverse pipetting. Tubes were vortexed and incubated for 15 min at room temperature in the dark. Erythrocytes were lysed by incubating with 450 µl 1xPharm Lyse™ (BD Biosciences), for 15min before analysing samples on a BD FACS Verse.

Statistics

The sample size was empirical for both the pilot and exploratory mechanistic study. All participants in the RESET CeD Study who had samples collected at Visit 5 and 28 were included in the analysis. Cytokine release (pg/ml) was normalized by dividing by the number of CD4+CD3+ T cells per well, or, to account for HLA class I restricted epitopes in CEFT and CD8+ T cell depletion studies, by the sum of CD4+CD3+ and CD8+CD3+ T cells per well. Non-parametric statistical tests were used to compare unpaired (Mann-Whitney U test) or paired data (Wilcoxon signed-rank test) without correction for multiple comparisons. ANOVA test was used to compare changes in biomarkers associated with treatment in the RESET CeD Study. For statistical tests, cytokine concentrations for signal values below the LLOD were treated as equal to the LLOD.

RESULTS

Exploratory Functional Assessments of Peripheral Blood Gluten-Specific CD4+ T Cells

The two CD patients (S0568 and S0211) in the pilot study each provided blood samples before and six days after commencing 3-day gluten challenge to assess CRAs and CD4+ T cell proliferation stimulated by gluten peptides (**Figure 1**). They were studied from 3 December to 11 December 2018. Both participants were HLA-DQ2.5+ and heterozygous for *HLA-DQA1*05* and *HLA-DQB1*02*. S0211 was a male aged 67 years on GFD for 20 years, and S0568 was a 62-year-old female on GFD for 17 years.

Gluten Peptide-Stimulated CD4+ T Cell Proliferation in PBMC

Dye-dilution proliferation assays are well described for detection of rare antigen-specific CD4+ T cells (27, 28). PBMC from blood

collected before and six days after gluten challenge were used in 8-day CTV dye-dilution proliferation assays. For PBMC assessed after patients had had a 3-day gluten challenge, CD4+ T-cells proliferated in response to each gluten peptide pool (NX, xNX and NXxNX), and the CEFT peptide pool (**Figures 2A–D**). For S0211, the fold-difference for NX compared to the control scrambled 8mer peptide pool was 5.7 before versus 77 after gluten challenge. In contrast, S0568's responses to NX and other gluten peptide pools were no different from control before gluten challenge, but after gluten challenge the NX-stimulated response was 57-times higher than control. Altogether, these findings indicated that the CTV dye-dilution proliferation assay was suitable for detection of gluten-specific CD4+ T cell responses, but may not be sufficiently sensitive in some CD patients without prior gluten challenge.

Gluten Peptide-Stimulated CRA in PBMC

With the aim of developing a simple but sensitive assay of gluten-specific CD4+ T cells, a 24-hour PBMC-based CRA using ECL to assess IL-2, IFN-γ, and IL-10 release was tested using six replicates for each condition with PBMC from blood collected after gluten challenge. Both patients showed significantly increased IL-2, IFN-γ, and IL-10 release stimulated by NX, xNX and NXxNX, and CEFT peptide pools compared to the negative control pool (**Figures 3A, B**). Overall, IL-2, IFN-γ and IL-10 release stimulated by NXxNX was always significantly higher than xNX ($p < 0.05$), but was significantly higher than NX only for IL-2 and IL-10 release by S0211 ($p < 0.05$) and not for any cytokine tested in S0568. Expressed as a ratio to the negative control, IL-2 release for gluten peptide pools (NX, xNX and NXxNX) were on average 2.8- or 7.5-times higher than for IFN-γ, and 15- or 10-times higher than for IL-10 in S0568 and S0211, respectively (**Figures 3C, D**). CRAs with PBMC separated from blood that had CD4+ or CD8+ T cells, or B cells depleted indicated that CD4+ T cells but not CD8+ T cells or B cells were necessary for NX and xNX-stimulated IL-2 release (**Figures 3E, F**). NX and xNX-stimulated IL-2 secretion was also reduced by incubating PBMC with anti-HLA-DQ antibody, but not by anti-HLA-DR antibody (**Figures 3E, F**). However, when the PBMC-based CRA was applied to cells isolated from blood collected before gluten challenge, only IL-2 release was significantly increased in PBMC stimulated with gluten peptide pools (**Figures 4A, B**), and neither IFN-γ or IL-10 release were detected (**Figures 4C–F**).

Collectively, these observations indicated this ECL-based CRA was capable of detecting IL-2, IFN-γ and IL-10 release in PBMC stimulated by gluten peptides. But without prior gluten challenge this CRA using PBMC could only detect relatively weak gluten peptide-stimulated IL-2-release. This fell short of the suggested minimal measure of T-cell function for clinical monitoring that should include both IL-2 and IFN-γ secretion (4).

Cytokine Release Stimulated by Gluten-Peptides in Whole Blood Compared to PBMC

We speculated that cytokine release in whole blood may be more efficient than using PBMC because of reduced delays and less manipulation of immune cells prior to stimulation with antigen. A portion of the heparinized blood collected from the two CD patients before and after gluten challenge was allowed to stand undisturbed at room temperature in the collection tube while the remainder was used to prepare PBMC. After PBMC were

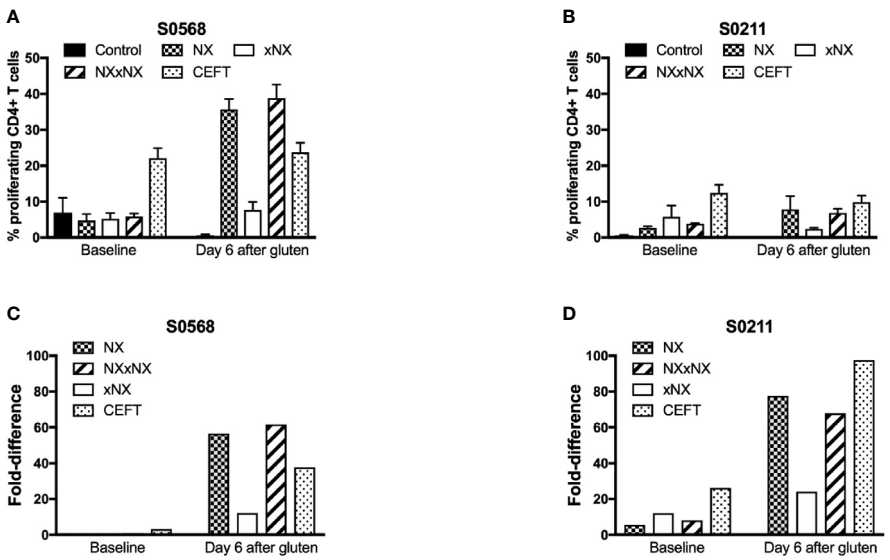

FIGURE 2 | CD4+ T cell proliferation assessed by CTV-dilution in fresh PBMC from two HLA-DQ2.5+ CD patients incubated for 8 days with peptide pools containing Nexvax2 peptides (NX), non Nexvax2 peptides (xNX) or both, or CEFT positive control peptides. Mean (SEM) percent proliferating CD4+ T cells **(A, B)** and mean fold-difference in proliferation compared to control **(C, D)** is shown for PBMC incubated with peptide mixes, data represent mean of triplicates.

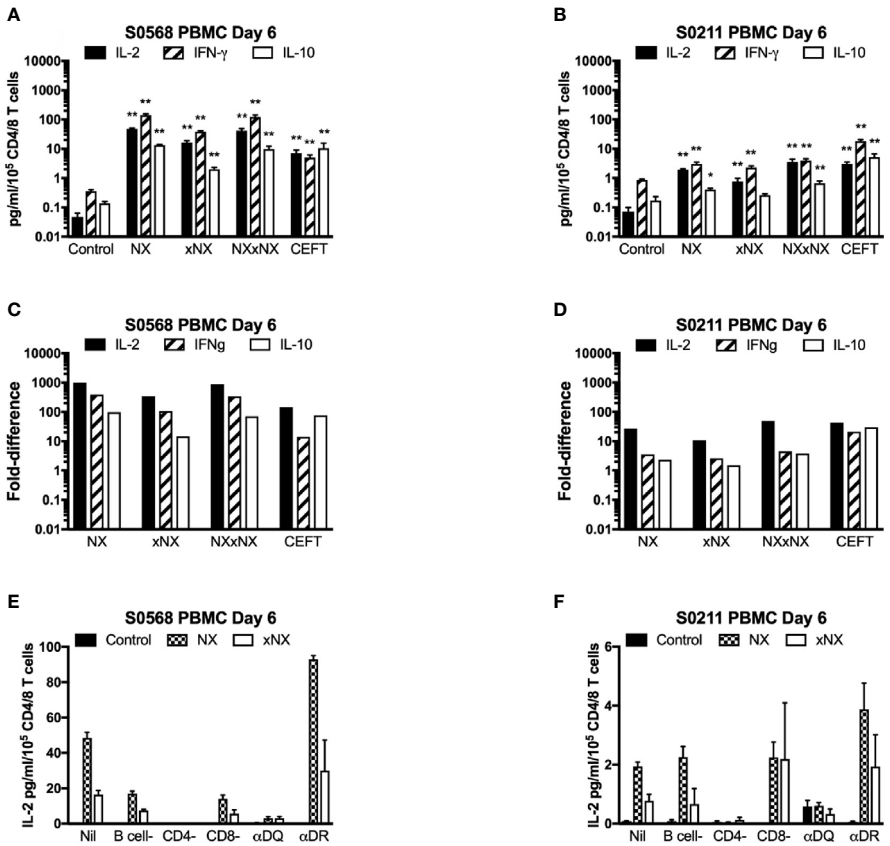

FIGURE 3 | Cytokine release in fresh PBMC from two HLA-DQ2.5+ CD patients. Concentrations **(A, B)** and fold-change relative to control **(C, D)** are shown for IL-2, IFN-γ, and IL-10 in media after 24-hour incubation of PBMC with gluten peptides or CEFT. Data are normalized for the number of live CD3+CD4+ and CD3+CD8+ T lymphocytes in PBMC and represent the mean + SEM of six replicate wells; Mann-Whitney U test was used to compare cytokine release for peptide mixes versus Control (statistical significance is indicated by *$p < 0.05$, or **$p < 0.01$). IL-2 release in PBMC is shown following depletion of B cells, CD4+ T cells or CD8+ T cells, or pre- and co-incubation with antibody that blocks epitope presentation by HLA-DQ or HLA-DR, data represent mean of duplicates **(E, F)**.

separated, the frequencies of CD4+CD3+ T cells, CD8+CD3+ T cells, CD19+CD20+ B cells, and CD14+ monocytes were assessed in PBMC and whole blood. After 3-hours, PBMC had been separated, resuspended in media, and cell density had been adjusted so that the number of CD4+ T cells per 225 µl (median: 0.19 million, range: 0.17 - 0.21 in a total of 0.5 million PBMC) was similar to the expected number of CD4+ T cells in 225 µl of matched, unseparated blood (median: 0.16 million, range: 0.13 - 0.21) (**Table 2**). This ensured that the immune cell population in each well incubated with gluten peptides was matched for the frequencies of responding CD4+ T cells and antigen presenting cells, and for delay since blood collection.

Before the 24-hour incubation with peptide mixtures, IL-2 concentrations were consistently below the lower level of detection, LLOD (0.04 pg/ml) in plasma from whole blood, whereas median IFN-γ and IL-10 concentrations were 2.4 and 0.27 pg/ml, respectively (**Table 2**). As expected, none of the three cytokines were detectable in media used to resuspend PBMC. After 24-hour incubation with control peptides, median IL-2, IFN-γ and IL-10 concentrations measured in plasma from the two whole blood samples collected from both CD donors (total of four blood samples studied) had modestly increased to 0.28, 4.2, 0.34 pg/ml, respectively, which were similar to their concentrations in media after 24-hour incubations of matched PBMC (0.59, 2.0, and 0.46 pg/ml, respectively) (**Table 2**). After normalizing for the frequency of live CD4+ T cells present in each well when incubation commenced, IL-2 release stimulated by NX relative to control was consistently higher in whole blood than matched PBMC incubations ($p < 0.05$) with median IL-2 release 10-times (range: 1.5 - 39) higher for whole blood than PBMC (**Figures 4A, B**). IFN-γ release stimulated by NX relative to control was also consistently higher in whole blood than matched PBMC incubations ($p < 0.01$) with median IFN-γ release 3.1-times (range: 2.1 - 23) higher for whole blood (**Figures 4C, D**). In contrast, IL-10 release was not consistently changed, and in two of the four blood samples, IL-10 release was significantly lower in whole blood than PBMC (**Figures 4E, F**). Other gluten peptide pools and CEFT also showed substantially greater IL-2 and IFN-γ release relative to control in whole blood than in matched PBMC (**Table 2**).

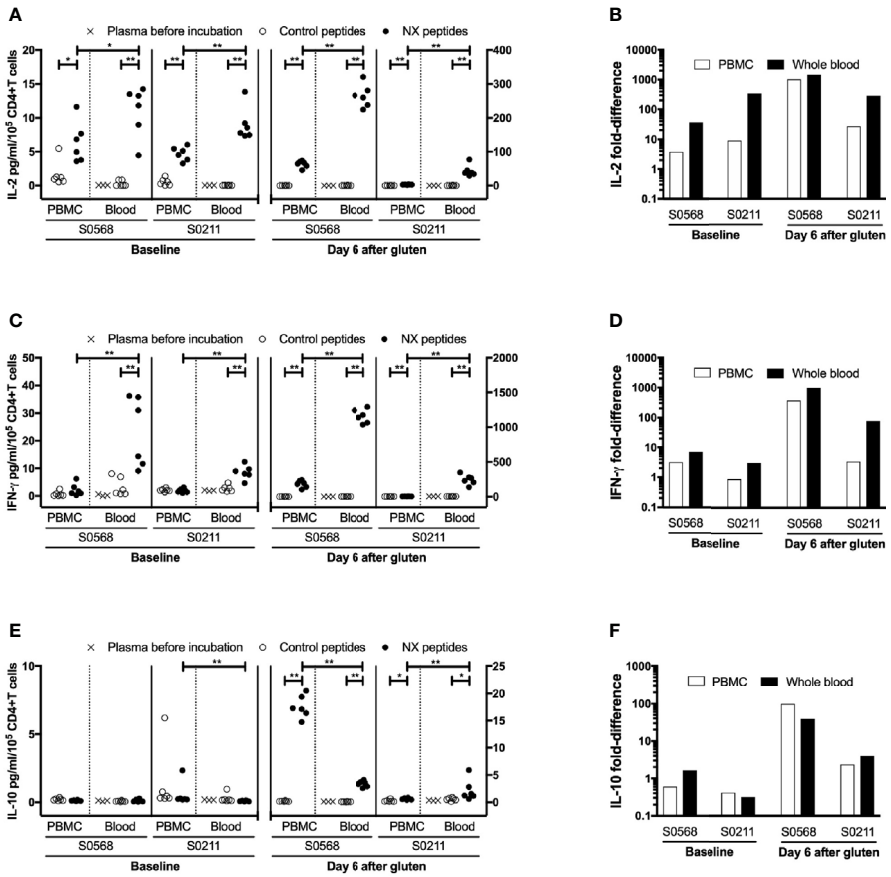

FIGURE 4 | Concentration and fold-difference relative to control for Nexvax2 (NX)-stimulated IL-2 **(A**, **B)**, IFN-γ **(C**, **D)** and IL-10 **(E**, **F)** release in PBMC and whole blood normalised for the number of live CD3+CD4+ T cells. Unseparated blood was allowed to stand at room temperature while PBMC were separated from a matched sample. Three hours after blood was collected, 225 µl aliquots of whole blood or 0.5 million PBMC in media were dispensed into microwells containing 25 ul PBS and test antigen. Plasma or media were separated after 24-hour incubation. For whole blood incubations, cytokine concentrations in plasma separated from whole blood before incubation with NX or control is also shown. Data points represent cytokine levels for each of six replicates assessed for each condition with blood collected before and six days after commencing gluten challenge for two coeliac disease patients. Mann-Whitney U test was used to compare NX-stimulated cytokine release in PBMC versus fresh whole blood; statistical significance is indicated by *$p < 0.05$, or **$p < 0.01$.

In addition to cytokine secretion in whole blood being more robust than with PBMC after gluten challenge, whole blood at baseline before gluten challenge also showed significantly increased IFN-γ as well as IL-2 release stimulated by NX, NXxNX and CEFT compared to control for both CD patients (**Figure 5**). At baseline, whole blood IL-2 release stimulated by NX was 37- and 351-times higher than control for S0568 and S0211, respectively, whereas whole blood IFN-γ release stimulated by NX was 16- and 3.1-times higher than control, respectively. However, none of the peptide pools except CEFT stimulated IL-10 release in whole blood at baseline. Whole blood IL-2 and IFN-γ release stimulated by NX at baseline were consistently significantly higher than for xNX ($p < 0.05$), but usually not significantly different from NXxNX.

Collectively, these findings indicated that using whole blood instead of PBMC substantially enhanced cytokine secretion stimulated by antigenic peptides, and allowed the ECL-based CRA to measure gluten peptide-stimulated IL-2 and IFN-γ release without requiring CD patients to undertake a gluten challenge.

Effects of Duration of Whole Blood Incubation on Cytokine Release

The kinetics of tetanus toxoid-stimulated whole blood cytokine release indicates that IL-2, IFN-γ, and IL-10 release begin only after six hours, and peak concentrations of IL-2, IFN-γ and IL-10 are reached at or after 24 hours, but assessments with longer incubations may be compromised by hemolysis (6). CRAs with whole blood plated into 96-well microplates without delay were used to test whether CRAs would be informative with incubations reduced to six hours. Blood incubated with NX for 24 hours compared to six-hours had 141- or 40-times higher plasma concentrations of IL-2, and 66- or 20-times higher plasma concentrations of IFN-γ for S0569 and S0211, respectively (**Figures 6A–C**). There were no significant differences between six- and 24-hour IL-2 or IFN-γ release for

TABLE 2 | Cytokine concentrations in plasma or media[†], assay dynamic range, and cell subset frequencies in matched 24-hour whole blood and PBMC cytokine release assays.

Patient		S0568				S0211			
Sample		Baseline		Day 6 after gluten		Baseline		Day 6 after gluten	
Matrix		Whole blood	PBMC	Whole blood	PBMC	Whole blood	PBMC	Whole blood	PBMC
Pre-incubation									
Cell frequencies per well	CD3+CD4+	128500	169000	205000	205500	182000	181000	130000	214500
	CD3+CD8+	56500	78500	76500	75000	100000	119500	69500	112000
	CD19+CD20+	48500	33000	62500	41500	60000	21500	34000	28500
	CD14+	32500	87000	52500	55000	35500	73000	37000	53000
IL-2	Plasma/media	ND	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD	<LLOD
pg/ml	LLOD	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
IFN-γ	Plasma/media	<LLOD	ND	1.11	<LLOD	3.64	<LLOD	5.7	<LLOD
pg/ml	LLOD	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88
IL-10	Plasma/media	0.12	ND	0.231	<LLOD	0.308	<LLOD	0.41	<LLOD
pg/ml	LLOD	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Post-incubation with peptides mixes									
IL-2	Control	0.39	2.9	0.37	0.13	0.05	0.94	0.19	0.23
pg/ml	NX	14	11	543	130	16	8.5	57	6.2
	xNX	3.0	4.1	154	44	7.7	3.8	30	2.5
	NXxNX	12	13	574	113	13	9.3	48	7.6
	CEFT	185	37	136	19	89	35	84	9.9
	LLOD	0.04	0.04	0.04	0.04	0.05	0.05	0.06	0.06
	ULOD	2940	2940	2940	2940	2940	2940	2940	2940
IFN-γ	Control	4.3	1.2	2.23	0.96	5.0	3.9	4.0	2.8
pg/ml	NX	30	3.8	2378	374	16	3.5	318	9.8
	xNX	11	2.9	591	103	7.7	3.4	129	7.2
	NXxNX	14	4.2	2307	328	13	3.0	178	13
	CEFT	362	37	245	14	366	181	264	59
	LLOD	0.49	0.49	0.49	0.49	0.49	0.49	0.47	0.47
	ULOD	2860	2860	2860	2860	2860	2860	2860	2860
IL-10	Control	0.09	0.34	0.17	0.37	0.51	2.5	0.71	0.55
pg/ml	NX	0.15	0.21	6.9	36	0.16	1.0	2.9	1.3
	xNX	0.16	0.22	2.2	5.4	0.44	1.3	2.3	0.84
	NXxNX	0.03	0.22	7.4	26	0.10	0.51	1.3	1.8
	CEFT	0.79	5.0	2.0	28	2.8	9.3	0.95	17
	LLOD	0.02	0.02	0.03	0.03	0.03	0.03	0.02	0.02
	ULOD	680	680	680	680	680	680	680	680

[†]*Mean of six replicate wells except for pre-incubation plasma/media that were assessed in triplicate.*
LLOD, lower limit of detection; ULOD, upper limit of detection.

control. These findings indicated gluten peptide-stimulated whole blood IL-2 and IFN-γ release largely occurred after six hours, which is notably slower than IL-2 release *in vivo* after CD patients consume gluten (13).

Effects of Delaying Incubation of Whole Blood on Cytokine Release

Delays of up to 24-hours before processing blood for functional T-cell assays are considered acceptable in multi-centre immunotherapy clinical trials, but validation studies have often utilized PBMC that may have been cryopreserved (5). To assess the effects of delaying incubation of whole blood with antigen, blood from both CD patients on day 6 after commencing gluten challenge was collected into 10 ml lithium-heparin vacutainers that remained capped at room temperature for three, eight or 27 hours before addition to 96 well microplates and commencing 24-hour incubation. Blood that was processed without delay was dispensed directly into microplates and commenced incubation with antigen within 25 minutes of collection. Frequencies of live cells for each immune cell subset were assessed in blood samples immediately before incubations commenced. Compared to blood processed without delay, the average NX-stimulated IL-2 release normalized for the frequency of living CD4+ T cells declined significantly to 74% after three hours delay, to 41% at eight hours, and to 37% by 27 hours (**Figure 6D**). Normalized NX-stimulated IFN-γ release also dropped significantly to 69% after three hours delay, to 34% at eight hours, and to 18% by 27 hours (**Figure 6E**). A similar reduction in NX-stimulated IL-10 release was also observed (**Figure 6F**). **Table S1** shows that the decline in IL-2, IFN-γ and IL-10 release with increasing delay in commencing incubation was similar for NX, xNX, NXxNX and CEFT.

These findings indicated that delays of more than three hours between collection and incubation of fresh blood with antigen were likely to alter the functional phenotype of immune cells and substantially reduce antigen-stimulated cytokine secretion.

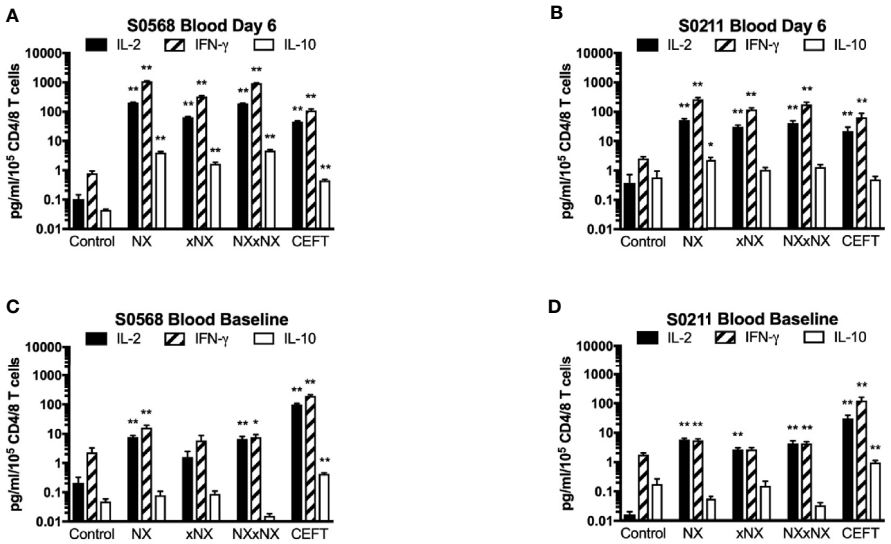

FIGURE 5 | Gluten peptide-stimulated 24-hour fresh whole blood IL-2, IFN-γ, and IL-10 release normalised for the number of live CD3+CD4+ and CD3+CD8+ T cells in individual microwells were compared to the negative control in samples from two HLA-DQ2.5+ CD patients: after gluten challenge **(A**, **B)** or at baseline before gluten challenge **(C**, **D)**. Bars represent the mean and SEM of six replicate wells. Mann-Whitney U test was used to compare gluten peptide-stimulated cytokine release versus Control; statistical significance is indicated by *p < 0.05, or **p < 0.01.

Monitoring Gluten Peptide-Stimulated T-Cells During Nexvax2 Immunotherapy

There were 23 participants in the RESET CeD study who enrolled in the supplementary sub-study from 20 December 2018 to 24 July 2019, but two were excluded as they did not have blood collected at both Visit 5 and 28. Demographics of the 12 participants who received Nexvax2 and 9 who received placebo treatment was similar, except that five among those receiving Nexvax2 were *HLA-DQB1*02* homozygotes compared to one receiving placebo (**Table 3**).

The ECL-based 24-hour whole blood IL-2, IFN-γ and IL-10 release assay normalized for the frequency of CD4+ T cells, and the 8-day CTV-dye dilution assay for CD4+ T-cell proliferation assay were used to monitor the effects of Nexvax2 immunotherapy. Overall, the median delay between blood collection and commencing incubation for CRA was 40 min

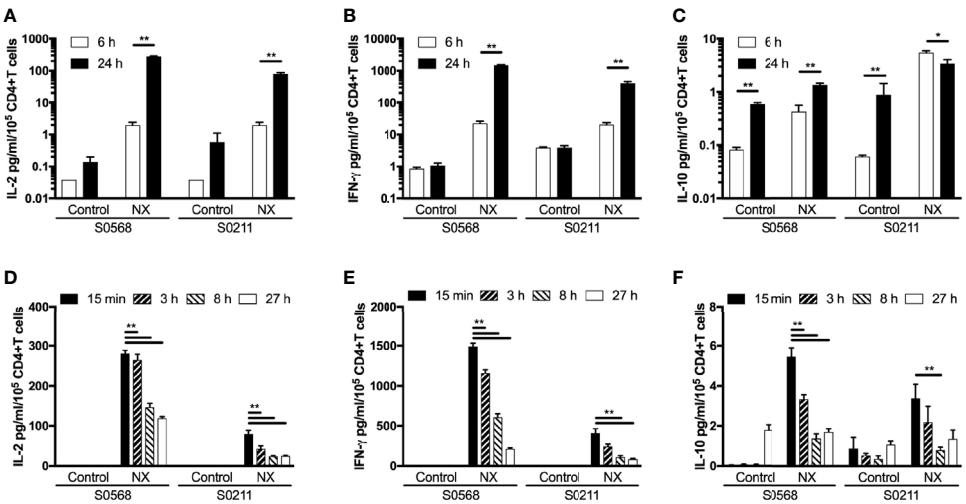

FIGURE 6 | Shortening the incubation period **(A**–**C)**, and varying the period blood was kept in collection tubes at room temperature **(D**–**F)** were assessed for their effects on gluten peptide-stimulated fresh whole blood IL-2, IFN-γ, and IL-10 release. Blood samples from two HLA-DQ2.5+ CD patients collected after gluten challenge were studied. Bars represent the mean and SEM of six replicate wells. Statistical significance by Mann-Whitney U test *p < 0.05, **p < 0.01.

TABLE 3 | Demographics and characteristics of RESET CeD Study participants.

Treatment	Nexvax2 (n=12)	Placebo (n=9)
Mean (SD) age in years	45 (11)	49 (16)
Number (%) females	8 (67)	4 (44)
Mean (SD) height in centimeters	171 (12)	176 (9)
Mean (SD) body mass in kilograms	81 (16)	80 (14)
Mean (SD) body mass index	28 (5)	26 (4)
Number (%) Caucasian	12 (100)	9 (100)
Number (%) positive for anti-TG2 IgA or DGP IgG	2 (17)	0 (0)
Number (%) positive for HLA-DQ2.5	12 (100)	9 (100)
Number (%) homozygous for *HLA-DQB1*02*	5 (42)	1 (11)
Secretory IgA deficiency	1 (8)	0 (0)
Median (range) years age at diagnosis CD	39 (18 - 54)	36 (22 - 61)
Median (range) years following GFD	8 (1 - 20)	10 (6 - 18)
Median (range) peak GloSS[†] after screening gluten challenge	8 (4 - 9)	8 (3 - 10)
Median (range) serum IL-2 4 h after screening gluten challenge (pg/ml)	6.3 (<0.5 - 36)	3.2 (<0.5 - 81)

[†]*GloSS is self-rated "global gastrointestinal symptom score" rated from no symptoms (0) to very severe (10) during the 6 hours after bolus food challenge with 10 g vital wheat gluten at screening.*

(range: 10 to 270 min), and the median difference between the delay at Visit 5 and Visit 28 for individual patients was 0 min (range: -50 to +95 min). At Visit 5 (n = 21), compared to the negative control, there were statistically significant increases in IL-2 and IFN-γ release, and CD4+ T cell proliferation stimulated by NX, NXxNX, and CEFT, and for IL-2 release and CD4+ T cell proliferation stimulated by xNX (**Figure 7**). Comparing Visit 5 and Visit 28 showed statistically significant reductions in NX-stimulated whole blood IL-2 and IFN-γ release, and CD4+ T cell proliferation in Nexvax2-treated patients, but not in placebo-treated patients (**Figure 8**, **Table S2**). In addition, xNX- and NXxNX-stimulated whole blood IL-2 and IFN-γ release fell significantly between Visit 5 and Visit 28 in Nexvax2-treated patients, albeit there was also a significant but isolated fall in xNX-stimulated IFN-γ release in the placebo group. Comparing the differences in biomarker measurements between Visit 5 and Visit 28 for Nexvax2 treatment compared to placebo, there were trends for reduction in NX- and NXxNX-stimulated IL-2 (p = 0.056, and p = 0.056, respectively by ANOVA test) and for NX-stimulated IFN-γ release (p = 0.0802, ANOVA test). Overall, Nexvax2 therapy appeared to have reduced gluten peptide-stimulated peripheral blood IFN-γ release so that it was no different from control, attenuated gluten peptide-stimulated peripheral blood IL-2 release and CD4+ T cell proliferation,

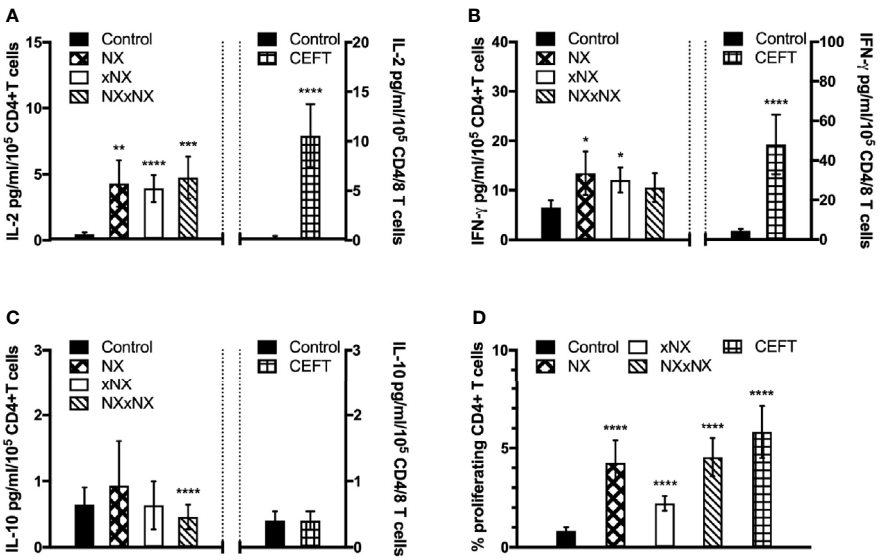

FIGURE 7 | Gluten-specific T cell responses prior to Nexvax2 administration. Gluten peptide-stimulated whole blood CRA **(A–C)** and CD4+ T cell proliferation measured by 8-day CTV-dye dilution assay before treatment **(D)** at Visit 5 in 21 patients randomized in the RESET CeD Study. Bars represent the mean and SEM. Wilcoxon signed-rank test was used to compare gluten peptide-stimulated cytokine release versus Control; statistical significance is indicated by *p < 0.05, **p < 0.01, ***p < 0.001, or ****p < 0.0001.

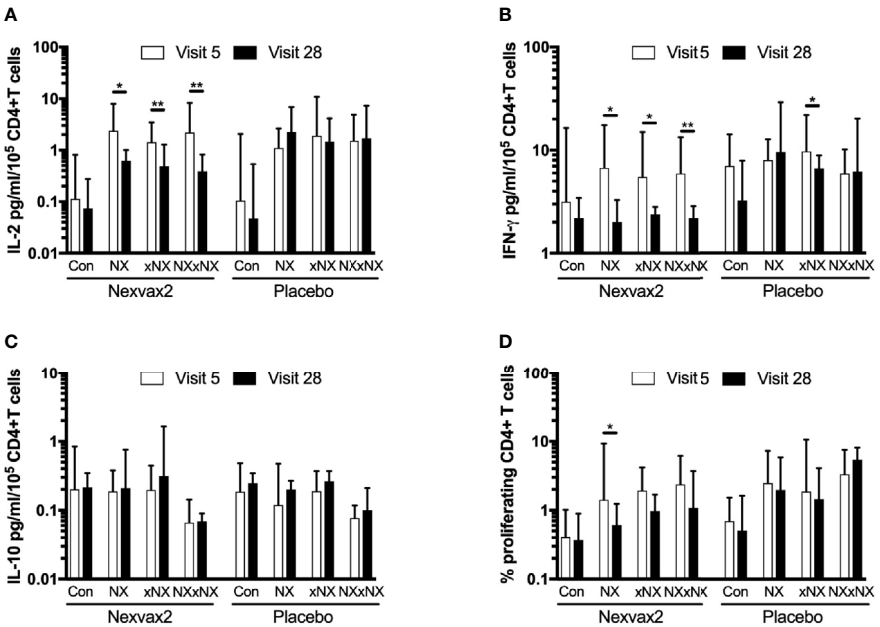

FIGURE 8 | Change in gluten peptide pool-stimulated whole blood CRA **(A–C)** and CD4+ T cell proliferation measured by 8-day CTV-dye dilution assay **(D)** over 12 weeks from before treatment at Visit 5 to Visit 28 during maintenance treatment in patients treated with Nexvax2 (n = 12) or placebo (n = 9) in the RESET CeD Study. Medians and interquartile ranges are indicated. Wilcoxon signed-rank test was used to compare Visit 5 to Visit 28; statistical significance is indicated by *$p < 0.05$, or **$p < 0.01$.

but did not enhance gluten peptide-stimulated peripheral blood IL-10 release.

DISCUSSION

In the present study and in a companion report (3), we used the MSD VPLEX multiplex ECL immunoassay for IL-2, IFN-γ and IL-10 to develop a highly sensitive whole blood CRA for rare peripheral blood gluten-specific CD4+ T cells. Here we demonstrate that standard practices for handling and processing blood can profoundly suppress antigen-stimulated IL-2 and IFN-γ release, which compromises detection of rare gluten-specific CD4+ T cells. The new whole blood CRA along with a dye-dilution proliferation assay were effective tools for monitoring gluten immunity in CD patients without needing a gluten food challenge to expand the pool of gluten-specific CD4+ T cells. These innovations allowed us to show that gluten peptide-stimulated whole blood IL-2 and IFN-γ release, and CD4+ T cell proliferation were significantly reduced in CD patients during treatment with Nexvax2 peptide immunotherapy, but not in placebo-treated CD patients. Interestingly, Nexvax2 therapy was associated with reduced ex vivo responses to gluten peptides present in Nexvax2 as well as an expanded pool of gluten peptides, which could support a mechanism of action involving direct suppression of Nexvax2-specific CD4+ T cells as well as indirect suppression of gluten-reactive CD4+ T cells not specific for epitopes in Nexvax2.

Previously, we and others have relied on 3-day gluten challenge to mobilize gluten-reactive CD4+ T cells in blood to monitor gluten-reactive CD4+ T cells by functional assays (22). Avoiding the need for gluten challenges in the immune monitoring sub-study associated with the RESET CeD Study was critical because patients had to comply with the RESET CeD Study protocol requiring no additional gluten exposures apart from the per-protocol masked gluten challenges required for the primary and secondary endpoints. PBMC had initially been preferred for CRAs because they could also be cryopreserved for later testing of CD4+ T cell function, staining with MHC class II-gluten peptide tetramers for flow cytometry assessments of surface phenotype, gene expression and DNA-based assays including T cell receptor gene analysis. However, finding that gluten peptides stimulated no detectable IFN-γ or IL-10 release, and only modest IL-2 release in PBMC without prior gluten challenge of donors prompted a detailed assessment of gluten peptide-stimulated cytokine release in whole blood.

IL-2 and IFN-γ release stimulated by gluten peptide pools and CEFT were normalized for the total numbers of CD4+ T cells or CD4+ and CD8+ T cells per well, respectively, which allowed direct comparisons between matched incubations of PBMC and whole blood. IL-2 and IFN-γ release stimulated by gluten peptide pools and CEFT in PBMC were typically one-tenth and one-third, respectively, the amounts measured using whole blood. The cause for reduced cytokine secretion by antigen-activated T cells in PBMC is unclear, but could relate to injury of reactive T cells due to physical manipulation, effects of Ficoll, or media

replacing plasma. Further investigations are required in larger numbers of blood samples to confirm these unexpected findings. Our observations could explain why CRAs using PBMC have been unable to detect rare antigen-specific CD4+ T cells, and position whole blood CRAs using high sensitivity immunoassays as an attractive new tool to study and monitor rare antigen-specific CD4+ T cells. In addition to the detrimental effects of separating PBMC from whole blood, this study also showed IL-2 and IFN-γ release stimulated by gluten peptide pools and CEFT were substantially reduced by delaying incubation of whole blood with antigen. Gluten peptide-stimulated IL-2 and IFN-γ release in blood allowed to stand for 24 hours at room temperature were reduced to one-third and one-fifth, respectively, the amount in blood that was incubated within 30 minutes of collection. This observation also challenges current practice in multicenter clinical trials that allows blood for functional T-cell assays to be processed at a central laboratory within 24 hours after collection.

Our finding that gluten peptide-stimulated whole blood IL-2 release is more pronounced than IFN-γ, and also IL-10 release, and is a sensitive marker for rare gluten-specific CD4+ T cells is in keeping with our parallel study that profiled cytokine release in CD patients when blood was incubated with a single immunodominant α-gliadin peptide identical to NEX-01 (3). In that study, we showed that the hierarchy of cytokines and chemokines released in whole blood stimulated by the α-gliadin peptide was IL-2 followed by IFN-γ, CXCL10/IP-10, CXCL9/MIG, IL-10, CCL3/MIP-1α, and TNF-α, in that order. We also showed that the whole blood assay was superior to other functional readouts of the antigen-specific T cell response such as the IFN-γ ELISpot, which is unable to detect gluten-specific T cell responses in treated CD patients without gluten challenge. The sensitivity of the whole blood IL-2 release assay appeared to be similar to MHC class II-gluten peptide tetramers, which have indicated the frequency of CD4+ T cells specific for epitopes in Nexvax2 is usually less than 10 per million peripheral blood CD4+ T cells in CD patients without prior gluten challenge (17, 19). A volume of 40 to 50 ml of blood is required for a single assessment by MHC class II-gluten peptide tetramers specific for epitopes in Nexax2 gluten peptides (17, 18), which contrasts with the whole blood CRA that detected Nexvax2 gluten peptide-stimulated IL-2 and IFN-γ release in 1.35 ml blood divided across six replicate wells each containing about 0.2 million CD4+ T cells. Therefore, in this study we estimate that an average total of about 12 gluten-specific CD4+ T cells distributed across six replicate wells was sufficient to monitor IL-2 and IFN-γ release stimulated by the pool of gluten peptides included in Nexvax2.

Finally, application of the whole blood CRA to monitor gluten-specific CD4+ T cells in the RESET CeD Study revealed that Nexvax2 treatment was associated with attenuation of gluten peptide-stimulated IL-2 and IFN-γ release, and CD4+ T cell proliferation. Statistical comparisons between the twelve Nexvax2 and nine placebo-treated patients in this sub-study almost reached significance for the reduction of IL-2 and IFN-γ release stimulated by gluten peptides in Nexvax2, and IL-2 release stimulated by the expanded pool of gluten peptides. This outcome was also unexpected, and may indicate that relatively small cohorts are needed to achieve statistical significance using whole blood CRAs to monitor antigen-specific immunotherapies in early clinical development. An explanation remains to be found for the discrepancy between significant reductions in ex vivo gluten peptide-stimulated IL-2 and IFN-γ release in whole blood, and the primary efficacy endpoint of the RESET CeD Study not being met. Self-reported digestive symptoms within one day after masked single-bolus gluten challenge compared to the pretreatment period was the primary endpoint. Patients ingested 10 grams of vital wheat gluten for the masked gluten challenge. This gluten challenge resulted in almost half of the placebo-treated patients in the RESET CeD Study experiencing vomiting between one to two hours, and was associated with median 20-fold increase of serum IL-2 at four hours (20). Serum IL-2 elevations correlated with nausea severity and vomiting (20), but the relationships between gluten exposure, symptoms, intestinal T cell activation, mucosal cytokine release, and systemic measures of gluten immunity including serum cytokines and responsiveness of peripheral blood gluten-specific CD4+ T cells remain to be determined. Nonetheless, the dose and/or duration of Nexvax2 administration was inadequate to suppress symptoms triggered by a food challenge administering almost half the usual daily gluten intake in an unrestricted diet as a single bolus.

A limitation of this work is that only two volunteers were assessed in the pilot study. However, findings from these two patients were consistent and demonstrated the capacity of the whole blood assay approach to detect gluten-specific T cells prior to a gluten challenge. Subsequent validation in the cohort of subjects assessed from the RESET CeD study strongly supported the sensitivity of the whole blood assay approach without gluten challenge. As gluten peptide-stimulated whole blood IL-2 and IFN-γ release was shown to be strongly affected by experimental conditions such as delayed incubation and overall incubation time, further work is required to understand the conditions supporting optimal assay performance. Future work should correlate whole blood IL-2 and IFN-γ release with assessment of PBMC by HLA-DQ2.5-gluten-peptide tetramers and flow cytometry in CD patients who have not been gluten challenged.

The whole blood CRA coupled with ultra-sensitive IL-2 and IFN-γ detection has promise as an immunomonitoring tool for clinical trials. In order to scale the protocol for multi-centre clinical trials, further refinements to address the challenges of blood transport delay from clinical sites distant from the laboratory will be important. For example, antigen-coated vacutainers could be employed for direct in-tube incubation of blood rather than multi-well plates, similar to that used in QuantiFERON-TB Gold *Mycobacterium tuberculosis* diagnostic kits, or the antigen could be added straight to blood in the collection tubes (22). This would expedite sample incubation at each trial site. Optimization of the antigens may also be useful.

In conclusion, rare gluten-specific CD4+ T cells can be detected and monitored using a simple whole blood CRA that utilizes a sensitive ECL immunoassay to measure IL-2 and IFN-γ release. Sensitivity of CRAs to detect peripheral blood gluten-

specific CD4+ T cells is markedly compromised by utilizing PBMC instead of whole blood, and by delays in incubating blood with antigen. Deploying this whole blood CRA to monitor effects of Nexvax2 immunotherapy was highly informative. This simple protocol in an optimized form may allow widespread utilization of this test to monitor the function of rare antigen-specific CD4+ T cells in clinical trials assessing vaccines and immunotherapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Research Ethics Committees at the Walter and Eliza Hall Institute of Medical Research, Melbourne Health, and Bellberry Limited. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RA, GG, LW, JD, and JT-D designed the studies. JT-D, SC, GB, KG, KN, and KT conducted clinical studies. MH, AR, SW, RZ, ES, and JD performed and analyzed immune assays. RA and GG provided data integration and analysis. RA wrote the manuscript and prepared the Tables and Figures. RA had full access to all the data in the study and had final responsibility for the decision to submit for publication. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.661622/full#supplementary-material

Supplementary Figure 1 | Cell subset FACS gating strategy for whole blood cell counts. 50 µl of whole blood was stained in TruCOUNT tubes and the number of each subset per µl of whole blood was calculated using the manufacturer's provided equation: (Number of events in region containing cells of interest/Number of events in absolute count bead region) × (Number of beads per test as per the TruCOUNT tube foil pouch/test volume in µl). This was used to determine cell numbers per well.

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REVIEW
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Interplay Between Gluten, HLA, Innate and Adaptive Immunity Orchestrates the Development of Coeliac Disease

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Several environmental, genetic, and immune factors create a "perfect storm" for the development of coeliac disease: the antigen gluten, the strong association of coeliac disease with HLA, the deamidation of gluten peptides by the enzyme transglutaminase 2 (TG2) generating peptides that bind strongly to the predisposing HLA-DQ2 or HLA-DQ8 molecules, and the ensuing unrestrained T cell response. T cell immunity is at the center of the disease contributing to the inflammatory process through the loss of tolerance to gluten and the differentiation of HLA-DQ2 or HLA-DQ8-restricted anti-gluten inflammatory $CD4^+$ T cells secreting pro-inflammatory cytokines and to the killing of intestinal epithelial cells by cytotoxic intraepithelial $CD8^+$ lymphocytes. However, recent studies emphasize that the individual contribution of each of these cell subsets is not sufficient and that interactions between these different populations of T cells and the simultaneous activation of innate and adaptive immune pathways in distinct gut compartments are required to promote disease immunopathology. In this review, we will discuss how tissue destruction in the context of coeliac disease results from the complex interactions between gluten, HLA molecules, TG2, and multiple innate and adaptive immune components.

Keywords: coeliac disease, villous atrophy, gluten, transglutaminase 2, HLA-DQ2/8, T lymphocytes

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INTRODUCTION

Coeliac disease (CeD) is a multifactorial intestinal immune-mediated disorder with autoimmune features that leads to inflammatory and destructive lesions in the proximal small intestine. CeD, similar to other organ-specific autoimmune disorders, is marked by its complexity both at the epidemiological and immunological levels, which translates into a spectrum of clinical manifestations (1). CeD is characterized by an infiltration of intraepithelial lymphocytes in the proximal part of the small intestine, crypt hyperplasia and the development of villous atrophy in the latest stages of the disease. In addition, CeD patients produce highly disease-specific antibodies against deamidated gluten peptides and the enzyme tissue transglutaminase 2 (TG2) (2–4). CeD is triggered by gluten consumption in genetically susceptible individuals carrying certain major histocompatibility complex (MHC) class II human leukocyte antigen (HLA) variants (5, 6). 90-95% of CeD patients carry the HLA-DQ2.5 variant (DQA1*05:01, DQB1*02:01) that confers the highest

risk of developing CeD while the remaining patients carry HLA-DQ2.2 (DQA1*02:01, DQB1*02:02) or HLA-DQ8 (DQA1*03, DQB1*03:02) (5, 7). However, while up to 40% of the general population in Western countries express one of the predisposing HLA molecules, the global prevalence of CeD is just 1% (8). This finding suggests that these HLA variants contribute to, but are not sufficient for, the development of the disease and that additional genetic and environmental factors are needed to mount a pathogenic immune response against gluten (9). In fact, the HLA locus which is the main inherited genetic susceptibility factor for CeD, only accounts for ~ 40% of the genetic variance of the disease. Hence, non-MHC susceptibility loci explaining ~ 15% of the disease risk (10–13), as well as additional environmental factors other than gluten, are thought to contribute to disease development. Among them are early life gastrointestinal infections, which have been associated with an increased risk of developing CeD in several cohorts of genetically susceptible children (14–16). Of particular interest are enteric viruses such as reovirus, norovirus and rotavirus, which are the most common causes of diarrheal disease in early childhood. Recurrent infections in young individuals with a permissive genetic background could interfere with the maturation of the mucosal immune system and the composition of the microbiome (17), and thus favor the subsequent induction of an inflammatory T cell responses and the loss of oral tolerance to dietary gluten (18, 19). Although much less documented, intestinal infections caused by bacteria such as *Campylobacter jejuni* or parasites such as *Giardia lamblia*, could also contribute to the onset or maintenance of the disease (20, 21). In strong support of a role of the microbial environment in promoting the development of CeD is the identification of microbially derived mimics of gliadin epitopes that can activate HLA-DQ2.5-restricted gliadin-specific T cells isolated from CeD patients (22).

The complexity of CeD is also reflected by the contribution of multiple immune pathways for the induction of the disease and intestinal tissue remodeling and destruction (23–25). It has been known for decades that HLA-DQ2 and HLA-DQ8 present TG2-deamidated gluten peptides to $CD4^+$ T cells in the intestinal lamia propria compartment, driving T_H1 differentiation (26–31). These gluten-specific T_H1 cells contribute to the inflammatory process through the production of the inflammatory cytokines Interferon (IFN)-γ (32) and Interleukin (IL)-21 (33). Yet, this gluten-specific adaptive immunity is not sufficient to promote the licensing of intraepithelial cytotoxic $CD8^+$ T cells (IE-CTLs) that are responsible for the destruction of distressed intestinal epithelial cells. This lack of sufficiency can be seen in potential CeD patients, who carry HLA-DQ2 or HLA-DQ8 and display adaptive immune responses against gluten (proxied by anti-TG2 and anti-endomysium antibodies) but lack villous atrophy (6, 34). In particular, potential CeD patients do not display an accumulation of IE-CTLs with an active killer phenotype (upregulated granzyme B expression, upregulated activating NK receptor expression, downregulated inhibitory NK receptor expression) and also lack upregulation of the pro-inflammatory cytokine IL-15 and the non-classical MHC class I stress molecules MICA/B and HLA-E in intestinal epithelial cells (25, 35, 36), immune features that are both required for the development of villous atrophy. Additionally no tissue destruction was observed in HLA-DQ2 or HLA-DQ8 humanized mice that develop anti-gluten immunity (37–39). Only in recent years has it become clear that the interplay between gluten-specific $CD4^+$ T cells and intraepithelial cytotoxic $CD8^+$ T cells, as well as the simultaneous activation of innate immune pathways in distinct gut compartments, are required to cause villous atrophy observed in the active form of the disease (**Figure 1**).

KEY ROLES OF TRANSGLUTAMINASE 2 AND HLA IN THE INITIATION OF THE GLUTEN-SPECIFIC ADAPTIVE IMMUNE RESPONSE

The identification of HLA-DQ2 or HLA-DQ8 restricted $CD4^+$ T cells in the lamina propria of CeD patients (26, 40) that preferentially recognize deamidated gluten peptides over native gluten peptides (41) stressed the connection between gluten, disease-associated HLA molecules and TG2 for the initiation of the pathogenic immune response. The generation of such a gluten-specific T cell response arises from the high affinity binding of gluten peptides post-translationally modified by TG2 to HLA-DQ2 or HLA-DQ8.

Two properties of gluten explain its ability to elicit a mucosal immune response. First, the high content of proline in gluten proteins makes the proteins resistant to degradation by intestinal proteases in the gut lumen (42). Second, the long undigested gluten-derived proteins are good substrates for the enzyme TG2, an ubiquitous and multifunctional enzyme expressed in many organs including the gut (43). It has been acknowledged for a long time that TG2 plays a key role in CeD pathogenesis as the enzyme is the target of autoantibodies that are highly-disease specific and used for the diagnostic work-up (2, 4). In addition, TG2 catalyzes the conversion of glutamine residues present in gluten peptides into glutamate (28–30). This deamidation process is key to initiate a pathogenic response in CeD as it promotes the generation of immunogenic peptides with negatively charged carboxylate residues that anchor with high affinity in the positively charged pockets of HLA-DQ2 or HLA-DQ8 binding grooves (5, 44).

In support of the role of TG2 in orchestrating mucosal immune responses to dietary gluten, TG2 is mostly found catalytically inactive in the intestine under physiological conditions but its expression and activity are increased in inflamed tissues and in cells with inflammatory stress (45). Interestingly, the administration of poly(I:C) (Polyinosinic:polycytidylic acid), which results in the rapid induction of villous atrophy that is the typical intestinal tissue injury observed in CeD patients, promotes TG2 activation (46). TG2 can be released into the gut lumen by small intestine enterocyte shedding, allowing TG2 to become catalytically active in the extracellular environment (47). This feature that allows the TG2 to be in close vicinity of gluten peptides could explain the formation of enzyme-substrate complexes between the two proteins

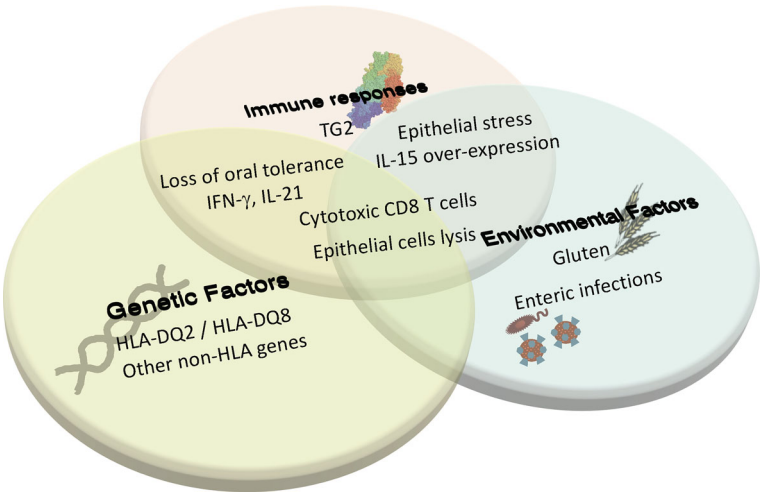

FIGURE 1 | Coeliac disease is a multifactorial complex autoimmune disorder that requires the interplay between genetics, innate and adaptive immunity, and environmental triggers to cause tissue destruction. In individuals with HLA-DQ2/DQ8, induction of adaptive immune response against gluten and the loss of oral tolerance to gluten can occur when there is environmentally triggered epithelial stress and IL-15 overexpression. This adaptive response to gluten and the associated cytokine production promotes further tissue stress, leading to the licensing of cytotoxic CD8 T cells to lyse epithelial cells and cause villous atrophy. Additional environmental triggers and immune factors yet to be determined are also thought to contribute to disease development.

that can bind to the B cell receptor of TG2-specific B cells, hence contributing to the generation of TG2-specific autoantibodies (24, 48, 49). In addition, using T84 enterocytic cell line as a model, it was shown that extracellular TG2 can be activated in a phosphatidylinositol-3-kinase-dependent mechanism by IFN-γ (50), the pro-inflammatory cytokine abundantly produced by gluten-specific T cells and released in the inflamed intestinal mucosa of CeD patients (31, 32). In accordance with a role for IFN-γ in TG2 activation, TG2 enzymatic activity can be triggered by the protein cofactor thioredoxin-1 (TRX) whose release from monocytic cells is also elicited by IFN-γ (51).

The requirement of gluten, predisposing HLA variant, TG2, and CD4 T cells to elicit the disease was formally demonstrated using a newly engineered DQ8-D^d-villin-IL-15tg mouse model of CeD that develops villous atrophy upon gluten exposure (23). Using this model, we showed that villous atrophy, anti-deamidated gluten peptide antibodies and T_H1 immunity recede on a gluten free diet and reoccur after gluten introduction. In addition, intestinal tissue destruction only occurred in mice carrying HLA-DQ8 and depletion of $CD4^+$ T cells or administration of TG2 inhibitors in gluten-fed animals prevented the development of villous atrophy (23). Hence, the priming of a gluten-specific immune response depends on the coordinated interaction between gluten, the coeliac predisposing HLA-DQ8 molecule, activated TG2, and $CD4^+$ T cells.

Interestingly, there is a gene dosage effect of the HLA-DQ2 or HLA-DQ8 allotypes in CeD whereby disease susceptibility depends on the HLA-DQ genotype and HLA homozygous individuals are at higher risk of developing the disease (52, 53). The haplotype HLA-DQ2.5 that binds and presents the full repertoire of gluten peptides, with many proline-rich α- and ω-gliadin-derived peptides that are protected from the degradation by gastrointestinal enzymes, confers a higher risk of developing CeD as compared to HLA-DQ8 that presents a smaller repertoire of immunogenic peptides more prone to proteolytic degradation and HLA-DQ2.2 that can only bind and present a few peptides (42, 54, 55). In addition, DQ2.5 has an increased ability to retain its peptide cargo as compared to DQ2.2. thanks to the presence of a polymorphism in the DQα chains allowing DQ2.5 to establish a hydrogen bond to the peptide main chain that stabilizes peptide-MHC complexes at the surface of antigen-presenting cells leading to sustained gluten peptides presentation to T cells (56). Interestingly, differences in the nature of HLA-DQ2.2 or HLA-DQ2.5-bound epitopes translates into a more diverse TCR repertoire generated in the context of HLA-DQ2.2, as compared to HLA-DQ2.5-mediated CeD and with a lower disease penetrance (57). Homozygosity for HLA-DQ2.5 that is linked to a heightened expression of HLA-DQ2.5 on the surface of antigen-presenting cells and increased antigen presentation is also more strongly associated with CeD as compared to heterozygosity (52, 55). Hence, the amounts of HLA-DQ-gluten peptides complexes correlates with the magnitude and breadth of the gluten-specific T cell response (52, 55). This suggests that CeD development and the ensuing intestinal tissue destruction will only occur when the T cell response has reached a certain magnitude to become pathogenic (35).

CD4 T CELLS AND THE PRO-INFLAMMATORY RESPONSE

Under homeostatic conditions, ingested dietary antigens induce oral tolerance, a state of local and systemic immune ignorance against orally ingested innocuous antigens (58, 59). However, in patients harboring the CeD-predisposing HLA-DQ2 or HLA-

DQ8 molecules, orally ingested gluten can initiate a gluten-specific pro-inflammatory T_H1 response rather than a tolerogenic response (35). In both adults and children, these gluten-specific CD4 T cells produce high levels of the pro-inflammatory cytokines IFN-γ and IL-21, hallmarks of T_H1 cells (32, 33, 60). As loss of oral tolerance to gluten is a preceding event for the development of villous atrophy in CeD patients (25, 32), much work has been done to uncover the mechanisms behind this loss of tolerance against gluten and how this pro-inflammatory T_H1 CD4 T cell response leads to villous atrophy.

One of the early culprits implicated in promoting an inflammatory *vs* tolerogenic T cell response to gluten is the cytokine interferon-α (IFN-α). IFN-α is a Type-1 interferon that is produced by almost all cells as an innate response to viral infection (61). Among its many immune effector-promoting roles, it has been shown to drive pro-inflammatory dendritic cells activation as well as to promote the differentiation of CD4 T cells to the T_H1 lineage (61, 62). The connection between IFN-α and CeD was made by Monteleone and colleagues, who identified a CeD-like enteropathy with villous atrophy and high intraepithelial lymphocytes infiltration in a chronic myeloid leukemia patient receiving an IFN-α treatment (63). The association between high expression of IFN-α and high levels of IFN-γ in CeD patients compared to controls suggested that IFN-α in CeD patients may be one factor leading to induction of a T_H1 response against gluten. It remains unclear what directly is driving the increase in IFN-α production, but recent studies have also implicated viral infection as a driver for loss of oral tolerance.

While viral infections, such as with adenovirus or hepatitis C, have long been known to be associated with a higher risk of developing CeD (64), only recently have viral infections been mechanistically shown to induce loss of oral tolerance to gluten and dietary antigens. Using the Type-I Lang (T1L) reovirus strain, and murine norovirus (MNV) that both infect the gut, Bouziat and colleagues demonstrated that both viruses were capable of mediating T_H1 responses to dietary antigens (18, 19). Type-1 IFN signaling was required for the blockade of peripheral regulatory T cells conversion while Interferon Regulatory Factor (IRF)1 expression was required for the induction of a T_H1 immunity characterized by the differentiation of IL-12p40-producing dendritic cells, the production of gluten-specific IgG2c antibodies in the serum, TG2 activation in the proximal small intestine and a delayed type hypersensitivity reaction to gluten, all hallmarks of loss of oral tolerance to gluten in virus-infected HLA-DQ8 transgenic mice (18, 19). Taken together, these studies demonstrated that viral infections can be triggers for loss of oral tolerance towards dietary antigens and T_H1-skewed responses to gluten.

Another major player implicated in the loss of oral tolerance to gluten is IL-15. The first signs that IL-15 may have been involved in the proinflammatory T_H1 response to gluten came with the finding that IL-15 is heavily upregulated in the lamina propria of active CeD patients, the effector site where dendritic cells will encounter gluten peptides (65, 66). Using HLA-DQ8 transgenic mice that overexpressed IL-15 in the lamina propria and mesenteric lymph nodes (DQ8-D^d-IL15tg mice) but not in the intestinal epithelium (38), we demonstrated that IL-15 overexpression in combination with retinoic acid altered the tolerogenic phenotype of intestinal dendritic cells and endowed them with a pro-inflammatory phenotype, hindering the development of $Foxp3^+$ regulatory T cells and instead promoting the differentiation of IFNγ-producing T_H1 cells. Additionally, these gluten-fed DQ8-D^d-IL15tg mice displayed elevated levels of anti-gliadin and anti-TG2 antibodies, mimicking potential CeD patients who display a loss of oral tolerance and the development of a T_H1 response to gluten in the absence of villous atrophy (38). In addition, IL-15 can block the immunosuppressive effects of TGF-β on CD4 and CD8 T cells by inhibiting Smad3-signalling and additionally render effector CD4 and CD8 T cells resistant to regulatory T cells-mediated suppression by activating PI3K-signaling (67, 68). Whether $Foxp3^+$ regulatory T cells play an active role in dampening harmful immune responses to gluten in the small intestine remains poorly understood. Although it was shown that $Foxp3^+$ regulatory T cells expand in the celiac lesion (69–71), it remains controversial whether regulatory T cells retain or loss their suppressive function (71–73). Moreover, regulatory $CD4^+$ T cells specific for immunodominant gluten peptides haven't been identified so far in the small intestine of genetically predisposed healthy individuals (74). Therefore, additional investigations are warranted to determine whether a regulatory response to gluten exists and whether an altered mucosal suppressive $CD4^+$ T cell response to gluten contributes to CeD pathogenesis.

In the context of CeD, T_H1 immunity is accompanied by the production of IFN-γ and IL-21 by mucosal gluten-specific $CD4^+$ T cells (32, 33, 60). The idea of crosstalk between lamina propria and epithelium mediated by cytokines was put forward several years ago based on *in vitro* observations (75). Indeed, it had been shown that IFN-γ released by stimulated mucosal T cells was required for the optimal killing of human colonic epithelial cell lines in *ex-vivo* cytotoxic assays (76). In addition, the incubation of intestinal tissue specimens with the supernatants from gluten-stimulated T cell clones or with IFN-γ lead to epithelial cell damage, and the cytotoxic effect of the supernatants could be counteracted by the addition of neutralizing IFN-γ (77). We recently confirmed the requirement of IFN-γ for the activation of cytotoxic intraepithelial lymphocytes and the ensuing development of villous atrophy *in vivo* using a relevant mouse model of CeD (23). Although the exact mechanism underlying this effect remains to be uncovered, it has been shown that local production of IFN-γ can promote the upregulation of the non-classical MHC class Ib molecule HLA-E on epithelial cells (78, 79) hence potentializing the expression of the ligand for the activating NK receptor CD94/NKG2C present on cytotoxic intraepithelial lymphocytes during disease development. Although it has been shown that IL-21 can increase the cytotoxicity of human intraepithelial lymphocytes (80), the administration of an IL-21R blocking antibody in our mouse

model didn't reveal any significant direct role of IL-21 in promoting cytotoxic properties on intraepithelial lymphocytes but instead demonstrated that IL-21 and IFN-γ both play a role in the development of anti-deamidated gluten peptides antibodies (23). Interestingly, IL-15 can promote IL-21 production in lamina propria cells (81) reinforcing the idea of the involvement of a cytokine network in CeD.

While IFN-α, viral infections and IL-15 overexpression in the lamina propria can induce a loss of oral tolerance and a pro-inflammatory T_H1 response to gluten, several studies in mice have demonstrated that the CD4 T cell response to gluten alone is nevertheless not sufficient to license intraepithelial lymphocytes and induce villous atrophy (23, 37, 38, 82). This is in accordance with observations in potential CeD patients who not only lack IL-15 and stress molecules expression on intestinal tissue cells, but also do not display an accumulation of intraepithelial cytotoxic T lymphocytes with an active killer phenotype, as seen in active CeD patients, despite the development of an inflammatory CD4 T cell response (25).

Interestingly, a distinct cytokine signature has been identified in the peripheral blood of treated CeD patients after oral gluten challenge (83, 84) or subcutaneous administration of T-cell stimulatory gluten peptides (85). Indeed, secretion of IL-2, IL-17A, TNF-α, IL-6 and IL-10 was detected as soon as 2h after gluten-re-exposure reflecting the rapid mobilization of gluten-specific memory CD4 T cells. Serum cytokine elevations, particularly IL-2 levels, correlated with the severity of acute digestive symptoms (83) and were specific to CeD (84, 86, 87), demonstrating a direct impact of gluten on the adaptive immune system in genetically susceptible individuals. Not only could serum cytokine release contribute to some extra-intestinal manifestations of CeD driven by inflammation, but it could be used as immune marker to diagnose and monitor the development of CeD.

INNATE IMMUNE RESPONSE TO GLUTEN AND EPITHELIAL STRESS

The contribution of innate immunity to CeD pathogenesis was suggested by the observation that non-HLA genomic regions associated with CeD harbor genes involved in stress pathways and innate immunity (5). The activation of innate immune pathways in different gut compartments, in particular at the level of the lamina propria or in the intestinal epithelium, has a substantial impact on the adaptive immune responses taking place in those same compartments. These responses include the loss of oral tolerance to gluten and the associated induction of T_H1 immunity, as well as the acquisition of lymphokine killer activity by intraepithelial lymphocytes, all of which contribute to disease development.

In vitro studies where gluten was used to stimulate duodenal biopsy samples, intestinal epithelial cells, monocytes, macrophages, and dendritic cells have shown that gluten may have innate immune stimulatory properties (88–94). Additionally, other molecules contained in wheat and related proteins could also drive immune cells activation, such as wheat amylase trypsin inhibitors (95, 96).

The gluten-derived α-gliadin peptide P31-43, which unlike the 33-mer (P55-87) and the 25-mer (P31-55) does not induce specific T cell responses in the celiac lesion, has been shown to activate innate immune pathways (90, 92, 97–99). However, the observed innate properties have been different across studies and we still need cross-validation. P31-43 peptide could induce enterocyte proliferation and actin rearrangements in an IL-15 and epithelial growth factor (EGF)-dependent manner, leading in particular to crypt hyperplasia, one of the characteristics of tissue remodeling seen in the celiac lesion (100–104). Other reported effects of gliadin on the innate immune system encompass cell structural changes, alterations in epithelial cells signaling, and induction of inflammatory and stress signals [reviewed in (100, 105)]. The finding that enterocytes from CeD patients have a stressed/inflamed phenotype and present a constitutive alteration in the intracellular vesicular trafficking provides an explanation as to why those cells are more sensitive to the effects of the P31-43 peptide (106–108). Indeed, due to sequence similarity between the P31-43 peptide and a region of hepatocyte growth factor regulated substrate (HRS) kinase - an essential protein involved in endocytic maturation-, P31-43 localizes in early endosomes and alters HRS-mediated maturation of early endosomes and the recycling pathway. The ensuing delayed vesicular trafficking leads to a reduction in the degradation of receptor tyrosine kinases including the receptor for EGF and promotes a sustained trans-presentation of IL-15 at the epithelial cell level [reviewed in (100, 105)].

Interestingly, a peptic-tryptic digest of gliadin or the P31-49-derived peptide can induce the upregulation of the expression of the stress-inducible MHC class I polypeptide-related molecules (MIC) *via* a pathway involving IL-15 (90). This is in accordance with the observation that intestinal epithelial cells in CeD patients express high levels of the MIC molecules (90, 109) and the non-classical MHC class I molecule HLA-E (79, 94). The expression of the inflammatory cytokine IL-15 can also be upregulated in whole biopsies, intestinal epithelial cells, or antigen-presenting cells from CeD patients upon gluten challenge or P31-49 derived peptide stimulation (65, 66, 78, 92, 110). The physiological consequences of the activation of immune pathways by gluten remain unclear given the fact that family members of CeD patients that lack an adaptive response to gluten, yet harbor the pre-disposing HLA-DQ2 and HLA-DQ8 and also show IL-15 upregulation in their intestinal compartment (25), retain normal intestinal morphology. However, the observation that, unlike active CeD patients, potential CeD patients -who display a gluten-specific adaptive immune response in the absence of tissue destruction- lack the innate epithelial stress response suggests that the alteration of the epithelial compartment is required for the development of villous atrophy (25). This observation is in accordance with the mechanism of epithelial cell destruction whereby activated intraepithelial TCRαβ lymphocytes mediate the killing of intestinal epithelial cells based on the recognition of stress signals such as non-classical MHC molecules and IL-15.

As discussed in more detail below, the acquisition of innate-like properties by intraepithelial lymphocytes is also driven by IL-15 (111).

In addition to the epithelial upregulation of IL-15, most CeD patients display a chronic upregulation of IL-15 in the lamina propria (66). IL-15 plays a critical role in the lamina propria as it can impart dendritic cells to initiate the polarization of inflammatory T_H1 responses and the loss of oral tolerance to gluten (38). This loss of oral tolerance to dietary gluten could also be triggered by type 1 interferons in individuals over-expressing IFN-α in lieu of IL-15 (63). Interestingly, the P31-43 peptide can trigger the expression of inflammatory mediators and increase cell death in a MyD88- and type 1 IFNs-dependent manner and this innate immune activation is enhanced by the TLR3 agonist poly(I:C) (112). This synergistic action of gluten peptides with poly(I:C) and the finding that poly(I:C) (46) or reovirus infection (19) promote the activation of TG2 suggests that multiple environmental hits can trigger and drive disease development. Because high levels of IL-15 can persist in a subset of patients on a gluten-free diet, it remains unclear what drives the excessive chronic upregulation of this innate cytokine. Similarly, both viral and bacterial infections could be the main source of type 1 interferons, yet how this expression is sustained remains to be determined (113).

The association between CeD susceptibility and single nucleotides polymorphisms in genes involved in microbial sensing has also pointed towards a role for bacterial microbes in triggering immune activation (5, 114). Many studies have noted differences in the microbiota composition between CeD patients, treated CeD patients on a gluten-free diet and healthy individuals [reviewed in (17)]. Gluten-degrading proteases produced by some opportunistic pathogens found in the duodenum of CeD patients such as *Pseudomonas aeruginosa*, can activate PAR-2 initiated inflammatory signaling pathways resulting in the expansion of intraepithelial lymphocytes (115). In addition, thanks to its elastase activity, *Pseudomonas aeruginosa* could contribute to the initiation of the disease by favoring the generation of immunogenic gluten peptides that can efficiently translocate through the intestinal barrier (116). *Neisseria flavescens*, abundantly present in the duodenal microbiome of CeD patients, could also contribute to the inflammatory response through its ability to endow a pro-inflammatory phenotype in dendritic cells (117). Dysbiosis is usually associated with a decrease in bacterial diversity and in the production of short-chain fatty acids such as butyrate, propionate and acetate that result from carbohydrate fermentation (118–121) and contribute to the maintenance of the gut homeostasis (122). Interestingly, a decrease in butyrate-producing bacteria such as *Bifidobacterium* or *Faecalibacterium prausnitzii* (123, 124) as well as alteration of the fecal metabolites patterns has been observed in children with CeD (125–127), yet these changes have not been observed in adult cohorts (128–130). These observations, together with the findings that genetically predisposed children carrying the HLA-DQ2 molecule present an altered gut microbiota composition, suggest that commensal bacteria could contribute early on to determining disease risk (131). In addition, because alterations in gut microbiota and fecal short-chain fatty acid composition can persist even when gluten is withdrawn, it is unclear whether dysbiosis could contribute to the initiation and enhancement of the disease or if changes in the microbiota reflect the ongoing local inflammation (15).

Overall, innate factors induced by gluten exposure and additional unknown triggers, perhaps of microbial origin, play a critical role in promoting the loss of oral tolerance to gluten and in altering intestinal epithelial cells that become the target of activated intraepithelial lymphocytes. However, studies comparing potential and active CeD patients as well as comparing mice expressing IL-15 in different gut compartments have shown that IL-15 and stress molecules overexpression in the epithelium need to be associated with adaptive immunity for villous atrophy to develop (23, 25).

DESTRUCTION OF EPITHELIAL CELLS BY CYTOTOXIC INTRAEPITHELIAL LYMPHOCYTES

In addition to the T_H1-skewed CD4 T cell response, another hallmark of CeD is the large accumulation of oligoclonal cytotoxic intraepithelial TCRαβ$^+$ CD8$^+$ lymphocytes (IE-CTLs) and TCRγδ$^+$ intraepithelial lymphocytes (78, 109, 132). These cells, and not the gluten-specific CD4$^+$ T cells, are the particular immune cell type thought to mediate the destruction of intestinal epithelial cells and directly lead to villous atrophy (23, 36). Their critical role in tissue destruction was not appreciated until the discovery that these cells are reprogrammed to express high levels of activating NK receptors and associated adaptor molecules. In healthy individuals, intraepithelial lymphocytes predominantly express the dimeric inhibitory CD94/NKG2A receptor with only low levels of the activating CD94/NKG2C and NKG2D receptors (78, 79, 133, 134). However, intraepithelial lymphocytes from CeD patients were found to undergo extensive NK cell-like reprograming, downregulating expression of the inhibitory CD94/NKG2A receptor and upregulating expression of the activating CD94/NKG2C and NKG2D receptors (78, 79, 133, 134). Furthermore, the CD94/NKG2C receptors in CeD patients are associated with the ITAM-bearing adaptor molecule DAP12, enabling cytokine secretion, proliferation and cytolytic activity in response to NK receptor ligands, even independently of TCR activation (79). The stress-inducible, non-classical MHC-like molecule HLA-E is the ligand for CD94/NKG2C and it is selectively upregulated on intestinal epithelial cells in CeD patients, allowing enterocytes to be targeted for killing by IE-CTLs (79). Upregulation of NKG2D on IE-CTLs in CeD patients, as well as upregulation of its adaptor molecule DAP10, was found to be directly caused by high levels of IL-15 on intestinal epithelial cells (109). This IL-15 mediated signaling not only upregulated NKG2D, but also acted in a co-stimulatory manner, synergizing with NKG2D signaling to enable TCR-independent cytolysis of targets expressing both

IL-15 and the stress-induced NKG2D ligands MICA/B (109, 134, 135). MICA/B, which are highly expressed in the intestinal mucosa of CeD, enable enterocytes to be excellent targets for IE-CTLs-mediated destruction (90). Taken together, the reprograming of $CD8^+$ intraepithelial lymphocytes into NK-like IE-CTLs with the ability to lyse target cells independently of TCR activation positions this cell type to be the direct mediator of tissue destruction in CeD.

It is important to note however that while no gluten-specific IE-CTLs have been identified in the intestinal epithelium of CeD patients, and IE-CTLs can kill intestinal epithelial cells in a TCR-independent manner, TCR specificity may still be playing a role in tissue destruction. Support for this idea comes from studies showing that inhibitory and activating NK receptor expression was associated with particular TCR specificities (133, 136). Additionally, signaling through CD94/NKG2C, NKG2D, and IL-15 receptors lowers the threshold for TCR activation (79, 109, 134). This co-stimulatory signaling could allow for low-affinity TCR-ligand interactions to activate TCR signaling, while under normal conditions the interaction would be too low affinity for activation. Evidence for this was shown in a study in which IL-15 expressing tumors were selectively controlled and killed by SIY-specific CTLs cells in a non-cognate but TCR-dependent fashion (137). Therefore, there still may be a potential role for TCR specificity among IE-CTLs as low affinity TCR interactions may be playing a part in tissue destruction along with TCR-independent cytolysis. Overall, although $TCR\alpha\beta^+$ IE-CTLs are not gluten specific, they destroy specifically intestinal epithelial cells expressing IL-15 and ligands for activating NK receptors.

While the frequencies of $TCR\alpha\beta^+$ $CD8^+$ IE-CTLs decrease when gluten is excluded from the diet (35), the expansion of $TCR\gamma\delta^+$ intraepithelial lymphocytes persists (132). Intriguingly, the composition of the tissue-resident $TCR\gamma\delta^+$ compartment is irreversibly altered by inflammation with the depletion of innate-like $V\gamma4^+/V\delta1^+$ intraepithelial lymphocytes and the expansion of gluten-sensitive IFN-γ-producing $V\delta1^+$ intraepithelial lymphocytes (138). Although the exact role of the naturally occurring tissue resident $TCR\gamma\delta^+$ intraepithelial lymphocytes that have both cytotoxic and tissue repair potential remains to be determined, their loss may lead to a defect in tissue healing and the protection against infections and tumors. Furthermore, the role of gluten-dependent production of IFN-γ by active CeD $V\delta1^+$ intraepithelial lymphocytes remains elusive.

A subset of adults with CeD go on to develop refractory coeliac disease (RCD), a rare CeD complication in which patients have persistent severe villous atrophy despite being on a strict gluten-free diet (139, 140). One of the hallmarks of RCD is the expansion of aberrant intraepithelial cytotoxic lymphocytes that lack surface CD3 expression ($sCD3^-$), express intracellular CD3 ($iCD3^+$), and display a highly activated NK cell-like phenotype (140). These aberrant IE-CTLs develop from hematopoietic stem cell-derived $CD103^+sCD3^-$ IELs that encounter high levels of IL-15 and Notch signals in the gut epithelium and develop gain-of-function JAK1 or STAT3 mutations (141). Since both JAK1 and STAT3 are involved in IL-15 signaling, these gain of function mutations lead to heightened IL-15 signaling in aberrant $iCD3^+$ IE-CTLs resulting in their expansion and survival through activation of anti-apoptotic signaling pathways (e.g. upregulation of anti-apoptotic factors Bcl-2 and Bcl-xl) (66, 142, 143). With IL-15 playing such a major role in the development of CeD and RCD, recent phase 2a clinical trials tested the impact of blocking IL-15 in CeD and RCD patients (144, 145). The trials did not show a significant difference in the primary clinical endpoints (improvement of the mucosal architecture in CeD and reduction in the proportion of aberrant intraepithelial lymphocytes in RCD). However, there were differences in some secondary endpoints, with treated RCD patients having fewer gastrointestinal symptoms and displaying less T cell receptor clonality than the placebo group (144), suggesting that blocking IL-15 may still be an option for treating RCD. There is a need to perform long-term follow-up studies in which the treatment duration will be significantly increased (duration of the anti-IL5 treatment in the published study was only ten weeks).

CONCLUSION

As discussed throughout this review, both innate and adaptive immune responses possibly connected by a cytokine network contribute to the immunopathogenesis of CeD. Each of the described cell types and their known mechanism of action are required to promote CeD but none of them individually is sufficient to culminate in the full-blown disease characterized by intestinal tissue destruction and remodeling. Observations in mice and humans have suggested or demonstrated the requirement for the simultaneous activation of distinct pathways in different gut locations, some cell-cell interactions, and the existence of a crosstalk between the lamina propria and the epithelial compartments (**Figure 2**).

First, the simultaneous analysis of IE-CTLs and intestinal epithelial cells features in patients encompassing the spectrum of the disease - i.e. family members, potential CeD patients and active CeD patients- has led to the hypothesis that the combination of epithelial stress associated with high IL-15 expression in enterocytes and an anti-gluten adaptive immune response induced in the lamina propria in the presence of inflammatory mediators such as IL-15 is needed for the development of villous atrophy (25). The cooperation between epithelial IL-15 and $CD4^+$ T cells to promote tissue destruction was also suggested by a study performed in mice overexpressing IL-15 in the gut epithelium and fed with the dietary antigen ovalbumin (146). The analysis of our DQ8-D^d-villin-IL-15g mice modeling CeD patients upon gluten oral challenge unequivocally confirmed that the development of villous atrophy requires the concomitant presence of epithelial stress and anti-gluten adaptive immunity (23).

Next, in agreement with the findings that $CD4^+$ T cells are required for the development of CeD, the prevention of villous atrophy in DQ8-D^d-villin-IL-15tg mice treated with an anti-CD4 depleting antibody, anti-IFNγ depleting antibody or TG2 inhibitors concomitantly to gluten administration also

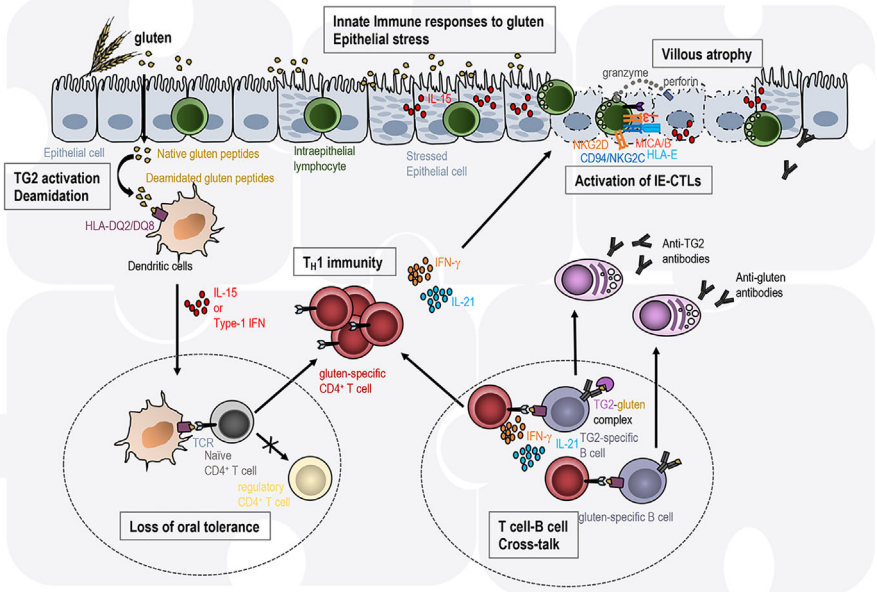

FIGURE 2 | Intestinal tissue destruction in coeliac disease results from the interplay between several immune pathways in distinct gut locations. Transglutaminase 2 (TG2)-deamidated gluten peptides bind with high affinity to the disease-associated HLA-DQ2 or HLA-DQ8 molecules on antigen-presenting cells. In an inflammatory context (presence of IL-15, type 1 IFN), dendritic cells acquire a pro-inflammatory phenotype and migrate to the mesenteric lymph nodes (left circle with dashed line). They present gluten peptides to naïve CD4 T cells and promote T cell differentiation into T_H1 effector T cells, while the induction of regulatory T cells involved in oral tolerance is abrogated. Anti-gluten $CD4^+$ T cells directly secrete IFN-γ or IL-21. Anti-gluten $CD4^+$ T cells are thought to provide help to gluten- and TG2-specific B cells in gut-associated secondary lymphoid organs (right circle with dashed line) leading to the production of IgA and IgG anti-gluten and anti-TG2 antibodies. In the presence of high IL-15 expression in the epithelium, intraepithelial lymphocytes acquire cytotoxic properties (activating NK receptors, release of the cytotoxic molecules granzyme B and perforin) and the ability to kill stressed epithelial cells expressing the ligands (HLA-E, MICA/B) for the NK receptors. Each of these immune events are required to promote coeliac disease but none of them individually is sufficient to promote intestinal tissue destruction. Hence the immunopathogenesis of coeliac disease is often presented as a jigsaw where each piece associated with one immune event needs to be connected to promote the disease.

underlined the existence of a cross-talk between T_H1 immunity in the lamina propria and the activation of IE-CTLs (23).

In addition, a recent study using a mouse model of CeD lacking B cells has demonstrated that B lymphocytes are required for the development of villous atrophy and the induction of a killer phenotype in IE-CTLs (147). The mechanisms underlying the exact contribution of B-cell mediated immune responses to the immunopathogenesis of CeD remains to be determined. Most of our current understanding of the role of antibodies is based upon *in vitro* experiments. Although anti-TG2 antibodies can exert several effects, no consensus has been reached regarding their potential pathogenic role [reviewed in (148)]. While cytokines produced by B cells, that need yet to be identified, could contribute to the inflammatory process in the CeD lesion, B lymphocytes and/or plasma cells themselves could contribute to disease pathogenesis through their role as antigen-presenting cells. Indeed, plasma cells were found to be the most abundant gluten peptide MHC-expressing cells in the lamina propria of CD patients (149). Human studies have also suggested that gluten $CD4^+$ T cells and B cells having internalized TG2-gluten complexes interact to promote the generation of anti-TG2 antibodies, whose formation rely on the presence of gluten (24, 48, 150, 151). This T cell-B cell crosstalk is supported by *in vitro* assays showing that transduced lymphoma B cells expressing HLA-DQ2.5 and binding catalytically active TG2 can activate gluten-specific hybridoma T cells in the presence of non-deamidated gluten peptides (151). However, whether this interaction also benefits $CD4^+$ T cells by promoting their activation and expansion helping to reach the threshold needed to reach a pathogenic T cell response remains to be determined.

T cell clones have proven to be an invaluable research tool to gain insights into the immune mechanisms underlying CeD pathogenesis. Early analysis of CD4 T cell clones in celiac patients uncovered T_H1-skewed gluten-specific CD4 T cells restricted to the disease-associated HLA-DQ2 and HLA-DQ8 molecules (26, 31, 40). Further interrogation of gut derived gluten-specific T cell clones helped to demonstrate that these clones respond to TG2-modified gluten peptides and that deamidation of these peptides leads to the strongest T cell response (29, 30). The usage of animal models of the disease combined with our ability to track gluten-specific T cells should help identify the intestinal location of the pathogenic cellular interactions described in this review, such as the B cell-T cell

crosstalk (37, 152). Leveraging single-cell approaches technologies to study human samples could also help identify additional understudied cell subsets such as innate lymphoid cells [reviewed in (138, 153)] that could participate in the establishment and maintenance of the disease.

Altogether, CeD represents a perfect example of a multifactorial complex autoimmune disorder. The priming of a gluten-specific inflammatory immune response depends on the coordinated interaction between gluten, the celiac predisposing HLA-DQ molecule, activated TG2, and $CD4^+$ T cells, while the alteration of epithelial cells expressing stress molecules, and the subsequent activation of IE-CTLs are all required to promote intestinal tissue destruction. How T lymphocytes in the lamina propria and cells present in the epithelial compartment communicate and where the cross-talk between distinct cell types occur is not yet understood and will certainly lead to the identification of signaling pathways that could potentially represent novel therapeutic targets.

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All authors contributed to the article and approved the submitted version.

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Apolipoprotein A4 Defines the Villus-Crypt Border in Duodenal Specimens for Celiac Disease Morphometry

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Histological evaluation of the small intestinal mucosa is the cornerstone of celiac disease diagnostics and an important outcome in scientific studies. Gluten-dependent injury can be evaluated either with quantitative morphometry or grouped classifications. A drawback of mucosal readings is the subjective assessment of the border where the crypt epithelium changes to the differentiated villus epithelium. We studied potential immunohistochemical markers for the detection of the villus-crypt border: apolipoprotein A4 (APOA4), Ki-67, glucose transporter 2, keratin 20, cytochrome P450 3A4 and intestinal fatty-acid binding protein. Among these, villus-specific APOA4 was chosen as the best candidate for further studies. Hematoxylin-eosin (H&E)- and APOA4 stained duodenal biopsy specimens from 74 adult patients were evaluated by five observers to determine the villus-to-crypt ratio (VH : CrD). APOA4 delineated the villus to crypt epithelium transition clearly, and the correlation coefficient of VH : CrD values between APOA4 and H&E was excellent (r=0.962). The VH : CrD values were lower in APOA4 staining ($p<0.001$) and a conversion factor of 0.2 in VH : CrD measurements was observed to make the two methods comparable to each other. In the intraobserver analysis, the doubled standard deviations, representing the error ranges, were 0.528 for H&E and 0.388 for APOA4 staining, and the ICCs were 0.980 and 0.971, respectively. In the interobserver analysis, the average error ranges were 1.017 for H&E and 0.847 for APOA4 staining, and the ICCs were better for APOA4 than for H&E staining in all analyses. In conclusion, the reliability and reproducibility of morphometrical VH : CrD readings are improved with the use of APOA4 staining.

Keywords: celiac disease, morphometry, duodenal biopsy, histology, gluten challenge, apolipoprotein A4, digital pathology, clinical trial

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INTRODUCTION

Celiac disease is an autoimmune disorder in which dietary gluten causes an immunological reaction manifesting as gradual development of small bowel mucosal damage (1). Small bowel damage consists of sequential and slow development of lymphocytosis, crypt hyperplasia and villus atrophy (2). Currently, the only treatment for celiac disease is a life-long gluten-free diet. However, dietary management is not sufficient for many patients with celiac disease, and up to 40% of patients suffer

from symptoms even on this diet (3). Additionally, the duodenal mucosa may not heal sufficiently on this diet, causing risks of complications and micronutrient deficiencies (4, 5). Interestingly, there are several ongoing gluten challenge studies assessing the efficacy of candidate drugs and vaccines for celiac disease (6). In these studies, it is of utmost importance to ensure that the drug, device, or vaccine protects against mucosal damage, as it is the only marker that is linked to the long-term health of the patient, risk of complications, and mortality (7–9).

Mucosal damage can be evaluated histologically with either categorical classifications or quantitative measurements. Categorical classifications such as the Marsh-Oberhuber and Corazza-Villanacci classifications are the most commonly used in routine clinical practice because of their ease of use (1, 10). These classifications combine the parameters of duodenal damage, intraepithelial lymphocyte (IEL) density, crypt depth (CrD) and villus height (VH) into a single class describing the level of mucosal damage. A more detailed analysis can be performed with the use of quantitative measurements such as the villus height-to-crypt depth ratio (VH : CrD) and density of CD3-positive IELs, which allow the detection of small but significant changes that are not detectable with categorical variables (11–13). Hence, it is preferred to use these continuous mucosal readouts separately for morphology and inflammation in rigorous scientific studies, such as in celiac disease drug/device/vaccine trials (12, 14).

Recent studies have shown poor reliability and reproducibility when using the results of grouped classifications in assessing duodenal specimens (15–19). There are several pitfalls in the assessment of duodenal biopsy specimens that explain these difficulties (11, 16, 17). An incorrect (tangential) cutting plane of the biopsy is currently a well-established source of error (11), but another fundamental problem is the definition of the border between differentiated villus enterocytes and the proliferating crypt epithelium (20). The distinction between small bowel villi and crypt epithelium can be made by the presence of fully differentiated microvilli revealed only by electron microscopy (**Figure 1**) (21). To date, specific markers for the villus-crypt border to be used in light microscopy have not been identified. Currently, the use of standard hematoxylin-eosin (H&E) staining makes it difficult to define exactly the epithelial transition zone determining the villus-crypt border. Understandably, as readers use their own experience in the assessment of the villus-crypt border, the results between readers have shown high interobserver variability (11, 16, 17, 19). The histopathological diagnosis (celiac disease vs normal) has even changed in up to 11% of cases when the samples have been reread (15). Even a small variation in the point where villus ends and crypt begins is multiplied when calculating the VH : CrD ratio, as it consists of two mutually dependent measurements (VH and CrD). Therefore, it would be of significant benefit to develop an objective marker of the villus-crypt border that would harmonize celiac disease diagnostics and increase measurement reliability and reproducibility. Hence, we studied several potential proteins to find an immunohistochemical marker that would define the exact border between villi and crypts.

MATERIALS AND METHODS

Patients and Biopsies

The material comprised 74 small intestinal mucosal specimens from 74 patients, which were obtained from a prospectively collected database and biobank maintained by our study group. Altogether, 6 specimens were obtained from newly diagnosed untreated celiac patients, 6 from patients on a gluten-free diet, 32 from patients who underwent gluten challenge (22) and 30 specimens from nonceliac controls. The mean age of the celiac patients was 57 years (range 15–63), and 63% were women. The mean age of the nonceliac controls was 57 years (range 17–86), and 52% of them were women. Small bowel biopsies were selected to represent variable stages of mucosal injury ranging from completely normal histology to overt mucosal atrophy and crypt hyperplasia. According to Marsh-Oberhuber grading (23), duodenal injury in the specimens was Marsh 0 (n=15), Marsh 1 (n=10), Marsh 2 (n=10), Marsh 3a (n=13), Marsh 3b (n=12) and Marsh 3c (n=9).

The forceps biopsy specimens were formalin-fixed and embedded in paraffin wax according to standard pathology practice. Standard 3- to 4-μm-thick sections were cut under a microscope to achieve the correct orientation and were then stained with hematoxylin and eosin. Slides were scanned as high-resolution whole slide images at a resolution of 0.17 μm per pixel (SlideStrider scanner, Jilab Inc., Tampere, Finland). Additional sections were cut and used for immunohistochemical (IHC) experiments. Figure panels and art work were created with Adobe Photoshop CS5 (Adobe Inc., CA, USA).

APOA4, Ki-67, GLUT2, KRT20, CYP3A4 and I-FABP Immunohistochemistry

We surveyed the existing genome-wide studies (4, 24) and the Human Protein Atlas (25) to identify candidate IHC markers that would preferentially label villi or crypt epithelium to define the villus-crypt border exactly. The most promising candidate proteins—apolipoprotein A4 (APOA4), antigen KI-67 (Ki-67), glucose transporter 2 (GLUT2), keratin-20 (KRT20), cytochrome P450 3A4 (CYP3A4) and intestinal fatty-acid binding protein (I-FABP)—were selected for preliminary staining experiments. The antibodies and their working dilutions are described in **Supplementary Table 1**. For all antibodies, a standard IHC staining protocol using high pH, heat-induced antigen retrieval (incubation at 121°C for 2 min in 0.01 Tris-EDTA buffer, pH 9.0), blocking of endogenous peroxidase activity (3% H_2O_2 for 5 min at RT), and a 60-min incubation with primary antibodies (60 min at RT) were used. Bound antibodies were visualized with anti-mouse/anti-rabbit peroxidase polymer and DAB chromogen (HistoFine kit, Nichirei Biosciences, Nichirei, Japan). Slides were counterstained with hematoxylin and mounted with DPX (Sigma-Aldrich, MO, USA). Immunohistochemical staining was carried out with an automated IHC-staining device (LabVision Autostainer; Thermo Fisher, Waltham, MA, USA). Slides were scanned as whole slide images.

After the selection of APOA4 for further analysis, the previously H&E stained and analyzed slides were soaked in

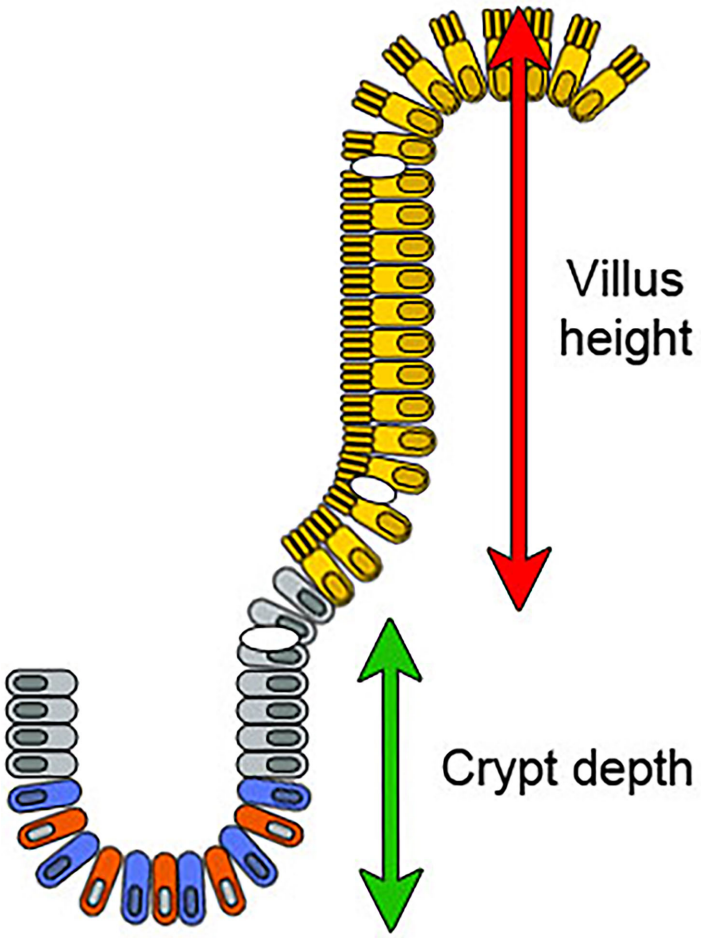

FIGURE 1 | A diagram of the intestinal epithelium in the villus-crypt axis. The crypt generates new cells that differentiate and migrate towards the tip of the villus. The crypt base columnar cells (blue) divide continuously and function as intestinal stem cells. Paneth cells (red) are also at the crypt bottom and nurse these stem cells. Above the stem cell zone is the zone of transit amplifying cells containing lineage-committed progenitor cells (gray). Fully mature absorptive epithelial cells displaying organized microvilli (villus enterocytes, in yellow) emerge from the crypt and move towards the villus tip. Goblet cells are present in both the crypts and villi (shown in white). Enteroendocrine cells are localized among mature enterocytes (not shown). The green and red arrows show villus height and crypt depth measurements in the model.

xylene for up to 3-4 days to dissolve the mounting medium and to detach the coverslips. Slides were then rehydrated and stained with APOA4 IHC as described above. The polyclonal APOA4 antibody was used for the stainings because its use is well documented and found to be rather specific for duodenum (25). The staining was also tested with monoclonal APOA4 antibody and its staining pattern appeared to be similar to that of the polyclonal antibody (not shown). Eosin was added to the counterstain to visualize the Paneth cells at the crypt bottom.

Digital Measurement of VH and CrD

All IHC-stained slides were scanned as whole-slide images as described above. The sections were viewed and analyzed with web-based client software (Celiac Slide Analyzer) according to our standard operating procedure (11, 24). The small intestinal mucosal VH : CrD was evaluated in all measurable (at least three) separate villus-crypt units, and the result was given as the average of the ratios. VH and CrD were measured digitally by drawing segmented lines whose lengths were calibrated to micrometers (24). Only well-oriented villus-crypt units in the samples, ie. perpendicular to the luminal surface, were allowed to be assessed.

Five academic observers 1, 2, 3, 4 and 5 (JT, JS, KS, AV, JI) analyzed all slides in a blinded fashion independently and were unaware of the clinical data or laboratory findings of the patients. Additionally, one evaluator evaluated the specimens twice with 1 month between the measurements (JT). The villus crypt units identified and measured on the H&E image were relocalized on the APOA4-IHC whole slide image. In the APOA4-stained specimens (digital images), VH : CrD measurements were performed using APOA4 labeling to define the border of the villus and crypt.

Statistics

Intraobserver and interobserver variations were analyzed by the Bland-Altman method, linear regression analyses, and intraclass correlation coefficients (ICCs) (26, 27). In the Bland-Altman method, the differences between two quantitative measurements are plotted against the averages of the two measurements, and the results are reported as the mean difference between the two measurements and limits of agreement, which are defined as the mean difference plus and minus twice the standard deviation of the differences. In the Bland-Altman plot, the x-axis shows the mean of the results of the two measurements, and the y-axis represents the absolute difference between the two measurements. The intraobserver, interobserver and intermethod agreement was assessed with ICC, and intermethod correlations were assessed by Pearson correlation analysis. Correlation coefficients were considered excellent (above 0.9), strong (0.7-0.9), moderate (0.4–0.6), weak (0.1–0.4) or negligible (0.0-0.1) (28). Quantitative data are expressed as the number of subjects (n), mean and ranges. A paired samples t-test was used to compare the means between groups.

RESULTS

In the comparison between APOA4, Ki-67, GLUT2, KRT20, CYP3A4 and I-FABP, APOA4 was chosen as the best candidate for further study (**Figure 2**). APOA4 labeling was specific for villus enterocytes and did not stain the crypt epithelium. The experiments with Ki-67, GLUT2, KRT20, CYP3A4 and I-FABP stainings yielded unsatisfactory results in demonstrating the villus-crypt border accurately (**Figure 2**). The Ki67-labeled proliferating crypt epithelium cells did not extend to the crypt-villus junction, rendering Ki67 staining unsuitable for our approach. In addition, proliferating IELs are also Ki-67 positive, interfering with the analysis. GLUT2 and KRT20 were stained in the villi, but the staining continued to some extent to the crypt. The CYP3A4 and I-FABP stainings were promising in healthy mucosa, however, in the damaged mucosa the stainings did not represent the villus-crypt junction. In the APOA4 staining (**Figure 2**), the villus-crypt border aligned properly, and the positively stained villus epithelium stopped abruptly, making the placement of the borderline easy. In damaged mucosa, long crypt basins can be misread as villi in H&E staining (**Figures 3D, G**), but with the aid of APOA4 staining (**Figures 3E, H**), it can be seen that the crypt extends up close to the lumen, resulting in a histological diagnosis of total villous atrophy in both cases.

There were 69 readable samples with at least 3 villus-crypt units for the intraobserver analysis among the 74 evaluated samples. Observers 2, 3, 4 and 5 identified 65, 64, 57, and 61 readable samples, respectively. Five samples were unreadable to all; in all others, at least two observers measured at least 3 villus-crypt units on the sample. The mean villus heights, crypt depths and VH : CrD values in H&E-stained and APOA4-stained specimens are presented in **Table 1**. APOA4 staining made the assessment of the villus-crypt border easier in difficult cases by marking an objective villus-crypt junction site (**Figure 3**, **Supplementary Figure 1**). There was constant excellent agreement among all observers between H&E and APOA4 staining (**Table 1**). When comparing VH : CrD measurements by all observers between the methods, the mean difference was 0.227 with limits of agreement from −0.302 to 0.756 (**Figure 4A**); the standard deviation (SD) was 0.529. There was a significant mean difference between the methods in villus height, crypt depth and VH : CrD measurements (**Table 1**). Logistic regression analysis (**Figure 4B**) indicated the following conversion equation between the two staining methods: VH : CrD in H&E = 0.2 + 1.01 * VH : CrD in APOA4.

Our main purpose was to study the reliability and reproducibility of VH : CrD measurements when using APOA4 IHC when compared with traditional H&E. For this comparison, we analyzed the same biopsy sections after destaining and restaining with APOA4. In the intraobserver VH : CrD analyses, the mean differences in the two measurement series were less than 0.1, ensuring that there was no systematic measuring error between the measurement series. In the intraobserver Bland-Altman plots (**Figures 4C, E**), the 95% limits of agreement ranged from -0.476 to 0.528 for H&E and -0.356 to 0.420 for APOA4. The 2xSD error range of the measurements was 0.528 for H&E and 0.388 for APOA4 staining of the same tissue sections. The intraobserver logistic regression analyses are shown in **Figures 4D, F**, and the ICCs are shown in **Table 2**. In the interobserver analyses, all VH : CrDs by all observers showed smaller SDs and better ICCs in APOA4 than in H&E staining (**Table 2**). The average error ranges in interobserver analyses were 0.519 in H&E and 0.432 in APOA4. The mean differences in the interobserver analyses, indicating the observer dependency of the measurements, ranged from 0.074 to 0.219 for H&E staining and from 0.067 to 0.251 for APOA4 staining (**Table 2**).

DISCUSSION

The present study shows that immunohistochemical staining of APOA4 defines the villus-crypt border by separating the differentiated villus epithelium and proliferating crypt epithelium. The villus-to-crypt ratios were analyzed with quantitative morphometry according to our standard operating procedure used in previous publications and gluten challenge trials (4, 5, 11, 12, 14, 24, 29). The correlation coefficients and Bland-Altman analyses showed excellent agreement between the results from APOA4 staining and the standard and validated H&E staining. Hence, APOA4 staining can be used as an objective marker of the villus-crypt border in analysis of the duodenal mucosal architecture in celiac disease. The addition of APOA4 staining to the immunohistochemistry workout is relatively easy because CD3 IHC staining of IELs is included routinely in translational celiac disease studies and clinical trials (2, 11, 12, 14, 29).

We adopted APOA4 as an immunohistochemical marker of the villus epithelium. Its function has not been linked to celiac

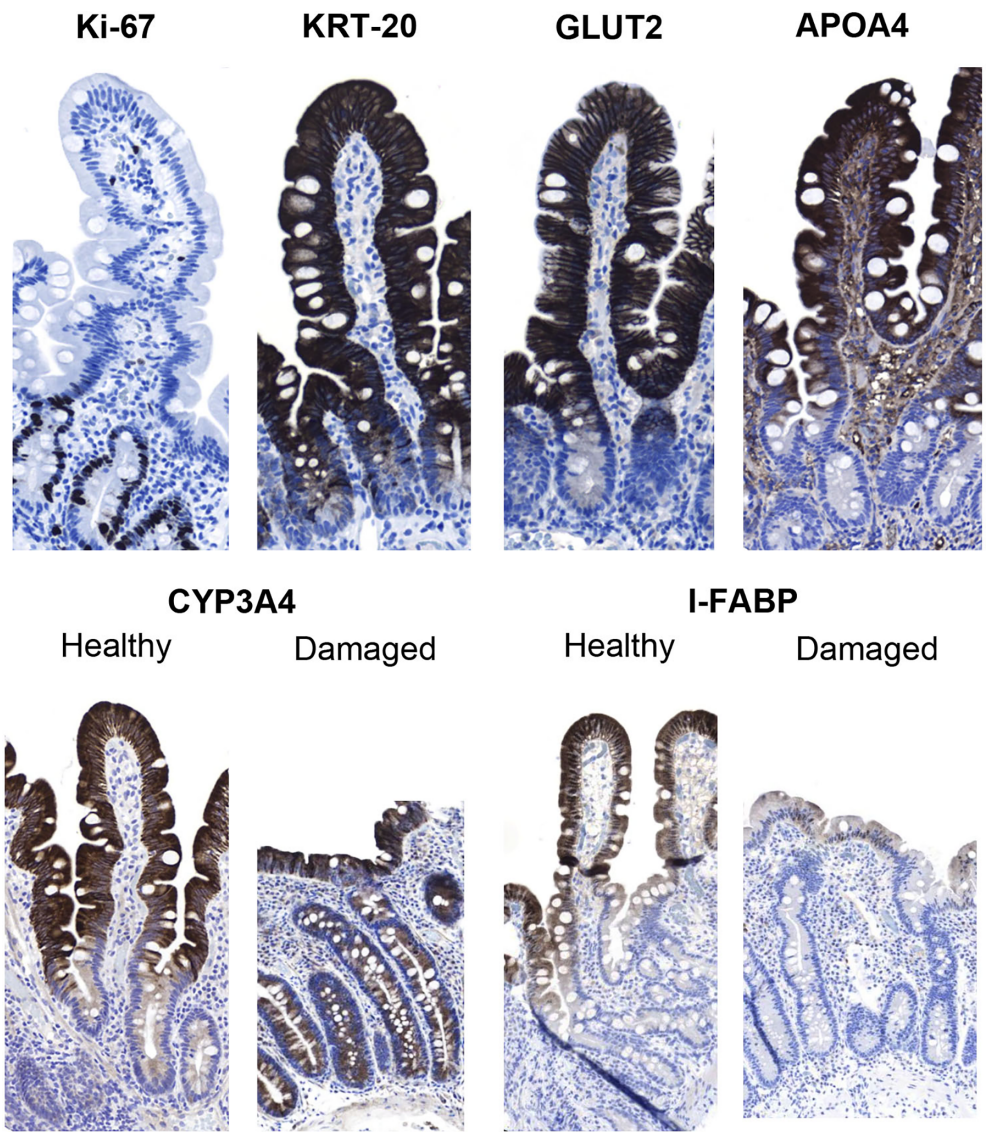

FIGURE 2 | Immunohistochemical analysis of the potential markers of the villus-crypt border in duodenal biopsy specimens. Ki-67 labels the crypt cells, but the labeling does not extend up to the villus-crypt border. Keratin 20 (KRT20) stains the villi but also extends to the crypt epithelium; thus, this marker cannot be used to define the villus-crypt border. The staining of glucose transporter 2 (GLUT2) resembles that of KRT-20, as it also extends to the crypt epithelium. In apolipoprotein A4 (APOA4) staining, the villus epithelium was strongly stained, while the crypt epithelium remained negative. Both cytochrome P450 3A4 (CYP3A4) and intestinal fatty-acid binding protein (I-FABP) looked promising in healthy control specimens but in damaged samples CYP3A4 also stained crypt cells and I-FABP then again disappeared almost completely from the sample. Magnification 200x, hematoxylin counterstain.

disease so far. It is a lipid-binding 46 kD glycoprotein that is almost exclusively synthesized in the absorptive enterocytes of the small intestine, packaged into chylomicrons, and secreted into intestinal lymph during fat absorption (30). APOA4 is involved in several physiological processes, such as lipid absorption and metabolism (31), antiatherosclerosis (32), anti-inflammatory agents (33), glucose homeostasis, and food intake (34). Previously, we showed that the mRNA expression levels of APOA4 are decreased in untreated celiac disease and after gluten challenge (4, 24). The decrease in APOA4 in the gluten-induced duodenal lesion in celiac disease showing villous atrophy and crypt hyperplasia is the logical result of the loss of mature absorptive villus epithelium, as shown in **Figures 2** and **3**.

The distinction of the border between villi and crypts is of utmost importance in assessing celiac disease biopsy specimens (2, 11, 20). Currently, the placement of this border is debatable and lacks scientific rationale in traditional analyses based on H&E staining. Ground truth differentiation between villi and crypt epithelium can be done only by transmission electron microscopy (20), but because microvilli are not visible in H&E

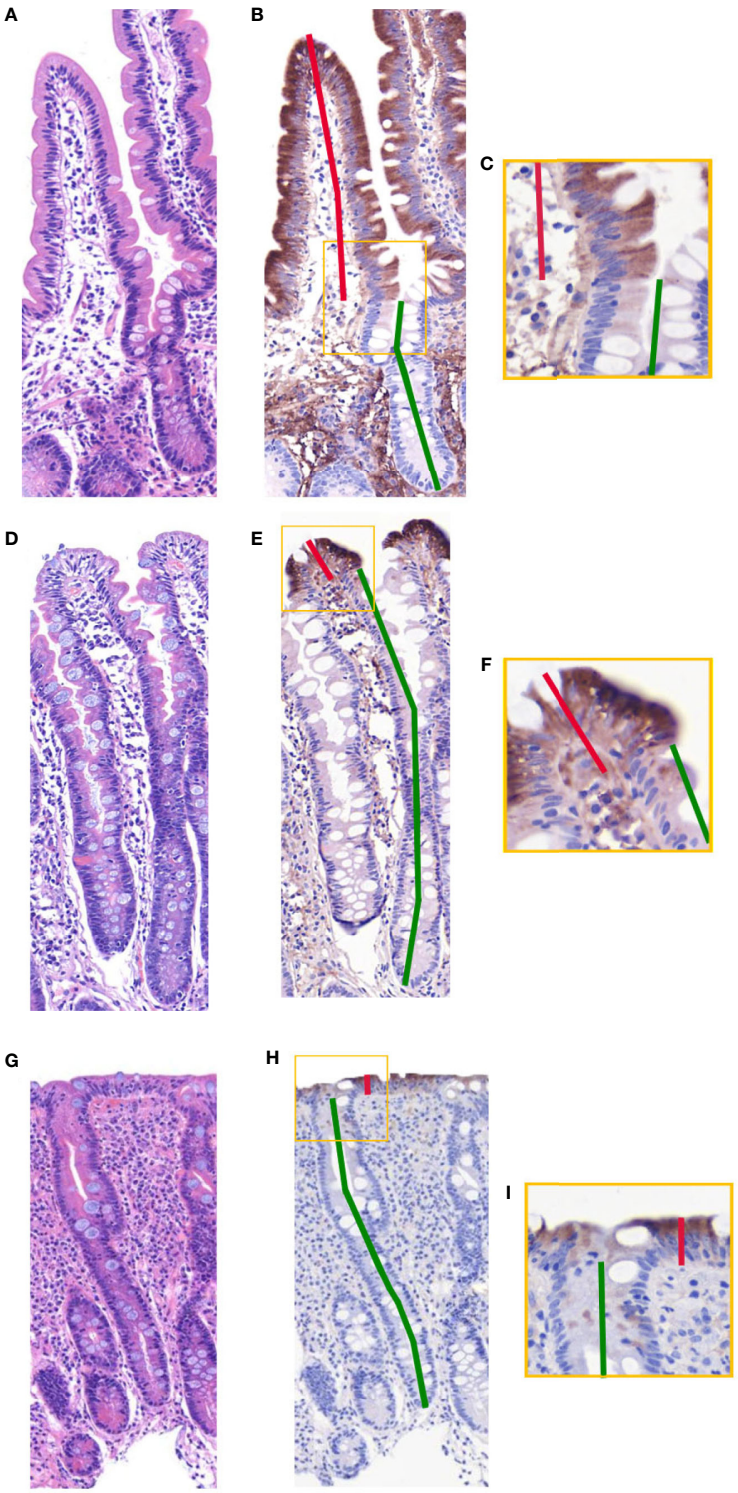

FIGURE 3 | Side-by-side comparison of duodenal specimens by traditional hematoxylin-eosin (H&E) and apolipoprotein A4 (APOA4) after restaining. **(A, D, G)** depict standard H&E-stained specimens, and panels **(B, E, H)** depict APOA4-stained specimens. The border between villi and crypts is clearly visible in APOA4-stained specimens, as also seen in closeups **(C, F, I)**. **(D–F)** and **(G–I)** present the common pitfall of a long crypt basin. This long crypt basin can be misread as villi in H&E staining **(D, G)**, but with the aid of APOA4 staining **(E, H)**, it is clear that the crypt extends up close to the lumen, rendering the histological diagnosis of total villous atrophy in both cases. The VH : CrD ratios in the samples are 1.5 in **(A–C)**, 0.1 in **(D–F)**, and 0.1 in **(G–I)**. Magnification 200x, hematoxylin and eosin counterstaining in **(B, E, H)**.

TABLE 1 | Comparison of villous height, crypt depth and villous height crypt depth ratio (VH : CrD) between hematoxylin-eosin (H&E) and apolipoprotein A4 (APOA4) stained specimens.

	Mean (range) in H&E, µm	Mean (range) in APOA4, µm	Mean difference	Correlation co-efficient
Villous height				
*Observer 1**	399 (23–790)	386 (19–793)	12.7**	0.988**
Observer 2†	381 (38–755)	366 (23–741)	14.6**	0.987**
Observer 3‡	377 (38–643)	350 (29–649)	27.2**	0.981**
Observer 4§	401 (43–668)	384 (22–655)	17.5**	0.978**
Observer 5¶	399 (35–685)	371 (23–675)	27.5**	0.972**
Total#	**391 (23–790)**	**372 (19–793)**	**19.7****	**0.981****
Crypth dept				
*Observer 1**	238 (121–458)	255 (130–525)	-16.7**	0.941**
Observer 2†	237 (130–447)	257 (137–558)	-20.2**	0.937**
Observer 3‡	231 (122–476)	258 (129–529)	-27.2**	0.947**
Observer 4§	237 (121–466)	255 (142–520)	-18.0**	0.925**
Observer 5¶	235 (131–534)	257 (143–522)	-22.5**	0.906**
Total#	**236 (121–534)**	**257 (129–558)**	**-21.0****	**0.928****
VH : CrD				
*Observer 1**	2.02 (0.10-4.11)	1.80 (0.10-3.84)	0.221**	0.979**
Observer 2†	1.90 (0.09-5.83)	1.73 (0.05-5.42)	0.167**	0.977**
Observer 3‡	1.93 (0.09-4.67)	1.63 (0.08-4.27)	0.300**	0.968**
Observer 4§	2.00 (0.09-4.41)	1.76 (0.06-3.91)	0.237**	0.914**
Observer 5¶	2.00 (0.07-5.21)	1.73 (0.05-4.72)	0.273**	0.962**
Total#	**1.97 (0.07-5.83)**	**1.73 (0.05-5.42)**	**0.233****	**0.962****

**n=69, †n=65, ‡n=64, §n=57, ¶n=61, #n=316, **p < 0.001.*

Bolded values represent the average value from the measurements of all observers together.

staining, researchers and pathologists use subjective pattern recognition to define the villus-crypt border according to the notch or a plateau usually seen at the border (**Figures 2A–C**). However, problems arise in celiac disease biopsies showing crypt hyperplasia in addition to villous atrophy. The long crypt collars or large open "basins" in a totally flat mucosal lesion (Marsh III) can be misinterpreted as villi (20, 35). In these samples, the notch or plateau was missing, and it was difficult to place the villus-crypt border (**Figure 2D**). In such instances, APOA4 staining provides a new possibility to define the villus-crypt border objectively and accurately (**Figures 2E, I**). The VH : CrD values were lower for APOA4 staining by a factor of approximately 0.2, indicating that the villus-crypt border appears somewhat lower with APOA4 staining than with H&E staining (**Table 1**, **Figure 4A**). For example, a VH : CrD value of 2.0, which is considered a borderline value for healed mucosa in celiac patients on a gluten-free diet (2, 5, 36), would equal 1.8 in APOA4 staining. We believe that with APOA4 staining, the reader has more confidence to place the border correctly and somewhat higher than in H&E staining, which might reflect the epithelial border better than in traditional H&E staining (see **Figure 2D–I**). Hence, APOA4 staining can be particularly helpful in borderline cases in which incorrect diagnoses may occur (15). The addition of eosin to the APOA4 staining

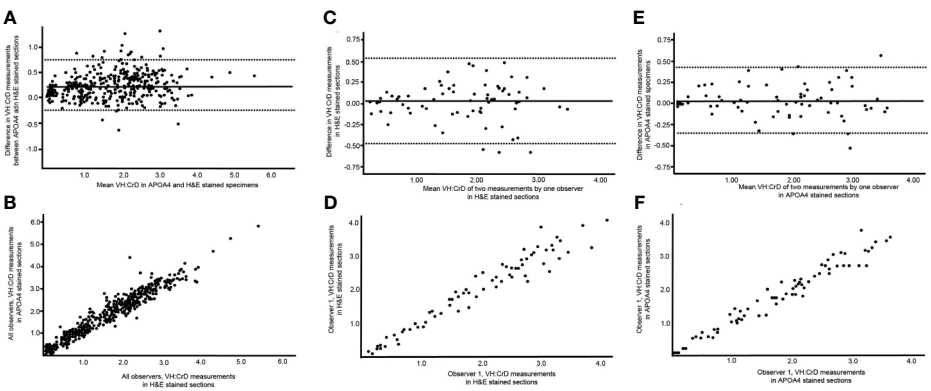

FIGURE 4 | Graphical illustrations of the reliability and reproducibility of the villus height crypt depth ratio (VH : CrD) in hematoxylin-eosin (H&E)- and apolipoprotein A4 (APOA4)-stained specimens. **(A, C)** show Bland-Altman plots, and **(B, D)** present the regression analyses for intraobserver analyses of VH : CrD in H&E and APOA4 staining, respectively. **(E, F)** show Bland-Altman plots and regression analysis between H&E and APOA4 staining in the VH : CrD measurements of all observers. The solid lines in **(A, B)** indicate the mean difference between the measurements, and the dashed lines correspond to the 95% limits of agreement.

TABLE 2 | Bland-Altman statistics with absolute values and intraclass correlation coefficients (ICC) for analysing agreement and repeatability in small-bowel mucosal villus height crypt depth ratio (VH : CrD).

	Mean difference (95% CI)	Standard deviation	ICC
VH : CrD in H&E			
*Intraobserver**	0.026 (-0.036 to 0.087)	0.256	0.971
Interobserver, Observer 2†	0.099 (-0.026 to 0.223)	0.491	0.897
Interobserver, Observer 3‡	0.074 (-0.040 to 0.188)	0.442	0.914
Interobserver, Observer 4§	0.219 (0.078 to 0.362)	0.534	0.862
Interobserver, Observer 5¶	0.127 (-0.032 to 0.286)	0.608	0.827
VH : CrD in APOA4			
*Intraobserver**	0.032 (-0.015 to 0.080)	0.198	0.980
Interobserver, Observer 2†	0.067 (-0.049 to 0.182)	0.445	0.905
Interobserver, Observer 3‡	0.172 (0.080 to 0.264)	0.357	0.937
Interobserver, Observer 4§	0.251 (0.139 to 0.364)	0.424	0.900
Interobserver, Observer 5¶	0.205 (0.074 to 0.336)	0.503	0.869

*CI, confidence interval. *n=69; †n=65; ‡n=64; §n=57; ¶n=61.*

procedure helps to identify the base of the crypt by staining the Paneth cells and thus ensuring that the entire crypt is considered.

In our study, APOA4 staining improved the reliability and reproducibility of VH : CrD measurements in celiac disease biopsy specimens in comparison to traditional H&E-stained sections. The standard deviations were smaller, and the ICCs were better both in intraobserver and in all interobserver analyses in APOA4-stained sections. Low interobserver agreement has been a concern in celiac disease histology (11, 15–17, 19). In the work by Werkstetter et al., two pathologists reviewed the same duodenal samples in a blinded manner, and in 11% of cases, the histological diagnosis changed from normal to celiac disease or vice versa (see Supplementary Table S21 in the article by Werkstetter et al.) (15). To remove such drastic problems in reading the samples, objective reading tools are needed for analysis of the duodenal mucosa to obtain reliable and reproducible results (2). Additionally, the use of the same reader or readers is essential to minimize variation in measurements, as interobserver analyses have significantly higher error ranges than intraobserver analyses, as also shown in this study. Hence, in our standard operating procedure, the sample is read by two or three blinded main readers, and then, in controversial results, a senior pathologist can counter this pitfall in second-opinion slide reading (11). The advantages of APOA4 in reliability and reproducibility is especially useful in pharmacological intervention studies in which small but significant changes in VH : CrD need to be observed (14, 29). In gluten challenge studies or when assessing the effect of a gluten-free diet with APOA4 staining, a conservative cutoff of a clinically relevant difference of 0.4 between the paired measurements was derived from the intraobserver Bland-Altman analysis.

When searching for a suitable immunohistochemical marker, we evaluated several candidate markers shown to be specific for either villus or crypt epithelium. Of these, the proliferating Ki-67-positive cells are increased due to the compensatory proliferation of epithelial cells in the duodenal crypts. The mRNA levels of Ki-67 predict mucosal damage well, as shown in a previous study (24, 37). The gene expression of GLUT2 and KRT20 showed significant reactions to gluten challenge in our previous study and was thus interesting prospects for the staining of the villus-crypt border (4). However, Ki-67, GLUT2 and KRT20 IHC staining was not optimal for defining the villus-crypt border by IHC, as shown in **Figure 2**. CYP3A4 and I-FABP have previously shown promise as blood biomarkers in predicting duodenal damage in celiac disease (38, 39). Both also looked promising as markers of villus-crypt border in healthy control samples, however, in damaged duodenal mucosa CYP3A4 also stained the crypt cells and I-FABP was almost completely absent from epithelium making these stainings unsuitable for this study. Based on epithelial differentiation, a direct microvillus marker, such as villin or CD10 (40), could be useful in our approach. However, villin and CD10 also stain the immature (forming) microvilli present in the crypt cells, making these cells unsuitable for VH : CrD assessments (20).

Previous studies have shown that the secretion of APOA4 into lymph is stimulated by lipid absorption (41) and that the plasma APOA4 correlates positively with plasma triglycerides (42). In addition, mRNA levels of APOA4 have been found to respond in a tissue specific-manner to a number of factors such as estrogen, thyroid hormone, corticosteroid and insulin (43, 44). These factors could also potentially affect APOA4 staining in duodenum, however, the effect of these on APOA4 staining in the small bowel has not been studied. A common pitfall in any IHC staining is also too weak staining intensity. In this study, the APOA4 staining was strong and had a clear cut-off for villus-crypt junction in wide variety of duodenal injuries (**Figure 3**, **Supplementary Figure 1**). Also, previously a decrease in mRNA expression of APOA4 has been shown to follow duodenal injury (4, 45). These findings provide support that the staining is not significantly affected by confounding factors. We titrated the antibody reagent carefully and obtained a nearly identical staining pattern with another APOA4 antibody (clone G-8). Despite potential pitfalls, APOA4 staining seemed to work in this controlled environment quite well.

APOA4 staining defines the villus crypt border accurately and objectively. The reliability and reproducibility of APOA4 is better than that of traditional H&E-stained specimens. APOA4 staining is easy to perform and allows coordinated analysis of the duodenal mucosa in celiac disease that has not been possible before. These findings are important for analyzing subtle differences in celiac disease biopsies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Ethics Committee of the Expert Responsibility area of Tampere University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JT, KV, MM, and JI: study concept and design. JT, AV, JS, KS, and JI: acquisition of data. JT, MM, and JI: analysis and interpretation of data. JT, MM, and JI: drafting of the manuscript. All authors were involved in the critical revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.713854/full#supplementary-material

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Conflict of Interest: JI is the chief execute officer of Jilab Inc. that produces diagnostic laboratory services for small-bowel diseases and a wide variety of cancers. JS and KS are employees of Jilab Inc. AV is employed by Fimlab Laboratories Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with the authors JT, MM, and JI.

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