The use of peripheral blood mononuclear cells in celiac disease diagnosis and treatment

Alma Kurki[1], Esko Kemppainen[1], Pilvi Laurikka[1], Katri Kaukinen[1, 2], Katri Lindfors[1]

1 Celiac Disease Research Center, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland

2. Department of Internal Medicine, Tampere University Hospital, Tampere, Finland

Corresponding author: Katri Lindfors, Celiac Disease Research Center, Faculty of Medicine and Health Technology, Tampere University, Arvo Ylpön katu 34, 33014 Tampere, Finland

Tel: +358 50 3186306

E-mail: katri.lindfors@tuni.fi

Funding: KK and KL was supported by the Academy of Finland, KK and KL by the Sigrid Juselius Foundation, KK by the Competitive State Research Financing of the Expert Area of Tampere University Hospital, and the Development Fund for Agriculture and Forestry (Makera, Ministry of Agriculture and Forestry of Finland). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Abstract

Intro: Celiac disease is characterized by an abnormal immune activation driven by the ingestion of gluten from wheat, barley, and rye. Gluten-specific $CD4^+$ T cells play an important role in disease pathogenesis and are detectable among peripheral blood mononuclear cells (PBMCs).

Areas covered: This review summarizes the use of celiac disease patient PBMCs in clinical applications focusing on their exploitation in the development of diagnostic approaches and novel drugs to replace or complement gluten-free diet.

Expert opinion: The most used PBMC-based methods applied in celiac disease research include ELISpot and HLA-DQ:gluten tetramer technology. ELISpot has been utilized particularly in research aiming to develop a celiac disease vaccine and in studies addressing the toxicity of different grains in celiac disease. HLA-DQ:gluten tetramer technology on the other hand initially focused on improving current diagnostics but in combination with additional markers it is also a useful outcome measure in clinical trials to monitor the efficacy of drug candidates. In addition, the technology serves well in the more detailed characterization of celiac disease-specific T cells, thereby possibly revealing novel therapeutic targets. Future studies may also reveal clinical applications for PBMC microRNAs and/or dendritic cells or monocytes present among PBMCs.

Keywords: celiac disease, diagnostics, ELISpot, gluten, HLA-DQ:gluten tetramer, treatment

Article highlights

- A major challenge in current celiac disease diagnostic methods is their reliability only during ingestion of gluten. In addition, an active search for novel treatment options to replace gluten-free diet (GFD) is ongoing. Gluten-specific $CD4^+$ cells present among peripheral blood mononuclear cells (PBMCs) are a distinguishing characteristic for the disease and could provide a useful tool for these purposes.

- The most commonly used methods utilizing celiac patients' PBMCs include ELISpot and HLA-DQ:gluten tetramer technology.

- ELISpot has been utilized particularly in vaccine development and in the identification of minimally toxic grains. However, for optimal performance it requires a short gluten challenge.

- HLA-DQ:gluten tetramers have been tested for their usefulness in celiac disease diagnostics. In addition, the technology is useful in the deeper characterization of the celiac disease T cell repertoire, which may reveal future therapeutic targets. Moreover, when combined with evaluation of other markers the tetramers are suitable for monitoring drug efficacy in clinical trials.

1. Introduction

Celiac disease is a chronic immune-mediated disorder characterized by abnormal immune activation in response to the ingestion of gluten from wheat, barley, and rye in a subset of genetically predisposed individuals. In patients with celiac disease, ingestion of gluten typically leads to duodenal mucosal damage comprising villous atrophy and crypt hyperplasia. In addition, increased infiltration of intraepithelial lymphocytes (IELs), as well as plasma cells and $CD4^+$ T cells in the lamina propria characterize the small bowel mucosal damage. The $CD4^+$ T cells arise from organized gut-associated lymphoid tissue after activation by gluten-derived peptides. Once activated in this inductive site, the $CD4^+$ T cells clonally expand and migrate to the effector site, the small intestinal mucosal lamina propria. While homing to the gut, $CD4^+$ T cells can be detected among peripheral blood mononuclear cells (PBMCs) in the peripheral circulation.

The activated $CD4^+$ T cells have a role in inducing the antibody response characteristic of celiac disease targeted against transglutaminase 2 (TG2) and deamidated gluten peptides [1,2]. The TG2 antibodies are exploited in celiac disease diagnosis either alone or in combination with histopathological analysis [3–5]. However, both small bowel biopsy and the serology-based diagnostic approaches are associated with several challenges and are reliable only when gluten is present in the diet.

The only treatment currently available for celiac disease is compliance with a strict lifelong gluten-free diet (GFD) excluding all food products containing wheat, barley, and rye. Although GFD is generally effective, it is associated with several challenges related to high cost, restrictive nature, and impaired quality of life. There is thus a need for alternative treatment forms and several drug pipelines are underway.

Because of the existing challenges in the diagnostics and treatment of celiac disease, novel approaches are needed. As PBMCs include the pathogenetically important $CD4^+$ T cells, and as these can be detected in the peripheral blood, they are of great interest in clinical applications. This review

summarizes the existing research utilizing celiac disease patient PBMCs in the development of novel diagnostic approaches and novel treatment strategies and evaluates their applicability as a clinical tool**.**

2. Celiac disease pathogenesis, current diagnostics and treatment

2.1 Celiac disease pathogenesis

The primary genetic risk for celiac disease is associated with major histocompatibility complex (MHC) class II genes encoding HLA-DQ receptors on the surface of antigen-presenting cells (APCs). The majority of patients carry HLA-DQ2.5 molecules while almost all other patients express HLA-DQ2.2 or HLA-DQ8. These molecules can bind antigenic gluten peptides, thus explaining their association with the disease risk (**Figure 1**) [6].

Gluten commonly refers to proteins of similar composition in wheat, rye, barley, and oats, although, strictly speaking, the term gluten is used to indicate the proline and glutamine rich proteins in wheat, while the homologue proteins in rye, barley and oat are referred to respectively as secalins, hordeins and avenins. In wheat, gluten consists of gliadins (α-, γ- and ω- gliadins) and glutenin, all of which are rich in proline and glutamine amino acids. The high proline content renders the proteins relatively resistant to proteolysis by gastrointestinal enzymes, leading to the presence of oligopeptides in the intestinal lumen [7]. Distinct glutamine residues in these peptides can serve as a substrate for TG2, which converts them into glutamic acid in a deamidation reaction, thereby increasing their affinity to HLA-DQ2.5 and -DQ8 molecules on APCs [8] (**Figure 1**). In the gut-associated lymphoid tissue, the APCs present the peptides to gluten-reactive T cells that recognize them by virtue of their T cell receptors (TCRs) and become activated. These activated T cells have an important role in the generation of TG2 and deamidated gliadin peptide antibody responses by offering cognate help to

TG2- and gluten-specific B cells inducing their differentiation into antibody-secreting plasma cells [9].

In the gut-associated lymphoid tissue, the activated gluten-specific $CD4^+$ T cells begin clonal expansion and appear circulating in peripheral blood. They carry gut-homing receptor α4β7-integrin [10,11] and will migrate back to the small intestinal mucosa infiltrating the lamina propria. There the cells function as effector cells secreting high levels of proinflammatory cytokines interferon (IFN)-γ, interleukin (IL)-2, and IL-21 [12] making the milieu susceptible to tissue destruction.

The development of the small bowel mucosal damage is thought to result from an interplay between the adaptive and innate immune responses described above. Although the details of the innate immune response are not completely understood, at least IL-15 is thought to play a decisive role. In celiac disease, IL-15 is upregulated in both the epithelium and the lamina propria [13]. While lamina propria expression of IL-15 is thought to induce T_H1 immunity [14], the epithelial expression of IL-15 has a crucial role in inducing a cytotoxic $CD8^+$ αβ phenotype in IELs [13] (**Figure 1**). This cytotoxic transformation by IL-15 involves the upregulation of the NKG2D receptor on $CD8^+$ IELs providing them with the ability to kill enterocytes by binding MHC class I chain-related gene (MIC) molecules and non-classical HLA-E molecules on enterocytes [15,16].

The gut-homing gluten-reactive $CD4^+$ T cells are not present in non-celiac disease individuals but are detectable, albeit at a low frequency, in the blood of treated celiac disease patients even after decades on GFD [11]. However, after short gluten challenge, their presence in blood circulation is increased considerably as existing T memory cells clonally expand. [11,17,18] The appearance of these circulating $CD4^+$ T cells after gluten exposure is used in research to develop more efficient and non-invasive diagnostic procedures and in the development of novel therapies for celiac disease.

2.2 Current diagnostics and its limitations

When celiac disease is suspected, investigations are usually initiated by serological tests measuring anti-TG2 and endomysial antibodies (EmA), immunoglobulin A class antibodies. Traditionally, the diagnosis is confirmed by histopathological analysis of duodenal mucosal biopsy samples, in which the markers of celiac disease are elevated number of IELs, villous atrophy, and crypt hyperplasia [3]. Indeed, the finding of villous atrophy in TG2 antibody or EmA positive is highly specific for celiac disease. In children, celiac disease diagnosis can be established on the basis of serological tests alone in case of high antibody titers [4]. There is evidence that the same criteria can be also be used reliably to diagnose celiac disease in adults [5,19], although in most countries biopsy is still mandatory.

Both serological tests and small-intestinal biopsy have limitations. As approximately 3 to 5% of celiac disease patients are seronegative, thus the antibody tests are not able to identify these patients [20]. The challenges associated with small-intestinal biopsy include possible patchiness of the damage and poor orientation, both of which may lead to erroneous diagnosis [21]. In addition, villous atrophy is not entirely specific for celiac disease and also occurs in other conditions [22].

Another limitation common to the conventional diagnostic methods is that they recognize celiac disease poorly if gluten has already been withdrawn from the diet [23]. In fact, GFD has gained popularity during recent years and individuals adopt GFD for various other reasons than celiac disease, either to treat symptoms or because the diet is believed to be otherwise beneficial [24]. In cases where intake of gluten has been reduced or GFD has been adhered to for at least three months, a period with normal amount of daily gluten intake is recommended before diagnostic tests [25]. Yet the actual amount and duration of gluten intake required to induce diagnostic changes in the majority of people with celiac disease remains poorly defined [26,27]. For a patient suffering from gluten-related symptoms, such a long period is challenging, and, in these cases, the diagnosis often remains

uncertain. Diagnostic assays allowing reliable celiac disease diagnosis also during GFD would therefore be welcomed.

2.3 Gluten-free diet and the need for novel alternative treatment modalities

The only available treatment for celiac disease is a lifelong, strict GFD, meaning exclusion from the diet of wheat, rye, barley, and products with added gluten. Pure oats are safe for most celiac disease patients [28,29], although the acceptance of oats as a part of GFD varies between countries [30]. The main benefits of the dietary treatment are the healing of small-intestinal mucosa, alleviation of symptoms, and improvement in quality of life [31–33]. In addition, the aim is to prevent celiac disease-associated complications [34,35].

However, GFD has well-known challenges. Some celiac disease patients continue to experience symptoms despite dietary treatment [36,37]. The reasons for these persistent symptoms are diverse, but continuous gluten intake seems to play a major role [37,38]. In addition, gut microbial dysbiosis has been suggested as a contributor to persistent symptoms [39]. Even though commercial products labeled as gluten-free may appear safe [40,41], there is a risk of cross-contamination in environments where both gluten-free and gluten-containing foods are prepared [42–44]. Thus, a strict GFD may be difficult to maintain. Moreover, GFD is burdensome and socially restrictive [45,46]. Gluten-free products are also more expensive, less nutritious, and their availability is limited compared with their gluten-containing counterparts [47–50]. In addition, there is a growing amount of data on the potentially adverse health implications of the highly restrictive GFD itself, such as adverse cardiovascular events, fatty liver disease and metabolic effects [50–52].

Due to these problems, there is a growing interest in novel treatments to complement or replace GFD. One approach would be the development of grains with reduced immunotoxicity either with selective breeding or genetic engineering [53,54]. However, the essential role of gluten in determining the

baking properties of wheat, and negative attitudes towards genetically modified foods have made this approach challenging. Several phase I and II clinical trials testing the efficacy of novel celiac disease drug candidates have been completed or are currently ongoing. These pipelines include, for example, restoring of oral tolerance to gluten, for example, by a vaccine [55] and degrading gluten in the intestinal lumen by peptidase treatment [56–58]. Interestingly, some of the treatment pipelines have exploited celiac disease PBMCs in proof-of-concept testing of the candidate drugs or as a study outcome.

3. PBMC applications in celiac disease

Human PBMCs comprise several classes of immune cells, including T cells (~70%), B cells (~15%), monocytes (~5%), dendritic cells (~1%), and natural killer (NK) cells (~10%) [59,60]. PBMCs are easily isolated from a peripheral blood sample by density-gradient centrifugation, whereafter they can be cultured or cryopreserved for further studies, making them an attractive research tool. Diagnostic methods exploiting PBMCs, particularly gluten-reactive T cells, aim to easily and consistently identify the cells that correlate with disease pathogenesis and provide tools for translational medicine. For example, it has been recently demonstrated that approximately 10% of the TCR α-, β-chain and paired αβ-chain sequences used in response to gluten is shared among patients [11]. The growing understanding of the phenotypes of these gluten-reactive cells can also help to improve the techniques to detect the cells of interest with higher sensitivity and specificity.

The role of PBMC microRNAs (miRNAs) in celiac disease has also been investigated to extend the knowledge of the disease at the molecular level. miRNAs are small non-coding RNA molecules that bind to complementary sequences in messenger RNAs, resulting in transcriptional silencing. A novel study reported that the expression of miRNAs related to inflammation processes (miRNA-146a, miRNA-155, miRNA-21 and miRNA-125b) in PBMCs was increased in both treated and untreated

celiac disease patients. The expression of these miRNAs does not appear to be gluten-dependent and their presence may thus signal increased susceptibility to celiac disease. [61]

In addition to gluten-reactive T cells, PBMCs may also harbor potentially disease-relevant B cells, dendritic cells and monocytes. Circulating dendritic cells and monocyte subsets expressing gut-homing profiles have been detected in celiac disease patients [62] along with anti-TG2 -producing memory IgA B cells that may be distinguishable even after a long-term GFD. [63]

Two techniques described below, the ELISpot and peptide-MHC (pMHC) tetramers, are often used in conjunction with patient-derived PBMCs. The tetramer technology, due to its high sensitivity, is well-suited *e.g.* for diagnostics and monitoring anti-gluten drug efficacy in clinical trials, while ELISpot provides less sensitivity but is ideal *e.g.* for cytokine-stimulation studies.

3.1 ELISpot

ELISpot (Enzyme-linked immunospot) assay is a quantitative and qualitative analytical technique utilizing specific antibodies for enumerating cells reactive to specific antigens present in a sample and has been mostly used to assess antigen-activated T cells and B cells. ELISpot is widely used in a variety of applications, including vaccine research [64], but it is also exploited in the diagnostics of tuberculosis [65]. ELISpot assays are very specific and sensitive in visualizing the cytokine production of individual antigen-specific T cells at single-cell level. The execution of the ELISpot assay is straightforward and it can be performed with both fresh and cryopreserved PBMCs [66]. Further, the ELISpot assay offers a simple and exceptionally valuable way to study the reactivity of T cells to a variety of epitopes at individual level as up to 100 peptides may be tested in parallel with cells isolated from a single 30ml peripheral blood sample [67].

In celiac disease applications researchers have mostly used the IFN-γ ELISpot assay. In this assay, the patient PBMCs are cultured with selected gluten peptides and the IFN-γ secreted by the activated

T cells is bound to the anti-IFN-γ antibody attached to the bottom of the dish (**Figure 2**). The bound IFN-γ is detected with enzyme-conjugated antibody, visualized by a color substrate, and a so-called spot is formed representing a single reactive cell. The number of spots is proportional to the strength of the immune response induced. [67] However, the number of gluten-reactive T cells in the peripheral blood of both treated and untreated celiac disease patients are rarely detected with ELISpot [17,18,68]. Therefore, in treated celiac disease patients, the detection of circulating gluten-reactive T cells with ELISpot requires a short 3-day gluten challenge. [17,68]

In terms of epitope mapping, the initial studies addressed a limited number of peptides in wheat but later the investigations expanded to cover all wheat peptides and other grain varieties, such as barley, rye, and oat [69–73]. A comprehensive gluten-reactive T cell epitope mapping study analyzed over 16,000 peptides from wheat, barley, and rye with HLA-DQ2.5+ celiac disease patient PBMCs using the IFN-γ ELISpot assay [71]. The study evaluated the most immunodominant HLA-DQ2.5 restricted T cell epitopes by using sequences found in peptide libraries existing at the time. In the study, celiac disease patients on a strict GFD underwent a short gluten challenge and consumed either wheat, barley, rye, or all combined, and their PBMCs were challenged with a variety of gluten peptides in an ELISpot assay. The assay revealed that the level of T cell reaction to certain peptides was dependent on the cereal ingested in the gluten challenge and that the immunogenicity varied individually between subjects. In addition, the T cells showed cross-reactivity to homologous epitopes. [71] Further, a panel of rye and barley peptides tested with ELISpot using PBMCs of HLA-DQ2.5+ children with celiac disease revealed immunodominant hordein and secalin peptides independent of wheat peptides [74]. The results from these studies have enabled the establishment of a hierarchy of immunogenic peptides by grading and comparing the strength of the immune response elicited and incidence between subjects. An extensive listing of reported HLA-DQ-restricted T cell epitopes has been published previously [75], and the grading and mapping of gluten epitopes has

recently been updated [76]. The diversity of the pathogenic epitopes identified seems to be limited, and many of the epitopes overlap even between different grains.

The concept of peptide-based therapy to restore immune tolerance to gluten in celiac disease was originated on the basis of the hierarchy established [71]. In this regard, a therapeutic vaccine consisting of three gluten peptides has been tested in clinical trials. Although, the results of the Phase I trials were promising [55,77], a multicenter Phase II trial was terminated due to interim results showing no significant protection against gluten-induced symptoms, the primary endpoint of the study [78].

In addition to vaccine development, ELISpot has been used in studying the toxicity of different grains, including oats, in celiac disease. Although oat is considered safe for celiac disease patients, the ingestion of gluten seems to induce cross-reactive T cell activation *in vitro* to normally non-immunogenic oat avenin peptides as demonstrated using ELISpot assay [73]. This cross-reactivity was observed with an oral barley challenge but not with wheat or rye, likely due to the striking similarity between barley hordein and oat avenin epitope. Further, oral oat challenge did not significantly activate avenin-specific T cells, even in large amounts. Therefore, it appears that grain cross-reactivity may have a significant impact on gluten-induced T cell responses. [73]

The establishment of the hierarchy of immunogenic gluten peptides has raised interest in developing possible non-toxic grain varieties safer for celiac disease patients. ELISpot may be utilized to identify such grain variants as in earlier studies with barley [54] and wheat [79,80]. Additionally, the sequencing of immunogenic peptides may open up the possibility for peptide modification of the antigenic sequence and thus the development of non-immunogenic grain varieties safe for celiac disease patients [81]. Understanding the epitope sequences could lead to a possibility to develop a ligand antagonist that could prevent the HLA-DQ binding and antigen presentation [82]. Further, ELISpot is a useful method for testing and developing therapeutic methods for celiac disease, as it monitors how gluten-specific T cell responses are altered to different epitopes. For example, T cell

IFN-γ secretion was shown to be inhibited by modifications to the immunodominant gliadin peptide [79]. The method has also been used as an outcome in assessing the potential of the hookworm *Necator americanus* to suppress the immunopathology induced by gluten [83]. Moreover, it has been exploited to study the efficacy of latiglutenase (previously known as ALV003), a mixture of two gluten degrading peptidases. However, despite promising results in the first Phase II clinical trial, in a trial with treated patients experiencing moderate or severe symptoms, latiglutenase did not improve histology or symptom scores when compared with placebo [56,84] and thus its efficacy will be further addressed in ongoing clinical trials.

ELISpot has also been proposed as a diagnostic tool to detect gluten-reactive T cells in circulation in celiac disease [85]. Based on the finding that a gluten-induced IFN-γ ELISpot response of PBMCs can only be achieved in treated celiac disease or dermatitis herpetiformis patients (a cutaneous manifestation of celiac disease) after a short gluten challenge, the assay seems highly specific and could offer diagnostic benefit in patients having initiated GFD prior to diagnosis [17,18,85,86]. However, the IFN-γ response detected by ELISpot varies considerably between individuals and the percentage of non-responding celiac disease patients has ranged between 8.3% and 47% in earlier studies [17,18,69,86]. Due to this varying sensitivity, the diagnostic value of ELISpot is therefore somewhat limited.

ELISpot can be supplemented by ELISA (Enzyme-linked immunosorbent assay), which can measure additional cytokines, proteins, or hormones secreted by PBMCs after exposure to a variety of grain peptides, for instance from maize and oats [87,88]. The utility of ELISA as a diagnostic tool in celiac disease has also been assessed [89]. In this study, the measurement of IFN-γ and IP-10 in gluten peptide stimulated whole blood collected after a 3-day oral gluten challenge revealed that both ELISA and ELISpot assays were 100% specific and the respective sensitivities were 85% and 94%.

ELISA is also used in combination with a BrdU cell proliferation assay, which in the case of PBMCs is useful for representing the induction of an immune response by measuring the initiation of clonal

expansion. It has been used supplementarily to ELISA and ELISpot testing in determining immunogenicity of grain peptides in celiac disease. The BrdU assay coupled with ELISA assay of several cytokines including TNF-α, IFN-γ, and IL-10 from celiac patient PBMC culture supernatants has been used in studies intended to block gliadin induced immune activation. One of the studies investigated whether antagonist peptides in durum wheat capable of inhibiting the abnormal immune response triggered by gliadin peptides could be a possible therapeutic strategy for CD [90]. Another study investigated modified wheat lines where gliadin expression was reduced with RNAi and the immune response was measured by applying ELISA and BrdU proliferation assay [91].

ELISpot and ELISA assays exploiting celiac disease patient PBMCs are useful tools in research aiming to develop improved diagnostics or alternative treatment forms. However, a limiting factor in their utilization is the fact that the number of gluten reactive cells in both treated and untreated celiac disease patients is below the detection limit of these assays [17] (**Table 1**). A short gluten challenge increases their number to a detectable level, but this increase is only transient and does not occur in all patients [69,85]. Therefore, PBMC-based ELISpot or ELISA is of limited value as a tool in clinical drug trials, but the approach could be exploited as a first-line screening tool of the efficacy of a drug prior to commencing the clinical trials.

3.2 Tetramers

The presence of gluten-reactive immune cells in celiac disease patients can be assessed using multiple methods. Serum profiling studies have recently suggested that rapid increase in the levels of circulating cytokines, most notably that of IL-2, only hours after single bolus oral gluten, could be used as a robust marker of gluten-specific T cell activation [92]. Yet, for the direct detection of the cells *per se*, the most sensitive method currently available is based on direct cell labeling and flow cytometry. The pMHC multimer technique makes use of the highly specific interactions between MHC molecules and restricted TCRs to stain antigen-specific T cells [93]. The interaction between a

single pMHC molecule and a TCR is typically very brief and of low affinity, but the stability can be increased by the multimerization of multiple pMHCs. The pMHC tetramers consist of four recombinant MHC molecules typically loaded with a single peptide bound to a streptavidin molecule and coupled with a fluorophore, which allows the visualization and enumeration of bound cells by conventional flow cytometry. This technology has expanded the studies of antigen-specific T cells in celiac disease since it allows direct *ex vivo* quantification and characterization of the bound T cell detected by its molecular antigen specificity. pMHC multimers can also be used as efficient screening tools even in cases where the MHC restrictions but not the epitopes are known. [94] MHC class I tetramers for detecting $CD8^+$ T cells have been widely utilized, yet the use of MHC class II tetramers for $CD4^+$ T cell studies has been hampered by the instability of MHCII molecules. Also, the prevalence of $CD4^+$ T cells of a given specificity appears to be present in much lower frequencies than that of $CD8^+$ T cells. The low frequency of $CD4^+$ T cells in peripheral tissues in particular is a challenge and different approaches such as magnetic bead enrichment have been applied to enumerate them for analysis [95].

The gluten:HLA-DQ tetramers predominantly used in celiac disease applications consist of four MHC class II HLA-DQ molecules covalently linked to a gluten peptide (**Figure 3**). Producing tetramers containing the celiac disease predisposing HLA-DQ2 molecules has proven challenging due to their instability when the peptide-binding groove is empty [94]. In 2001, Quarsten and colleagues produced recombinant HLA-DQ2:gluten (DQ2.5-glia-α1a, DQ2.5-glia-α2, or γ- gliadin I) tetramers for the first time using a baculovirus expression system. These novel HLA-DQ2:gluten tetramers were able to activate gluten peptide-specific T cell clones derived from small intestinal biopsies with signs of immunogenic differences in the elicited T cell responses between the bound peptides. [96] Since then, the pMHCII complexes used in celiac disease research have mostly been limited to HLA-DQ2.5 and HLA-DQ8 molecules and the most immunodominant gliadin epitopes [10,11,27,97–99]. Tetramer research should preferably be conducted with a broader range of DQ8

epitopes and HLA-DQ2.2 epitopes to expand the research to include all celiac disease-associated HLA-types. HLA-DQ2 tetramers consisting of gliadin epitopes DQ2.5-glia-α1a or DQ2.5-glia-α2 have been successfully used to detect gluten-specific T cells amongst the PBMCs of celiac disease patients after a short-term gluten challenge but not in controls [68]. The frequency of gluten-reactive T cells in patients was found to vary between 1:1,000 to 1:5,000 among all $CD4^+$ T cells, amounting to approximately 1:5,000 to 1:25,000 among all PBMCs [68].

One of the major advantages of the pMHCII tetramer technique over the conventional ELISpot method or histology after a short gluten challenge is diagnostic sensitivity. In patients with biopsy-proven celiac disease on GFD, the tetramer technology proved to be a more sensitive disease biomarker after 3 or 6-day gluten challenge than a small bowel histopathological analysis after four days or two weeks on gluten consumption [27,97]. Further improvements and modifications of the HLA-DQ:gluten tetramer test performance aim to eliminate the need for even a short gluten challenge and to enhance its diagnostic potential. For this, bead-enrichment without gluten challenge was applied to enumerate DQ2.5-glia-α1a and DQ2.5-glia-α2 tetramer bound T cells in PBMCs of untreated and treated celiac disease patients and control individuals for enhanced flow cytometry detection [10]. The gliadin tetramer bound T cells were successfully enumerated with bead-enrichment *ex vivo*, without the inclusion of gluten challenge and the detected median frequency of gut-homing gliadin-tetramer bound $CD4^+$ effector memory T cells was significantly higher in celiac disease patients (approximately five gluten-responsive cells per one million total $CD4^+$ T cells in treated patients and 16 per one million in untreated patients) than in controls (0.01 cells per one million total $CD4^+$ T cells). These cells likely reflect an antigen-driven, celiac disease-associated T-cell response, and there is a potential for using this parameter as an additional diagnostic criterion for celiac disease. Quantification of gut-homing, gluten-specific memory T cells in the peripheral blood, visualized with tetramers, may thus be used to distinguish celiac disease patients from healthy individuals. [10] HLA-DQ:gluten tetramers could also bind to few $CD4^+$ T memory cells of healthy

non-celiac HLA-DQ2.5+ subjects, but the binding appeared to be mainly unspecific as the T cell phenotype is different (not Tre or Tr1) from those of celiac disease individuals [100].

A recent study further improved the tetramer assay and included a wider variety of immunodominant gluten epitopes bound to pMHCII tetramers pooled together. Without including a gluten challenge, the study tested 143 HLA-DQ2.5+ subjects including 62 subjects with celiac disease on GFD, 19 subjects with self-reported gluten sensitivity on GFD and 10 subjects with celiac disease on gluten-containing diet. For subjects on a normal gluten-containing diet, sensitivity (1.00 [95% CI 1.00–1.00]) and specificity (0.90 [95% CI 0.83–0.98]) of the HLA-DQ:gluten tetramer test was comparable to the accuracy of celiac disease-specific antibody tests, thus providing a new and less invasive supplement to existing tests. [99] Importantly, the tetramer test identified subjects with celiac disease on GFD with 97% sensitivity (95% CI 0.92-1.00) and 95% specificity (95% CI 0.84-1.00) when compared to controls and is thus promising as a diagnostic tool for subjects having adopted GFD.

Further characterization of the T cells detected by HLA-DQ:gluten tetramers may also be valuable for detecting new potential biomarkers for celiac disease follow-up or diagnosis. The gluten-specific T cells emerging after gluten challenge have upregulated expression of CD38 [101]. Furthermore, the CD38 expression was significantly higher in untreated celiac disease patients than in treated patients and achieved a test accuracy similar to that of a serology test [99]. The expression of CD38 has thus been investigated as a possible biomarker for gluten re-exposure in celiac disease or as a diagnostic marker. CD38 expression in HLA-DQ:gluten tetramer-positive memory T cells is not dependent on the duration of the gluten challenge and the increase of expression is consistent [102]. Thus, CD38 expression has potential for use as a GFD compliance parameter, and also suits well for monitoring drug efficacy in clinical trials.

Thus far, the use of tetramers has been limited to known gluten epitopes able to form kinetically stable pMHCII tetramers to be used in detection with flow cytometry (**Table 1**). Native gluten peptides able to form stable pMHCII are yet to be identified and thus tetramers consist of the deamidated

counterparts and some of the antigen-specific T cells possibly significant for disease pathogenesis may therefore go undetected. [100] Because of these limitations, further research on gluten pMHCII technique is needed to expand the peptide repertoire of clinically usable epitopes and MHCII molecules to detect all T cells that significantly affect celiac disease. The frequency of antigen-specific T cells may in some cases be underestimated when relying solely on pMHC tetramer staining and therefore other complementary techniques can still be beneficial [94]. Thus, the pMHCII tetramer technique still needs additional PBMC analysis techniques to support its performance.

4. Conclusions

The current diagnostics of celiac disease and therapy with GFD require improvement and PBMCs offer an easily accessible tool for this purpose. Currently, ELISpot is mainly utilized in the identification of suitable gluten-peptides for vaccine development and to assess the toxicity of different grains and cultivars, but it is also a useful tool to monitor pharmacological therapies modulating gluten T cell response (**Table 1**). Advanced HLA-DQ:gluten tetramer technology has been proven to be a promising method for celiac disease diagnosis, particularly if GFD has been initiated. Moreover, HLA-DQ:gluten tetramer technology has been useful in the identification of the T cell repertoire in celiac disease, which is an attractive future target for therapy. As both ELISpot and HLA-DQ:gluten tetramer tests have limitations, their use in diagnostics and novel drug testing calls for methodological improvement or for them to be combined with other tests or markers.

5. Expert opinion

Celiac disease has become a worldwide health problem in the past few decades with an increasing incidence among children and adults. If untreated, celiac disease is a debilitating disease, and adhering to the only known effective treatment, a lifelong GFD regimen, is difficult to achieve and has a significantly negative effect on quality of life. It is thus evident that there is a high demand for new

forms of therapy. The HLA-alleles predisposing to celiac disease are universal but the immune response to celiac antigens is ultimately shaped by our individual genetics, and further advances in the development of diagnostic tools paves the way for developing more personalized forms of therapeutics. For example, we still know little about how different patient-specific T cell repertoires correlate with disease severity and prognosis in refractory celiac disease, a severe but rare form of celiac disease that does not respond to treatment with GFD. Also of particular interest is how different T cell receptor alleles are implicated in the development of celiac disease in general. Interestingly, increased frequencies of T cells of the same phenotype have been discovered in patients with celiac disease, systemic lupus erythematosus and patients with systemic sclerosis [103]. Whether this overlap also extends to other autoimmune disorders remains to be ascertained. Nonetheless, such observations are of great relevance for the development of immunotherapeutics targeting selectively only disease-causing cells.

In order to develop more effective and targeted treatments for celiac disease and also other autoimmune disorders, disease-specific T cells and their cognate antigens need to be better characterized. During the past two decades, the conventional ELISA-based applications have been partially replaced by pMHC tetramers and flow cytometry for the detection of $CD8^+$ and $CD4^+$ T cells. This technology enables rapid and simultaneous screening of a large number of disease-relevant T cells specific to a predefined set of antigens in the context of MHC classes I and II. Furthermore, the significant improvement in the sensitivity of detecting immune cells has made it possible to detect circulating T cells even without prior gluten challenge. This improvement in sensitivity is particularly crucial since the proportion of relevant T cells among all immune cells is several fold lower in circulation than in the inflamed intestinal epithelium. The ability to detect and identify circulating disease-relevant cells effectively is also crucial for recruiting patients to participate in clinical research since venipuncture is not considered a very invasive procedure. While the evaluation of

mucosal condition after a long-term treatment or exposure to detoxified grains still remains the gold standard to assess the efficacy of any therapeutic drugs or gluten detoxification strategies, circulating immune cells can provide an easily attainable source of surrogate markers of disease progression. Finally, the tetramer technology in combination with e.g. deep sequencing has proven superior to more conventional methods of studying disease etiology, but there is still room for improvement in terms of tetramer target specificity and sensitivity. The most obvious ways to improve pMHC tetramer staining are optimizing the choice and use of fluorophores and higher order of pMHC multimerization.

In addition to tetramer technology, the vast progress in deep sequencing methods during the past two decades has greatly increased our understanding of the properties and repertoire of celiac disease relevant immune cells. For example, the characterization of specific TCR repertoires by sequencing may provide more profound insight into the function and structure of the gluten-reactive T cells driving disease pathogenesis and also provide tools for diagnosis and management as well as targets for novel treatment strategies. The role of PBMC miRNAs has also been tentatively investigated to extend our knowledge of the disease at the molecular level [61] and further research could elucidate the role of miRNAs in celiac disease pathogenesis and their position as relevant biomarkers for celiac disease diagnosis regardless of the stage of the disease.

Finally, PBMCs harbor multiple disease-relevant cell types in addition to gluten-reactive T cells, such as monocytes, B cells and dendritic cells. [62] Gliadin-derived peptides appear to induce changes in the dendritic cell subtype phenotype and function [62] and memory B cells may be stimulated *in vitro* to induce production of antibodies against TG2 even in patients on a long-term GFD [63]. Further research on B cells and dendritic cells in celiac disease may thus prove them to be useful biomarkers for the diagnosis of the disease and compliance with GFD.

References

[1] Dieterich W, Ehnis T, Bauer M, et al. Identification of tissue transglutaminase as the autoantigen of celiac disease. Nat Med. 1997;3:797–801.

[2] Aleanzi M, Demonte AM, Esper C, et al. Celiac disease: antibody recognition against native and selectively deamidated gliadin peptides. Clin Chem. 2001;47:2023–2028.

[3] Ludvigsson JF, Bai JC, Biagi F, et al. Diagnosis and management of adult coeliac disease: Guidelines from the British society of gastroenterology. Gut. 2014;63:1210–1228.

[4] Husby S, Koletzko S, Korponay-Szabó I, et al. European Society Paediatric Gastroenterology, Hepatology and Nutrition Guidelines for Diagnosing Coeliac Disease 2020. J Pediatr Gastroenterol Nutr. 2020;70:141–156.

[5] Popp A, Kivelä L, Fuchs V, et al. Diagnosing Celiac Disease: Towards Wide-Scale Screening and Serology-Based Criteria? Gastroenterol Res Pract. 2019;2019:2916024.

[6] Sollid LM. The roles of MHC class II genes and post-translational modification in celiac disease. Immunogenetics. 2017;69:605–616.

[7] Shan L, Qiao S-W, Arentz-Hansen H, et al. Identification and Analysis of Multivalent Proteolytically Resistant Peptides from Gluten: Implications for Celiac Sprue. J Proteome Res. 2005;4:1732–1741.

[8] Molberg Ø, Mcadam SN, Körner R, et al. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. Nat Med. 1998;4:713–717.

[9] Stamnaes J, Sollid LM. Celiac disease: Autoimmunity in response to food antigen. Semin Immunol. 2015;27:343–352.

[10] Christophersen A, Ráki M, Bergseng E, et al. Tetramer-visualized gluten-specific CD4+ T cells in blood as a potential diagnostic marker for coeliac disease without oral gluten challenge. United Eur Gastroenterol J. 2014;2:268–278.

**** First study to demonstrate that tetramer technology enables the detection of gluten-reactive T cells in peripheral blood without preceding oral gluten challenge.**

[11] Risnes LF, Christophersen A, Dahal-Koirala S, et al. Disease-driving CD4+ T cell clonotypes persist for decades in celiac disease. J Clin Invest. 2018;128:2642–2650.

[12] Jabri B, Sollid LM. T Cells in Celiac Disease. J Immunol. 2017;198:3005–3014.

[13] Setty M, Discepolo V, Abadie V, et al. Distinct and Synergistic Contributions of Epithelial Stress and Adaptive Immunity to Functions of Intraepithelial Killer Cells and Active Celiac Disease. Gastroenterology. 2015;143:681–691.

[14] Abadie V, Kim SM, Lejeune T, et al. IL-15, gluten and HLA-DQ8 drive tissue destruction in coeliac disease. Nature. 2020;578:600–604.

[15] Hüe S, Mention J-J, Monteiro RC, et al. A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. Immunity. 2004;21:367–377.

[16] Meresse B, Chen Z, Ciszewski C, et al. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. Immunity. 2004;21:357–366.

[17] Anderson RP, van Heel DA, Tye-Din JA, et al. T cells in peripheral blood after gluten challenge in coeliac disease. Gut. 2005;54:1217–1223.

[18] Camarca A, Radano G, Di Mase R, et al. Short wheat challenge is a reproducible in-vivo assay to detect immune response to gluten. Clin Exp Immunol. 2012;169:129–136.

[19] Fuchs V, Kurppa K, Huhtala H, et al. Serology-based criteria for adult coeliac disease have excellent accuracy across the range of pre-test probabilities. Aliment Pharmacol Ther. 2019;49:277–284.

[20] Schiepatti A, Sanders DS, Biagi F. Seronegative coeliac disease: Clearing the diagnostic dilemma. Curr Opin Gastroenterol. 2018;34:154–158.

[21] Taavela J, Koskinen O, Huhtala H, et al. Validation of morphometric analyses of small-intestinal biopsy readouts in celiac disease. PLoS One. 2013;8:e76163.

[22] Schiepatti A, Sanders DS, Zuffada M, et al. Overview in the clinical management of patients with seronegative villous atrophy. Eur J Gastroenterol Hepatol. 2019;31:409–417.

[23] Silvester JA, Kurada S, Szwajcer A, et al. Tests for Serum Transglutaminase and Endomysial Antibodies Do Not Detect Most Patients With Celiac Disease and Persistent Villous Atrophy on Gluten-free Diets: a Meta-analysis. Gastroenterology. 2017;153:689-701.e1.

[24] Lerner BA, Green PHR, Lebwohl B. Going Against the Grains: Gluten-Free Diets in Patients Without Celiac Disease-Worthwhile or Not? Dig Dis Sci. 2019;64:1740–1747.

[25] Bruins MJ. The clinical response to gluten challenge: A review of the literature. Nutrients. 2013;5:4614–4641.

[26] Leffler D, Schuppan D, Pallav K, et al. Kinetics of the histological, serological and symptomatic responses to gluten challenge in adults with coeliac disease. Gut. 2013;62:996–1004.

[27] Sarna VK, Skodje GI, Reims HM, et al. HLA-DQ:gluten tetramer test in blood gives better detection of coeliac patients than biopsy after 14-day gluten challenge. Gut. 2018;67:1606–1613.

[28] Pinto-Sánchez MI, Causada-Calo N, Bercik P, et al. Safety of Adding Oats to a Gluten-Free Diet for Patients With Celiac Disease: Systematic Review and Meta-analysis of Clinical and Observational Studies. Gastroenterology. 2017;153:395-409.e3.

[29] Lionetti E, Gatti S, Galeazzi T, et al. Safety of Oats in Children with Celiac Disease: A Double-Blind, Randomized, Placebo-Controlled Trial. J Pediatr. 2018;194:116-122.e2.

[30] Lindfors K, Ciacci C, Kurppa K, et al. Coeliac Disease. Nat Rev Dis Prim. 2019;5:1–37.

[31] Murray JA, Watson T, Clearman B, et al. Effect of a gluten-free diet on gastrointestinal symptoms in celiac disease. Am J Clin Nutr. 2004;79:669–673.

[32] Casellas F, Rodrigo L, Vivancos JL, et al. Factors that impact health-related quality of life in adults with celiac disease: a multicenter study. World J Gastroenterol. 2008;14:46–52.

[33] Jericho H, Sansotta N, Guandalini S. Extraintestinal Manifestations of Celiac Disease: Effectiveness of the Gluten-Free Diet. J Pediatr Gastroenterol Nutr. 2017;65:75–79.

[34] Ilus T, Kaukinen K, Virta LJ, et al. Incidence of malignancies in diagnosed celiac patients: a population-based estimate. Am J Gastroenterol. 2014;109:1471–1477.

[35] Zylberberg HM, Lebwohl B, RoyChoudhury A, et al. Predictors of improvement in bone mineral density after celiac disease diagnosis. Endocrine. 2018;59:311–318.

[36] Laurikka P, Salmi T, Collin P, et al. Gastrointestinal Symptoms in Celiac Disease Patients on a Long-Term Gluten-Free Diet. Nutrients. 2016;8:429.

[37] Sansotta N, Amirikian K, Guandalini S, et al. Celiac Disease Symptom Resolution: Effectiveness of the Gluten-free Diet. J Pediatr Gastroenterol Nutr. 2018;66:48–52.

[38] Stasi E, Marafini I, Caruso R, et al. Frequency and Cause of Persistent Symptoms in Celiac Disease Patients on a Long-term Gluten-free Diet. J Clin Gastroenterol. 2016;50:239–243.

[39] Francavilla R, Cristofori F, Vacca M, et al. Advances in understanding the potential therapeutic applications of gut microbiota and probiotic mediated therapies in celiac disease. Expert Rev Gastroenterol Hepatol. 2020;14:323–333.

[40] Gibert A, Kruizinga AG, Neuhold S, et al. Might gluten traces in wheat substitutes pose a risk in patients with celiac disease? A population-based probabilistic approach to risk estimation. Am J Clin Nutr. 2013;97:109–116.

[41] Thompson T, Simpson S. A comparison of gluten levels in labeled gluten-free and certified gluten-free foods sold in the United States. Eur J Clin Nutr. 2015;69:143–146.

[42] Lerner BA, Phan Vo LT, Yates S, et al. Detection of Gluten in Gluten-Free Labeled Restaurant Food: Analysis of Crowd-Sourced Data. Am J Gastroenterol. 2019;114:792–797.

[43] Weisbrod VM, Silvester JA, Raber C, et al. Preparation of Gluten-Free Foods Alongside Gluten-Containing Food May Not Always Be as Risky for Celiac Patients as Diet Guides Suggest. Gastroenterology. 2020;158:273–275.

[44] Halmos EP, Di Bella CA, Webster R, et al. Gluten in "gluten-free" food from food outlets in Melbourne: A cross-sectional study. Med J Aust. 2018;209:42–43.

[45] Wolf RL, Lebwohl B, Lee AR, et al. Hypervigilance to a Gluten-Free Diet and Decreased Quality of Life in Teenagers and Adults with Celiac Disease. Dig Dis Sci. 2018;63:1438–1448.

[46] Leinonen H, Kivelä L, Lähdeaho M-L, et al. Daily Life Restrictions are Common and Associated with Health Concerns and Dietary Challenges in Adult Celiac Disease Patients Diagnosed in Childhood. Nutrients. 2019;11:1718.

[47] Panagiotou S, Kontogianni MD. The economic burden of gluten-free products and gluten-free diet: a cost estimation analysis in Greece. J Hum Nutr Diet Off J Br Diet Assoc. 2017;30:746–752.

[48] Lee AR, Wolf RL, Lebwohl B, et al. Persistent Economic Burden of the Gluten Free Diet. Nutrients. 2019;11:399.

[49] Punshon T, Jackson BP. Essential micronutrient and toxic trace element concentrations in gluten containing and gluten-free foods. Food Chem. 2018;252:258–264.

[50] Ciccone A, Gabrieli D, Cardinale R, et al. Metabolic Alterations in Celiac Disease Occurring after Following a Gluten-Free Diet. Digestion. 2019;100:262–268.

[51] Reilly NR, Lebwohl B, Hultcrantz R, et al. Increased risk of non-alcoholic fatty liver disease after diagnosis of celiac disease. J Hepatol. 2015;62:1405–1411.

[52] Lebwohl B, Cao Y, Zong G, et al. Long term gluten consumption in adults without celiac disease and risk of coronary heart disease: prospective cohort study. BMJ. 2017;357:j1892.

[53] García-Molina MD, Giménez MJ, Sánchez-León S, et al. Gluten Free Wheat: Are We There? Nutrients. 2019;11:487.

[54] Tanner GJ, Howitt CA, Forrester RI, et al. Dissecting the T-cell response to hordeins in coeliac disease can develop barley with reduced immunotoxicity. Aliment Pharmacol Ther. 2010;32:1184–1191.

[55] Daveson AJM, Ee HC, Andrews JM, et al. Epitope-Specific Immunotherapy Targeting CD4-Positive T Cells in Celiac Disease: Safety, Pharmacokinetics, and Effects on Intestinal Histology and Plasma Cytokines with Escalating Dose Regimens of Nexvax2 in a Randomized, Double-Blind, Placebo-Controlled. EBioMedicine. 2017;26:78–90.

[56] Lähdeaho M-L, Kaukinen K, Laurila K, et al. Glutenase ALV003 attenuates gluten-induced mucosal injury in patients with celiac disease. Gastroenterology. 2014;146:1649–1658.

[57] Cavaletti L, Taravella A, Carrano L, et al. E40, a novel microbial protease efficiently detoxifying gluten proteins, for the dietary management of gluten intolerance. Sci Rep. 2019;9:1–11.

[58] Wolf C, Siegel JB, Tinberg C, et al. Engineering of Kuma030: A Gliadin Peptidase That Rapidly Degrades Immunogenic Gliadin Peptides in Gastric Conditions. J Am Chem Soc. 2015;137:13106–13113.

[59] Autissier P, Soulas C, Burdo TH, et al. Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans. Cytometry A. 2010;77:410–419.

[60] Kleiveland CR. Peripheral Blood Mononuclear Cells. In: Verhoeckx K, Cotter P, López-Expósito I, et al., editors. Impact Food Bioact Heal Vitr ex vivo Model. Cham: Springer International Publishing; 2015. p. 161–167.

[61] Bascuñán KA, Pérez-Bravo F, Gaudioso G, et al. A miRNA-Based Blood and Mucosal Approach for Detecting and Monitoring Celiac Disease. Dig Dis Sci. 2019;65:1982–1991.

[62] Escudero-Hernández C, Martín Á, de Pedro Andrés R, et al. Circulating Dendritic Cells from Celiac Disease Patients Display a Gut-Homing Profile and are Differentially Modulated by Different Gliadin-Derived Peptides. Mol Nutr Food Res. 2020;64:1–10.

[63] Snir O, Mesin L, Gidoni M, et al. Analysis of Celiac Disease Autoreactive Gut Plasma Cells and Their Corresponding Memory Compartment in Peripheral Blood Using High-Throughput Sequencing. J Immunol. 2015;194:5703–5712.

[64] Yung BS, Pugh H, Generotti AA, et al. Novel IFN-γ ELISpot reveals robust T cell responses elicited after influenza nucleoprotein DNA vaccination in New Zealand White rabbits. Vaccine. 2019;37:903–909.

[65] Richeldi L, Losi M, Cerri S, et al. Using ELISpot technology to improve the diagnosis of tuberculosis infection: From the bench to the T-SPOT.TB assay. Expert Rev Respir Med. 2008;2:253–260.

[66] Kreher CR, Dittrich MT, Guerkov R, et al. CD4+ and CD8+ cells in cryopreserved human PBMC maintain full functionality in cytokine ELISPOT assays. J Immunol Methods. 2003;278:79–93.

[67] Anthony DD, Lehmann P V. T-cell epitope mapping using the ELISPOT approach. Methods. 2003;29:260–269.

[68] Ráki M, Fallang L-E, Brottveit M, et al. Tetramer visualization of gut-homing gluten-specific T cells in the peripheral blood of celiac disease patients. Proc Natl Acad Sci USA. 2007;104:2831–2836.

**** First study to report that HLA-DQ2:gluten tetramers were able to detect gluten-specific T cells in the peripheral blood of celiac disease patients.**

[69] Anderson RP, Degano P, Godkin AJ, et al. In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. Nat Med. 2000;6:337–342.

*** First study to detect gluten-reactive T cells in the peripheral blood of celiac disease patients by ELISpot after a short gluten challenge.**

[70] Arentz-Hansen H, Fleckenstein B, Molberg Ø, et al. The Molecular Basis for Oat Intolerance in Patients with Celiac Disease. PLoS Med. 2004;1:e1.

[71] Tye-Din JA, Stewart JA, Dromey JA, et al. Comprehensive, quantitative mapping of T cell epitopes in gluten in celiac disease. Sci Transl Med. 2010;2:41ra51.

**** The most comprehensive gluten-reactive T cell epitope mapping study with over 16,000 peptides from wheat, barley, and rye exploiting the IFN-γ ELISpot assay.**

[72] Vader LW, Stepniak DT, Bunnik EM, et al. Characterization of Cereal Toxicity for Celiac Disease Patients Based on Protein Homology in Grains. Gastroenterology. 2003;125:1105–1113.

[73] Hardy MY, Tye-Din JA, Stewart JA, et al. Ingestion of oats and barley in patients with celiac disease mobilizes cross-reactive T cells activated by avenin peptides and immuno-dominant hordein peptides. J Autoimmun. 2015;56:56–65.

[74] Hardy MY, Russell AK, Pizzey C, et al. Characterisation of clinical and immune reactivity to barley and rye ingestion in children with coeliac disease. Gut. 2019;gutjnl-2019-319093.

[75] Sollid LM, Qiao S-W, Anderson RP, et al. Nomenclature and listing of celiac disease relevant gluten T-cell epitopes restricted by HLA-DQ molecules. Immunogenetics. 2012;64:455–460.

[76] Sollid LM, Tye-Din JA, Qiao S-W, et al. Update 2020: nomenclature and listing of celiac disease-relevant gluten epitopes recognized by CD4(+) T cells. Immunogenetics. 2020;72:85–88.

*** Updated listing of all identified celiac disease-relevant T cell activating gluten epitopes to date.**

[77] Goel G, King T, Daveson AJ, et al. Epitope-specific immunotherapy targeting CD4-positive T cells in coeliac disease: two randomised, double-blind, placebo-controlled phase 1 studies. lancet Gastroenterol Hepatol. 2017;2:479–493.

[78] Truitt KE, Anderson RP. Editorial: a non-dietary treatment for coeliac disease—two steps forward, one step back? Authors' reply. Aliment Pharmacol Ther. 2019;50:956–957.

[79] Anderson RP, van Heel DA, Tye-Din JA, et al. Antagonists and non-toxic variants of the dominant wheat gliadin T cell epitope in coeliac disease. Gut. 2006;55:485–491.

[80] Picascia S, Camarca A, Malamisura M, et al. In Celiac Disease Patients the In Vivo Challenge with the Diploid Triticum monococcum Elicits a Reduced Immune Response Compared to Hexaploid Wheat. Mol Nutr Food Res. 2020;64:1–9.

[81] Grover J, Chhuneja P, Midha V, et al. Variable Immunogenic Potential of Wheat: Prospective for Selection of Innocuous Varieties for Celiac Disease Patients via in vitro Approach. Front Immunol. 2019;10:84.

[82] Silano M, Vincentini O, De Vincenzi M. Toxic, Immunostimulatory and Antagonist Gluten Peptides in Celiac Disease. Curr Med Chem. 2009;16:1489–1498.

[83] Daveson AJ, Jones DM, Gaze S, et al. Effect of Hookworm Infection on Wheat Challenge in Celiac Disease – A Randomised Double-Blinded Placebo Controlled Trial. PLoS One. 2011;6:e17366.

[84] Murray JA, Kelly CP, Green PHR, et al. No Difference Between Latiglutenase and Placebo in Reducing Villous Atrophy or Improving Symptoms in Patients With Symptomatic Celiac Disease. Gastroenterology. 2017;152:787-798.e2.

[85] Picascia S, Mandile R, Auricchio R, et al. Gliadin-Specific T-Cells Mobilized in the Peripheral Blood of Coeliac Patients by Short Oral Gluten Challenge: Clinical Applications. Nutrients. 2015;7:10020–10031.

[86] Kalliokoski S, Mansikka E, de Kauwe A, et al. Gliadin-Induced Ex Vivo T-Cell Response in Dermatitis Herpetiformis: A Predictor of Clinical Relapse on Gluten Challenge? J Invest Dermatol. 2020;15:1867-1869.e2.

[87] Ortiz Sánchez JP, de la Barca AMC. Effect of Maize Prolamins on Peripheral Blood Mononuclear Cells from Celiac Disease Patients. Immunome Res. 2016;12.

[88] Silano M, Benedetto R Di, Maialetti F, et al. Avenins from different cultivars of oats elicit response by coeliac peripheral lymphocytes. Scand J Gastroenterol. 2007;42:1302–1305.

[89] Ontiveros N, Tye-Din JA, Hardy MY, et al. Ex-vivo whole blood secretion of interferon (IFN)-γ and IFN-γ-inducible protein-10 measured by enzyme-linked immunosorbent assay are as sensitive as IFN-γ enzyme-linked immunospot for the detection of gluten-reactive T cells in human leucocyte antigen (HLA)-DQ2·5(+) -associated coeliac disease. Clin Exp Immunol. 2014;175:305–315.

[90] Silano M, Di Benedetto R, Trecca A, et al. A decapeptide from durum wheat prevents celiac peripheral blood lymphocytes from activation by gliadin peptides. Pediatr Res. 2007;61:67–71.

[91] Sánchez-León S, Giménez MJ, Comino I, et al. Stimulatory response of celiac disease peripheral blood mononuclear cells induced by RNAi wheat lines differing in grain protein composition. Nutrients. 2019;11:2933.

[92] Goel G, Tye-Din JA, Qiao S-W, et al. Cytokine release and gastrointestinal symptoms after gluten challenge in celiac disease. Sci Adv. 2019;5:eaaw7756.

[93] Altman JD, Moss PAH, Goulder PJR, et al. Phenotypic analysis of antigen-specific T lymphocytes. Science (80-). 1996;274:94–96.

[94] Christophersen A. Peptide-MHC class I and class II tetramers: From flow to mass cytometry. Hla. 2020;95:169–178.

[95] Jansen DTSL, Ramnoruth N, Loh KL, et al. Flow cytometric clinical immunomonitoring

using peptide-MHC class II tetramers: Optimization of methods and protocol development. Front Immunol. 2018;9:1–10.

[96] Quarsten H, McAdam SN, Jensen T, et al. Staining of Celiac Disease-Relevant T Cells by Peptide-DQ2 Multimers. J Immunol. 2001;167:4861–4868.

[97] Brottveit M, Ráki M, Bergseng E, et al. Assessing possible celiac disease by an HLA-DQ2-gliadin tetramer test. Am J Gastroenterol. 2011;106:1318–1324.

[98] Dahal-Koirala S, Risnes LF, Christophersen A, et al. TCR sequencing of single cells reactive to DQ2.5-glia-α2 and DQ2.5-glia-ω2 reveals clonal expansion and epitope-specific V-gene usage. Mucosal Immunol. 2016;9:587–596.

[99] Sarna VK, Lundin KEA, Mørkrid L, et al. HLA-DQ–Gluten Tetramer Blood Test Accurately Identifies Patients With and Without Celiac Disease in Absence of Gluten Consumption. Gastroenterology. 2018;154:886-896.e6.

[100] Christophersen A, Risnes LF, Bergseng E, et al. Healthy HLA-DQ2.5 + Subjects Lack Regulatory and Memory T Cells Specific for Immunodominant Gluten Epitopes of Celiac Disease. J Immunol. 2016;196:2819–2826.

[101] du Pré MF, Van Berkel LA, Ráki M, et al. CD62Lneg CD38+ expression on circulating CD4+ T cells identifies mucosally differentiated cells in protein fed mice and in human celiac disease patients and controls. Am J Gastroenterol. 2011;106:1147–1159.

[102] Zühlke S, Risnes LF, Dahal-Koirala S, et al. CD38 expression on gluten-specific T cells is a robust marker of gluten re-exposure in coeliac disease. United Eur Gastroenterol J. 2019;7:1337–1344.

[103] Christophersen A, Lund EG, Snir O, et al. Distinct phenotype of CD4+ T cells driving celiac disease identified in multiple autoimmune conditions. Nat Med. 2019;25:734–737.

Table 1. Advantages and limitations of ELISpot and HLA-DQ:gluten tetramer assays as tools in celiac disease diagnostics and development of novel therapeutics for celiac disease

		ELISpot	HLA-DQ:gluten tetramers
DIAGNOSTICS	Advantages	Minimally invasive	Minimally invasive
		Simple and sensitive for assessing the number of reactive cells	No need for gluten challenge
		Allows rapid immune analysis at an individual level	Sensitive analysis also with pooled epitopes
		Possible with less than 30ml of blood	Enables further characterization of the reactive T cells
	Limitations	High individual variability in responses	Current tetramer constructs are limited and do not cover all celiac disease-relevant molecules
		Requires at least 4-week GFD period prior to a short gluten challenge or *in vitro* mitogenic expansion	Requires considerable volume of blood (50–150ml) and large number of cells (1–4 million)
		Characterization of activated cells not possible	Tetramer production and analysis is challenging
		Dependent on the stimulus used	
DEVELOPING NOVEL TREATMENTS	Advantages	Allows identification of most immunogenic peptides	Enables further characterization of the reactive T cells
		Enables investigation of less toxic grains and strains	May offer novel therapeutic targets on disease-specific cells
		Simple monitoring of immune responses following the treatment	Offers novel biomarkers for GFD compliance
		Simple identification of non-toxic peptides for an individual	
		Possible with less than 30ml of blood	
	Limitations	Requires at least 4-week GFD period prior to a short gluten challenge	Current tetramer constructs are limited and do not cover all celiac disease-relevant molecules
		Characterization of activated cells not possible	Requires considerable volume of blood (50–150ml) and large number of cells (1–4 million)
		Dependent on the stimulus used	Tetramer production and analysis is challenging

GFD; gluten-free diet

Figure legends

Figure 1. Pathogenesis of the celiac disease. Ingested gluten is not fully degraded in the intestinal lumen and the resulting peptides gain access to the lamina propria. The peptides serve as a substrate for transglutaminase 2 (TG2) enzymes that catalyzes their deamidation, thereby increasing their affinity to HLA-DQ2 and -DQ8 molecules on antigen-presenting cells (APCs). Gluten-bound APCs progress to gut-associated lymphoid tissue, where they activate gluten-specific $CD4^+$ T cells, which have an important role in stimulating a celiac type B cell response. The activated B cells and the clonally expanded T cells migrate via peripheral circulation back to the small intestinal mucosa. There they function as effector cells: The T cells secreting proinflammatory cytokines and the plasma cells differentiated from B cells antibodies against deamidated gluten peptides and TG2. The proinflammatory cytokines secreted by activated T cells and increased levels of interleukin-15 (IL-15) secreted by distressed epithelial cells promote the transformation intraepithelial lymphocytes (IELs) into cytotoxic cells that mediate destruction of enterocytes promoting the development of villous atrophy.

Figure 2. Interferon-γ ELISpot applied in celiac disease research. (1) The bottom of a well is coated with a primary interferon-γ (IFN-γ) binding antibody. (2) Thereafter, the peripheral blood mononuclear cells (PBMCs) are incubated with a peptide from the cereals to be investigated. The cells activated by the peptide will secrete IFN-γ, which will be bound to the primary anti-IFN γ antibody. (3) The cells and peptides are washed away and biotinylated anti-IFN-γ detection antibody is added, followed by the addition of alkaline phosphatase-conjugated streptavidin, which will bind to the biotin on the detection antibody. Finally, a detection substrate is added which will bind and react with the alkaline phosphatase to form color. Each spot visible in the well represents a single activated IFN-γ secreting T cell specific to the peptide investigated.

Figure 3. The HLA-DQ:gluten tetramer test used in celiac disease research. The tetramers used in celiac disease applications have been produced by a baculovirus expression system. The engineered

recombinant α- and β- HLA-DQ chains have covalently bound gluten peptide in the binding groove to ensure the formation of a stable HLA-DQ:gluten monomer molecule. Biotinylation further increases the stability of the HLA-DQ:gluten monomer molecule and the biotin serves as a high-affinity binding site for the fluorescent-labeled streptavidin. Multiple monomers bind to a streptavidin, forming an HLA-DQ:gluten tetramer. The simultaneous binding to the antigen-specific T cell receptors (TCRs) on a T cell surface increases the sensitivity of the multimer technology. The antigen-specific T cells bound to the tetramer are detected with flow cytometry by the fluorophore bound to the streptavidin (SA).

Figure 1.

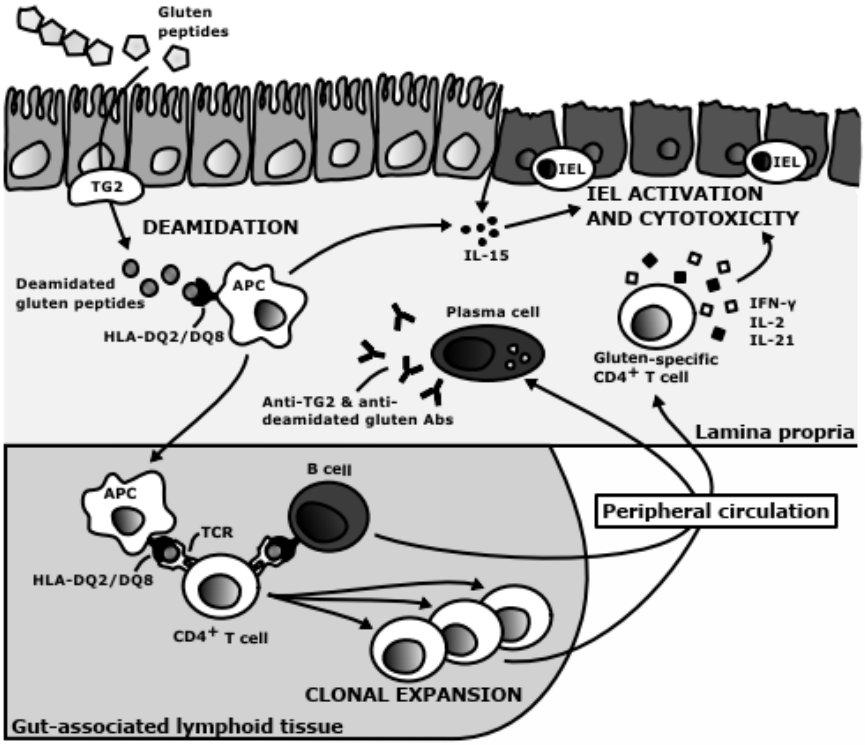

Gluten peptides
TG2
DEAMIDATION
IEL
IEL
IEL ACTIVATION AND CYTOTOXICITY
IL-15
Deamidated gluten peptides
APC
HLA-DQ2/DQ8
Plasma cell
Anti-TG2 & anti-deamidated gluten Abs
IFN-γ
IL-2
IL-21
Gluten-specific CD4+ T cell
Lamina propria
APC
B cell
TCR
HLA-DQ2/DQ8
CD4+ T cell
Peripheral circulation
CLONAL EXPANSION
Gut-associated lymphoid tissue

Figure 2

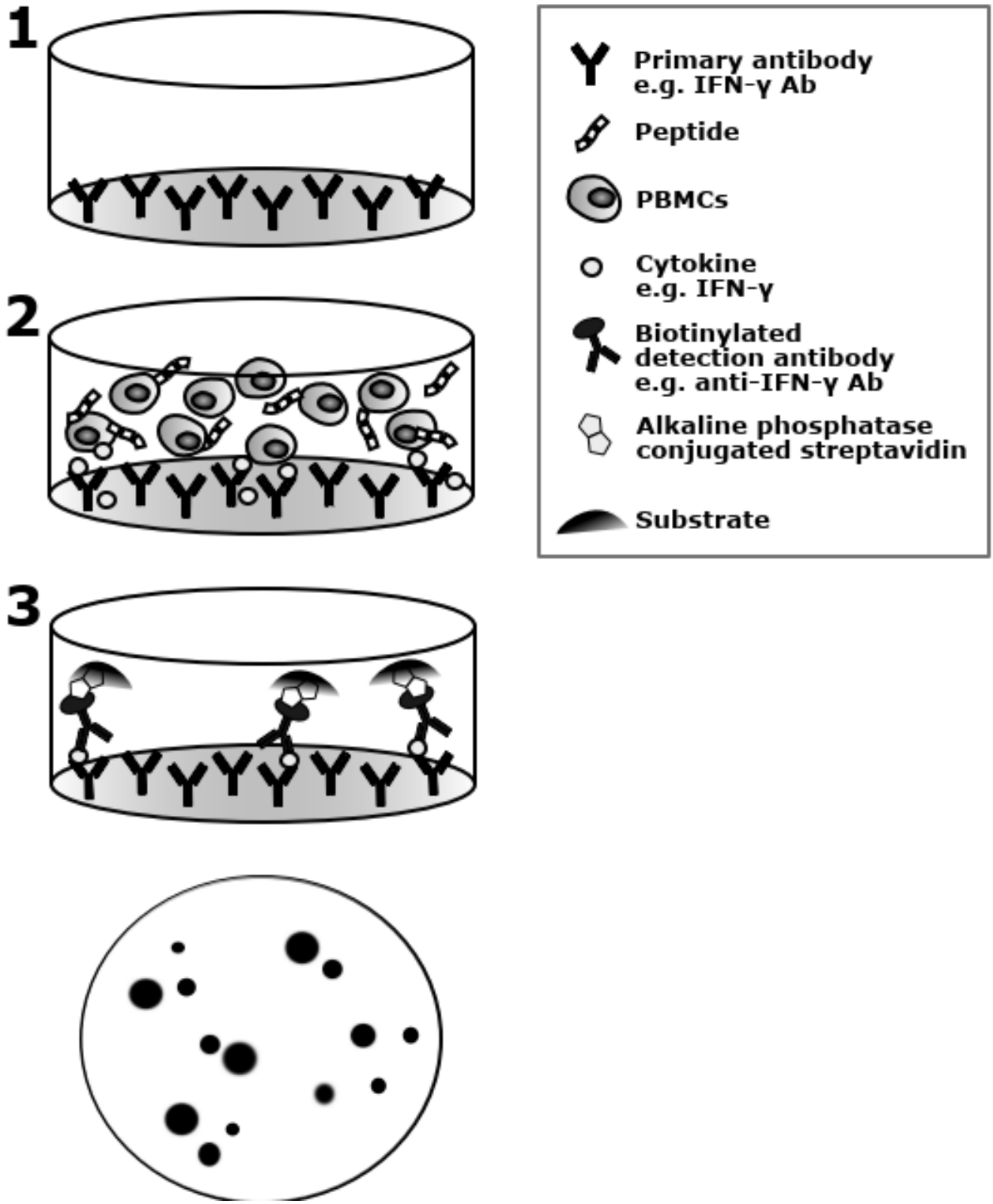
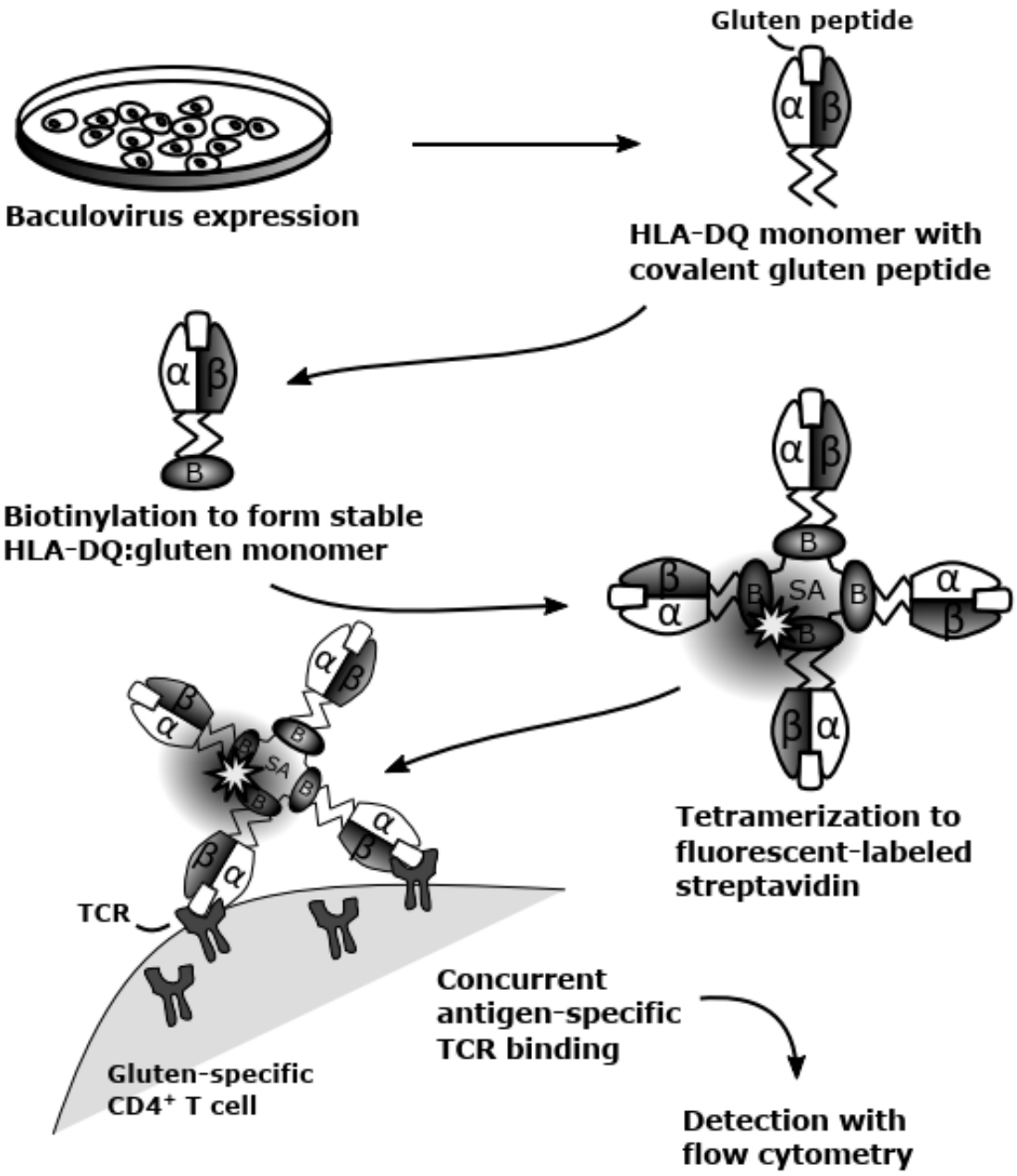

1
2
3
Primary antibody
e.g. IFN-γ Ab
Peptide
PBMCs
Cytokine
e.g. IFN-γ
Biotinylated
detection antibody
e.g. anti-IFN-γ Ab
Alkaline phosphatase
conjugated streptavidin
Substrate

Figure 3.


Gluten peptide
Baculovirus expression
HLA-DQ monomer with
covalent gluten peptide
Biotinylation to form stable
HLA-DQ:gluten monomer
Tetramerization to
fluorescent-labeled
streptavidin
TCR
Concurrent
antigen-specific
TCR binding
Gluten-specific
CD4+ T cell
Detection with
flow cytometry