GLUTEN-REACTIVE CD4$^+$ T CELLS IN CELIAC DISEASE

Doctoral thesis by

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2012

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1321

ISBN 978-82-8264-426-4

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

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Acknowledgements

This work was undertaken at the Department of Immunology, Rikshospitalet, during the period 2007-2011. I was financed by a three-year fellowship obtained via the Norwegian Coeliac Society from the Norwegian ExtraFoundation for Health and Rehabilitation (EXTRA funds). I also received support from the South-Eastern Norway Regional Health Authority. I am grateful to these funding bodies for giving me the opportunity to carry out my research. Thank you also to all co-authors who have contributed to this work.

I want to express my gratitude to my supervisor Ludvig M. Sollid for excellent guidance. His skillful tutoring, together with his ability to apply his broad knowledge in immunology to specific issues, have been invaluable. I have truly understood why "the devil is in the details" is his favorite idiom.

I would like to thank all the current and past members of the CD group. Thank you in particular to Marie for her impressive patience in introducing an ignorant MD to the lab world and to Bjørg for her kind help. Thank you to my triathlon competitors at IMMI (Rasmus, Roberto, Silja and Ole Audun) for pushing me to train harder, the T-cell people (Ingrid, Eirik, Asbjørn, Shuo-Wang and Stig) for understanding the frustration and joy involved in T-cell culture and my office mates (Elin, Ulrike, Lars-Egil, Jorunn, Muluneh and Øyvind) for great discussions, shared joy and frustration. Thank you to Melinda for her energy and informal co-supervision, Siri for vivid discussions and enthusiasm and Luka for his advices and friendship. Thank you also to Maria, Burkhard, Knut, Fleur, Omri, Anders, Hao, Axel, Astrid, Ann-Christin, Tahira, Louise and all those I might have forgotten to mention! Thank you also for broken ribs, head injuries and black eyes, all liberally given and received during "ballgames" in this period.

I would further like to thank my entire family for supporting me during this period of my life. Thank you to Bjarne for hiring Kristina (although he did not know that she would become my future girlfriend) and to Kristina for being strict, kind or adorable when necessary.

Abbreviations

APC	Antigen-presenting cells
Bcl6	B-cell lymphoma 6
CD	Celiac disease
CD3, CD4, CD8, CD28	Cluster of differentiation 3, 4, 8 and 28 respectively
CXCL	Chemokine (C-X-C motif) ligand
EATL	Enteropathy-associated T-cell lymphoma
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
FoxP3	Forkhead box P3
HLA	Human leukocyte antigen
IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
IL	Interleukin
MHC	Major histocompatibility complex
NK	Natural killer
RCD	Refractory celiac disease
ROR	RAR-related orphan receptor
Tfh	Follicular T helper cells
Th	T helper
TBX-21	T-box 21
TCC	T-cell clone
TCL	T-cell line
TCR	T-cell receptor
TG2	Transglutaminase 2
TGF	Transforming growth factor

List of papers

Paper I

Bodd M, Ráki M, Tollefsen S, Fallang LE, Bergseng E, Lundin KE and Sollid LM.
HLA-DQ2-restricted gluten-reactive T cells produce IL-21 but not IL-17 or IL-22.
Mucosal Immunology 2010(3):594-601

Paper II

Bodd M, Kim CY, Lundin KE and Sollid LM.
T-cell response to gluten in patients with HLA-DQ2.2 reveals requirement of peptide-MHC stability in celiac disease.
In press *Gastroenterology* 2012

Paper III

Bodd M, Tollefsen S, Bergseng E, Lundin KE and Sollid LM.
Evidence that HLA-DQ9 confers risk to celiac disease by presence of DQ9-restricted gluten-specific T cells.
In press *Human Immunology* 2012

Introduction

The immune system

The immune system is a complex system responsible for protecting the body from harmful microorganisms and malignant cells. In some circumstances the immune system can also have detrimental effects and lead to chronic inflammatory reactions. The immune system is traditionally divided into an innate and an adaptive component. Upon entrance of a pathogen, the innate immune system is triggered first. Innate immune cells like macrophages and neutrophils recognize conserved microbial structures or stress-induced self molecules using an invariant set of receptors (pattern recognition receptors).[1] Later on, the adaptive immune system, dependent on T and B lymphocytes is triggered. The adaptive response takes longer time to initiate but has memory, meaning that the response will be more rapid after encounter with the same antigen on the next occasion[2] (an antigen is a substance recognized by the adaptive immune system). In general, each lymphocyte expresses only a unique antigen receptor on its surface, but the total repertoire of antigens recognized by the pool of lymphocytes is enormous.

B lymphocytes recognize antigen directly using their cell-surface B-cell receptor. After activation, B lymphocytes proliferate and differentiate into plasma cells that secrete large amounts of the soluble version of the B-cell receptor (antibody), or into memory B cells. Memory B cells respond more rapidly after encounter with the same antigen.

Using their T-cell receptors (TCRs), T lymphocytes recognize antigen presented on specialized major histocompatibility complex (MHC) molecules, expressed on antigen-presenting cells (APC). Mature T cells that have not yet met their specific antigen (naïve T cells) recirculate between blood and peripheral lymphoid organs. APC engulfing antigen in the periphery, migrate to lymphoid organs where they can get in contact with naïve T cells. Activated naïve T cells will then proliferate and give rise to effector and memory T lymphocytes.

T lymphocyte maturation and activation

Progenitor T cells migrate from the bone marrow to the thymus where maturation of these cells (thymocytes) takes place. Maturation involves

rearrangements of TCR gene segments. This process is responsible for the great diversity of TCRs generated. Only thymocytes with a TCR that has sufficient affinity to self-peptide-MHC will survive (positive selection).[3] Thymocytes whose TCR has too strong affinity to self-peptide-MHC are eliminated (negative selection).[4] The avidity model proposes that the strength of signaling determines whether the cell undergoes negative or positive selection.[5]

After thymic maturation, a few T cells express a γδ heterodimer, while the majority express an αβ heterodimer. The αβ positive T cells express either the co-stimulatory molecule cluster of differentiation 4 (CD4) or CD8. Schematically, $CD8^+$ T cells are cytotoxic T cells and kill the cells they recognize, while $CD4^+$ T cells, also called T helper cells (Th), are master regulators of the adaptive immune response and important both for B-cell responses and $CD8^+$ T-cell responses.

To be activated, naïve T cells require additional signals in addition to TCR stimulation, provided by costimulatory molecules and cytokines. Only after perceiving a "danger" signal through their pattern recognition receptors do APC upregulate costimulatory molecules and become efficient activators of T cells.[6] In the absence of costimulation, TCR stimulation seems to induce a state of nonresponsiveness (anergy).[7]

$CD4^+$ T cell subsets

An important role of effector $CD4^+$ T cells is to produce cytokines. Cytokines are proteins released by cells that affect the behavior of cells that bear receptors for these proteins. The term "Interleukin" (IL) was originally chosen to name cytokines produced by and acting on leukocytes. The term now refers to cytokines with far more diverse functions and origins. The cytokine environment plays an essential role in deciding the differentiation of naïve $CD4^+$ T cells into various Th subsets[8] (Figure 1).

Since the discovery that $CD4^+$ T cells could be divided into Th1 and Th2 cells,[9] several subtypes of $CD4^+$ T cells have been uncovered. In addition to Th1 and Th2 cells, regulatory T cells, Th17 and follicular T helper cells (Tfh) are now recognized as distinct Th subsets. These Th subsets can be distinguished based on transcription factors, surface molecules and the cytokines produced.[8]

Th1 cells express the transcription factor T-box 21 (TBX-21)[10] and produce IFN-γ. This subset plays a role in protection against intracellular pathogens and was

until recently considered the main player in autoimmunity. Th2 cells express the transcription factor GATA-3,[11] produce IL-4, IL-5 and IL-13. This subset plays a role in protection against parasites and is particularly important in allergy. Regulatory T cells express the transcription factor forkhead box P3 (FoxP3),[12] produce IL-10 and transforming growth factor (TGF)-β, and have a dampening effect on the immune response. Th17 cells express the transcription factor RAR-related orphan receptor (ROR)γt (RORC in humans)[13] and produce IL-17A, IL-17F, IL-21 and IL-22. IL-17 seems particularly important in defense against Candida infections.[14] The last years' considerable research on Th17 cells have shown that not only Th1 cells but also Th17 cells play an important role in several chronic inflammatory disorders.[15] The relative contribution of the Th1 and Th17 subsets in various diseases is still controversial.[16] Tfh cells express the transcription factor B-cell lymphoma 6 (Bcl6),[17, 18] produce IL-21 and express the surface molecule chemokine (C-X-C motif) receptor 5 (CXCR5). Tfh cells are important in formation of germinal centers and regulate B-cell differentiation into plasma cells and memory B cells.[19] The relationship of Tfh cells to other Th subset is an ongoing debate.[19] Other subsets, such as the Th22[20, 21] and Th9 subsets[22] characterized by the production of IL-22 and IL-9 respectively, have also been proposed.

Importantly, recent findings suggest that T cells are more plastic than previously understood. While the concept of Th subsets remains useful, subsets do not appear to be end-stage phenotypes and the phenotype of Th cells is probably a dynamic process.[23]

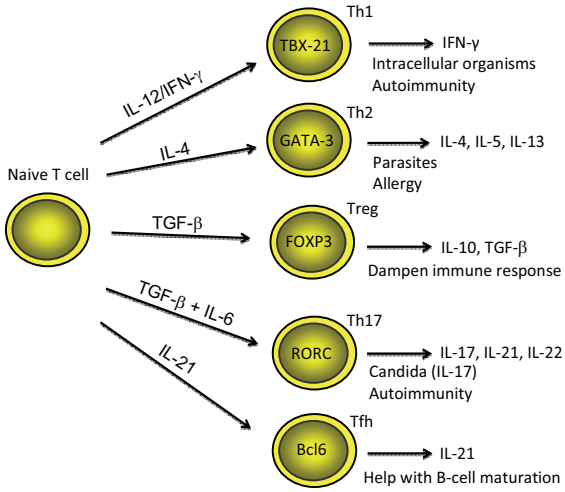


Figure 1: Simplistic representation of the different CD4$^+$ T cell subsets. *The figure shows the cytokine environment that favors the differentiation of Th subsets, the transcription factors expressed, cytokines produced and some selected functions.*

Th17 cytokines

Given our interest in cytokines produced by Th17 cells, I will discuss this in greater detail. As mentioned, Th17 cells produce the cytokines IL-17A, IL-17F, IL-21 and IL-22. These so-called Th17 cytokines exert a variety of different effects and are not produced exclusively by Th17 cells.

IL-17A and IL-17F have proinflammatory properties and act on a variety of cell types to induce the expression of other cytokines (tumor necrosis factor, IL-1β, IL-6, granulocyte macrophage colony-stimulating factor), chemokines (chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL8, CXCL10) and metalloproteases. IL-17A and IL-17F are key cytokines for the recruitment, activation and migration of neutrophils.[15] IL-17 is also produced by other cell types, such as γδ T cells,[24] neutrophils,[25] CD8$^+$ T cells[15, 26] and the newly described innate lymphoid cells.[27]

IL-21 has been shown to induce differentiation of naïve T cells to Th17 cells, in an autocrine fashion in synergy with TGF-β.[28-30] IL-21 is also produced by Tfh cells and in lower amounts by Th1 cells (in humans).[31] The cytokine also has multiple

other functions, and stimulates $CD8^+$ T cells, natural killer (NK) cells[32] and exerts important effects on B cells and plasma cells.[33]

IL-22 has both pro- and anti-inflammatory properties, possibly dependent on the inflammatory environment.[34]

IL-23 is important for maintenance of Th17 cells,[35] but also has important effects on non-T cells.[36]

The immune response in the gut

The induction of the adaptive immune response in the gut takes place in organized gut associated lymphoid tissue (consisting of Peyer's patches and isolated lymphoid follicles) or draining mesenteric lymph nodes. The effector sites of the gut are localized in the epithelium and lamina propria, where intrusion of pathogens mainly takes place. The intestinal mucosa is continuously exposed to a large amount of microorganisms and food antigens, not least because of the very large surface area of the gut. Thus, not very surprisingly, a large number of immune cells are found in the gut. In healthy individuals, these immune cells maintain a balance between tolerance to harmless agents (oral tolerance) and inflammation towards pathogenic agents. Clonal anergy, deletion and the generation of antigen-specific regulatory T cells are involved in the process of oral tolerance.[37] The maintenance of tolerance possibly relates to a tolerogenic microenvironment. In the steady state, epithelial cells seem to condition dendritic cells into a non-inflammatory state.[38, 39] Oral tolerance to the food antigen gluten is broken in celiac disease (CD), a disease that will be discussed in more detail later.

MHC

Function

The MHC complex is called human leukocyte antigen (HLA) complex in humans. The HLA complex is a gene-dense region localized to the short arm of chromosome 6. The classical HLA class I and class II genes encode surface molecules essential for antigen presentation to T cells. HLA class I molecules are expressed on all nucleated cells and usually present intracellular peptides to $CD8^+$ T cells. HLA class II molecules are constitutively expressed on professional APC, like B cells,

dendritic cells and macrophages and present mainly endocytosed antigen to $CD4^+$ T cells. Cross-presentation, which is presentation of endocytosed antigen on HLA class I molecules, can however also occur.[40] Loading of peptides onto HLA class II is facilitated by the chaperone molecule HLA-DM.

MHC class II structure

As CD is associated with certain MHC class II molecules, we will focus on MHC class II. MHC class II molecules are heterodimers of α and β glycoprotein chains. There are three pairs of HLA class II genes in humans, namely HLA-DR, -DQ and -DP. Both the α-chains and the β-chains of DQ and DP molecules are polymorphic. Thus for these molecules, αβ heterodimers can be encoded both in *cis* (on the same chromosome) and *trans* (on two different chromosomes), but pairing restrictions exist.[41] Since the first crystal structure of an MHC class II molecule was determined in 1993,[42] the structure of several MHC class II molecules bound to different peptides has been determined. The open ends of MHC class II molecules allow peptides to extend out of the binding groove. The peptide-binding groove of the MHC class II molecule is formed by a floor provided by a β-sheet and walls formed by two α helices. Two main principles have traditionally been described for binding of peptides to MHC class II.[43] First, hydrogen bonds are formed from conserved MHC residues to the peptide backbone. Second, polymorphic residues from MHC class II interact with peptide side chains at position P1, P4, P6 and P9 (and sometimes P7). The side chains of these so-called anchor residues interact with distinctive pockets in the binding groove.[43] A third, less commonly mentioned principle, is the formation of hydrogen-bonds between polymorphic residues of MHC and the peptide backbone.[44] Upon binding to HLA class II, the peptide assumes a conformation similar to a polyproline type II helix,[45] so that some peptide side chains are directed towards MHC (P1, P4, P6, P7 and P9) and others towards the TCR.

The HLA nomenclature

The beginning of the HLA allele name specifies the locus within the gene region (e.g, DQA1). The allele name is followed by an asterisk. Several numbers, each separated by a colon, follow (e.g, DQA1*05:01:01:01). The first number following the asterisk refers to the allele group and is often similar to the serotype.

The second number refers to specific alleles encoding for proteins with different sequences. The third number refers to alleles that differ by synonymous nucleotide substitutions. Alleles differing in the fourth number have sequence polymorphisms in introns or 5' and 3' untranslated regions.[46]

HLA molecules can be named according to the HLA alleles by which they are encoded. This is generally determined by genomic typing. HLA molecules can also be named by their β chain, followed by their α chain, separated by a dot (e.g, DQ2.5). The strong linkage disequilibrium (see below) between HLA-DR and -DQ often enables the HLA-DR and -DQ genotypes to be determined with a fairly high certainty by serologic HLA-DR and -DQ typing.

The nomenclature can be exemplified by HLA-DQ2 (DQ2) (Figure 2). The antibody which initially defined the DQ2 molecules recognizes the β chain encoded by DQB1*02 alleles. Three DQ2 heterodimers can actually be distinguished: the *cis*-encoded DQ2.5, the *trans*-encoded DQ2.5 and the *cis*-encoded DQ2.2. DQ2.5 is encoded in *cis* on the DR3-DQ2 haplotype, or in *trans* on the DR7-DQ2/DR5-DQ7 haplotypes. DQ2.2 is encoded in *cis* on the DR7-DQ2 haplotype.

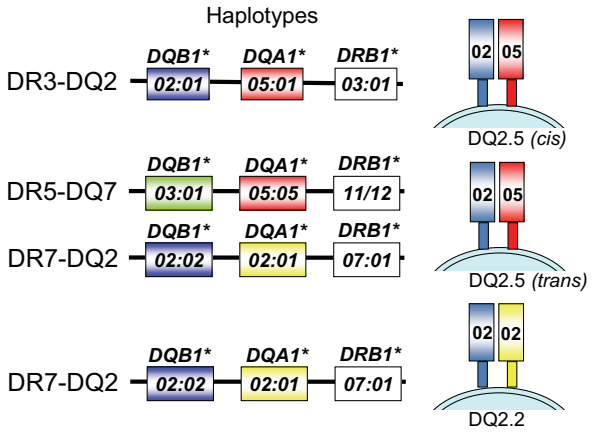


Figure 2: HLA nomenclature, exemplified with HLA-DQ2 *(Adapted from Abadie et al.*[47]*).*

MHC and disease association

Two aspects of the MHC region are worth mentioning. First, the region is highly polymorphic.[48] Second, many alleles show very strong linkage disequilibrium

(non-random association of alleles). This is a major problem when trying to identify disease-predisposing MHC genes. Many autoimmune or chronic inflammatory diseases, such as diabetes, rheumatoid arthritis, ankylosing spondylitis and narcolepsy, show an association with certain HLA types.[49] The differential predisposition to diseases of various HLA molecules is believed to be mainly related to their different peptide-binding properties. This may affect the TCR repertoire generated during thymic selection or the selection of peptides presented to T cells in the periphery. One disease that demonstrates a strong MHC association is CD.

Celiac disease

Clinical features

CD is a chronic inflammatory disease of the small intestine triggered by gluten proteins from wheat, barley and rye. Malabsorption can develop and may lead to weight loss, vitamin deficiencies, osteoporosis and anemia.[50] However, as shown by screening studies, many patients have mild or no symptoms and remain undiagnosed.[51] The prevalence of CD is about 1% in general western populations[52] and is possibly increasing.[53] CD can present at all ages. The initial detection of CD is usually based on the presence of antibodies specific for the enzyme transglutaminase 2 (TG2). A definite diagnosis has up to now been based on histological findings in small intestinal biopsies.[54] However, according to recently launched criteria, CD can be diagnosed in children without biopsy assessment.[55] The only current available treatment of CD is a life-long gluten-free diet, which is efficient in the vast majority of patients. A rare complication is refractory CD (RCD), with persistent malabsorptive symptoms and villous atrophy despite strict adherence to a gluten-free diet. RCD can be categorized into RCD1 and RCD2.[56] The latter, characterized by abnormal clonal intraepithelial lymphocytes gives a high risk of enteropathy-associated T-cell lymphoma (EATL). CD patients seem to have an increased risk of cancer (in particular lymphoma) and premature death, that may possibly be reduced by adhering to a strict gluten-free diet.[57]

Histological changes

The histological changes of the celiac lesion were initially graded by Marsh,[58] a grading later modified by Oberhuber et al[59] to grades ranging from Marsh 0 to 3.

According to Oberhuber, Marsh 0 refers to a normal histology. Marsh 1 refers to an increased number of intraepithelial lymphocytes. Marsh 2 refers to crypt hyperplasia and an increased number of intraepithelial lymphocytes. Marsh 3a, 3b and 3c refers to villous atrophy of some degree. Although, not very strong, there seems to be a certain correlation between clinical features and histological changes.[60, 61]

Genetics

The high concordance rate between monozygotic twins (70-75%)[62] suggests a strong genetic factor in CD that can be estimated to account for 50-90% of the variance in liability to the disease.[63]

The genetic loci identified, of which HLA is the most important, can account for over 50% of the genetic variance in CD.[64] The individual non-HLA genes identified so far, together probably account for only up to 14% of the genetic variance in CD (assuming a heritability of 50%).[64] Some of the missing heritability is believed to be caused by highly penetrant rare variants, common variants with small effects or possibly epistatic interactions between risk genes.[47] Interestingly, many of the predisposing genes identified in chronic inflammatory diseases (including CD) are shared, suggesting common biological mechanisms.[65]

The great majority of CD patients express DQ2.5, which can be encoded in *cis* or in *trans* (see HLA nomenclature above).[66] The mature *cis*- and *trans*-encoded DQ2.5 proteins differ only in one residue localized membrane proximally and thus unlikely to affect peptide-binding. The risks for CD of *cis*- and *trans*-encoded DQ2.5 are indeed similar.[47] Most of the patients not expressing DQ2.5 express DQ8 or carry either the α-chain or the β-chain variants of DQ2.5 (Table 1).[67] The patients expressing only the β chain of DQ2.5 express DQ2.2, a molecule highly homologous to DQ2.5. The relative risk of developing CD for patients expressing DQ2.2 without any other predisposing HLA genes is much lower than that of CD patients expressing DQ2.5. A possible reason for this was recently suggested[44] (see discussion). This was further examined in Paper II, in which we studied the gluten T-cell response in CD patients with DQ2.2 not expressing other CD predisposing HLA molecules.

Another observation is that, in contrast to many HLA-DQ molecules, the CD predisposing HLA molecules DQ2 and DQ8 both lack aspartic acid (alanine instead) in position 57 of the β chain. HLA-DQ9.3 (hereafter DQ9 unless specified differently) differs from DQ8 only in this particular position and is not considered associated with

CD.[68] In paper III, however, we identified gluten-reactive T cells restricted by DQ9 in a CD patient, thus suggesting that DQ9 may be involved in the T-cell response to gluten in CD.

Table 1: **Description and naming of HLA-DQ molecules that are associated with celiac disease and which are used as antigen presenting elements for CD4$^+$ T cells of celiac disease patients *(From Sollid et al.[69]).***

HLA-DQ molecule	Encoded by DQA1*	Encoded by DQB1*	Risk	Expression in *cis* or *trans*	Part of common *cis* haplotype
HLA-DQ2.5	05	02	High	*cis, trans*	DR3DQ2
HLA-DQ2.2	02	02	Low	*cis, (trans)*	DR7DQ2
HLA-DQ2.3	03	02	Likely low †	*trans, (cis)* §	
HLA-DQ7.5	05	03:01	Very low	*cis (trans)*	DR5DQ7
HLA-DQ8	03	03:02	Low	*cis*	DR4DQ8
HLA-DQ8.5	05	03:02	Likely low †	*trans, (cis)* §	

† *Risk for celiac disease has not been established in population studies.*

§ *Molecule can also be encoded in cis on some rare haplotypes.*

Transglutaminase 2

With the discovery in 1997 that the, until then, elusive endomysial auto-antigen was TG2,[70] a role of this enzyme in the pathogenesis of CD was rapidly uncovered. It was shown that TG2 increases the immunogenicity of gluten peptides, by catalyzing the conversion of glutamine to glutamic acid, in a process called deamidation.[71, 72] The introduction of negative charges in certain positions increases the binding affinity of gluten peptides to the disease associated DQ2 and DQ8 molecules, thereby increasing T-cell activation.[71, 73] TG2 also has the ability to cross-link proteins, in a process called transamidation. The specificity of TG2 is affected by C-terminal proline (P) residues and TG2 typically recognizes glutamine (Q) residues in the Q-X-P sequence (X is any amino acid).[74, 75]

The inflammatory reaction

The inflammatory reaction of CD involves cells of both the innate and adaptive immune system. The adaptive immune response is perhaps best understood. Gluten-reactive CD4$^+$ T cells are likely to be essential in the pathogenesis of CD as such cells are isolated only from the small intestine of CD patients but not healthy

controls[76], and are restricted by the disease predisposing DQ2 and DQ8 molecules.[77, 78] The cytokine production by gluten-reactive $CD4^+$ T cells is dominated by IFN-γ, although several other cytokines are produced, including IL-21 (Paper I), IL-4[79] and IL-10.[80]

$CD8^+$ T cells also seem to be involved in the pathogenesis of CD. Two studies have suggested that gluten-specific $CD8^+$ T cells can be found in the small intestine of CD patients.[81, 82] Nevertheless it is likely that the main involvement of $CD8^+$ T cells is mediated through their expression of innate receptors. Increased IL-15 in CD induces increased expression of NK cell receptors on these $CD8^+$ T cells, which can then recognize non-classical MHC molecules on the surface of epithelial cells and induce apoptosis of epithelial cells.[83-85]

The presence of autoantibodies against the enzyme TG2 is an autoimmune feature of CD. A high frequency of TG2-specific plasma cells can be visualized directly in the celiac lesion.[86] The dependency of a gluten-containing diet for production of TG2-specific antibodies, and the absence of TG2-specific T cells in the celiac lesion have led to the hapten-carrier model.[87] The model proposes that TG2 transamidates gluten peptides to itself (or to another surface molecule on the cell). This would facilitate uptake of gluten peptide by TG2-specific B cells and enable T-cell help by gluten-specific T cells. Whether TG2-specific B cells contribute to disease development is still unknown. Gliadin-specific antibodies are also commonly found in CD and some of the epitopes recognized have been identified.[88]

The possible innate effects of gluten are less well understood. While several gluten peptides with alleged innate effects have been reported,[89] one particular α-gliadin peptide, the p31-43 peptide has been most studied. Among several effects, this peptide interestingly induces IL-15.[84, 90, 91]

HLA tetramer staining of antigen-specific T cells

The development of MHC-peptide multimers has greatly facilitated the detection of antigen-specific T cells.[92] MHC-peptide multimers generally consist of up to four MHC-peptide complexes, multimerized on streptavidin (conjugated to a fluorochrome for identification) to overcome the low affinity of the TCR-peptide/MHC interaction. Although the valency of these MHC multimers is variable (most will usually be trimers or tetramers),[93] they are commonly referred to as MHC

tetramers. We will continue using the term “HLA tetramer”. Gluten-reactive T cells can be visualized directly in peripheral blood after a short-term gluten challenge of treated $DQ2^{+}$ CD patients using HLA-DQ2 gluten-peptide tetramers.[94] Such T cells can also be visualized in peripheral blood of untreated and treated patients, using a bead-based enrichment protocol (Christophersen et al., unpublished observations) and directly in biopsy material of CD patients (unpublished data).

The cereal antigen gluten

Gluten is the mass remaining when washing dough to remove starch and other water soluble constituents. In practice the term refers only to the remaining proteins (accounting for around 80% of this mass).[95] Historically, gluten referred only to wheat proteins. The term is now being used to include also proline and glutamine-rich proteins from barley, rye and oat. Wheat gluten can be divided into the alcohol soluble gliadins and the alcohol insoluble glutenins. Glutenins can further be subdivided into low and high-molecular weight glutenins. Gliadins have traditionally been subdivided into α-, β-, γ-, and ω-gliadins, according to their electrophoretic mobility. Sequence similarities have however later revealed that α- and β- gliadins belong to one group.[95] As common wheat (*Triticum aestivum*) is hexaploid and because of extensive gene duplication, a high number of highly homologous proteins are found in common wheat. The gluten proteins of barley, rye and oat are called hordeins, secalins and avenins respectively.[96] The high content of proline makes gluten proteins highly resistant to digestion by enzymes in the digestive tract[97, 98] and influences the selective targeting of glutamines by the enzyme TG2.

Gluten T-cell epitopes

CD patients react to sequences in wheat (mostly sequences in gliadin, but also in glutenin), barley and rye.[99] These sequences of barley and rye are often homologous to those found in wheat.[100, 101] Although oats are generally considered safe for CD patients,[102] some patients are apparently intolerant,[103] and oat-specific T cells have been isolated from small intestinal biopsies of CD patients.[104]

The nomenclature of gluten epitopes recognized by CD patients has been inconsistent and different groups have used different names for the same epitopes. A process of standardizing the nomenclature of gluten T-cell epitopes is ongoing[69] and I

will use this nomenclature (Table 2). Since the initial discovery of the first gluten epitope in 1998[105] and later the DQ2.5-glia-α1a and DQ2.5-glia-α2 epitopes,[73] many gluten epitopes have been described.[106-109] The DQ2.5-glia-α1, DQ2.5-glia-α2, DQ2.5-glia-ω1, DQ2.5-glia-ω2, DQ2.5-hor-1 and DQ2.5-sec-1 are dominant DQ2.5-restricted gluten epitopes.[101, 107] Three DQ8-restricted gluten epitopes have also been described, of which the dominant epitope is the DQ8-glia-α1.[110-112] We recently also identified the first DQ2.2-restricted gluten epitope, which appears to be a dominant DQ2.2-restricted gluten epitope (Paper II).

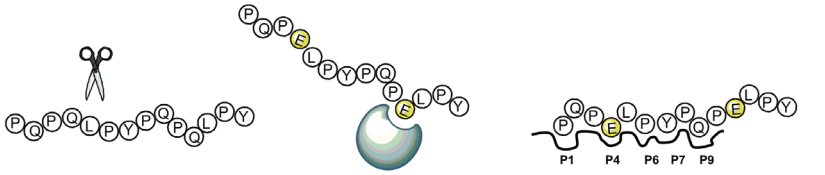
Table 2: **Overview of DQ2.5, DQ2.2 and DQ8-restricted gluten T-cell epitopes described to date *(From Sollid et al.).***

Epitope†	Previous names	Peptide-binding register 1	2	3	4	5	6	7	8	9
DQ2.5 restricted epitopes										
DQ2.5-glia-α1a	DQ2-α-I, α9	P	F	P	Q	P	E	L	P	Y
DQ2.5-glia-α1b	DQ2-α-III	P	Y	P	Q	P	E	L	P	Y
DQ2.5-glia-α2	DQ2-α-II, α2	P	Q	P	E	L	P	Y	P	Q
DQ2.5-glia-α3	glia-α20	F	R	P	E	Q	P	Y	P	Q
DQ2.5-glia-γ1	DQ2-γ-I	P	Q	Q	S	F	P	E	Q	Q
DQ2.5-glia-γ2	DQ2-γ-II, γ30	I	Q	P	E	Q	P	A	Q	L
DQ2.5-glia-γ3	DQ2-γ-III	Q	Q	P	E	Q	P	Y	P	Q
DQ2.5-glia-γ4a	DQ2-γ-IV	S	Q	P	E	Q	E	F	P	Q
DQ2.5-glia-γ4b	DQ2-γ-VIIc	P	Q	P	E	Q	E	F	P	Q
DQ2.5-glia-γ4c	DQ2-γ-VIIa	Q	Q	P	E	Q	P	F	P	Q
DQ2.5-glia-γ4d	DQ2-γ-VIIb	P	Q	P	E	Q	P	F	C	Q
DQ2.5-glia-γ5	DQ2-γ-VI	Q	Q	P	F	P	E	Q	P	Q
DQ2.5-glia-ω1	DQ2-ω-I	P	F	P	Q	P	E	Q	P	F
DQ2.5-glia-ω2	DQ2-ω-II	P	Q	P	E	Q	P	F	P	W
DQ2.5-glut-L1	glutenin-17	P	F	S	E	Q	E	Q	P	V
DQ2.5-glut-L2	glutenin-156	F	S	Q	Q	Q	E	S	P	F
DQ2.5-hor-1	Hor-α9, Hα9	P	F	P	Q	P	E	Q	P	F
DQ2.5-hor-2	Hor-α2, Hα2	P	Q	P	E	Q	P	F	P	Q
DQ2.5-hor-3	Hor-I	P	I	P	E	Q	P	Q	P	Y
DQ2.5-sec-1	Sec-α9, Sα9	P	F	P	Q	P	E	Q	P	F
DQ2.5-sec-2	Sec-α2, Sα2	P	Q	P	E	Q	P	F	P	Q
DQ2.5-ave-1a	Av-α9A	P	Y	P	E	Q	E	E	P	F
DQ2.5-ave-1b	Av-α9B, 1490	P	Y	P	E	Q	E	Q	P	F
DQ2.2 restricted epitopes										
DQ2.2-glut-L1	glutenin-17	P	F	S	E	Q	E	Q	P	V
DQ8 restricted epitopes										
DQ8-glia-α1	DQ8-α-I	E	G	S	F	Q	P	S	Q	E
DQ8-glia-γ1a	DQ8-γ-Ia	E	Q	P	Q	Q	P	F	P	Q
DQ8-glia-γ1b	DQ8-γ-Ib	E	Q	P	Q	Q	P	Y	P	E
DQ8-glut-H1	HMW-glutenin	Q	G	Y	Y	P	T	S	P	Q

†*In the names of the epitopes, the following short terms denote the proteins of origin: glia-α: α-gliadin; glia-γ: γ-gliadin; glia-ω: ω-gliadin; glut-L: low molecular weight glutenin; glut-H: high molecular weight glutenin; hor: hordein; sec: secalin; ave: avenin. Glutamate residues (E) formed by TG2-mediated deamidation which are important for recognition by T cells are shown in bold. Additional glutamine residues also targeted by TG2 are underlined.*

What determines whether a gluten peptide can initiate a T-cell response?

Several factors determine whether a gluten peptide can initiate a T-cell response (Figure 3). Important factors are (1) proteolytic stability, (2) sustained binding to MHC and (3) targeting by TG2. (1) Proteolytic stability of gluten peptides is maintained by a high content of proline.[97, 98] The amount of protein containing the antigenic peptide prior to digestion is also likely to be important. (2) Sustained binding of peptides to MHC is essential to enable priming of naïve T cells in peripheral lymph nodes.[44, 113] As previously mentioned, two principles are particularly important for peptide binding to MHC. The first principle is hydrogen bond formation between conserved MHC residues and the main chain of the peptide. Prolines, which are particularly common in gluten peptides, would eliminate the hydrogen bonds from MHC to the peptide main chains (in P2, P4, P6 and P9). Hence, in DQ2, prolines are generally not present in P2, P4, P7 and P9 (the absence of proline at P7 instead of P6 possibly relates to pocket preferences).[109, 114] A similar pattern is seen for DQ8, with the difference that proline is penalizing also in P1.[111] The second principle is side chain interaction with the HLA pockets. DQ2 has a preference for a negative charge in P4, P6 and P7.[115, 116] DQ8 has a preference for a negative charge in P1 and P9.[117, 118] (3) These negative charges are introduced by the conversion of glutamine residues to glutamic acid by TG2. Peptide fragments harboring T-cell epitopes are generally good targets of TG2.[119]



Resistance to intestinal proteolysis (proline-rich regions)

Selective force by TG2

Sufficient affinity to DQ2 or DQ8
-Pocket interactions
(negative charges in certain positions)
-Main chain interactions
(abscence of proline in certain positions)

Figure 3: Selection of gluten epitopes *(modified from Sollid et al.[120]).*

Aim of thesis

Gluten-reactive $CD4^+$ T cells are key players in CD. We aimed to study the phenotype of these cells and to investigate how HLA shapes the specificity of the T-cell response to gluten in CD.

At the time of initiation of this project, Th17 cells were a newly described T-cell subset. Th17 cells were shown to be associated with chronic inflammation in several mice models. We therefore aimed to investigate whether some gluten-reactive T cells had the phenotype of Th17 cells.

DQ2.2 is highly homologous to DQ2.5, but gives a much lower risk for CD. The $CD4^+$ T cell response to gluten in DQ2.5 patients is well characterized, while the one in DQ2.2 patients had not been previously investigated. We wanted to study the gluten T-cell response in patients expressing DQ2.2 but no other CD predisposing HLA molecules.

Finally, we serendipitously observed that $CD4^+$ T cells from one CD patient recognized gluten peptides presented on DQ9. DQ9 has previously not been considered a risk factor for CD. Interestingly DQ9 differs from the CD-associated DQ8 molecule in one particularly MHC residue (β57). This residue is considered essential for the association of DQ8 with CD. The aim was to understand more about the role of DQ9 and thereby the β57 polymorphism for predisposition to CD.

Methodological considerations

Patients

For these studies, we recruited adult patients with a CD diagnosis according to the American Gastroenterological Association Institute's recommendations.[54] The patients underwent endoscopy and duodenal biopsies were sampled. The studies were approved by the regional ethics committee. Fresh small intestinal biopsies enable the culture of living cells obtained from the site of inflammation. Some patients underwent a 3 days oral gluten challenge before we collected blood samples on day 6.[121] This method enables direct visualization of gluten-specific T cells in the peripheral blood of CD patients.[94] While the gluten challenge is generally well tolerated, some patients do experience clinical symptoms.

T-cell culture

Most T-cell lines (TCLs) were generated by incubating duodenal biopsies with gluten overnight. The biopsies were chopped into pieces the next day and the TCLs subsequently expanded in a polyclonal manner.[122] T-cell clones (TCCs) were generated by limiting dilution. Importantly, although the antigen specificity is preserved, the phenotype of $CD4^+$ T cells has been shown to be plastic after long term *in vitro* culture.[23] In particular, Th17 cells can switch to Th1 cells.[123, 124] We controlled for this by assessing the cytokine production of $CD4^+$ T cells with unknown specificity, to ascertain that the culture conditions enabled maintenance of the Th17 phenotype (Paper I).

T-cell proliferation assays

To examine the reactivity of $CD4^+$ T cells we used a ^3H- thymidine incorporation assay. In this assay, antigen-specific T cells are identified by proliferation after presentation of antigen by APC. Differential requirements for proliferation of certain $CD4^+$ T cell subsets compared with others (such as regulatory T cells)[125] could introduce a potential bias.

To investigate the T-cell response to gluten in CD patients (Paper II and III), we used Epstein-Barr virus (EBV) transformed B-cell lines as APC. As establishment

of autologous EBV B-cell lines takes time, we instead often used HLA homozygous EBV-transformed B-cell lines obtained through HLA workshops. Cell lines matched for the patient's most CD relevant HLA haplotype were used as APC. Thus, presentation of gluten peptides by less CD relevant HLA molecules was not systematically assessed. In some cases, this could have lead to interesting findings gone unreported. In Paper II, for instance, two patients also expressed the DQ2.3 transdimer (DQA1*03/DQB1*02) which has been shown to be able to present gluten peptides.[111] This was not investigated further in this paper.

As complex cereal antigen in paper II, we used a chymotrypsin digest of wheat gluten. We did not examine the T-cell responses to barley, rye or oat, although T-cell epitopes would possibly be expected to be found in also these cereals. Such sequences are often homologous between cereals.[100, 101] Noteworthy however, we found no homologous sequences to the dominant DQ2.2-restricted epitope (DQ2.2-glut-L1) in barley or rye (paper II).

Traditional T-cell proliferation assays do not take into account the affinity of peptide to MHC (see discussion). Consequently, the peptide sequence inducing the initial T-cell activation *in vivo* could be a different (probably homologous) sequence from the one inducing activation *in vitro.* This is in particular true for the antigen gluten, as many highly homologous sequences are present.

ELISA/Bioplex analysis

We investigated the cytokine production of gluten-reactive T cells after stimulation. Of notice, various modes of stimulation may lead to different cytokine production profiles. For instance, stimulation with anti-CD3/anti-CD28 may lead to a much higher IL-10 production than stimulation with phorbol-12-myristate-13-acetate/ionomycin (Ingrid Olsen, unpublished observations). We therefore stimulated the T cells in various ways before examining cytokine production by enzyme-linked immunosorbent assay (ELISA) or Bioplex analysis. Defining a high and low cytokine producer is not straightforward and including positive and negative controls in the assays is important.

Peptide-binding assays

The relative peptide-binding affinity was assessed in a competitive binding assay by measuring IC_{50} values (half maximal inhibitory concentration). The IC_{50} value measures the amount needed of the peptide of interest to inhibit 50% of the binding of a biotinylated indicator peptide. Briefly, DQ molecules were captured (using anti-DQ antibodies) from lysates of homozygous EBV-transformed B-cell lines expressing the DQ molecule of interest. The indicator peptide was then incubated together with the peptide of interest.[126] Of notice, this assay is suited to compare the relative binding affinity of peptides to a given HLA molecule, but not to different HLA molecules.

Peptide binding to MHC was also assessed using two different peptide-MHC off-rate assays. These methods can be used to compare binding of peptides to different MHC molecules. In the first method, recombinant MHC molecules are loaded with a fluorescently labeled peptide. Dissociation of this peptide is then measured.[44] In the second method, APC are loaded with peptide before washing away free peptide. Proliferation of antigen-specific T cells at different time points after wash-off is used to estimate off-rate.[44] This second method is less accurate and a TCC specific to the actual peptide-MHC complex is needed. On the other hand, generation of recombinant MHC molecules is not required.

Flow-cytometry

Flow-cytometry was used to identify and characterize $CD4^+$ gluten-reactive T cells. The advantage of flow-cytometry is its ability to detect and characterize rare cell populations accurately. The method is sensitive to improper gating and artifacts. The use of appropriate controls is therefore essential.

We used DQ2-gliadin tetramers (see Introduction) to visualize gluten-specific T cells in intestinal TCLs or in peripheral blood. The soluble DQ2 molecules were loaded with a peptide harboring the DQ2-glia-α1a, the DQ2-glia-α2 epitope or with an endogenous peptide (serving as negative control). T cells binding these tetramers can be identified by flow-cytometry and further characterized. The identification of gluten-specific T cells using this method is highly specific, but enables the identification of T cells specific to only defined gluten T-cell epitopes. Further,

despite being peptide-MHC specific, some TCCs might not stain, likely because of a high off-rate of some TCRs to peptide-MHC.[127]

The generation of recombinant DQ2 molecules currently requires the synthesis of new protein for each DQ2-peptide complex of interest. The reason for this is that the peptide must be covalently linked to the β chain of DQ2 for the stability of the molecule to be maintained. We are currently trying to develop a method that enables exchange of peptide on the surface of DQ2. The idea is to cleave off the covalently bound peptide, and then enzymatically attach a peptide of interest using the enzyme Sortase A (Bergseng et al, ongoing project).

Identification of gluten T-cell epitopes

Gluten is very complex and we do not have access to a panel of gluten peptides covering all known gluten proteins. Instead, to identify gluten epitopes, we used the strategy of reducing the complexity of a gliadin digest that stimulated the relevant TCCs. Fractionation of the gliadin digest was performed in two dimensions, namely first by size (gelfiltration chromatography) and then by hydrophobicity (reversed-phase high performance liquid chromatography). T-cell stimulating fractions were tested by mass-spectrometry. Peptide sequences identified by mass-spectrometry that were common to all T-cell stimulating fractions were synthesized and tested for their T-cell stimulatory capacity. The disadvantage of the method is that sufficient amounts of a peptide must be present after fractionation to enable detection by mass spectrometry. Many highly homologous sequences in gliadin proteins also make a sufficient separation of peptides difficult.

Summary of papers

Paper I

In this study we investigated whether gluten-reactive $CD4^+$ T cells produce Th17 cytokines. We examined gluten-reactive T cells isolated from the small intestine or visualized in peripheral blood of CD patients. We found that gluten-reactive T cells do not seem to produce the typical Th17 cytokines IL-17 or IL-22. Interestingly however, we found that the gluten-reactive T cells produce the pro-inflammatory cytokine IL-21.

Paper II

A high off-rate of the common DQ2.5-restricted gluten epitopes was found on DQ2.2.[44] Consequently, we hypothesized that T cells from CD patients with DQ2.2 without other HLA risk genes would not recognize these common DQ2.5-restricted epitopes, but epitopes showing sustained binding to DQ2.2. We investigated the $CD4^+$ T-cell response to gluten in such patients. We identified a dominant epitope that was not commonly recognized by DQ2.5 CD patients without DQ2.2. The epitope showed sustained binding to DQ2.2. We investigated the basis for stable binding of the dominant DQ2.2-restricted epitope to this molecule. Our findings underscore the importance of kinetic stability of peptide-MHC in determining T-cell responses in CD.

Paper III

DQ9 differs from DQ8 only in position β57. This position has been suggested to be critical for the association of DQ8 to CD and type 1 diabetes. DQ9 has been claimed not to be associated with CD. In this paper we investigated the gluten response in a DR7-DQ2/DR9-DQ9 heterozygous CD patient. Unexpectedly, we found many gluten-reactive T cells restricted by DQ9. We characterized the DQ9-restricted gluten response in this patient in detail and identified a dominant DQ9-restricted epitope. We further investigated the binding of gluten peptides to DQ8 and DQ9. The findings suggest that DQ9 can be involved in the pathogenesis of CD.

Discussion

Functional difference between DQ2.2 and DQ2.5

DQ2.2 and DQ2.5 are homologous molecules and differ only in ten amino acids in their membrane distal domains. The binding motifs of DQ2.2 and DQ2.5 are also fairly similar.[128] The question was how this small difference could translate into the large risk difference observed between DQ2.2 and DQ2.5 for CD. In particular it seemed paradoxical that despite the large risk difference, DQ2.2 expressing APC could present peptide *in vitro* to gluten-reactive T cells isolated from CD patients expressing DQ2.5.[109, 126] Fallang and Bergseng[44] recently proposed that the difference in risk relates to a polymorphism at position 22 of the α chain. Tyrosine in α22 of DQ2.5 is necessary to maintain a hydrogen-bonding network to the main chain of the peptide. In DQ2.2, this tyrosine is changed to a phenylalanine, which is unable to form this hydrogen bonding network. The result is that common DQ2.5-restricted gluten epitopes show less sustained binding to DQ2.2 than to DQ2.5. This difference would explain the risk difference between DQ2.2 and DQ2.5.[44]

As sustained binding of peptides to MHC was hypothesized to be critical, we predicted that T cells from CD patients with DQ2.2 would not recognize the common epitopes recognized by T cells of DQ2.5 patients. Instead epitopes which bound well to DQ2.2 would be recognized. To investigate this, we studied the gluten T-cell response in CD patients carrying DQ2.2 but no other HLA risk genes (Paper II).

T-cell response in DQ2.2 patients

DQ2.2 gives a much lower risk than DQ2.5 for CD, but up to 5% of CD patients express DQ2.2 without other predisposing HLA genes.[67] Interestingly, no difference in disease severity was observed between DQ2.2 and DQ2.5 patients.[60] We found that DQ2.2 patients without other predisposing HLA genes do have gluten-reactive $CD4^+$ T cells in their small intestine (Paper II). Most gluten-reactive T cells from such patients recognized an epitope, named DQ2.2-glut-L1. This epitope has previously been described in the literature as a DQ2.5-restricted gluten epitope (glut-17). Noteworthy, we only found T-cell responses towards this epitope in DQ2.5 patients who also expressed DQ2.2. TCCs reactive to this epitope have only been isolated from one CD patient expressing DQ2.5, but not DQ2.2,[106] so this must be rare. Stronger binding of the DQ2.2-glut-L1 epitope to DQ2.2 than to DQ2.5 can most likely explain this observation. Further, in DQ2.2 patients that did not express DQ2.5, we found no T cells reactive to the common DQ2.5-restricted gluten epitopes. Thus, the stability of different peptides to DQ2.2 and DQ2.5 controls the generation of T-cell responses in DQ2.2 and DQ2.5 patients. This supports the hypothesis we put forward. Sustained antigen binding to MHC seems critical in determining T-cell responses in CD and hence possibly in setting the threshold for disease initiation.

Mechanism for sustained binding of DQ2.2-glut-L1 to DQ2.2

We found that a serine in P3 of the dominant DQ2.2-restricted epitope is important for sustained binding to DQ2.2. We modeled the binding of the DQ2.2-glut-L1 epitope to DQ2.2. The model suggests that the side chain of this peptide (in P3) can interact with DQ2.2. This interaction can possibly compensate for the loss of the hydrogen-bonding network from α22 to the main-chain of the peptide that is lost in DQ2.2. The absence of proline in P3 was proposed to be essential for efficient antigen presentation by DQ2.2,[126] but this was contended in a later study.[109] Our findings suggest that the preference of serine at P3, rather than the absence of proline is essential for strong binding of the DQ2.2-glut-L1 epitope to DQ2.2. A preference of a serine in P3 however was not seen for DQ2.5 and this possibly relates to space constraints imposed by the tyrosine in α22.

Why does DQ2.2 give a lower risk than DQ2.5 for celiac disease?

The gluten epitope DQ2.2-glut-L1 shows sustained binding to DQ2.2 and binds better to DQ2.2 than to DQ2.5. Why is the risk of DQ2.2 for CD then so much lower than that of DQ2.5?

One contributory factor could be the lower amounts of the DQ2.2-glut-L1 epitope apparently present in digested gluten compared with the dominant DQ2.5-restricted gluten epitopes (Paper II). This could be caused either by a lower amount of protein harboring this sequence or by a lower proteolytical stability of this peptide. The latter is supported by the presence of a chymotrypsin cleavage site within the sequence of the DQ2.2-glut-L1 epitope.

Further, an anchor residue in P3 (serine, aspartate or threonine) seems important for sustained binding to DQ2.2, but not DQ2.5.[128] Hence, fewer different peptides are likely to fulfill the stricter criteria necessary to become a good DQ2.2 binder. In order to examine this, we searched in a tailored gluten database (containing all identified gluten proteins from wheat) with the predicted peptide-binding motif of DQ2.2 ([Either QFYWILMSP]-[No P]-[S or T]-[No P]-[Any]-[Either PEQD]-[No P]-[Any]-[No P]; at least one P and one Q present). In fact, among the unique proteins containing this pattern, many (over 15%) contained sequences homologous to the DQ2.2-glut-L1 epitope.

The use of soluble MHC molecules to enrich for high affinity peptide binders is a promising method to identify dominant T-cell epitopes in infectious and autoimmune diseases.[129] Our group has started using recombinant soluble DQ molecules to enrich for high affinity peptide binders in a complex gluten digest treated with TG2. Astonishingly, almost half the peptides identified from the gluten digest after enrichment with soluble DQ2.2 contained the DQ2.2-glut-L1 epitope (Dørum et al., unpublished data).

Possibly, initiation of CD is a matter of threshold. Somehow the “advantage” of a new anchor in P3 in DQ2.2 is lower than the “disadvantage” of losing a hydrogen-bonding network to the peptide main chain (Figure 4).

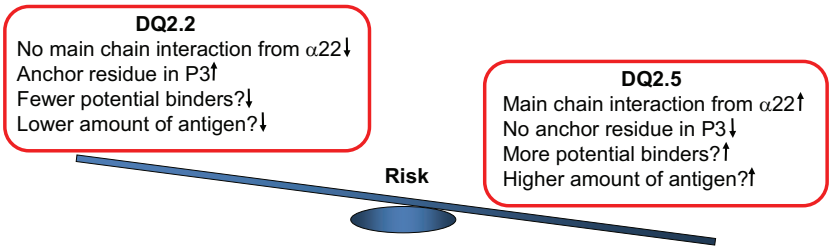


Figure 4: Increased risk of DQ2.5 compared with DQ2.2 for CD. *The loss of the main chain interaction to the peptide leads to a greater risk reduction than the risk increase of a potential anchor residue in P3 (arrows up/down refer to increased/reduced risk).*

The concept of threshold of HLA

The concept of a threshold effect of HLA in CD was already proposed in 2003.[126] The idea is supported by epidemiologic data showing that the risk for CD is increased in homozygous compared with heterozygous DQ2 patients.[130] In additional support of this concept, is the higher frequency of low-risk HLA class II genes (including DQ2.2) in latent CD (high antibody titers, but no histological changes) compared with full-blown CD.[131, 132] The association of DQ2 homozygosity with RCDII and EATL[133] also suggests that the degree of inflammation is somehow correlated with disease severity.

Kinetic stability of peptide-MHC

Our findings suggest that kinetic stability of peptide-MHC complexes has important effects on the specificity of T-cell responses in CD. Kinetic stability affects both the hierarchy of peptides loaded onto MHC[134] and the half-life of peptide-MHC on the cell surface. The latter impacts the number of peptide-MHC complexes that reach the lymph node on the surface of the APC. Interestingly, in a mouse model, peptide off-rate on MHC was a crucial factor in determining the CD8 T cells' decision to stop and form long-lived contacts with dendritic cells.[113] Another study showed that *in vivo* priming in the presence of competitive T-cell responses to unrelated peptides, lead to an aborted expansion of T cells reactive to peptide-MHC complexes

with poor kinetic stability.[135] Two alternative scenarios could thus explain the differential T-cell response in DQ2.2 and DQ2.5 patients. In the first scenario, the patients generate an initial response towards peptides with a lower kinetic stability to MHC. This response is later on inhibited by the presence of peptides with higher kinetic stability. In the second scenario, no initial response is generated towards epitopes with a low affinity to MHC.

Whether CD should be called an autoimmune disease or not is a matter of semantics. Autoantibodies are found, but the disease-driving $CD4^+$ T cells recognize a foreign antigen. In contrast to CD, many autoimmune diseases seem to be driven by autoreactive T cells. Somehow, these autoreactive T cells escape negative selection. A low affinity of peptides to MHC has been suggested as a potential mechanism.[136] In non-obese diabetic mice (model of type 1 diabetes), the 9-23 peptide of the insulin β chain may be an important autoantigen.[137] This peptide shows a very low affinity to the relevant MHC molecule.[138] Thus, while kinetic stability seems to be an important factor in determining the specificity of the T-cell response in CD, the transferability of these findings to autoimmunity is not obvious.

DQ9 as a risk factor for celiac disease

Both DQ2 and DQ8 lack aspartic acid in β57 (non-Asp β57) as both carry alanine in this position. This has been proposed to be particularly important for their association with CD. In contrast to DQ8, DQ9 has not been considered a risk factor for CD,[68] although the genetic epidemiological evidence for this seems to be lacking. The presence of aspartic acid in β57 of DQ9 is the only difference between DQ8 and DQ9. Interestingly, we found that DQ9-restricted gluten-reactive T cells could be isolated from the small intestine of a CD patient expressing DQ9 and DQ2.2 (Paper III). This shows that DQ9 can be implicated in the T-cell response to CD. We identified a dominant DQ9-restricted gluten epitope, DQ8-glut-H1, also recognized by CD patient expressing DQ8, but uncommonly.

The non-Asp β57 molecule DQ8 has a preference for a negative charge in P9, while DQ9 has no such preference.[68] It has also been proposed that the importance of the DQ8 β57 polymorphism relates to the ability of this molecule to interact with a negatively charged TCR, upon binding a native gluten peptide[139] (Figure 5). As

expected, our binding data of gluten peptides confirmed that DQ9 is unable to harness a negative charge in P9. Further, the DQ8-glut-H1 epitope bound better than two more dominant DQ8-restricted gluten epitopes to DQ9. This could possibly explain why a T-cell response is generated to this epitope and not to the two more dominant DQ8-restricted epitopes, which bound less well to DQ9. Although we only studied the T-cell response from one CD patient expressing DQ9, it is tempting to speculate that only the DQ8-glut-H1 epitope would bind sufficiently well to DQ9 to initiate a gluten response in CD patients expressing DQ9.

Interestingly, in Western populations, type 1 diabetes shows an association with the non-Asp β57 DQ molecules encoded by the DR3-DQ2 and DR4-DQ8 haplotypes.[140, 141] However, this association is not seen in Japan, where the β57 Asp positive DQ9 on the DR9-DQ9 haplotype is more common and actually seems to be associated with type 1 diabetes.[142]

We demonstrate that DQ9 may contribute to CD development, but does DQ9 give a lower risk for CD than DQ8? Understanding this would help to uncover the importance of the DQ β57 polymorphism in CD. None of the patients in a study investigating a large number of CD patients expressed DR9-DQ9 without other predisposing HLA genes.[67] However, this haplotype is rare in Western populations[143] and concluding based on epidemiologic studies is therefore difficult. The extreme rarity of CD in Japan,[144] despite the fact that the DR9-DQ9 haplotype is found in up to 15% of individuals,[142] may suggest a lower risk for DQ9 than DQ8. However, scarcity of CD in Japan could also be related to the fact that the main cereal in the Japanese diet is rice and not wheat, or to other genetic factors. On a more speculative basis, it seems plausible that, as with DQ2.2 and DQ2.5, DQ9 gives a lower risk than DQ8 for CD, as fewer gluten peptides potentially bind well to DQ9.

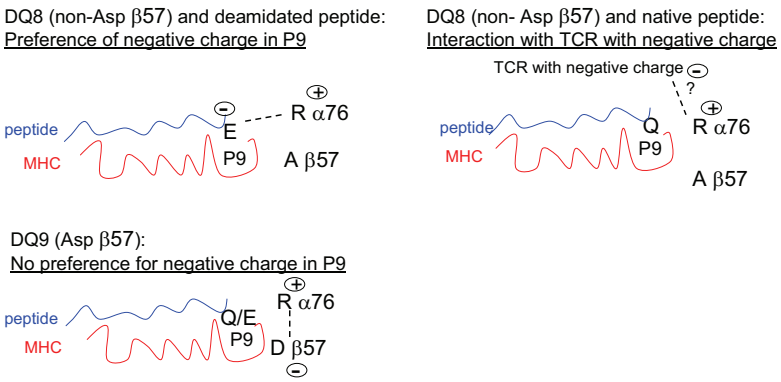


Figure 5: Impact of the DQ8 β57 polymorphism on binding of gluten peptides and interaction with TCR *(schematic representation). R: arginine; D: aspartate; A: alanine; Q: glutamine; E: glutamic acid; Peptide in blue and MHC in red; Hydrogen bonds represented as dotted lines; Positive charges represented with a +, negative charges with a -.*

Importance of transdimers

When investigating the gluten response in HLA heterozygous individuals, taking into account the formation of DQ heterodimers is important. As mentioned, the polymorphism of both DQA1 and DQB1 enables the formation of unique DQ molecules encoded by alleles positioned in *trans*. It has been reported that, in addition to the two *cis*-encoded DQ heterodimers, both *trans*-encoded DQ heterodimers formed in DQ2/DQ8 heterozygous CD patients can present gluten peptides and that their binding motifs are different from the two *cis*-encoded DQ molecules.[111, 145, 146] In paper III, we investigated the T-cell response to gluten in the context of the *trans*-encoded DQ9.2 molecule and the *cis*-encoded DQ9.3 molecule in a given CD patient. We found that both these MHC molecules can present peptide to DQ9-restricted gluten-reactive TCCs. DQ9.3 presented the identified gluten peptide most efficiently to the TCCs, and we believe this is likely to be the (most) disease relevant molecule. Interestingly, from this patient, expressing also DQ2.2 in *cis* and DQ2.3 in *trans*, we recently isolated TCCs that recognized an epitope which could be presented by both the DQ2.2 and the DQ2.3 transdimer (unpublished data). It is possible that other transdimers than those described until now could be involved in presenting gluten

peptides in CD, although this is probably very limited by pairing restrictions.[41] Interestingly, transdimer formation between DQ2 and DQ8 has been reported as a possible explanation for the increased risk observed in individuals with DR3-DQ2/DR4-DQ8 for type 1 diabetes.[147]

The pitfall of T-cell proliferation assays

Traditional *in vitro* T-cell proliferation assays, in which APC, T-cells and antigen are mixed together at the start of the assay, do not take into account the off-rate of gluten peptides to MHC molecules. APC from DQ2.2 expressing individuals are generally able to present peptide to gluten-reactive T cells from DQ2.5 expressing individuals *in vitro*, and vice a versa. This means that the affinity of these peptides to both DQ2.2 and DQ2.5 is sufficient to activate T cells *in vitro*. Further DQ2.2 and DQ2.5 are so homologous that the TCRs of these TCCs recognize peptide presented on both MHC molecules. Still, the longer off-rate of dominant DQ2.5-restricted gluten epitopes on DQ2.5 than on DQ2.2, and vice a versa, results in T-cell responses towards different epitopes *in vivo* in DQ2.2 and DQ2.5 patients. So, while the *in vitro* T-cell response in a standard T-cell proliferation assay is similar, the *in vivo* activation and expansion of T cells differs. This emphasizes the importance of categorizing an epitope only in the context of the HLA molecules expressed by the patient.

Assessing cytokine production by gluten-reactive T cells (Paper I)

The drawbacks of organ cultures

Gluten-reactive T cells appear to be key players in the celiac lesion and determining their phenotype is important. Many of the studies investigating cytokine production of gluten-reactive T cells rely on an organ culture system. In such organ culture experiments, small intestinal biopsies from treated or untreated CD patients are cultured in the presence of medium (control) or gluten antigen and the production of cytokines from the biopsy is assessed (using for instance flow-cytometry, real-time polymerase chain reaction or western-blot).[148-150] A weakness of many of these studies relates to the large interbiopsy variation, which does not always seem to be taken sufficiently into account (Tollefsen et al., unpublished data). Further, the cytokine producing cells are not necessarily antigen specific T cells, as a bystander activation of antigen unspecific cells can potentially be observed. We have performed pilot

experiments in which we sorted proliferating and/or activated T cells four to seven days after stimulation of whole biopsies (or single-cell suspension) with a TG2-treated digest of gluten. Activated and proliferating cells were mainly seen in CD patients after antigen stimulation. These cells were sorted by flow-cytometry and cultured. Still, only a small fraction of the T cells (less than 10%) were found to be antigen-specific upon retesting. Although organ cultures usually assess cytokine production after a shorter time span, our findings indicate that interpretation of the specificity of the T cells activated in an organ culture must be done with caution.

Conflicting data on IL-17 production by gluten-reactive T cells

Instead of using organ cultures, we studied gluten-reactive TCLs, generated from small intestinal biopsies, and visualized the gluten-reactive T cells with DQ2-tetramers. We found that gluten-reactive T cells did not seem to produce IL-17 (or IL-22). Nevertheless, IL-17 mRNA is increased in the celiac lesion[150] and an increased frequency of $CD4^+$ T cells producing IL-17 is found in the mucosa of untreated CD patients.[149] The source of IL-17 could therefore be hypothesized to be $CD4^+$ T cells with unknown specificity and/or other cells than $CD4^+$ T cells.

Our findings are apparently contradictory to the more recent findings of Fernandez et al., which found gliadin-specific T cells producing IL-17 in CD patients.[151] There were however several key differences between our studies. First, we generated TCLs from treated CD patients, whereas they generated TCLs from untreated patients. Second, they added IL-23 to their culture medium, which favors *in vitro* expansion of Th17 cells, and cultured Th17 enriched cells separately, in the presence of gliadin. A small proportion of gliadin-specific Th17 cells in this sample may thus have been highly expanded, even though their initial frequency could have been very low. A weakness of our study is a possible bias in the selection of the TCLs used for testing cytokine production. We chose TCLs with a good proliferative response to gluten. Thus, we can not exclude that in these TCLs, gluten-reactive T cells have proliferated more than other T cells *in vitro*. A more extensive proliferation of the gluten-reactive T cells could affect the phenotype of these cells to a larger degree than that of other cells used as an internal control of the culture conditions of the TCL.

We are currently validating direct tetramer staining of biopsy material and could possibly combine this with intracellular staining for cytokine production. This

should eliminate the potential problems associated with long term *in vitro* culturing. To further examine the phenotype of gluten-reactive T cells, we have also initiated a study to investigate the transcription factors of gluten-reactive T cells visualized in peripheral blood of CD patients after a short gluten-challenge. Preliminary data show a dominant Th1 phenotype with high TBX-21 expression (unpublished data).

Production of IL-21 by gluten-reactive T cells

We found that gluten-reactive T cells, visualized in intestinal TCLs using tetramer staining, produce IFN-γ and IL-21. Interestingly, the IL-2/IL-21 locus is associated with CD[152] and IL-21 seems to control the production of the pro-inflammatory cytokines IFN-γ[148] and IL-17[149] in the celiac lesion. The cytokine IL-21 has also been shown to have an important role in B-cell differentiation in germinal centers and one could envisage that this cytokine contributes to the production of TG2-specific antibodies.[153]

The pathogenic role of gluten-reactive T cells

We have performed extensive studies of gluten-reactive T cells. How well established is their pathogenic role for CD initiation? Compelling evidence suggests that gluten-reactive $CD4^+$ T cells are required for initiation of CD: Gluten-reactive T cells are only found in the small intestine of CD patients and not in the intestine of healthy controls,[76] and, moreover, there is a strong MHC class II association with CD. One can speculate that these T cells also initiate the large intraepithelial infiltration of $CD8^+$ T cells characteristic of CD. Indeed, in a mouse genital viral infection model, IFN-γ produced by $CD4^+$ T cells, induced secretion of cytokines exerting a chemotactic effect on $CD8^+$ T cells.[154] As previously discussed (see "inflammation in CD"), also the autoantibody response to TG2 appears to be controlled by gluten-reactive T cells and such antibodies are only found in individuals with the CD predisposing HLA genes.[155] Nevertheless, CD is not induced in DQ2 transgenic mice also expressing gliadin-specific TCRs.[156, 157] Thus, in mice models, the appropriate MHC restriction (DQ2) and gluten-reactive T cells alone seem insufficient to trigger CD. Importantly however, little activation of naïve T cells in mesenteric lymph nodes was seen after feeding these mice with gluten antigen.[156, 157] The failure of these mice to develop a CD-like enteropathy may relate to the absence of background genes that

substitute the human non-MHC CD susceptibility genes, as well as to differences in the gut physiology between man and mouse. Still, while gluten-reactive T cells in the celiac lesion seem necessary, it remains to be shown that activation of these cells is sufficient for initiation of CD.

Peptide vaccination for therapy

A gluten-free diet is the only established treatment in CD, but can be difficult to sustain, has high costs and may be a social burden.[158] However, given the efficiency of a gluten-free diet in the treatment of CD, therapeutic alternatives should have little adverse effects. Based on our growing understanding of the pathogenesis of CD, several alternative approaches have been investigated, such as the digestion of gluten epitopes by exogenous enzymes, transglutaminase inhibitors, DQ2-blockers and different cytokine-blockers.[159] A highly attractive option would be to target the antigen-specific T cells directly by restoring tolerance to gluten. In allergic diseases, subcutaneous injection therapy using a mixture of complex proteins can restore tolerance to the immunogenic antigens and is an established treatment modality. Gluten proteins are unfortunately poorly soluble in water and also contain peptides with possible innate effects. Therefore, peptide-based vaccination, using dominant gluten epitopes has been proposed instead of protein-based vaccination as a possible therapy in CD. A safety study of peptide vaccination using 3 dominant DQ2.5-restricted epitopes in DQ2.5 expressing CD patients has already been undertaken (Nexvax2). Designing a vaccine containing only dominant T-cell epitopes could be sufficient. Indeed, tolerance induction after peptide vaccination is believed to occur through generation of regulatory T cells, and spreading of tolerance has been described using peptide vaccination.[160, 161] Characterization of the epitopes recognized by gluten-reactive T cells of DQ2.2 expressing CD patients is important with regard to eventually designing a peptide vaccine to also this patient group.

Future perspectives

To evaluate the validity of our model of the DQ2.2-DQ2.2-glut-L1 complex, an attempt to solve the crystal structure of this complex will be made (Bergseng et al., ongoing project). One of the current limitations of T-cell studies of gluten-reactive T

cells in CD is the necessity to culture the T cells, or to expose the patient to a short-term gluten challenge. To avoid this, we have developed a staining protocol to visualize gluten-reactive T cells directly in samples from small intestinal mucosa of untreated and treated CD patients expressing DQ2.5 using DQ2-tetramers (unpublished data). Whole transcriptome analysis of gluten-reactive T cells isolated directly from the small intestine could enable further characterization of these important cells. It would also be interesting to investigate whether any gluten-reactive T cells can be visualized in the small intestine of healthy individuals expressing DQ2.5, and if so, whether they have a regulatory phenotype. Preliminary data indicate that a small number of both memory and naïve gluten-reactive T cells can be visualized in peripheral blood of healthy individuals and CD patients (Christophersen et al., unpublished data). It will be interesting to find out whether these memory T cells of healthy individuals have a regulatory phenotype and whether they home to the small intestine.

Conclusion

CD is an important model for understanding the pathogenesis of chronic inflammatory diseases. Gluten-reactive $CD4^+$ T cells, producing pro-inflammatory cytokines such as IFN-γ and IL-21, seem to be central in the pathogenesis. The threshold for disease initiation of CD is partly determined by the MHC genes of the individual. This relates to the importance of stable peptide binding to MHC in determining T-cell responses (as shown with DQ2.2 and DQ2.5; and more speculatively with DQ8 and DQ9). Why is it then so that the great majority of patients with the predisposing MHC genes do not develop CD? Other risk genes, many of which are immune-related explain some of this gap. The rest possibly relates to environmental factors or “bad luck”. Whether an active regulatory mechanism (such as antigen-specific regulatory T cells) is found in healthy individuals expressing the risk MHC genes, or whether the absence of a response to gluten is simply a matter of ignorance, remains unknown. Understanding more of the T-cell biology in CD could help us decipher what initiates the disease and possibly open new therapeutic avenues.

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Erratum

Paper I:

Introduction, second paragraph, "T_R" should be removed.

'TCL KT CDE3' should in all instances in paper read 'TCL KT CD3'

Figure 1: The order of the bars in the panel showing IL-21 production was inadvertently interchanged. Further, TCC 430.1.1.142 is α-I restricted, not α-II restricted. These errors do not affect the conclusion drawn from this figure. The correct figure is displayed below:

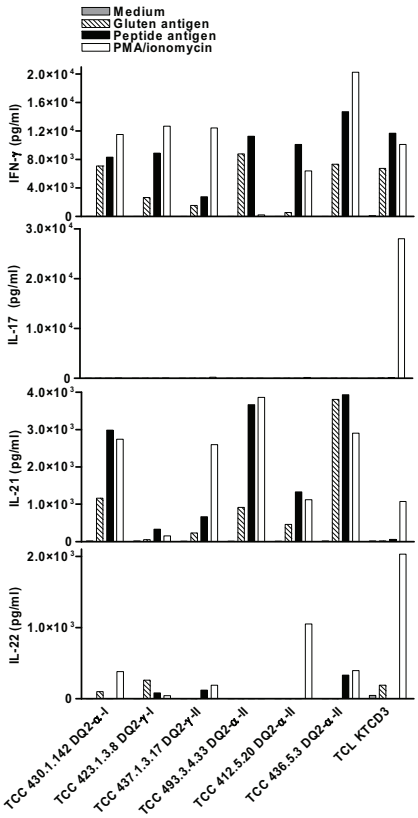

Figure 2: 'pg/ml^{-1}' as indicated on the y-axis should read 'pg/ml'.

Table 1, row 1: 'TCL 548.A.1.4' should read 'TCL 48.A.1.4'.

Figure 4 and Figure 5: In the legends of both figures ‘18-24h’ should read ‘5h’.

I

II

III

Evidence that HLA-DQ9 confers risk to celiac disease by presence of DQ9-restricted gluten-specific T cells

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We describe the gluten T-cell response of a DR7DQ2/DR9DQ9 heterozygous celiac disease patient CD555. Interestingly, this patient had T cells recognizing gluten in the context of HLA molecules of both haplotypes. For the DR9DQ9 haplotype, DQ9 was identified as the antigen-presenting molecule. As DQ9 carries aspartate at DQ β57 but is otherwise identical to DQ8 and not considered associated with celiac disease, we aimed to characterize this DQ9-restricted T-cell response in detail. By fractionation of pepsin-trypsin digested gliadin we identified an epitope stimulatory for several T-cell clones. This epitope was identical to an epitope (DQ8-glut-1) previously identified in DQ8 patients. In CD555, this was the dominant DQ9-restricted epitope, while no T-cell response was found towards two other DQ8-restricted epitopes. These findings correlated with peptide binding data demonstrating that this epitope bound better to DQ9 than the two other DQ8-restricted epitopes. Whereas glutamine to glutamate exchange at P9 improved binding of all three epitopes to DQ8, no such effect was observed for DQ9. The differential ability of DQ8 and DQ9 to harness a negatively charged anchor at P9 may result in fewer potential gluten epitopes in DQ9 patients. Our data further indicate that DQ9 is a susceptibility factor for celiac disease.

1. INTRODUCTION

The absence of aspartate at position β57 is considered a particularly important functional feature of the type 1 diabetes and celiac disease (CD) associated HLA-DQ8 molecule [1-3]. HLA-DQ9 (DQA1*03/DQB1*03:03) differs from HLA-DQ8 (DQA1*03/DQB1*03:02) only in position β57 where DQ8 has alanine and DQ9 has aspartate. This difference leads to the loss of a salt bridge between arginine α76 and aspartate β57 of DQ8 and hence to a preference of negatively charged peptides in P9 of the peptide binding groove of DQ8, but not of DQ9 [3-5]. In support of the importance of the β57 polymorphism it has been stated that DQ9 is not associated with CD [5]. It has recently been proposed that the remarkable association of DQ8 to CD is related to the ability to recruit negatively charged TCR and that this is linked to the β57 polymorphism [2]. However the effect of this polymorphism on peptide-MHC interaction has not been fully investigated.

Gluten-reactive $CD4^+$ T cells are found in the intestinal mucosa of CD patients but not of healthy controls [6]. Most intestinal gluten-reactive T cells respond to gluten peptides only after conversion by the enzyme transglutaminase 2 (TG2) of certain glutamine residues to glutamic acid [7]. In this paper, we found that DQ9-restricted gluten-reactive $CD4^+$ T cells could be isolated from the small intestine of a CD patient expressing DQ9 and DQ2.2. We established T-cell clones and identified the epitope recognized. We further investigated the binding of gluten peptides to DQ8 and DQ9. Our findings suggest that DQ9 can be implicated in CD, and they shed light on the importance of the HLA-DQ β57 polymorphism for disease initiation.

2. SUBJECTS AND METHODS

2.1. Subject

Intestinal biopsies were obtained on two separate occasions from an adult female patient (CD555). She carried the serological HLA type DR7DR9/DQ2DQ3 and genomic DQ type DQA1*02:01/03:02 and DQB1*02/03:03. Based on known linkage disequilibria, she is very likely to carry the DR7DQ2 and DR9DQ9 haplotypes. She had a diagnosis of CD from 1999 which was in accordance with the American Gastroenterology Association guidelines [8] with a positive endomysium antibody test and histology showing Marsh 3C. Upon accidental exposure to gluten the patient has experienced abdominal pain and diarrhea. As a child she suffered from abdominal pain and failure to thrive. Biopsies were taken both in 2004 as part of routine follow-up and in 2007 as part of a study examining the gluten response in patients with DQ2.2 (Bodd et al., Gastroenterology, in press). She was on a gluten-free diet and was well-treated on both occasions. In 2010, the patient also accepted to undergo an oral bread challenge (see below). The regional committee for medical research ethics had approved the relevant protocols, and the patient gave written consents before participating.

2.2. Oral bread-challenge

An oral gluten challenge was performed as previously described [9,10]. In brief, the patient ingested four slices of white bread daily for 3 days and blood was drawn on day 6 followed by peripheral blood mononuclear cell isolation by density gradient centrifugation. 4×10^5 peripheral blood mononuclear cells and various peptides were added to wells of 96-well plates and incubated overnight at 37°C. Single-cell secretion of interferon-γ was detected using an interferon-γ ELISPOT assay [9,10].

2.3. Antigen

As complex cereal antigens we used chymotrypsin digested gluten (hereafter referred to as gluten) and pepsin and trypsin digested gliadin (Sigma, 9007-90-3) [11]. Treatment with TG2 was performed as previously described [11]. Peptides were purchased from GLS Biochem, EZ Biolabs or were synthesized in house. The sequences of the synthetic gluten peptides used can be found in Supplementary Table 1.

2.4. Fractionation of gliadin digest

Fractionation of pepsin and trypsin digested gliadin was done by gel filtration and reverse phase HPLC. The gliadin digest was first fractionated by gel filtration (Äkta; Superdex peptide 10/300 GL column; GE healthcare; 1ml/min in milli-Q water). A fraction stimulating T-cell clone (TCC) 555.A.1.4.S.6 was then further fractionated by reverse phase HPLC (Agilent 1100, Zorbax 300SB-C18 column using an acetonitrile gradient from 5-100%; 1ml/min; 0.1% trifluoroacetic acid). A T-cell stimulating fraction (fraction 14) was then analyzed by LC-MS/MS (Q-TOF; Bruker Daltonics). Peptides were separated on an analytical column (150mm x 0.075 mm) packed with 100Å C18 3.5μm particles (G&T Septech). Data were acquired using microTOF control v2.0 and processed using DataAnalysis v3.4. The data were analyzed using the Mascot search engine and Proteome Discoverer software version 1.0 (Thermo Scientific) using an in house built database of *Triticum aestivum* derived proteins.

2.5. T-cell assays

Generation of gluten-reactive T-cell lines (TCL) and proliferative T-cell assays were performed as previously described [11]. Irradiated B lymphoblastoid B-cell lines (B-LCL) 9050 (DQA1*02:01/ DQB1*02:02; DQ2.2), 9092 (DQA1*03:01/ DQB1*03:02; DQ8), 9076 (DQA1*03:02/DQB1*03:03; DQ9.3), 9102 (DQA1*03:03/DQB1*02:02; DQ2.3) and 9052 (DQA1*02:01/DQB1*03:03; DQ9.2) were used as antigen-presenting cells (APC). Notably, the mature DQα chains encoded by DQA1*03:02 and DQA1*03:03 are identical, but different from DQA1*03:01 at residue α160 (aspartate instead of alanine), which is unlikely to affect peptide binding and T-cell recognition. To assess HLA restriction of the TCC, APC were incubated with anti-DQ (SPV-L3), anti-DR (B8.11) or anti-DP (B7/21) antibodies (final concentration of 20μg/ml) for 30 min prior to addition of antigen. In experiment from figure 2D, the APC were incubated with peptide overnight before free peptide was washed away and T cells were added.

2.6. Peptide MHC off-rate assay

Functional peptide off-rate assays were performed as previously described [12]. In brief, antigen-pulsed irradiated APC were incubated for various time points without antigen before adding T cells and measuring T-cell proliferation.

2.7. Competitive binding assay

Peptide binding was measured in a competitive binding assay as described previously, using the B-LCL 9092 and 9076 as source of HLA-DQ8 and HLA-DQ9 molecules, respectively [13,14]. IC_{50} values (half maximal inhibitory concentration) were established as the concentration of peptide required to inhibit the binding of an indicator peptide (biotin-PEG-FESTGNLIAPEYG; used at 0.2μM for DQ8 and 2μM for DQ9) by 50%. Three independent 4-fold titration experiments were performed.

2.8 Analysis of HLA-DQ expression

Staining of B-LCL 9076 and 9052 was performed using a monoclonal antibody to HLA-DQ (clone FN81, IgG2a, PE conjugated; Diatec) or an IgG2a isotype control antibody conjugated with PE. Analysis was performed on a FACSCalibur (BD Biosciences).

3. RESULTS

3.1. Identification of DQ9-restricted epitope

The gluten response in one CD patient (CD555) expressing both DQ2.2 and DQ9 was examined. A total of ten intestinal gluten-reactive TCL were generated. The TCL were initially tested for recognition of TG2 treated gluten presented by homozygous B-LCL carrying DR7DQ2 and DR9DQ9 haplotypes. Six gluten-reactive TCL made a positive response with DR7DQ2 APC of which four also made a response with DR9DQ9 APC. Five TCL (B1.1, A.1.1.S, A.1.4.S, B.2.2, A.0.4.S) made a response with DR9DQ9 APC but only weakly with DR7DQ2 APC (Figure 1). These TCL were considered particularly interesting, and we generated gluten-reactive TCC from three of these TCL. Three gluten-reactive TCC were obtained from TCL 555.A.1.4.S, one from TCL 555.A.1.1.S (originally from same biopsy) and eleven from TCL 555.B.2.2 (different biopsy from different time point). The TCC made a response to gluten in the context of DR9DQ9 but not DR7DQ2 APC (Figure 2A). Using DR9DQ9 APC, the proliferative response could be inhibited by an anti-DQ specific monoclonal antibody, but not by anti-DR or anti-DP specific monoclonal antibodies (Figure 2B). We thus conclude that the TCC were restricted by DQ9. The TCC proliferated similarly to titrated amounts of native and TG2-treated gluten (data not shown).

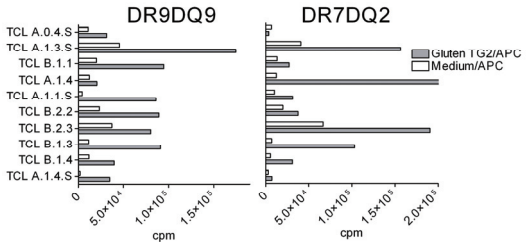

Figure 1: Identification of DQ9-restricted T-cell responses to gluten in CD555, a celiac disease patient heterozygous for DQ2.2 and DQ9. The figure shows proliferation responses of several gluten-reactive T-cell lines stimulated with DR7DQ2 (B-LCL 9050) or DR9DQ9 (B-LCL 9076) expressing antigen-presenting cells and transglutaminase (TG2)-treated gluten. Proliferation was assessed by [^{3}H] thymidine incorporation and is measured in counts per minute (cpm; mean of duplicates).

3.2. Recognition of DQ8-glut-1 epitope by DQ9-restricted T cells

We performed fractionation of a pepsin-trypsin digest of gliadin and obtained several T-cell stimulating fractions for TCC 555.A.1.4.S.6 (Supplementary Figure 1). One fraction (fraction 14) was subjected to mass spectrometry analysis. Several masses were present in the fraction, but only the sequence of one peptide, a 35-mer, was identified with high certainty (qQGYYPTSPQQPGQGQQLGQGQPGYYPTSQQPGQK, q is pyroglutamate). This peptide harbored the sequence (underlined) of the previously described DQ8-glut-1 (or glutenin) epitope [15], indicating glutenin contamination of the commercial gliadin preparation. Glutamine in P1 of this epitope can be targeted by TG2 (for deamidation) [15] and DQ9 exhibits the preference of a negative charge in P1[5]. Hence, the peptide variant with glutamate at P1 should be biologically relevant and we used this peptide in later experiments. Reactivity of the generated gluten-reactive TCC to the DQ8-glut-1 epitope (underlined) was established by testing the synthetic peptide Ac-QEGYYPTSPQQSG (Figure 2C). All 15 generated DQ9-restricted T-cell clones from altogether 3 TCL were reactive to the DQ8-glut-1 epitope.

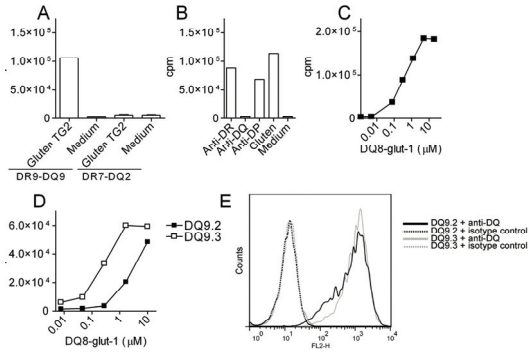

Figure 2: Several gluten-reactive T-cell clones (TCC) generated are DQ9-restricted and they recognize the previously described DQ8-glut-1 epitope. Figure A shows proliferation responses of TCC 555.A.1.1.13 stimulated with DR9DQ9 (B-LCL 9076) or DR7DQ2 (B-LCL 9050) expressing antigen-presenting cells and transglutaminase-treated gluten or medium. Figure B shows proliferation responses of TCC 555.A.1.1.13 in the presence of blocking antibodies and gluten, gluten alone or medium, and DR9DQ9 expressing antigen-presenting cells. Figure C shows proliferation of this TCC stimulated with DR9DQ9 expressing antigen-presenting cells and peptide containing the DQ8-glut-1 epitope (Ac-QEGYYPTSPQQSG). Figure D shows proliferation of TCC 555.B.2.2.45 after stimulation with APC encoding DQ9.3 (encoded in *cis* by the patient) or DQ9.2 (encoded in *trans* by the patient), loaded with peptide containing the DQ8-glut-1 epitope. The results are representative of the testing of five TCC. Figure E shows flow cytometric analysis of expression of HLA-DQ on the surface of the DQ9.2 and DQ9.3 expressing APC. Proliferation was assessed by [^{3}H] thymidine incorporation, measured in counts per minute (cpm) (mean of duplicates).

3.3 Presentation of DQ8-glut-1 epitope by a DQ9 variant encoded in *trans* by CD555

In addition to the *cis*-encoded HLA-DQ molecules DQ2.2 (DQA1*02:01/DQB1*02:02) and DQ9.3 (DQA1*03:02/ DQB1*03:03), patient CD555 can express the two *trans*-encoded HLA-DQ molecules DQ2.3 (DQA1*03:02/ DQB1*02:02) and DQ9.2 (DQA1*02:01/ DQB1*03:03). While the TCC did not respond to the DQ8-glut-1 epitope when presented by a homozygous B-LCL expressing DQ2.3 (data not shown), the TCC responded when presented by a homozygous B-LCL expressing DQ9.2 although with a tenfold lower sensitivity than when presented by a DQ9.3 expressing APC (Figure 2D). When stained with an anti-DQ antibody, the expression of HLA-DQ molecules by these two APC was found to be similar (Figure 2E).

3.4. Importance of DQ8-glut-1 epitope in this patient

Two other gluten epitopes, namely the DQ8-glia-α-1 and the DQ8-glia-γ-1 epitope, are commonly recognized by celiac disease patients with DQ8. We therefore carefully examined whether the TCL that made gluten-specific responses with DR9DQ9 APC, could harbor T cells which were specific for these epitopes. None of the seven tested DQ9-restricted gluten-reactive TCL responded to peptides representing the DQ8-glia-α-1 or the DQ8-glia-γ-1 epitopes, whereas they made responses to the DQ8-glut-1 epitope (Figure 3A). This response pattern was maintained in TCL established from intestinal biopsies taken with 3 years' interval (Figure 3A). We also examined by ELISPOT the response to these epitopes in peripheral blood after a short gluten challenge and found that the DQ8-glut-1 epitope gave a titratable IFN-γ response which was much stronger than the response to the two other common DQ8 gluten epitopes (Figure 3B). Taken together the results indicate that patient CD555 made persistent T-cell responses to the DQ8-glut-1 epitope in the context of DQ9.

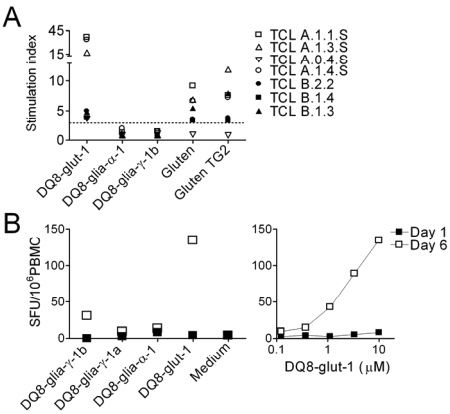

Figure 3: Compared with two other common DQ8 gluten epitopes, the DQ8-glut-1 epitope appears to be a dominant epitope in this patient. Figure A shows proliferation responses of seven T-cell lines (TCL) from CD555 stimulated with DQ9 (DQ9.3) expressing antigen-

presenting cells and peptides containing the DQ8 gluten epitopes, gluten or transglutaminase 2 (TG2)-treated gluten. Open symbols represent TCL generated from biopsies taken in 2004 while closed symbols represent TCL generated from biopsies taken in 2007. TCL B.2.2 was generated from a different biopsy than TCL B.1.3 and TCL B.1.4; TCL A.1.1.S, TCL A.1.3.S and TCL A.1.4.S from a different biopsy than TCL A.0.4.S. Proliferation was assessed by [^{3}H] thymidine incorporation and is expressed in stimulation index (cpm with antigen stimulation/cpm with medium stimulation; mean of duplicates). A cut-off of 3 was chosen as a significant stimulation index (dotted line). Figure B shows response in peripheral blood after a short-term gluten challenge as measured by IFN-γ ELISPOT (average of triplicates; Spot forming units (SFU) per 10^6 peripheral blood mononuclear cells). Left panel shows response towards peptide harboring DQ8 gluten epitopes including DQ8-glut-1 epitope (Ac-QEGYYPTSPQQSG) or medium and right panel shows response against titrated amounts of the DQ8-glut-1 peptide. For the peptide sequences, see Supplementary Table 1.

3.5. Sustained antigen presentation

As sustained antigen-binding appears crucial for the initiation of a gluten response [12], we wanted to investigate whether this epitope shows sustained binding to DQ9. By using a T-cell based off-rate assay, we found that the DQ8-glut-1 epitope was effectively presented by DQ9 (DQ9.3) even after 96h (Figure 4).

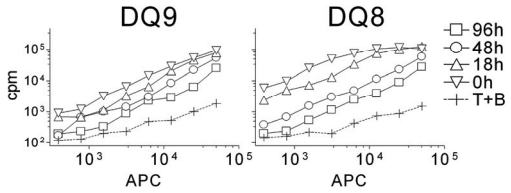

Figure 4: The DQ8 glutenin epitope shows sustained binding to DQ9. The figure shows proliferation responses of T-cell clone 555.A.1.4.S.32 stimulated with irradiated DQ8 or DQ9 (DQ9.3) expressing antigen-presenting cells loaded for 2 h with peptide (Ac-QEGYYPTSPQQSG) containing the DQ8-glut-1 peptide or medium (T+B). The antigen-presenting cells were then washed and incubated for various time points followed by addition of T cells. The experiment is representative of two separate experiments. Proliferation was assessed by [^{3}H] thymidine incorporation, measured in counts per minute (cpm) (mean of duplicates).

3.6. Binding of various DQ8 epitopes to DQ9

We performed a competitive binding assay for peptide binding to DQ9 (DQ9.3) and found that the DQ8-glut-1 epitope had a tenfold lower IC_{50} value (half maximal inhibitory concentration; hence higher affinity) than the DQ8-glia-α-1 epitope, whereas the DQ8-glia-γ-1 epitope had an even lower affinity (Table 1). This could explain why the DQ8-glut-1 epitope appears to be recognized over the two other DQ8 gluten epitopes in this patient.

Table 1: Affinity measurements of known DQ8 gluten epitopes for interaction with DQ9 (DQ9.3) or DQ8 in competitive binding assays. Affinity is measured as IC_{50} values (half maximal inhibitory concentration).

		IC_{50} (μM)	
	Peptide sequence	DQ8	DQ9
DQ8-glut-1	Ac-QEGYYPTSPQQSG	4.3	3
DQ8-glut-1 (E)	----------E------	1.6	5.4
DQ8-glia-α-1	SGEGSFQPSQQNPQ	12.9	36
DQ8-glia-α-1 (E)	----------E-----	2	21.6
DQ8-glia-γ-1b	FPEQPQQPYPQQPQQ	>160	>160
DQ8-glia-γ-1b (E)	----------E--------	6.8	>160

3.7. Effect of glutamine to glutamate exchange for peptide binding to DQ8 and DQ9

In a competitive binding assay, using P9 glutamine to glutamate substitutes, we found improved binding of all three epitopes for binding to DQ8, but no such effect for any of the epitopes for binding to DQ9 (Table 1). Further we found that for DQ8, the DQ8-glut-1 variant with a glutamine residue at P9 had an affinity intermediate between that of the deamidated forms of the DQ8-glia-α-1 and the DQ8-glia-γ-1 epitopes. We also examined the response of one of the DQ8-glut-1 reactive TCC from patient CD555 to different P1 and P9 (glutamine→glutamate) variants of the DQ8-glut-1 epitope (Figure 5). The TCC proliferated well in response to native peptide. A negative charge at P1 seemed slightly beneficial for recognition by this TCC. On the other hand, the TCC recognized this epitope less well when a negative charge was present at P9. This probably relates to TCR specificity issues as the affinities of the native and deamidated variant of DQ8-glut-1 for binding to DQ9 are fairly similar (Table 1) and suggests that this TCC was initially primed by the peptide variant with a Q at P9.

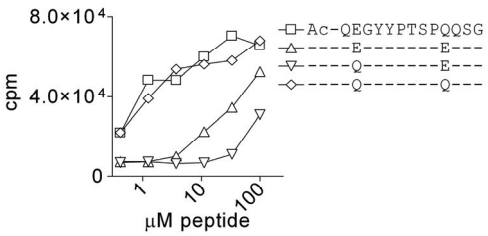

Figure 5: Lower proliferation of DQ8-glut-1 reactive T-cell clone from CD555 when stimulated with peptide with a negative charge at P9. The figure shows proliferation responses of TCC 555.A.1.4.S.32 stimulated with P1 and P9, Q or E variants of DQ8-glut-1 epitope and irradiated DQ9 (DQ9.3) antigen-presenting cells. Proliferation was assessed by [^{3}H] thymidine incorporation, measured in counts per minute (cpm) (mean of duplicates).

4. DISCUSSION

In this study we show for the first time the presence of DQ9-restricted gluten-reactive T cells in the small intestine of a CD patient. As this patient was heterozygous for DQ2.2 and DQ9, it is still unknown whether DQ9 on its own can initiate CD. We have found the presence of DQ2.2-restricted gluten-reactive T-cell clones in this patient which recognize the DQ2.2-glut-1 epitope (Bodd et al., Gastroenterology, in press). Further, this patient can form the transdimer DQ9.2 (DQA1*02:01/DQB1*03:03), that also activated the gluten-reactive DQ9-restricted T cells. This transdimer shares the β chain with the *cis* encoded DQ9.3 molecule (DQA1*03:02/DQB1*03:03). While both DQ9.2 and DQ9.3 could activate the gluten-reactive T cells, the *cis*-encoded DQ9.3 appeared more efficient. Altogether, even though studies of HLA-DQ homozygous patients may have been more informative, our study of this heterozygous patient indicates that DQ9 (in particular DQ9.3) can be implicated in a pathogenic gluten response in CD.

The DR9DQ9 haplotype is very rare in Caucausian populations (< 1%) [16], and thus it is hard by population genetics to determine whether DQ9 is a risk factor for CD. Interestingly, in a study of a large series of CD patients [17], one of the four patients who did not carry the known risk factors DQ2.5, DQ8, or either the α or β chain of DQ2.5, expressed DQ9.3 (DQA1*03:02/DQB1*03:03) encoded in *trans* and DQ9.2 (DQA1*02:01/DQB1*03:03) encoded in *cis*. In such a setting functional studies may provide important hints to unravel risk factors. This is exemplified by studies of DQ7 and DQ8 as risk factors for CD. DR4 haplotypes confer risk in DQ2-negative CD patients, but initially it was unclear whether the risk was associated with DR4DQ7, DR4DQ8 or both haplotypes [18]. A study of a DR4DQ7/DR4DQ8 heterozygous patient provided a clue to this issue [19]. The gluten-reactive T cells established from gut biopsies of this patient only recognized gluten in the context of DQ8 but not DQ7, thus indicating that DQ8 is the susceptibility factor. This conclusion has later been confirmed in population genetics studies [20]. Similar to the above described study, our current observation indicates that DQ9 is a susceptibility factor for CD.

The gluten epitope recognized by the patient expressing DQ9 was identified as the DQ8-glut-1 epitope, which has been previously identified in DQ8 patients, but does not appear to be a common epitope in patients of this category [15,21]. All DQ9-restricted gluten-reactive TCC generated from three TCL (two of which generated from biopsies taken years apart) and all seven TCL (generated from in total four different biopsies) responding to gluten after stimulation with DQ9 expressing APC responded to this peptide. Thus, the DQ8-glut-1 epitope appears to be the dominant DQ9-restricted gluten epitope in this patient. In line with the importance of sustained antigen presentation for initiating a gluten response [12], we found that the epitope shows sustained binding to DQ9. Further, we showed a strong DQ9-restricted T-cell response to the DQ8-glut-1 epitope, while no response was found against two other common DQ8 gluten epitopes. Although we must be cautious to conclude on the basis of one patient, we propose that this is likely due to a higher affinity of the DQ8-glut-1 epitope for binding to DQ9 than the other two DQ8 epitopes, as measured in a competitive binding assay.

Affinity measurements of gluten peptide interaction with DQ8 and DQ9 also confirmed, as previously modeled [5], the importance of a negative charge at P9 of gluten peptides for binding to DQ8, but not to DQ9. Interestingly we found that the DQ8-glut-1 epitope binds fairly well also to DQ8, compared with two other common DQ8 epitopes, even with the presence of a glutamine at P9. This might explain why the response to the DQ8-glut-1 epitope is deamidation-independent in DQ8 CD patients [21]. This also explains why some DQ8-glut-1 reactive TCC from DQ8 patients recognize the peptide much better with a glutamine than with a glutamate at P9, suggesting that the T cells were initially primed by the peptide variant with a glutamine at this position [5].

We have recently shown that the lower risk for CD of DQ2.2 compared with DQ2.5 relates to stricter constraints for binding of gluten peptides to DQ2.2 than to DQ2.5 [12] (Bodd et al., Gastroenterology, in press). Our findings suggest a similar mechanism for DQ8 and DQ9 as for DQ2.5 and DQ2.2. Indeed, the loss of the advantage of a negative charge at P9 for DQ9 possibly restricts the number of potential epitopes binding sufficiently well to initiate a T-cell response. In conclusion, we report the presence of DQ9 -restricted gluten-reactive T cells recognizing the DQ8-glut-1 epitope, an epitope previously described to be recognized by DQ8 patients, in the small intestine of a CD patient expressing DQ9. This epitope appears to be the dominant DQ9-restricted epitope in this patient and binds particularly well to DQ9 compared with other DQ8 gluten epitopes. Our findings indicate that the lost potential benefit of a negative charge at P9 possibly restricts the number of peptides binding well to DQ9 and hence the threshold for disease initiation. Our findings shed light on the association of the DQ8 β57 polymorphism with immunological diseases.

ACKNOWLEDGEMENTS

This work received financial support from the Norwegian Extra Foundation for Health and Rehabilitation through EXTRA funds, the Novo Nordisk Foundation and the Research Council of Norway. The authors thank Bjørg Simonsen for help with performing the competitive peptide binding assays and Maria Stensland (Proteomics Core Facility, Oslo University Hospital) and Hao Yu for excellent technical assistance. The authors also thank the celiac disease patient who donated biological material to the study and Professor Frits Koning for critical reading of the manuscript.

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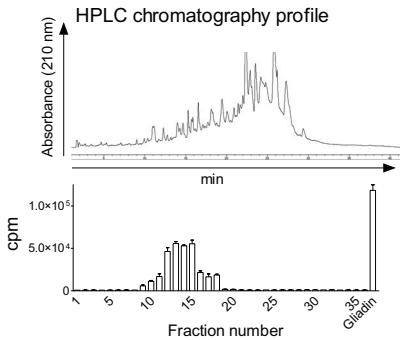
SUPPLEMENTARY MATERIAL

Supplementary table 1: Overview of peptides used in the study. Acetylated peptides where used to avoid the formation of pyroglutamate.

Epitope	Sequence	Comment
DQ8-glut-1	Ac-QEGYYPTSPQQSG	Most frequently used DQ8-glut-1 peptide in manuscript.
DQ8-glia-α1	SGEGSFQPSQQNPQ	
DQ8-glia-α1 (E)	SGEGSFQPSQENPQ	
DQ8-glia-γ1b	FPEQPQQPYPQQP	
DQ8-glia-γ1b (E)	FPEQPQQPYPEQPQQ	
DQ8-glia-γ1a	PQTEQPQQPFPQPQ	
DQ8-glut-1, P1 and P9 (E)	Ac-QEGYYPTSPEQSG	
DQ8-glut-1, P9 (E)	Ac-QQGYYPTSPEQSG	
DQ8-glut-1, No E	Ac-QQGYYPTSPQQSG	



Supplementary figure 1: T-cell proliferation of TCC 555.A.1.4.S.6 against transglutaminase-treated pepsin-trypsin digested gliadin and high-performance liquid chromagraphy (HPLC) separated fractions (from 1-36) from a stimulatory fraction of pepsin-trypsin digested gliadin separated by gel-filtration.