PEPTIDE BINDING TO HLA-DQ2 AND DEVELOPMENT OF BLOCKING AGENTS FOR THE TREATMENT OF CELIAC DISEASE

Doctoral thesis by

Elin Bergseng

Institute of Immunology

Faculty Division Rikshospitalet

University of Oslo

2007

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 673

ISBN 978-82-8072-750-3

Cover: Inger Sandved Anfinsen.
Printed in Norway: AiT e-dit AS, Oslo, 2008.

Produced in co-operation with Unipub AS.
The thesis is produced by Unipub AS merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

Unipub AS is owned by
The University Foundation for Student Life (SiO)

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

The work presented in this thesis was carried out at the Institute of Immunology (IMMI), University of Oslo, Rikshospitalet during the period 2003-2007. This study has been supported by National Institutes of Health (NIH) from April 2003 to December 2004 as part of a collaborative project with Stanford University and the Norwegian Foundation for Health and Rehabilitation (EXTRA funds) from January 2005 to December 2007. I am most grateful for this support. The Research Council of Norway provided financial support for a stay at Stanford University as a visiting researcher (from November 2005 to April 2006). The additional funding received from Medinnova and Norsk Farmaceutisk Selskaps Stipendiefond is also highly appreciated.

First of all I want to express my gratitude to my supervisor Professor Ludvig M. Sollid for brilliant supervision and continuous support throughout the work of this thesis. I also want to thank him for giving me the opportunity to work on excellent projects in collaboration with outstanding scientists. One of the scientists I have worked with is Professor Chaitan Khosla at Stanford University. I had the opportunity to stay in his lab for 5 months. I am very grateful for his hospitality during my stay, and for his generous sharing of knowledge and exceptional skills.

I would like to acknowledge the contributions of all my other co-authors, Jiang Xia, Chu-Young Kim, Shuo-Wang Qiao, Øyvind Molberg, Günther Jung, Burkhard Fleckenstein, Matthew Siegel, John Sidney and Alessandro Sette.

A special thanks goes to Tore Jensen and Marie Kongshaug Johannesen for training me in the lab and answering all my questions, Shuo-Wang Qiao who introduced me to the peptide binding assay, Hanne Quarsten who introduced me to molecular biology and expression of recombinant soluble DQ2 molecules in the baculovirus expression system and Lars-Egil Fallang for our many discussions which have been stimulating and extremely valuable. I also want to thank the other members of the “CD group” for the great working environment and helpful discussions: Jorunn Stamnæs, Ulrike Jüse, Luka Mesin, Ann-Christin Røberg Beitnes, Silja S. Amundsen, Melinda Ráki, Stig Tollefsen, Helene Arentz-Hansen, Øyvind Molberg, Anna Hayman Robertson, Bjørg

Simonsen, Elisabeth Reed Eng, Gry Namløs Nordang, Marit Sandvik, Knut E. A. Lundin, Eirik H. Halvorsen, Sylvie Pollmann, Anders Fallang, Burkhard Fleckenstein, Maria Ekman, Siri Dørum, Astrid Tutturen, Marit Jørgensen and Anders Holm.

I also want to thank all the nice colleagues at IMMI.

Finally, I would like to express my gratitude to my family and friends for encouragement and support. I especially want to thank my husband Bent Ove Barmoen Rinden for his patience and support.

Oslo, December 2007

Elin Bergseng

ABBREVIATIONS

APC	antigen presenting cell
APL	altered peptide ligand
CDR	complementarity determining region
CLIP	class II-associated invariant chain peptide
ER	endoplasmatic reticulum
GFD	gluten-free diet
HLA	human leukocyte antigen
HMW	high molecular weight
HPSEC	high performance size exclusion chromatography
IEL	intraepithelial lymphocyte
IFN-γ	interferon-γ
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
LMW	low molecular weight
MHC	major histocompatibility complex
MIIC	MHC class II compartments
PEG	polyethylene glycol
PEP	prolyl endopeptidase
TCC	T cell clone
TCR	T cell receptor
TG2	transglutaminase 2

LIST OF PAPERS

Paper I

Bergseng E., Xia J., Kim C.-Y., Khosla C., and Sollid L.M. (2005). Main chain hydrogen bond interactions in the binding of proline-rich gluten peptides to the celiac disease-associated HLA-DQ2 molecule. *J Biol Chem.* 280:21791-6

Paper II

Qiao S.W., **Bergseng E.**, Molberg Ø., Jung G., Fleckenstein B., and Sollid L.M. (2005). Refining the rules of gliadin T cell epitope binding to the disease-associated DQ2 molecule in celiac disease: importance of proline spacing and glutamine deamidation. *J Immunol.* 175:254-61

Paper III

Xia J.[*], **Bergseng E.**[*], Fleckenstein B., Siegel M., Kim C.-Y., Khosla C., and Sollid L.M. (2007). Cyclic and dimeric gluten peptide analogues inhibiting DQ2-mediated antigen presentation in celiac disease. *Bioorg Med Chem.* 15:6565-73

[*]The authors contributed equally to this work.

Paper IV

Bergseng E., Sidney J., Sette A., and Sollid L.M. Binding of gluten T cell epitopes to various HLA class II molecules. *Manuscript submitted*

INTRODUCTION

The immune system is a complex network of organs, cells and molecules that has evolved to protect the body from invading pathogenic microorganisms and cancer. At the same time the immune system can be detrimental and cause disease as seen in autoimmune diseases. The immune system is traditionally divided into two parts: the innate and the adaptive immune system. The innate immune system provides the first line of defense against infection and most components including phagocytic cells, complement and natural killer cells, are present before the onset of infection. The innate immune response responds rapidly, but in contrast to the adaptive immune system it does not have an immunological memory. The adaptive immune response is dependent on antigen specific receptors on the surface of B and T lymphocytes. This response takes days to develop as there are few specific lymphocytes for the invading pathogen that have to undergo clonal expansion. Following elimination of the pathogen, a subset of the cells remains as memory cells that respond faster and stronger to a subsequent encounter with the pathogen.

Major histocompatibility complex (MHC) molecules are expressed on most cells in the body. In humans these molecules are called human leukocyte antigen (HLA). The function of MHC molecules is to display peptide antigens to T cells. MHC class I molecules are expressed on nearly all nucleated cells, while MHC class II molecules are constitutively expressed on professional antigen presenting cells (APC). The APCs include dendritic cells, B cells, macrophages and thymic epithelial cells. Peptides from the cells' own proteins or from bacteria, virus or ingested antigen are presented on the MHC molecules. These peptide-MHC complexes are scanned by circulating T cells. When a naïve T cell recognizes a peptide-MHC complex, a signaling cascade is initiated and the T cell is activated. Activated T cells can mediate their effects through cytotoxic mechanisms or through cytokine production. In humans, three isotypes of class II molecules are expressed: HLA-DR, HLA-DQ and HLA-DP.

Autoimmune diseases are characterized by an immune mediated damage of cells or tissues. The causes of autoimmune diseases are unknown. However both genetic and

environmental factors are involved. For most diseases, like type 1 diabetes, rheumatoid arthritis and multiple sclerosis the environmental factor is unknown. For celiac disease, which has several autoimmune features, the antigen is known to be gluten. Gluten is an exogenous antigen and celiac disease may therefore also be categorized as food hypersensitivity or allergic disease. The MHC is the major genetic susceptibility factor for autoimmunity. It is located on the short arm of chromosome 6 (6p21.3). The extended HLA complex contains 252 genes and spans 7.6 Mb (Horton et al., 2004). A recent genome wide association study on several autoimmune diseases revealed that HLA is by far the most important susceptibility factor for many of these diseases (The Wellcome Trust Case Control Consortium, 2007). Yet, the molecular mechanism for the HLA association for most diseases is unknown. Celiac disease has become a valuable model for the study of HLA associated diseases. In such a model the primary HLA association and the antigen should be known and the antigen specific T cell should be possible to isolate and grow. In celiac disease all these criteria are fulfilled. There is a strong association with HLA-DQ2 and -DQ8, gluten is found to be the antigen and gluten specific T cells from the intestine of celiac disease patients can be cultured from biopsies.

The goal of my work has been to further investigate how peptides bind to HLA-DQ2 and to start developing peptide blockers for the treatment of celiac disease.

MHC class II molecules

Structure of MHC class II molecules and peptide binding

The MHC class II molecules are membrane-bound glycoproteins consisting of two subunits, the 29 kDa α-chain and the 32 kDa β-chain. Each chain consists of two extracellular domains (α_1 and α_2, β_1 and β_2), a transmembrane region and a short cytoplasmic domain. The membrane distal domains (α_1 and β_1) form the peptide binding groove which consists of eight strands of anti-parallel β-sheets with two antiparallel α-helical regions overlaying them. The deep cleft formed between the α-helices accommodates the peptide. In contrast to MHC class I molecules, the MHC class II molecules have a peptide binding site with open ends allowing peptides with variable length to bind (Madden, 1995). Studies have shown that peptides eluted from

MHC class II molecules are normally between 10 and 34 amino acids long (Rudensky et al., 1991; Hunt et al., 1992; Chicz et al., 1993). Their function is to bind and present fragments of degraded proteins to $CD4^+$ T cells (Germain, 1994). The determination of the three-dimensional structure by x-ray crystallography has revealed that peptides are bound in an extended conformation within a groove formed by the α- and β-chain of the MHC class II molecule, adopting a polyproline type II helical conformation (Stern et al., 1994; Jardetzky et al., 1996). A seven stranded β-sheet represents the floor of the binding groove and the sides are formed by two long α-helices. Two mechanisms are involved in the stable binding of peptides to the MHC class II molecule. Hydrogen bonding between highly conserved residues of the MHC class II molecule and carbonyl oxygen and amide nitrogen of the peptide backbone is one mechanism (Stern et al., 1994; Jardetzky et al., 1996). The other mechanism includes the extensive polymorphic residues situated around the peptide binding site which results in the formation of pockets that can bury amino acid side chains of the bound peptide at the P1, P4, P6, P7 and P9 positions (Stern et al., 1994). These polymorphisms in MHC class II molecules are responsible for the allele specific peptide binding motifs for each molecule. The side chains of residues P2, P5 and P8 are found to point outward in the direction of the T cell receptor (TCR) (Stern et al., 1994) (Figure 1).

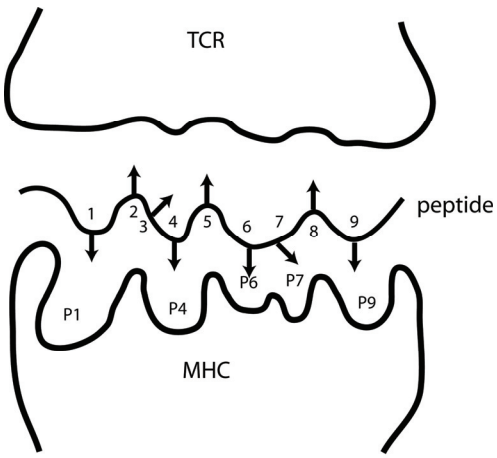

Figure 1. *Schematic representation of peptide, MHC and TCR.*

Antigen processing

The APCs display a complex of peptide and MHC class II molecule for recognition by $CD4^+$ T cells (Babbitt et al., 1985). These peptides usually derive from exogenous antigens which can be internalized in APC through receptor-mediated endocytosis, phagocytosis or macropinocytosis (Watts, 1997). The antigens are unfolded and degraded into peptides within the endocytic pathway by low pH, reducing conditions and proteolytic activity in the endosomes (Jensen, 1995).

The MHC class II αβ heterodimer is assembled in the endoplasmic reticulum (ER) where it associates with the chaperone molecule invariant chain (Ii), which ensures correct folding of the MHC class II molecule (Cresswell, 1996). A trimer of Ii associates with three MHC class II molecules to form a nonameric complex $(\alpha\beta Ii)_3$ (Roche et al., 1991). This complex is transported from the ER, through the Golgi apparatus to late endosomal vesicles called MHC class II compartments (MIIC) (Peters et al., 1991). This transport is directed by targeting signals in the cytoplasmic domain of Ii (Bakke and Dobberstein, 1990). In these vesicles, Ii is proteolytically degraded leaving a short fragment called CLIP (class II-associated Ii peptide) bound to the binding groove of the MHC class II molecule. CLIP contributes to MHC stability and prevents loading of endogenous peptides in the ER (Roche and Cresswell, 1990; Busch et al., 1996). The release of CLIP is catalyzed by HLA-DM and is required for binding of antigenic peptides (Denzin and Cresswell, 1995; Sherman et al., 1995). The MHC class II-peptide complex is subsequently transported to the cell surface for inspection by $CD4^+$ T cells. HLA-DM is a non-classical MHC class II molecule, which is unable to bind peptides due to a closed peptide binding groove. In addition to catalyzing the release of CLIP, HLA-DM stabilizes the empty form of the MHC class II molecule (Kropshofer et al., 1997) and edits the repertoire of bound peptides. A second non-classical MHC class II molecule, called HLA-DO is associated with HLA-DM (Liljedahl et al., 1996) and acts as a negative regulator of HLA-DM (Denzin et al., 1997; van Ham et al., 1997; Liljedahl et al., 1998).

Peptide binding to HLA-DQ2

The peptide binding motif of HLA-DQ2 was first characterized by sequencing of peptides eluted from affinity purified molecules (van de Wal et al., 1996; Vartdal et

al., 1996) and by binding experiments with substituted and truncated variants of high affinity binding peptides (Johansen et al., 1996; van de Wal et al., 1996; Vartdal et al., 1996). The results demonstrated a preference for negatively charged anchor residues in the P4, P6 and P7 pockets and bulky hydrophobic residues in P1 and P9.

The first x-ray crystal structure of a MHC class II molecule was reported in 1993 (Brown et al., 1993). Since then more than 20 crystal structures have been solved. Kim et al solved the x-ray crystal structure of HLA-DQ2 complexed with a gluten peptide, QLQPFPQPELPY (DQ2-α-I-gliadin peptide) with a resolution of 2.2Å (Kim et al., 2004). This study provided definite information about the interaction between peptide and HLA-DQ2. In Figure 2, the binding site of HLA-DQ2 with its pockets is shown with and without the DQ2-α-I-gliadin peptide. Despite the presence of four Pro residues in the peptide, the critical hydrogen bonds between HLA-DQ2 and the peptide are retained. As Pro residues are not able to participate in amide-mediated hydrogen bonds, the positioning is such that they do not interfere with the hydrogen bonds. The amide mediated hydrogen bonds are seen in positions 2, 4, 6 and 9. In crystal structures of other MHC class II molecules there is an amide mediated hydrogen bond also in position 1 (Stern et al., 1994; Ghosh et al., 1995; Fremont et al., 1996; Dessen et al., 1997; Fremont et al., 1998; Scott et al., 1998; Smith et al., 1998; Latek et al., 2000; Lee et al., 2001; He et al., 2002; Liu et al., 2002b; Zhu et al., 2003; Siebold et al., 2004; Henderson et al., 2007). This bond is not seen in HLA-DQ2 since there is a Pro residue in this position. The crystal structure also revealed that the Glu residue at P6 is an important anchor residue due to its participation in an extensive hydrogen bonding network involving Lys-β71.

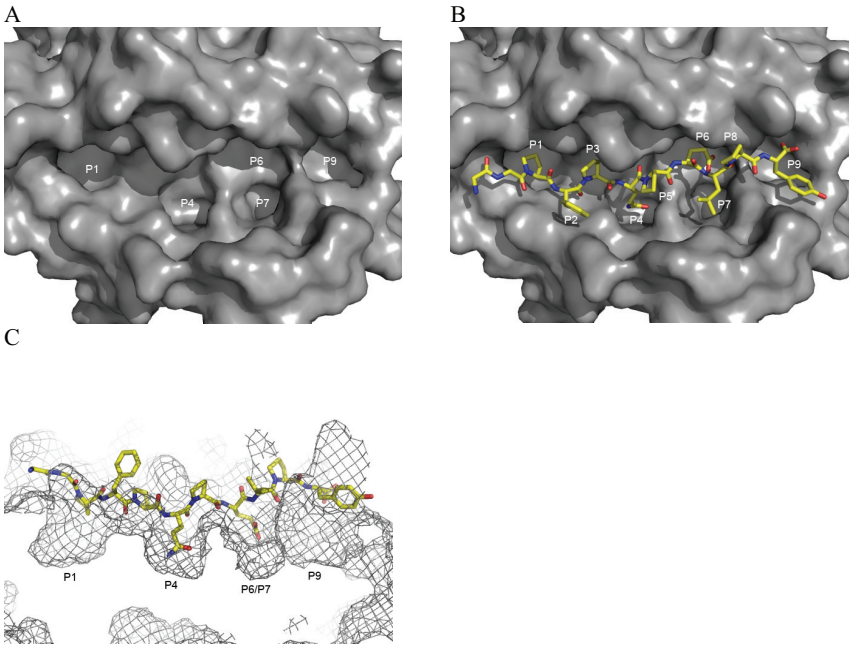

Figure 2. *Pockets in the peptide binding groove of HLA-DQ2. A. The surface of the HLA-DQ2 peptide binding groove with the pockets labeled after the amino acid side chain accommodated. B. DQ2-α-I-gliadin peptide bound in the binding groove. The side chains of P2, P5 and P8 extend in the direction of the TCR. Carbon atoms are yellow, nitrogen atoms are blue and oxygen atoms are red. C. Side view of the peptide binding groove. The surface is shown as a grey mesh. Figures were generated using PyMol (DeLano Scientific LLC).*

T cell recognition

Most T lymphocytes express an αβ TCR. These T cells can be separated into two subsets on the basis of the cell-surface markers CD4 and CD8. The $CD8^+$ T cells usually recognize peptides presented by MHC class I molecules, whereas $CD4^+$ T cells recognize peptides presented by MHC class II molecules.

The TCR is a heterodimer consisting of either α- and β-chains or γ- and δ-chains linked by a disulfide bond. Each chain is composed of variable and constant Ig-like domains, a transmembrane domain and a short cytoplasmic tail. Only a minority of the T cells has a TCR made up of γ- and δ-chains and the function of these γδ T cells is not yet completely understood. The αβ TCR binds the peptide-MHC complex through complementarity determining regions (CDR) present in their variable domains.

Activation of $CD4^+$ T cells take place via interaction of the TCR with a peptide bound to a MHC class II molecule on an APC. As the peptide is bound in a polyproline type II helical conformation, only a few of the peptide side chains are available for direct interaction with the TCR. The other side chains are accommodated in pockets in the MHC class II molecule. Recognition of a peptide-MHC complex is the first of two signals required for full T cell activation. The second signal is provided by a variety of co-stimulatory molecules expressed on the surface of T cells such as CD28, CTLA-4 or CD40L which interact with their counterparts on the APC. This second signal may strengthen or weaken the TCR signal. When a T cell is fully activated it becomes less dependent on the co-stimulatory signals on later restimulation.

Based on the outcome of the interaction between TCR and peptide-MHC, peptides can be classified as agonists, weak agonist, partial agonist, antagonist or null compounds. An altered peptide ligand (APL) is a peptide where single or multiple amino acid replacements cause a different activation of the T cells as a result of the modified interaction with the TCR. Agonists induce full T cell stimulation, while an antagonist do not induce any function and block this stimulation by modifying signaling pathways downstream of the TCR. Weak agonists are able to induce full T cell stimulation at high concentrations while partial agonists induce some but not all T cell functions (e.g. cytokine secretion without proliferation) (Sloan-Lancaster and Allen, 1996; Bielekova and Martin, 2001).

Celiac disease

Clinical aspects

Celiac disease is a chronic inflammatory disorder of the small intestine induced by dietary proteins in wheat, rye and barley. It was first described by Samuel Gee in 1888 (Gee, 1888). The chronic inflammation of the small intestine results in the celiac lesion, characterized by villous atrophy, crypt hyperplasia and infiltration of lymphoid cells in the epithelium and lamina propria (Trier, 1991). These pathological findings are reversed on withdrawal of gluten from the diet. The symptoms in early childhood typically include chronic diarrhea, abdominal distension, failure to thrive and malabsorption (Mäki and Collin, 1997). When the disease presents later in life, the symptoms tend to be more vague and they include weight loss, diarrhea, constipation and extraintestinal symptoms like anemia, fatigue, neurological symptoms and behavioral disturbances as depression and irritability (Fasano and Catassi, 2001). The fact that many patients have only mild or no symptoms leads to the high frequency of undiagnosed patients. Untreated celiac disease is associated with complications such as infertility, osteoporosis and intestinal malignancies (Mäki and Collin, 1997) in addition to increased mortality (Corrao et al., 2001; Fasano and Catassi, 2001).

Untreated celiac disease patients have increased levels of serum antibodies for gluten and transglutaminase 2 (TG2) and testing of these are utilized to predict celiac disease. However, a positive serologic test has to be confirmed by the findings of typical histological alterations of the small intestinal mucosa by a biopsy for a definite diagnosis of celiac disease. Development of sensitive serological tests has made screening studies possible. These studies have demonstrated that the prevalence of celiac disease may be as high as 1:100 in Caucasians (Mäki et al., 2003; Dube et al., 2005).

Genetic factors

Celiac disease develops as a result of interplay between genetic and environmental factors. The genetic factor was evident from clinical observations of multiple cases of celiac disease within families, with about 10% of first-degree relatives being affected (Petronzelli et al., 1997) and a high rate of concordance (75%) for celiac disease

among monozygotic twins (Greco et al., 2002; Nistico et al., 2006). HLA-DQ2 and -DQ8 have been identified as the key genetic risk factors in celiac disease (Sollid and Thorsby, 1993). It has been estimated that the HLA genes account for around 50% of the genetic heritability of celiac disease (Sollid and Lie, 2005). More than 90% of celiac disease patients express the HLA-DQ2.5 heterodimer (DQA1*05/DQB1*02) compared to a prevalence of 30% among the general population (Sollid et al., 1989; Sollid and Thorsby, 1993). Most of the remaining individuals express HLA-DQ8 (DQA1*0301/DQB1*0302). The celiac disease associated HLA-DQ2.5 can be encoded in *cis* (on one chromosome) by DQ alleles of the DR3-DQ2 haplotype, or in *trans* (across both chromosomes) by the DR5-DQ2 and DR7-DQ2 haplotypes. Among the few celiac disease patients who are neither HLA-DQ2.5 nor -DQ8 there are some patients carrying HLA-DQ2.2 (DQA1*0201/DQB1*0202) from the DR7-DQ2 haplotype (Karell et al., 2003). In the membrane distal domains HLA-DQ2.2 and -DQ2.5 have identical sequence in their β chains while the α chains differ by ten amino acids.

As celiac disease only occurs in a small portion of the population carrying HLA-DQ2 and -DQ8, additional factors clearly contribute. This means that HLA-DQ2 and -DQ8 are necessary but not sufficient for development of celiac disease and has led to a hunt for other candidate genes. A variety of candidate regions have been identified (Djilali-Saiah et al., 1998; Greco et al., 2001; Liu et al., 2002a; Woolley et al., 2002; van Belzen et al., 2003; Monsuur et al., 2005), but until recently no susceptibility genes other than HLA-DQ have been identified and convincingly replicated. Recently a genome-wide association study by van Heel et al (van Heel et al., 2007) identified genetic elements predisposing to celiac disease in a region on chromosome 4q27 that contains the *IL2*, *IL21*, *TENR* and *KIAA1109* genes. In addition Alizadeh and coworkers found that homozygosity for a variant of the *FcgRIIa* gene predisposes to celiac disease (Alizadeh et al., 2007). Both of these findings need to be replicated. Based on the current knowledge it seems reasonable to assume that a number of different genes predispose to celiac disease development, and that the constellation of predisposing genes may vary between the individual celiac disease patients.

Gluten – the environmental factor

Gluten is obviously the critical environmental factor as the disease goes into remission when gluten is eliminated from the diet. The triggering factor of celiac disease was found to be gluten more than 50 years ago by Dicke and coworkers (Dicke et al., 1953). Gluten is the storage protein of wheat and is responsible for the unique baking properties of wheat. It is traditionally divided into two groups based on solubility in aqueous alcohol, gliadins (soluble fraction) and glutenins (alcohol insoluble fraction). Gliadins are further classified into three groups, α-, γ- and ω-gliadins, based on their amino acid sequences (Wieser et al., 1987) and glutenins into high molecular weight (HMW) and low molecular weight (LMW) glutenin subunits.

The closely related proteins in rye and barley are secalins and hordeins, respectively. Collectively, these storage proteins in cereal grains are often named prolamins and they contain a high percentage of Pro and Gln residues. Based on the primary amino acid sequence there are many similarities between the gliadins and glutenins with many repetitive sequences. It has been shown that both gliadins and glutenins are toxic for the celiac disease patients (Ciclitira et al., 1984; Lundin et al., 1993; Lundin et al., 1994; Molberg et al., 1997; van de Wal et al., 1999; Vader et al., 2002b; Molberg et al., 2003; Spaenij-Dekking et al., 2005b; Dewar et al., 2006).

Avenin is the prolamin fraction of oats. Whether oats could be included in a gluten free diet or not has been controversial and several clinical studies have indicated that oats in the diet is safe for most celiac disease patients (reviewed in Haboubi et al., 2006). However there are studies showing that some celiac disease patients have avenin-reactive mucosal T cells (Arentz-Hansen et al., 2004).

Pathogenesis – involvement of adaptive and innate immunity

Celiac disease is the result of an abnormal $CD4^+$ T cell initiated immune response to wheat gluten (Figure 3). Gluten reactive $CD4^+$ T cells can be isolated and cultured from small intestinal biopsies of celiac disease patients, but not from healthy controls (Lundin et al., 1993; Lundin et al., 1994; Molberg et al., 1997). Upon recognition of gluten peptides, these T cells are activated and a cytokine response strongly dominated by interferon-γ (IFN-γ) is induced (Nilsen et al., 1998). These T cells are exclusively restricted by HLA-DQ2 and -DQ8. It was demonstrated by Ráki et al that a unique subset of dendritic cells is responsible for the activation of gluten reactive T cells in the celiac lesion (Ráki et al., 2006).

A growing number of gluten derived epitopes recognized by T cells from the intestinal mucosa of celiac disease patients have been described (Sjöström et al., 1998; van de Wal et al., 1998b; Arentz-Hansen et al., 2000; Arentz-Hansen et al., 2002; Vader et al., 2002b). Most of these epitopes are dependent on a conversion of Gln to Glu residues for T cell recognition. This process, which is called deamidation, is mediated by the enzyme TG2. The introduction of a negatively charged residue increases the binding affinity for DQ2 (Molberg et al., 1998; van de Wal et al., 1998a). Notably, the T cell recognition seems to be more sensitive to the Gln to Glu exchange than what can be explained by the increased binding alone (Arentz-Hansen et al., 2000). TG2 shows a specificity for selected Gln residues in the gluten peptide (Fleckenstein et al., 2002; Vader et al., 2002a). It was demonstrated by Arentz-Hansen et al that the gliadin epitopes cluster in Pro-rich regions. The high Pro content makes them exceptionally resistant to gastric, pancreatic and intestinal proteases and peptidases (Hausch et al., 2002; Shan et al., 2002; Shan et al., 2005).

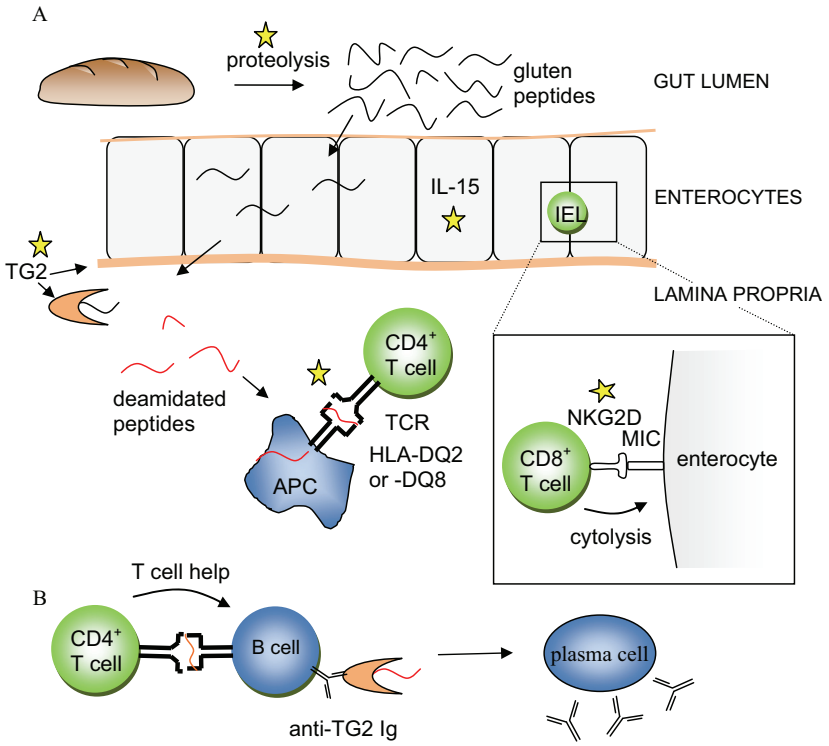

Figure 3. *The celiac lesion. A. Gluten is digested into amino acids and peptides by luminal and brush-border enzymes. Some Pro-rich fragments of gluten are resistant to proteolysis and these peptides can be transported across the epithelium into the lamina propria where they are deamidated by TG2. $CD4^+$ T cells in the lamina propria recognize deamidated gluten peptides presented by HLA-DQ2 or -DQ8 molecules on the surface of APC. There is infiltration of intraepithelial lymphocytes in the epithelium. These $CD8^+$ T cells express NK cell receptors like NKG2D and are able to kill enterocytes that express stress induced molecules like MICA. Gluten stimulates the expression of IL-15 which can induce upregulation of NKG2D and MICA. A yellow star indicates possible treatment strategies. B. Model of mechanism for production of antibodies to TG2. Complexes of gluten peptide and TG2 are formed during the deamidation reaction. These complexes can be bound by immunoglobulin (Ig) on the surface of TG2-specific B cells and endocytosed. The deamidated gluten peptide can bind to HLA-DQ2 or -DQ8 and be presented to $CD4^+$ T cells. The T cell*

recognition will provide help signals to the B cells necessary for secretion of antibodies.

More recent studies suggest that the innate immune system is important in celiac disease pathogenesis. A hallmark of celiac disease is an increase in the number of intraepithelial lymphocytes (IEL), most of which are $CD8^+$ cytotoxic T cells. These IELs have been shown to mediate the destruction of the enterocytes in celiac disease (Hüe et al., 2004; Meresse et al., 2006). Interleukin (IL)-15 is also implicated in this process. This cytokine can be induced by gliadin (Maiuri et al., 2000; Maiuri et al., 2003; Mention et al., 2003). Gliadin and the α-gliadin derived peptide, p31-49 are shown to induce expression of MICA on epithelial cells, probably via IL-15 (Hüe et al., 2004). Up-regulation of NKG2D on the IELs is also mediated via IL-15 (Roberts et al., 2001; Meresse et al., 2004). The interaction between NKG2D and MICA leads to enterocyte killing, but no proliferation or cytokine secretion (Hüe et al., 2004). Recently Meresse et al demonstrated that CD94-NKG2C recognizing HLA-E on epithelial cells is expressed on IELs in celiac disease patients (Meresse et al., 2006). The stress-induced molecule HLA-E is induced by IFN-γ (Perera et al., 2007), which in turn is produced by gluten reactive $CD4^+$ T cells in lamina propria and by the IELs. This interaction between CD94-NKG2C and HLA-E can induce cytokine secretion and proliferation, as well as cytolysis, and helps to explain the IFN-γ production and the expansion of IELs.

Treatment of celiac disease

The only available treatment of celiac disease today is to adhere to a life long gluten-free diet (GFD). Strict compliance is essential for mucosal recovery and prevention of complications. Since gluten is a very common ingredient in our diet, this presents a big challenge for celiac disease patients and adherence to GFD is estimated to be only 40-80% (Högberg et al., 2003; Leffler et al., 2007). Furthermore, the gluten-free products are often more expensive and many find the diet unpalatable. A large fraction of the celiac disease patients cannot adhere to this strict gluten-free diet. Especially those who are asymptomatic may find it hard. There are also some patients that do not respond to dietary therapy. They therefore have a strong desire for alternative therapies. If a treatment was available so that some gluten could be

ingested, their quality of life would improve. However, to be on a gluten-free diet is safe and effective and this must be taken into account for new therapies.

AIMS OF THE STUDY

The focus of this thesis has been to characterize peptide binding to HLA-DQ2 in detail and to develop blocking agents for the treatment of celiac disease.

The specific aims of the present studies were to:

1. Investigate the relative contribution of each main chain amide hydrogen bond interaction between HLA-DQ2 and peptide.
2. Examine the hydrogen bond between HLA-DQ2 and the P1 amide nitrogen in peptides without Pro at this position.
3. Characterize HLA-DQ2.5 binding frames of DQ2 gluten T cell epitopes
4. Investigate peptide binding of DQ2 gluten T cell epitopes to HLA-DQ2.2
5. Design and synthesize cyclic peptides for the treatment of celiac disease
6. Examine the blocking peptides for binding to HLA-DQ2 and inhibition of T cell recognition of antigen
7. Investigate which HLA class II molecules the DQ2 gluten T cell epitopes recognized by T cells of celiac disease patients are able to bind to.

SUMMARY OF PAPERS

Paper I

Main chain hydrogen bond interactions in the binding of proline-rich gluten peptides to the celiac disease-associated HLA-DQ2 molecule

Elin Bergseng, Jiang Xia, Chu-Young Kim, Chaitan Khosla, Ludvig M. Sollid

This study demonstrates that there are hydrogen bonds important for binding of peptides to HLA-DQ2 at positions P2 and P4. There are also hydrogen bonds at P6 and P9, but these are less important, and there is no evidence for a hydrogen bond in position P1. These observations explain the non-random positioning of Pro in gluten T cell epitopes when bound to the HLA-DQ2 molecule. They also explain why HLA-DQ2 can accommodate Pro residues at position P1 with no penalty despite the lack of hydrogen bond to the peptide backbone. This is a unique feature of HLA-DQ2 and is likely a key parameter for preferential binding of Pro-rich peptides and the development of celiac disease. The information of the paper is also useful for the design of agents that block presentation of peptides by HLA-DQ2 which potentially can be used for the treatment of celiac disease.

Paper II

Refining the rules of gliadin T cell epitope binding to the disease-associated DQ2 molecule in celiac disease: importance of proline spacing and glutamine deamidation

Shuo-Wang Qiao, Elin Bergseng, Øyvind Molberg, Günther Jung, Burkhard Fleckenstein, Ludvig M. Sollid

In this study two novel epitopes in γ-gliadin were characterized and fine mapping of the HLA-DQ2.5 binding frame of these and three previously reported epitopes were undertaken. Alignment of the gluten T cell epitopes revealed Pro residues in positions P1, P3, P5, P6 and P8, but never in positions P2, P4, P7 and P9. It was also shown that several Gln residues in these epitopes were targeted by TG2, but only Glu residues in positions P4, P6 and rarely P7 was important for T cell recognition. In addition, nine DQ2 gluten T cell epitopes were screened for binding to HLA-DQ2.2

and presentation by HLA-DQ2.2 APC. The majority of these epitopes were recognized by T cells and this ability was not related to the absence of Pro in the P3 pocket as suggested earlier. However, the HLA-DQ2.5 presentation is more efficient than HLA-DQ2.2 presentation of the DQ2.5 gluten T cell epitopes and this could be the reason for the differential risk associated with these two molecules.

Paper III

Cyclic and dimeric gluten peptide analogues inhibiting DQ2-mediated antigen presentation in celiac disease

Jiang Xia*, Elin Bergseng*, Burkhard Fleckenstein, Matthew Siegel, Chu-Young Kim, Chaitan Khosla, Ludvig M. Sollid

*These authors contributed equally to the work.

In this study cyclic and dimeric peptides were studied as blockers of HLA-DQ2 -mediated antigen presentation in celiac disease. Our results demonstrate proof-of-principle of these peptides for inhibition of HLA-DQ2-mediated presentation of gluten T cell epitopes. These blocking agents therefore represent a promising class of compounds for MHC blockers. The future challenge in relation to celiac disease will be to generate compounds that bind to DQ2 with higher affinities.

Paper IV

Binding of gluten T cell epitopes to various HLA class II molecules

Elin Bergseng, John Sidney, Alessandro Sette, Ludvig M. Sollid

In this study we aimed to shed light on peptide determinant selection by HLA-DQ2 as a factor to explain the strong HLA association in celiac disease. A panel of peptides representing DQ2 gluten T cell epitopes was tested for binding to various HLA class II molecules. Three different experimental approaches were used for assessing binding. The results demonstrated that the gluten T cell epitopes mainly bind to HLA-DQ2 and not to other HLA class II molecules. This contributes to explaining the strong HLA association with HLA-DQ2 in celiac disease.

METHODOLOGICAL CONSIDERATIONS

Peptide binding assays

Competitive MHC class II peptide binding assay

We have used a traditional competitive inhibition assay with detergent solubilized MHC class II molecules (Buus et al., 1986) or recombinant soluble HLA-DR3 molecule (Quarsten et al., 2001; Fallang et al., 2007) in our studies to assess the binding efficiency of peptides. This is an indirect assay where the binding capacity of the peptide is measured as the ability to inhibit binding of a ^{125}I-labeled indicator peptide. The high affinity indicator peptide was labeled by the chloramine T method (Greenwood and Hunter, 1963). The affinity purified MHC class II molecules were incubated with radiolabeled indicator peptide in the presence of increasing concentration of peptide to be tested, a mixture of protease inhibitors and a citrate-phosphate pH buffer at 37°C as described by Johansen et al (Johansen et al., 1994). Complexes of peptide and MHC class II molecule were separated from free peptides by a spin column chromatography technique (Buus et al., 1995). The results are given as the IC_{50} value, which is the concentration of the peptide needed to inhibit 50% of the binding of the high affinity radiolabeled indicator peptide. The lower the IC_{50} value, the better the peptide bind to the MHC class II molecule. In the experiments where recombinant soluble HLA-DR3 molecules were used, HLA-DM was added to increase the peptide exchange and thereby reducing the amount of MHC class II molecules needed for achieving 10-15% binding of the indicator peptide. Under equilibrium conditions where [labeled indicator peptide] < [MHC class II molecule] and $IC_{50} \geq$ [MHC class II molecule], the measured IC_{50} values are reasonable approximations of the K_D values. Due to a sustained release of endogenously bound peptides from the naturally purified MHC class II molecules, complete equilibrium has likely not been reached in many of these assays, which is a weakness in the assessment of peptide binding.

For comparison of peptides that have been tested on different days or with different preparations of affinity purified MHC class II molecules, relative binding values

appear to be more accurate and consistent than comparing the IC_{50} values, since IC_{50} values varies between two experiments.

Peptide binding in an antibody based catch assay with cell lysates

Peptide binding was also assayed in an antibody based catch assay modified from Stepniak et al (Stepniak et al., 2005). This assay was used to test a panel of biotinylated gluten T cell epitopes for binding to various MHC class II molecules. MHC class II molecules in cell lysates of B-lymphoblastoid cell lines were caught either with a DQ-specific antibody (SPV-L3), a DR-specific antibody (L243) or a DP-specific antibody (B7/21). Binding of biotinylated gluten peptides was measured by time-resolved fluorometry using europium-labeled streptavidin. This assay is less labor some and less accurate than the competitive MHC class II peptide binding assay described above. However, it has the ability to screen many different MHC class II molecules including those of the DRB3, DRB4 and DRB5 loci. The amount of MHC class II molecules in the different cell lines was not quantified. Comparison between cell lines and MHC class II molecules should therefore be done with caution.

Competitive inhibition in an antibody based catch assay

This assay is similar to the competitive MHC class II peptide binding assay described above. However, while the unbound peptides are separated from bound peptides by a spin column chromatography technique in the assay described above, MHC binding of the radiolabeled peptide was determined by capturing peptide-MHC complexes on LB3.1 (anti-HLA-DRA) antibody coated Lumitrac 600 plates and measuring bound cpm.

Peptide exchange assay

Recombinant soluble HLA-DQ2 molecules were treated with thrombin to release the covalently linked DQ2-α-I-gliadin peptide and incubated with fluorescein-conjugated peptide in a 25:1 ratio at 37°C in PBS with a citrate-phosphate buffer (pH 5.5 or pH 7.3). Peptide binding was measured by high performance size exclusion chromatography (HPSEC). The fluorescence signal was recorded and the peak areas

corresponding to peptide-MHC complex and the free peptide were used to calculate the percentage of bound peptide. In this assay, directly labeled peptides are required.

Dissociation experiments

In the dissociation experiment, detergent solubilized HLA-DQ2 molecules were loaded with a radiolabeled peptide. These peptide-DQ2 complexes were isolated by the spin column chromatography technique (Buus et al., 1995) and then incubated at pH 5.2 at 37°C. Samples were taken at various time points and separated on the spin columns. The fraction of bound peptide was calculated. The experiments were performed without a competing unlabeled peptide. However, the effect of competing unlabeled peptide in excess was examined and the dissociation rates were found to be similar.

T cell proliferation assay

T cell proliferation as a result of T cell receptor recognition of antigen was assessed in a ^{3}H-thymidine incorporation assay. Fixed or γ-irradiated HLA-DR3/DQ2 homozygous Epstein Barr virus transformed B lymphoblastoid cell lines were used as APC. Antigen and APC were incubated overnight to allow for presentation of the peptide before T cells were added. In the T cell blocking experiments, the blocking peptide and antigen were incubated overnight with APC. These assays were commonly performed with fixed APC. However, we showed in paper III that a dimeric peptide (peptide 8) was blocking antigen presentation with irradiated APC. In the blocking experiments a T cell clone (TCC) with a specificity other than the one the peptide was based on were chosen to avoid the possibility of altered peptide ligand phenomena. For the cyclic peptides based on the DQ2-α-I-gliadin epitope in paper III, a DQ2-γ-II specific TCC was chosen for testing. An experiment with an HLA-DR3 restricted TCC specific for *Mycobacterium tuberculosis* 65-kDa Hsp 3-13 epitope was included to test for unspecific effects in the blocking assay. An HLA-DR3 restricted T cell clone was chosen since the APC express both HLA-DR3 and -DQ2.

Expression of recombinant soluble peptide-MHC class II molecules in a baculovirus expression system

Recombinant soluble HLA-DQ2 and -DR3 molecules were expressed in the baculovirus expression system as previously described (Quarsten et al., 2001; Fallang et al., 2007). A complementary Fos/Jun leucine zipper dimerization motifs replaced the transmembrane region of the α and β chains and a peptide was covalently linked to the N-terminus of the β chain by a linker including a thrombin site. The molecules were expressed in expresSF^{+} serum free insect cells to avoid contamination of serum proteins. Prior to use in the peptide binding assays, the molecules were treated with thrombin.

Peptide synthesis of cyclic peptides

The linear peptides were synthesized by Fmoc/PyBOP chemistry on TentaGEL SRAM resin using an automated synthesizer. The N-termini were acetylated before the peptides were cyclized on the resin. The Lys residues were protected with 1-(4,4-dimethyl-2,6-dioxycyclohex-1-ylidene)ethyl (Dde) groups, which was selectively removed with 1% hydrazine in DMF. The peptides were cyclized by coupling dicarboxy polyethylene glycol (PEG) between the two Lys residues. In peptide **4** (terminology from paper III) bis-dPEG7-acid (HO2CCH2CH2(CH2CH2O)7CO2H) with a defined length was used to cyclize the peptide, while peptide **5** was cyclized by poly(ethyleneglycol)bis(carboxymethyl)ether (HO2CCH2(OCH2CH2)OCH2CO2H) with an average MW of 600. A mix of the dicarboxylic acid, PyBOP, diisopropylethylamine and the peptide on resin were incubated overnight. A completed reaction was verified by a negative ninhydrin test. Peptides were deprotected and cleaved from the resin with 95% TFA and 2.5% water and 2.5% triisopropylsilan as scavengers. Precipitation of peptides was carried out by the addition of icecold *tert*-butyl methyl ether and thereafter the peptides were dissolved in water and lyophilized. The peptides were analyzed by reverse phase HPLC and MALDI-TOF mass spectrometry. Peptides were dissolved, aliquoted and lyophilized before storage at -20°C. Notably, peptide 5 contains a mixture of peptides with various length of the PEG linker.

GENERAL DISCUSSION

HLA association in celiac disease

The HLA association in celiac disease can be explained by the ability of HLA-DQ2 to bind the Pro- and Gln-rich gluten peptides that have survived the gastrointestinal digestion and have been deamidated by TG2. It has been demonstrated that HLA-DQ2 has a preference for binding peptides with amino acids with negative charges at the relative positions P4, P6 and P7 (Johansen et al., 1996; van de Wal et al., 1996), while HLA-DQ8 has a preference for binding peptides with negatively charged residues at relative positions P1 and P9 (Kwok et al., 1996; Godkin et al., 1997; Moustakas et al., 2000; Lee et al., 2001; Suri et al., 2005; Tollefsen et al., 2006). Gluten proteins naturally have remarkably few negatively charged amino acids. The HLA-DQ2 restricted presentation of gluten peptides was therefore a mystery until the observation of preferential recognition of deamidated gluten peptides by T cells of celiac lesions (Sjöström et al., 1998), and the discovery of TG2 being able to deamidate gluten peptides (Molberg et al., 1998; van de Wal et al., 1998a). In the x-ray crystal structure of HLA-DQ2 complexed with the DQ2-α-I-gliadin peptide, it was shown that the Glu in P6 participates in a hydrogen bonding network involving Lys-β71 of HLA-DQ2 (Kim et al., 2004). The Lys-β71 is positioned so that it can also interact with negatively charged residues in the P4 or P7 positions.

A network of hydrogen bonds between conserved MHC residues and the peptide main chain carbonyl oxygen and amide nitrogen groups contributes to much of the energy for binding of peptides to MHC class II molecules. Amide nitrogen hydrogen bonds are commonly in positions P1, P2, P4, P6 and P9 (reviewed in Nelson and Fremont, 1999). In paper I we demonstrated that the hydrogen bonds in position P2 and P4 are most important for binding in HLA-DQ2, while the hydrogen bonds in P6 and P9 made smaller contributions. Pro residues in the peptide are not able to engage in amide hydrogen bonding. As the DQ2-α-I-gliadin peptide has a Pro residue in P1, a hydrogen bond is therefore not seen in this position in the x-ray crystal structure. The Pro cannot act as a hydrogen bond donor in the peptide due to its tertiary nitrogen. However, we did not find any evidence of a hydrogen bond in this position when testing peptides without Pro at this position. This may explain why DQ2 can

accommodate Pro residues in this position without any penalty. As discussed in paper I, the reason for this could be the deletion of residue 53 in the α chain of HLA-DQ2. To further investigate this, a recombinant soluble HLA-DQ2 molecule with either Arg or Gly inserted in this position was constructed and expressed (Bergseng et al, unpublished). This may restore the hydrogen bond and a longer dissociation of the peptide is therefore expected. Unfortunately, the dissociation of the peptides tested was very fast. The insertion of an amino acid may possibly change the three dimensional structure of the molecule, thereby disrupting the binding groove. The positioning of Pro residues in the gluten peptides is important to avoid loss of any of the hydrogen bonds. In paper II it was demonstrated that Pro residues are found at positions P1, P3, P5, P6 and P8 and never at positions P2, P4, P7 and P9. This non random positioning of Pro residues explains the ability of DQ2 to accommodate the Pro-rich gluten peptides without any loss of binding energy with negatively charged anchor residues at P4, P6 or P7.

Alternative treatments for celiac disease

Detailed knowledge of the molecular mechanisms of celiac disease should allow us to rationally design new therapeutic agents to treat the disease. As HLA-DQ2 and -DQ8 are common among the general population and almost all celiac disease patients express HLA-DQ2 or -DQ8, these molecules are necessary but not sufficient for the development of celiac disease. This indicates that activation of gluten specific $CD4^+$ T cells by presentation of gluten peptides in context of HLA-DQ2 or -DQ8 is one of the key features in celiac disease development. Interference with activation of gluten specific T cells should therefore control the disease, and one can envisage several possible avenues to accomplish this (Green and Jabri, 2003; Sollid and Khosla, 2005).

To be acceptable, the therapies for celiac disease have to be safe, effective and affordable since a dietary alternative is already available. Potential treatment strategies can be divided into two classes, modifying gluten or modifying the immune response to gluten. To grow genetically modified wheat lacking the gluten T cell epitopes is one alternative. Another strategy is to breed wheat cultivars with low or absent levels of harmful gluten proteins with the baking quality of wheat species

commonly used today (Frisoni et al., 1995; Molberg et al., 2005; Spaenij-Dekking et al., 2005a; Spaenij-Dekking et al., 2005b). The gluten reactive T cells from intestinal biopsies can recognize a broad repertoire of gluten peptides and these differs from patient to patient. Wheat has complex genetics and all epitopes are not known. It will be difficult to delete all the genes encoding T cell stimulatory epitopes and still retain the baking quality. Genetic modifications also raise concerns about safety and ethics. The search for naturally occurring wheat variants with fewer T cell epitopes than common bread wheat used today could be a better starting material for detoxification.

Enzyme supplementation that can cleave the gluten peptides into short inactive sequences is also an approach for detoxification of gluten. Several endopeptidases have been described. A prolyl endopeptidase (PEP) that cleaves Pro-rich peptides was the first to be described (Shan et al., 2002; Hausch et al., 2002; Piper et al., 2004; Marti et al., 2005; Matysiak-Budnik et al., 2005). This enzyme is going to be tested in clinical trials in combination with a cysteine endoprotease (EP-B2) (Siegel et al., 2006; Gass et al., 2007). Another prolyl endopeptidase which has been described to be efficient is AN-PEP from *Aspergillus niger* (Stepniak et al., 2006). However, enzyme therapy has some disadvantages as well. The gluten in normal food may not be easily accessible and thus hard to degrade, so the efficiency of the oral proteases is questionable.

There are also several possibilities to modify the immune response to gluten. Blocking the binding site of HLA-DQ2 and -DQ8 will prevent the presentation of gluten peptides and thereby also T cell activation (Xia et al., 2006; Siegel et al., 2007; Kapoerchan et al., 2007). Peptide blockers will possibly have few side effects since many healthy individuals are homozygous for HLA alleles. Thus, immunosuppression or infections are unlikely. Inhibition of TG2 will prevent the production of deamidated immunogenic epitopes (Siegel et al., 2007; Siegel and Khosla, 2007). Inhibitors of TG2 may create unpredicted adverse effects since TG2 has a diverse biological role. However, TG2 knockout mice appeared to have a normal phenotype (De Laurenzi and Melino, 2001; Nanda et al., 2001). In addition, the efficiency is questionable since there are gluten T cell epitopes which seems not to be dependent on deamidation (Vader et al., 2002b; Dewar et al., 2006). Silencing of the gluten reactive T cells by soluble HLA-peptide complexes is another alternative, but this is

complicated by the broad repertoire of gluten T cell epitopes. It has also been suggested to induce tolerance by intranasal administration of gluten (Maurano et al., 2001; Senger et al., 2003). Cytokine therapy targeting IL-15, IL-10 and IFN-γ is also a possibility. IL-15 and IFN-γ play important roles in the celiac disease pathogenesis and their neutralization may have a therapeutic effect (Nilsen et al., 1995; Mention et al., 2003). Also, recombinant IL-10 may possibly down regulate the Th1 response dominating in celiac disease (Mulder et al., 2001; Salvati et al., 2005). Other possible targets are chemokines and adhesion molecules. These molecules assist in the recruitment of inflammatory cells to inflamed sites. Blockade of these molecules may therefore be an approach to reduce the number of inflammatory cells. However, an increased susceptibility to infections may be a potential side effect of this therapy. Other options may be to use NK receptor antagonists or zonulin antagonists (Fasano et al., 2000; Hüe et al., 2004; Meresse et al., 2004; Drago et al., 2006).

In paper III we studied cyclic and dimeric peptides as peptide blockers of HLA-DQ2. Blocking of peptide presentation by HLA molecules has been a suggested therapy for other HLA associated diseases (Sette et al., 1991). It is well documented that peptide blockers can effectively interfere with T cell activation both *in vitro* and *in vivo* (Adorini et al., 1988; Sakai et al., 1989; Hurtenbach et al., 1993). Interference of HLA class II restricted antigen presentation by altered peptide ligands (antagonists) has also been explored (Sloan-Lancaster and Allen, 1996; de Haan et al., 2005), but this approach would not be feasible for celiac disease due to the many gluten-derived T cell epitopes and the polyclonal nature of T cells recognizing the same epitope. This therapeutic concept of inhibiting antigen presentation to T cell was not developed further mainly because the delivery of the peptide based drug into the affected organs, like joints of rheumatoid arthritis and β cells in the pancreas in type 1 diabetes, proved difficult (Ishioka et al., 1994). However, the situation in celiac disease is different. The peptide blockers will be delivered onto the intestinal mucosa in parallel with the gluten peptides to out-compete. This is much easier compared with delivery to antigen presenting cells of the joints in rheumatoid arthritis or the islet β cells of the pancreas in type 1 diabetes.

Peptide based compounds as drugs

Peptides offer several advantages over small molecules (increased specificity and lower toxicity) and antibodies (better tissue penetration due to smaller size) as potential therapeutics. Nevertheless a number of key issues have hampered their use. In most cases peptides have poor in vivo stability, poor pharmacokinetics and poor bioavailability (Soares et al., 2007). Thus, most peptides cannot be administered orally as they are rapidly inactivated by gastrointestinal enzymes and poorly absorbed. Solubility may also be a problem depending on the amino acid sequence. In addition they are expensive to produce.

As gluten is ingested, it is subject to extensive proteolysis in the gastrointestinal tract by gastric, pancreatic and intestinal brush border membrane enzymes. It has, however, been shown that the gluten T cell epitopes are extremely stable against proteolysis (Hausch et al., 2002; Shan et al., 2002; Shan et al., 2005). The identification of a 33-mer peptide containing multiple copies of known T cell epitopes DQ2-α-I, DQ2-α-II and DQ2-α-III, were shown to survive extensive proteolysis (Shan et al., 2002). To overcome the problem of proteolytical instability, we have used a gliadin peptide as a starting point for our peptide blockers.

Gluten peptides must cross the intestinal epithelial barrier and reach the lamina propria where they are presented by antigen presenting cells to $CD4^+$ T cells. How this happen is still unclear. The intestinal epithelial barrier is normally almost impermeable to macromolecules. However, it has been demonstrated that untreated celiac disease patients have increased intestinal permeability (Hamilton et al., 1982) with a compromised tight junction system (Madara and Trier, 1980; van Elburg et al., 1993; Schulzke et al., 1998). It has been suggested that up-regulation of zonulin, a molecule claimed to affect tight junction function, could at least partly be responsible for the increased gut permeability (Fasano et al., 2000; Wang et al., 2000). Gliadin peptides are also shown to be taken up by enterocytes by endocytosis (Friis et al., 1992). Zimmer et al has demonstrated that the gliadins are translocated to late endosomes or lysosomes in untreated celiac disease patients (Zimmer et al., 1995). Later they showed that gliadin peptides were located in vacuoles and Golgi complexes in enterocytes of celiac disease patients with active disease (Zimmer et al., 1998).

Another alternative by which gluten peptides could be taken up is through direct sampling from the lumen by dendritic cells which is shown in mice (Rescigno et al., 2001; Niess et al., 2005). M cells and defects in the intestinal transport are also possible routes through which gliadin could be taken up. The uptake of the peptide blocker will probably use the same entrance as the gluten peptides.

Peptide blockers

A peptide blocker should not serve as a TCR ligand for any T cell as it then could elicit hypersensitivity reactions. Conceptually there are two ways this can be achieved. The peptide can be big and bulky thereby avoiding the docking of any TCR onto the peptide-MHC complex. Alternatively, the peptide can be small and invisible thereby allowing the TCR to dock onto the peptide-MHC complex, but avoiding TCR recognition (Figure 4).

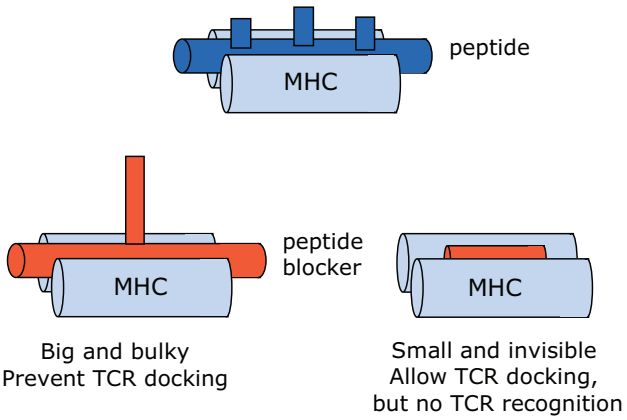

Figure 4. *Schematic representation of peptide and peptide blockers in the peptide binding groove of the HLA class II molecule.*

In paper III, two types of sterically hindered peptides were studied as peptide blockers; cyclic and dimeric peptides. The concept of cyclic peptides as peptide blockers is not new. It has been described by Bouvier et al for MHC class I molecules

(Bouvier and Wiley, 1996). It is unlikely that any TCR could dock onto a cyclic peptide when bound to an MHC molecule, and thus there should not be a problem of hypersensitivity reactions to cyclic blockers. Another advantage is that cyclic peptides are more proteolytically stable than the linear peptide (Matsoukas et al., 2005).

In paper III we demonstrated that our peptide blockers bind to HLA-DQ2, they inhibit T cell proliferation and they are specific for HLA-DQ2. However, the peptide blockers appear not to have sufficient efficiency to be used as therapeutic agents. This may be achieved by increasing binding affinity for HLA-DQ2. A peptide blocker needs to be potent since several studies have demonstrated that very few peptide-MHC complexes (1-400) can trigger antigen-specific T cells (Demotz et al., 1990; Harding and Unanue, 1990; Kimachi et al., 1997; Reay et al., 2000; Irvine et al., 2002).

Final comments

The use of peptide blockers as therapeutic compounds is favorable due to their easy access to the small intestine. However, the poor pharmacokinetic properties like stability and bioavailability of peptides as drugs has to be overcome. Thus a peptide blocker should bind with high affinity to DQ2, be stable against proteolysis and not be recognized by any T cell. To use a gluten peptide as starting point for a peptide blocker could solve the problem with proteolytic instability of peptides. The possibility of a hypersensitivity reaction to occur is reduced by using cyclic peptides as peptide blockers. The major problem is to increase the binding affinity of the peptide blocker. An effective peptide blocker should most likely have an affinity which is at least 100-fold higher than the gluten peptides. Several groups are working on how to increase the binding affinity. If such a high affinity and proteolytically resistant peptide is found, the concept of peptide blockers could be ready for testing in clinical trials.

REFERENCES

Adorini L, Muller S, Cardinaux F, Lehmann PV, Falcioni F and Nagy ZA (1988). In vivo competition between self peptides and foreign antigens in T-cell activation. *Nature* **334**:623-625.

Alizadeh BZ, Valdigem G, Coenen MJ, Zhernakova A, Franke B, Monsuur A, van Riel PL, Barrera P, Radstake TR, Roep BO, Wijmenga C and Koeleman BP (2007). Association analysis of functional variants of the *FcgRIIa* and *FcgRIIIa* genes with type 1 diabetes, celiac disease and rheumatoid arthritis. *Hum Mol Genet* **16**:2552-2559.

Arentz-Hansen H, Fleckenstein B, Molberg Ø, Scott H, Koning F, Jung G, Roepstorff P, Lundin KEA and Sollid LM (2004). The molecular basis for oat intolerance in patients with celiac disease. *PLoS Med* **1**:84-92.

Arentz-Hansen H, Körner R, Molberg Ø, Quarsten H, Vader W, Kooy YM, Lundin KE, Koning F, Roepstorff P, Sollid LM and McAdam SN (2000). The intestinal T cell response to α-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. *J Exp Med* **191**:603-612.

Arentz-Hansen H, McAdam SN, Molberg Ø, Fleckenstein B, Lundin KE, Jørgensen TJ, Jung G, Roepstorff P and Sollid LM (2002). Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. *Gastroenterology* **123**:803-809.

Babbitt BP, Allen PM, Matsueda G, Haber E and Unanue ER (1985). Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature* **317**:359-361.

Bakke O and Dobberstein B (1990). MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell* **63**:707-716.

Bielekova B and Martin R (2001). Antigen-specific immunomodulation via altered peptide ligands. *J Mol Med* **79**:552-565.

Bouvier M and Wiley DC (1996). Antigenic peptides containing large PEG loops designed to extend out of the HLA-A2 binding site form stable complexes with class I major histocompatibility complex molecules. *Proc Natl Acad Sci U S A* **93**:4583-4588.

Brown JH, Jardetzky TS, Gorga JC, Stern LJ, Urban RG, Strominger JL and Wiley DC (1993). Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* **364**:33-39.

Busch R, Cloutier I, Sékaly RP and Hämmerling GJ (1996). Invariant chain protects class II histocompatibility antigens from binding intact polypeptides in the endoplasmic reticulum. *EMBO J* **15**:418-428.

Buus S, Sette A, Colon SM, Jenis DM and Grey HM (1986). Isolation and characterization of antigen-Ia complexes involved in T cell recognition. *Cell* **47**:1071-1077.

Buus S, Stryhn A, Winther K, Kirkby N and Pedersen LO (1995). Receptor-ligand interactions measured by an improved spun column chromatography technique. A high efficiency and high throughput size separation method. *Biochim Biophys Acta* **1243**:453-460.

Chicz RM, Urban RG, Gorga JC, Vignali DA, Lane WS and Strominger JL (1993). Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J Exp Med* **178**:27-47.

Ciclitira PJ, Evans DJ, Fagg NL, Lennox ES and Dowling RH (1984). Clinical testing of gliadin fractions in coeliac patients. *Clin Sci (Lond)* **66**:357-364.

Corrao G, Corazza GR, Bagnardi V, Brusco G, Ciacci C, Cottone M, Sategna GC, Usai P, Cesari P, Pelli MA, Loperfido S, Volta U, Calabro A and Certo M (2001). Mortality in patients with coeliac disease and their relatives: a cohort study. *Lancet* **358**:356-361.

Cresswell P (1996). Invariant chain structure and MHC class II function. *Cell* **84**:505-507.

de Haan EC, Moret EE, Wagenaar-Hilbers JPA, Liskamp RMJ and Wauben MHM (2005). Possibilities and limitations in the rational design of modified peptides for T cell mediated immunotherapy. *Mol Immunol* **42**:365-373.

De Laurenzi V and Melino G (2001). Gene disruption of tissue transglutaminase. *Mol Cell Biol* **21**:148-155.

Demotz S, Grey HM and Sette A (1990). The minimal number of class II MHC-antigen complexes needed for T cell activation. *Science* **249**:1028-1030.

Denzin LK and Cresswell P (1995). HLA-DM induces CLIP dissociation from MHC Class II $\alpha\beta$ dimers and facilitates peptide loading. *Cell* **82**:155-165.

Denzin LK, SantAngelo DB, Hammond C, Surman MJ and Cresswell P (1997). Negative regulation by HLA-DO of MHC class II-restricted antigen processing. *Science* **278**:106-109.

Dessen A, Lawrence CM, Cupo S, Zaller DM and Wiley DC (1997). X-ray crystal structure of HLA-DR4 (DRA*0101, DRB1*0401) complexed with a peptide from human collagen II. *Immunity* **7**:473-481.

Dewar DH, Amato M, Ellis HJ, Pollock EL, Gonzalez-Cinca N, Wieser H and Ciclitira PJ (2006). The toxicity of high molecular weight glutenin subunits of wheat to patients with coeliac disease. *Eur J Gastroenterol Hepatol* **18**:483-491.

Dicke WK, Weijers HA and van de Kamer JH (1953). Coeliac disease. II. The presence in wheat of a factor having a deleterious effect in cases of coeliac disease. *Acta Paediatr* **42**:34-42.

Djilali-Saiah I, Schmitz J, Harfouch-Hammoud E, Mougenot JF, Bach JF and Caillat-Zucman S (1998). CTLA-4 gene polymorphism is associated with predisposition to coeliac disease. *Gut* **43**:187-189.

Drago S, El Asmar R., Di Pierro M., Clemente MG, Tripathi A, Sapone A, Thakar M, Iacono G, Carroccio A, D'Agate C, Not T, Zampini L, Catassi C and Fasano A (2006). Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scand J Gastroenterol* **41**:408-419.

Dube C, Rostom A, Sy R, Cranney A, Saloojee N, Garritty C, Sampson M, Zhang L, Yazdi F, Mamaladze V, Pan I, Macneil J, Mack D, Patel D and Moher D (2005). The prevalence of celiac disease in average-risk and at-risk Western European populations: a systematic review. *Gastroenterology* **128**:S57-S67.

Fallang LE, Roh S, Holm A, Bergseng E, Bandyopadhyay A, Fleckenstein B, Yoon T, Mellins ED and Sollid LM. HLA-DQ2 interacts atypically with both invariant chain and HLA-DM. Submitted.

Fasano A and Catassi C (2001). Current approaches to diagnosis and treatment of celiac disease: an evolving spectrum. *Gastroenterology* **120**:636-651.

Fasano A, Not T, Wang W, Uzzau S, Berti I, Tommasini A and Goldblum SE (2000). Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. *Lancet* **355**:1518-1519.

Fleckenstein B, Molberg Ø, Qiao SW, Schmid DG, von der Mülbe F, Elgstøen K, Jung G and Sollid LM (2002). Gliadin T cell epitope selection by tissue transglutaminase in celiac disease. Role of enzyme specificity and pH influence on the transamidation versus deamidation process. *J Biol Chem* **277**:34109-34116.

Fremont DH, Hendrickson WA, Marrack P and Kappler J (1996). Structures of an MHC class II molecule with covalently bound single peptides. *Science* **272**:1001-1004.

Fremont DH, Monnaie D, Nelson CA, Hendrickson WA and Unanue ER (1998). Crystal structure of I-A(k) in complex with a dominant epitope of lysozyme. *Immunity* **8**:305-317.

Friis S, Dabelsteen E, Sjöström H, Noren O and Jarnum S (1992). Gliadin uptake in human enterocytes. Differences between coeliac patients in remission and control individuals. *Gut* **33**:1487-1492.

Frisoni M, Corazza GR, Lafiandra D, De Ambrogio E., Filipponi C, Bonvicini F, Borasio E, Porceddu E and Gasbarrini G (1995). Wheat deficient in gliadins: promising tool for treatment of coeliac disease. *Gut* **36**:375-378.

Gass J, Bethune MT, Siegel M, Spencer A and Khosla C (2007). Combination enzyme therapy for gastric digestion of dietary gluten in patients with celiac sprue. *Gastroenterology* **133**:472-480.

Gee S (1888). On the coeliac disease. *St Bart Hosp Rep* **24**:17-20.

Germain RN (1994). MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell* **76**:287-299.

Ghosh P, Amaya M, Mellins E and Wiley DC (1995). The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. *Nature* **378**:457-462.

Godkin A, Friede T, Davenport M, Stevanovic S, Willis A, Jewell D, Hill A and Rammensee HG (1997). Use of eluted peptide sequence data to identify the binding characteristics of peptides to the insulin-dependent diabetes susceptibility allele HLA-DQ8 (DQ 3.2). *Int Immunol* **9**:905-911.

Greco L, Babron MC, Corazza GR, Percopo S, Sica R, Clot F, Fulchignoni-Lataud MC, Zavattari P, Momigliano-Richiardi P, Casari G, Gasparini P, Tosi R, Mantovani V, De VS, Iacono G, D'Alfonso A, Selinger-Leneman H, Lemainque A, Serre JL and Clerget-Darpoux F (2001). Existence of a genetic risk factor on chromosome 5q in Italian coeliac disease families. *Ann Hum Genet* **65**:35-41.

Greco L, Romino R, Coto I, Di Cosmo N, Percopo S, Maglio M, Paparo F, Gasperi V, Limongelli MG, Cotichini R, D'Agate C, Tinto N, Sacchetti L, Tosi R and Stazi MA (2002). The first large population based twin study of coeliac disease. *Gut* **50**:624-628.

Green PHR and Jabri B (2003). Coeliac disease. *Lancet* **362**:383-391.

Greenwood FC and Hunter WM (1963). Preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem J* **89**:114-123.

Haboubi NY, Taylor S and Jones S (2006). Coeliac disease and oats: a systematic review. *Postgrad Med J* **82**:672-678.

Hamilton I, Cobden I, Rothwell J and Axon AT (1982). Intestinal permeability in coeliac disease: the response to gluten withdrawal and single-dose gluten challenge. *Gut* **23**:202-210.

Harding CV and Unanue ER (1990). Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation. *Nature* **346**:574-576.

Hausch F, Shan L, Santiago NA, Gray GM and Khosla C (2002). Intestinal digestive resistance of immunodominant gliadin peptides. *Am J Physiol Gastrointest Liver Physiol* **283**:G996-G1003.

He XL, Radu C, Sidney J, Sette A, Ward ES and Garcia KC (2002). Structural snapshot of aberrant antigen presentation linked to autoimmunity: The immunodominant epitope of MBP complexed with I-A(u). *Immunity* **17**:83-94.

Henderson KN, Tye-Din JA, Reid HH, Chen Z, Borg NA, Beissbarth T, Tatham A, Mannering SI, Purcell AW, Dudek NL, van Heel DA, McCluskey J, Rossjohn J and Anderson RP (2007). A structural and immunological basis for the role of human leukocyte antigen DQ8 in celiac disease. *Immunity* **27**:23-34.

Högberg L, Grodzinsky E and Stenhammar L (2003). Better dietary compliance in patients with coeliac disease diagnosed in early childhood. *Scand J Gastroenterol* **38**:751-754.

Horton R, Wilming L, Rand V, Lovering RC, Bruford EA, Khodiyar VK, Lush MJ, Povey S, Talbot CC, Jr., Wright MW, Wain HM, Trowsdale J, Ziegler A and Beck S (2004). Gene map of the extended human MHC. *Nat Rev Genet* **5**:889-899.

Hüe S, Mention JJ, Monteiro RC, Zhang SL, Cellier C, Schmitz J, Verkarre V, Fodil N, Bahram S, Cerf-Bensussan N and Caillat-Zucman S (2004). A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. *Immunity* **21**:367-377.

Hunt DF, Michel H, Dickinson TA, Shabanowitz J, Cox AL, Sakaguchi K, Appella E, Grey HM and Sette A (1992). Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-Ad. *Science* **256**:1817-1820.

Hurtenbach U, Lier E, Adorini L and Nagy ZA (1993). Prevention of autoimmune diabetes in non-obese diabetic mice by treatment with a class II major histocompatibility complex-blocking peptide. *J Exp Med* **177**:1499-1504.

Irvine DJ, Purbhoo MA, Krogsgaard M and Davis MM (2002). Direct observation of ligand recognition by T cells. *Nature* **419**:845-849.

Ishioka GY, Adorini L, Guery JC, Gaeta FCA, Lafond R, Alexander J, Powell MF, Sette A and Grey HM (1994). Failure to demonstrate long-lived MHC saturation both in vitro and in vivo - Implications for therapeutic potential of MHC-blocking peptides. *J Immunol* **152**:4310-4319.

Jardetzky TS, Brown JH, Gorga JC, Stern LJ, Urban RG, Strominger JL and Wiley DC (1996). Crystallographic analysis of endogenous peptides associated with HLA-DR1 suggests a common, polyproline II-like conformation for bound peptides. *Proc Natl Acad Sci U S A* **93**:734-738.

Jensen PE (1995). Antigen unfolding and disulfide reduction in antigen presenting cells. *Semin Immunol* **7**:347-353.

Johansen BH, Buus S, Vartdal F, Viken H, Eriksen JA, Thorsby E and Sollid LM (1994). Binding of peptides to HLA-DQ molecules: peptide binding properties of the disease-associated HLA-DQ(α1*0501, β1*0201) molecule. *Int Immunol* **6**:453-461.

Johansen BH, Vartdal F, Eriksen JA, Thorsby E and Sollid LM (1996). Identification of a putative motif for binding of peptides to HLA-DQ2. *Int Immunol* **8**:177-182.

Kapoerchan VV, Wiesner M, Overhand M, van der Marel GA, Koning F and Overkleeft HS (2007). Design of azidoproline containing gluten peptides to suppress CD4(+) T-cell responses associated with Celiac disease. *Bioorg Med Chem* Epub ahead of print doi:10.1016/j.bmc.2007.10.091.

Karell K, Louka AS, Moodie SJ, Ascher H, Clot F, Greco L, Ciclitira PJ, Sollid LM and Partanen J (2003). HLA types in celiac disease patients not carrying the

DQA1*05-DQB1*02 (DQ2) heterodimer: results from the european genetics cluster on celiac disease. *Hum Immunol* **64**:469-477.

Kim CY, Quarsten H, Bergseng E, Khosla C and Sollid LM (2004). Structural basis for HLA-DQ2-mediated presentation of gluten epitopes in celiac disease. *Proc Natl Acad Sci U S A* **101**:4175-4179.

Kimachi K, Croft M and Grey HM (1997). The minimal number of antigen-major histocompatibility complex class II complexes required for activation of naive and primed T cells. *Eur J Immunol* **27**:3310-3317.

Kropshofer H, Arndt SO, Moldenhauer G, Hammerling GJ and Vogt AB (1997). HLA-DM acts as a molecular chaperone and rescues empty HLA-DR molecules at lysosomal pH. *Immunity* **6**:293-302.

Kwok WW, Domeier ML, Raymond FC, Byers P and Nepom GT (1996). Allele-specific motifs characterize HLA-DQ interactions with a diabetes-associated peptide derived from glutamic acid decarboxylase. *J Immunol* **156**:2171-2177.

Latek RR, Suri A, Petzold SJ, Nelson CA, Kanagawa O, Unanue ER and Fremont DH (2000). Structural basis of peptide binding and presentation by the type I diabetes-associated MHC class II molecule of NOD mice. *Immunity* **12**:699-710.

Lee KH, Wucherpfennig KW and Wiley DC (2001). Structure of a human insulin peptide-HLA-DQ8 complex and susceptibility to type I diabetes. *Nat Immunol* **2**:501-507.

Leffler DA, Edwards-George J, Dennis M, Schuppan D, Cook F, Franko DL, Blom-Hoffman J and Kelly CP (2007). Factors that influence adherence to a gluten-free diet in adults with celiac disease. *Dig Dis Sci* Epub ahead of print doi:10.1007/s10620-007-0055-3.

Liljedahl M, Kuwana T, Fung-Leung WP, Jackson MR, Peterson PA and Karlsson L (1996). HLA-DO is a lysosomal resident which requires association with HLA-DM for efficient intracellular transport. *EMBO J* **15**:4817-4824.

Liljedahl M, Winqvist O, Surh CD, Wong P, Ngo K, Teyton L, Peterson PA, Brunmark A, Rudensky AY, Fung-Leung WP and Karlsson L (1998). Altered antigen presentation in mice lacking H2-O. *Immunity* **8**:233-243.

Liu J, Juo SH, Holopainen P, Terwilliger J, Tong X, Grunn A, Brito M, Green P, Mustalahti K, Maki M, Gilliam TC and Partanen J (2002a). Genomewide linkage analysis of celiac disease in Finnish families. *Am J Hum Genet* **70**:51-59.

Liu XQ, Dai SD, Crawford F, Fruge R, Marrack P and Kappler J (2002b). Alternate interactions define the binding of peptides to the MHC molecule IA(b). *Proc Natl Acad Sci U S A* **99**:8820-8825.

Lundin KE, Scott H, Fausa O, Thorsby E and Sollid LM (1994). T cells from the small intestinal mucosa of a DR4, DQ7/DR4, DQ8 celiac disease patient preferentially recognize gliadin when presented by DQ8. *Hum Immunol* **41**:285-291.

Lundin KE, Scott H, Hansen T, Paulsen G, Halstensen TS, Fausa O, Thorsby E and Sollid LM (1993). Gliadin-specific, HLA-DQ(α1*0501,β1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. *J Exp Med* **178**:187-196.

Madara JL and Trier JS (1980). Structural abnormalities of jejunal epithelial cell membranes in celiac sprue. *Lab Invest* **43**:254-261.

Madden DR (1995). The three-dimensional structure of peptide-MHC complexes. *Annu Rev Immunol* **13**:587-622.

Maiuri L, Ciacci C, Auricchio S, Brown V, Quaratino S and Londei M (2000). Interleukin 15 mediates epithelial changes in celiac disease. *Gastroenterology* **119**:996-1006.

Maiuri L, Ciacci C, Ricciardelli I, Vacca L, Raia V, Auricchio S, Picard J, Osman M, Quaratino S and Londei M (2003). Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. *Lancet* **362**:30-37.

Mäki M and Collin P (1997). Coeliac disease. *Lancet* **349**:1755-1759.

Mäki M, Mustalahti K, Kokkonen J, Kulmala P, Haapalahti M, Karttunen T, Ilonen J, Laurila K, Dahlbom I, Hansson T, Hopfl P and Knip M (2003). Prevalence of celiac disease among children in Finland. *N Engl J Med* **348**:2517-2524.

Marti T, Molberg O, Li Q, Gray GM, Khosla C and Sollid LM (2005). Prolyl endopeptidase-mediated destruction of T cell epitopes in whole gluten: chemical and immunological characterization. *J Pharmacol Exp Ther* **312**:19-26.

Matsoukas J, Apostolopoulos V, Kalbacher H, Papini AM, Tselios T, Chatzantoni K, Biagioli T, Lolli F, Deraos S, Papathanassopoulos P, Troganis A, Mantzourani E, Mavromoustakos T and Mouzaki A (2005). Design and synthesis of a novel potent myelin basic protein epitope 87-99 cyclic analogue: enhanced stability and biological properties of mimics render them a potentially new class of immunomodulators. *J Med Chem* **48**:1470-1480.

Matysiak-Budnik T, Candalh C, Cellier C, Dugave C, Namane A, Vidal-Martinez T, Cerf-Bensussan N and Heyman M (2005). Limited efficiency of prolyl-endopeptidase in the detoxification of gliadin peptides in celiac disease. *Gastroenterology* **129**:786-796.

Maurano F, Siciliano RA, De GB, Luongo D, Mazzeo MF, Troncone R, Auricchio S and Rossi M (2001). Intranasal administration of one alpha gliadin can downregulate the immune response to whole gliadin in mice. *Scand J Immunol* **53**:290-295.

Mention JJ, Ben Ahmed M, Begue B, Barbe U, Verkarre V, Asnafi V, Colombel JF, Cugnenc PH, Ruemmele FM, McIntyre E, Brousse N, Cellier C and Cerf-Bensussan N (2003). Interleukin 15: A key to disrupted intraepithelial lymphocyte homeostasis and lymphomagenesis in celiac disease. *Gastroenterology* **125**:730-745.

Meresse B, Chen ZG, Ciszewski C, Tretiakova M, Bhagat G, Krausz TN, Raulet DH, Lanier LL, Groh V, Spies T, Ebert EC, Green PH and Jabri B (2004). Coordinated

induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity* **21**:357-366.

Meresse B, Curran SA, Ciszewski C, Orbelyan G, Setty M, Bhagat G, Lee L, Tretiakova M, Semrad C, Kistner E, Winchester RJ, Braud V, Lanier LL, Geraghty DE, Green PH, Guandalini S and Jabri B (2006). Reprogramming of CTLs into natural killer-like cells in celiac disease. *J Exp Med* **203**:1343-1355.

Molberg Ø, Flaete NS, Jensen T, Lundin KEA, Arentz-Hansen H, Anderson OD, Uhlen AK and Sollid LM (2003). Intestinal T-cell responses to high-molecular-weight glutenins in celiac disease. *Gastroenterology* **125**:337-344.

Molberg Ø, Kett K, Scott H, Thorsby E, Sollid LM and Lundin KE (1997). Gliadin specific, HLA DQ2-restricted T cells are commonly found in small intestinal biopsies from coeliac disease patients, but not from controls. *Scand J Immunol* **46**:103-108.

Molberg Ø, McAdam SN, Körner R, Quarsten H, Kristiansen C, Madsen L, Fugger L, Scott H, Noren O, Roepstorff P, Lundin KE, Sjöström H and Sollid LM (1998). Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat Med* **4**:713-717.

Molberg Ø, Uhlen AK, Jensen T, Flaete NS, Fleckenstein B, Arentz-Hansen H, Raki M, Lundin KE and Sollid LM (2005). Mapping of gluten T-cell epitopes in the bread wheat ancestors: implications for celiac disease. *Gastroenterology* **128**:393-401.

Monsuur AJ, de Bakker PI, Alizadeh BZ, Zhernakova A, Bevova MR, Strengman E, Franke L, van't SR, van Belzen MJ, Lavrijsen IC, Diosdado B, Daly MJ, Mulder CJ, Mearin ML, Meijer JW, Meijer GA, van OE, Wapenaar MC, Koeleman BP and Wijmenga C (2005). Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. *Nat Genet* **37**:1341-1344.

Moustakas AK, van de WY, Routsias J, Kooy YM, van VP, Drijfhout JW, Koning F and Papadopoulos GK (2000). Structure of celiac disease-associated HLA-DQ8 and non-associated HLA-DQ9 alleles in complex with two disease-specific epitopes. *Int Immunol* **12**:1157-1166.

Mulder CJ, Wahab PJ, Meijer JW and Metselaar E (2001). A pilot study of recombinant human interleukin-10 in adults with refractory coeliac disease. *Eur J Gastroenterol Hepatol* **13**:1183-1188.

Nanda N, Iismaa SE, Owens WA, Husain A, Mackay F and Graham RM (2001). Targeted inactivation of Gh/tissue transglutaminase II. *J Biol Chem* **276**:20673-20678.

Nelson CA and Fremont DH (1999). Structural principles of MHC class II antigen presentation. *Rev Immunogenet* **1**:47-59.

Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, Vyas JM, Boes M, Ploegh HL, Fox JG, Littman DR and Reinecker HC (2005). CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* **307**:254-258.

Nilsen EM, Jahnsen FL, Lundin KE, Johansen FE, Fausa O, Sollid LM, Jahnsen J, Scott H and Brandtzaeg P (1998). Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. *Gastroenterology* **115**:551-563.

Nilsen EM, Lundin KE, Krajci P, Scott H, Sollid LM and Brandtzaeg P (1995). Gluten specific, HLA-DQ restricted T cells from coeliac mucosa produce cytokines with Th1 or Th0 profile dominated by interferon gamma. *Gut* **37**:766-776.

Nistico L, Fagnani C, Coto I, Percopo S, Cotichini R, Limongelli MG, Paparo F, D'Alfonso S, Giordano M, Sferlazzas C, Magazzu G, Momigliano-Richiardi P, Greco L and Stazi MA (2006). Concordance, disease progression, and heritability of coeliac disease in Italian twins. *Gut* **55**:803-808.

Perera L, Shao L, Patel A, Evans K, Meresse B, Blumberg R, Geraghty D, Groh V, Spies T, Jabri B and Mayer L (2007). Expression of nonclassical class I molecules by intestinal epithelial cells. *Inflamm Bowel Dis* **13**:298-307.

Peters PJ, Neefjes JJ, Oorschot V, Ploegh HL and Geuze HJ (1991). Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* **349**:669-676.

Petronzelli F, Bonamico M, Ferrante P, Grillo R, Mora B, Mariani P, Apollonio I, Gemme G and Mazzilli MC (1997). Genetic contribution of the HLA region to the familial clustering of coeliac disease. *Ann Hum Genet* **61**:307-317.

Piper JL, Gray GM and Khosla C (2004). Effect of prolyl endopeptidase on digestive-resistant gliadin peptides in vivo. *J Pharmacol Exp Ther* **311**:213-219.

Quarsten H, McAdam SN, Jensen T, Arentz-Hansen H, Molberg Ø, Lundin KE and Sollid LM (2001). Staining of celiac disease-relevant T cells by peptide-DQ2 multimers. *J Immunol* **167**:4861-4868.

Ráki M, Tollefsen S, Molberg O, Lundin KE, Sollid LM and Jahnsen FL (2006). A unique dendritic cell subset accumulates in the celiac lesion and efficiently activates gluten-reactive T cells. *Gastroenterology* **131**:428-438.

Reay PA, Matsui K, Haase K, Wulfing C, Chien YH and Davis MM (2000). Determination of the relationship between T cell responsiveness and the number of MHC-peptide complexes using specific monoclonal antibodies. *J Immunol* **164**:5626-5634.

Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP and Ricciardi-Castagnoli P (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* **2**:361-367.

Roberts AI, Lee L, Schwarz E, Groh V, Spies T, Ebert EC and Jabri B (2001). NKG2D receptors induced by IL-15 costimulate CD28-negative effector CTL in the tissue microenvironment. *J Immunol* **167**:5527-5530.

Roche PA and Cresswell P (1990). Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature* **345**:615-618.

Roche PA, Marks MS and Cresswell P (1991). Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature* **354**:392-394.

Rudensky AY, Preston-Hurlburt P, Hong SC, Barlow A and Janeway CA, Jr. (1991). Sequence analysis of peptides bound to MHC class II molecules. *Nature* **353**:622-627.

Sakai K, Zamvil SS, Mitchell DJ, Hodgkinson S, Rothbard JB and Steinman L (1989). Prevention of experimental encephalomyelitis with peptides that block interaction of T cells with major histocompatibility complex proteins. *Proc Natl Acad Sci U S A* **86**:9470-9474.

Salvati VM, Mazzarella G, Gianfrani C, Levings MK, Stefanile R, De GB, Iaquinto G, Giardullo N, Auricchio S, Roncarolo MG and Troncone R (2005). Recombinant human interleukin 10 suppresses gliadin dependent T cell activation in ex vivo cultured coeliac intestinal mucosa. *Gut* **54**:46-53.

Schulzke JD, Bentzel CJ, Schulzke I, Riecken EO and Fromm M (1998). Epithelial tight junction structure in the jejunum of children with acute and treated celiac sprue. *Pediatr Res* **43**:435-441.

Scott CA, Peterson PA, Teyton L and Wilson IA (1998). Crystal structures of two I-A(d)-peptide complexes reveal that high affinity can be achieved without large anchor residues. *Immunity* **8**:319-329.

Senger S, Luongo D, Maurano F, Mazzeo MF, Siciliano RA, Gianfrani C, David C, Troncon R, Auricchio S and Rossi M (2003). Intranasal administration of a recombinant alpha-gliadin down-regulates the immune response to wheat gliadin in DQ8 transgenic mice. *Immunol Lett* **88**:127-134.

Sette A, Wentworth P and Grey HM (1991). Major histocompatibility complex binding peptides: a target for therapeutic development. *Curr Opin Biotechnol* **2**:877-881.

Shan L, Molberg Ø, Parrot I, Hausch F, Filiz F, Gray GM, Sollid LM and Khosla C (2002). Structural basis for gluten intolerance in celiac sprue. *Science* **297**:2275-2279.

Shan L, Qiao SW, Arentz-Hansen H, Molberg Ø, Gray GM, Sollid LM and Khosla C (2005). Identification and analysis of multivalent proteolytically resistant peptides from gluten: implications for celiac sprue. *J Proteome Res* **4**:1732-1741.

Sherman MA, Weber DA and Jensen PE (1995). DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. *Immunity* **3**:197-205.

Siebold C, Hansen BE, Wyer JR, Harlos K, Esnouf RE, Svejgaard A, Bell JI, Strominger JL, Jones EY and Fugger L (2004). Crystal structure of HLA-DQ0602 that protects against type 1 diabetes and confers strong susceptibility to narcolepsy. *Proc Natl Acad Sci U S A* **101**:1999-2004.

Siegel M, Bethune MT, Gass J, Ehren J, Xia J, Johannsen A, Stuge TB, Gray GM, Lee PP and Khosla C (2006). Rational design of combination enzyme therapy for celiac sprue. *Chem Biol* **13**:649-658.

Siegel M and Khosla C (2007). Transglutaminase 2 inhibitors and their therapeutic role in disease states. *Pharmacol Ther* **115**:232-245.

Siegel M, Xia J and Khosla C (2007). Structure-based design of α-amido aldehyde containing gluten peptide analogues as modulators of HLA-DQ2 and transglutaminase 2. *Bioorg Med Chem* **15**:6253-6261.

Sjöström H, Lundin KE, Molberg Ø, Körner R, McAdam SN, Anthonsen D, Quarsten H, Noren O, Roepstorff P, Thorsby E and Sollid LM (1998). Identification of a gliadin T-cell epitope in coeliac disease: general importance of gliadin deamidation for intestinal T-cell recognition. *Scand J Immunol* **48**:111-115.

Sloan-Lancaster J and Allen PM (1996). Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. *Annu Rev Immunol* **14**:1-27.

Smith KJ, Pyrdol J, Gauthier L, Wiley DC and Wucherpfennig KW (1998). Crystal structure of HLA-DR2 (DRA*0101, DRB1*1501) complexed with a peptide from human myelin basic protein. *J Exp Med* **188**:1511-1520.

Soares AF, Carvalho RA and Veiga F (2007). Oral administration of peptides and proteins: nanoparticles and cyclodextrins as biocompatible delivery systems. *Nanomed* **2**:183-202.

Sollid LM and Khosla C (2005). Future therapeutic options for celiac disease. *Nat Clin Pract Gastroenterol Hepatol* **2**:140-147.

Sollid LM and Lie BA (2005). Celiac disease genetics: current concepts and practical applications. *Clin Gastroenterol Hepatol* **3**:843-851.

Sollid LM, Markussen G, Ek J, Gjerde H, Vartdal F and Thorsby E (1989). Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer. *J Exp Med* **169**:345-350.

Sollid LM and Thorsby E (1993). HLA susceptibility genes in celiac disease: genetic mapping and role in pathogenesis. *Gastroenterology* **105**:910-922.

Spaenij-Dekking L, Kooy-Winkelaar Y and Koning F (2005a). The Ethiopian cereal tef in celiac disease. *N Engl J Med* **353**:1748-1749.

Spaenij-Dekking L, Kooy-Winkelaar Y, van VP, Drijfhout JW, Jonker H, van SL, Smulders MJ, Bosch D, Gilissen LJ and Koning F (2005b). Natural variation in toxicity of wheat: potential for selection of nontoxic varieties for celiac disease patients. *Gastroenterology* **129**:797-806.

Stepniak D, Spaenij-Dekking L, Mitea C, Moester M, de RA, Baak-Pablo R, van VP, Edens L and Koning F (2006). Highly efficient gluten degradation with a newly

identified prolyl endoprotease: implications for celiac disease. *Am J Physiol Gastrointest Liver Physiol* **291**:G621-G629.

Stepniak D, Vader LW, Kooy Y, Van Veelen PA, Moustakas A, Papandreou NA, Eliopoulos E, Drijfhout JW, Papadopoulos GK and Koning F (2005). T-cell recognition of HLA-DQ2-bound gluten peptides can be influenced by an N-terminal proline at p-1. *Immunogenetics* **57**:8-15.

Stern LJ, Brown JH, Jardetzky TS, Gorga JC, Urban RG, Strominger JL and Wiley DC (1994). Crystal-structure of the human class-II MHC protein HLA-DR1 complexed with an influenza-virus peptide. *Nature* **368**:215-221.

Suri A, Walters JJ, Gross ML and Unanue ER (2005). Natural peptides selected by diabetogenic DQ8 and murine I-A(g7)molecules show common sequence specificity. *J Clin Invest* **115**:2268-2276.

The Wellcome Trust Case Control Consortium (2007). Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* **447**:661-678.

Tollefsen S, Arentz-Hansen H, Fleckenstein B, Molberg Ø, Raki M, Kwok WW, Jung G, Lundin KE and Sollid LM (2006). HLA-DQ2 and -DQ8 signatures of gluten T cell epitopes in celiac disease. *J Clin Invest* **116**:2226-2236.

Trier JS (1991). Celiac sprue. *N Engl J Med* **325**:1709-1719.

Vader LW, de Ru A, van der WY, Kooy YM, Benckhuijsen W, Mearin ML, Drijfhout JW, van Veelen P and Koning F (2002a). Specificity of tissue transglutaminase explains cereal toxicity in celiac disease. *J Exp Med* **195**:643-649.

Vader W, Kooy Y, van Veelen P, de Ru A, Harris D, Benckhuijsen W, Pena S, Mearin L, Drijfhout JW and Koning F (2002b). The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. *Gastroenterology* **122**:1729-1737.

van Belzen MJ, Meijer JW, Sandkuijl LA, Bardoel AF, Mulder CJ, Pearson PL, Houwen RH and Wijmenga C (2003). A major non-HLA locus in celiac disease maps to chromosome 19. *Gastroenterology* **125**:1032-1041.

van de Wal Y, Kooy Y, van Veelen P, Pena S, Mearin L, Papadopoulos G and Koning F (1998a). Cutting edge: Selective deamidation by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity. *J Immunol* **161**:1585-1588.

van de Wal Y, Kooy YM, Drijfhout JW, Amons R and Koning F (1996). Peptide binding characteristics of the coeliac disease-associated DQ(α1*0501, β1*0201) molecule. *Immunogenetics* **44**:246-253.

van de Wal Y, Kooy YM, van Veelen P, Vader W, August SA, Drijfhout JW, Pena SA and Koning F (1999). Glutenin is involved in the gluten-driven mucosal T cell response. *Eur J Immunol* **29**:3133-3139.

van de Wal Y, Kooy YM, Van Veelen PA, Pena SA, Mearin LM, Molberg Ø, Lundin KE, Sollid LM, Mutis T, Benckhuijsen WE, Drijfhout JW and Koning F (1998b). Small intestinal T cells of celiac disease patients recognize a natural pepsin fragment of gliadin. *Proc Natl Acad Sci U S A* **95**:10050-10054.

van Elburg RM, Uil JJ, Mulder CJ and Heymans HS (1993). Intestinal permeability in patients with coeliac disease and relatives of patients with coeliac disease. *Gut* **34**:354-357.

van Ham SM, Tjin EP, Lillemeier BF, Grüneberg U, van Meijgaarden KE, Pastoors L, Verwoerd D, Tulp A, Canas B, Rahman D, Ottenhoff TH, Pappin DJ, Trowsdale J and Neefjes J (1997). HLA-DO is a negative modulator of HLA-DM-mediated MHC class II peptide loading. *Curr Biol* **7**:950-957.

van Heel DA, Franke L, Hunt KA, Gwilliam R, Zhernakova A, Inouye M, Wapenaar MC, Barnardo MC, Bethel G, Holmes GK, Feighery C, Jewell D, Kelleher D, Kumar P, Travis S, Walters JR, Sanders DS, Howdle P, Swift J, Playford RJ, McLaren WM, Mearin ML, Mulder CJ, McManus R, McGinnis R, Cardon LR, Deloukas P and Wijmenga C (2007). A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. *Nat Genet* **39**:827-829.

Vartdal F, Johansen BH, Friede T, Thorpe CJ, Stevanovic S, Eriksen JE, Sletten K, Thorsby E, Rammensee HG and Sollid LM (1996). The peptide binding motif of the disease associated HLA-DQ (α1* 0501, β1* 0201) molecule. *Eur J Immunol* **26**:2764-2772.

Wang W, Uzzau S, Goldblum SE and Fasano A (2000). Human zonulin, a potential modulator of intestinal tight junctions. *J Cell Sci* **113**:4435-4440.

Watts C (1997). Capture and processing of exogenous antigens for presentation on MHC molecules. *Annu Rev Immunol* **15**:821-850.

Wieser H, Mödl A, Seilmeier W and Belitz HD (1987). High-performance liquid chromatography of gliadins from different wheat varieties: Amino acid composition and N-terminal amino acid sequence of components. *Z Lebensm Unters Forsch* **185**:371-378.

Woolley N, Holopainen P, Ollikainen V, Mustalahti K, Maki M, Kere J and Partanen J (2002). A new locus for coeliac disease mapped to chromosome 15 in a population isolate. *Hum Genet* **111**:40-45.

Xia J, Siegel M, Bergseng E, Sollid LM and Khosla C (2006). Inhibition of HLA-DQ2-mediated antigen presentation by analogues of a high affinity 33-residue peptide from α2-gliadin. *J Am Chem Soc* **128**:1859-1867.

Zhu YR, Rudensky AY, Corper AL, Teyton L and Wilson IA (2003). Crystal structure of MHC class II I-A(b) in complex with a human CLIP peptide: Prediction of an I-A(b) peptide-binding motif. *J Mol Biol* **326**:1157-1174.

Zimmer KP, Naim H, Weber P, Ellis HJ and Ciclitira PJ (1998). Targeting of gliadin peptides, CD8, α/β-TCR, and γ/δ-TCR to Golgi complexes and vacuoles within celiac disease enterocytes. *FASEB J* **12**:1349-1357.

Zimmer KP, Poremba C, Weber P, Ciclitira PJ and Harms E (1995). Translocation of gliadin into HLA-DR antigen containing lysosomes in coeliac disease enterocytes. *Gut* **36**:703-709.

ERRATA

Paper I:
Page 21792, right column, line 14 in Results
The correct should be:
(23.3- and 35.6-fold, respectively)

Paper IV:
Page 1
The title should be:
Analysis of the binding of gluten T-cell epitopes to various human leukocyte antigen class II molecules

Page 6 line 10
The correct should be:
The antibodies used in this study are L243 (anti-DR) [13], B8.11 (anti-DR) [14], LB3.1 (anti-DRA) [15], SPV-L3 (anti-DQ) [16], 2.12.E11 (anti-DQ2) [17] and B7/21 (anti-DP) [18].

Page 6 line 19
The correct should be:
Detergent solubilized DQ2.5 (DQA1*0501, DQB1*0201) and DR3 (DRA*0101, DRB1*0301) molecules were purified from the Epstein-Barr virus-transformed B-LCL, 9023 VAVY and CD114, as described previously [19] using specific mAbs, 2.12.E11 and B8.11, respectively.