INVESTIGATING CELIAC DISEASE USING RECOMBINANT SOLUBLE MHC CLASS II MOLECULES

Doctoral thesis by
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TABLE OF CONTENTS

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Oslo, February 2009

Lars-Egil Fallang

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ABBREVIATIONS

AIRE	Autoimmune regulator gene
APC	Antigen presenting cells
BCR	B-cell receptor
B-LCL	B-lymphoblastoid cell line
CD	Celiac disease
CLIP	Class II associated invariant chain peptide
DC	Dendritic cell
EBV	Epstein Barr virus
EE	Early endosomes
ER	Endoplasmic reticulum
GILT	IFN-γ inducible thiol reductase
HLA	Human leukocyte antigen
HLA I/II	HLA class I/II
IEL	Intraepithelial lymphocytes
IFN-γ	Interferon-γ
Ig	Immunoglobulin
Ii	Invariant chain
LIP	Leupeptin induced peptide (The p22 fragment of Ii)
MHC	Major histocompatibility complex
MHC I/II	MHC class I/II
MIIC	MHC class II compartment
PPII	Type II polyproline
SLIP	small LIP (The p10 fragment of Ii)
TCR	T-cell receptor
TG2	Transglutaminase 2
TM	Transmembrane

LIST OF PAPERS

Paper I

Fallang L-E., Roh S., Holm A., Bergseng E., Yoon T., Fleckenstein B., Bandyopadhyay A., Mellins E.D., Sollid L.M. Complexes of two cohorts of CLIP peptides and HLA-DQ2 of the autoimmune DR3-DQ2 haplotype are poor substrates for HLA-DM. *J Immunol.* 2008; 181(8):5451-61.

Paper II

Fallang L-E., Bergseng E., Hotta K., Berg-Larsen A., Kim C-Y., Sollid L.M. HLA-DQ2 disease association depends on a polymorphic residue hydrogen bonding to peptide main chain. *Manuscript submitted.*

Paper III

Raki M., **Fallang L-E**., Brottveit M., Bergseng E., Quarsten H., Lundin K.E., Sollid L.M. Tetramer visualization of gut-homing gluten-specific T cells in the peripheral blood of celiac disease patients. *Proc Natl Acad Sci U S A.* 2007; 104(8):2831-6.

INTRODUCTION

The immune system is a fascinating and complex system of molecules, cells and organs that has evolved to protect the body against invading pathogens, such as bacteria, virus and parasites. The immune responses that are initiated upon pathogen entry are divided into innate immunity and adaptive immunity. The innate immune response is the first line of defense, and is an early non-specific response by white blood cells, or leukocytes, that recognize non-self entities common to microbes. Innate responses include recruitment of immune cells to the site of infection for pathogen removal, inflammation, and activation of the complement cascade. The innate response is also responsible for activating the adaptive response, initiated by professional antigen presenting cells (APC). These cells engulf and process pathogens and subsequently present pathogen-derived fragments on the cell surface. T lymphocytes (T cells) of the adaptive immune system recognize the fragment by means of their antigen specific T-cell receptors (TCR). Upon recognition, these lymphocytes get activated and evolve into effector cells, resulting in a pathogen specific response much more potent than the initial innate response. At this stage the innate and adaptive response synergize to clear the infection by effector T cells killing pathogen infected cells directly, or by cytokine production which stimulates other cells of the immune system, like macrophages and B lymphocytes (B cells). The B cell, another cell of the adaptive immune system, has surface expressed immunoglobulin (Ig) as antigen specific B-cell receptors (BCR). Upon activation, these cells secrete soluble versions of the BCR, called antibodies, which are necessary to combat the pathogen. After the pathogen has been cleared, effector cells are removed except for a subset of the lymphocytes, termed memory cells, which allow for a quicker response to a subsequent infection of the same pathogen.

The APCs present protein fragments, or peptides, to circulating T lymphocytes via certain proteins of the major histocompatibility complex (MHC) to. These surface expressed proteins, termed human leukocyte antigen (HLA) in humans, are divided into two classes. MHC class I molecules (MHC I) are expressed by all nucleated cells. These molecules generally present self or foreign peptides from the interior of the cells to cytotoxic $CD8^+$ T cells. The MHC class II molecules (MHC II) are selectively

expressed by epithelial cells in the thymus, and professional APCs, including dendritic cells, macrophages and B cells. These molecules mainly present endocytosed proteins.

The MHC molecules are of great importance for the body's ability to detect an intrusion of foreign entities and initiate a response towards these pathogens. However, these molecules may also elicit undesirable immune responses, exemplified by MHC presentation of self peptides that may lead to autoimmune disease. A common feature of many autoimmune diseases, like rheumatoid arthritis, type 1 diabetes and systemic lupus erythematosus, is the lacking identity of the disease-causing antigen, which makes studying these diseases cumbersome and difficult. Celiac disease (CD), however, has a known antigen in the form of gluten, which becomes more immunogenic after transglutaminase 2 (TG2) modification. The chronic intestinal inflammation, a hallmark of this disease, is the result of the activation of T cells recognizing gluten peptides presented by HLA-DQ2.5 (DQ2.5) or HLA-DQ8 (DQ8) molecules. Much is known about the role of particularly DQ2.5 to CD. However, this molecule is associated with numerous other autoimmune diseases as well, indicating a possible uniqueness of this molecule. This thesis focuses on the underlying mechanism for the selective celiac disease association with DQ2.5, but also the detection of gluten specific T cells in peripheral blood, by means of soluble recombinant MHC molecules.

MHC II molecules

MHC I and MHC II are both important molecules in the immune response. However, as DQ2.5 is a class II molecule, I will focus on this class of MHC in this introduction. The MHC II molecules are membrane bound heterodimeric glycoproteins encoded in a gene complex on the short arm of chromosome 6 (6p21.3), encompassing ~3.6Mb. Three isoforms of peptide binding MHC II exist in humans, termed HLA-DP, -DQ, and -DR. These molecules have differential cellular expression levels, where DQ and DP show approximately ten-fold and hundred-fold lower expressions compared to DR, respectively. All three isoforms consist of an α-chain of 35kDa, and a β-chain of 28kDa.With the exception of the locus encoding for the α-chain of DR, termed

DRA1, the genes encoding for DRB1, DQA1, DQB1, DPA1 and DPB1 are highly polymorphic. Each chain consists the membrane proximal domain, the membrane distal domain, a transmembrane domain, and a short cytoplasmic tail. The membrane distal domain is what constitutes the peptide binding groove, with a β-sheet providing the floor and two α-helixes forming the walls of the groove. This is based on the crystal structure of a diverse set of MHC molecules, pioneered by Brown *et al* [1]. The MHC II molecule has an open ended groove, meaning that even though the groove itself accompany a core of 9 residues, longer peptides (10-34 residues) are allowed to bind with ends protruding from the groove[2]. The crystal structure also revealed two main principles of peptide binding: (1) Hydrogen bonding between MHC backbone or conserved residues and the main chain carbonyl oxygen and amide nitrogen groups of the peptide, and (2) "pocket-anchor" interactions, where the side chains of peptides interact with polymorphic MHC residues[3-5]. The former principle provides a universal affinity for peptides of variable sequence, while the latter principle determines the differential peptide specificities for the different MHC molecules. In addition to the conserved hydrogen bonding network, there are also MHC specific hydrogen bonding to the peptide, which further determines the binding affinity. Upon binding to the MHC molecule, the peptide conforms to a type II polyproline (PPII) helix[4,6,7]. This allows for extensive interaction of the peptide side chains with both the MHC pockets (peptide residues in position P1, P4, P6, P7 and P9) and the TCR of the T cell (residues in P2, P5 and P8) (Fig. 1).

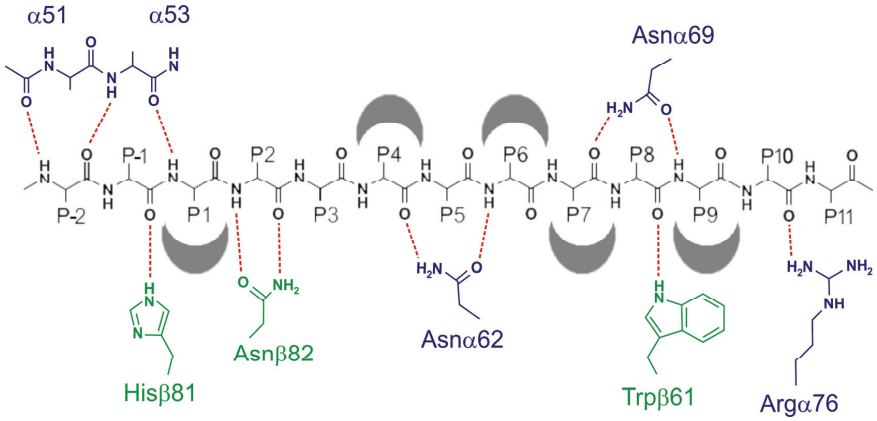

Figure 1: Schematic representation of the conserved hydrogen bonding involved in MHC II peptide binding*. MHC II interaction with peptides (in black) includes accommodation of peptide side chains*

(denoted "P") in MHC II "pockets" (in grey), and conserved hydrogen bonding (red dashed lines) between peptide backbone and MHC II main chain or conserved residues. Residues of the MHC II α-chain and β-chain are represented in blue and green, respectively. The peptide side chains are numbered according to their position relative to MHC II pocket 1. The peptide has not been depicted in a PPII helix for simplicity. The figure is based on HLA-DR1, and has been modified from [8].

HLA nomenclature

Human leukocyte antigens were first described in the mid 1950's, through the discovery of antibodies recognizing donor leukocyte antigens in blood transfusion recipients. During the next two decades the vast increase of identified leukocyte antigens revealed the requirement for a standardized nomenclature. This was resolved over a series of international histocompatibility workshops, and the term HLA was coined in 1975[9]. As of 1999, more than 220 genes have been characterized in the HLA region[10], and a vast number of polymorphisms have been described[11]. The resulting HLA nomenclature is therefore complex and is based both on the original method of serotyping, but also genotyping, which better distinguishes the polymorphisms in HLA. The limitations associated with serotyping can be exemplified with DQ2 (Fig. 2). This molecule was identified by using an antibody that recognizes the β-chain (DQB1*02). However, because this β-chain can dimerize with two different α-chains (DQA1*0501 and DQA1*0201), genotyping is required to discriminate between the two resulting forms of DQ2. The extended term DQ2.2 and DQ2.5 was therefore introduced for these two molecules, where the last digit refers to the α-chain of the allele[12].

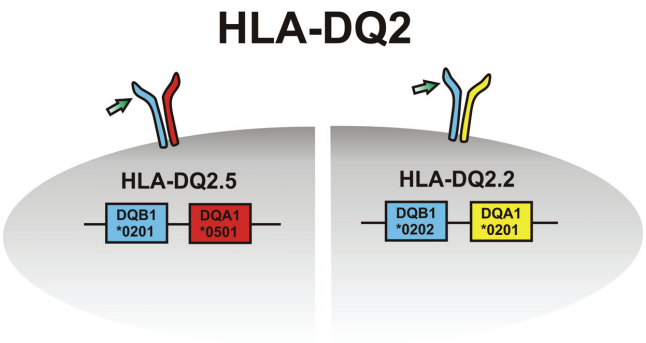

Figure 2: HLA nomenclature based on serotype vs. genotype*. The DQ2 serotype does not distinguish between the two DQ2-variants DQ2.2 and*

*DQ2.5 due to antibody recognition of the DQB1*02 molecule (green arrow). The genotyping, however, reveals two different DQ2 alleles based on their different α-chains (DQA1*0501 for DQ2.5 and DQA1*0201 for DQ2.2).*

The naming of an HLA molecule is here exemplified by the HLA-DQA1*0501 molecule of the DQ2.5 allele. The first part of the name, "HLA" specifies the gene region. The second part, DQA1, specifies the locus within this gene region, while the first two digits following the asterix, "05", refers to the allele group, and is often similar to the serotype. The following two digits, "01", refer to the specific allele within the allele group. Variations within the gene that do not cause amino acid changes are denoted by additional following digits[13].

Antigen processing and presentation

MHC II molecules assemble in the endoplasmic reticulum (ER) with the help of the chaperone molecule Invariant chain (Ii). The resulting complex forms a nonamer consisting of three molecules of each α-, β- and Ii-chains. The Ii serves three major purposes: (1) Assuring assembly and proper folding of the MHC II molecules[14], (2) preventing premature loading of peptides located in the ER by occupying the antigen binding groove with a short sequence termed Class II associated Ii peptide (CLIP)[15], and (3) translocation of the nonameric αβIi complex out of the ER, via the trans-Golgi-apparatus, and to the multivesicular MHC class II containing compartment (MIIC), which is a specialized late endosomal compartment[16,17]. The translocation is mediated by transport signals located in the N-terminal cytoplasmic tail of the Ii molecule[16,17]. Due to alternative initiation of translation, Ii has several N-terminally truncated isoforms, which consequently affects the transport signaling. The actual route of transport therefore depends on the isoform of the Ii that chaperones the MHC II molecule, causing the αβIi nonamer to either directly translocate to the MIIC[18,19], or use an indirect pathway by way of the plasma membrane and/or entering the early endosomes prior to arriving at the MIIC[20,21]. The simultaneous presence of all these pathways have been suggested to be of advantage to the cell by ensuring access to potential foreign antigens present in many different intracellular compartments[20,22]. During transition from the early endosomes to the MIIC the compartments gradually become more acidic, and degradation of Ii by proteases of the aspartyl and cathepsin

family causes the αβIi nonameric complex to disassemble, leaving CLIP in the peptide binding groove. The MHC II-CLIP complex is now available for peptide antigen exchange.

Internalized antigens are trafficked through the endocytic pathway to the MIIC. They are processed by the same protease machinery as Ii, following an initial denaturing by the enzyme GILT (interferon-γ (IFN-γ) inducible thiol reductase)[23], making the resulting peptide fragments available for MHC II binding. However, the binding of antigenic peptides to MHC II is dependent on the release of CLIP, a process shown to be accelerated by the molecule HLA-DM[24-26]. This non-classical MHC II molecule interacts with the MHC II-CLIP complex which results in conformational changes that causes destabilization and subsequent release of CLIP. This peptide receptive state of the MHC II molecule, is maintained by DM until a peptide with sufficient affinity is bound, upon which the DM-MHC interaction ceases[25,27]. The MHC II-peptide complex is then transported to the cell surface, by a still poorly understood mechanism, for the recognition of $CD4^+$ T cells of appropriate specificity. The entire pathway is depicted in Figure 3.

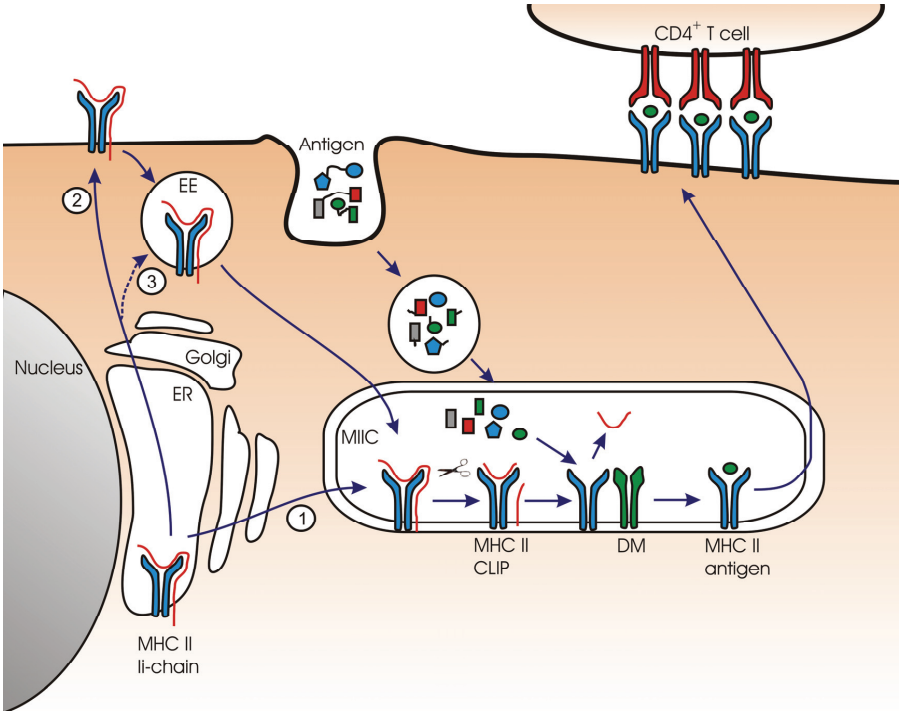

Figure 3 Antigen processing pathway*. MHC II molecules assemble in the ER with the chaperone Ii in a nonamer complex. The nonamer translocates to the MIIC via three possible pathways: 1) Directly to the MIIC; 2) via the cell surface to the early endosomes (EE) prior to MIIC entry; or 3) directly to EE prior to MIIC entry. Once in the MIIC, proteases digest Ii, leaving CLIP in the binding groove. Internalized antigen is degraded by the same protease machinery, and is ready for MHC II binding. MHC II-CLIP complex interacts with HLA-DM, which removes CLIP and allows for internalized degraded peptide antigens to bind. The MHC II-peptide complex migrates to the cell surface for recognition by CD4$^+$ T cells.*

MHC II presentation of peptides is not, however, critically dependent on antigen internalization and processing. Surface exchange of antigens have been demonstrated for peptides of various lengths up to 39 amino acids [28-30], and even larger proteins have been reported to be processing independent[31]. This mechanism is DM independent and requires the presence of low affinity MHC II-peptide complexes for efficient exchange.

Accessory molecules in antigen presentation

Invariant chain

Ii is a type II transmembrane protein expressed mainly by APCs, but also by other cell types during inflammation. Ii comes in different N-terminal truncated isoforms, termed p33, p35, p41 and p43, based on their molecular weight[32]. The isoforms are the result of alternative translational initiation, or alternative splicing and come in a major form, termed p33 (33kDa), and a minor form, termed p35 (35kDa). The p41 (41kDa) and p43 (43kDa) isoforms have an additional internal cystein-rich exon, but are otherwise identical to the shorter variants. All isoforms contain di-leucine transport motifs causing endosome translocation, while the p35/p43 variants have an additional di-arginine motif causing ER retention in the absence of MHC II. In addition, studies show that the p35/p43 isoforms are directly transported to the MIIC, while the p33/p41 isoforms are responsible for the cell surface/early endosome targeting[21]. Recently, a distinct new role for p41 as a cathepsin inhibitor have been suggested[33].

The Ii molecule is able to associate with a divergent set of MHC molecules by not only accommodating CLIP in the binding groove, but also through association with

regions outside the groove with sequences both C- and N-terminally of the CLIP sequence[34-36]. As the nonameric complex moves through the endocytic pathway, a pool of proteases sequentially cleave the Ii in a C-terminal to N-terminal direction, revealing size intermediates termed p22 (LIP) and p10 (SLIP)[37]. The additional enzymes involved in these processes are not known and appear to be redundant. However, the release of CLIP from the transmembrane and cytosolic domains of the Ii is less redundant and attributed to the enzymes cathepsin S or L, depending on the cell type.

The importance of Ii is demonstrated in knock out studies done in mice, showing faulty assembly and folding, and retention of the MHC II in the ER, which ultimately results in reduced amounts of MHC II on the cell surface[38,39]. The absence of Ii would therefore reduce the effectiveness of antigen presentation, and culminate in a decrease in the $CD4^+$ T cell number and diversity[40]. Ii has also been implicated in other cellular functions. The remnant N-terminal fragment after cathepsin S degradation of SLIP has been associated with B cell differentiation, through its implication in the activation of the transcription factor NF-κB[41]. Another recent study, by Faure-André *et al*, revealed a role for Ii in the migrational control of DC, suggested to increase the ability of the DC to encounter and process antigens in the tissues[42]. Ii has also been implicated in the intracellular trafficking of the FcRn receptor[43]. The function and relevance of this association is, however, not understood.

HLA-DM and HLA-DO

HLA-DM was discovered in the early nineties when E. Mellins, D. Pious and colleagues studied cells with antigen processing defects[44]. The defect was later linked to a mutation in the MHC region, causing loss of HLA-DM expression[45]. However, the molecule is not directly linked to the presentation of the peptides, as this non-classical MHC II molecule is unable to bind peptides due to a closed binding groove[46]. Instead, it transiently interacts with MHC II molecules, allowing the exchange of CLIP for antigenic peptides. Studies involving the HLA-DR1 allele demonstrate that the lateral face of DM interacts with the side of the DR1 molecule that accompanies the N-terminus of the bound peptide[47,48]. The proposed mechanism of peptide release involves a conformational change of the MHC II molecule due to

the DM interaction. The resulting "floppy" conformation is caused by H-bond disruption between MHC residues and peptide backbone, shown to be located around pocket 1 of the MHC II binding groove (residues α51-53 and β81)[8,46,47,49-51]. Once CLIP is removed, HLA-DM functions as a peptide editor by keeping the MHC molecule in a peptide receptive conformation until a peptide of sufficient affinity binds[52,53]. Lower affinity peptides will have the same faith as that of CLIP. This system of selection ensures that the cell presents a smaller set of immunodominant peptides instead of a wide variety of peptides of moderate affinity.

Studies in mice where the murine variant of HLA-DM (H2-M) was knocked out revealed an unaltered quantity of MHC II. However, the peptides presented were mainly CLIP peptides, thus demonstrating a severely hampered ability to display a variety of antigens. This results in an inefficient intrathymic positive selection of $CD4^+$ T cells, revealing a slight decrease in quantity and greatly reduced diversity of T cells[54,55]. The intrathymic negative selection was also affected, as seen by the appearance of autoreactive T cells[56]. Another similar study has further demonstrated that mice lacking H-2M display a more promiscuous population of $CD4^+$ T cells, recognizing numerous antigenic epitopes instead of a few immunodominant epitopes[57].

HLA-DO is another non-classical MHC class II molecule believed to have a closed binding groove due to its inability to bind peptides[58]. It is principally expressed in mature B cells[59], but more recent studies also report its expression in primary mature dendritic cells[60]. HLA-DO is critically dependent on its interaction with HLA-DM, which ensures its assembly, stability, and eventual transport out of the ER[61]. Its effects on antigen presentation are mediated through this interaction by negatively regulating the activity of HLA-DM[62,63], though one study also reports positive regulation[64]. The biological benefit of DO is unknown. One suggested effect of the inhibition of DM is a preference for the presentation of exogenous over endogenous antigens by MHC II[65]. This is attributed in part by the ability of DO to limit the activity of DM to that of the late endosomes, in a pH dependent manner[66]. This favours loading of peptides in MIIC, which, in the case of B cells, are enriched due to antigen specific BCR internalization. The DO:DM ratio has also been reported to be lower in the late endocytic pathway[67]. Mice knockout studies of the murine variant of

HLA-DO (H2-O) reveal a weakened ability to present foreign antigen, an impaired humoral immune response, and increased presence of autoimmune antibodies as the mice ages[65]. This supports the role of DO in maximizing the ability of the mature B cell or DC to favourably present exogenous over endogenous antigens.

Professional antigen presenting cells

All cells, except red blood cells, have the ability to present antigens on their surface by means of MHC molecules. However, APCs also present antigens by means of MHC II molecules, although a transient MHC II expression may occur in other cells as well. The APCs include dendritic cells, macrophages, and B cells.

Dendritic cells

The dendritic cell (DC), named for its characteristic cellular projections, was identified in 1973 by Steinman *et al*[68]. There are two distinct types of DCs, the myeolid DC and the plasmacytoid DC. The myeloid DC is the primary cell that activates naïve T cells, and is therefore of particular importance for the initiation of an immune response. Immature DCs survey the tissues in search for foreign entities, and undergo a process of maturation upon recognition/activation. The maturation process involves a down-regulation of antigen uptake, and up-regulation of antigen presentation, exemplified by a ten fold increase in MHC half-life[69]. This long half-life, combined with the functional irreversibility of the bound antigen[70], thus increases the capability of the DCs to activate T cells. The expression of chemokine receptor CCR7 is also induced, which enables the DC to migrate to the lymph node via the lymph[71]. Upon arrival, the DC encounter and activate antigen specific naïve T cells, which subsequently undergo maturation and proliferation[72].

The exact function of the other major subtype, the plasmacytoid DCs, is unclear, However, this cell has been associated with a large production of type I interferons in response to virus infection[73].

Macrophages

Macrophages are large mononuclear phagocytotic cells located in tissues. These leukocytes are important scavengers, removing necrotic and apoptotic material. However, they also play an important role in the immune system, contributing in innate immunity by engulfing and destroying encountered pathogens[74]. Upon pathogenic activation they assist the adaptive part of the immune system as APCs by upregulating antigen processing mechanisms and MHC II expression[75]. Unlike DCs, however, the activated macrophages do not migrate to the lymph nodes, but rather remain in the tissue for stimulation of tissue recruited mature T cells and mediate immune cell recruitment through cytokine secretion.

B cells

B cells differ from the other types of APCs in that they are mainly located in peripheral lymphoid organs in their naïve state. They are also less promiscuous, but more effective with regards to antigen internalization. The primary mechanism of antigen uptake is through the BCR, which specifically binds the antigen. Sufficient stimulation by BCR cross linking causes antigen internalization and subsequent up-regulation of the antigen processing mechanisms[76]. However, complete activation of the B cell is dependent on T-cell interaction, implying that B cell activation of naïve T cells is limited, although possible[76,77]. Mature B cells secrete pathogen specific antibodies, which are of dire importance for the adaptive immune response because of the antibodies ability to opsonize and neutralize pathogens.

T-cell activation

T cells originate from the bone marrow, but migrate to the thymus where they undergo maturation and selection. This involves the surface expression of a functional αβ heterodimeric TCR and the subsequent positive and negative selection based on TCR recognition of self-peptides presented on MHC molecules. Upon maturation, the naïve T cells are allowed to enter the blood stream and circulate through the secondary lymphoid organs in search for antigen presented by APC[78].

There are two subsets of T cells, $CD4^+$ and $CD8^+$, named by their associated co-receptor molecules. $CD4^+$ T cells are activated by pathogenic antigen presented by APCs expressing MHC II, and the resulting effector cells are involved in regulating the humoral and cellular immune system. $CD8^+$ T cells, on the other hand, are activated by MHC I-antigen complex and their effector functions include the recognition and killing of pathogen infected cells. The activation of naïve T cells consists of two major signals, 1) the peptide MHC recognition by the TCR, and 2) the co-stimulatory molecule CD28 interacting with B7 molecules on the DC[79]. The combined signal results in activation and maturation of the naïve T cell into effector or memory T cells. The effector cells have a direct role in the ongoing immune response and migrate to the site of infection, where they are activated upon appropriate antigenic stimulation. The memory cells, on the other hand, are quiescent until a subsequent infection occurs, by which a faster response may be elicited.

The migration of T cells from the peripheral lymphoid organs to the site of the infection, also called "homing", is mediated by the expression of a combination of adhesion and chemoattractant receptors[80]. These receptors allow the cells to attach to the capillary wall at the site of infection and subsequently enter the tissue through a process called diapedesis.

Autoimmunity

The development and maturation of lymphocytes involves a variety of selection processes to ensure that the resulting diverse repertoire can respond to all encountered pathogens. There is a pitfall associated with this powerful process, because with higher diversity comes higher frequency of self-reactive lymphocytes. These self-reactive lymphocytes, however, are normally eliminated due to several tolerogenic mechanisms, the primary mechanism being negative selection in the bone marrow or thymus, termed central tolerance. The intrathymic elimination of autoreactive T cells is the most stringent process and is critically dependent on T cell exposure to all potential self antigens. This is made possible by the expression of the transcription factor Autoimmune Regulator gene (AIRE)[81,82]. AIRE, expressed by stromal cells in the thymus, allows for the thymic expression of, and therefore also T-cell tolerance

towards tissue specific antigens that are normally restricted to specialized peripheral organs[83]. The negative selection is largely assisted by thymic DCs, which are thought to acquire tissue specific antigen from the stromal cells[83]. As some autoreactive T cells do escape this primary selection, there are also lymphoid and non-lymphoid associated peripheral tolerogenic mechanisms that can eliminate these mature T cells as well. The system is not perfect, however, and under certain conditions, like that of an infection, autoreactive lymphocytes may emerge[84]. This may lead to autoimmunity, caused by an abnormal B- and T-cell recognition of self antigens, leading to autoantibody production by B cells and possibly autoimmune disease.

The involvement of autoreactive T (and B) cells in autoimmune disease implicates a role of HLA in the disease mechanism, due to the requirement of peptide presentation. Coincidentally, most, if not all autoimmune diseases are associated with one or more HLA alleles[85]. A list of autoimmune diseases and their HLA association is given in table 1.

Table 1: Examples of HLA-associated autoimmune diseases

Disease	HLA	Pts[a]	Ctrls[a]	RR[b]
Ankylosing spondylitis	B27	> 95	9	> 150
Subacute thyroiditis	B35	70	14	14
Psoriasis vulgaris	Cw6	87	33	7
Graves disease	DR3	65	27	4
Myasthenia gravis	DR3	50	27	2
Addisons disease	DR3	69	27	5
Rheumatoid arthritis	DR4(some)	81	33	9
Juvenile idiopathic arthiritis	DR8	38	7	8
Celiac disease	DQ2 (+DQ8)	92	28	30
Narcolepsy	DQ6(02)	> 95	33	> 40
Multiple sclerosis	DQ6(02)	86	33	12
Type 1 diabetes	DQ8(+)	81	23	14
Type 1 diabetes	DQ6(02)	< 0.01	33	0.02

Antigen frequencies in a Norwegian population. (RR): Relative risk, i.e. how many times more frequent the disease is in those having the corresponding HLA molecule compared to those lacking it. Not included is the RR of DQ2.5 with type 1 diabetes. Table from Thorsby, et al [11].

The disease association can either confer susceptibility for, or protection from the disease. In the case of type 1 diabetes, several alleles confer susceptibility for the disease DQ2, DQ8, DR4.1 and DR4.5 confer susceptibility, while DQ6 and DR4.3 do not, as reviewed in[86]. Although the HLA association is known, the underlying molecular mechanism for this association is often elusive. This can be contributed to the fact that these diseases are commonly polygenic diseases that are also influenced by environmental factors[86]. Another complicating factor is the frequently unknown autoantigen(s), which makes the studying of these diseases more difficult. As previously mentioned, CD is an autoimmune disease with a strong linkage to HLA and with a known antigen, gluten, and the following section will describe this disease in more detail.

Celiac disease

History and clinical aspects

Celiac disease (CD) is a complex inflammatory disorder of the small intestine caused by an inappropriate immune response to dietary proteins in wheat, barley and rye. The disease was first described by S. Gee in 1888[87], and later refined in the 1950s by W. Dicke who demonstrated that a factor in wheat, later defined as gluten, attributed to the onset of the disease[88].

The gluten sensitive enteropathy, as a result of the chronic inflammation, is characterized by villous atrophy, crypt hyperplasia, and increased infiltration of lymphocytes in the epithelium and lamina propria[89]. The clinical symptoms of the disease include typical intestinal features, such as chronic diarrhea, failure to thrive and distended abdomen, but also atypical extraintestinal features such as osteoporosis, anemia and ataxia[90]. However, the disease can also be asymptomatic[91]. Except in the case of refractory celiac disease[89,92], the enteropathy and symptoms disappear upon removal of the precipitating grain from the diet. In the absence of treatment, however, the disease may lead to gastrointestinal lymphomas, the development of associated autoimmune diseases[90], and death[93].

The diagnostics of CD include serological testing and intestinal biopsy analysis. The former detects for the presence of disease associated antibodies in blood having specificity for gluten, endomysium and for TG2[94,95]. The IgA autoantibodies for TG2 has a very high disease sensitivity and specificity (95-99%)[96,97] and is most commonly applied. However, upon a positive serological screening, the definite diagnosis is done by performing morphology analysis on intestinal biopsies. The only safe and effective treatment of celiac disease to date is the adherence to a life-long gluten free diet.

Genetic factors

The prevalence for CD is reported to be ~1% in the western population[98], and there is a distinct familial tendency of disease development indicative of a genetic component in addition to the environmental factor. This is supported by the high rate of pairwise concurrence (75%) found in monozygotic twins, compared to the lower rate observed for dizygotic twins and in first degree relatives (20% and 10%, respectively)[99]. The primary genetic component in CD is HLA, accounting for about 50% of the genetic predisposition[100]. More than 90% of the patients express DQ2.5 (DQA1*0501/DQB1*0201)[101], while most of the remaining express DQ8 (DQA1*0301/DQB1*0302)[102]. The DQ2.5 allele can either be expressed in cis (α and β-chain located on the same chromosome), as in the DR3-DQ2 haplotype, or in trans, (α and β chains located on separate chromosomes) as in the case of DR7-DQ2 and DR5-DQ7 heterozygotes. A minority of the non-DQ2.5 and -DQ8 CD patients express the DQ2.2 (DQA1*0201/DQB1*0202) allele of the DR7-DQ2 haplotype[103].

DR3-DQ2 is a common haplotype expressed by 20-30% of the normal population, suggesting the involvement of additional genetic components. A recent genome wide association study determined the 4q27 region, containing IL-2 and IL-21 as (non-HLA) genes predisposing for CD[104]. There was also a weak association with the previously reported ICOS-CTLA4 region[105], while the previously indicated CD susceptibility gene MYO9B[106], did not show association. A follow up study found seven new chromosomal regions containing genes involved in immune responses[107], which have been replicated[108]. Together with the 4q27 region, these eight regions are estimated to account for 3-4% of the CD heritability, which emphasizes the polygeneity.

HLA-DQ2 and gluten

Two major contributors of celiac disease are the genetic component DQ2.5 and the environmental component gluten. These factors have a unique interplay based on several aspects, which causes activation of an immune response. Gluten is the major storage protein in wheat, and can be divided into two major groups, glutenins and gliadins, based on their alcohol solubility. These proteins are unusually high in their proline and glutamine content[109]. Two features of gluten contribute to the unique interplay with DQ2.5. First, due to their high proline content, they are highly resistant to gastrointestinal enzymatic degradation, allowing for larger peptide fragments to pass through the epithelium into the lamina propria. One such peptide was found through mimicking intraluminal enzymatic conditions, which revealed a proline rich and highly stimulatory fragment of 33 residues[110]. Second, the combination of high proline and glutamine content also makes these peptide fragments efficient targets for the enzyme TG2[111-113]. This multifunctional enzyme modifies neutral glutamines to negatively charged glutamic acids through a process called deamidation.

The resulting proline-rich deamidated gluten peptides are well suited for binding to DQ2.5, as described below. Several early DQ2.5 peptide binding studies identified five major peptide binding pockets in positions P1, P4, P6, P8 and P9, displaying a preference for negative charges in position P4, P6 and P7 of the peptide[114-117]. The molecular basis for this negative preference was largely solved by x-ray crystal structure analysis of DQ2.5[118]. This revealed that lysine in position β71 creates a positive region in the groove accommodating position P4, P6 and P7 of the peptide. The structure analysis and later reports also revealed a unique ability of DQ2.5 to accommodate numerous proline residues without loss of binding affinity[119]. One relatively unique feature of DQ2.5 concerns the accommodation of proline in position P1 without energetic penalty[120]. This is likely due to a deletion of residue α53 in DQ2.5, which is normally part of the conserved hydrogen bond network between the MHC and peptide backbone. Interestingly, the presence of proline in position P1 of gluten antigenic epitopes is a common feature[121-123]. However, DQ2.5 shares many of the above mentioned features with the non-celiac associated DQ2.2 allele. These molecules have a virtually identical β-chain, but have slightly different α-chains diverging by only ten amino acids in the membrane distal region[124]. This is demonstrated by their highly similar peptide binding motifs[116,125], and further

supported by their similar ability to activate gluten specific T cells, though with less efficiency in the case of DQ2.2[12,119]. This disparate disease association has remained an enigma, though theories have been postulated. One theory concerns the slightly inferior gluten presentation by DQ2.2 resulting in an unattainable threshold of T-cell activation[12,119]. One proposed reason for the inferior presentation is a possible lower tolerance for accommodating proline in position P3 of the peptide for DQ2.2[12]. However, conflicting results oppose this latter theory[119].

Pathogenesis of celiac disease

The celiac lesion is associated with an abnormal influx of lymphocytes to the lamina propria and epithelium in response to ingested gluten. The lamina propria lymphocytes are dominated by $CD4^+$ T cells that specifically recognize gluten peptides presented by DQ2.5/DQ8 expressing APCs[126,127]. The gluten peptides presented by the APC have undergone TG2 mediated deamidation, causing higher HLA binding affinity and drastically increases the level of T cell activation[122,128,129]. Upon activation, the T cells secrete large amounts of cytokines dominated by the pro-inflammatory cytokine IFN-γ[130].

Another aspect of the celiac lesion is the flattening of the mucosa. This is probably the result of enterocyte killing by the CD associated infiltrated intraepithelial lymphocytes (IELs). This is proposed to be orchestrated by the high amounts of IL-15 associated with CD[131,132]. The presentation of α-gliadin peptide p123-132 by HLA-A2 expressing enterocytes has recently been reported to activate gluten specific $CD8^+$ IELs, causing up-regulation of IFN-γ, granzyme and FASL, and subsequent enterocyte apoptosis[133,134]. The presence of IL-15 is suggested to promote this cytotoxic effect by lowering the TCR activation threshold through NKG2D up-regulating, which acts like a co-receptor for the TCR[135]. Interestingly, IL-15 production in active CD has been reported to be inducible by α-gliadin peptide p31-43[136]. This peptide, via IL-15, appears to cause not only an up-regulation of the activating natural killer receptor NKG2D on the IELs, but also its ligand, MICA, on the enterocyte[137]. The interaction between these molecules is suggested to provide a TCR independent mechanism of inducing epithelial apoptosis[135]. A third mechanism of enterocyte killing may be mediated by another NK receptor, the CD94-NKG2C

expressed by the IEL[138]. This receptor interacts with the stress induced molecule HLA-E on the enterocyte, thus causing TCR independent apoptosis, cytokine secretion and extensive IEL proliferation. Interestingly, HLA-E expression is induced by IFN-γ[139]. This cytokine is produced not only by the IELs, but also by the gluten reactive $CD4^+$ T cells in the lamina propria, thus providing a possible bridging of the innate and adaptive immune response of CD.

Soluble MHC II molecules

The in-depth study of MHC II molecules, involving the intricacies of peptide binding and T-cell interaction, is simplified by using MHC II molecules in a cell-free system. However, this was originally considered difficult due the requirement of a lipid containing environment. This problem was solved in the late 1980's by treating isolated MHC II molecules with papain which cleaved off the hydrophobic transmembrane (TM) region[140]. The disadvantages of this method included non-specific papain cleavage and a lacking option of modifying the MHC II molecule. This was solved a few years later by transfecting cells with modified cDNA from MHC II, thus allowing for molecular tailoring. The first reported recombinant molecules were still initially membrane bound, where the TM region was replaced by a cleavable GPI-linker[141]. Stern *et al* omitted the TM region altogether in his insect cell produced soluble HLA-DR1 molecules[142]. The truncation of other MHC II molecules were, however, relatively unstable due to the lack of bound peptide, as the removal of the TM excluded the molecules from the normal antigen processing pathway. This was solved by covalently linking a peptide to the β-chain that stabilized the binding-groove[143]. A thrombin site was included in the linker, permitting the subsequent peptide release. A subset of MHC II molecules, however, did not assemble properly in the absence of the TM region. To resolve this issue a leucine zipper dimerization motif was added to mimic the TM domain, which facilitated the MHC II folding[144]. The combining of the above techniques allowed for the expression of numerous MHC II molecules, including DQ2.5[145]. The versatility of the molecules were further increased by performing peptide exchange on a thrombin cleaved MHC II-peptide construct, thus enabling the production of numerous molecules from a single MHC-peptide complex, as reported by Day *et al*[146].

Both eukaryotic, and prokaryotic expression systems have been used for soluble recombinant MHC II production[142,144,147]. A common eukaryotic production system utilizes insect cells, which produces pre-folded molecules with post-translational modifications like glycosylation. Prokaryotic production systems, on the other hand, are easy to handle and rapidly produce vast amounts of protein. However, the required *in vitro* folding of the molecules has proven difficult and has to be tailored for each molecule. Also, the lacking of post-translational glycosylation in prokaryotes may pose problems, though functional molecules have been produced[147,148].

The applications of soluble MHC II molecules are numerous and their implementations have been critical in the elucidation of the mechanisms behind various HLA related immune processes. In terms of celiac disease, soluble recombinant DQ2.5 and DQ8 molecules tethered with a gluten peptide were used to solve their x-ray crystal structures, which allowed predictions as to why these HLA alleles preferentially bind TG2-treated gluten[118,149]. Soluble molecules have also been implemented in the designing of peptide blockers[150,151] (Juse *et al*, unpublished material) and in the successful targeting of CD specific $CD4^+$ T cells by using tetramers of soluble DQ2.5 molecules with linked gluten peptides[145].

MHC tetramer technology

The detection and analysis of the antigen specific T cells was revolutionized with the introduction of MHC tetramers[152]. This flow cytometry-based technology utilized tetramers consisting of four MHC I-peptide complexes multimerized on a fluorescently labelled streptavidin molecule, to identify antigen specific $CD8^+$ T cells. The tetramer technology was later applied to MHC II molecules and the identification of antigen specific $CD4^+$ T cells. However, due to the combined low frequency of disease related $CD4^+$ T cells and the low TCR-MHC II affinity, these tetramers are associated with low success rates[153-155]. The key to the tetramer technology is the overcoming of the inherent low affinity of a single MHC-TCR interaction by creating an MHC multimer, which increases the avidity of the interaction[156,157]. The molecules are therefore engineered to have a tail-linked biotin that associates with one of the

four biotin binding pockets of streptavidin. This ensures the binding groove exposure of all MHC molecules, though the rigidity and structure of streptavidin prevents more than three simultaneous TCR interaction points[158]. Since the introduction of the tetramer, additional multimers, such as pentamer and octamers have also been created with the purpose of increasing the avidity[159,160]. In celiac disease, the known identity of several gluten T-cell epitopes facilitated the successful production of MHC II tetramers that recognized disease relevant $CD4^+$ T-cell lines and clones[145].

AIMS OF THE STUDY

This thesis focuses on two main aspects; 1) the molecular mechanism behind the celiac disease association of DQ2.5, and 2) the detection of gluten specific T cells in peripheral blood, both aspects in which include the extensive use of soluble recombinant HLA II molecules. Firstly, DQ2.5 is associated not only with celiac disease, but also to various other autoimmune diseases. We sought to find the molecular basis for this extensive association by studying aspects of peptide binding and presentation to DQ2.5, and comparing this with the highly similar, but non-disease associated DQ2.2. Secondly, we aimed to use HLA II tetramers to detect and characterize gluten specific T cells in peripheral blood of treated CD patients after a short gluten challenge.

SUMMARIES OF THE INDIVIDUAL PAPERS

Paper I

This study investigates the conspicuous finding that peptide elution from DQ2.5-expressing B-LCL is associated with high levels of two cohorts of invariant chain fragments. The cohorts consist of the established CLIP1, but also an alternate CLIP2, with a slightly shifted binding frame as determined in this study. By using soluble recombinant HLA II molecules in dissociation studies, we determined that the CLIP peptides had relatively high intrinsic stability for DQ2.5, with a dissociation rate two fold slower than CLIP1 from DR3. Interestingly, dissociation in the presence of DM revealed a mere 16-28 fold increase in dissociation for CLIP1 and CLIP2 from DQ2.5, respectively, compared to the ~250 fold increase as seen for CLIP1-DR3. An additional set of peptides of varying stability for DQ2.5 was tested and the DM effect in relation to intrinsic peptide stability was compared with other HLA alleles from previous studies. This comparison revealed a clear inefficiency of DM for DQ2.5. The abnormal DM interaction was also observed in studies involving B-LCLs where the absence of DM neither changed the conformation, cell surface expression, nor the high CLIP association of DQ2.5. A possible contributing factor to the observed high CLIP content on DQ2.5 was found to be the competitive inhibition of DM by the co-expressed DR3.

Paper II

This paper aims to describe the differential association of DQ2.5 and DQ2.2 with celiac disease. The study builds on paper I, comparing the CLIP1 content in peptide elutions from DQ2.5 expressing cells with that of the non-celiac associated, but highly homologous DQ2.2. Conspicuously, DQ2.2 had a very low CLIP1 phenotype. Dissociation studies using soluble DQ2.5/2-CLIP1 complexes revealed this low CLIP1 phenotype was not due to an increased DM efficiency, but rather a drastically lower intrinsic stability of CLIP1 for DQ2.2. Upon further dissociation studies with mutant DQ2 molecules, we determined that DQα22, out of the total ten polymorphic residues, was the cause of the differential stability for CLIP1. This polymorphism determines the presence (DQ2.5) or absence (DQ2.2) of a hydrogen bond to the peptide backbone, which therefore affects the binding of all peptides to DQ2. The

functional relevance of this finding was determined via a functional off-rate study, where peptide-pulsed APCs were incubated in the absence of peptides for various time points prior to T cell addition. The results showed that DQ2.5-expressing APCs retained the ability to activate T cells much longer than the DQ2.2 counterpart. This increases the opportunity of T cell/antigen encounter, thus influencing the initiation of the anti-gluten T-cell response. The findings in this paper display the potential importance of these polymorphic MHC to peptide backbone hydrogen bonds in peptide binding. Furthermore, this presents a possible link for the extensive association of DQ2.5 to several autoimmune diseases, as the generally low affinity auto-antigens will gain kinetic stability and thereby open for T-cell response initiation.

Paper III

The goal of the study was to investigate whether or not gluten specific T cells were detectable in peripheral blood after a gluten challenge of treated CD patients. Following a three day gluten challenge (160 g white bread) on ten DQ2.5^{+} celiac disease patients and six controls who were all previously on a gluten free diet, we were, by using HLA II peptide tetramers, able to detect DQ2-α-I and DQ2-α-II gliadin specific T cells in the celiac disease patients, but not in the controls. The findings were confirmed by quantifying IFN-γ in ELISPOT assays. The identified T cells were phenotypically characterized and found to express the gut homing integrin β_7, which concurs with the expected intestinal destination. The cells were otherwise expressing a heterogeneous set of markers. The results of the study opens for the use of MHC class II tetramers as a diagnostic tool for diseases where the antigen and MHC restriction element is known.

METHODOLOGICAL CONSIDERATIONS

Soluble HLA II-peptide molecules

The water soluble HLA II molecules used in this work were made up of various gene components combined into expression plasmids, as previously described[145]. The membrane distal part of the HLA α- and β-chains had C-terminally linked complementary Fos/Jun leucine zipper dimerization motifs. The β-chain was further modified by the C-terminal addition of a biotinylation sequence, which allows the attachment of a single biotin molecule by means of biotin ligase (BirA) from E.coli. Additionally, a peptide was covalently attached via a thrombin site containing linker at the N-terminus of the β-chain (Fig. 4a). The gene-expression plasmids were transfected into ExpreSF^{+} insect cells using a baculovirus transfection system and proteins were expressed in a serum free medium to avoid contaminating serum proteins. The proteins were isolated and purified by immunoprecipitation. In the case of tetrameric complexes, the soluble HLA II-peptide molecules were first biotinylated by the BirA enzyme and then allowed to multimerize on a streptavidin backbone via the four biotin specific binding sites (Fig. 4b).

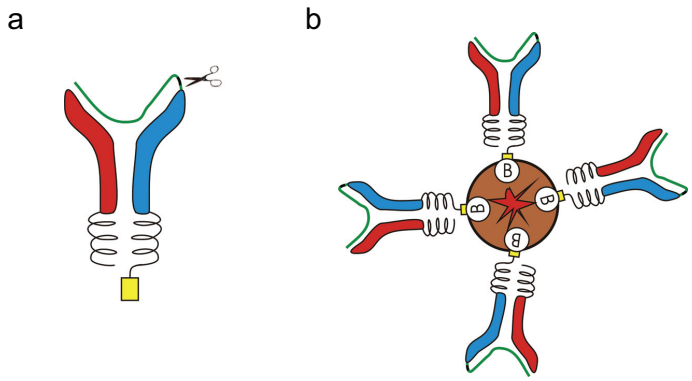

Figure 4 Schematic presentation of a single and tetrameric soluble HLA II–peptide molecule. a.) *The membrane distal part of the α-chain (red) and β-chain (blue) is dimerized via a leucine zipper (in black). The peptide (in green) is covalently attached to the β-chain via a linker containing a thrombin cleavage site (scissors). A BirA site (in yellow) is included at the end of the β-chain for subsequent biotinylation and tetramer assembly.* ***b.)*** *The biotinylated (marked "B") MHC II – peptide molecule multimerized on a streptavidin molecule (in brown) with fluorescent dye (red star).*

HLA II-peptide interaction assays

Soluble HLA II-peptide dissociation assay

This dissociation assay uses soluble HLA II-peptide molecules to measure the kinetic stability of a bound peptide. In short, the molecules were thrombin cleaved to allow for peptide release and incubated for various timepoints before separating the bound from the released peptides. The still-bound peptides were eluted in the presence of an internal standard of known quantity and analyzed on a MALDI-TOF MS instrument. The relative quantity of remaining peptide in each sample was determined by comparing the ionization peak intensity of the peptide with that of the internal standard, and the results were fitted to a single exponential decay curve. This system has the obvious advantage of eliminating the peptide loading step. The permanent close proximity of the peptide to the groove would limit the possible intermediate binding of peptides, as seen for double exponential decay curves. Also, with regards to CLIP, the tethering of CLIP to the MHC II is likely the most physiologically relevant manner to measure the kinetic stability as it simulates the manner in which Ii assembles to MHC II in the ER. Notably, the loading of CLIP onto detergent solubilized DQ2.5 for off-rate measurements was unsuccessful (data not shown).

Another advantage of using water soluble truncated molecules over detergent solubilized full length molecules in dissociation studies is the reported increase in peptide dissociation due to the presence of detergent[161]. Additionally, the detergent effect was proposed to be allele dependent, which would complicate the comparison of dissociation rates between alleles. Notably, in the case of DQ2.5, the α-I-gliadin peptide showed a comparable dissociation rate in both systems (detergent solubilized DQ2.5 off-rate shown in[120]).

Fluid-phase peptide binding/exchange assay

To assess the binding efficiency of peptides to HLA II, we used a traditional inhibition assay as previously described[162]. This assay measures the ability of a peptide to inhibit, or out-compete, the binding of a radioactively labelled indicator peptide, thus giving an indication of the binding capacity. This assay was applied in the CLIP2 binding frame determination utilizing a combination of N- and C-terminal peptide truncations, together with a lysine scan, which takes advantage of the

molecule's ability to accommodate a lysine residue in position 5 of the peptide, but not in positions 4 and 6.

A similar assay was applied to determine the differential DM efficiency on DR3 and DQ2.5 using water soluble HLA II-CLIP complexes. This assay measured the ability of a titrated amount of DM to exchange CLIP1 for a high affinity radioactively labelled indicator peptide. In addition, the ability of DR3 to competitively inhibit DM-DQ2.5 interaction was assessed by utilizing this assay, but in this case a titrating amount of un-cleaved DR3-CLIP molecules was added. The assay with reversed roles of the molecules was also completed. This assay attempts to mimic the in vivo situation where DR3 is present in ten fold excess[140]. However, the inability to displace the peptide from the HLA II molecule may have an unknown effect on the DM-HLA II interaction.

Solid-phase peptide exchange assay

The level of peptide exchange was also assayed in an antibody based catch assay modified from Stepniak *et al*[163]. This assay was used to explore the effect of DM on DQ2.5 compared to that of DR3 in cells. Lysates of hemizygous DQ2.5/DR3 Epstein Barr virus (EBV) transformed B lymphoblastoid cell lines (B-LCL) either expressing or not expressing DM, was added to wells with DQ-specific or DR-specific antibodies. Biotinylated high affinity peptides were added and following incubation, the amount of exchanged peptide was measured by time-resolved fluorometry using europium-labeled streptavidin. The *intra-allelic* differences in spontaneous exchange with biotinylated peptides were used as a measure of DM effect, as DM favorably exchanges CLIP for higher affinity peptides. This assay is quick and requires little work; however the quantity of the bound HLA II is unknown, although the *intra-allelic* relative quantity was measured by using a secondary biotinylated anti-DQ or -DR antibody. However, since this is only a relative measure of HLA II quantity, *inter-allelic* comparisons cannot be performed with this assay.

CLIP quantification

The CLIP phenotype assessment was completed in EBV transformed B-LCLs. However, this analysis could have benefited from being completed in dendritic cells, because of the important role of DCs in naïve T-cell activation and positive/negative thymic selection. However, due to the difficulties associated with culturing of these cells, the readily available BLCLs were used in this work.

B-LCL peptide elution and MS quantification of CLIP

The immunoprecipitation of HLA II molecules from B-LCLs were completed essentially as described[114]. The HLA II molecules were acid eluted and peptides were isolated by use of a size exclusion filter. The CLIP peptides were identified by MS/MS analysis. To assess the CLIP1 phenotype of APCs, peptides were analyzed on the MALDI-TOF MS. One limitation of this method was the inability to detect CLIP2 due to the low ionization of these peptides. However, the high ionizing efficiency of CLIP1 allowed for a rough comparison of relative CLIP1quantity in cells by comparing their peak intensity with that of the other endogenous peptides. To accurately quantify the CLIP1 and CLIP2 peptides, we labeled the eluted peptides and CLIP indicator peptides with heavy and light versions of isotope-coded protein labels (ICPL), followed by LC-MS analysis. The labeling allowed us to separate the eluted peptides from the indicator peptides on the MS spectrum without affecting the LC retention time or the ionization efficiency of the peptides. Quantification was completed by peak intensity comparisons of the eluted peptides with the indicator peptides of known quantity. One problem that emerged with the quantification was the occurrence of oxidated form of the peptides. However, as this appeared to be the result of the centrifugation and lyophilization process, we assessed that the level of oxidation would be similar for eluted peptides and indicator peptides.

Flow cytometric assessment of CLIP1 phenotype

The assessment of the CLIP phenotype through flow cytometry analysis is a time efficient and easy way to assess the level of CLIP expression in reference to the level of DQ expression on the cell surface. However, there are several disadvantages of this method. The employed CLIP antibody (cerCLIP1.1) recognizes CLIP peptides bound

to all alleles expressed by the APC. In addition, the antibody only recognizes a limited number of the CLIP1 variants, and no CLIP2 variants. In the case of the B-LCL transfectants in paper II there were no interfering DR alleles, thus allowing for differential surface CLIP amount assessment. The latter issue, however, could have an impact on the CLIP1 phenotype assessment if different alleles have variable preference for CLIP1 variants. However, in our case, the MS analysis supported the results obtained by flow cytometry.

T cells

Tetramer staining of T cells

The tetramers used in this study consisted of streptavidin conjugated sDQ2.5-α-I gliadin or sDQ2.5-α-II gliadin complexes. In addition, a negative control tetramer was also used where DQ2.5 was tethered to a self-peptide (a peptide originating from an HLA I molecule found to be a dominating self-ligand for DQ2.5[115,117]). $CD4^+$ T cells isolated from peripheral blood were co-stained with tetramers and conjugated monoclonal antibodies specific for various surface markers, and subsequently analyzed by flow cytometry. The specificity of the tetramers was also determined by staining a T cell specific for the α-I gliadin epitope with a tetramer presenting the α-II gliadin epitope, and *vice versa*. This test displayed a very low cross-reactivity and background staining (Fig. 5).

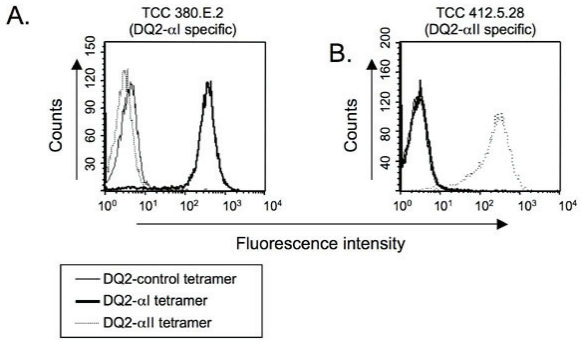

Figure 5 Specificity-testing of tetramers*. Two T-cell clones were tested for cross-reactivity. The T-cell clones were only stained with the tetramer that contained their specific epitope. The control tetramer showed background staining. This figure is taken from article III (Supplementary Information, Fig. 6).*

The high specificity of these tools reduces the likelihood of obtaining false positives, which shows their applicability in the identification of antigen specific T cells of low frequency.

T-cell proliferation assays

The T-cell assays in this work measured the ^{3}H-thymidine incorporation of proliferating T cells as a result of TCR recognition of APC-presented gluten antigen. The APCs used were γ-irradiated B-LCLs either 1) DR3/DQ2.5 homozygous, or 2) DQ-/DR-deleted cells that were retrovirally transfected with wild type or mutated DQ2.2/DQ2.5 full length molecules. The T-cell clones were derived from various gluten reactive T cells isolated from DQ2.5^{+} celiac disease patients. We performed two types of assays: 1) The standard assay, with peptide incubation of APC followed by direct addition of T cells[119], and 2) the functional off-rate assay, where the peptide-pulsed APC was incubated for various time points in the absence of peptide prior to T-cell addition[70].

For certain gluten peptides, the traditional T-cell assay did not reveal a difference between the T-cell activation capability of DQ2.2- and DQ2.5-expressing APCs. In contrast, the functional off-rate assay displayed dramatic time-dependent differences in the T-cell activation capability of the same APCs. These differences reflect the kinetic stability variations of the peptide-HLA complexes, indicating that this assay is a better measure of the physiological relevance of the antigen.

DISCUSSION

Soluble recombinant HLA II molecules as tools in disease analysis

HLA II molecules are major players of the immune system through their presentation of endogenous and exogenous peptides on APCs. The presentation of processed fragments of pathogens alerts the immune system of its presence, thus allowing for a powerful response by pathogen specific T cells. Autoimmune diseases display a similar immune reaction, as most of these are associated with peptide-presenting HLA molecules[85]. This association implicates two interrelated main aspects of the reaction: 1) The uptake, processing and presentation of the antigen on HLA molecules, and 2) the subsequent interaction of T cells specific for the HLA-antigen complex. A third aspect of HLA involvement is the role in thymic selection of T cells, which upon faulty regulation may yield an increase in autoimmune T cells.

Water soluble recombinant MHC II molecules are constructed in a variety of ways, but common to all is the removal of the hydrophobic transmembrane region. One major advantage of these molecules is the detergent independence, which allows for easier handling and a wider specter of applications. These include the determination of peptide affinity for HLA molecules via association and dissociation studies, and also the significance of polymorphic residues for this affinity through mutational studies. In addition, the elucidation of the mechanism behind DM's peptide editing function, and the proposed DM interaction site on HLA were largely dependent on these tools. The solving of numerous crystal structures were also facilitated by these molecules, which subsequently allowed for modeling of the TCR-MHC interaction. Furthermore, the use of HLA tetramers allows for not only the identification and quantification of disease specific T cells, but also the isolation of these cells to further characterize their effector functions.

The ancestral HLA haplotype 8.1, comprising of the HLA-A1, -B8, -DR3, and -DQ2.5 alleles, is associated with more than 30 autoimmune diseases and is therefore termed the "autoimmune haplotype"[164,165]. Notably, for several of the diseases the major HLA association have been mapped to the DR3-DQ2 interval[166,167]. In the case of CD, the role of DQ2.5 is largely defined by its unique ability to bind and present

proline rich deamidated gluten peptides to gluten specific $CD4^+$ T cells. However, the extensive association with autoimmune diseases suggests that this molecule may have unique properties that go beyond its ability to bind proline rich sequences. Paper I and II attempts to unravel this uniqueness through extensive use of recombinant soluble MHC II molecules.

DQ2.5 and DM inefficiency

A unique property of DQ2.5 is the previously reported high abundance of two cohorts of CLIP peptides among natural ligands eluted from APC-isolated DQ2.5 molecules[115,117]. A high level of CLIP-expressing HLA II on APCs is not a common trait, and has been associated with loss of DM expression[168]. This was addressed in paper I, by developing a peptide dissociation assay using soluble recombinant DR3 and DQ2.5 molecules tethered to a peptide by a cleavable linker. This method gives ~100% loading of CLIP and thus simulates the Ii loading in the ER. The dissociation assay was completed in the presence or absence of DM, which allowed us to determine that the underlying mechanism for the CLIP-high phenotype of DQ2.5 was indeed due to DM inefficiency, combined with a relatively high kinetic stability of CLIP. By using the same molecules in a DM competitive inhibition assay, we also found that DR3 was able to out-compete DQ2.5 for DM interaction, which would further diminish the effects of DM. However, the mechanism for the low DM interaction was not determined during this work. On the other hand, it is interesting to speculate whether or not the naturally occurring deletion of residue α53 in DQ2.5 may be involved. The proposed DM induced conformational change in HLA II is suggested to be mediated by a "lever action" through hydrophobic interactions between the protruding phenylalanine in position α51 (α51F) and corresponding residue in DM[46,51]. This may move the extended α51-53 strand, thus destabilizing the peptide by breaking the associated hydrogen bonds. The possible effects of the α53 deletion can be illustrated with crystal structures of DR3-CLIP[6] and DQ2.5-α-I gliadin[118], by comparing their presumed DM interacting site (Fig. 6) (Notably, the proposed DM interaction site was determined by biochemical studies using the HLA-DR1 allele[47,48]). In contrast to the α53-sufficient DR3, the α53 deletion in DQ2.5 shortens the extended strand, thus leaving the hydrophobic α51F residue "hidden"

behind the hydrophilic arginine in position α50 (α50R). This hydrophobic to hydrophilic surface change could have an impact on both the association to and the "lever action" of DM on DQ2.5.

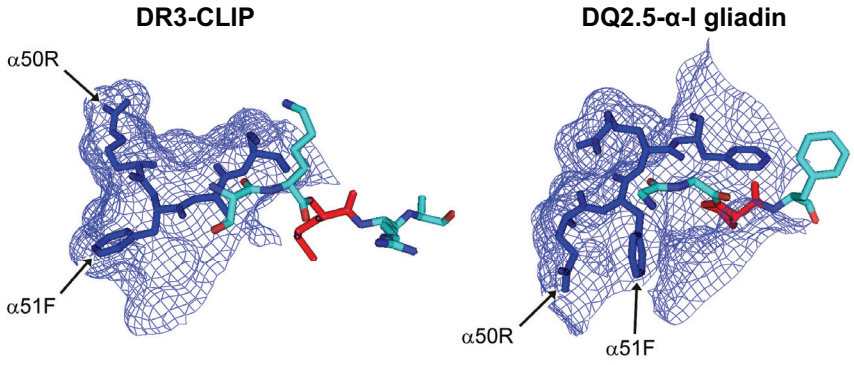

Figure 6 Proposed DM interacting residue α51F is exposed for DR3, but hidden for DQ2.5. *The alpha-chain of DR3 and DQ2.5 (in blue) with the N-terminal end of the CLIP (DR3) and α-I gliadin (DQ2.5) peptide (in turquoise). Position P1 of the peptide is in red. Figures were generated using PyMol (DeLano Scientific LLC).*

To further investigate the potential role of the α53 deletion on DM efficiency, recombinant soluble DQ2.5 molecules that contained insertions of either arginine or glycine in this position were constructed and expressed. However, the peptide dissociation from these molecules was extremely quick, indicating that the insertion may have altered the binding groove conformation (Fallang *et al*, unpublished results). Further studies must therefore be completed to investigate the role of the α53 deletion, or to pinpoint other causal residue(s).

The low DM efficiency of DQ2.5 has potential implications for peptide exchange and antigen presentation in the periphery. Moreover, it may affect thymic selection and T-cell tolerance. This is supported by studies done with mice either lacking DM, or expressing a single MHC II-peptide complex. These studies reported changes in the $CD4^+$ T-cell population, including a reduction in numbers and diversity, a more promiscuous ligand recognition, and increased occurrence of autoreactive cells[55-57,169-171]. These effects were suggested to be the result of inefficient positive and negative thymic selection. It is therefore tempting to speculate that the DM insensitivity and CLIP abundance found with DQ2.5 can lead to a limited $CD4^+$ T-cell repertoire

containing promiscuous and autoreactive T cells, thus offering a possible explanation to the disease association of the DR3/DQ2 haplotype.

Contrary to our findings, there have been reports that MHC II displaying low CLIP affinity is associated with autoimmune diseases, as reviewed by Busch *et al*[172]. One example is the positive or negative association of various DR4 alleles with rheumatoid arthritis, which correlates with their CLIP affinity[173]. However, the same study reports exceptions to this linkage. Busch *et al* therefore proposes that the mechanism for disease association differs between alleles of high or low CLIP affinity. This is further supported by dissociation studies from paper II, which show that the non-CD associated DQ2.2 has low CLIP kinetic stability.

Differential association of DQ2.5 and DQ2.2 with celiac disease – Molecular aspects

The importance of HLA polymorphisms with regards to disease association was originally assessed in the one-dimensional context of linear sequences, thus limiting the accuracy of the predicted effect. However, the emergence of crystal structures of soluble recombinant HLA molecules with tethered peptides allowed for more refined predictions[1]. These three-dimensional models display whether or not polymorphisms affect the TCR docking and/or the peptide binding to HLA by altering the hydrogen bonding or binding groove conformation. The predicted involvement of polymorphic residues in peptide binding is normally based on "pocket-anchor" interactions, involving peptide side chains and polymorphic HLA residues[86].

Of two variants of DQ2, DQ2.5 shows high association with celiac disease, while the highly similar variant DQ2.2 does not. As their peptide binding properties are highly similar and they both have the ability to activate gluten specific T cells, the differential association has remained an enigma. Previous structural analyses of DQ2.5 have focused on the uniqueness of the lysine in position 71 of the β-chain (β71K)[86,118]. This positively charged residue mediates a preference for negative charges in positions P4, P6 and P7 of the peptide, which is common in TG2-modified gliadin epitopes. However, since DQ2.2 has virtually the same β-chain as DQ2.5 (DQB1*0201 and DQB1*0202 differ only at residue 135 in the membrane proximal

domain), the β71K residue does not explain their differential CD association. Structural analysis of DQ2.5 reveals that the two molecules differ in their α-chains by ten polymorphic residues in the ligand binding domain (Fig. 7). Six of these residues are located in the proposed DM interaction site, thus making a differential DM peptide editing a possible candidate. This speculation was initially supported by findings in paper II showing that the CLIP amount was very low among natural ligands eluted from DQ2.2 isolated from B-LCLs. However, dissociation experiments using soluble DQ2.5-CLIP molecules with the six polymorphic residues changed to the corresponding residues of DQ2.2, did not significantly alter the DM effect. The theory was further weakened by the subsequent observation that the dissociation rate of CLIP from DQ2.2 was thirty-six folds faster than for DQ2.5, and that they displayed a similarly low effect of DM. Interestingly, both molecules have the α53 deletion, which underscores the potential importance of this deletion in the observed low DM interaction of both molecules.

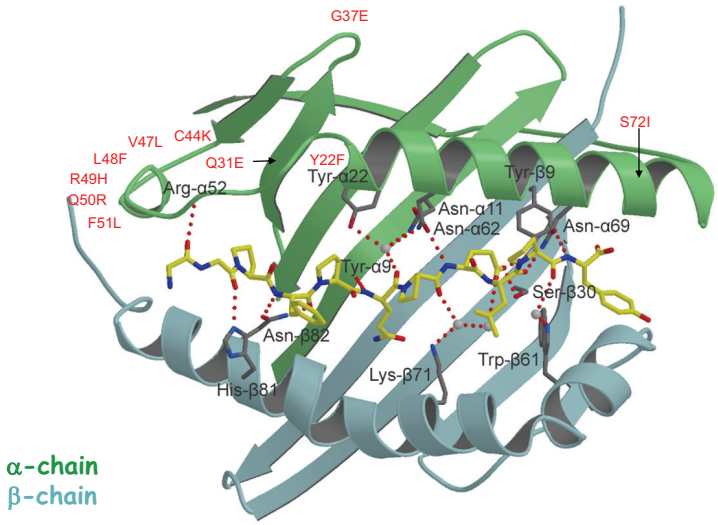

Figure 7. DQ2.5-α-I gliadin complex displaying polymorphic amino acid residues of DQ2.5 and DQ2.2. *The polymorphic residues are displayed in red lettering. The figure also depicts the extensive hydrogen bonding between the HLA and peptide. Figure modified from*[118]

The lacking role of DM led us to analyze the remaining four polymorphisms. The crystal structure revealed an obvious candidate in the tyrosine in position 22 (α22Y)

due to its involvement in hydrogen bonding, via a water molecule, to the peptide backbone[118] (Fig. 7). DQ2.2 accommodates a phenylalanine in this position, which lacks the hydroxyl required for this hydrogen bond network. To study this polymorphism, we performed dissociation assays using soluble CLIP-linked DQ2.2 and DQ2.5 molecules containing switched residues in α22. Strikingly, the switching of α22 caused a reversal of CLIP kinetic stability, thus displaying the importance of this hydrogen bond. The same assay using constructs linked to a gliadin peptide mirrored the result of CLIP, thus connecting the finding to celiac disease. The observations were further confirmed by transfecting α22 mutant DQ2.5 and DQ2.2 full length molecules into DQ- and DR-devoid B-LCLs, which displayed a reversal of the DQ2.5 and DQ2.2 CLIP phenotype.

The hydrogen bonding between polymorphic MHC residues and peptide backbone, though prevalent in both DQ and DR alleles, has been given little attention with regards to the influence on peptide binding. One reason for the neglect may be their frequent bonding via water molecules that are not always visible due to the requirement of high resolution crystal structures. Due to the peptide backbone involvement, this hydrogen bond will have a universal effect on all ligands irrespective of sequence. The impact on the peptide affinity is potentially huge and the inclusion of these bonds may therefore prove crucial when predicting the effects of polymorphisms.

Differential association of DQ2.5 and DQ2.2 with celiac disease – Physiological aspects

In terms of celiac disease, the functional relevance of the differential peptide kinetic stability observed with the α22 polymorphism is likely related to the ability of the dendritic cell to retain antigen for prolonged presentation. DCs that endocytose antigen in the tissue travel to lymph nodes for antigen presentation to T cells. This potentially long transition time presents a requirement for sufficient peptide affinity as suggested by Lanzavecchia *et al*[70]. The same author showed that immunogenic peptides are functionally bound irreversibly in that the half life of the complex is higher than the rate of MHC II turnover. To assess the proposed functional effect of

the α22 polymorphism on the two DQ2 molecules, we adopted the functional off-rate assay done by Lanzavecchia *et al*[70]. This assay measures the effect of peptide dissociation on the ability of the APC to present antigen to T cells, thus mimicking the transit time of the DC traveling from tissue to lymph node. In order to directly relate our findings to celiac disease, we pulsed APCs with gluten peptides and measured their ability to activate gluten specific T cells. As expected, DQ2.5 displayed an efficient presentation of gliadin peptides over time, while the T-cell stimulatory capacity of DQ2.2 diminished rapidly for all gluten peptides tested. In addition, a reversal of the stimulatory capacity was observed when using α22-switched DQ2.5 and DQ2.2 expressing transfectants as APCs, thus verifying the causative role of α22. This finding presents evidende that the differential association of the two DQ2 variants with celiac disease may be due to differences in their inherent kinetic stability of gluten peptides. Credence to this notion has been obtained in an *in vivo* study in mice of MHC I and $CD8^+$ T cells[174]. In this study, Henrickson *et al* demonstrated that a small difference in peptide kinetic stability (t½ 2.4 h vs. 6 h) of two peptides, differing only at the P9 residue, translated into an enormous (30,000 fold) difference in antigenicity in the lymph nodes. This was assayed by injection of CFSE labeled T cells into mice 18 hours after footpath injection of DCs pulsed with either of the two peptides. The dramatic observed effect was ascribed to changes in the dynamic interaction between the naïve T cell and DCs, which was found crucial for the transition of the T cell from a naïve state to a state of activation and subsequent proliferation. These observations underscore the relevance of our reported peptide stability differences between the DQ2 variants in terms of celiac disease association. It is plausible that the transition time associated with DC migration to lymph nodes, combined with the disparate kinetic stabilities of gluten peptides for DQ2.5 and DQ2.2, may result in antigen density differences that would ultimately affect the T cell-DC interaction dynamics and subsequent naïve T cell activation. However, once the anti-gluten T-cell response is initiated, the activation of the effector T cells in the lamina propria would not be affect by these kinetic stability differences. The anti-gluten immune response of the few $DQ2.2^+$ and the $DQ2.5^+$ CD patients would therefore be indistinguishable.

Our work, and the work by Lanzavecchia[70], Henrickson[174] and others, imply that in the quest for disease precipitating antigens, the importance of kinetic stability for the MHC must compliment the specificity of the T-cell epitope in order to find the grail.

Detection of disease specific T cells in peripheral blood of celiac disease patients

Soluble MHC I molecules in fluorescently labeled tetramers have been extensively utilized as tools to identify and characterize disease specific T cells. However, the use of soluble MHC II molecules have proven difficult due to the combined low frequency of disease related $CD4^+$ T cells and the low TCR-MHC II affinity[154,155]. This appeared to be the case for celiac disease as well, as initial efforts using tetramers to detect gluten specific T cells in the tissue of treated or untreated celiac disease patients were unsuccessful (Quarsten *et al*, unpublished results). In paper III, however, based on a study by Anderson *et al*[175], we were able to detect gluten-specific $CD4^+$ T cells in peripheral blood with DQ2.5-α-I/α-II gliadin tetramers after doing a short gluten challenge of treated celiac patients. By co-staining with specific antibodies and the tetramers, we were also able to phenotypically characterize these IFN-γ producing T cells, which revealed the gut-homing associated β_7 integrin as a common marker. The imprinting of gut-homing on T cells has been contributed to DCs located in gut-associated secondary lymphoid organs[176,177]. Therefore, the identified T cells likely represent activated naïve or central memory T cells in transit from secondary lymph nodes to the small intestinal lamina propria. Characterization of other phenotypic markers revealed a great heterogeneity. This may represent the various states of differentiation of the T cells following the previous antigen stimulation, as suggested by Roman *et al*[178].

The diagnosis of celiac disease is based on a combination of several techniques. However, the only accepted method is biopsy analysis of the small intestine, where the level of villous atrophy, crypt hyperplasia and IEL infiltration determines disease severity/presence, as graded by the Marsh scoring system[179]. However, this analysis has certain pitfalls. The affected mucosa of a celiac patient is not always significantly altered, and the potential patchiness of the celiac lesion may lead to difficulties

making the correct diagnosis[179]. The serological detection of IgA antibodies towards deamidated gluten and TG2 is another tool for CD diagnosis, which both display similarly high sensitivity and specificity for celiac disease[180]. The presence of anti-TG2 in celiac patients has been detected even in the absence of significantly altered mucosa[181], thus revealing the importance of serology. However, these tests are not fool proof, as false negative results occur. The known predisposition of celiac patients to IgA deficiency further complicates the matter[182]. There is also a cohort of CD patients that are on a gluten free diet without a proper clinical work up. Therefore, there are likely healthy individuals among the treated CD patients that could benefit from being re-diagnosed. However, the re-diagnosis of such patients requires at least a month-long gluten challenge in order for clinical signs to reoccur, presenting the need for an improved method. The use of DQ2.5 tetramers, as described in paper III, may therefore represent a potential diagnostic tool to distinguish the treated healthy individuals from the CD patients.

FINAL COMMENTS

The work in this thesis demonstrates that soluble recombinant HLA II molecules can be powerful tools in assessing the molecular basis for disease association, and potentially also in disease diagnostics. Interestingly, our findings have extended implications in the understanding of peptide-HLA interaction. Peptide binding affinity for HLA II molecules have been mainly attributed to two established principles involving conserved hydrogen bonds and "pocket-anchor" interactions. We show that a third principle, involving hydrogen bonds between polymorphic HLA II residues and the peptide backbone, is a major determinant of the peptide-HLA II interaction. The significance of this bond was exemplified in our work as its presence or absence likely plays a role in the observed differential celiac disease association with two DQ2 alleles, due to peptide kinetic stability differences. The role of such hydrogen bonds may also be important in the HLA-association for other diseases, and should be investigated. However, in order to ensure the validity of our results, the *in vitro* findings must be transferred to an *in vivo* situation. This will be our focus in future studies.

ERRATA

Paper I

In the **Results** section under the first subhead, *In cells, CLIP is released from DR3 more efficiently than from DQ2*, the corrected text in the second half of the first paragraph should read as follows: "CLIP was detected in association with both DQ2 and DR3 molecules at the 8-h postsynthesis time point. In the presence of DM (8.1.6 cells), the CLIP:DR3 ratio is significantly lower at 8 h of chase (23% of 9.5.3), whereas the change in the CLIP:DQ2 ratio is more modest (66% of 9.5.3; Fig. 1*B* and densitometric analysis in *C*). Thus, DQ2-CLIP complexes appear more resistant than DR3-CLIP to DM-mediated peptide exchange. Formally, DM resistance could result from a thermodynamic block (reduced DM binding to DQ2-CLIP) or a kinetic block (reduced ability of DM to release CLIP from DQ2). The finding that increased DM (9.5.3-DM) further reduces DQ2-CLIP levels at 8 h (23% of levels in 9.5.3) suggests that the block is thermodynamic. At all levels of DM, CLIP removal is more efficient from DR3 than from DQ2 (Figs. 1, *B* and *C*)."

The corrected legend for Fig. 1 should read as follows: "**FIGURE 1**. The efficiency of Ii peptide release from DQ2 is lower than from DR3 and related to the level of HLA-DM. *A*, Intracellular DM staining of the indicated cell lines using anti-HLA-DM dimer Ab. *B*, 9.5.3, 8.1.6, and DM transfectant of 9.5.3 (9.5.3-DM) cells were pulsed for 1 h with [^{35}S]methionine/cysteine and then chased in label-free medium. Cell lysates (3 x 10^6 cell equivalents/lane) were immunoprecipitated with anti-DQ mAb, SPV-L3 (*top*), or anti-DR mAb, L243 (*bottom*), at the indicated times (hours) of chase and then analyzed by SDS-PAGE. Representative images from one of two independent experiments are shown. *C*, Densitometry analysis calculating ratios of CLIP to class II band intensity at 8 h from images shown in *B*. *D*, Assessment of cell surface CLIP-class II accumulation by FACS analysis. Indicated cell lines (9.5.3: DR3, DQ2, DP4, DM-null; 9.22.3: DQ2, DP4, DM+; 3.1.3: DQ1, DP4, DM+; 5.2.4: DP4, DM-null) were stained with anti-CLIP (CerCLIP.1; *left panel*) and anti-DQ (SPV-L3; *right panel*) followed by a secondary Ab (FITC-conjugated goat anti-mouse IgG). Control staining (without primary Ab: dotted lines) is comparable on all cell

lines and histogram for one representative cell line is shown in each panel. CLIP:DQ ratios were calculated as $MFI_{CerCLIP} - MFI_{isotype} \div MFI_{SPVL3} - MFI_{isotype}$: CLIP:DQ1 = 1:75.9; CLIP:DQ2 = 1:11.3; $p < 0.04$. Representative data from one of more than three experiments are shown."

REFERENCES

1. Brown, J. H., T. S. Jardetzky, J. C. Gorga, L. J. Stern, R. G. Urban, J. L. Strominger, and D. C. Wiley. 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* 364: 33-39.

2. Chicz, R. M., R. G. Urban, J. C. Gorga, D. A. Vignali, W. S. Lane, and J. L. Strominger. 1993. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J Exp Med* 178: 27-47.

3. Nelson, C. A., and D. H. Fremont. 1999. Structural principles of MHC class II antigen presentation. *Rev Immunogenet* 1: 47-59.

4. Stern, L. J., J. H. Brown, T. S. Jardetzky, J. C. Gorga, R. G. Urban, J. L. Strominger, and D. C. Wiley. 1994. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature* 368: 215-221.

5. Sant, A. J., C. Beeson, B. McFarland, J. Cao, S. Ceman, P. W. Bryant, and S. Wu. 1999. Individual hydrogen bonds play a critical role in MHC class II: peptide interactions: implications for the dynamic aspects of class II trafficking and DM-mediated peptide exchange. *Immunol Rev* 172: 239-253.

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

7. Jardetzky, T. S., J. H. Brown, J. C. Gorga, L. J. Stern, R. G. Urban, J. L. Strominger, and D. C. Wiley. 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.

8. Stratikos, E., D. C. Wiley, and L. J. Stern. 2004. Enhanced catalytic action of HLA-DM on the exchange of peptides lacking backbone hydrogen bonds between their N-terminal region and the MHC class II alpha-chain. *J Immunol* 172: 1109-1117.

9. WHO Nomenclature Committee. 1975. Nomenclature for factors of the HLA system. *Bull Wld Hlth Org* 52.

10. The MHC sequencing consortium. 1999. Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium. *Nature* 401: 921-923.

11. Thorsby, E., and B. A. Lie. 2005. HLA associated genetic predisposition to autoimmune diseases: Genes involved and possible mechanisms. *Transpl Immunol* 14: 175-182.

12. Vader, W., D. Stepniak, Y. Kooy, L. Mearin, A. Thompson, J. J. van Rood, L. Spaenij, and F. Koning. 2003. The HLA-DQ2 gene dose effect in celiac disease is directly related to the magnitude and breadth of gluten-specific T cell responses. *Proc Natl Acad Sci U S A* 100: 12390-12395.

13. The Anthony Nolan Research Institute. 2009. HLA nomeclature - http://www.ebi.ac.uk/imgt/hla/nomenclature/index.html.

14. Anderson, M. S., and J. Miller. 1992. Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules. *Proc Natl Acad Sci U S A* 89: 2282-2286.

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

16. Lotteau, V., L. Teyton, A. Peleraux, T. Nilsson, L. Karlsson, S. L. Schmid, V. Quaranta, and P. A. Peterson. 1990. Intracellular transport of class II MHC molecules directed by invariant chain. *Nature* 348: 600-605.

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

18. Peters, P. J., J. J. Neefjes, V. Oorschot, H. L. Ploegh, and H. J. Geuze. 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.

19. Benaroch, P., M. Yilla, G. Raposo, K. Ito, K. Miwa, H. J. Geuze, and H. L. Ploegh. 1995. How MHC class II molecules reach the endocytic pathway. *EMBO J* 14: 37-49.

20. Roche, P. A., C. L. Teletski, E. Stang, O. Bakke, and E. O. Long. 1993. Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization. *Proc Natl Acad Sci U S A* 90: 8581-8585.

21. Warmerdam, P. A., E. O. Long, and P. A. Roche. 1996. Isoforms of the invariant chain regulate transport of MHC class II molecules to antigen processing compartments. *J Cell Biol* 133: 281-291.

22. Hiltbold, E. M., and P. A. Roche. 2002. Trafficking of MHC class II molecules in the late secretory pathway. *Curr Opin Immunol* 14: 30-35.

23. Maric, M., B. Arunachalam, U. T. Phan, C. Dong, W. S. Garrett, K. S. Cannon, C. Alfonso, L. Karlsson, R. A. Flavell, and P. Cresswell. 2001. Defective antigen processing in GILT-free mice. *Science* 294: 1361-1365.

24. Denzin, L. K., and P. Cresswell. 1995. HLA-DM induces clip dissociation from MHC class II αβ dimers and facilitates peptide loading. *Cell* 82: 155-165.

25. Sloan, V. S., P. Cameron, G. Porter, M. Gammon, M. Amaya, E. Mellins, and D. M. Zaller. 1995. Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature* 375: 802-806.

26. Sherman, M. A., D. A. Weber, and P. E. Jensen. 1995. DM enhances peptide binding to class-II MHC by release of invariant chain-derived peptide. *Immunity* 3: 197-205.

27. Denzin, L. K., C. Hammond, and P. Cresswell. 1996. HLA-DM interactions with intermediates in HLA-DR maturation and a role for HLA-DM in stabilizing empty HLA-DR molecules. *J Exp Med* 184: 2153-2166.

28. Kovac, Z., and R. H. Schwartz. 1985. The molecular basis of the requirement for antigen processing of pigeon cytochrome c prior to T cell activation. *J Immunol* 134: 3233-3240.

29. Ria, F., B. M. C. Chan, M. T. Scherer, J. A. Smith, and M. L. Gefter. 1990. Immunological activity of covalently linked T-cell epitopes. *Nature* 343: 381-383.

30. Qiao, S. W., E. Bergseng, O. Molberg, J. Xia, B. Fleckenstein, C. Khosla, and L. M. Sollid. 2004. Antigen presentation to celiac lesion-derived T cells of a 33-mer gliadin peptide naturally formed by gastrointestinal digestion. *J Immunol* 173: 1757-1762.

31. Lee, P., G. R. Matsueda, and P. M. Allen. 1988. T cell recognition of fibrinogen. A determinant on the A alpha-chain does not require processing. *J Immunol* 140: 1063-1068.

32. Strubin, M., C. Berte, and B. Mach. 1986. Alternative splicing and alternative initiation of translation explain the four forms of the Ia antigen-associated invariant chain. *EMBO J* 5: 3483-3488.

33. Mihelic, M., A. Dobersek, G. Guncar, and D. Turk. 2008. Inhibitory fragment from the p41 form of invariant chain can regulate activity of cysteine cathepsins in antigen presentation. *J Biol Chem* 283: 14453-14460.

34. Thayer, W. P., L. Ignatowicz, D. A. Weber, and P. E. Jensen. 1999. Class II-associated invariant chain peptide-independent binding of invariant chain to class II MHC molecules. *J Immunol* 162: 1502-1509.

35. Stumptner, P., and P. Benaroch. 1997. Interaction of MHC class II molecules with the invariant chain: role of the invariant chain (81-90) region. *EMBO J* 16: 5807-5818.

36. Siebenkotten, I. M., C. Carstens, and N. Koch. 1998. Identification of a sequence that mediates promiscuous binding of invariant chain to MHC class II allotypes. *J Immunol* 160: 3355-3362.

37. Watts, C. 2004. The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. *Nat Immunol* 5: 685-692.

38. Bikoff, E. K., L. Y. Huang, V. Episkopou, M. J. van, R. N. Germain, and E. J. Robertson. 1993. Defective major histocompatibility complex class II assembly, transport, peptide acquisition, and CD4+ T cell selection in mice lacking invariant chain expression. *J Exp Med* 177: 1699-1712.

39. Viville, S., J. Neefjes, V. Lotteau, A. Dierich, M. Lemeur, H. Ploegh, C. Benoist, and D. Mathis. 1993. Mice lacking the MHC class II-associated invariant chain. *Cell* 72: 635-648.

40. Wong, P., and A. Y. Rudensky. 1996. Phenotype and function of CD4+ T cells in mice lacking invariant chain. *J Immunol* 156: 2133-2142.

41. Matza, D., A. Kerem, and I. Shachar. 2003. Invariant chain, a chain of command. *Trends Immunol* 24: 264-268.

42. Faure-Andre, G., P. Vargas, M. I. Yuseff, M. Heuze, J. Diaz, D. Lankar, V. Steri, J. Manry, S. Hugues, F. Vascotto, J. Boulanger, G. Raposo, M. R. Bono, M. Rosemblatt, M. Piel, and A. M. Lennon-Dumenil. 2008. Regulation of dendritic cell migration by CD74, the MHC class II-associated invariant chain. *Science* 322: 1705-1710.

43. Ye, L., X. Liu, S. N. Rout, Z. Li, Y. Yan, L. Lu, T. Kamala, N. K. Nanda, W. Song, S. K. Samal, and X. Zhu. 2008. The MHC class II-associated invariant chain interacts with the neonatal Fc{gamma} receptor and modulates its trafficking to endosomal/lysosomal compartments. *J Immunol* 181: 2572-2585.

44. Mellins, E., L. Smith, B. Arp, T. Cotner, E. Celis, and D. Pious. 1990. Defective processing and presentation of exogenous antigens in mutants with normal HLA class II genes. *Nature* 343: 71-74.

45. Mellins, E., S. Kempin, L. Smith, T. Monji, and D. Pious. 1991. A gene required for class II-restricted antigen presentation maps to the major histocompatibility complex. *J Exp Med* 174: 1607-1615.

46. Mosyak, L., D. M. Zaller, and D. C. Wiley. 1998. The structure of HLA-DM, the peptide exchange catalyst that loads antigen onto class II MHC molecules during antigen presentation. *Immunity* 9: 377-383.

47. Busch, R., A. Pashine, K. C. Garcia, and E. D. Mellins. 2002. Stabilization of soluble, low-affinity HLA-DM/HLA-DR1 complexes by leucine zippers. *J Immunol Methods* 263: 111-121.

48. Stratikos, E., L. Mosyak, D. M. Zaller, and D. C. Wiley. 2002. Identification of the lateral interaction surfaces of Human Histocompatibility Leukocyte Antigen (HLA)-DM with HLA-DR1 by formation of tethered complexes that present enhanced HLA-DM catalysis. *J Exp Med* 196: 173-183.

49. Narayan, K., C. L. Chou, A. Kim, I. Z. Hartman, S. Dalai, S. Khoruzhenko, and S. Sadegh-Nasseri. 2007. HLA-DM targets the hydrogen bond between the histidine at position β81 and peptide to dissociate HLA-DR-peptide complexes. *Nat Immunol* 8: 92-100.

50. Chou, C. L., and S. Sadegh-Nasseri. 2000. HLA-DM recognizes the flexible conformation of major histocompatibility complex class II. *J Exp Med* 192: 1697-1706.

51. Doebele, R. C., R. Busch, H. M. Scott, A. Pashine, and E. D. Mellins. 2000. Determination of the HLA-DM interaction site on HLA-DR molecules. *Immunity* 13: 517-527.

52. Weber, D. A., B. D. Evavold, and P. E. Jensen. 1996. Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. *Science* 274: 618-621.

53. Grotenbreg, G. M., M. J. Nicholson, K. D. Fowler, K. Wilbuer, L. Octavio, M. Yang, A. K. Chakraborty, H. L. Ploegh, and K. W. Wucherpfennig. 2007. Empty class II Major Histocompatibility Complex created by peptide photolysis establishes the role of DM in peptide association. *J Biol Chem* 282: 21425-21436.

54. Fung-Leung, W. P., C. D. Surh, M. Liljedahl, J. Pang, D. Leturcq, P. A. Peterson, S. R. Webb, and L. Karlsson. 1996. Antigen presentation and T cell development in H2-M-deficient mice. *Science* 271: 1278-1281.

55. Martin, W. D., G. G. Hicks, S. K. Mendiratta, H. I. Leva, H. E. Ruley, and L. Van Kaer. 1996. H2-M mutant mice are defective in the peptide loading of class II molecules, antigen presentation, and T cell repertoire selection. *Cell* 84: 543-550.

56. Surh, C. D., D. S. Lee, W. P. Fung-Leung, L. Karlsson, and J. Sprent. 1997. Thymic selection by a single MHC/peptide ligand produces a semidiverse repertoire of CD4+ T cells. *Immunity* 7: 209-219.

57. Nanda, N. K., and E. K. Bikoff. 2005. DM peptide-editing function leads to immunodominance in CD4 T cell responses in vivo. *J Immunol* 175: 6473-6480.

58. Kropshofer, H., G. J. Hammerling, and A. B. Vogt. 1999. The impact of the non-classical MHC proteins HLA-DM and HLA-DO on loading of MHC class II molecules. *Immunol Rev* 172: 267-278.

59. Chen, X., O. Laur, T. Kambayashi, S. Li, R. A. Bray, D. A. Weber, L. Karlsson, and P. E. Jensen. 2002. Regulated expression of human Histocompatibility Leukocyte Antigen (HLA)-DO during antigen-dependent and antigen-independent phases of B cell development. *J Exp Med* 195: 1053-1062.

60. Chen, X., L. M. Reed-Loisel, L. Karlsson, and P. E. Jensen. 2006. H2-O expression in primary dendritic cells. *J Immunol* 176: 3548-3556.

61. Liljedahl, M., T. Kuwana, W. P. Fung-Leung, M. R. Jackson, P. A. Peterson, and L. Karlsson. 1996. HLA-DO is a lysosomal resident which requires association with HLA-DM for efficient intracellular transport. *EMBO J* 15: 4817-4824.

62. Denzin, L. K., D. B. Sant'Angelo, C. Hammond, M. J. Surman, and P. Cresswell. 1997. Negative regulation by HLA-DO of MHC class II-restricted antigen processing. *Science* 278: 106-109.

63. Fallas, J. L., H. M. Tobin, O. Lou, D. Guo, D. B. Sant'Angelo, and L. K. Denzin. 2004. Ectopic expression of HLA-DO in mouse dendritic cells diminishes MHC class II antigen presentation. *J Immunol* 173: 1549-1560.

64. Kropshofer, H., A. B. Vogt, C. Thery, E. A. Armandola, B. C. Li, G. Moldenhauer, S. Amigorena, and G. J. Hammerling. 1998. A role for HLA-DO as a co-chaperone of HLA-DM in peptide loading of MHC class II molecules. *EMBO J* 17: 2971-2981.

65. Chen, X., and P. E. Jensen. 2008. MHC class II antigen presentation and immunological abnormalities due to deficiency of MHC class II and its associated genes. *Exp Mol Pathol* 85: 40-44.

66. Liljedahl, M., O. Winqvist, C. D. Surh, P. Wong, K. Ngo, L. Teyton, P. A. Peterson, A. Brunmark, A. Y. Rudensky, W. P. Fung-Leung, and L. Karlsson. 1998. Altered antigen presentation in mice lacking H2-O. *Immunity* 8: 233-243.

67. Gondre-Lewis, T. A., A. E. Moquin, and J. R. Drake. 2001. Prolonged antigen persistence within nonterminal late endocytic compartments of antigen-specific B lymphocytes. *J Immunol* 166: 6657-6664.

68. Steinman, R. M., and Z. A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137: 1142-1162.

69. Cella, M., A. Engering, V. Pinet, J. Pieters, and A. Lanzavecchia. 1997. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* 388: 782-787.

70. Lanzavecchia, A., P. A. Reid, and C. Watts. 1992. Irreversible association of peptides with class II MHC molecules in living cells. *Nature* 357: 249-252.

71. Dieu-Nosjean, M. C., A. Vicari, S. Lebecque, and C. Caux. 1999. Regulation of dendritic cell trafficking: a process that involves the participation of selective chemokines. *J Leukoc Biol* 66: 252-262.

72. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245-252.

73. Facchetti, F., W. Vermi, D. Mason, and M. Colonna. 2003. The plasmacytoid monocyte/interferon producing cells. *Virchows Arch* 443: 703-717.

74. Aderem, A., and D. M. Underhill. 1999. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* 17: 593-623.

75. North, R. J. 1978. The concept of the activated macrophage. *J Immunol* 121: 806-809.

76. Rodriguez-Pinto, D. 2005. B cells as antigen presenting cells. *Cell Immunol* 238: 67-75.

77. Bouaziz, J. D., K. Yanaba, G. M. Venturi, Y. Wang, R. M. Tisch, J. C. Poe, and T. F. Tedder. 2007. Therapeutic B cell depletion impairs adaptive and autoreactive CD4+ T cell activation in mice. *Proc Natl Acad Sci U S A* 104: 20878-20883.

78. Starr, T. K., S. C. Jameson, and K. A. Hogquist. 2003. Positive and negative selection of T cells. *Annu Rev Immunol* 21: 139-176.

79. Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* 356: 607-609.

80. Johansson-Lindbom, B., and W. W. Agace. 2007. Generation of gut-homing T cells and their localization to the small intestinal mucosa. *Immunol Rev* 215: 226-242.

81. Finnish-German APECED Consortium. 1997. An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat Genet* 17: 399-403.

82. Nagamine, K., P. Peterson, H. S. Scott, J. Kudoh, S. Minoshima, M. Heino, K. J. E. Krohn, M. D. Lalioti, P. E. Mullis, S. E. Antonarakis, K. Kawasaki, S. Asakawa, F. Ito, and N. Shimizu. 1997. Positional cloning of the APECED gene. *Nat Genet* 17: 393-398.

83. Kyewski, B., and L. Klein. 2006. A central role for central tolerance. *Annu Rev Immunol* 24: 571-606.

84. Ercolini, A. M., and S. D. Miller. 2009. The role of infections in autoimmune disease. *Clin Exp Immunol* 155: 1-15.

85. Shiina, T., H. Inoko, and J. K. Kulski. 2004. An update of the HLA genomic region, locus information and disease associations: 2004. *Tissue Antigens* 64: 631-649.

86. Jones, E. Y., L. Fugger, J. L. Strominger, and C. Siebold. 2006. MHC class II proteins and disease: a structural perspective. *Nat Rev Immunol* 6: 271-282.

87. Gee, S. On the coeliac disease. 1888., 17-20.

88. Dicke, W. K., H. A. Weijers, and J. H. van de Kamer. 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.

89. Trier, J. S. 1991. Celiac sprue. *N Engl J Med* 325: 1709-1719.

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

91. Ferguson, A., E. Arranz, and S. O'Mahony. 1993. Clinical and pathological spectrum of coeliac disease--active, silent, latent, potential. *Gut* 34: 150-151.

92. Trier, J. S., Z. M. Falchuk, M. C. Carey, and D. S. Schreiber. 1978. Celiac sprue and refractory sprue. *Gastroenterology* 75: 307-316.

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

94. Sollid, L. M., and H. Scott. 1998. New tool to predict celiac disease on its way to the clinics. *Gastroenterology* 115: 1584-1586.

95. Lerner, A., V. Kumar, and T. C. Iancu. 1994. Immunological diagnosis of childhood coeliac disease: comparison between antigliadin, antireticulin and antiendomysial antibodies. *Clin Exp Immunol* 95: 78-82.

96. Dieterich, W., E. Laag, H. Schopper, U. Volta, A. Ferguson, H. Gillett, E. O. Riecken, and D. Schuppan. 1998. Autoantibodies to tissue transglutaminase as predictors of celiac disease. *Gastroenterology* 115: 1317-1321.

97. Sulkanen, S., T. Halttunen, K. Laurila, K. L. Kolho, I. R. Korponay-Szabo, A. Sarnesto, E. Savilahti, P. Collin, and M. Maki. 1998. Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. *Gastroenterology* 115: 1322-1328.

98. van Heel, D. A., and J. West. 2006. Recent advances in coeliac disease. *Gut* 55: 1037-1046.

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

100. Sollid, L. M., and B. A. Lie. 2005. Celiac disease genetics: current concepts and practical applications. *Clin Gastroenterol Hepatol* 3: 843-851.

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

102. Spurkland, A., L. M. Sollid, I. Polanco, F. Vartdal, and E. Thorsby. 1992. HLA-DR and -DQ genotypes of celiac disease patients serologically typed to be non-DR3 or non-DR5/7. *Hum Immunol* 35: 188-192.

103. Karell, K., A. S. Louka, S. J. Moodie, H. Ascher, F. Clot, L. Greco, P. J. Ciclitira, L. M. Sollid, and J. Partanen. 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.

104. van Heel, D. A., L. Franke, K. A. Hunt, R. Gwilliam, A. Zhernakova, M. Inouye, M. C. Wapenaar, M. C. N. M. Barnardo, G. Bethel, G. K. T. Holmes, C. Feighery, D. Jewell, D. Kelleher, P. Kumar, S. Travis, J. R. Walters, D. S. Sanders, P. Howdle, J. Swift, R. J. Playford, W. M. McLaren, M. L. Mearin, C. J. Mulder, R. McManus, R. McGinnis, L. R. Cardon, P. Deloukas, and C. Wijmenga. 2007. A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. *Nat Genet* 39: 827-829.

105. Hunt, K. A., D. P. McGovern, P. J. Kumar, S. Ghosh, S. P. Travis, J. R. Walters, D. P. Jewell, R. J. Playford, and D. A. van Heel. 2005. A common CTLA4 haplotype associated with coeliac disease. *Eur J Hum Genet* 13: 440-444.

106. Monsuur, A. J., P. I. de Bakker, B. Z. Alizadeh, A. Zhernakova, M. R. Bevova, E. Strengman, L. Franke, S. R. van't, M. J. van Belzen, I. C. Lavrijsen, B. Diosdado, M. J. Daly, C. J. Mulder, M. L. Mearin, J. W. Meijer, G. A. Meijer, O. E. van, M. C. Wapenaar, B. P. Koeleman, and C. Wijmenga. 2005. Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. *Nat Genet* 37: 1341-1344.

107. Hunt, K. A., A. Zhernakova, G. Turner, G. A. R. Heap, L. Franke, M. Bruinenberg, J. Romanos, L. C. Dinesen, A. W. Ryan, D. Panesar, R. Gwilliam, F. Takeuchi, W. M. McLaren, G. K. T. Holmes, P. D. Howdle, J. R. F. Walters, D. S. Sanders, R. J. Playford, G. Trynka, C. J. J. Mulder, M. L. Mearin, W. H. M. Verbeek, V. Trimble, F. M. Stevens, C. O'Morain, N. P. Kennedy, D. Kelleher, D. J. Pennington, D. P. Strachan, W. L. McArdle, C. A. Mein, M. C. Wapenaar, P. Deloukas, R. McGinnis, R. McManus, C. Wijmenga, and D. A. van Heel. 2008. Newly identified genetic risk variants for celiac disease related to the immune response. *Nat Genet* 40: 395-402.

108. Romanos, J., D. Barisani, G. Trynka, A. Zhernakova, M. T. Bardella, and C. Wijmenga. 2009. Six new coeliac disease loci replicated in an Italian population confirm association with coeliac disease. *J Med Genet* 46: 60-63.

109. Shewry, P. R., and A. S. Tatham. 1990. The prolamin storage proteins of cereal seeds: structure and evolution. *Biochem J* 267: 1-12.

110. Shan, L., O. Molberg, I. Parrot, F. Hausch, F. Filiz, G. M. Gray, L. M. Sollid, and C. Khosla. 2002. Structural basis for gluten intolerance in celiac sprue. *Science* 297: 2275-2279.

111. Piper, J. L., G. M. Gray, and C. Khosla. 2002. High selectivity of human tissue transglutaminase for immunoactive gliadin peptides: implications for celiac sprue. *Biochemistry* 41: 386-393.

112. Fleckenstein, B., O. Molberg, S. W. Qiao, D. G. Schmid, M. F. von der, K. Elgstoen, G. Jung, and L. M. Sollid. 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.

113. Parrot, I., P. C. Huang, and C. Khosla. 2002. Circular dichroism and nuclear magnetic resonance spectroscopic analysis of immunogenic gluten peptides and their analogs. *J Biol Chem* 277: 45572-45578.

114. Johansen, B. H., S. Buus, F. Vartdal, H. Viken, J. A. Eriksen, E. Thorsby, and L. M. Sollid. 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.

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

116. van de Wal, Y., Y. M. Kooy, J. W. Drijfhout, R. Amons, G. K. Papadopoulos, and F. Koning. 1997. Unique peptide binding characteristics of the disease-associated DQ(α1*0501, β1*0201) vs the non-disease-associated DQ(α1*0201, β1*0202) molecule. *Immunogenetics* 46: 484-492.

117. Vartdal, F., B. H. Johansen, T. Friede, C. J. Thorpe, S. Stevanovic, J. E. Eriksen, K. Sletten, E. Thorsby, H. G. Rammensee, and L. M. Sollid. 1996. The peptide binding motif of the disease associated HLA-DQ (α1* 0501, β1* 0201) molecule. *Eur J Immunol* 26: 2764-2772.

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

119. Qiao, S. W., E. Bergseng, O. Molberg, G. Jung, B. Fleckenstein, and L. M. Sollid. 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: 254-261.

120. Bergseng, E., J. Xia, C. Y. Kim, C. Khosla, and L. M. Sollid. 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: 21791-21796.

121. Arentz-Hansen, H., S. N. McAdam, O. Molberg, B. Fleckenstein, K. E. Lundin, T. J. Jorgensen, G. Jung, P. Roepstorff, and L. M. Sollid. 2002. Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. *Gastroenterology* 123: 803-809.

122. Arentz-Hansen, H., R. Korner, O. Molberg, H. Quarsten, W. Vader, Y. M. C. Kooy, K. E. A. Lundin, F. Koning, P. Roepstorff, L. M. Sollid, and S. N. McAdam. 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.

123. Sjostrom, H., K. E. Lundin, O. Molberg, R. Korner, S. N. McAdam, D. Anthonsen, H. Quarsten, O. Noren, P. Roepstorff, E. Thorsby, and L. M. Sollid. 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.

124. Sollid, L. M., and E. Thorsby. 1993. HLA susceptibility genes in celiac disease: genetic mapping and role in pathogenesis. *Gastroenterology* 105: 910-922.

125. Johansen, B. H., T. Jensen, C. J. Thorpe, F. Vartdal, E. Thorsby, and L. M. Sollid. 1996. Both α and β chain polymorphisms determine the specificity of the disease-associated HLA-DQ2 molecules, with beta chain residues being most influential. *Immunogenetics* 45: 142-150.

126. Lundin, K. E., H. Scott, T. Hansen, G. Paulsen, T. S. Halstensen, O. Fausa, E. Thorsby, and L. M. Sollid. 1993. Gliadin-specific, HLA-DQ(alpha 1*0501,beta 1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. *J Exp Med* 178: 187-196.

127. Lundin, K. E., H. Scott, O. Fausa, E. Thorsby, and L. M. Sollid. 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.

128. Molberg, O., S. N. McAdam, R. Korner, H. Quarsten, C. Kristiansen, L. Madsen, L. Fugger, H. Scott, O. Noren, P. Roepstorff, K. E. Lundin, H. Sjostrom, and L. M. Sollid. 1998. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat Med* 4: 713-717.

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

130. Nilsen, E. M., F. L. Jahnsen, K. E. Lundin, F. E. Johansen, O. Fausa, L. M. Sollid, J. Jahnsen, H. Scott, and P. Brandtzaeg. 1998. Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. *Gastroenterology* 115: 551-563.

131. Di, S. A., R. Ciccocioppo, F. Cupelli, B. Cinque, D. Millimaggi, M. M. Clarkson, M. Paulli, M. G. Cifone, and G. R. Corazza. 2006. Epithelium derived interleukin 15 regulates intraepithelial lymphocyte Th1 cytokine production, cytotoxicity, and survival in coeliac disease. *Gut* 55: 469-477.

132. Mention, J. J., A. M. Ben, B. Begue, U. Barbe, V. Verkarre, V. Asnafi, J. F. Colombel, P. H. Cugnenc, F. M. Ruemmele, E. McIntyre, N. Brousse, C. Cellier, and N. Cerf-Bensussan. 2003. Interleukin 15: a key to disrupted intraepithelial lymphocyte homeostasis and lymphomagenesis in celiac disease. *Gastroenterology* 125: 730-745.

133. Gianfrani, C., R. Troncone, P. Mugione, E. Cosentini, M. De Pascale, C. Faruolo, S. Senger, G. Terrazzano, S. Southwood, S. Auricchio, and A. Sette. 2003. Celiac disease association with CD8+ T cell responses: Identification of a novel gliadin-derived HLA-A2-restricted epitope. *J Immunol* 170: 2719-2726.

134. Mazzarella, G., R. Stefanile, A. Camarca, P. Giliberti, E. Cosentini, C. Marano, G. Iaquinto, N. Giardullo, S. Auricchio, A. Sette, R. Troncone, and C. Gianfrani. 2008. Gliadin activates HLA class I-restricted CD8+ T cells in celiac disease intestinal mucosa and induces the enterocyte apoptosis. *Gastroenterology* 134: 1017-1027.

135. Meresse, B., Z. G. Chen, C. Ciszewski, M. Tretiakova, G. Bhagat, T. N. Krausz, D. H. Raulet, L. L. Lanier, V. Groh, T. Spies, E. C. Ebert, P. H. Green, and B. Jabri. 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.

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

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

138. Meresse, B., S. A. Curran, C. Ciszewski, G. Orbelyan, M. Setty, G. Bhagat, L. Lee, M. Tretiakova, C. Semrad, E. Kistner, R. J. Winchester, V. Braud, L. L. Lanier, D. E. Geraghty, P. H. Green, S. Guandalini, and B. Jabri. 2006. Reprogramming of CTLs into natural killer-like cells in celiac disease. *J Exp Med* 203: 1343-1355.

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

140. Gorga, J. C., V. Horejsi, D. R. Johnson, R. Raghupathy, and J. L. Strominger. 1987. Purification and characterization of class II histocompatibility antigens from a homozygous human B cell line. *J Biol Chem* 262: 16087-16094.

141. Wettstein, D. A., J. J. Boniface, P. A. Reay, H. Schild, and M. M. Davis. 1991. Expression of a class II major histocompatibility complex (MHC) heterodimer in a lipid-linked form with enhanced peptide/soluble MHC complex formation at low pH. *J Exp Med* 174: 219-228.

142. Stern, L. J., and D. C. Wiley. 1992. The human class II MHC protein HLA-DR1 assembles as empty alpha beta heterodimers in the absence of antigenic peptide. *Cell* 68: 465-477.

143. Kozono, H., J. White, J. Clements, P. Marrack, and J. Kappler. 1994. Production of soluble MHC class II proteins with covalently bound single peptides. *Nature* 369: 151-154.

144. Kalandadze, A., M. Galleno, L. Foncerrada, J. L. Strominger, and K. W. Wucherpfennig. 1996. Expression of recombinant HLA-DR2 molecules. Replacement of the hydrophobic transmembrane region by a leucine zipper dimerization motif allows the assembly and secretion of soluble alpha beta heterodimers. *J Biol Chem* 271: 20156-20162.

145. Quarsten, H., S. N. McAdam, T. Jensen, H. Arentz-Hansen, O. Molberg, K. E. Lundin, and L. M. Sollid. 2001. Staining of celiac disease-relevant T cells by peptide-DQ2 multimers. *J Immunol* 167: 4861-4868.

146. Day, C. L., N. P. Seth, M. Lucas, H. Appel, L. Gauthier, G. M. Lauer, G. K. Robbins, Z. M. Szczepiorkowski, D. R. Casson, R. T. Chung, S. Bell, G. Harcourt, B. D. Walker, P. Klenerman, and K. W. Wucherpfennig. 2003. Ex vivo analysis of human memory CD4 T cells specific for hepatitis C virus using MHC class II tetramers. *J Clin Invest* 112: 831-842.

147. Altman, J. D., P. A. Reay, and M. M. Davis. 1993. Formation of functional peptide complexes of class II major histocompatibility complex proteins from subunits produced in Escherichia coli. *Proc Natl Acad Sci U S A* 90: 10330-10334.

148. Cunliffe, S. L., J. R. Wyer, J. K. Sutton, M. Lucas, G. Harcourt, P. Klenerman, A. J. McMichael, and A. D. Kelleher. 2002. Optimization of peptide linker length in production of MHC class II/peptide tetrameric complexes increases yield and stability, and allows identification of antigen-specific CD4+T cells in peripheral blood mononuclear cells. *Eur J Immunol* 32: 3366-3375.

149. Henderson, K. N., J. A. Tye-Din, H. H. Reid, Z. Chen, N. A. Borg, T. Beissbarth, A. Tatham, S. I. Mannering, A. W. Purcell, N. L. Dudek, D. A. van Heel, J. McCluskey, J. Rossjohn, and R. P. Anderson. 2007. A structural and immunological basis for the role of human leukocyte antigen DQ8 in celiac disease. *Immunity* 27: 23-34.

150. Xia, J., E. Bergseng, B. Fleckenstein, M. Siegel, C. Y. Kim, C. Khosla, and L. M. Sollid. 2007. Cyclic and dimeric gluten peptide analogues inhibiting DQ2-mediated antigen presentation in celiac disease. *Bioorg Med Chem* 15: 6565-6573.

151. Juse, U., B. Fleckenstein, E. Bergseng, and L. M. Sollid. 2008. Soluble HLA-DQ2 expressed in S2 cells copurifies with a high affinity insect cell derived protein. *Immunogenetics*.

152. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. Heyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274: 94-96.

153. Homann, D., L. Teyton, and M. B. Oldstone. 2001. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat Med* 7: 913-919.

154. Mallet-Designe, V. I., T. Stratmann, D. Homann, F. Carbone, M. B. A. Oldstone, and L. Teyton. 2003. Detection of low-avidity CD4+ T cells using recombinant artificial APC: Following the antiovalbumin immune response. *J Immunol* 170: 123-131.

155. Crawford, F., H. Kozono, J. White, P. Marrack, and J. Kappler. 1998. Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. *Immunity* 8: 675-682.

156. Boniface, J. J., J. D. Rabinowitz, C. Wulfing, J. Hampl, Z. Reich, J. D. Altman, R. M. Kantor, C. Beeson, H. M. McConnell, and M. M. Davis. 1998. Initiation of signal transduction through the T cell receptor requires the multivalent engagement of peptide/MHC ligands [corrected]. *Immunity* 9: 459-466.

157. Cochran, J. R., T. O. Cameron, and L. J. Stern. 2000. The relationship of MHC-peptide binding and T cell activation probed using chemically defined MHC class II oligomers. *Immunity* 12: 241-250.

158. McMichael, A. J., and C. A. O'Callaghan. 1998. A new look at T cells. *J Exp Med* 187: 1367-1371.

159. Bakker, A. H., and T. N. Schumacher. 2005. MHC multimer technology: current status and future prospects. *Curr Opin Immunol* 17: 428-433.

160. Guillaume, P., D. F. Legler, N. Boucheron, M. A. Doucey, J. C. Cerottini, and I. F. Luescher. 2003. Soluble major histocompatibility complex-peptide octamers with impaired CD8 binding selectively induce Fas-dependent apoptosis. *J Biol Chem* 278: 4500-4509.

161. Buelow, R., S. Kuo, L. Paborsky, K. J. Wilson, and J. B. Rothbard. 1994. Detergent-enhanced dissociation of endogenous peptides from PI-DRB1*0401. *Eur J Immunol* 24: 2181-2185.

162. Buus, S., A. Stryhn, K. Winther, N. Kirkby, and L. O. Pedersen. 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.

163. Stepniak, D., L. W. Vader, Y. Kooy, P. A. van Veelen, A. Moustakas, N. A. Papandreou, E. Eliopoulos, J. W. Drijfhout, G. K. Papadopoulos, and F. Koning. 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.

164. Price, P., C. Witt, R. Allcock, D. Sayer, M. Garlepp, C. C. Kok, M. French, S. Mallal, and F. Christiansen. 1999. The genetic basis for the association of the 8.1 ancestral haplotype (A1, B8, DR3) with multiple immunopathological diseases. *Immunol Rev* 167: 257-274.

165. Lechler, R., and A. Warrens. 2000. *HLA in health and disease*. Academic, London.

166. Fernando, M. M., C. R. Stevens, P. C. Sabeti, E. C. Walsh, A. J. McWhinnie, A. Shah, T. Green, J. D. Rioux, and T. J. Vyse. 2007. Identification of two independent risk factors for lupus within the MHC in United Kingdom families. *PLoS Genet* 3: 2109-2121.

167. Nejentsev, S., J. M. M. Howson, N. M. Walker, J. Szeszko, S. F. Field, H. E. Stevens, P. Reynolds, M. Hardy, E. King, J. Masters, J. Hulme, L. M. Maier, D. Smyth, R. Bailey, J. D. Cooper, G. Ribas, R. D. Campbell, D. G. Clayton, and J. A. Todd. 2007. Localization of type 1 diabetes susceptibility to the MHC class I genes HLA-B and HLA-A. *Nature* 450: 887-892.

168. Mellins, E., P. Cameron, M. Amaya, S. Goodman, D. Pious, L. Smith, and B. Arp. 1994. A mutant human histocompatibility leukocyte antigen DR molecule associated with invariant chain peptides. *J Exp Med* 179: 541-549.

169. Ignatowicz, L., J. Kappler, and P. Marrack. 1996. The repertoire of T cells shaped by a single MHC/peptide ligand. *Cell* 84: 521-529.

170. Lee, D. S., C. Ahn, B. Ernst, J. Sprent, and C. D. Surh. 1999. Thymic selection by a single MHC/peptide ligand: autoreactive T cells are low-affinity cells. *Immunity* 10: 83-92.

171. Singh, N., and L. Van Kaer. 1999. Unexpected reactivities of T cells selected by a single MHC-peptide ligand. *J Immunol* 163: 3583-3591.

172. Busch, R., C. H. Rinderknecht, S. Roh, A. W. Lee, J. J. Harding, T. Burster, T. M. Hornell, and E. D. Mellins. 2005. Achieving stability through editing and chaperoning: regulation of MHC class II peptide binding and expression. *Immunol Rev* 207: 242-260.

173. Patil, N. S., A. Pashine, M. P. Belmares, W. Liu, B. Kaneshiro, J. Rabinowitz, H. McConnell, and E. D. Mellins. 2001. Rheumatoid arthritis (RA)-associated HLA-DR alleles form less stable complexes with class II-associated invariant chain peptide than non-RA-associated HLA-DR alleles. *J Immunol* 167: 7157-7168.

174. Henrickson, S. E., T. R. Mempel, I. B. Mazo, B. Liu, M. N. Artyomov, H. Zheng, A. Peixoto, M. P. Flynn, B. Senman, T. Junt, H. C. Wong, A. K. Chakraborty, and U. H. von Andrian. 2008. T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. *Nat Immunol* 9: 282-291.

175. Anderson, R. P., P. Degano, A. J. Godkin, D. P. Jewell, and A. V. Hill. 2000. In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. *Nat Med* 6: 337-342.

176. Mora, J. R., M. R. Bono, N. Manjunath, W. Weninger, L. L. Cavanagh, M. Rosemblatt, and U. H. von Andrian. 2003. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* 424: 88-93.

177. Stagg, A. J., M. A. Kamm, and S. C. Knight. 2002. Intestinal dendritic cells increase T cell expression of alpha4beta7 integrin. *Eur J Immunol* 32: 1445-1454.

178. Roman, E., E. Miller, A. Harmsen, J. Wiley, U. H. von Andrian, G. Huston, and S. L. Swain. 2002. CD4 effector T cell subsets in the response to influenza: Heterogeneity, migration, and function. *J Exp Med* 196: 957-968.

179. Rostom, A., J. A. Murray, and M. F. Kagnoff. 2006. American Gastroenterological Association (AGA) Institute technical review on the diagnosis and management of celiac disease. *Gastroenterology* 131: 1981-2002.

180. Sugai, E., H. Vazquez, F. Nachman, M. L. Moreno, R. Mazure, E. Smecuol, S. Niveloni, A. Cabanne, Z. Kogan, J. C. Gomez, E. Maurino, and J. C. Bai. 2006. Accuracy of testing for antibodies to synthetic gliadin-related peptides in celiac disease. *Clin Gastroenterol Hepatol* 4: 1112-1117.

181. Salmi, T. T., P. Collin, O. Jarvinen, K. Haimila, J. Partanen, K. Laurila, I. R. Korponay-Szabo, H. Huhtala, T. Reunala, M. Maki, and K. Kaukinen. 2006. Immunoglobulin A autoantibodies against transglutaminase 2 in the small intestinal mucosa predict forthcoming coeliac disease. *Aliment Pharmacol Ther* 24: 541-552.

182. Rittmeyer, C., and J. M. Rhoads. 1996. IgA deficiency causes false-negative endomysial antibody results in celiac disease. *J Pediatr Gastroenterol Nutr* 23: 504-506.