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Title: A genome-wide cell biological analysis of genes involved in MHC class II antigen presentation

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Chapter 1: Introduction

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Towards a systems understanding of MHC class II
antigen presentation

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The molecular details of antigen processing and presentation by MHC class II molecules have been studied extensively for almost three decades. Although the basics of these processes were laid out some ten years ago, the recent years have revealed many details and provided new insights into its control and specificity. MHC molecules employ various biochemical reactions in order to achieve successful presentation of antigenic fragments to the immune system. Here we present a timely evaluation of the biology of antigen presentation and a survey of issues considered unresolved. The continuing flow of new details into the biology of MHC class II antigen presentation is exciting and builds a system involving several cell biological processes, which is being discussed in this review.

Major Histocompatibility class I and II molecules (MHC-I and MHC-II) are similar in function: they present peptides at the cell surface to CD8⁺ and CD4⁺ T cells, respectively. These peptides originate from different sources — intracellular for MHC-I and exogenous for MHC-II — and are obtained via different pathways [1]. In this review, we focus on MHC-II antigen presentation. We start with a description of the basic pathways, followed by recent additions in the field to arrive at a systems description of antigen presentation by MHC-II molecules.

MHC-II, like and unlike MHC-I molecules

MHC-I and MHC-II molecules overlap in a number of characteristics: high polymorphism, similar 3D structure due to the fact that they originate from one common founder gene by simple gene duplication, location in one gene locus and presentation of peptides to the immune system. Yet these molecules show a different tissue distribution and differ in the types of antigenic peptides presented as a result of their different cell biology. Here, we discuss how the MHC-II antigen presentation pathway differs from MHC-I to present fragments generated at different locations in cells.

Like MHC-I, MHC-II is encoded by three polymorphic genes (*HLA-DR*, *HLA-DQ* and *HLA-DP* in humans) that bind different peptides. Many MHC-II alleles are the strongest genetic markers for several autoimmune diseases, possibly due

to the peptides they present [2]. Although the different alleles appear to associate differentially with the chaperone HLA-DM (see later) [3], the effects of MHC-II polymorphism on their cell biology is poorly studied when compared to MHC-I. The MHC-II pathway described below is mainly based on studies of HLA-DR and mouse MHC-II (I-A and I-E). Of note, the pathway may differ in details for other MHC-II molecules.

The basics of MHC-II antigen presentation

While MHC-I is ubiquitously expressed, MHC-II molecules are primarily expressed by professional APCs, such as dendritic cells (DCs), macrophages and B cells. It has been concluded from the work of many groups that the transmembrane α - and β -chains of MHC-II are assembled in the ER and associate with the invariant chain (Ii). The resulting MHC-II-Ii complex is transported to late endosomal compartments, termed MIIC (MHC class II compartment). Here Ii is digested, leaving a residual class II-associated Ii peptide (CLIP) in the peptide-binding groove of MHC-II. In the MIIC, MHC-II requires HLA-DM (H2-DM in mice) to facilitate the exchange of the CLIP fragment for a specific peptide derived from proteins degraded in the endosomal pathway. MHC-II is then transported to the plasma membrane to present its peptide cargo to CD4⁺ T cells (Figure 1). In B cells, a modifier of HLA-DM is expressed called HLA-DO (H2-O in mice) which associates

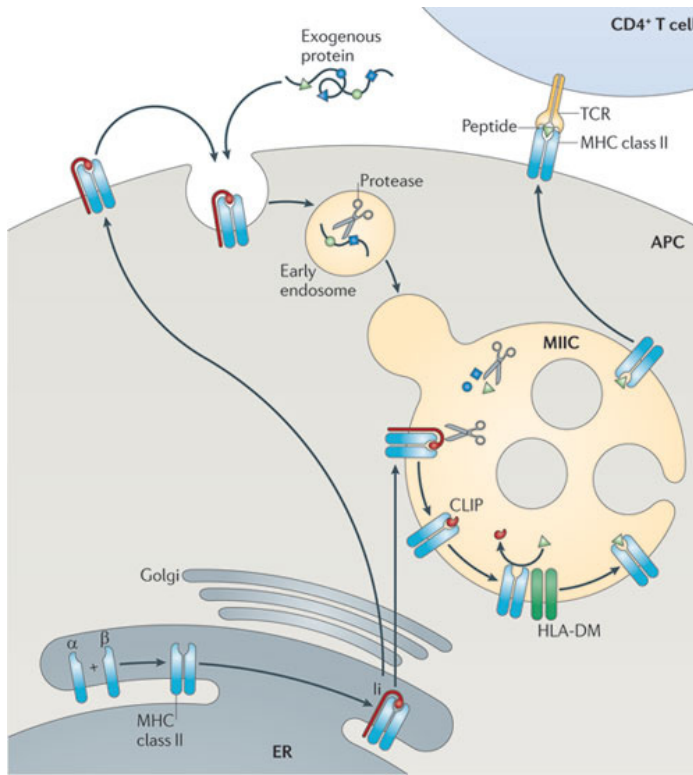


Figure 1 | **The basic MHC class II antigen presentation pathway.** MHC-II α - and β -chains assemble in the ER and form a complex with the Ii. The MHC-II/Ii heterotrimer is transported through the Golgi to the MIIC, either directly and/or via the plasma membrane. Endocytosed proteins and the Ii are here degraded by resident proteases. The CLIP fragment of Ii remains in the peptide-binding groove of MHC-II and is exchanged for proper peptide with the help of the dedicated chaperone HLA-DM. MHC-II is then transported to the plasma membrane for presentation of antigenic fragments to CD4⁺ T cells.

with HLA-DM and restricts HLA-DM activity to more acidic compartments thus modulating peptide binding to MHC-II [4]. Cross-presentation aside, MHC-I presents peptides of cytosolic origin, whereas MHC-II carries peptides derived from antigens degraded in the endocytic pathway. Their combined specificities cover antigens from almost all cellular compartments. However, essential differences in the pathways complicate this basic paradigm. In addition, various issues are less well understood and no numbers to calculate the reaction efficiencies leading to MHC-II peptide loading have been reported.

The complexity of the MHC-II pathway

MHC-II expression

Unlike MHC-I, the expression of MHC-II is restricted to APCs. However, MHC-II expression can be induced by IFN γ and

other stimuli in non-APCs, including mesenchymal stromal cells [5], fibroblasts and endothelial cells [6], and in epithelial cells and enteric glial cells in Crohn's disease [7,8] and eosinophilic esophagitis [9]. Also dermatoses, such as psoriasis [10], can induce MHC-II expression by keratinocytes [11]. Non-APCs may express MHC-II in the absence of co-stimulatory molecules that may drive or attenuate local T cell responses. The question is how expression of MHC-II is controlled in APC and non-APCs.

The master regulator of MHC-II expression is class II transactivator (CIITA). CIITA is recruited by the MHC-II enhanceosome (which contains cyclic-AMP-responsive-element-binding protein (CREB), nuclear transcription factor Y (NFY) and the regulatory factor X (RFX) complex) to the X1, X2, Y-box elements at the MHC-II locus (reviewed in [12]).

CIITA expression is regulated in a more complex manner, yielding CIITA isoforms I, III and IV [13,14], which are expressed in different cell types. Transcriptional regulation of MHC-II in DCs is controlled by an additional layer of regulation. In immature DCs, four factors (PU.1, IRF8, NF- κ B and SP1) bind to the type I CIITA promoter resulting in high CIITA transcription and, as a result, high MHC-II transcription. During DC maturation, this complex is replaced by a complex containing PR domain zinc finger protein 1 (PRDM1) and B-lymphocyte-induced maturation protein 1 (BLIMP1) that inhibits CIITA transcription [15] (Figure 2B). In addition, CIITA requires phosphorylation [16,17] and mono-ubiquitination [18,19] before being active as the MHC-II transcription factor in APCs. By combining the results of a genome-wide small interfering RNA (siRNA) screen with quantitative PCR, five upstream regulators of CIITA (CDCA3, RMND5B, CNOT1, MAPK1 and PLEKHA4) were recently identified. By determining how these factors controlled the expression of each other, a complex feedback mechanism in control of CIITA and MHC-II transcription was uncovered [20] (Figure 2A). In fact, a complex transcriptional feed-back mechanism is the only mechanism possible to explain how a master regulator of transcription (CIITA) is controlled by the next factor that is controlled by the next *ad infinitum*. However, the factors constituting the feed-back mechanism should also be controlled. Further systems biology analyses showed that feed-back control of CIITA expression is determined by the combined activities of transforming growth factor b (TGF β) signalling and chromatin modifications leading to MHC-II transcription in APCs [20]. Tissue specific regulation of MHC-II expression is then the consequence of two general terms; chromatin modifications that include epigenetics, and signalling by external factors. The latter has

been noticed earlier as a series of cell types only express MHC-II under inflammatory conditions (see later). In summary, transcription of MHC-II is controlled by the master regulator CIITA, which in turn is regulated by post-translational modifications and factors mainly, but not exclusively, active in immune cells. Under defined conditions of signalling and chromatin modifications, CIITA and MHC-II can be expressed in non-immune cells, often in response to infections or inflammation.

MHC-II transport from ER to the MHC

Although both MHC-I and MHC-II are assembled in the ER, MHC-I needs to be loaded with peptide to leave the ER, whereas MHC-II associates with Ii [21]. Four different splice variants of Ii exist, with variation in the cytoplasmic tail (the p33 and p35 variants) or inclusion of an additional exon encoding a protease inhibitor cystatin (the p43 and p45 variants) [22,23]. While the α and β chain of MHC-II are ER-bound, the assembled MHC-II $\alpha\beta$ heterodimers is already slowly leaving the ER, which is further accelerated by Ii binding. It is believed that the CLIP region of Ii blocks the peptide-binding groove of MHC-II, thus preventing binding of other peptides in the ER. Indeed, the levels of endogenous antigen presentation is higher in Ii knockout mice [24], but biochemical analyses of the same mice suggest this is not an efficient process, as most MHC-II are not converted into a stable peptide-loaded form in the absence of Ii [25,26]. Yet, particular antigens can access MHC-II after TAP-dependent translocation in the ER [27] but the vast majority of peptides will fail to enter MHC-II due to Ii.

The cytoplasmic tail of Ii contains two classical di-leucine sorting motifs that direct MHC-II to endosomal compartments (Figure 2C). These sorting motifs are recognized by the sorting adaptors AP1 (a *trans*-Golgi network

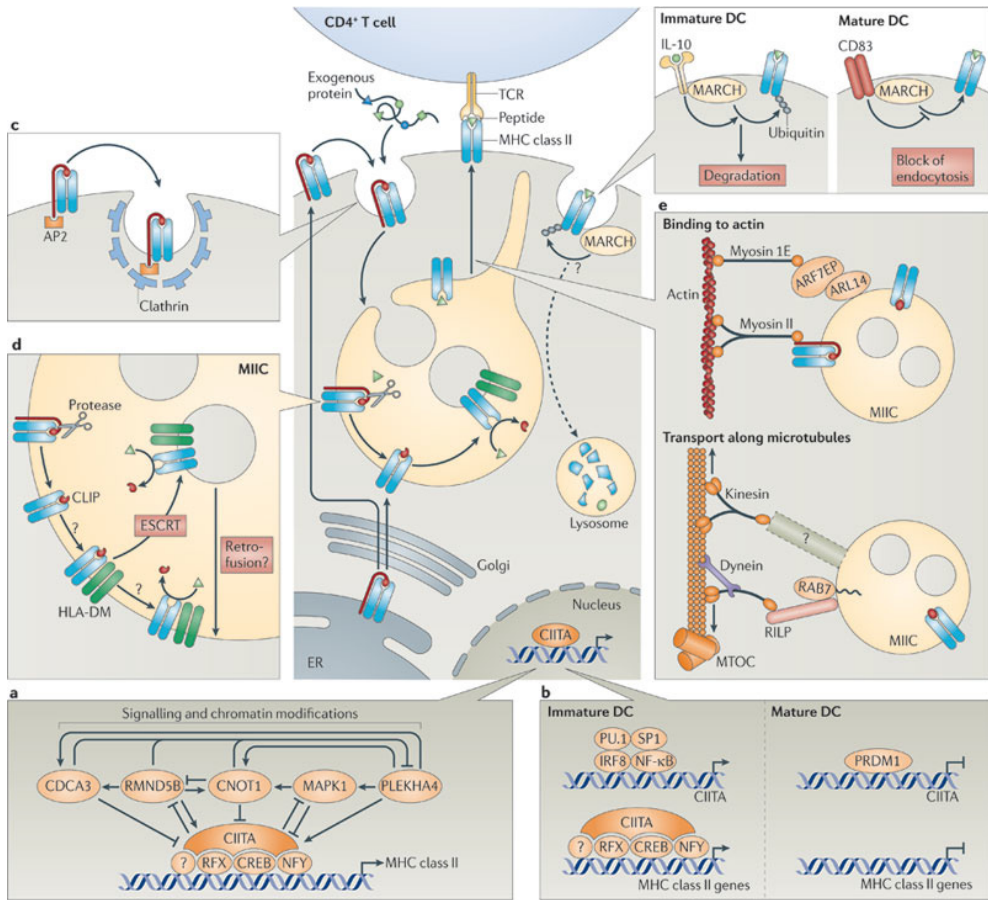


Figure 2 | **Complexity of the MHC class II antigen presentation pathway.** Insights in the various steps of the MHC-II pathway are shown in different boxes projected on the basic pathway of Figure 1. (A) MHC-II transcription is controlled by master regulator CIITA ensuring tissue specific expression. CIITA is controlled by a feedback loop of factors that are subsequently controlled by two general processes: (TGFβ) signaling and chromatin modifications. (B) CIITA expression is differentially controlled in imDC (via an activating transcriptional complex) and mDC (via an inhibitory complex). Consequently, CIITA (with other factors) induces transcription of MHC-II in imDC unlike in mDC. (C) Adaptor proteins binding the Ii are known. AP-2 drives internalization of MHCII-Ii complexes via CCV at the plasma membrane for endocytosis and transport to the MIIC. (D) In the MIIC, Ii is degraded and MHC-II interacts with HLA-DM. Most HLA-DM and MHC-II locate and interact in the internal structures formed by the ESCRT machinery. The mechanism of back-fusion of internal DM and MHC-II to the limiting membrane is hypothetical. (E) MHC-II or its tubular extensions are transported by the microtubule-based motor proteins dynein and kinesin. These have receptors on the MIIC, like RAB7-RILP for the dynein motor. The final step involves actin-based myosin motors that interact with the MIIC via Ii (MYOII) or the GTPase ARL14 (MYO1e). (F) In imDCs, internalization of MHC-II from the plasma membrane may require the ubiquitin ligase MARCH1 which is controlled by IL10. CD83 on mDC prevents this ubiquitin modification of MHC-II which stabilizes MHC-II cell surface expression.

adaptor) and AP2 (a plasma membrane adaptor) [28]. Ii may direct MHC-II directly from the *trans*-Golgi network to MIIC or

by endocytosis from the plasma membrane. Endocytosis may be preferred in human cervical carcinoma cells (HeLa cells) and

immature DCs (AP2 dependent) [29,30], whereas direct sorting may be dominant in mature DC (AP1 dependent) [31]. In summary, Ii is essential for various steps in the life of MHC-II, but may take different routes to its final destination, which is the endosomal pathway where Ii is degraded before MHC-II finally acquires its final peptide.

The MHC-II peptide-loading compartment

While MHC-I binds peptides in a partially folded state stabilized by chaperones in the ER, this is probably different for MHC-II, as endosomes are not known for contributing to folding. Where MHC-II peptide loading occurs has been a matter of debate since the MIIC was visualized by electron microscopy in 1990 [32]. At that time, the MIIC was shown to contain MHC-II and Ii, to be multilamellar in morphology, to be acidic and to contain lysosomal proteases and CD63, which defined it as late endosomal [32]. Other structures were subsequently identified and a revised definition for the MIIC was required. The MHC-II chaperone HLA-DM was found to localize in late endosomes [33], where it stabilizes MHC-II either bound to or devoid of the CLIP peptide (thus preventing aggregation and degradation of MHC-II) until high affinity peptides bind [34] (Figure 2D). The late endosomal tetraspanin proteins [35] which interact with HLA-DM and MHC-II, and probably induce the formation of a proteinacious network, were identified, as were the proteases cathepsin S and cathepsin L that degrade Ii [36]. An *in vitro* reconstitution experiment defined the molecules minimally required for the MIIC as MHC-II, HLA-DM and cathepsins [37] and the combined data suggest that a late endosomal structure with (at least) these three factors would fulfill the criteria for the MIIC.

A complicating factor is that the MIIC is

not homogeneous but exists in multiple morphologies (multivesicular, mixed and multilamellar) that may represent different maturation states. MHC-II, HLA-DM and other molecules are located mainly in the internal structures of the MIIC and have to be ubiquitinated and sorted by the endosomal sorting complex required for transport (ESCRT) machinery on the limiting membrane [38]. Fluorescence resonance energy transfer (FRET) studies have suggested that HLA-DM interacts with MHC-II on the internal vesicles of the MIIC and not on the limiting membrane [39]. The internal vesicles carrying MHC-II and HLA-DM are thought to fuse back to the limiting membrane of MIIC to prevent secretion in the form of exosomes and to be embedded in the plasma membrane. This process of 'retrofusion' has not yet been defined. Another model proposes that peptide-loaded MHC-II appearing on the plasma membrane in DCs originate from the limiting membrane and that MHC-II on internal vesicles are destined for degradation [40]. However, as most of the MHC-II is found on internal vesicles, a major loss of MHC-II would be expected to occur, but this was not observed in biochemical experiments [41]. The molecular mechanisms of retrofusion (if any) need to be defined to resolve this issue.

Although the intracellular location for peptide loading of MHC-II seems to be in the MIIC, many issues have yet to be resolved. These include the entry of MHC-II via earlier endosomes into the MIIC and the functional role of Ii to mediate fusion of early endosomes [42] and regulate intracellular transport of MHC-II [43]. MHC-II will probably present different peptides when sampling these in different parts of the endosomal pathway with different help of HLA-DM [44]. Finally, degradation of antigens is strongly delayed in immature DCs possibly as a mechanism to store

antigens for presentation over long periods of time [45]. Whereas the minimal MIIC has been defined, the consequences of different MIIC morphology, different proteolytic activities, controlled acidification during DC maturation, retrofusion and other processes need to be defined for a more complete understanding of the intracellular process of MHC-II antigen loading.

Regulation of MHC-II transport from MIIC towards the plasma membrane

Late endosomal compartments such as MIIC are not typical recycling structures, yet MHC-II, HLA-DM, tetraspanins and other molecules are transported from the MIIC to the plasma membrane. The content of MIIC, including MHC-II, is released after a specific time period. This release is controlled by factors such as cholesterol, cytosolic pH, kinases and GTPases.

Fast transport of MIIC and other vesicles is driven by the microtubule-based motors dynein (for inward transport) and the kinesin family (for outward transport), whereas slow vesicle transport involves the actin-based myosin motor family. Motor proteins require vesicle receptors that are subsequently controlled by other processes. The molecular basis for this part of cell biology is largely undefined with few exceptions. Inward transport of MIIC by the dynein motor is controlled by the RAB7-interacting lysosomal protein (RILP) on MIIC (Figure 2E), which is further controlled by the cholesterol-sensor OSBP-related protein 1L (ORP1L) and the ER-resident protein VAMP-associated protein A (VAPA) [46]. This may explain the effect of cholesterol on MHC-II antigen presentation [47].

DCs may be unique in that MHC-II transport from MIIC is regulated by maturation signals, which induce higher MHC-II surface expression at the cost of the intracellular pool of MHC-II [48,49]. Lipopolysaccharide triggers the formation

of tubules that originate from MIIC in DCs, generating a complex network of moving vesicles and tubules that may all fuse to the plasma membrane [50-52]. What controls MHC-II transport in DCs? Two actin-based motors have been implicated. The common actin motor myosin II (MYOII) may interact with Ii to control MHC-II transport in DCs [53] (Figure 2E). Another pathway controlling MHC-II transport in DCs was identified using an integrated siRNA and cell biology screen. First, siRNAs affecting MHC-II expression were defined, then downregulation of the target genes upon maturation of DCs was determined and the finally remaining candidates were silenced in immature DCs. Some of these induced redistribution of MHC-II corresponding to matured DCs, while the cells remained immature in respect to other activation markers [20]. The candidates included GTPase ADP-ribosylation factor-like protein 14 (ARL14; also known as ARF7) that locates on MIIC, recruits the effector ARF7EP, which acts as a receptor for the motor protein myosin1E (MYO1E) [20 and Chapter 2 of this thesis]. This pathway controls MHC-II export in DCs (Figure 2E). How maturation signals by LPS control these pathways is unclear, yet they may show some resemblance to the induced secretion of other lysosome-related organelles, such as cytolytic granules, melanosomes and Weibel-Palade bodies [54].

The end of an MHC-II molecule

Similar to MHC-I, MHC-II do not have an infinite life. However, MHC-II is relatively stable (it has already survived late endosomal conditions) and does not dissociate at the plasma membrane. In addition, the half-life of MHC-II greatly increases upon DC maturation [48,49]. How is it then finally degraded? MHC-II (like MHC-I) can be ubiquitin modified by MARCH E3 ligases, namely MARCH1 [55]. Since

the expression levels of MARCH1- and ubiquitination of MHC-II – decrease when DCs mature, ubiquitination was proposed to control MHC-II half-life [56]. Interleukin-10 (IL-10) downregulates surface expression of MHC-II and controls the expression of MARCH1 [57,58]. In addition, the co-stimulatory molecule CD83 is highly expressed by mature DCs and inhibits the interaction between MARCH1 and MHC-II, thereby preventing MHC-II ubiquitination [59]. These observations suggest a causal link between ubiquitination and MHC-II half-life (Figure 2D). However, this link has recently been challenged. Mice engineered to express MHC-II with mutations that prevent its ubiquitination still show normal antigen presentation by MHC-II, although MHC-II expression at the plasma membrane was slightly elevated [60]. Therefore, MHC-II ubiquitination may be involved in sorting within the endosomal pathway rather than endocytosis and degradation [45,61].

In summary, MHC-II is extraordinary stable but still displays cell type-specific half-lives. The control of MHC-II degradation has not been established but could involve ubiquitination [60]. Most likely, MHC-II ends like any other lysosomal protein by lysosomal proteolysis, but the exact mechanism is unresolved.

The Systems of MHC-II antigen presentation

Although the system of antigen presentation is understood at a high level of detail, this in fact only represents sketches of the total biology. For a further understanding, modern technologies such as siRNA screens allow genome-wide consideration of relevant molecular relationships. This can yield comprehensive lists of new molecules involved in any process. An integration of siRNA data with flow cytometry, microscopy and transcriptional information from qPCR and microarray yielded various

novel pathways, placing novel GTPases and motor proteins in the control of MHC-II transport [20]. Such experimental data sets can be integrated with others derived from siRNA screens, genetic screens, expression and protein-protein interaction data bases to build pathways in silico. These pathways then have to be experimentally validated to avoid noise in our understanding of the MHC-I and MHC-II antigen presentation pathway.

Outside-in signalling by MHC-II

MHC-II mediate inside-out signalling when presenting peptides to T cells, but recent data suggest that MHC-II also functions as a signalling receptor, resulting in outside-in signalling (reviewed in [62]). This can lead to apoptosis of activated APCs and results in the termination of immune responses [63]. By contrast, engagement of MHC-II on melanoma cells by its ligand lymphocyte activation gene 3 (LAG3) expressed by infiltrating lymphocytes can prevent cell death by activating survival pathways [64]. Since MHC-II has short cytoplasmic tails without detectable signalling motifs, adaptor molecules must be involved to transduce the outside-in signals [62]. Toll-like receptor (TLR) activation induces the association of CD40 and Bruton's tyrosine kinase (BTK) with intracellular MHC-II, resulting in prolonged BTK activation and TLR signalling-specific gene transcription [65]. In addition to CD40, the B cell receptor complex components CD79a and CD79b [66], the IgE receptor [67] and CD19 [68] have been reported to be involved in MHC-II-associated signal transduction. Signalling through MHC-II is a new concept and consequences of this have to be revealed in the future.

Conclusions and perspectives

The biology of MHC-II has been studied extensively due to their fundamental role in controlling immune responses and their

involvement in transplantation, infection, vaccination and autoimmunity. Understanding MHC-II antigen presentation can be – and in fact already is – translated into treatment options [69-71]. Deeper understanding of antigen presentation by MHC-II should result in additional targets for therapeutic manipulation of the immune system.

Many groups have recently uncovered new steps in the antigen processing and presentation system. However, many unknowns and controversies remain. Whether immunodominance of peptides can be predicted and why particular MHC-II alleles are associated with autoimmune diseases is mostly unclear (except for the known link between gluten, HLA-DQ2 and HLA-DQ8 and celiac disease [72]) but we hope they will be resolved in the coming years.

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