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INVITED REVIEW ARTICLE

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Revisiting nonclassical HLA II functions in antigen presentation: Peptide editing and its modulation

Miguel Álvaro-Benito | **Christian Freund**

Laboratory of Protein Biochemistry, Institute für Chemie und Biochemie, Department of Biology, Chemistry, Pharmacy, Freie Universität Berlin, Berlin, Germany

Correspondence
Miguel Álvaro-Benito, Laboratory of Protein Biochemistry, Institute für Chemie und Biochemie, Department of Biology, Chemistry, Pharmacy, Freie Universität Berlin, Berlin, Germany.
Email: m.alvaro@fu-berlin.de

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The nonclassical major histocompatibility complex of class II molecules (ncMHCII) HLA-DM (DM) and HLA-DO (DO) feature essential functions for the selection of the peptides that are displayed by classical MHCII proteins (MHCII) for CD4$^+$ T$_h$ cell surveillance. Thus, although the binding groove of classical MHCII dictates the main features of the peptides displayed, ncMHCII function defines the preferential loading of peptides from specific cellular compartments and the extent to which they are presented. DM acts as a chaperone for classical MHCII molecules facilitating peptide exchange and thereby favoring the binding of peptide-MHCII complexes of high kinetic stability mostly in late endosomal compartments. DO on the other hand binds to DM blocking its peptide-editing function in B cells and thymic epithelial cells, limiting DM activity in these cellular subsets. DM and DO distinct expression patterns therefore define specific antigen presentation profiles that select unique peptide pools for each set of antigen presenting cell. We have come a long way understanding the mechanistic underpinnings of such distinct editing profiles and start to grasp the implications for ncMHCII biological function. DM acts as filter for the selection of immunodominant, pathogen-derived epitopes while DO blocks DM activity under certain physiological conditions to promote tolerance to self. Interestingly, recent findings have shown that the unexplored and neglected ncMHCII genetic diversity modulates retroviral infection in mouse, and affects human ncMHCII function. This review aims at highlighting the importance of ncMHCII function for CD4$^+$ T$_h$ cell responses while integrating and evaluating what could be the impact of distinct editing profiles because of natural genetic variations.

KEYWORDS
CD4$^+$ T cell epitope, HLA-DO, HLA-DM, MHC class II, nonclassical MHCII, peptide editing, peptidome

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1 | INTRODUCTION

Classical human leukocyte antigens of class II (HLAII) also called major histocompatibility complex (MHCII)

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proteins display antigenic peptides, primarily from extracellular proteins, at the cell surface of professional antigen presenting cells (pAPCs). The pool of peptides presented by MHCII proteins (the "immunopeptidome") is surveyed by $CD4^+$ T_h cells. Noteworthy, although a typical pAPC displays around 10^5 surface-peptide-MHCII molecules in steady state, a very low number of pMHCII complexes (10-200) loaded with immunogenic peptides are capable of providing the trigger for activation of cognate T cells.[1,2] Therefore, even subtle changes in the immunopeptidome composition can have the potential to affect $CD4^+$ T_h responses. In this context, the immune system has evolved and optimized a complex and regulated peptide exchange mechanism that ensure the optimal display of peptide antigens under steady-state conditions and upon immunological challenges on different types of pAPCs. These mechanisms of peptide-repertoire selection rely on the differential expression of two nonclassical MHCII proteins (ncMHCII): HLA-DM (DM) and HLA-DO (DO). DM and DO map to the same genetic locus as classical MHCIIs but they are of very limited polymorphism and presumably do not bind peptides themselves. DM acts as a chaperone with a MHCII-peptide exchange catalyst function expressed in all pAPCs. DM function selects peptides with high kinetic stability thereby promoting the cell surface display of long-lived peptide-MHCII complexes (pMHCII), usually named immunodominant $CD4^+$ T_h cell epitopes when they raise immune responses. This function contributes to selection of pathogen-derived peptides upon infection promoting T cell immunity toward the pathogen. DO on the other hand is a MHCII-substrate mimic that binds tightly to DM and inhibits its function, primarily in B cells and thymic epithelial cells. DO function is restricted to specific developmental conditions of B cells,[3] and to the thymic environment where T cells undergo negative and positive selection.[4] In both cases, DO has been proposed to broaden the immunopeptidome facilitating the development of tolerance to self-antigens. Thus, while the biological function of antigen presentation is mainly carried out by MHCII, ncMHCII have evolved to influence peptide selection. Ultimately, ncMHCII function governs whether and which antigen specific T cells will become activated.

2 | CLASSICAL AND NONCLASSICAL HLAII GENES

MHCII molecules, classical and nonclassical, are heterodimers, and the combination of the polypeptide variants encoded by alpha (A) and beta (B) genes yields the functional molecules called allotypes. The A and B genes encoding for MHCII proteins are arranged as pairs, with HLA-DR and HLA-DO being the exception to this rule. There is only one HLA-DRA gene and for each individual there are potentially several HLA-DRB genes. Thus, DRA dimerizes with any of the polypeptides encoded by functional HLA-DRB genes for an individual. HLA-DOA and DOB genes are spaced by several genes related to MHC class I antigen presentation (see general architecture of the HLAII locus in Figure 1A). HLA genes arose from a common ancestor through gene duplication events.[5] Hughes and collaborators analyzed the sequence of MHCII genes and proposed that while HLAII genes are subject of balancing selection, nonclassical genes undergo purifying or directional selection.[6] The most important evolutionary pressure acting on HLA genes is the interaction of the host with pathogens.[7] Thus, the highly polymorphic nature of classical MHCII is an advantage to overcome infections, while one could assume that ncMHCII genes have been optimally evolved to perform their functions. For ncMHCII, despite the rather low polymorphic degree recent findings from our lab,[8,9] and from Denzin-Golovkina[10] have shown that ncMHCII genetic variants are functionally distinct. Whether or not specific ncHLAII genetic variants have been selected for based on their functional profiles, and whether these variants indeed contribute to or provide any evolutionary advantage that has been fixed over time remains to be determined.

The MHC features a very strong linkage disequilibrium (LD), hence HLA genes associate in blocks that do not segregate upon recombination. These blocks are considered as haplotypes which have been studied mostly for their association to diseases, mainly autoimmunity.[11] Haplotypes described to date include DR and DQ genes, while haplotype segregation of DP genes has only been recently scored in a systematic manner in the context of unrelated hematologic stem cell transplantation (HSCT).[12] DP genes have been traditionally neglected from haplotype analysis because of the presence of a recombination hotspot in the HLA class II locus that leads to a decreased LD between the genes on each side of *DMB* and *DQB*.[13] However, DP mismatches have been related to survival rates and clinical conditions after unrelated HSCT.[14,15] Similarly, the potential contribution of ncMHCII variants to haplotypes have been traditionally ignored. In this case, the very low variation profiles and our ignorance on their distinct functional profiles would be the main argument for their exclusion from haplotype analysis. Recent findings nevertheless have shown the impact of distinct functional profiles of ncMHCII on T cell activation[9] and even responses to retroviral infections.[10] Thus, the inclusion of both, DP and ncMHCII genes in haplotype analysis, already proposed in the late 1990s,[16] could provide additional insights into relevant physiological and clinical processes. Noteworthy,

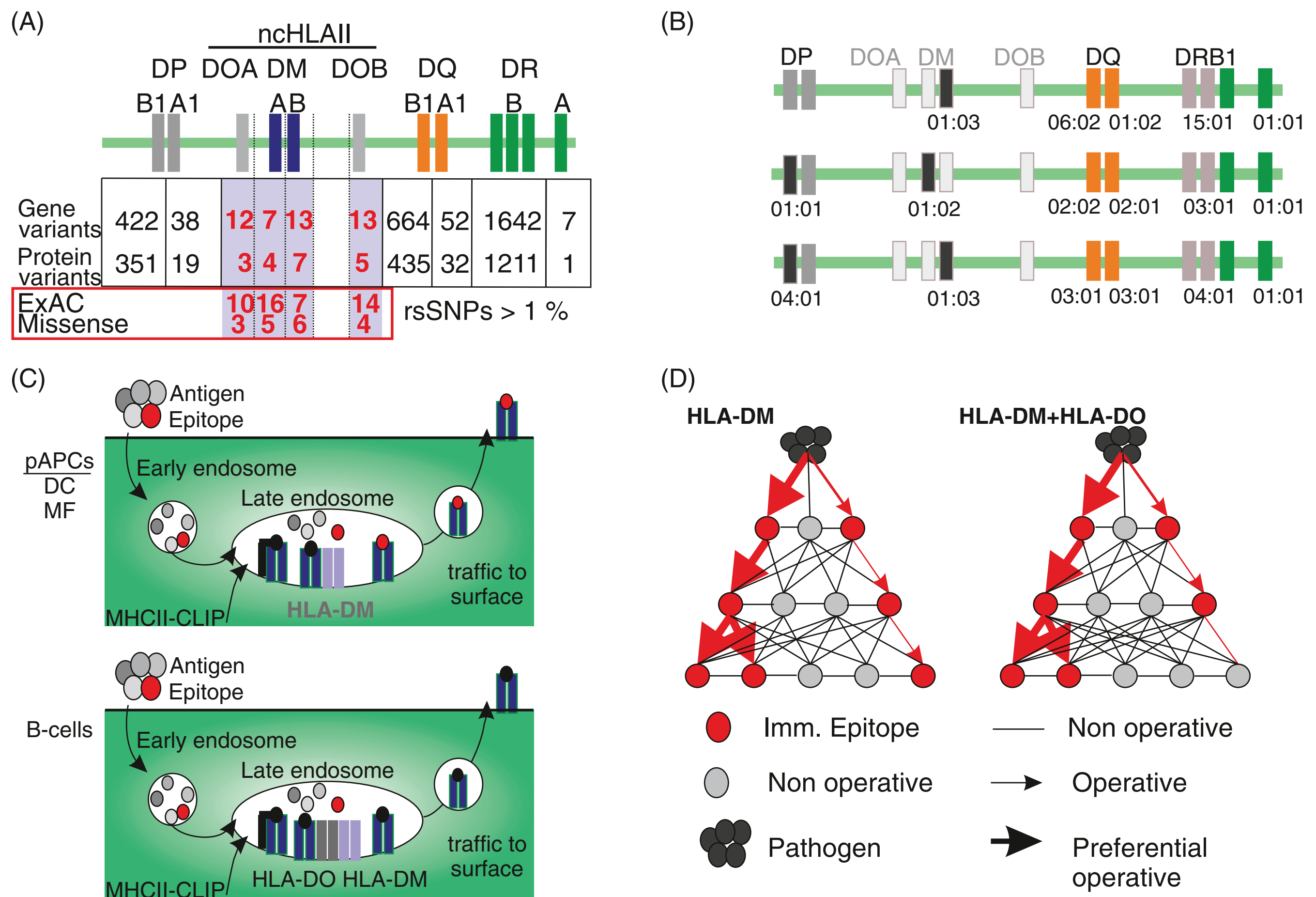

FIGURE 1 Overview of ncMHCII genetics and functions. A, The number of HLAII gene and protein variants with a frequency higher than 1% is shown below a nonscaled representation of the MHCII locus (IPD-IMGT/HLA database).[118] Colored boxes correspond to HLA genes. The total number of variants reported by the ExAC consortia (ExAC)[119] as well as the number of missense variants with frequencies higher than 1% are also indicated. B, Schematic representation of the HLAII locus, including classical HLAII and ncHLAII. The strong linkage disequilibrium in the HLA region defines ancestral HLA haplotypes. These haplotypes consist of a specific combination of HLAII genes (indicated below the corresponding boxes). C, Overview of the cellular processes, and main route resulting in antigen presentation by MHCII in pAPCs and B cells. DM activity in pAPCs results in the presentation of high affinity antigenic peptides, while its inhibition by DO in B-cells impairs peptide exchange, thus in most MHCII allotypes this results in the presentation of CLIP. D, Model network of MHCII antigen presentation adapted from Miller et al[31] to indicate the impact of DM and DO function. Antigens derived from pathogens undergo different processing routes. Each represented by the connections that lead to the final display in the lower row. For a specific pathogen-derived immunodominant epitope (Imm epitope, red) the presence of DM (left) may allow loading onto MHCII by different distinct pathways. For instance, in the presence of DO (right), the Imm. (immunodominant) Epitope may only be presented through one of the candidate routes. Note that the depicted model will represent the impact of DM and DM+DO for single peptides or epitopes and thus, it does not represent the predicted impact for the immunopeptidome. CLIP, class II invariant chain associated peptide; HLAII, human leukocyte antigens of class II; MHCII, major histocompatibility complex of class II molecules; nc, nonclassical; pAPCs, professional antigen presenting cells

with the exception of *DOA* all ncMHCII and DP genes are considered to be part of the same block in the HLA region. Of particular interest for this review, the modulatory effect on MHCII presentation imposed by allelic variants of ncMHCII could affect epitope selection and subsequent physiological processes (Figure 1B).

Classical and the ncHLAII HLA-DM genes are expressed constitutively in pAPCs, but could also be induced in response to cytokines in epithelial cells or even T cells. In all cases, the expression of these genes is dependent on the Class II Trans Activator (CIITA), the master regulator for MHCII expression. Ubiquitously expressed transcription factors bind to conserved elements on the MHCII promoters (W, X, X2, Y boxes) and the CIITA acts as a platform bridging all these elements to facilitate HLA transcription. Different promoters of the CIITA are responsible for constitutive and induced HLA expression and have been reviewed by Reith and colleagues.[17] Interestingly, neither IFNγ nor IL10, but IL4 are able to induce DOA or DOB expression.[18]

Furthermore, CIITA itself is not sufficient to induce DOB expression although it is required for its maximal expression. Thus, while DM follows the same expression pattern as that from MHCII, DO seems to undergo a specific program, not yet fully understood.

3 | GENERAL MECHANISMS OF ANTIGEN PRESENTATION BY MHCII MOLECULES

The cellular processes that result in the display of antigenic peptides on the cell surface of APCs for T cell surveillance are collectively known as antigen presentation.[19] At a minimum these mechanisms include: the regulated gene expression of MHCII molecules; the control of their recycling rate between the surface and intracellular compartments; antigen internalization and/or proteolytic degradation; and finally, the active selection of peptides for presentation by ncMHCII.

To date, there is the canonical pathway, as well as several nonconventional pathways described for antigen presentation, which in sum, account for the display of specific peptide repertoires. In the canonical pathway, described for pAPCs not expressing DO, classical MHCII proteins are synthesized in the ER and their binding groove is chaperoned by a specific fragment of the CD74/invariant chain (Ii) termed class II invariant chain associated peptide (CLIP). Ii not only chaperones the MHCII's binding groove but also contributes to their sorting to the Golgi apparatus and subsequently to the cell surface for their reinternalization to late endosomal compartments. Once in late endosomal compartments, MHCII proteins encounter antigens mostly internalized by bulk liquid phase endocytosis. The function of proteolytic activities degrades most of the invariant chain, and the available antigens. In these compartments the peptide-exchange activity of DM contributes to release CLIP, and facilitates the loading of higher affinity antigenic peptides.[20] This canonical pathway includes receptor-mediated endocytosis by the B Cell Receptor, Fc receptors, or even MHCII. DM therefore assumes a central role in antigen presentation as it affects peptide exchange by all classical MHCII molecules (DP, DQ, and DR) in every pAPC (Figure 1C). DO function, however, has only been observed to date in particular cellular subsets including B and thymic epithelial cells,[4] leading as a direct consequence to the increased presentation of CLIP peptides on these cells. (Figure 1C). However, besides this rough distinction there are considerable differences between the expression patterns of ncMHCII between subsets of prototypic APCs[21,22] whose potentially different contribution to CD4$^+$ T$_h$ cell immune responses has not yet been fully investigated.

Alternative pathways of antigen presentation have been shown to be extremely important for infections[23] or even autoimmunity.[24-26] Peptides or unfolded proteins can bind to MHCII at the cell surface where they are directly displayed for T cell recognition.[27] Similarly autophagocytosis can deliver cytosolic proteins to endosomal compartments for MHCII antigen presentation.[28] Exosome transfer of MHCII molecules, and their cargoes further diversifies the potential conditions for antigen presentation.[29] Recent findings even show a direct loading of MHCII molecules in the ER and Golgi supported by alternative chaperones as described below.[30] Overall, a very attractive, and perhaps more realistic picture of MHCII antigen presentation will include interconnections between all these different routes and processes as proposed by the Eisenlohr lab for viral infections[31,32] (Figure 1D).

4 | CLASSICAL MHCII PROTEINS, THE NEED FOR AND THE CONSEQUENCES OF PEPTIDE EDITING

Classical HLAII proteins are not the primary focus of this review, although to comprehend DM and DO function it is essential to describe fundamental aspects of their biochemistry and cell biology. Their biological function is the display of peptides available for binding, thereby providing CD4$^+$ T$_h$ cells with a global picture of the immune condition of the individual. HLA-DP, −DQ, and -DR are heterodimers, with a membrane distal domain built up by the two subunits that define the peptide-binding groove.[33] Noteworthy most of the polymorphisms found in the genes encoding these proteins lie at, or close to the structural elements defining the binding groove; therefore defining the nature of the peptides presented to T cells, but also the interaction with DM.[34] MHCII molecules bind peptides of 13 to 25 amino acids in length.[33] Peptide residues stick through their lateral chains into MHCII cavities defined on the binding groove. The most N-terminal residue of the peptide buried into one of these cavities defines the so-called P1 pocket, and subsequent pockets toward the C-terminal region of the peptide are named consecutively up to P9. Thus, the preference in binding specific residues in each pocket define the so-called binding motifs for each allotype conventionally used to predict peptide binding to MHCII molecules in silico.

A common aspect to all MHCII, is that they are initially chaperoned in the ER by the Ii. While the C-terminal region of Ii is involved in cellular trafficking, the CLIP region chaperones the binding groove of MHCII preventing the premature binding of peptides in the ER (reviewed in Reference 35). The relative affinity between

MHCII and CLIP varies considerably between MHCII allotypes, reaching up to 4-log-fold differences in IC50 values[36] (Figure 2A). This feature makes a clear distinction between classical MHCIIs and their behavior as antigen presenting molecules. Thus, MHCII allotypes with a high affinity for CLIP require either high antigenic peptide concentrations or HLA-DM function to exchange CLIP, whereas low affinity binders do not require DM function to exchange this peptide. An intriguing exception to the abovementioned MHCII-peptide binding rules and the MHCII interaction with CLIP arise from the results of our own lab. Structural and biochemical investigations demonstrated the class II invariant chain associated peptide (CLIP) could bind in two different orientations to the common HLA-DR1 (*DRA*01:01-DRB1**01:01) MHCII molecule.[37] To date, the biological relevance of these findings has not been investigated. Other relevant exceptions to the well-defined binding of peptides to MHCII molecules that should be taken into account are register shifting[26] and the differential binding of post-translationally modified peptides.[38]

The discovery of DM function and its importance for peptide-repertoire selection is described below. However, it is worth mentioning here that, similar to the allotype-specific requirements for the CLIP chaperoning of the binding groove, MHCII molecules are also considerably different in their ability to interact with DM and therefore exchange peptides in a DM-catalyzed manner (Figure 2B). The increase of peptide dissociation rates dependent on the presence and the absence of DM was described by Jensen and collaborators[39] as HLA-DM susceptibility. Mellins and colleagues provided the first systematic evidence for the influence of both, MHCII polymorphisms and the bound peptide on DM-susceptibility,[40] and further proved MHCII residues influencing the interplay between DM-MHCII.[41] Together with Sollid's lab, they prove that a natural deletion in DQA, Ser53, found in DQ2 results in a DM-insensitive MHCII.[42] The influence of the peptide bound to an MHCII on DM susceptibility has been consistently connected with the region of the P1 pocket, first related to pocket occupation,[43] and later to the establishment of conserved

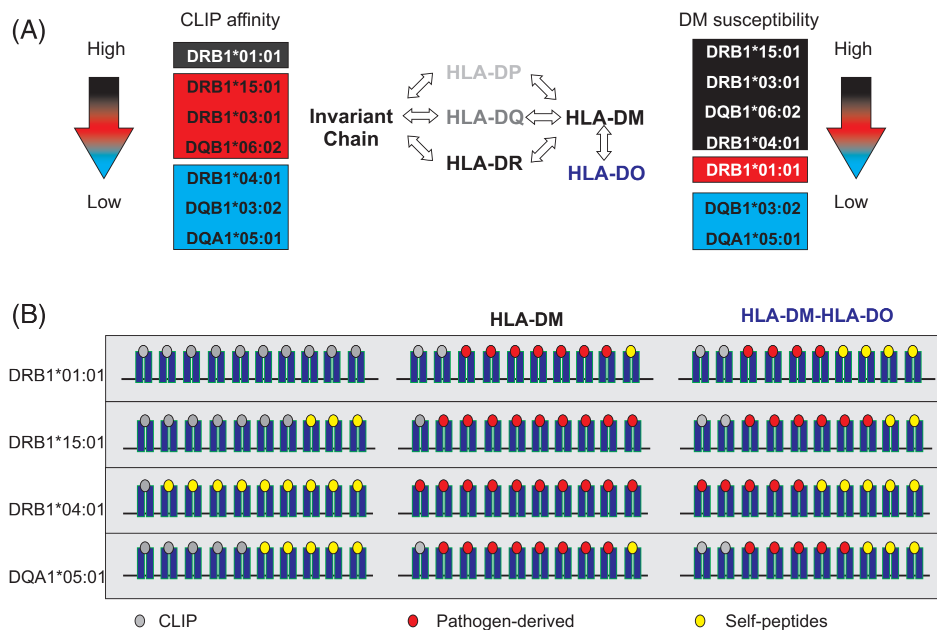

FIGURE 2 Interplay between MHCII and ncMHCII. A, Schematic representation of the interplay of classical and ncMHCII highlighting the two most relevant features that define the extent of editing as well as its requirement. CLIP affinity and intrinsic DM-susceptibility. Classical MHCII molecules (in the middle) interact with invariant chain and DM directly, while the latter two do not interact with each other. Note that only one chain is indicated for DR allotypes since all of them share the same DRA. In case of DQ allotypes: DQB1*06:02 has been studied combined with DQA1*01:02 (DQ6), and DQA1*05:01 with DQB1*02:01 (DQ2). Also, to date DO is only assumed to interact with DM. B, Expected impact of ncMHCII function on the immunopeptidome upon immunological challenge (eg, infection) for different MHCII allotypes with known CLIP affinity and DM susceptibility in three different conditions, the absence of ncMHCII, the presence of only DM, and the presence of DM-DO. The pool of pMHCII represents a theoretical distribution of abundances based on qualitative arguments of three types of peptides indicated in the legend below the figure. The ability of MHCII molecules to bind pathogen derived peptides in the absence of DM is set to null for clarity although depending on the infection, pathogen-derived peptides could be selected for presentation. CLIP, class II invariant chain associated peptide; HLAII, human leukocyte antigens of class II; MHCII, major histocompatibility complex of class II molecules; nc, nonclassical; pAPCs, professional antigen presenting cells; pMHCII, peptide-MHCII

hydrogen bonds.[44] While partial dissociation of the peptide from this region was shown to be critical for DM interaction[45] anchoring of the peptide to this region and substituting residues located in the P9 pocket[46] challenged this view. Structural and biochemical analysis coupled to extensive molecular dynamics simulations have allowed us to define that indeed the dynamics of the pMHCII complex are responsible for the DM susceptibility.[47]

Noteworthy, most of these conclusions have been drawn from studies using DR and only a few allotypes of DQ strongly associated to autoimmunity (eg, DQ2 and DQ8 related to celiac disease and type 1 diabetes, or DQ6 related to multiple sclerosis and narcolepsy). A similar behavior for DP proteins was expected based on the sequence and structural similarities. However, the distinct DM and Ii requirements of some alleles of DP in cellular settings to form stable complexes and egress from the ER[48] pinpointed potential dissimilarities. Recently, it has been proposed that a particular polymorphism of DP, DPBG84 results in a low CLIP affinity, which subsequently relies on peptide loading and editing by chaperones of the MHCI pathway.[30] While extremely undogmatic, the contextualization of these findings into general adaptive immune responses will be required to define their physiological relevance.

5 | PEPTIDE EDITING BY HLA-DM

Mellins et al originally showed that genetic elements in the central region of the HLAII were essential for the presentation of exogenous antigens[49] and further mapped the defective phenotype to *DMA* and *DMB* genes.[50,51] The same result was additionally confirmed by the Cresswell using a different experimental model.[52] Cresswell's, Jensen's and Mellins' labs proved basically at the same time the ability of DM to dissociate CLIP from MHCII molecules. This function was extended to any peptide with a suboptimal pocket occupation or low affinity. The observation that DM worked at substochiometric concentrations in biochemical experiments[53] and in cells,[54] together with the Michaelis-Menten kinetics of the peptide exchange reaction in the presence of DM[55] led researchers to consider this protein an enzyme. However, the ability to recover MHCII molecules from aggregated states or keep them in a peptide-receptive state in the absence of peptide classified it as a chaperone.[56] DM samples rare conformational states of peptide-MHCII complexes,[47] and also interacts transiently with MHCII with partially dissociated peptides.[45]

Human HLA-DM[57] and its murine homolog H2-DM[58] have been crystalized. Both proteins are structurally very similar to classical MHCII proteins although they bear substantial differences in the apical domains conforming the binding groove of classical MHCIIs, likely impeding the binding of any peptide to DM. Over more than two decades researchers sought to define the molecular mechanisms resulting in DM-mediated peptide exchange. However, despite extensive mutagenesis analysis mapping the lateral interaction of DM and DR in the early 2000s,[59] it took more than 10 years and a considerable protein engineering effort to solve the 3-D structure of the DM-DR complex.[34] The 3-D structure solved by Wucherpfennig's group in two different states has facilitated the proposal of a reaction mechanism for the DM-catalyzed peptide exchange of MHCII in which several highly conserved residues in DM and MHCII are involved. As mentioned in the previous section and reviewed in[33] DM seems to recognize the partial dissociation of the peptide from the P1 pocket and sense rare conformations in tightly bound peptide-MHCII complexes.

DM functions optimally at low-endosomal pH[53] which was proposed to result from a pH sensing mechanism confirmed partly by CD spectroscopy upon shift of the pH of DM in solution.[60] The three acidic residues in the so-called acidic patch of DM (DMBE8, DMBD31, and DMBE46 in humans) were suggested in the first 3-D structure to be responsible for this pH-sensing.[58] Indeed, the 3D structure of DM in complex with DR1 confirmed that DMB-E46 and D31 undergo a considerable rearrangement upon interaction with DR1 which is further modulated by the protonation state of the carboxyl groups. Thus, at high pH deprotonation of D31 results in a loss of a hydrogen bond that rearranges the DM structure and interacts in a less favorable manner with DR1. Recently, we have shown that a naturally encoded DMB variant (*DMBD31V present in DMB*01:07*) confers a considerably less pH-sensitive activity.[9]

HLA-DM traffics independently of its interaction with any other MHCII or the invariant chain through the cell. The presence of a cytoplasmic sorting signal on DMB facilitates its cellular sorting into vesicles and its recycling through endosomal compartments.[61] The subcellular localization of DM and its catalytic pH-dependency suggests that DM is preferentially located at and more active in late endosomal compartments, which in pAPCs are termed class II MHCII compartments (CIIM) or multivesicular bodies (MVB).[62] DM nevertheless is also found in all endocytic compartments where it is supposed to contribute to a greater or lower extent to peptide editing, and for some cell types even at the cell surface.[63] Although DM activity at the surface is usually ignored, it has been shown that overexpression of a mutant which is retained at the cell surface can indeed favor extracellular peptide biding.[64] Similarly, our recent description of a DM allotype with a considerably higher

activity at neutral pH may have a significant impact on peptide editing at the cell surface.[9]

Unanue's lab revealed the importance of the distribution and pH-dependency of DM, showing that distinct editing in different cellular compartments lead to the formation of pMHCII isomers with the ability to trigger different types of T cells.[24,25] Thus, type A CD4$^+$ T_h cells recognize isomers formed in the presence of active DM while type B CD4$^+$ T_h cells only recognize isomers formed in the absence of DM. The preferential display of each isomer in physiologically relevant contexts, namely the thymus (type A) and the pancreas (type A and B) was then correlated to the onset of CD4$^+$ T_h cell responses in autoimmune diabetes.[26] Many other types of experiments have illustrated how DM function and even its expression levels result in the increased or decreased presentation of specific CD4$^+$ T_h cell epitopes although the only true rule that one could consider is, that DM will select the pMHCII complexes of highest kinetic stability from the pool of available peptides (Figure 2B middle).

The field of mass spectrometry (MS) has facilitated the study of the influence of DM function for the display of antigenic peptides at a more global level.[65-67] These studies shed light on the preferential cellular compartments for peptide loading of MHCII in the presence of DM. Recent technical improvements have facilitated deeper deconvolution of DM's influence in peptide selection for the immunopeptidome. In this regard, we have recently reported the influence of different expression levels of DM for the immunopeptidome of cell lines expressing the high-CLIP affinity and high DM susceptible allotype DR3 (*DRB1*03:01*) developing a processing algorithm and pipeline for improved analysis of this type of data.[68] The Jensen lab has also applied cell lines to directly dissect the influence of DM in the immunopeptidome of DQ in the most systematic immunopeptidome analysis performed up to date of DM function for these molecules.[69] In general, the outcome of these investigations is that DM reduces the abundance of CD74/CLIP-related peptides and it facilitates the loading of peptides with higher predicted affinity and optimal pocket occupation from the endocytic pathway. Finally, a recent study making use of MS, has shown that considering DM function and implementing it into epitope prediction tools enhances cancer epitope prediction.[70]

6 | HLA-DO AS MODULATOR OF DM ACTIVITY

Studying DO function has been considerably challenging. DOA and DOB genes are spaced by many other HLA genes, which led to a very late discovery of its functional heterodimer. Besides, DO expression is restricted to B cells and thymic epithelial cells, suggesting a subtler function than that of DM. Furthermore, DO protein is considerably unstable, defaulting any attempt to define its biochemical activity. Indeed, DO only egress the ER[71] when it is bound to DM, and recombinant expression for in vitro experimentation is only facilitated upon introducing a P11A mutation that likely stabilizes the protein. Interestingly, this particular mutation also facilitates the egress of DO from the ER in cells independently of DM.[72]

Denzin and collaborators were able to show that DO blocks DM activity in removing CLIP from MHCII molecules. They used DO/DM purified from cellular extracts and expressed this molecule in the T2 cell line.[73] One year later Kropshofer and collaborators showed that the presence of DO favored the selection of specific peptides by DR molecules rather than simply inhibiting CLIP dissociation.[74] More recently, Larry Stern's group has shown by crystallography and kinetic measurements of peptide exchange that DO acts as a substrate mimic that binds tightly to DM blocking its catalytic activity on MHCII.[75] This study also showed that DO´s groove lacks critical hydrogen-bond donors, making peptide binding to DO unlikely.[75] In this context, Sadegh-Nasseri's group has reported an intriguing observation consisting on the direct interaction between DO and DR molecules in a peptide-receptive conformation, which would promote the direct selection of peptides.[76] Several studies have co-precipitated DM-DO-DR complexes[74,77,78] proposing a direct interaction of DM-DO complexes with MHCII as well. Whether or not these findings are specific to the experimental set up described, or they are representative of a more universal peptide exchange mechanism remains to be proven in systematic manner.

In nonoverexpressing systems (eg, B-LCLs or primary cells), DO does not completely block DM activity. Thus, under steady-state conditions, all DO present is bound to DM while there is still around 50% of free DM from the total pool of this molecule.[3] DM-DO complexes egress the ER and recycle between the plasma membrane and the MIIC because of the cytoplasmic tail signal in DMB.[79] Interestingly, while DM-DO complexes seem to localize to the peripheral membrane of MIIC, free DM localizes to the MVB of these structures.[79] Perhaps, such distribution is owed to the DOB cytoplasmic tail, which is known to be responsible for tuning the intracellular distribution of DO.[80] Denzin and Cresswell suggested that MHCII present in each of these MIIC substructures would therefore be loaded with a differential set of peptides and account for a particular contribution to the net-immunopeptidome.[81] A recent study using CRISPR/Cas9 to Knock-out DO in the LG1 B-LCL has concluded that DO presence leads to a broadened immunopeptidome

containing a smaller fraction of kinetically stable high affinity pMHCII complexes (Figure 2B right).[82]

DO influences the presence of epitopes of sources internalized via receptor mediated endocytosis, and in particular by the B cell receptor.[83] Rather than a direct interaction or DO-mediated peptide loading, the pH dependency of the DM-DO interaction would result in the dissociation of the complex at low pH. This mechanism will result in DO degradation and an endosomal-focused DM activity.[83] However, DM-DO interaction, once established, has proven to be pH-independent in vitro[84]. Although these findings might not apply to a cellular environment in the presence of the transmembrane domains, the mechanism for DM-DO dissociation that precedes DM sorting into different structures in MVB remains unsolved. To date, only a conformational change on DOB dependent on the interaction with DMA that facilitates DO egress from the ER has been defined at the cellular level using monoclonal antibodies.[85] An alternative, yet hypothetical mechanism states that an intracellular signaling event, likely triggered by TLR signaling would be responsible for the dissociation of DM-DO complexes.[81]

Another interesting aspect of DO refers to its particular expression pattern. DO is only expressed once B cell development is complete, and its cellular levels are considerably reduced during B-cell entry in germinal centers.[3] Noteworthy, this decrease in DO levels does not seem to be correlated to differences in DOB transcription levels,[86] and seems to be essential for B cells to gain access to T cell help in this process.[87] Besides B cells and thymic epithelial cells, DO expression has been also reported in some subsets of DCs[21]. As in B cells, in these cells maturation also yields an increase in DO expression.[21]

In summary, it seems that DO has evolved to promote tolerance to self by inhibiting the presentation of self-peptides other than CLIP in mature cells. This inhibition would have important consequences on shaping the self-peptidome during positive and negative selection of thymocytes, during B cell maturation in the germinal centers, and on T cell activation by DCs.

7 | SMALL MOLECULES IN "PEPTIDE EDITING"

Almost two decades ago Falk and collaborators demonstrated that small molecules acting as hydrogen bond donors could influence peptide exchange from MHCII.[88] Afterwards the concept was exploited to generate the so-called molecular loading enhancers (MLEs) and to show that in some circumstances their function depended on specific features of the MHCII allotypes.[89,90] More importantly, such small molecules have been used in in vivo experimentation demonstrating a great capacity to work under physiological conditions.[91] In recent years we have seen how such small molecules could contribute not only to favor peptide exchange but also to interfere directly with peptide binding for MHCI with important consequences for immune reactions as reviewed in[92]. Thus, crossing the line from a transiently interacting MLE to a stable MHCII groove-binding molecule potentially causing adverse drug reactions is of critical importance in attempts to manipulate the MHCII immunopeptidome by small molecules.

Noteworthy, the Wucherpfennig group reported that there are small molecules capable of tuning DM function.[57] Certain fluorinated compounds are able to increase the ability of DM to catalyze peptide exchange. Interestingly, the authors proposed that these small molecules interact with DM within a region that has been mapped a posteriori to the interface between DM-DR. Two key aspects remain unclear: the molecular mechanism of these molecules to interfere with DM function, and whether they also influence DM-DO interaction. While their application in vivo, is challenging, using these molecules for in vitro experimentation could lead to additional molecular insights and a better understanding of DM and DO function.

8 | BIOLOGICAL IMPLICATIONS OF PEPTIDE EDITING

The function of DM and DO has been evaluated and correlated to clinical conditions mostly upon investigation in settings restricted to specific peptides. The influence of DM has been studied in the context of the editing of epitopes related to autoimmunity, allergy or infection (eg, GAD65, CII, Insulin, BetV1, MTB) and for DO, on the display of self-antigens related to allogenic stem cell transplantation (reviewed in Reference 93). While extremely appealing, the sometimes-contradictory effects of DM and/or DM/DO complicate the assessment of a preferential function on editing epitopes related to a particular disease. It is very unlikely that a clinical condition is only driven by the presentation of one single peptide by an MHCII.

Several murine models have been used to understand the physiological relevance of DM and DO (see Table 1). These models have shown that DM and DO are essential for proper thymic selection, for adequate immune responses to pathogens, and for the development of autoimmune phenotypes. Thus, DM and DO function together for the optimization of antigen selection

TABLE 1 Murine models for studying DM and DO function

Model	CLIP affinity	DM-susceptibility	Consequences	Ref.
H2-b $DM^{-/-}$	I-A high I-E low	Expected high Unknown	T cell selection altered	95-97,106
H2-d $DM^{-/-}$	I-A very high I-E low	Expected high Unknown	Altered T cell repertoires	99,110
H2-K $DM^{-/-}$	I-A very low I-E low	Unknown Unknown	T cell selection not altered Change in immunodominance	99
H2-g7 $DM^{-/-}$	I-A very low	Very low	Protected from diabetes High Treg numbers	101
H2-b $DO^{-/-}$	I-A high I-E low	Expected high Unknown	Immunodeficient	103,104
H2-g7 +DO	I-A low	Very low	Protected from diabetes No altered profile of Tregs	104
H2-j DO^{null}	Not defined	Not defined	Protected from retroviral infection Neutralizing antibodies	10

Note: Described murine models in which DM and DO function has been studied. Haplotype, affinity for CLIP (relative), DM susceptibility of each MHCII, and the main observed consequences are indicated. References for each of the models are also given.
Abbreviations: CLIP, class II invariant chain associated peptide; MHCII, major histocompatibility complex of class II molecules.

favoring effective responses to pathogens while preserving self-reactivity at low levels. Of note, the MHCII locus in the mouse shows relevant differences with regard to humans. Mice only have *H2-A*, or *H2-A and H2-E* genes (orthologues of DQ and DR, respectively), and there are two *H2-DMb* genes which seems to be differentially expressed in response to cytokines but yield functionally active H2-DM heterodimers (reviewed in Reference 93).

The global impact of peptide editing for T cell development, and in particular of DM function, is more pronounced on MHCII backgrounds with high affinity for CLIP. In this context, positive and negative selection are mainly driven by MHCII-CLIP stability and $CD4^+$ T_h numbers in peripheral tissues are considerably reduced (up to 50%) as a result of defective positive selection in H2-M-deficient mice. Furthermore, the pool of $CD4^+$ T_h cells available in peripheral tissues reacts strongly to the peptidome displayed by pAPCs of syngeneic wild-type mice in mixed lymphocyte reactions.[94-96] This is indicative of altered negative selection in the absence of H2-DM. In case of haplotypes (single MHCII or mixed) with low MHCII-CLIP affinity, thymic selection seems to be less affected for conventional $CD4^+$ T_h cells and consequently the extent of reactivity to syngenic, or MHCII-matched strains is more variable.[97-99] In these low MHCII-CLIP affinity backgrounds, however, it has been shown that the frequency of T_{reg} in peripheral tissues is higher in the absence of DM.[100,101] The implications of the lack of DO for thymic selection has been studied only in the murine H2-b background (high MHCII-CLIP affinity)[102,103] and only Perraudeau and collaborators reported a slight increase in the total number of $CD4^+$ T_h cells in lymph nodes of KO animals (9.4% total cells) compared to wild-type (7.6%) animals.[103] Following a completely different strategy to understand protein function, the Denzin lab ectopically expressed human DO in murine DCs on the diabetogenic (low affinity MHCII-CLIP) background H2-Ag[7] of the NOD mouse and reported neither influence on T cell numbers nor selective reactivities.[104]

The impact of DM and DO on $CD4^+$ T_h-mediated immune mechanisms has been evaluated in different contexts. Following the original reports on strong reactivities of T cells from wt mice to cells of syngenic mice missing DM,[94-96,105] van Kaer and collaborators pinpointed the importance of the peptide-repertoire associated to MHCII for allo-reactivity.[106] Subsequently it was shown that DM loss has a dramatic effect on murine cardiac transplantation.[107] In case of DO, there is no direct report assessing the influence of lack of function for allo-responses or transplantation conditions. Their influence in infection is better documented. DM focuses and restricts the immune response towards a very limited number of high-stability peptides, which are called "immunodominant".[108] The Bikoff lab[109] determined that for H2-$DM^{-/-}$ mice, not only is the immunodominance of antigenic peptides broader when DM is absent, but the entire immune response shifts from I-A^d presentation in the wild-type to I-E^d presentation in the KO mouse. Furthermore DM seems to be responsible for murine control of *Mycobacterium tuberculosis* infection under laboratory conditions.[110] A unifying conclusion

from all of these studies is that in the haplotypes bearing MHCII allotypes with a low affinity for CLIP, the antigen-presenting function seems to be less affected. Tourne et al.[111] also described that $DM^{-/-}$ mice were able to respond to certain viral infections, although antibody responses were generally less efficient than in the wild type mouse. The Eisenlohr laboratory has demonstrated that only some viral epitopes require the presence of DM for presentation, and that efficient immune responses rely mostly on the antigen processing of endogenously synthesized virions.[23] In the case of bacterial infection, however, DM seems to be required for an appropriate immune response.[110] The requirement of DM function for mounting efficient antibody responses to pathogens was later evaluated, and the absence of DM was associated with an impaired ability to form germinal centers for B-cell maturation.[112]

There are considerable physiological differences between humans and mice regarding antigen presentation and/or T cell responses although the fundamental mechanisms affected by altered editing in these models, are likely to apply to humans as well. To date fundamental research questions, such as the impact of peptide editing in T cell selection, have been solved using inbred strains. However, recent findings by Denzin and Golovkina have demonstrated an impact of genetic variations of ncMHCII for adaptive immune responses. A *H2-Ob* null allele, found in the I/LnJ strain protects animals from retroviral infections by facilitating the production of neutralizing antibodies.[10] To what extent these findings correlate and/or apply to humans is difficult to predict although they demonstrated the different function of human DO depending on allelic variants of *DOB*. In particular, rs144814623 found in a gain of function *DOB* variant (high DM inhibition) is in LD with rs4273729 "A" found in *DQA2* alleles that have been associated with viral persistence while s4273729 "G" is related to viral clearance. We have been able to demonstrate that human DM allelic variants impose distinct functional profiles that also affect epitope display and T cell activation[9] albeit these variants have not been yet put into any clinical context.

9 | GENETIC ASSOCIATION STUDIES IN HUMANS TO UNDERSTAND ncMHCII FUNCTION

Classical HLA class II allelic variants have been linked to autoimmune disorders, allergies and other clinical conditions in which $CD4^+$ T_h cells play important roles. The main mechanism accounting for the contribution of these allelic variants to health and disease lies on their ability to bind and present antigenic peptides recognized by reactive $CD4^+$ T_h cells. In this context, the discovery of ncMHCII, and their function, motivated genetic association studies evaluating a direct link between ncMHCII genes, *DMA* and/or *DMB* mostly, and several autoimmune-related disorders. Despite statistically sound outcomes (reviewed by us in Reference 93), there was no clear correlation for any genetic variant of ncMHCII to disease. The low frequency of allelic variants of ncMHCII genes impede the assessment through conventional association studies with low number of samples.

Genome wide association studies of big datasets could overcome these problems. Interestingly, in recent reports it has been shown that certain natural variations in ncMHCII genes with frequencies higher than 5% are linked to particular disease outcomes as for instance rheumatoid arthritis[113] and systemic lupus erythematosus.[114] Other approaches have shown a correlation between specific ncMHCII genes and autoimmune diseases[115] as well as between allelic variants and viral infections.[116] However, very low frequencies mask associations unless specific considerations are taken into account.[117] The comparison of the functional features of any of the genetic variants related to disease with those un-related could contribute to determine the biological or physiological relevance of peptide editing. This includes those polymorphisms affecting gene expression that have already been detected in associations to diseases.[113,114] Thus, besides the identification of causative variants for diseases, the next biggest issue faced in the field is the development of reliable and physiologically relevant functional assays. On the one hand, we should consider appropriate cellular models based on physiologically relevant expression levels of MHCII and ncMHCII proteins to solve basic mechanistic questions. On the other hand, animal models should incorporate human transgenes or being reconstituted with the complete human immune system. Alternatively, we could also draw conclusions by engineering murine backgrounds in which specific ncMHCII functions are tuned.

10 | CONCLUDING REMARKS

DM and DO function has been characterized at the molecular, cellular and organismic level using a handful of representative MHCII allotpyes. This research has been fundamental in determining that the impact of peptide editing on T cell responses, and hence adaptive immunity, significantly depends on the MHCII allotype. While we are now starting to have a clearer picture on their function, one of the biggest problems for scoring the impact of peptide editing in a broad sense, is the

distinct behavior of MHCII allotypes. Systematic analysis of peptide editing on individual MHCII allotypes should contribute to defining models for studying the influence of peptide editing. Adding to complexity, most (immunopeptidomic) studies so far have not taken into account actual human haplotypes, neglecting nonadditive effects of peptide loading and exchange under competitive conditions. Further key remaining questions in DM and DM-DO peptide editing include: (a) how the DM-DO ratio is regulated in cells under steady state and maturation conditions; (b) if the DM-catalyzed peptide exchange shown for DR applies to other MHCII allotypes. Furthermore, recent findings from our lab and from Denzin-Golovkina have pinpointed the impact of natural variations of ncMHCII on peptide-MHCII editing. Indeed, the study by Denzin-Golovkina have even shown the tremendous impact these processes have in immune responses to pathogens. Since it is very hard to extrapolate findings in murine models to human biology, other experimental models are required to evaluate the impact of natural variations in ncMHCII genes for human immunology. It remains to be unraveled whether natural variations on ncMHCII genes might differentially modulate peptide editing of specific haplotypes. In this case, genetic studies in conjunction with functional characterization of natural variants in appropriate experimental models will be essential.

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CONFLICT OF INTEREST

The authors have declared no conflicting interests.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Miguel Álvaro-Benito https://orcid.org/0000-0002-6019-3567

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AUTHOR BIOGRAPHIES

Miguel Álvaro-Benito is a senior postdoctoral researcher in the group of Christian Freund at the department of Biochemistry of the Freie Universität Berlin. He studied Biochemistry and graduated in Molecular Biology at the Universidad Autónoma de Madrid. Trained on biotechnology and structural biology he shifted his career towards the understanding of peptide editing at the molecular and cellular level. His main research interest is the interplay of the HLA genetic background and functional profiles that relate to clinical relevant conditions, particularly for accessory molecules essential for antigen presentation.

Christian Freund is a Professor of Biochemistry at Freie Universität Berlin. He studied Chemistry in Düsseldorf and München and graduated at the Max Planck Institute for Biochemistry in Martinsried in affiliation with LMU München. After two postdocs at the University of Zürich and Harvard Medical School he received the Biofuture prize from Germany's Federal Ministry for Education and Research in the year 2000, allowing him to establish his own group. His main interests are in the field of protein structure and dynamics, especially in the field of molecular structural immunology, including antigen presentation by MHC class II molecules and T cell receptor proximal signaling.

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