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REVIEW

What to do with HLA-DO/H-2O two decades later?

Robin Welsh[1] · Nianbin Song[1] · Scheherazade Sadegh-Nasseri[1,2]

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Abstract

The main objective of antigen processing is to orchestrate the selection of immunodominant epitopes for recognition by CD4 T cells. To achieve this, MHC class II molecules have evolved with a flexible peptide-binding groove in need of a bound peptide. Newly synthesized MHC-II molecules bind a class II invariant chain (Ii) upon synthesis and are shuttled to a specialized compartment, where they encounter exogenous antigens. Ii serves multiple functions, one of which is to maintain the shape of the MHC-II groove so that it can readily bind exogenous antigens upon dissociation of the Ii peptide in MHC- II compartment. MIIC contains processing enzymes, one or both accessory molecules, HLA-DM/H2-M (DM) and HLA-DO/H2-O (DO), and optimal denaturing conditions. In a process known as "editing," DM facilitates the dissociation of the invariant chain peptide, CLIP, for exchange with exogenous antigens. Despite the availability of mechanistic insights into DM functions, understanding how DO contributes to epitope selection has proven to be more challenging. The current dogma assumes that DO inhibits DM, whereas an opposing model suggests that DO fine-tunes the epitope selection process. Understanding which of these, or potentially other models of DO function is important, as DO variants have been linked to autoimmunity, cancer, and the generation of broadly neutralizing antibodies to viruses. This review therefore attempts to evaluate experimental evidence in support of these hypotheses, with an emphasis on the less discussed model, and to explore intriguing questions about the importance of DO in biology.

Keywords HLA-DO · HLA-DM · HLA-DR · MHC class II · Antigen processing · H-2O

Class II accessory molecules

DO is a non-classical MHC-II-like molecule that is highly conserved in warm-blooded vertebrates. Like DM, DO is an α/β heterodimer which does not bind to peptides. While genes encoding the DO molecule were first identified in the late 1980s, and its restricted expression to thymic medulla and B cells was reported in the early 1990s, it took another decade or so before its contributions to class II antigen processing were investigated (Karlsson et al. 1991; Trowsdale and Kelly 1985). This is in contrast to the discovery of DM, a molecule that was identified much later, yet mechanistically understood more readily. In fact, the total number of DO-focused published papers to date, including *Review* articles, remains less than 200. Work from our group and several others highlight the importance of understanding the relationship of DO to DM and to MHC-II molecules. The current understanding about DO can be distilled into two working hypotheses. In one model, DO forms a tight complex with DM in order to prevent DM from removing the invariant chain peptide CLIP, and in the other, DO differentially affects the presentation of structurally diverse peptides and acts as a second chaperone together with DM to fine-tune MHC-II repertoire selection. Table 1 briefly summarizes evidence in support of these hypotheses.

Robin Welsh and Nianbin Song contributed equally to this work.

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✉ Scheherazade Sadegh-Nasseri
ssadegh@jhmi.edu

[1] Graduate Program in Immunology and Department of Pathology, Johns Hopkins University, Baltimore, MD, USA

[2] Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

DM

Before discussing the evolution and our understanding of DO function, we must consider the evolutionarily older MHC-II co-chaperone, DM. DM is a non-polymorphic MHC-II-like molecule that does not bind peptides (Mosyak et al. 1998),

Table 1 A comparison of published evidence for the two hypotheses proposing mechanism of DO function

DO binds to DM to inhibit its activity	DO works together with DM in fine tuning MHC-II repertoire selection
1. Less CLIP removal and peptide loading with DR by DM/DO complexes than DM alone (Denzin et al. 1997).	1. Enhanced influenza peptide HA (307–319) binding to DR1 or DR4 with DM/DO complexes (Kropshofer et al. 1998).
2. DO binds to DM at the same interface with which DM contacts DR1 (Guce et al. 2013).	2. DM/DO complexes co-precipitated with DR (Kropshofer et al. 1998).
3. Overexpression of DO in dendritic cells protected NOD.DO mice from development of diabetes (Yi et al. 2010).	3. DM/DO complexes are better at keeping empty DR4 active at low pH (Kropshofer et al. 1998).
4. No spontaneous autoimmune phenotypes have been observed in DO-KO mice (Gu et al. 2013).	4. DO can interact directly with DR molecules in peptide-receptive conformation (Poluektov et al. 2013b).
	5. DO-KO mice did not show reduced MHCII-CLIP levels (Brocke et al. 2003; Liljedahl et al. 1998; Perraudeau et al. 2000)
	6. Different DOβ variants, despite having different effects on MHCII-CLIP levels, all bind to DM (Denzin et al. 2017).
	7. DO has a more dramatic effect on the presentation of epitopes from antigens internalized by the B cell receptor, rather than fluid phase endocytosis (Alfonso et al. 2003).
	8. Naïve DO-KO mice spontaneously produce high titers of antinuclear antibodies (Gu et al. 2013).

but is necessary for the efficient displacement of CLIP from the MHC groove (Cresswell 1996; Denzin and Cresswell 1995; Denzin et al. 1996, 1994; Ghosh et al. 1995; Mellins et al. 1994, 1991; Morris et al. 1994; Riberdy et al. 1992; Spies et al. 1990) and its exchange for exogenous peptides (Kropshofer et al. 1996). Over the past two decades, we have learned a considerable amount about the structure and function of DM. DM senses and interacts with the empty P1 pocket of DR1 and induces conformational changes that disrupt H bonds between the peptide and the binding groove, leading to dissociation of the bound peptide (Belmares et al. 2003; Chou et al. 2008; Chou and Sadegh-Nasseri 2000; Doebele et al. 2000; Kropshofer et al. 1997; Marin-Esteban et al. 2004; Narayan et al. 2007, 2009; Pu et al. 2004; Sadegh-Nasseri et al. 2008; Stratikos et al. 2004; Ullrich et al. 1997; Zarutskie et al. 2001). Removal of the bound peptide generates a receptive conformation (Natarajan et al. 1999; Rabinowitz et al. 1998) that can readily scan suitable stretches of partially folded antigens or large antigenic fragments (Hartman et al. 2010; Kim et al. 2014). This process continues until an optimal peptide is selected from the denatured protein antigen for further trimming and presentation to specific T cells.

Part of this process of DM editing can be gleaned from recent structural studies of DM. The transient interaction of DM with DR molecules (Narayan et al. 2009) made co-crystallization of DM/DR complexes a challenge until recently. Anders et al. cleverly designed a DR1/peptide complex using a truncated peptide that acted as a barrier to the closing of the hydrophobic P1 pocket of the DR1 groove, keeping the empty P1 open. As a result, DM could form stable complexes with the open groove of DR1 and was co-crystallized (Anders et al. 2011; Painter et al. 2011; Pos et al. 2012). Consistent with previous reports, the 3-D structure of DM/DR1 revealed that during the interaction, a tryptophan residue within the P1 area of DR1, had rotated out of the groove. The accompanied conformational changes and the potential disruption of multiple H bonds would likely lead to destabilization of the bound peptide and its dissociation. Thus, while the DM/DR1 crystal structure does not reveal the initiation of the process, based on cumulative data one might summarize the contributions of DM to peptide exchange as follows: DM induces a dynamic MHC-II conformational change in pMHC-II complexes when the P1 pocket is not fully occupied, enhancing peptide dissociation, and mediates peptide exchange. This provides a molecular mechanism whereby DM efficiently targets and promotes the dissociation of peptides that lack a suitable sidechain to fill the P1 pocket of DR1, editing them for more stable peptides. Such peptides are defined here as "DM sensitive." Peptides that carry a suitable sidechain to fill the P1 pocket of DR1 and can resist DM-mediated dissociation are defined here as "DM resistant." Hence, in addition to the removal of CLIP, DM helps in the selection of immunodominant epitopes.

A role for DM in immunodominance has been established by both cellular (Lich et al. 2003; Nanda and Bikoff 2005) and extensive biochemical studies by Yin and Stern (Yin et al. 2014). Other studies using a cell-free antigen processing system reported that DM increases the abundance of the immunodominant pMHC-II for a given antigen (Kim et al. 2014; Kim and Sadegh-Nasseri 2015). Recent studies have added another layer of complexity by providing evidence that DM may fail to cause dissociation of a peptide that carries a non-optimal P1-fitting residue if the P9-fitting residue forms a tight fit (Yin et al. 2014). Notably, most of our mechanistic understanding of DM is based mainly on DM interactions with only a few DR alleles, especially DR1. Moreover, some MHC-II alleles such as I–E^k and HLA-DQ (DQ2) (Fallang et al. 2008; Nguyen et al. 2017) are not good substrates for DM (Koonce et al. 2003; Wolf et al. 1998). One explanation for the failure of I–E^k reactivity with DM might be the fact that its P1 pocket is partially filled (Fremont et al. 2002). This phenotype resembles a mutant form of DR1 (Gb86Y) described by our group that does not interact with DM (Chou and Sadegh-Nasseri 2000). Similarly, DQ2 has a hydrogen bond network located at the bottom of the peptide-binding groove that renders the residues in this region relatively immobile (Nguyen et al. 2017).

DO

Biochemical and in vitro studies

In the first functional characterization of DO, Denzin et al used lysates from several lymphoma-derived cell lines to purify DM/DO complexes or DM alone. These lysates were used to evaluate peptide exchange with CLIP (Denzin et al. 1997). From in vitro peptide binding assays, the authors concluded that presence of DM/DO led to a reduced binding of the antigenic peptide to DR3 molecule compared to DM alone. Therefore, DO could be having an inhibitory effect on DM. The authors also transfected cell lines with DO constructs and observed enhanced levels of surface MHC-II-CLIP expression, again suggesting that DO may serve to inhibit DM function and lead to more CLIP rather than antigenic peptide presentation. This study put forward measuring MHC-II-CLIP surface expression as an indirect method for assessment of DO function in cells. Indeed, this method has been adopted by many groups in transfection studies where either wild type (WT) or mutated DO and DM constructs were used to transfect various cell lines in order to assess the functionality of DO (Denzin et al. 2017, 1997; Fallas et al. 2004; Glazier et al. 2002; Jiang et al. 2015; Kremer et al. 2012; Yi et al. 2010; Yoon et al. 2012). These early findings nucleated the basis for the current dogma that DO inhibits DM function.

An alternative function for DO on peptide binding of various alleles of MHC-II was proposed around the same time, by Kropshofer et al. using both recombinant full-length and cell-isolated DO and DM proteins. Utilizing a series of previously identified DR4-binding peptides and testing them for binding to DR4 in the presence or absence of DM and DO, the authors reported that DO altered the DR4 peptide-binding profile (Kropshofer et al. 1998). They also showed that including DO together with DM increased the overall binding of the well-studied influenza peptide HA (307–319) to DR1 and to DR4, although the enhancing effects of DO diminished if DR3 was used.

The complexity of DO impacting the presentation of different peptides was directly examined by Poluektov et al, who proposed an alternative model for DO function. The group produced and purified soluble recombinant DO and tested its impact on association and dissociation kinetics of different peptides to soluble DR1 molecules in the presence and absence of soluble DM (Poluektov et al. 2013a). Notably, (a) DO affected only peptide *association*, not pMHC *dissociation*; (b) DO did not inhibit DM; but rather, DO interacted directly with DR molecules in a peptide-receptive conformation, (c) DO increased the quantities of pMHCII complexes when the peptides were DM-insensitive, and (d) DO reduced the number of formed pMHCII complexes when peptides were DM-sensitive. In light of our understanding that DM mainly induces pMHC dissociation, and DO only affects peptide association, it is reasonable to interpret these data in a model wherein DO needs DM for generation of the peptide-receptive MHCII conformation, to which DO binds. Binding of DO to MHCII could stabilize the complex in its open conformation and would allow a more fine-tuned epitope selection (Fig. 1) (Poluektov et al. 2013b).

A few prior studies showing a direct interaction of DO with DR molecules support such a model by Poluektov et al. In one case, the beta chain of DO was found to directly interact with DR (Papadimitriou et al. 2008), and in other studies (Hammond et al. 1998; Kropshofer et al. 1998), DO/DM complexes could be co-precipitated with DR molecules in purified class II peptide-loading compartments. These data suggest that DO may coexist with DM and DR as multimers (Gondre-Lewis et al. 2001; Hammond et al. 1998). If this is the case, and if persistent inhibition by DO of DM activity would impair proper antigen presentation, then these findings call into question whether DO is truly an inhibitor of DM for removal of CLIP. Another example of DO affecting epitope selection came from Brocke et al., who knocked down H2-Oα and β chain genes in a murine B cell line and found that decreased DO expression was associated with an increased expression of epitopes derived from various protein antigens (Brocke et al. 2003). These findings, along with other studies showing that DO regulates peptide presentation in different subsets of dendritic cells (Hornell et al. 2006), suggested that DO may be important for presenting peptides from different sources (self vs non-self) (Chen et al. 2006).

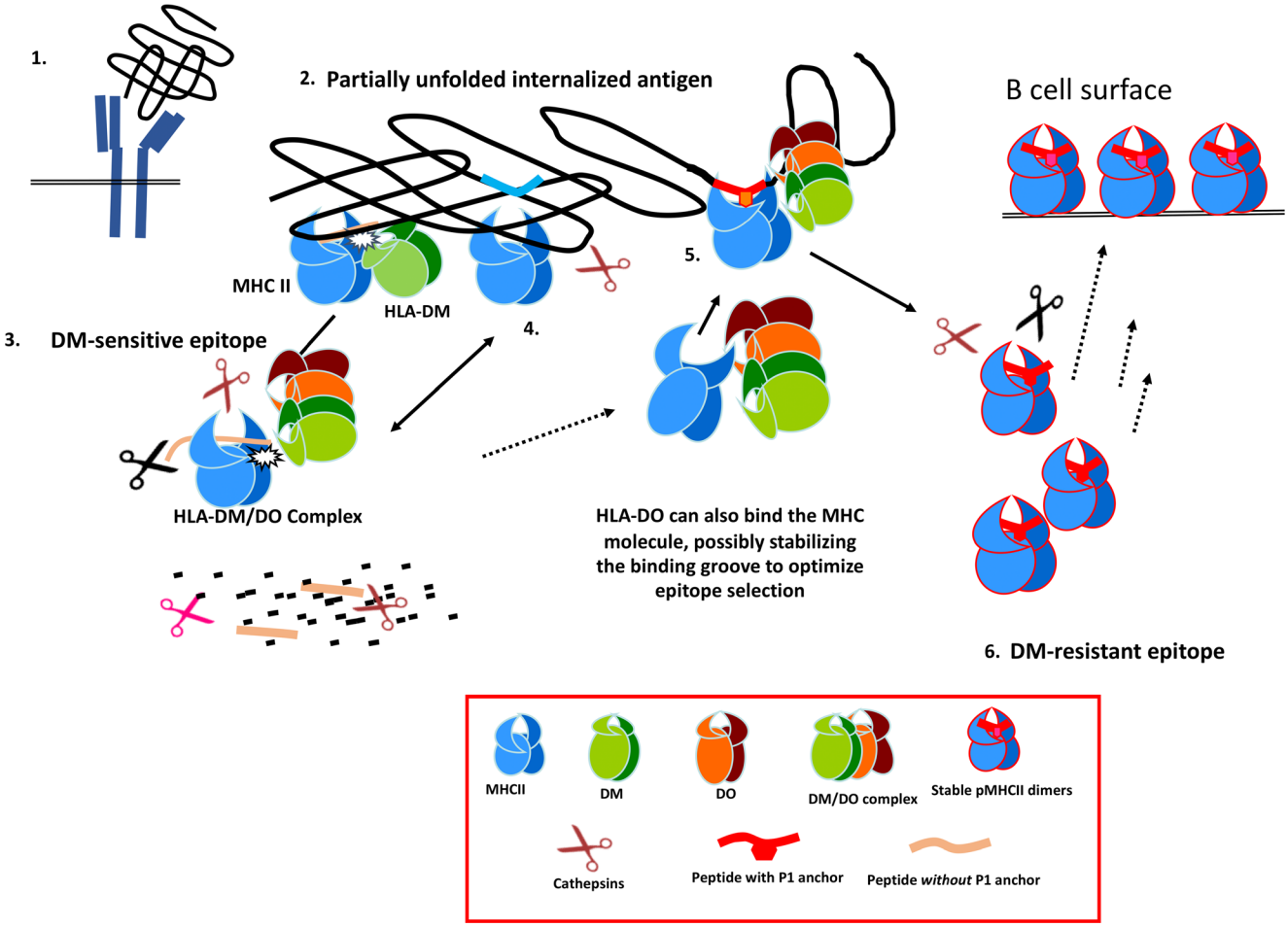

Fig. 1 B cells internalize antigens via the BCR receptor and shuttle the antigen to the MHC Class II compartment in (1). Under denaturing conditions, internalized antigen will become partially unfolded, revealing possible epitopes to which peptide receptive MHCII molecules can bind (2). Epitopes will be edited by HLA-DM for the best fitting ones (3). While peptide-receptive MHCII continues scanning the unfolded antigen for a good binding epitope (4), HLA-DO can further widen the MHC II groove and help DM to optimize epitope selection (5). Once a peptide with a good P1 anchor is loaded, the complex becomes DM-resistant and is shuttled to the surface of B cells for recognition by cognate $CD4^+$ T cells (6)

Efforts to better understand DO function have led to revealing structural studies of the protein. Guce et al. co-crystalized the DM/DO complex (Guce et al. 2013) and found that (a) the 3-D structure of DO interacting with DM is very similar to that of DM interacting with HLA-DR1; (b) DO has a rather open and accessible peptide-binding groove, similar to DR, and in contrast to DM which has a closed peptide-binding groove; and (c) DO binds DM at the same interface that DM interacts with DR1 (Pos et al. 2012). Altogether, the combined information gained from solving the two 3-D structures was suggestive of a model whereby DO can act as a competitive inhibitor for DM in interacting with MHCII (Denzin and Cresswell 2013; Guce et al. 2013). While this is a feasible model, it is important to keep in mind that while the interaction between DM and DO is relatively stable, the interaction of DM with MHCII is transient (Chou and Sadegh-Nasseri 2000; Narayan et al. 2009; Sadegh-Nasseri et al. 2012, 2010). The DM/DR1 complex was elegantly stabilized under highly stringent conditions to allow for crystallization, which included generating an open and peptide-receptive DR1 conformation (Anders et al. 2011). Moreover, the DM/DO co-crystal structure revealed a rather open and accessible DO groove (Guce et al. 2013), which could possibly bind the MHCII. Mutational studies agreed with the crystal structures and showed that some previously identified amino acid residues known to disturb the DM/DR interaction (Pashine et al. 2003) mapped almost entirely to the DM/DO interface (Yoon et al. 2012). While the interface of DM interacting with DR might be the same as that of DM interacting with DO, this notion does not exclude the possibility of either molecule interacting with DR independently. Nonetheless, opposing evidence from Pos et al. revealed that two previously described DM mutants, DM βHis141 and βSer142, which reportedly interfered with DO interacting with DM, had

no effects on the ability of DM to facilitate peptide binding to DR1 adding to the confusion about how the three molecules interact (Pos et al. 2012).

DO and pH effects

pH may be another important factor impacting DM/DO functions. Kropshofer et al. observed that at low pH, DR4 binds to the DM/DO complex with a higher affinity than DM alone. DM/DO complexes were also better at keeping empty DR4 peptide-receptive at pH values lower than 5.0 when compared to DM only. Based on these findings, the authors proposed that DO might protect DM from being denatured from prolonged exposure to low pH, which is typical of where these molecules naturally function. These findings were partially challenged by a recent study using several DO mutant proteins. Work by Jiang et al. suggests that DO forms complexes with DM to protect DM function in late endosomal pH of 4.6–5.0, but to inhibit DM at a pH range greater than 5.0 (early endosomal pH) (Jiang et al. 2015). Put together, a clear correlation between DO/DM functionality and pH remains to be discovered.

Evidence documenting DO function in vivo

Elucidating the in vivo function of DO has been even more challenging. To evaluate the in vivo role of DO, H2-O$\alpha^{-/-}$ (DO-KO) mice were created by two different laboratories (Liljedahl et al. 1998; Perraudeau et al. 2000). Based on the current dogma from transfection experiments described earlier, where DO inhibits DM, one would expect DO-KO mice to show increased DM activity as measured by reduced levels of MHC-CLIP on the surface of APCs. However, DO-KO mice did not show reduced MHCII-CLIP levels (Brocke et al. 2003; Fallas et al. 2007; Liljedahl et al. 1998; Perraudeau et al. 2000, 2013). Other studies, which focused specifically on either B cells or CD11c^{+} DCs, detected a slight reduction in CLIP levels in DO-KO mice (Denzin et al. 2017; Gu et al. 2013). Thus, a clear model of DO from these cellular models is still elusive. Additional insights into the in vivo function of DO came from investigations into the route of antigen uptake by APCs. Using DO-KO or DO-WT B cells and OVA and HEL as model antigens, Alfonso et al showed that loss of DO had more noticeable effects on the presentation of epitopes from antigens internalized by the B cell receptor versus those from fluid-phase endocytosis (Alfonso et al. 2003).

The role of DO in autoimmunity and its evolutionary importance

To determine if DO might have a role in susceptibility to autoimmune diseases, Yi et al. developed a non-obese diabetic mouse (NOD.DO) that expressed HLA-DO under CD11c promoter control (Yi et al. 2010). Interestingly, NOD.DO were protected from developing spontaneous diabetes, which was hypothesized to be due to decreased DM function in DCs leading to decreased presentation of an unknown diabetogenic epitope. The results could also be interpreted as a decrease in the presentation of a DM-sensitive autoantigen peptide in the presence of DO. In another study, Gu et al. found that naïve DO-KO mice spontaneously produced high titers of antinuclear antibodies (ANA), but did not develop a detectable autoimmune phenotype (Gu et al. 2013). To date, no other studies have reported the development of any spontaneous autoimmune phenotype in DO-KO mice.

More recently, human genome-wide association studies (GWAS) have correlated the occurrence of rheumatoid arthritis (RA) to several single-nucleotide polymorphisms (SNP) in DO genes (Okada et al. 2016; Reynolds et al. 2010). Additional association studies have also suggested multiple links between some SNPs in DO genes and various diseases such as, type I diabetes (Santin et al. 2009), HCV infection (Huang et al. 2015; Yao et al. 2018), and non-small cell lung cancer (Pu et al. 2014). However, none of these studies investigated how those SNPs could impact the activities of DO. This is of particular interest since most SNPs identified, like those in van Lith et al (van Lith and Benham 2006) study, were found to reside in the non-coding region of DO genes. Contributions of DO in protection from exogenous pathogens were also recently reported. Using a newly generated H-2O$\beta^{-/-}$ mice, Denzin et al found that the absence of DO led to an increased production of broadly neutralizing antibodies (bNAbs) against a mouse mammary tumor virus (MMTV) (Denzin et al. 2017). The group also showed through bioinformatic analysis that the human DOB gene is in linkage disequilibrium with the HLA-DQA2-DQB2 locus, which has been associated with HCV or HBV persistence (Chang et al. 2014; Duggal et al. 2013). The authors thus proposed that the DOB gene is co-inherited with DQA2-B2 gene and could possibly contribute to HCV persistence due to decreased bNAb production. A loss of DO leading to the production of bNAbs is interesting since DO levels decrease when B cells enter germinal centers (GC) and recover upon exiting the germinal center (Brocke et al. 2003; Chalouni et al. 2003; Chen et al. 2002; Fallas et al. 2007). These findings, together with the fact that DO and germinal centers appear roughly around the same time in evolution (Flajnik 2018), suggest that DO may be involved in regulating the germinal center reaction.

Future questions

As discussed above, although some progress has been made towards understanding the physiological role of DO, the functions of DO are unclear and many questions remain unanswered. While the DM sequence appeared first in amphibians

(tetrapods) (Dijkstra et al. 2013), DO developed in warm-blooded mammals (Flajnik 2018). What evolutionary benefits could the appearance of DO provide over a system with just DM alone? Why is DO mainly expressed in B cell, thymic medullary epithelial cells (mTECs) (Douek and Altmann 1997), and to a less appreciable level in certain subpopulations of dendritic cells (DC) (Chen et al. 2006; Hornell et al. 2006)? Is the regulated expression of DO linked to the activation status of the cells? If DO is important for maintaining proper negative selection, why is it expressed at lower levels in perinatal mTEC, but high in adult mTEC (Yang et al. 2015)? What role does DO play in regulating germinal center reaction? How do the non-coding SNPs in DO contribute to the pathogenesis of diabetes, RA, HCV, and non-small cell lung cancer? These questions are important and complex, potentially linking DO to thymic education, clearance from pathogens, and autoimmune diseases.

A comparison of MHC class I chaperones, Tapasin and TAPBPR (TAP-binding protein related), with MHC class II chaperones presents an intriguing message, as there seems to be a great deal of similarities. Tapasin has been shown to edit peptides in a manner similar to how DM edits peptides for MHC class II (Sadegh-Nasseri et al. 2008). The field also has new mechanistic understandings of how TAPBPR protein interacts with MHC-I, where a scoop loop of TAPBPR stabilizes an open MHC-I groove for facilitating peptide exchange (Jiang et al. 2017; McShan et al. 2018; Thomas and Tampe 2017; van Hateren et al. 2017). This characteristic of TAPBPR resembles a mechanism proposed for DO in interaction with MHCII by Poluektov et al. (Poluektov et al. 2013a, 2013b). TAPBPR and tapasin work cooperatively in the peptide-loading complex to ensure high-affinity peptide binding to MHC-I molecules. MHC-II molecules may also need DM and DO for the same purpose, as nature likes to repeat itself.

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