Human Immunology 80 (2019) 15–31

Contents lists available at ScienceDirect

Human Immunology

journal homepage: www.elsevier.com/locate/humimm

# On the structure–function of MHC class II molecules and how single amino acid polymorphisms could alter intracellular trafficking

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ARTICLE INFO

*Keywords:*
MHC class II molecules
CD74
Invariant chain
Iip35
Di-arginine
Ubiquitin
MARCH1
Antigen presentation
Endoplasmic reticulum
Polymorphism

ABSTRACT

Classical HLA class II molecules are highly polymorphic heterodimeric transmembrane proteins encoded by a polygenic cluster on chromosome 6. Polymorphic residues in the membrane-distal domains ensure that a large collection of microbial peptides can be bound in the human population. Still, the HLA-DR, -DP and -DQ isotypes show a high degree of conservation in their overall tertiary and quaternary structures, in line with their common function in T cell receptor activation. Interestingly, the primary structure of the intracellular domains are highly divergent between isotypes and they also show allotypic variations. The functional impact of these differences remains to be fully appreciated. Here, we address the role of the MHC class II cytoplasmic tails in intracellular trafficking. First, the emphasis will be on the interplay between the cytoplasmic domains of classical human MHC class II molecules and those of the invariant chain chaperone (CD74) isoforms. Then, we will examine the importance of the highly conserved β-chain cytoplasmic lysine residue in the ubiquitin-driven trafficking of MHC class II molecules. These considerations should help understand the potential functional impact of sequence variations that may arise in the cytoplasmic tails and transmembrane domains of MHC class II molecules.

## 1. Introduction

The Major Histocompatibility Complex (MHC) is called H-2 in mice and HLA in humans. It is divided in three main regions encoding antigens of the class I, II and III. MHC class II molecules are responsible for activating the adaptive immune system, which is ultimately responsible for the production of memory cells capable of reacting swiftly upon subsequent exposures to a given microbial insult [1]. The cornerstone of this arm of immunity is the pool of $CD4^+$ helper T (Th) cells. These bear T cell receptors complementary to MHC class II molecules associated with peptides of self or nonself origin. In Th cells, APCs bearing MHC class II molecules filled with foreign peptides will trigger different signaling cascades. The ensuing cytokine production and cell–cell interactions lead to antibody production and activation of cytotoxic effector cells, complementing the initial innate immune response with new sets of weapons.

The MHC has evolved into a very sophisticated polymorphic and polygenic locus in order to maximize the chances that some individuals in the population will mount an immune response against any incoming new pathogen [2]. Importantly, expression of these genes is co-dominant, resulting in the display of many different molecules. As self-/non-self-discrimination is based on the intricate interactions with T cell receptors and co-receptors, the ligand function of MHC molecules must operate in a defined framework and comply to somewhat rigid evolutionary structural constraints [3–5]. As discovered by Wiley, Strominger and collaborators upon solving the 3D structure of MHC class I and class II molecules, most of the polymorphic residues in the membrane-distal domains are lining the peptide-binding groove and affect only minimally the overall quaternary structure [6].

While MHC class I molecules mainly bind newly synthesized, endogenous byproducts of the proteasome that have been translocated to the ER by the TAP transporters, MHC class II is responsible for binding

*Abbreviations:* APC, antigen presenting cell; BcR, B cell receptor; BiP, binding immunoglobulin protein; CIITA, Class II transactivator; CLIP, Class II-associated Ii chain peptide; COP, coat protein; DC, dendritic cell; ER, endoplasmic reticulum; ERGIC, ER-golgi intermediate compartment; FPR, fluorescent photobleaching recovery; HLA, human leukocyte antigen; IL1RAP, interleukin-1 receptor associated protein; MHC, major histocompatibility complex; MARCH, membrane-associated RING-CH; MIIC, MHC II compartments; MitAP, mitochondrial antigen presentation; FcRn, neonatal Fc receptor; NleA, non-LEE-encoded effector A; NMR, nuclear magnetic resonance; PKC, protein kinase C; SPPL2a, signal peptide peptidase 2a; SAPs, single amino acid polymorphisms; SNPs, single nucleotide polymorphisms; TcR, T cell receptor; TAPA-1, target of antiproliferative antibody-1; TLR, toll-like receptor; TM, transmembrane; TRAIL, tumor necrosis factor-related apoptosis inducing ligand

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https://doi.org/10.1016/j.humimm.2018.10.001
Received 27 July 2018; Received in revised form 25 September 2018; Accepted 1 October 2018
Available online 12 October 2018

exogenous antigens [7,8]. In line with their highly specialized functions in antigen presentation, MHC class II molecules must sample endocytic compartments in order to collect a large range of peptides, mostly of self-origin [9]. Then, the peptide-loaded molecule must be displayed at the plasma membrane in order to be scanned by attracted T cells searching for invaders. The convoluted intracellular journey of MHC class II molecules would be impossible without the help of chaperones. Indeed, expressed on their own, class II molecules would swiftly bind ER peptides and exit through the default pathway [10–12]. Not only should such peptide binding in the ER be prevented, MHC molecules must be guided to endosomes. Granting them access to the endocytic pathway through the evolutionary inclusion of a cytosolic targeting signals or through the addition in the Golgi of mannose-6-phosphate (M6P) for recognition by M6P receptors does not appear to be the key as it would impair the subsequent display at the cell surface [13]. Rather, the system has evolved to rely on the invariant chain (Ii; CD74) chaperone to escort MHC class II molecules to endosomes [14].

In the ER, Ii folds into the peptide groove of MHC class II molecules. It encodes sorting signals to endosomes, where it is degraded to free the peptide binding groove and to prevent further retention of MHC molecules inside the cell [15,16]. Although the MHC class II molecules are then able to exit to the cell surface in association with the class II-associated invariant chain peptides (CLIP), Ii degradation allows their binding to HLA-DM and -DO. These two endosomal non-classical MHC class II molecules modulate the release of CLIP and binding of high affinity peptides to classical MHC class II molecules (reviewed in [17]).

Once at the plasma membrane and depending on the cell type and physiological conditions, a variable fraction of MHC class II molecules undergoes cycles of internalization and relocation at the surface. As these molecules are extremely stable in late endosomes, the homeostatic balance of their cellular content is guaranteed by the stochastic but precipitated degradation of a fraction of the molecules through an active process involving ubiquitination of the cytoplasmic tail [18]. Such post-translational modification tags MHC class II molecules for degradation by diverting them from the recycling loop and sending them “deeper” into the endocytic pathway.

Here, we will address the role of the intracellular domains of human HLA class II molecules in intracellular trafficking. The structure–function analysis of these cytoplasmic tails, especially in light of their interactions with Ii and E3 ubiquitin ligases, will help understanding the potential functional impact of single amino acid polymorphisms (SAPs) resulting from single nucleotide polymorphisms (SNPs) [19].

## 2. MHC class II domains: structure, function and polymorphism

### 2.1. Organization of the extracellular domains

The crystal structure of the extracellular portion of the classical MHC class II molecule HLA-DR has been resolved about 25 years ago by Brown, Strominger, Wiley and collaborators [20]. MHC class II molecules are composed of two non-covalently- associated type I transmembrane glycoproteic chains (α and β) [21]. The mature α and β chains are composed of roughly 230 amino acids and migrate as proteins of about 29–34 KDa when analyzed by electrophoresis on denaturing polyacrylamide gels [22,23]. Basically, a peptide-binding nest is formed by the membrane distal α1 and β1 domains. Its floor comes from the juxtaposition of the α and β chain-encoded N-terminal regions into a single sheet. These antiparallel β-strands support on each side an α-helix that delimits the walls of the nest [20]. The α-helix and β-sheet portions of each domain are encoded on a single exon and its evolutionary origin is still debated. Similar domain folds have been described in only few proteins, such as IL-8, but their precise organization suggests that they are not related to MHC molecules [24]. As this peptide binding groove fold is rarely seen in MHC-unrelated molecules, the origin of the ancestor structure(s), if any, remains obscure and could have been the result of coding as well as non-coding sequence recombinations [25]. The peptide-binding structure is supported by two membrane-proximal domains (α2 and β2), each consisting of two β-pleated sheets hold together by a di-sulfide bond and arranged in the form of a barrel. This C1-set Ig-like domain is found in some members of the immunoglobulin superfamily, such as the TcR [26]. It is still unclear if all Ig-like domains originate from a single common prototype or if some have an independent origin that arose from convergent evolution [25]. Noteworthy, the quaternary structures of the non-classical MHC class II molecules HLA-DM and -DO are very similar to that of their classical counterpart [27,28]. Still, DM and DO are confined to the endocytic pathway, they do not bind peptides and have acquired completely different yet specialized functions in the antigen presentation process [17]. Even more surprising is the fact that the MHC fold is the structural basis for molecules such as CD1, which presents lipids, or such as the neonate Fc receptor (FcRn), which transports immunoglobulins [29].

Human classical MHC class II molecules include three different isotypes termed HLA-DR, -DP and -DQ. The primary structures of these isotypes show overall homologies reaching 68% when comparing the β chains of DR and DQ [30]. Still, the quaternary structures determined by X-ray crystallography are very similar [31] and, in many documented cases, allow for the generation of mixed isotypic pairs [32]. Apart from DRα, for which there are only a few alleles, the MHC class II α and β chains are highly polymorphic. For example, thousands of DRβ coding sequences have been identified so far [33]. Many polymorphic residues are concentrated around the peptide-binding groove and ensure that a large number of microbial peptides can be recognized by the human population. Polymorphism also concerns residues with side-chains pointing up from the groove, diversifying the families of TcRs being selected [34]. While important isotypic primary sequence differences are found in all extracellular domains, allelic polymorphisms are more concentrated in the peptide binding region. Some key amino acids appear to be conserved in all isotypes, even between class I and class II and even between species [35]. A mutation in these scaffold amino acids will likely affect the structure and function dramatically. Otherwise, there is a lot of variability and new SNPs might not affect folding.

In line with their role in antigen presentation, MHC class II molecules must interact with different partners. For example, MHC class II molecules form a complex with the TcR and the CD4 co-receptor at the surface of T cells in order to regulate signal strength. CD4 being monomorphic, the interaction site on the β2 domain of MHC molecules must be conserved. However, we have shown that allotypic and isotypic differences can fine tune the interaction with CD4 and the ensuing T cell response [36]. For example, we found that DRB1*0401 interacts better with CD4 than DRB5*0101. Similarly, variability outside the peptide-binding groove was shown to affect the binding of bacterial superantigens [37]. Our results demonstrated that DRw53 binds staphylococcal enterotoxin A (SEA) and SEE poorly as compared to DR1 due to the presence of a tyrosine residue at position β81 [38]. Importantly, such SAPs can indirectly modulate the associated peptide repertoire (immunopeptidome) if modulating the interaction with HLA-DM or -DO [39,40]. Of note, new molecules capable of interacting with MHC class II proteins are identified at a regular pace. These include CD40 [41], tetraspanins [42] and the BcR [43]. The structural features involved in these contacts are being characterized but the polymorphic nature of MHC class II molecules is likely to be associated with differential functional responses in the population following, for example, triggering of TLR4 [41].

### 2.2. Transmembrane and cytoplasmic regions

The length of transmembrane domains can impact the sorting and lipid domain partitioning of integral proteins [44]. Early work by Kaufman and Strominger suggested that the large extracellular NH2-terminal domains of HLA-DR were followed by small intramembranous

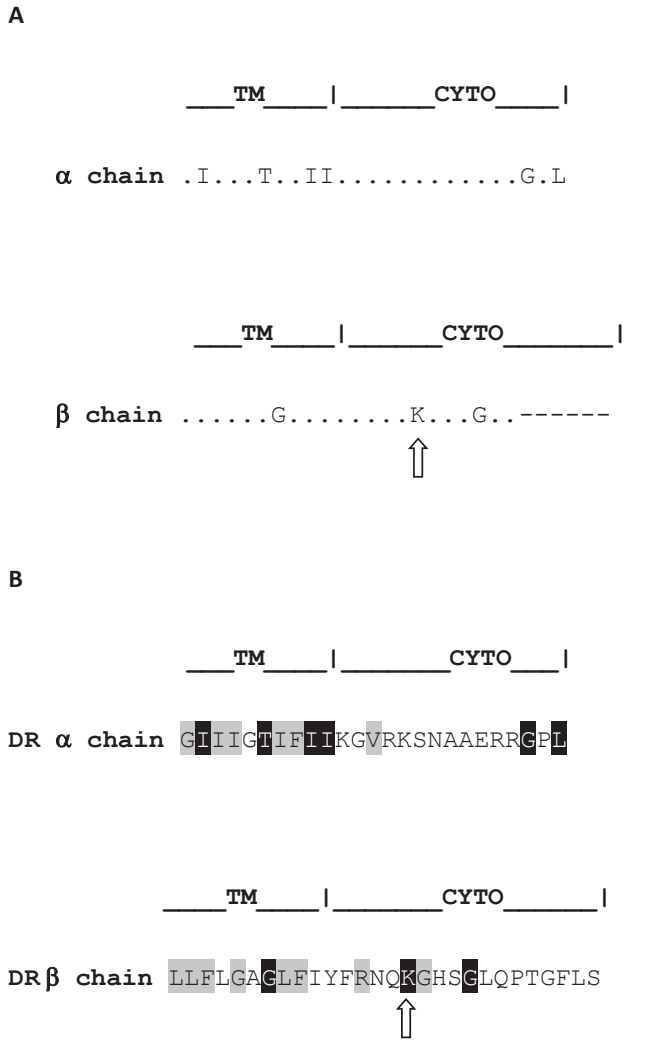


**Fig. 1.** Conserved amino acids between the cytoplasmic tails of mouse (I-A and I-E) and human (HLA-DR, -DP and -DQ) MHC class II molecules. (A) Partial amino acid sequence of MHC class II α and β chains. Only residues that are fully conserved in all mouse and human isotypes and allotypes are shown. Lines indicate residues that are simply absent in DP, and thus not conserved. Arrow indicates the conserved β-chain cytoplasmic lysine. This K residue (at position 235 in the mature DRB1*0101) was not found in HLA-DQB2 isoform 2 but while DQB2 was shown recently to be expressed in Langerhans cells [297], there is no indication that this isoform 2 is functional. Sequence data was from [46]. (B) Sequence of the DRA*01- and DRB1*0101- encoded C-terminal regions. Residues conserved in all mouse and human allotypes and isotypes are shown in black boxes. Residues which are similar in terms of charge or hydrophobicity are highlighted in grey.

and intracellular regions [45]. The core of these 20 or so membrane-embedded amino acid domains are highly conserved in classical MHC class II molecules [46]. Since the original description the alpha-helical nature of the transmembrane regions of α and β chains, numerous studies have provided in vitro and in vivo structural and functional evidence for a role of these domains in the folding of MHC class II molecules [47–50]. The importance of such sequence conservation for interacting with other molecules that could regulate intracellular trafficking, such as Ii, will be addressed below.

While in mice and humans the MHC class II α gene exon 4 encodes both the transmembrane and cytoplasmic regions, the amino acids of the β chain cytoplasmic domain come from two or even three different exons [51–55]. The polymorphic nature of the cytoplasmic tails of MHC class II molecules was recently addressed in an excellent review by Harton et al. on the immunological functions of the membrane proximal domains [46]. The cytoplasmic domain boundaries can be readily determined based on the conservation of the TM regions. The cytoplasmic tails of MHC class II α and β chains vary between 10 and 18 amino acids in length and there are also important allotypic and isotypic variations in their primary sequence (see for example [53]). Fig. 1 shows the conserved amino acids in the cytoplasmic tails of the mouse and human MHC class II molecules, based on the analysis of Harton et al. [46].

The transmembrane/cytoplasmic regions of mice and human MHC class II molecules have been ascribed many different functions. Their importance in the capacity of MHC class II molecules to transduce intracellular signals is addressed in detail by Harton [298] in this special issue. Below, we will discuss experimental evidence for a role of the transmembrane and cytoplasmic domains in fine-tuning MHC class II intracellular trafficking.

## 3. Trafficking of immature MHC class II molecules

Early experiments identified a monomorphic polypeptide, called Ii, which co-immunoprecipitated with MHC class II molecules [56]. For an excellent historical perspective on the discovery of Ii, see this short comment by Ron Germain [57]. A large body of literature demonstrates that Ii assists, at different degrees depending on the isotype and allotype studied, in the folding and transport of MHC II molecules [58–61]. For example, mouse I-$A^b$ is much more dependent on Ii for its folding than the I-A molecule of the k haplotype [58]. As MHC restriction of the $CD4^+$ T cell response implies that a given TcR recognizes simultaneously both the antigen and the MHC class II molecule, Ii must dissociate at some point in order to allow immature MHC class II molecules to bind peptides and acquire a compact conformation [62]. The term “immature” can refer to different conformations of MHC class II molecules. For one, immaturity can denote MHC class II molecules that have not yet replaced CLIP for a T cell epitope in the endocytic pathway. However, we prefer to call immature those MHC class II molecules that are still associated to any part of Ii, except for CLIP. Indeed, the crystal structure of the CLIP/DR3 complex is very similar to the one of DR/$HA_{307-318}$, for example [63]. Also, the trafficking of CLIP-bound MHC class II molecules is not likely to be peculiar (except if they enter lysosomes where their sensitivity to acidic pH will cause their degradation [64]). In other words, maturity marks the frontier where the MHC class II molecule has dissociated from the cytoplasmic tail of Ii and becomes independent of a direct interaction with Ii for its trafficking. However, this definition does not take into account the specific scenario where mature peptide-MHC class II complexes are indirectly influenced by free Ii at the plasma membrane [65]. Below, we will first describe the behaviour of mature MHC class II molecules in the early secretory pathway in the absence of Ii. Then, we will address the interplay between MHC molecules and Ii in the sorting of immature complexes. There have been reports of mutations in the extracellular domains of mouse and human MHC class II molecules that affect transport and cell surface expression (see for example [66]). The structural and functional consequences of such polymorphisms can be diverse and dependent or not on chaperones such as Ii and HLA-DM (reviewed by Busch et al., [67]). Here, we will concentrate on the role of the cytoplasmic regions in the intracellular sorting of immature MHC class II molecules.

### 3.1. Ii-independent transport

It is well established that cells expressing MHC class II molecules also express Ii, the latter being found in excess [68]. Accordingly, there is no firm evidence that HLA class II molecules could traffic on their own under physiological conditions. Also, Ii and MHC class II molecules show multiple contact sites, making unlikely the possibility that a SAP could prevent the interaction to take place [61,63,69]. Still, many groups have overexpressed MHC class II molecules in artificial APCs in

the absence of Ii, shedding light on the importance of the latter in MHC class II folding and trafficking. Also, the fate of MHC class II molecules in Ii-deficient mice as well as in cultured cells that have been knocked-out or knocked-down for Ii revealed interesting features of the interplay between the two molecules.

The assembly of MHC II α and β chains is essential for surface expression of either component of the heterodimer. Non-assembled chains, certain allotypes or some haplotype mismatched pairs, such as $A\alpha^{d}A\beta^{k}$, expressed in the absence of Ii are doomed to remain in the ER in association with ubiquitous chaperones, such as BiP [70,71]. Indeed, early after biosynthesis or in the absence of Ii, MHC class II molecules have a tendency to aggregate [71,72]. This phenomenon is most likely independent of the cytoplasmic tails since recombinant soluble MHC class II molecules secreted from insect cells also form aggregates and these can be dissociated by the addition of peptides [73]. Still, depending on the α/β combination or the cell type, a pool of MHC class II molecules will acquire ER peptides (or polypeptides) [58,74]. This has been shown in artificial APCs as well as in cells from Ii-deficient mice [10,74]. Once the peptide binding site is occupied, these complexes will be delivered to the cell surface through the default secretory pathway [75]. Thus, Ii is not a prerequisite for ER egress of classical and non-classical MHC class II molecules [76–78]. Interestingly, Ii knockdown in a brain metastatic melanoma cell line did not downregulate MHC class II expression at the surface [79]. Once at the plasma membrane, by and large, these classical MHC class II complexes will behave just like their mature counterpart and their fate will be described below in Section 4. Of note, Ii can also interact with peptide-MHC class II complexes, owing to the presence of groove-independent binding sites [65,80–82]. Such a safety net would allow efficient endosomal sorting and DM encounter of MHC class II molecules that have fortuitously bound an unstable ER polypeptide in their groove. The discrepancies between these studies and others, which concluded that binding of ER peptides would preclude Ii association [83,84], appears to be due to the use of different detergents [81]. Interestingly, the group of Hirano has shown that a SAP at position 84 of DPβ ($HLA\text{-}DP^{84Gly}$) prevented the binding of CLIP to the groove [85]. However, this allele can still interact with Ii and it constitutively presents endogenous peptides generated by the class I antigen processing machinery.

### 3.2. Ii-dependent transport

Ii is dispensable for the association of most αβ chain combinations but it can support the folding of haplotype mismatched pairs in transfected cells [86]. In more physiological settings where a plethora of classical and non-classical chains are co-expressed, Ii was shown to prevent aberrant associations [87]. A supramolecular complex forms in the endoplasmic reticulum (ER) and Ii-encoded signals play a key role in its trafficking. Numerous reviews have analyzed the structural basis behind the capacity of Ii to divert the complex from the default pathway and take MHC class II molecules to the endosomes (see for example [15,88,89]). Below, we will briefly decode the common and isoform-specific signals embedded into the cytoplasmic tail of Ii. Then, we will address the structural features of MHC class II molecules that have an influence on the functionality of these Ii-specific motifs.

#### 3.2.1. Structure-function of Ii

Ii is a type II transmembrane glycoprotein with its N-terminus in the cytosol [90,91]. It may be non-polymorphic and evolutionary conserved, but Ii is not so much invariant in terms of its protein structure. Four isoforms, p33, p35, p41 and p43, have been described in humans [92,93]. Iip33 is the prototypic and most abundant form, containing all the basic structural and functional elements needed to chaperone MHC class II molecules. Iip35 and p43 bear an N-terminal cytoplasmic extension of 16 amino acids that arises from the use of an alternative upstream start codon (Fig. 2). These last two isoforms appeared late in evolution and were only found in primates [94]. Iip41 and p43 both contain an additional lumenal domain encoded by the exon 6b, which is subjected to alternative splicing [92]. This exon encodes a thyroglobulin-like cysteine-rich domain that could potentially regulate the activity of cathepsins [93,95,96]. As it can regulate Ii proteolysis and possibly antigen presentation in general, this domain is likely to restrain the flow of MHC class II molecules [97,98]. In mice, the proportions of Iip41 appear to increase in dendritic cells (DCs) and Langerhans cells as compared to macrophages [99]. Interestingly, in trouts, the isoform bearing the thyroglobulin-like domain is not generated by alternative splicing but is encoded by a separate paralogous gene [100]. All isoforms of Ii include a trimerisation domain in their lumenal portion [101,102]. Through this domain, but also the transmembrane section, Ii isoforms stochastically associate into mixed trimers [68,69,84,103–105]. It was estimated that about half of the trimers were p33 homotrimers whereas the other half were heterotrimers that incorporated one or two p35 isoform [106]. It is useful to mention that some early studies referred to human Ii p33 and p35 as p31 and p33, respectively (see for example [107]).

Whereas the structures of dozens of MHC class II molecules have been determined by X-ray crystallography, the 3D structure of soluble Ii has not yet been resolved. This is most likely due to the disordered lumenal segments of the protein [108]. These include the CLIP region, first described by the group of Cresswell [109]. CLIP constitutes a nested set of peptides found temporarily in the groove of MHC II class II molecules following the degradation of Ii. Albeit with different affinity and even registers, CLIP participates in the interaction of Ii with most allotypes and isotypes [39,59,110].

All isoforms share a common cytoplasmic region of 29 amino acids (Fig. 2). Close to the TM domain, a cysteine residue was found to be palmitoylated [111,112]. For the sake of clarity and because p41 and 43 are usually less abundant and similar to the other isoforms in their cytoplasmic regions, we will only discuss below the trafficking of Iip33 and p35. The portion of the cytoplasmic domain common to all Ii isoforms has been shown by different groups to play a predominant role in the endosomal localization of MHC class II molecules [7,107,113–117]. More specifically, mutation of two cytosolic leucine-based motifs affected the intracellular distribution of the complex [114]. The functionality of these motifs depends on their capacity to multimerize [118]. Interestingly, nuclear magnetic resonance (NMR) spectroscopy studies have revealed that the cytoplasmic tails form trimers in solution, possibly also explaining the capacity of Ii to fuse endosomes and create large compartments [119]. Endoglycosidase sensitivity assays coupled to the subcellular localization study of deletions mutants in the cytoplasmic tail of human p33 suggested that the sorting motifs diverts Ii from the Golgi complex [107]. Accordingly, in the absence of MHC class II molecules, mouse Ii was found to reach endosomes from the ER through a distinct path involving autophagy [120].

The N-terminal 16 amino acid extension specific to Iip35 and p43 contains intricate functional motifs implicated in the regulation of ER egress (Fig. 2). The R-R-R-S-R sequence creates multiple functional di-arginine motifs (RxR or RR) that can individually act as ER retention signals [121]. Di-arginine motifs are found in many proteins forming quaternary structures (reviewed in [122]). For example, a functional GABA(B) receptor depends on the formation of a heterodimeric complex between GB1 and GB2. The presence of an R-based signal in GB1 provides a trafficking checkpoint by preventing the premature ER egress of any unassembled moiety [123].

Di-arginine motifs are extremely potent in their ability to prevent premature ER egress and their activity is regulated by adjacent amino acid sequences. Iip35 is phosphorylated by PKC, principally on serine 8, and this modification is critical for overcoming the di-arginine motif [106,124–126]. Interestingly, Kuwana et al. found that phosphorylation does not require co-expression of MHC class II molecules and pull-down experiments demonstrated that 14-3-3 interacts with Ii in a phosphorylation-dependent manner [126]. The 14-3-3 family of cytosolic proteins consists of adaptors capable of homo/hetero/

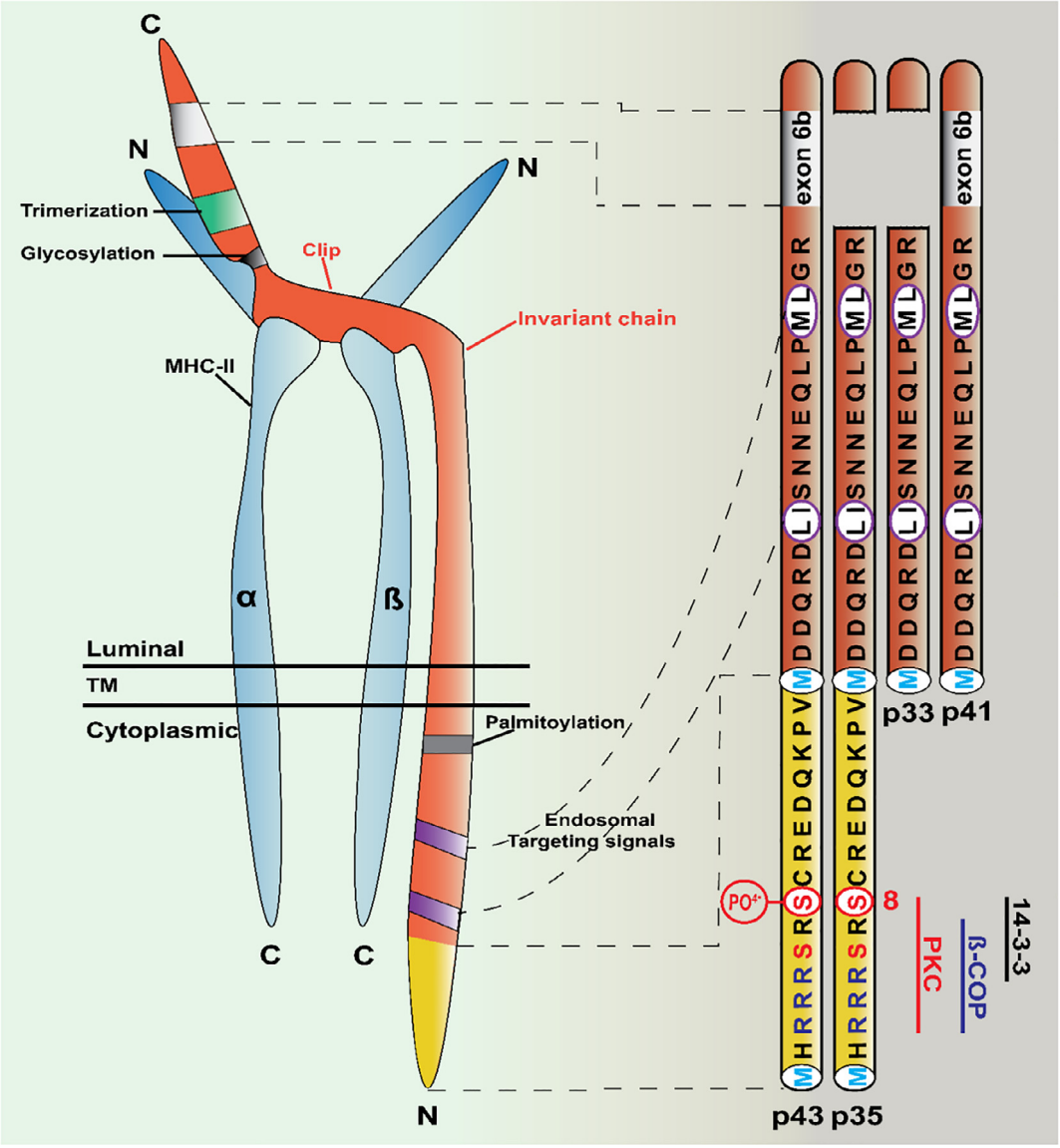


**Fig. 2.** Schematic representation of the MHC class II/Ii complex and the various Ii isoforms. The primary structure of the N-terminal region of the different human CD74 isoforms is shown in a single-letter code. The 16 amino acid extension of p35 and p43 is shown in yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dimerization and which regulate processes such as intracellular transport, signal transduction, apoptosis etc. [127]. Synthetic peptides corresponding to the p35-specific N-terminal extension were used by O'Kelly et al. to define functional guidelines governing the activity of the di-arginine motif. It was found in rat brain extracts that βCOP, but not 14-3-3β, binds a non-phosphorylated version of the peptide [128]. βCOP is part of the COPI coatomer, which is responsible for the retrograde transport of proteins from the ERGIC to the ER [129]. However, binding of βCOP was reduced on the phosphorylated version of the Iip35 peptide and this correlated with strong binding of 14-3-3β. Thus, a consensus "release" motif ($_5R_6SX_8S^P$) recognized by 14-3-3β overlaps the di-arginine "retention" motif and binds β-COP (Fig. 2). Accordingly, serial immunoprecipitations of Iip35 transfected in COS-7 cells showed the mutually exclusive binding of β-COP and 14–3-3β on Ii trimers [128]. This last result is somewhat surprising however as only about 50% of Iip35 is phosphorylated at any given time (at least in B cells [106]), suggesting that each Iip35 trimer would statistically contain both phosphorylated and non-phosphorylated subunits. Thus, one must conclude from these experiments that once an Iip35 subunit is phosphorylated and bound by 14-3-3β, β -COP can no longer access the other non-phosphorylated subunits of the trimer. This was not directly addressed in the O'Kelly paper and such a scenario is incompatible with our results [130,131] showing that all di-arginine motifs in a trimer must be directly "concealed" to allow forward trafficking (reviewed in [132]). An alternative explanation is that phosphorylation occurs simultaneously on all p35 subunits in a given trimer, preventing the long-term mixing of differentially phosphorylated moieties. Future studies on the trafficking of Iip35 should aim at clarifying these important issues. Still, a general spatiotemporal model now prevails whereby 14-3-3β binding to the PKC-phosphorylated serine 8 in the ER sterically prevents βCOP-mediated retrograde transport from the ERGIC of p35-containing Ii trimers [128].

Why does p35 encode an ER retention signal? There is still no definitive answer to this question, but one can speculate that the di-arginine motif guarantees the formation of Ii trimers. If that was the case, phosphorylated p35-including trimers would be released from the early secretory pathway. However, this does not happen in transfected cells [133]. Co-expression of MHC class II molecules is a prerequisite for preventing ER retention of Iip35. Thus, the most logical explanation is that Iip35 forces the recycling of Ii trimers between the ERGIC and the

ER to maximize the chances of binding MHC class II molecules [130,131]. If other species devoid of Iip35 have evolved alternative mechanisms to retain immature MHC class II molecules in the ER remains to be addressed. As mentioned above, the absolute need for MHC class II molecules in the trafficking of Iip35 has been overlooked in the past. While mechanistically we still do not know all the hurdles faced by p35 on its way out of the ER, the next section will address the structural basis for the positive role of MHC class II molecules in forward trafficking.

### *3.2.2. Interplay between II and MHC class II molecules in the early secretory pathway*

Using a variety of mutant cell lines devoid of HLA class II molecules, Cresswell and collaborators have shown by immunofluorescence microscopy that the bulk of Ii is sequestered in the ER [68]. Using different biochemical methods, they further showed that Ii forms trimers and that their glycans remained unprocessed and sensitive to endoglycosidase H. However, in MHC class $II^+$ cells, the presence of Ii trimers provokes the disaggregation of MHC class II molecules and the formation of an ordered nonameric complex composed of three Ii and three αβ heterodimers [62,72,104]. Alternatively, at least for some allotypes such as HLA-DR, evidence suggests the initial formation of a complex between isolated α or β chains and Ii. Only then would the complementary MHC class II chains adhere to the structure [134,135]. It was originally postulated that Iip33, or Iip33 bound to isolated α or β chains that failed to form a heterodimer, would be transported to autophagosomes for degradation [107].

While Ii has a major impact on the trafficking of MHC class II molecules, it appears that the reverse is also true. For example, as just mentioned, MHC class II molecules diverts Ii from an autophagy-based pathway. It is not clear what structural features, or lack thereof, of the αβ dimer are responsible for this [120]. Also, it was shown that truncation of the MHC II cytoplasmic tails can have a dramatically negative impact on the acquisition of endoglycosidase-H resistance and plasma membrane expression, even in the presence of Ii [136,137]. However, the role of the tails in this context is not clear as these observations may be allotype-specific and linked to proper heterodimer folding.

One area where the importance of human MHC class II molecules has been more extensively documented is alleviation of the Iip35-mediated ER retention, raising important questions as to the molecular mechanism involved. As mentionned above, this is extremely relevant to the field of antigen presentation but also to the cell biology of membrane protein trafficking in general. As Iip33 is the most abundant isoform, it is unlikely that homotrimers of Iip35 could be formed [106,132]. Thus, following the stochastic association of isoforms, we can assume that mixed Ii trimers will contain one or sometimes two p35 units. Recently, it was proposed that due to structural constraints, only one MHC class II molecule could bind to an Ii trimer [138], thereby challenging the long-accepted existence of nonamers [139]. A corollary to such pentamer-only model is that the MHC class II molecule does not need to be in direct contact (through a CLIP/groove interaction) with Iip35 in order to overcome retention. Otherwise, any complex incorporating two p35 would be prevented from gaining access to the plasma membrane due to the presence of a “MHC class II-free” di-arginine motif. We have investigated this issue and showed that a free Iip35 ER retention motif cannot be overcome in *trans* [140]. Masking must occur in *cis* and requires a direct interaction between Ii and the MHC class II molecule. This result suggests that Iip35, by analogy with other R-X-R-containing proteins, favors formation of high order complexes [130]. The reason why, at least in humans, the cell evolved a mechanism to favor the formation of larger structures is nebulous. The possibility remains that this is a collateral effect to the fact that p35 provides an advantage in antigen uptake in the late secretory pathway (see below).

The fact that overcoming the retention motif requires a direct interaction between MHC class II and Ii suggests that these cytoplasmic tails are intertwined. This raises the question of the structural requirements allowing such masking, especially in light of the great isotypic sequence variability in both the α and β domains (Fig. 1). We had already addressed this issue by assessing the relative importance of the α and β chains. By expressing truncated HLA-DR chains with Iip35 in MHC class II-negative HeLa cells, we have shown that the DRβ cytoplasmic tail is the functional entity. Truncation mutants and an alanine scan of this tail revealed that only three residues (Y-F-R) next to the TM are needed (Fig. 1). Moreover, the sequence Y-A-A is equally functional, confirming that no specific sequence is required and in line with the ability of all isotypes to mask the R-X-R motif [141,142]. In this context, it is highly unlikely that SAPs in the cytoplasmic tails of MHC class II molecules could alter p35-oriented trafficking in the early secretory pathway. Many questions remain regarding the role of MHC class II molecules in inactivating the di-arginine motif of Iip35. If they are not required for Ii phosphorylation and 14-3-3β binding, then why can’t a MHC II-free Ii trimer with one phosphorylated p35 subunit escape ER retention? This crucial issue requires clarification. Only then will it be possible to design experiments aimed at understanding the role of MHC class II molecules. The efficacy of such a short class II β sequence argues against a trivial steric hindrance mechanism where the cytoplasmic tail blocks access to β-COP. Still, as the Ii cytoplasmic domain could fold on the same plane as the plasma membrane, the MHC class II β chain could also interact with 14-3-3β.

Finally, another observation has raised questions as to a possible differential trafficking of p35-including complexes. Indeed, we have found that co-transfection of NleA, a virulence factor of enteropathogenic *E. coli*, prevented the surface display of MHC class II molecules expressed with p35, but not with p33 [131]. As NleA binds Sec24 and compromises the protein coat forming transport vesicles, these experiments suggest that Iip35, but not p33, exits the ER in COPII vesicles [143]. The implications of this finding regarding the immunopathogenicity of *E. coli* and antigen presentation in general are not entirely clear. More experiments will be needed to decisively conclude that trafficking of the two isoforms diverge at this early checkpoint.

### *3.2.3. Trafficking of Ii/MHC class II complexes in the late secretory pathway*

The exact nature of the compartments involved in the generation and acquisition of peptides has been the subject of intense debates in the early days. The study of different species, antigens and cell types are all factors that can certainly explain some of the discrepancies observed between laboratories. Nevertheless, the importance of these issues sparked the interest of many researchers and the cell biology principles underlying antigen processing/presentation were rapidly set (see [144] for an excellent overview of the functional cellular compartments of interest to immunologists). The concepts have been refined over the years, for example following the demonstration of MHC ubiquitination (see below). Also, the biological significance of mechanisms such as cross-presentation, autophagy and mitochondrial antigen presentation (MitAP) pathways highlights the importance of genuine cell biology research in our understanding of immune reactions [145–147]. Briefly, the endocytic pathway must serve at least four principal functions during the lifespan of an MHC class II molecule. First, Ii must be degraded to free the peptide-binding groove. Then, peptides must be generated and loaded with the help of HLA-DM in an acidic environment. Third, those class II that made their way to the plasma membrane can be endocytosed and recycled to allow peptide acquisition or exchange. Finally, MHC class II molecules must be terminated in lysosomal compartments or simply shed outside the cell as part of exosomes [148].

As mentioned above, the efficiency of immune surveillance is maximized by the existence of two separate antigen presentation pathways. MHC class II molecules present exogenous antigens and must ultimately intersect with material engulfed from the extracellular

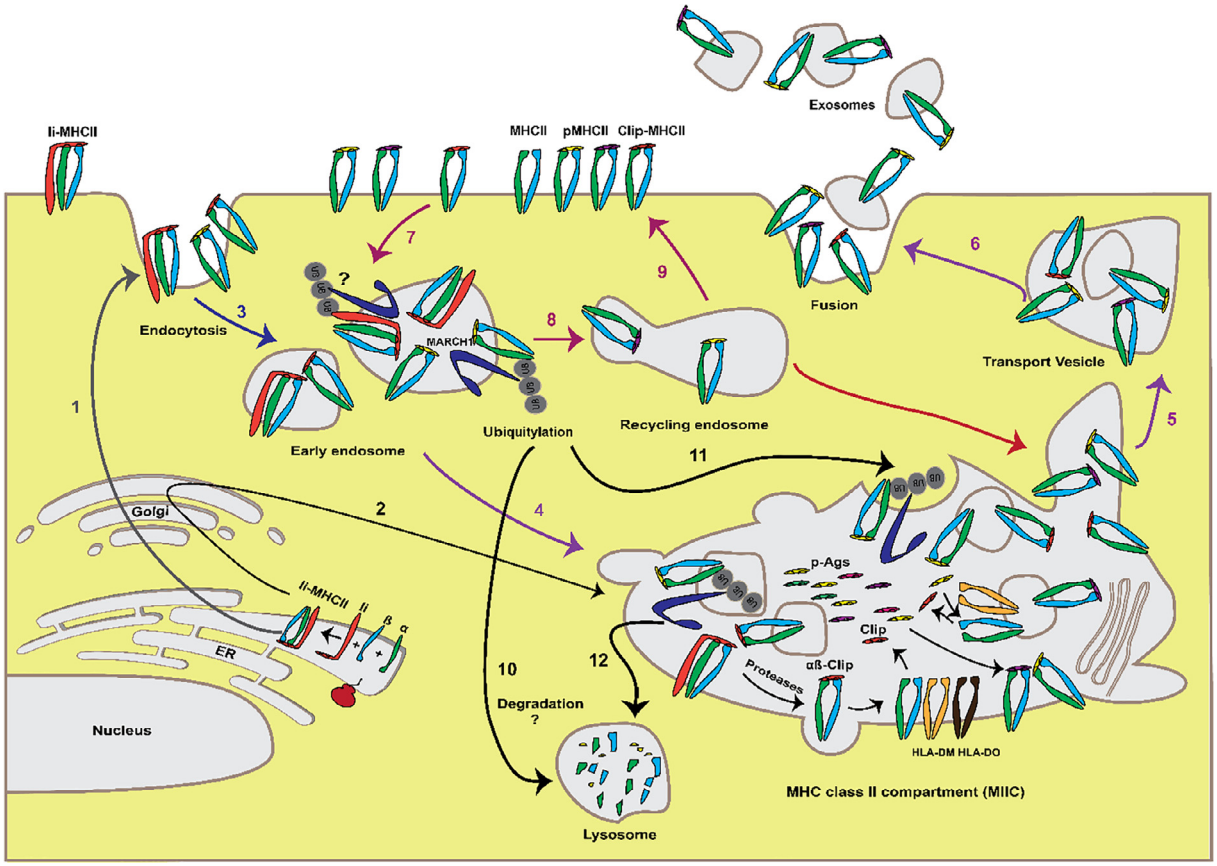


**Fig. 3.** MHC II intracellular trafficking and impact of ubiquitination on the subcellular localization of MHC class II molecules. After synthesis and association of the MHC class II α and β chains with Ii in the ER, the complex reaches the cell surface directly from the Golgi (1) or after transiting in endosomes (2). Once at the surface, αβ-Ii is endocytosed and gains access to sorting/early endosomes (3). The αβ-Ii complex reaches the MIICs where Ii is degraded to generate the αβ-Clip complex (4). Some αβ-CLIP complexes can move towards the surface in transport vesicles or tubules (5) but most will interact with HLA-DM to exchange CLIP for a more stable peptide. Then, the MHC class II-peptide complex is transported to the plasma membrane for the presentation of epitopes to T cells, or shed as part of exosomes (5 and 6). Once at the plasma membrane, MHC class II molecules are internalized (7) and recycled back to the cell surface (8,9). MARCH1 expression in early endosomes prevents recycling and diverts MHC class II molecules toward late compartments for their degradation (10,11,12).

milieu. For this process to be efficient, a single compartment regrouping classical MHC molecules, degraded antigens and the dedicated chaperone HLA-DM must exist. Early immunofluorescence microscopy work by Pletscher and Pernis has shown that crosslinking IgM at the surface of B cells resulted in their internalization into endocytic compartments that also contained MHC class II molecules [149]. Later, Brodsky and collaborators used electron microscopy to show that MHC class II molecules, Ii and internalized immunoglobulins all co-localize in protease-containing endosomes [150]. These MHCII-rich endocytic compartments of antigen presenting cells are called MHCII compartments (MIICs) (Fig. 3). They are close relatives of lysosomes but mildly acidic and devoid of mannose 6-phosphate receptors, which are typically found in late endosomes [151]. MIICs come in different flavors, some showing a multivesicular or multilamellar organization, while others display mixed characteristics [152]. Also reside in these compartments molecules such as cathepsins and HLA-DM, which are needed to breakdown Ii, generate peptides and load T cell epitopes onto MHC class II molecules [78,153–155]. Indeed, Rudensky, Blum and collaborators used the YAe monoclonal antibody, which recognizes the complex between I-$A^b$ and a peptide derived from the class II Eα chain, to show in murine B cells that MHC class II-peptide complexes are generated in MIICs [156]. For a comprehensive description of the trafficking of MHC class II molecules and peptide acquisition in the various types of compartments in the late secretory pathway, the reader is invited to consult the excellent reviews by the group of Paul Roche [157,158].

While some Ii-free MHC class II molecules move from the trans-Golgi to the plasma membrane through the default pathway, the route followed when the two molecules are in association has been the subject of controversies. Experimental evidence acquired over the past three decades supports the existence of three main corridors by which Ii reaches MIICs. Two all-intracellular routes lead to lysosomes from the TGN, either directly or through early endosomes, and these appear to be entirely dependent on the cytoplasmic tail of Ii [9,159,160]. Another path to the endocytic compartments implies a trek at the plasma membrane. It was estimated that $10^5$ Ii molecules are expressed at the surface of two human B cell lymphoma cell lines [161]. Internalization is mediated by clathrin-coated vesicles following the binding of cargo molecules to the AP-2 adaptor [162,163]. Rapid endocytosis to MIICs is dependent on the Ii cytoplasmic tail and mediated by the leucine-based motifs [117,164,165]. Interestingly, MHC class II/Iip35 complexes are internalized more rapidly than p33, to a point where accumulation of the former at the plasma membrane could only be detected when blocking endocytosis with a dominant-negative form of dynamin [126].

Of importance here is the role, if any, played by the MHC class II once Ii has assumed control. By and large, MHC class II molecules appear to sit back and relax, enjoying the free ride afforded by the Ii chaperone. The αβ cytoplasmic tails do not appear to influence trafficking at this stage and a truncated MHC class II ends up in endosomes [64]. The dominant nature of Ii makes it unlikely that SAPs in the cytoplasmic domains of MHC class II molecules could affect trafficking of the immature complex after exiting the Golgi. Still, given the functional

interplay between the β chain cytoplasmic tail and Iip35 (see above), it could be interesting to compare the affinity of the AP1 and AP2 medium chains for p33 versus p35 complexes [166].

Once internalized, and always under the guidance of Ii, the complex will successively enter a series of compartments to ultimately reach late, acidic endosomes where Ii is degraded and CLIP is exchanged [144]. Ii greatly influences membrane behavior in the endocytic pathway of APCs (reviewed in [16,89]). Early work has shown that Ii induces the formation of large endosomes, a function dependent on its capacity to trimerize [101,114,167]. Formation of these large endocytic structures, which is inhibited by Iip35, coincides with a delay in the transport of fluid phase markers and of MHC class II molecules [65,168,169]. Other functions of Ii, such as the regulation of cell migration or the regulation of gene expression following the ultimate cleavage by SPPL2a, appear to be intrinsic and not directly influenced by the MHC class II molecules [170-172].

## 4. Trafficking of mature MHC class II molecules

Ii retains MHC class II molecules in endosomes and undergoes proteolytic degradation by the sequential action of proteases [116]. The sequence of cleavage events needed to degrade Ii and release MHC class II molecules has been studied in many species and cell types. It can be summarized by the initial release of the C-terminal portion by non-cysteine proteases, such as asparagine endopeptidase (AEP) [173]. Subsequent cutting events by SPPL2a and cysteine proteases, including cathepsin S, lead to the release of the biologically active intracellular Ii fragment [171,174,175]. Even though inhibiting cysteine proteases has a differential effect on the maturation of MHC class II depending on the haplotype, it is most likely related to the variable affinity of the peptide-binding groove for Ii and, thus, independent of the MHC II cytosolic domains [176].

The stepwise removal of Ii discharges classical MHC class II molecules, which are then free to interact with HLA-DM (reviewed in [177]). This chaperone is responsible for removing CLIP and maintaining MHC class II molecules in an open conformation favorable to the acquisition of a putative T cell epitope [178–180]. Once the peptide is stably anchored in the groove, the structure adopts a “compact” conformation [181,182]. This complex, resistant to SDS denaturation, was originally described by Dornmair et al. after analyzing non-boiled cell lysate samples on Western blots [183]. The binding of a stable peptide and formation of a compact complex is not a pre-requisite for leaving the endocytic pathway. In HLA-DM- or H2-DM-deficient cells, CLIP-loaded MHC class II molecules were detected at the surface [78,109,184–188]. Also, in various HLA-DM$^+$ cell lines, primary cells and tumors, substantial amounts of CLIP-associated MHC class II molecules have been detected at the plasma membrane. These complexes are usually called “floppy” and migrate slightly more slowly than compact species when analyzed by electrophoresis on SDS-polyacrylamide gels under non-boiled conditions [183]. The term floppy also applies to other non-compact MHC class II complexes, independent of the origin of the peptide [189]. These most likely represent a slightly open conformation, in line with the demonstration of energy transfer between two peptides bound to one floppy MHC class II molecule [190]. Of note, polymorphisms affect the SDS-resistance of CLIP-MHC class II complexes [179,191,192]. Cell-surface CLIP appears to play important physiological roles, such as Th2 cell polarization, and could represent a useful diagnostic or prognostic factor in cancer [193–198]. Finally, a substantial fraction of the pool of cell surface MHC class II molecules was shown to include complexes that are empty and readily fillable with exogenously supplied peptides [199]. All these floppy, empty and compact forms are not under the control of Ii anymore. Their whereabouts and fate are addressed below.

### 4.1. Cell surface expression and endocytosis

There are probably many explanations for the quantitative fluctuations observed in the expression of various MHC class II conformational species at the plasma membrane, including trivial ones such as the sensitivity of the detection methods and cell type differences. Also, their relative abundance is certainly affected by allotypic and isotypic variations in the strength of the interaction and ratio between MHC class II molecules and HLA-DM, the function of which is modulated by its inhibitor HLA-DO [200]. In any case, the interactions between MHC class II molecules and HLA-DM are transient and polymorphisms that fine-tune the binding affinity are not likely to be located in the cytosolic domains and to modulate trafficking per se [28]. Still, the cytosolic domains could alter the sorting of MHC class II molecules in their search for HLA-DM. Indeed, the work of Neefjes and collaborators has put to light interesting features of the sophisticated mechanism by which HLA-DM contacts classical MHC class II molecules to catalyze peptide exchange [201]. It appears that multivesicular MIICs, also called multivesicular bodies (MVBs), are the preferred sites where peptide exchange occurs. However, FRET experiments between HLA-DR and -DM demonstrated that for this exchange to happen, the two molecules must gain access to internal vesicles. Indeed, experimental conditions that eliminate these intravesicular membranes, such as chloroquine treatment, prohibits contacts between the two types of MHC class II molecules. HLA-DM resides in these late compartments, a characteristic that is linked to the presence of a tyrosine-based sorting motif (Y-x-x-ϕ) in the DMβ cytoplasmic domain [202]. The mechanism by which classical class II molecules enters MVBs is not known but may be linked, as described below, to its ability to interact with tetraspanins. Ubiquitination operates in structures close to the plasma membrane to target mature MHC class II molecules, indicating that it would not play a role in the intravesicular sorting and HLA-DM encounter (see below). A SAP resulting in the inclusion of a tyrosine near the C-terminus of MHC class II molecules could create a functional Y-x-x-ϕ motif, thereby undoubtedly impairing surface expression. Interestingly, apart from the one at the boundary of the DRβ TM region, there is a remarkable absence of tyrosine residues in the cytosolic domains of classical MHC class II molecules (Fig. 1).

On the one hand, a large fraction of unstable complexes will most likely never make it to the cell surface. From MIICs, they may be randomly sent deeper into the endocytic pathway where they do not withstand the harsh conditions [64]. On the other hand, MHC class II molecules with a peptide on board must reach the cell surface. Specialized cell-type specific membranous structures responsible for the endosome to plasma membrane transfer have been described and this transport can occur as a default solution at steady-state. Such pathway is usually referred to as “retrograde transport” in light of the fact that lysosomal compartments are generally the end of the journey for most proteins and that specialized structures must thus be utilized to regulate this phenomenon [203]. However, it is clearly a step forward for MHC class II molecules in their quest for cognate TcRs. Resting APCs in the periphery, such as B cells and immature DCs, must display MHC class II molecules at steady-state. This is critical for maintaining the pool of peripheral CD4 T cells [204]. However, immature DCs must also capture low doses of self-antigens to delete specific T cells and induce antigen-specific unresponsiveness or tolerance in central lymphoid organs and in the periphery [205]. Thus, at all time, a pool of MHC class II molecules can reach the cell surface following the fusion of the external membrane of MVBs and the plasma membrane [206,207]. Based on the brefeldin A sensitivity of the pathway delivering peptide/MHC class II complexes at the surface, Pond and Watts highlighted an important role for vesicular intermediates in this process [208]. These mechanisms probably also exist in epithelial cells expressing ectopically MHC class II molecules and Ii.

MHC class II molecules do not travel solo as they were shown to be incorporated into endosomal tetraspanin webs, which include

molecules such as CD82 [42,209]. Since the first demonstration of an association between HLA-DR and TAPA-1 [210], many studies have addressed the structural and functional properties of tetraspanins in antigen presentation (reviewed in [211]). Nearly all members of this superfamily of proteins encode some sort of leucine- or tyrosine-based motif that could influence the sorting of associated MHC class II molecules [212]. Most likely, MHC class II molecules get incorporated into these multiprotein membrane domains by interacting with other proteins through their TM regions, possibly implicating palmitoylation of the highly conserved α chain cysteine residue [213]. Also, MHC class II molecules incorporate lipid rafts as early as in the Golgi and travels with Ii inside these microdomains [214]. The cytoplasmic tails of MHC class II molecules are not required to enter lipid rafts [215,216]. However, preventing palmitoylation by changing the TM cysteine residue strongly reduced their incorporation [217,218]. A mutation affecting this key residue would have important consequences on trafficking, peptide acquisition and the capacity of a given MHC class II molecule to stimulate T cells [219]. Interestingly, HLA-DPβ chains also have a cysteine residue in their TM region and it was postulated that such isotypic variation could result in stronger raft association and increased avidity, compensating for an overall lower protein expression [217].

Many groups have addressed the developmental regulation of antigen processing, principally in DCs [203]. For example, in maturing DCs, the activation of the vacuolar proton pump enhances lysosomal acidification to allow more efficient antigen degradation and peptide loading [220,221]. Such studies are inseparable from the regulated trafficking of MHC class II molecules. In DCs, transport of peptide-MHC class II complexes is tightly regulated (reviewed in [222,223]). Activation/maturation-dependent transport from the endocytic pathway to the plasma membrane is based on an intricate network of vesicular and tubular membrane structures [224]. Indeed, in addition to the transcriptional regulation of MHC genes occurring in response to activation signals (such as those delivered by TLRs), cellular mechanisms involving membrane dynamics are triggered and assist in the need for a greater output of MHC class II molecules. Early studies in mouse and human DCs described a maturation-dependent movement toward the plasma membrane of the endolysosomal pool of MHC class II molecules [225,226]. Elucidation of the mechanistic basis for this trafficking was confounded by the fact that ubiquitination of MHC class II molecules and the tight maturation-dependent regulation of the responsible E3 ubiquitin ligase had not been described at the time (see next section).

Motor proteins ensure trafficking of organelles and rely on microtubules and actin fibers. Ii has been shown to bind myosin II and this actin-based motor could couple MIICs to the plasma membrane [170,227]. Interestingly, myosin II interaction was lost in the absence of Ii, suggesting that the cytoplasmic tails of MHC class II molecules are not involved [227]. During DC maturation, MHC class II molecules are transported by default in tubules that extend up to the plasma membrane [228]. In line with these results, it was shown that delivery of MHC class II molecules from endosomes/lysosomes to the plasma membrane is independent of their α and β cytosolic domains [64,229]. Again, these observations make it unlikely that SAPs could preclude cell surface expression of MHC class II molecules at this level. A separate pathway, based on myosin 1E, has recently been characterized in maturing DCs but so far it does not appear to depend on interactions with Ii or MHC class II [230].

Mature MHC class II molecules that reached the plasma membrane are subjected to mechanical forces and thus not static [231]. Perturbations of MHC class II surface organization in the various microdomains described above can affect lateral or rotational diffusion in the lipid bilayer and impact T cell activation (reviewed in [232]). Interactions with the actin cytoskeleton can affect oligomerization, capping and signals transduction upon cell activation, as well as modulate molecular motions at steady-state. Using different constructs of mouse I-A/I-E isotypes, various cell lines and techniques, such as fluorescence photobleaching recovery (FPR) and time-resolved phosphorescence anisotropy, many laboratories have addressed the role of the cytoplasmic domains in the molecular motion of MHC class II molecules. While an early study has shown that truncation of I-$A^k$ cytoplasmic tails increased translational diffusion [233], other reports suggested that these tails have little effect on the intramembrane mobility of MHC class II molecules [136,234,235]. Other studies have looked at the association with the detergent-insoluble cytoskeleton or directly at the interaction with actin filaments. Chia et al. have shown that both the transmembrane and cytoplasmic regions of MHC class II molecules are involved in the cytoskeleton association [236]. In line with these results, we found that deletion of either the DR α or β chain cytoplasmic tails could not prevent the association with the cytoskeleton [237]. In these circumstances, it was surprising to learn that mutation of a three-amino acids stretch (Gly-His-Ser) to alanines in DRβ could abrogate actin binding upon MHC class II crosslinking. This short motif and adjacent residues are not found in DRα. The fact that the DPβ cytoplasmic tail does not allow interaction with the actin cytoskeleton may explain some of the isotypic differences in the signaling capability of HLA class II [238].

The half-life of MHC class II molecules, based on functional and biochemical assessments, was found to vary between 15 and 50 h [239]. This suggests a particularly stable halt at the plasma membrane and/or the efficient recycling of endocytosed molecules. Around 1990, using various cell lines and experimental conditions, many groups proposed the existence of alternative routes, independent of newly synthesized MHC class II molecules, and which lead to the presentation of exogenous antigens. At the same time, it was found that mature MHC class II molecules can be internalized rapidly from the plasma membrane. This was not the case for MHC class I, suggesting that such a pathway intersected the compartments responsible for antigen degradation [240]. A seminal study by Long and collaborators showed that truncation of either the DR α or β chain prevented the presentation of peptides from two exogenously supplied native antigens (MBP and haemagglutinin H3) [241]. This correlated with the absence of internalization from the surface of these transfected fibroblasts. Interestingly, DQ was reported to be stable at the surface of B cells, suggesting that there might be isotype-specific functions associated with rapid internalization [242]. The fate of surface MHC class II molecules depends on the cell type, the isotype/allotype and the physiological conditions tested. Roche and collaborators recently showed in a variety of human cell types that MHC class II molecules are endocytosed via a clathrin-, AP-2-, and dynamin-independent pathway. Then, they enter tubular endosomes positive for Arf6, Rab35 and EHD1, which are involved in recycling [243]. The techniques used by the different laboratories to assess internalization/recycling are numerous and have yield variable results (see the discussion in [244]). Also, as discussed below, ubiquitination is an important variable that was not accounted for in early experiments. Still, the group of Germain identified a cytoplasmic leucine-based motif in HLA-DR (Phe-Leu) and -DQ (Leu-Leu) as well as in their mouse counterparts [245]. Once mutated in I-$A^k$β, internalization and presentation of two HEL epitopes were reduced. Interestingly, both the DRB4 and DRB5 genes of the DR51 and DR53 haplotypes, respectively, bear variants of the leucine-based motif (Leu-Val or Leu-Leu) [246]. More work will be needed to understand the possible functional impact of isotypic variations in this region. For example, such β chain-encoded leucine-based motif, which is absent in DP, is needed to concentrate MHC class II molecules at the basolateral side of polarized epithelial cells, allowing activation of intraepithelial T cells [247]. Altogether, these results demonstrate that SAPs in the cytosolic parts of the MHC class II molecules could alter or even create motifs capable of fine tuning antigen presentation through altered trafficking from the plasma membrane.

### 4.2. Ubiquitin-dependent intracellular retention and degradation

Even though mature Ii-free MHC class II molecules are destined for the plasma membrane, the display of peptides must be regulated at the post-translational level in certain physiological conditions. Immature DCs expose only a limited number of complexes at steady-state. Coupled to the low expression of co-stimulatory molecules, this is propitious to the development of immune tolerance [205]. On the other hand, mature DCs that have received danger signals must increase quantitatively and qualitatively the collection of T cell epitopes. This implies the prolonged cell surface display of peptide/MHC class II complexes at the plasma membrane.

Many different potential mechanisms have been investigated in an effort to explain the intracellular accumulation of MHC class II molecules in immature mouse and human DCs and their apparent relocalisation at the plasma membrane upon DC maturation [248]. It was concluded that this phenomenon was mainly controlled by the rates of MHC class II synthesis and endocytosis [249]. Then, the discovery by the group of Mellman in 2006 that MHC class II molecules were ubiquitinable provided a strong molecular basis for their maturation-dependent differential cellular distribution [18]. In immature DCs, some MHC class II molecules are ubiquitinated on their cytoplasmic lysine residue and forced to remain inside the cell. Upon maturation, this specific ubiquitination stops and the number of surface peptide-MHC class II complexes increases dramatically [18,250]. MARCH1, which is expressed in immature DCs and down-regulated by activation signals such as LPS, is responsible for this phenotype [251,252]. In mouse $CD11b^+$ conventional DCs, MARCH1 downregulation appears to be under the control of the transcription factor IRF4 [253].

MARCH1 is a member of the E3 ubiquitin ligase family called “membrane-associated RING-CH” [254–256]. MARCH1-deficient mice do not show gross phenotypic defects but have an important reduction in the number of thymus-derived regulatory T cells [257]. Recently, the group of Shin provided mechanistic foundation for this observation by demonstrating the sphingolipid-related disruption of lipid rafts and of the tetraspanin web on DCs. This is due to overcrowding of the cell surface microdomains by non-ubiquitinable MHC class II molecules, ultimately impairing T cell engagement [258].

Not only is MARCH1 involved in the regulated MHC class II expression in DCs, it is also responsible for the IL-10-induced sequestration of MHC class II molecules inside monocytes and macrophages [259–261]. MARCH1 also plays an important role in B cells. Its expression is high in follicular cells and naïve cells down-regulate MARCH1 upon activation [262,263]. A recent elegant study demonstrated that MARCH1 levels are high in centroblasts of the germinal center dark zone. This allows a rapid turnover of MHC class II molecules and resets the peptidome before interaction with follicular helper T cells in the light zone [264]. Altogether, these results define MARCH1 as a major regulator of mature MHC class II recycling, sequestration and degradation. Of note, some aspects of innate immunity are perturbed in MARCH1-deficient mice. We have shown that the response to LPS was exacerbated but this was independent of MHC class II ubiquitination [265].

MARCH1 expression was originally thought to be restricted to hematopoietic cells but specific siRNAs were shown to up-regulate the insulin receptor levels in HeLa cells in a MARCH1- and ubiquitin-dependent fashion [266]. MARCH1, which is barely detectable at the protein level, is very potent and its expression seems tightly regulated. A close homologue, MARCH8, appears to be constitutively expressed and also able to modulate levels of MHC class II molecules [267]. As such, it could play a role in the homeostatic control of MHC class II expression, as seen for the interleukin-1 receptor accessory protein (IL1RAP) or the tumor necrosis factor-related apoptosis inducing ligand (TRAIL) [268,269]. Interestingly, bacteria and viruses, such as Salmonella, Francisella and KHSV, have developed strategies to down-regulate MHC class II expression through the modulation of MARCH1/8 [270–272].

MARCH1 and MARCH8 have been detected at the plasma membrane but they are mostly found in the early endosomes [273]. Accordingly, the impact on MHC class II endocytosis is minimal (reviewed in [274]). Instead, these MARCH proteins appear to intercept their targets as they cycle between the cell surface and early endosomes, diverting their route toward late endosomes/lysosomes [275,276]. Ubiquitination promotes degradation of peptide-MHC II complexes and this is dependent on lysosomal proteolytic activity [18]. The di-leucine targeting motif of Ii prevents degradation of immature complexes by promoting their clathrin-dependent endocytosis into MARCH1-negative endosomes [273,275]. The type/length of ubiquitin chains will likely depend on the cell type and physiological context (cytokines, danger signals etc). We have shown that MARCH1 overexpressed in HEK293 kidney epithelial cells causes the intracellular retention of co-transfected HLA-DR but their degradation does not appear to be dramatically increased. However, the same type of experiments performed in CIITA-transfected HeLa cells showed that MARCH1 overexpression resulted in an important reduction in the total amount of MHC class II molecules, as assessed by flow cytometry of permeabilized cells or on Western blots of total cell lysates [277]. Accordingly, in more physiological cells types, the group of Mellman showed that the intracellular distribution of the modified MHC class II molecules is a function of the ubiquitin chain length [278].

The precise road and the chaperones involved in the ubiquitination-dependent trafficking have yet to be fully characterized. Ubiquitination is not a prerequisite to reach internal vesicles of MVBs or to be shed in exosomes [277,279]. It is not known if ubiquitination speeds up the degradation of MHC class II molecules by preventing recycling to the plasma membrane or if the ubiquitin modification actively tags for degradation by bringing the complex in more acidic and harsh lysosomal compartments. As mentioned above, the fact that some ubiquitin chains are sufficient to prevent cell surface display without provoking degradation goes against a model where there is continuous, stochastic delivery of a pool of internalized MHC II molecules into some degradation-competent compartments. All these considerations point to a critical role of ubiquitination in homeostatic and inducible expression of MHC class II molecules. Thus, it is not surprising that the membrane-proximal cytoplasmic lysine residue is highly conserved during evolution. In fact, a sequence alignment of the cytoplasmic domains of the β chain reveals that this lysine is the only conserved residue between isotypes and allotypes of different species (Fig. 1). This lysine residue is even found in non-classical HLA-DM and -DO β chains, in line with studies showing regulation of their expression by MARCH1 [280,281]. Given the great variability in the nature of the amino acids surrounding the lysine, it is likely that no contextual positioning of the lysine is required. Finally, the fact that mutation of this conserved lysine abrogates MHC class II downregulation argues against ubiquitination of other types of amino acids. The ubiquitination of non-lysine residues was originally observed for MIR1, an E3 ubiquitin ligase encoded by Kaposi's sarcoma-associated herpesvirus, which down-regulates MHC class I molecules lacking cytoplasmic lysine residues [282]. The cytoplasmic domain of MHC class II α chains also show great diversity. While ubiquitination of the α chain has been shown to occur, the β chain is clearly the most important [283]. Thus, although a thorough scanning mutagenesis has not been reported, SNPs modifying amino acids around the lysine are not likely to have a dramatic impact on ubiquitination of HLA class II. Also, the poor conservation around the lysine suggests that the main interaction site with E3 ubiquitin ligases is located in other parts of the protein. Indeed, different groups have implicated the conserved MHC class II transmembrane domains, suggesting that SAPs in this region could have an indirect effect on MHC class II trafficking by affecting the strength of interaction with MARCH family members[284–286]. Altogether, these results suggest that impaired ubiquitination of a mutant class II molecule could affect its trafficking and have an impact on the immunopeptidome displayed on

APCs expressing MARCH1/8.

## 5. Concluding remarks

Whereas dozens of crystal structures of soluble MHC class II α1, α2, β1, β2 complexes have been resolved, the conformation of the cytoplasmic regions remains speculative. The structure of the Ii cytoplasmic domain has been the subject of investigations and it was shown by NMR spectroscopy that it forms an almost coplanar trimer, probably parallel to the membrane [119]. The Ii cytoplasmic tail helps shape the endocytic pathway and might also fine tune its own trafficking by regulating the expression of genes involved in antigen presentation [172]. Indeed, it is now well established that the cleaved intracellular N-terminal domain of Ii enters the nucleus where it acts as a transcriptional regulator [16]. MHC class II molecules are also able to transduce signals through the activation of kinases such as PKC, which might in turn affect their trafficking [287]. Knowing how the cytoplasmic domains of Ii and MHC class II molecules affect the folding of one another would help understand the interplay with the intracellular sorting machineries.

HLA genes are strongly associated with the development of autoimmune pathologies [288]. While the underlying mechanism points to the polymorphic nature of the antigen binding groove, it was also proposed that SAPs in other regions of the molecule could alter the peptidome by modulating the interaction with chaperones [39]. Similarly, mutations affecting one of the multiple trafficking steps of MHC class II molecules described above could have drastic consequences on the selection of T cell epitopes. Indeed, there are examples of pathologies where the intracellular distribution of MHC class II molecules is affected. For instance, the polarized distribution of MHC class II molecules in intestinal epithelial cells appears to be altered in patients with coeliac disease [289,290]. Just like the aberrant sub-cellular distribution of these molecules in Chediak-Higashi disease [291,292], the relocalisation is likely the indirect consequence of profound alterations in the endocytic pathway. Still, these observations highlight the potential negative impact of tempering with MHC class II trafficking.

Ubiquitination-driven trafficking represents the sorting event most dependent on a specific MHC class II cytoplasmic amino acid. Hardly no ubiquitin can be detected on MHC class II molecules in B cells from MARCH1-deficient animals [262]. The same applies to activated DCs that down-regulated MARCH1, as well as to cell expressing transfected HLA-DR ectopically [293]. Degradation of MHC class II molecules in these conditions is most likely independent of direct ubiquitination. The exact cellular pattern of expression of MARCH1/8 remains to be fully characterized but most cell types express very little of these proteins. Thus, non-professional APCs (various epithelial cells, fibroblasts, endothelial cells, keratinocytes, some tumors, hepatocytes etc) expressing low levels of constitutive or inflammation-induced MHC class II must also rely on a ubiquitin-independent mechanism for their elimination. A fraction of the MHC class II molecules must at some point passively and stochastically end up in lysosomal compartments, probably dragged by other proteins in microdomains. Also, plasma membrane shedding could play a role in their disappearance [294].

The structure of the MHC class II cytoplasmic domains is highly divergent and apart from the conserved lysine residue, no amino acid or structural characteristic should be critical for function. On an evolutionary standpoint, this lysine appears to be indispensable, allowing for a rapid and efficient control over MHC class II expression. Given the long half-life of these molecules in the harsh environment of late endosomes/lysosomes, a mere transcriptional regulation does not seem to provide the turnover speed required during affinity maturation of antibodies in germinal centers or to quickly respond to IL-10 [260,264].

The polygenic and polymorphic nature of the classical MHC class II molecules provides a better defense system, widening the breadth of foreign proteins that can be recognized. All the encoded proteins fulfill similar functions, but they have different peptide binding specificities and their activity is not redundant per se. Indeed, all isotypes can orchestrate specific responses against a variety of microbes. However, there are obvious structural constraints that limit the amino acid sequence variability, such as the need for MHC class II molecules to, at least minimally, associate with peptide-exchange chaperone HLA-DM and be recognized by the TcR. In the face of the impressive diversity of the MHC class II sequences, it appears that the “invariant” chain gene is under negative selection pressure and intended to assist the trafficking of *all* alleles. Ii brings MHC class II molecules into a wide range of endocytic compartments where antigen capture can occur. The exact nature of these vesicles (from early endosomes to lysosomes) is certainly influenced by the cell type studied. As MHC class II molecules must sample a variety of compartments, there is no need for a stringent localization signal to take over once Ii is degraded. As mentioned above, DR and DQ have leucine-based motifs that modulate internalization kinetics and the acquisition of certain antigens. A SAP inactivating or reinforcing these motifs would have a limited quantitative effect on surface expression but could modulate, positively or negatively, the presentation of specific antigens. In addition, there is the possibility of a SAP introducing a cytoplasmic tyrosine residue. This could create a Y-x-x-φ motif due to the presence of bulky hydrophobic residues in alpha and beta chains. Interestingly, a tyrosine in the tail of MHC class I molecules was shown to fine tune intracellular trafficking and play a role in cross-presentation [295]. In CD1 molecules, the presence of a functional cytoplasmic tyrosine motif is required to reach and scan MIICs for their lipidic ligands [296]. Depending on the position of the SAP, the tyrosine-based motif may be more or less stringent and minimally reduce surface expression. Although difficult to assess, it remains to be demonstrated if sequence variations in the MHC class II cytoplasmic tails can explain the link with the development of certain autoimmune disease or the resistance to specific microbes. Peptidomic analyses of variant MHC class II molecules bearing mutations in their cytoplasmic tails could help understanding the importance of these domains. Thus, SAPs could have an important impact on a specific immune response or establishment of tolerance toward self-peptides. More studies will be needed to assess the possible subtle differences in intracellular trafficking imparted directly or indirectly by the cytoplasmic domains.

## Acknowledgements

We thank James Drake for critical reading of the manuscript. JT is supported by Grants from the Natural Sciences and Engineering Research Council of Canada (NSERC; Grant # RGPIN/4821), the Canadian Cancer Society (CCS: Grant # 704818) and the Canadian Institutes for Health Research (CIHR; Grant # MOP 136802). JT holds the Saputo Research Chair.

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