

MHC Class II Antigen Presentation by Dendritic Cells Regulated through Endosomal Sorting

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For the initiation of adaptive immune responses, dendritic cells present antigenic peptides in association with major histocompatibility complex class II (MHCII) to naïve CD4⁺ T lymphocytes. In this review, we discuss how antigen presentation is regulated through intracellular processing and trafficking of MHCII. Newly synthesized MHCII is chaperoned by the invariant chain to endosomes, where peptides from endocytosed pathogens can bind. In nonactivated dendritic cells, peptide-loaded MHCII is ubiquitinated and consequently sorted by the ESCRT machinery to intraluminal vesicles of multivesicular bodies, ultimately leading to lysosomal degradation. Ubiquitination of newly synthesized MHCII is blocked when dendritic cells are activated, now allowing its transfer to the cell surface. This mode of regulation for MHCII is a prime example of how molecular processing and sorting at multivesicular bodies can determine the expression of signaling receptors at the plasma membrane.

Dendritic cells (DCs) are present throughout peripheral tissues, where they constitutively sample their environment for the presence of pathogens and diseased cells through uptake of soluble and particulate matter by endocytic mechanisms, including clathrin-mediated endocytosis, phagocytosis, macropinocytosis, and trogocytosis. Trogocytosis refers to uptake of membrane and associated molecules from one cell by another. After endocytic uptake, both environmental “self” proteins and proteins from pathogenic origin can be processed into peptides for loading onto major histocompatibility complex (MHC) molecules. Peptides can be generated either by lysosomal proteases in the

endocytic pathway, or by proteasomes when endocytosed proteins are transferred across the endosomal membrane into the cytosol. Thus, generated peptides may associate intracellularly with either MHC class I (MHCI) or MHC class II (MHCII) molecules, and in that context can be transferred to and displayed at the plasma membrane. MHC–peptide complexes can be recognized by T cells upon migration of DCs to lymphoid tissues (Guermónprez et al. 2002). In the absence of danger signals, DCs remain in a “resting” or “immature” state and display endogenous “self” peptides to maintain peripheral tolerance (Steinman et al. 2003; Schmidt et al. 2012). However, DCs also survey their

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environment with a collection of innate pattern-recognition receptors (PRRs), including Toll-like receptors (TLR), C-type lectins, and nucleotide oligomerization domain (NOD)-like receptors, which collectively recognize a wide array of conserved pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), with the latter representing (altered) “self” molecules that are released by dying cells or expressed by tumor cells. DCs that are activated through their PRRs or by inflammatory cytokines differentiate into phenotypes that can stimulate adaptive immune responses (Reis e Sousa 2006; Joffre et al. 2009). Characteristic features of DC differentiation or “maturation” include a transient increase in phagocytosis and macropinocytosis for efficient antigen uptake, increased surface expression of costimulatory molecules (e.g., CD86, CD80, CD40), and enhanced potential to migrate from peripheral tissues to the local lymphoid tissues for interaction with T cells (West et al. 2004; Reis e Sousa 2006). Several other stimuli, for example, TNF- α , can drive alternative DC maturation programs that result in tolerogenic rather than immunogenic DCs (Menges et al. 2002; Tan and O’Neill 2005; Cools et al. 2007; Maldonado and von Andrian 2010).

MHC molecules direct antigen specificity for adaptive immunity toward invading pathogens and malignant cells. MHCI on DCs predominantly helps elimination of infected and malignant cells through activation of antigen-specific CD8⁺ cytotoxic T cells. MHCI-driven cell killing by cytotoxic T cells, however, also requires licensing by DCs through MHCII-dependent activation of CD4⁺ helper T cells. In addition, MHCII on DCs serves to mount humeral immune responses and to instruct regulatory T cells and memory T cells. In contrast to MHCII, MHCI is expressed by nearly all cell types, and in nonprofessional antigen-presenting cells is exclusively loaded with peptides that are generated from cytosolic proteins by the ubiquitin/proteasome system. Cytosolic peptides can be translocated into the lumen of the endoplasmic reticulum (ER) for loading onto MHCI with the help of a dedicated peptide-loading complex (Cresswell et al. 2005). Pep-

tide-loaded MHCI is then transported out of the ER via the Golgi apparatus to the plasma membrane, where it is stably exposed. Infected cells that display pathogen-derived peptides on MHCI can be killed by cytotoxic T cells that specifically recognize relevant MHCI-peptide complexes. A unique feature of DCs is their ability to also present peptides from endocytosed material via MHCI, a process called “cross-presentation.” Cross-presentation by DCs is essential for the activation of naïve T cells to drive MHCI-restricted immune responses against tumor cells and cells other than DCs that are infected by pathogens. The mechanisms by which peptides from exogenously acquired proteins are generated and delivered to MHCI molecules in DCs have been discussed elsewhere (Amigorena and Savina 2010; Segura and Villadangos 2011; Joffre et al. 2012) and are beyond the scope of this review.

Although MHCI is expressed by all cells, expression of MHCII is restricted mainly to “professional” antigen-presenting cells (APCs), including DCs, macrophages, and B cells (Guermonprez et al. 2002; Trombetta and Mellman 2005). However, constitutive MHCII expression by non-APCs in the absence of costimulatory molecules, for example, by epithelial cells, has an important role in maintaining peripheral tolerance (Krupnick et al. 2005; Kreisel et al. 2010). Yet other cell types can be induced to express MHCII by certain stimuli, for example, by the cytokine IFN- γ (Pober et al. 1983; Geppert and Lipsky 1985; Romieu-Mourez et al. 2007; Mulder et al. 2011). On the transcriptional level, MHCII expression is controlled by a complex of DNA-binding proteins, also referred to as the MHCII enhanceosome, and activation of this complex requires association with the MHCII transactivator (CIITA) (Jabrane-Ferrat et al. 2003; Choi et al. 2011).

Peptides that bind to MHCII molecules are generated within the endocytic tract by limited proteolysis of endocytosed protein (complexes), or alternatively, of cytosolic material that entered the endocytic tract via autophagy (Dengjel et al. 2005; Crotzer and Blum 2010; Kondylis et al. 2013). In professional antigen-presenting cells, the organelles at which MHCII peptide



loading occurs mainly constitute late endosomes, although lysosomes have also been implicated (Geuze 1998). Together, these endocytic compartments are also referred to as MHCII compartments (MIICs) (Peters et al. 1991; Kleijmeer et al. 1997, 2001). DCs constitutively synthesize MHCII, which, in the absence of pathogen, are all loaded with “self” peptides in MIICs. In the absence of danger signals, however, cell-surface exposure of peptide loaded MHCII (pMHCII) is generally kept low through rapid sorting to and degradation in lysosomes. Limited exposure of self-peptide-loaded MHCII, in the absence of costimulatory signals, provides cues that support immune tolerance. Contrary to immature DCs, activated DCs efficiently convert to stable expressors of newly formed pMHCII complexes at their plasma membrane to increase the chance of interaction with TCRs on CD4⁺ T cells. The distinctive mechanisms and pathways for MHCI and MHCII antigen loading and trafficking have recently been compared in several excellent reviews (Neeffjes et al. 2011; Blum et al. 2013; Mantegazza et al. 2013). The present review focuses in depth on mechanisms and pathways that regulate and determine the expression of pMHCII complexes at the plasma membrane of DCs.

BIOSYNTHESIS OF MHCII

MHCII consists of a noncovalently associated complex of two type I transmembrane glycoproteins, designated α chain and β chain (Fig. 1). The α chain of MHCII has a molecular weight of 33–35 kDa and contains two sites for *N*-linked glycosylation. The β chain has a slightly lower molecular weight of 25–30 kDa and contains one amino-linked glycosylation site. Both chains have a short carboxy-terminal cytoplasmic and a single transmembrane domain. Together, their exoplasmic domains form a groove that can accommodate a large variety of peptides of 13–25 amino acids in length. Longer unfolded protein stretches have also been shown to bind, but at low frequencies, and these are prone to be trimmed after binding to MHCII (Watts 2012). The variance in peptide binding is to ensure immune responses toward many distinct

antigens. Peptides require, however, specific and correctly spaced anchor residues for stable binding to a particular haplotype of MHCII. Individuals express multiple types of the polymorphic MHCII complexes (in humans encoded by the HLA-DR, DP, and DQ loci, and in mice, I-A and I-E), each covering a spectrum of peptides. In addition, MHCII polymorphism within populations of organisms supplies distinct peptide-binding specificities that further increase the potential to fight invading pathogens.

MHCII-associated peptides are largely derived from proteins (complexes) that have been hydrolyzed in endosomal or lysosomal compartments (Amigorena et al. 1995; Castellino and Germain 1995; Lutz et al. 1997). Although MHCII α chains and β chains already assemble and form peptide-binding sites in the ER, they capture peptides only after arriving at the endocytic pathway. Premature peptide loading is prevented by the invariant chain (Ii, also called CD74) (Teyton et al. 1990; Busch et al. 1996). Ii binds the MHCII peptide-binding groove with a region in its luminal domain that is called CLIP (for class-II-associated invariant chain peptide) (Rudensky et al. 1991; Chicz et al. 1992; Riberdy et al. 1992; Sette et al. 1992; Freisewinkel et al. 1993). Normally, Ii is synthesized in excess over MHCII, ensuring Ii rather than peptide binding to MHCII (Kvist et al. 1982; Sekaly et al. 1986; Marks et al. 1990; Pieters et al. 1991). Peptides that are transferred into the ER by TAP, for potential binding to MHCI, indeed have poor potential to bind MHCII (Bikoff et al. 1993; Viville et al. 1993; Elliott et al. 1994; Romagnoli and Germain 1994; Busch et al. 1996). Ii is encoded outside the MHC gene region, but its expression, like MHCII, is under the control of CIITA (Reith et al. 2005). Ii is a monomorphic type II transmembrane protein, hence its name, but alternative initiation sites and splicing result in multiple forms that are named after their relative molecular weight. The shortest form of Ii, p31 in mice and p33 in man, is expressed most prominently. Because of alternative splicing, mice and humans also express p41 and p43, respectively (Strubin et al. 1986; Koch et al. 1987; O’Sullivan et al. 1987). The additional sequence in their exoplasmic domain has protease inhibitory ac-

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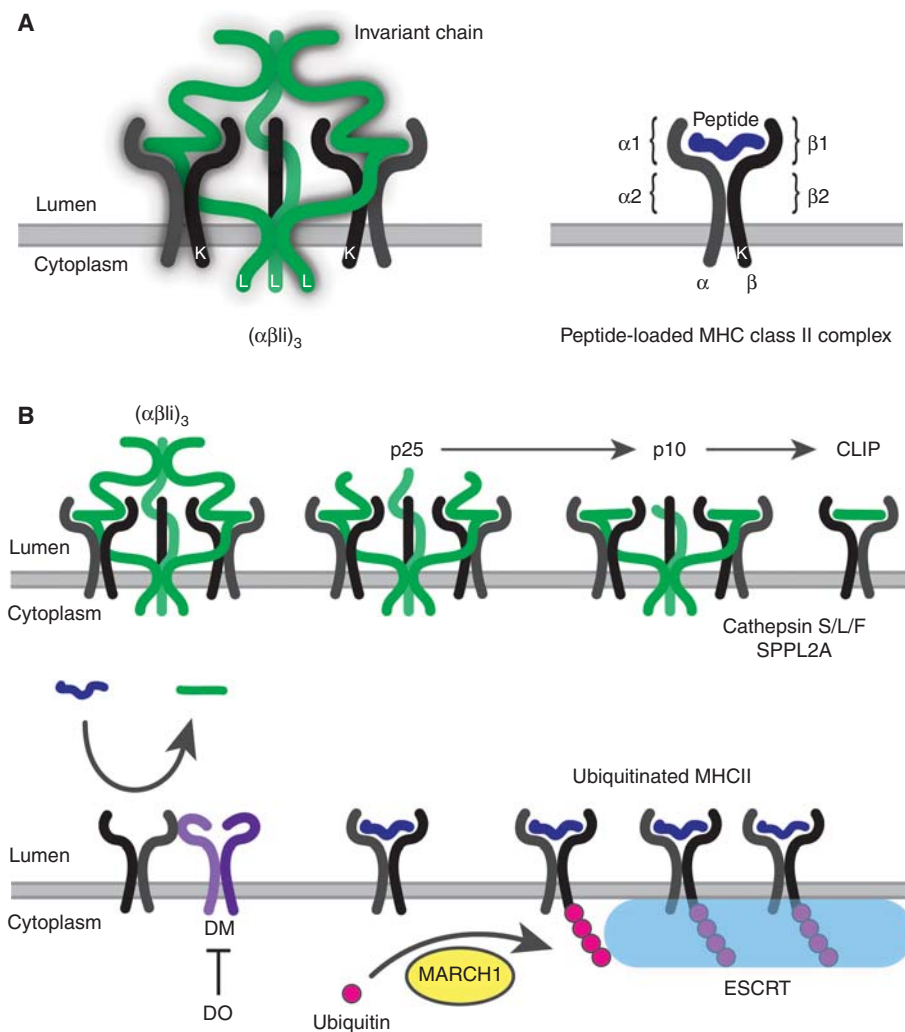


Figure 1. MHCII processing. (A) On the *left*, a nonameric $(\alpha\beta Ii)_3$ MHCII complex with trimerized Ii (green), MHCII α (gray), and β (black). Leucine-based sorting motifs in Ii (L) and the site for MHCII β ubiquitination (K) are indicated. (Gray bar) The membrane. On the *right*, pMHCII with bound peptide (blue) and the $\alpha 1$, $\alpha 2$, $\beta 1$, and $\beta 2$ domains of the α and β chains. (B) Schematic representation of progressive Ii processing, MHCII peptide loading, and ubiquitination. The sites of action for cathepsin S/L/F, SPPL2A, DM, DO, MARCH1, and ESCRT are indicated, and their roles are explained in the text.

tivity especially to the cysteine protease cathepsin L (Koch et al. 1989; Bevec et al. 1996; Mihelič et al. 2008) and influences the antigen-processing capacity of endosomes (Peterson and Miller 1992). In humans, but not in mice, an alternative start codon provides two additional Ii isoforms, p35 and p45, carrying a cytoplasmic extension that is shielded only after correct folding of the MHCII–Ii complex and, when exposed, serves

as an ER-retention motif. After synthesis in the ER, Ii forms trimers in which all forms of Ii can be incorporated, although the p31/33 form is most prominently present (Lamb and Cresswell 1992; Arunachalam et al. 1994). Trimerization of Ii is promoted by intermolecular associations of transmembrane and luminal domains (Bijlmakers et al. 1994; Gedde-Dahl et al. 1997; Ashman and Miller 1999). In the ER, three

MHCII $\alpha\beta$ heterodimers associate with one Ii trimer, generating nonameric $(\alpha\beta Ii)_3$ complexes (MHCII–Ii) (Fig. 1A) (Lamb and Cresswell 1992). MHCII $\alpha\beta$ dimers are stabilized by association with Ii; absence of Ii results in accumulation of misfolded MHCII molecules in the ER (Anderson and Miller 1992; Schaiff et al. 1992; Marks et al. 1995). Ii not only assists MHCII folding and covers the peptide-binding site of MHCII, but also chaperones MHCII via the Golgi and *trans*-Golgi network (TGN) to endosomes (Fig. 2). Both a direct route from the TGN to endosomes (Neefjes et al. 1990; Benaroch et al. 1995; Warmerdam et al. 1996; Liu et al. 1998) and an indirect transport pathway via the plasma membrane (Roche et al. 1993; Bremnes et al. 1994; Wang et al. 1997; Dugast et al. 2005; McCormick et al. 2005) are involved. Newly synthesized MHCII–Ii is directed to the endocytic system with the aid of two classical leucine-based sorting motifs (Bonifacino and Traub 2003) that are embedded within the Ii cytoplasmic domain (Pieters et al. 1993; Odorizzi et al. 1994; Rodionov and Bakke 1998; Hofmann et al. 1999). These motifs can interact with the cytosolic *trans*-Golgi-network-associated protein-sorting complex AP1 (Salamero et al. 1996) and with the plasma membrane adaptor AP2 (Rodionov and Bakke 1998; Hofmann et al. 1999). The cytoplasmic extension of the human p35 isoform can be phosphorylated by protein kinase C, a requirement that may be essential for chaperoning MHCII directly from the TGN to endosomes (Spiro and Quaranta 1989; Anderson and Roche 1998; Anderson et al. 1999), and in human cells, the AP1-directed pathway may become important particularly during DC maturation (Santambrogio et al. 2005). MHCII–Ii complexes that are deposited on the plasma membrane are rapidly taken up by clathrin-mediated endocytosis, and this process is largely driven by recognition of the two leucine-based motifs encoded in the cytoplasmic domain of Ii by the clathrin adaptor AP2 (Dugast et al. 2005; McCormick et al. 2005). In any case, MHCII–Ii must pass transferrin receptor-positive early endosomes to reach subsequent endocytic compartments where antigen loading occurs (Benaroch et al. 1995; Pond and Watts 1999). In

endosomes, MHCII is loaded with peptides only after Ii is degraded by proteolysis (Jones et al. 1979; Owen et al. 1981; Charron et al. 1983; Rudd et al. 1985; Roche and Cresswell 1990, 1991; Teyton et al. 1990). Ii is degraded by multiple proteases in defined sequential cleavage steps, starting at the carboxy-terminal luminal domain, until only the CLIP fragment is left, still occupying the peptide-binding groove of MHCII (Hsing and Rudensky 2005). Intermediate Ii degradation products ranging from 25 kDa to \sim 10 kDa (p25–p10) have been identified (Fig. 1B), and these accumulated when proteolytic activity of endosomes was inhibited (Neefjes and Ploegh 1992; Amigorena et al. 1995; Villadangos et al. 2000). Processing of Ii appears to be based on redundant protease activities (Costantino et al. 2008) with the exception of the cleavage of the p10 intermediate degradation product into CLIP. This requires cathepsin S (Riese et al. 1996; Driessen et al. 1999; Nakagawa et al. 1999; Shi et al. 1999) or cathepsins L and F (Nakagawa et al. 1998; Shi et al. 2000), depending on the cell type. Degradation of the remaining transmembrane fragment requires the intramembrane endopeptidase SPPL2A (Beisner et al. 2013; Bergmann et al. 2013; Schneppenheimer et al. 2013). Although early studies proposed that Ii degradation is regulated and enhanced in response to DC activation (Pierre and Mellman 1998), subsequent studies proved that Ii processing is a constitutive process and not rate limiting for antigen presentation by MHCII (Villadangos et al. 2001, 2005; El-Sukkari et al. 2003; ten Broeke et al. 2010).

PEPTIDE LOADING ONTO MHCII

Newly synthesized MHCII meets its ligands—peptides that originate from the proteolysis of endocytosed antigens—in the same endocytic compartment where Ii is also degraded. In DC endosomes and phagosomes, the activity of pH-sensitive lysosomal proteases allows hydrolysis of endocytosed antigens into peptides, whereas full degradation of peptides is prevented by multiple mechanisms (Watts 2012). Peptide loading of MHCII generally proceeds only after proteolytic processing of Ii into CLIP. As an exception,

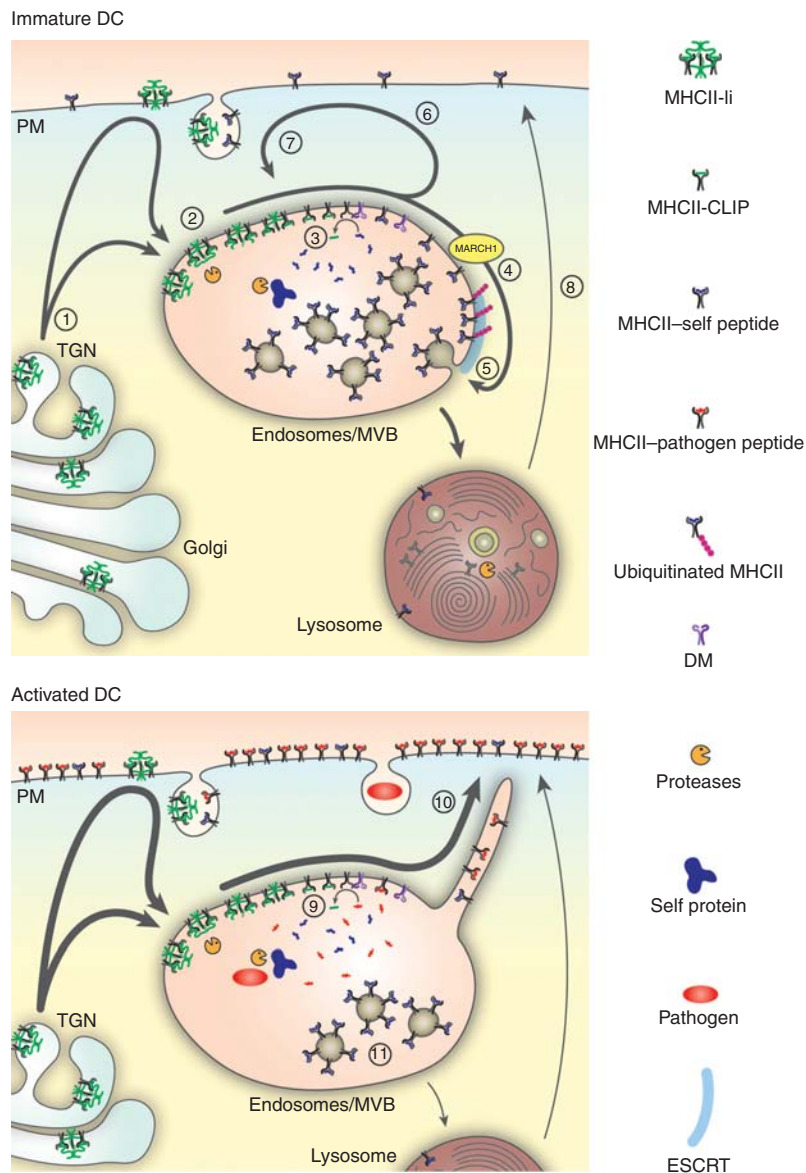


Figure 2. MHCII trafficking. The symbols are explained on the *right* and are equivalent to those in Figure 1. In immature DCs (*top* graph), newly synthesized MHC–Ii complexes are transferred via the Golgi and *trans*-Golgi network (TGN) to the endocytic pathway (1), either directly or via the plasma membrane, driven by leucine-based sorting signals contained within the Ii. In the endosomal system, Ii is degraded constitutively in a stepwise fashion (2), resulting in removal of the Ii-encoded sorting signals. The last remnant of the Ii, CLIP, is substituted by other peptides present within the endosomal lumen, facilitated by DM (3). Once the Ii is removed, pMHCII can be ubiquitinated by MARCH1 (4), driving its sorting to ILVs (5), probably through interactions with ESCRT. pMHCII at the endosomal delimiting membrane may also escape to the plasma membrane (6) from where it can be endocytosed (7) to encounter a new opportunity for sorting into ILVs. pMHCII that is sorted to ILVs is ultimately degraded after fusion of the MVB with the lysosome. Some MHCII may be transferred from the MVB-delimiting membrane to the lysosomal-delimiting membrane and from there to the plasma membrane to present lysosomal-generated peptides (8). In activated DCs (*bottom* graph), biosynthesis of MHC–Ii is temporarily up-regulated (thick arrows). Ii is processed with similar kinetics as in immature DCs, but liberated MHCII dimers are no longer ubiquitinated (9). Consequently, newly generated pMHCII is now redirected via tubular extensions from MIIC to the plasma membrane (10), whereas pMHCII that was already sorted to ILV before DC activation is still directed to lysosomes for degradation (11).

however, peptide loading onto some MHCII haplotypes does not necessarily require full processing of Ii into CLIP (Villadangos et al. 1997). In MIICs, the exchange of CLIP for other peptides is facilitated by the nonclassical MHC molecule HLA-DM (H2-M in mice) (Denzin and Cresswell 1995; Sherman et al. 1995; Sloan

et al. 1995; Kropshofer et al. 1996; Weber et al. 1996), but the degree of DM requirement for peptide loading is dependent on the MHCII isotype (Fig. 3) (van Lith et al. 2010). HLA-DM (DM) is structurally similar to MHCII but lacks the ability to bind peptides. DM can associate with MHCII to provoke dissociation of low-aff-

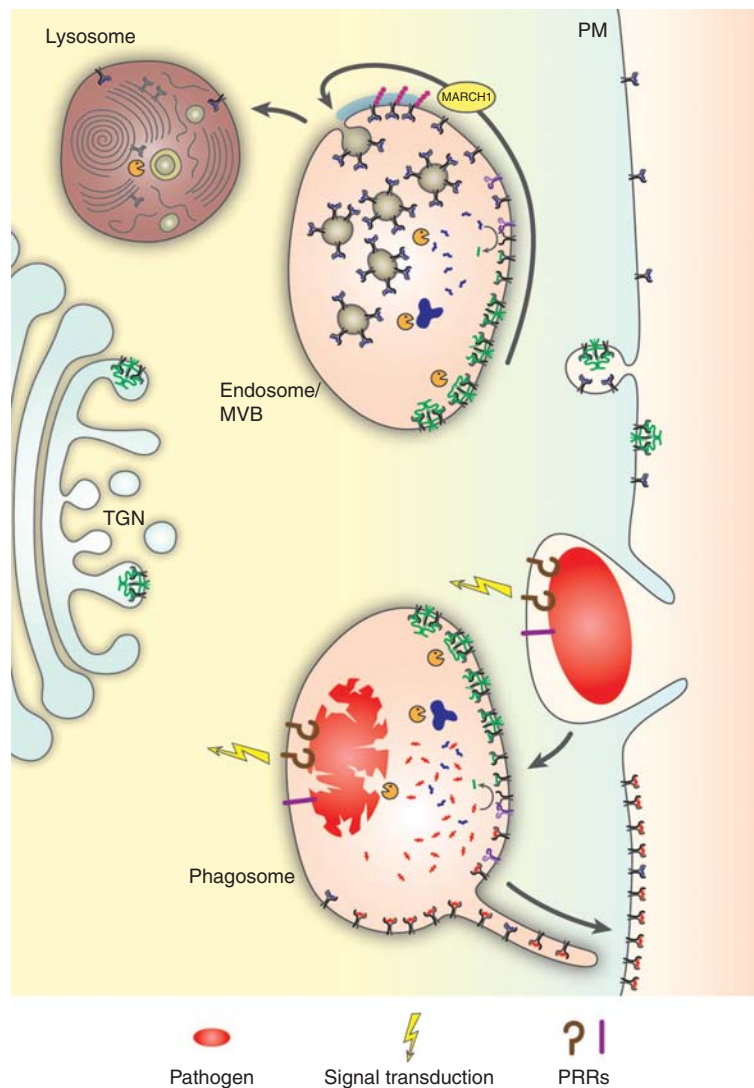


Figure 3. Autonomous function of phagosomes in MHCII processing. The symbols are similar to those in Figures 1 and 2; additional symbols are indicated at the *bottom*. PRR signaling from phagosomes may stimulate selective recruitment of pMHCII from those compartments to the plasma membrane (*bottom* structure), hypothetically through interference with MHCII ubiquitination selectively at those phagosomes. Transfer of pMHCII from pathogen-loaded phagosomes to the plasma membrane can occur unidirectionally, for example, toward immunological synapses between DC and T cells. MHCII in MIIC other than phagosomes located within the same cell may still be ubiquitinated and consequently targeted for lysosomal degradation (*top* structure).

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finity peptides (such as CLIP), after which another peptide can bind (Schulze and Wucherpennig 2012). During such peptide exchange, the MHCII-binding groove is temporarily empty, a state that is rather unstable and prone to aggregation (Germain and Rinker 1993; Rabinowitz et al. 1998). DM prevents this aggregation and stabilizes empty MHCII molecules, keeping them in a peptide-receptive state (Kropshofer et al. 1997; Grotenbreg et al. 2007; Anders et al. 2011). This important DM-catalyzed editing process favors the generation of high-affinity pMHCII complexes and is facilitated by the acidic milieu in late endosomes. Another non-classical MHC molecule involved in the regulation of MHCII antigen presentation is HLA-DO (H2-O in mice) (Denzin et al. 2005; Karlsson 2005). Like DM, HLA-DO (DO) is structurally similar to MHCII. DO forms stable complexes with DM directly after its synthesis in the ER and is transported as such to the endosomal system (Liljedahl et al. 1996). DO inhibits the peptide-editing function of DM (Denzin et al. 1997; van Ham et al. 1997) particularly at mild acidic pH values (Kropshofer et al. 1998; Liljedahl et al. 1998; van Ham et al. 2000). DO thus inhibits DM function early in the endocytic pathway but allows peptide editing by DM in more acidic, late endocytic compartments. In immature DCs, DO is thought to increase the range of presented peptides, therewith facilitating tolerance induction to a broad range of self peptides (Yi et al. 2010). DO expression is down-regulated in activated B cells and DCs, enhancing DM-catalyzed peptide loading under inflammatory conditions (Glazier et al. 2002; Hornell et al. 2006; Draghi and Denzin 2010; Porter et al. 2011).

TRAFFICKING OF pMHCII

As a result of Ii degradation, Ii-encoded sorting signals are removed, and the nonameric MHCII-Ii complex ($\alpha\beta\text{Ii}$)₃ dissociates into MHCII $\alpha\beta$ dimers. From this point on, MHCII may either be sorted to lysosomes for degradation or be transported to the cell surface (Wubbolts et al. 1996; Théry et al. 1998). As endosomal MIICs mature, they are transported

toward the cell center along microtubules (Wubbolts et al. 1999) by a dynein motor complex that is controlled by Rab7-interacting lysosomal protein (RILP) and the cholesterol sensor ORP1L (Rocha et al. 2009). In immature DCs, MHCII predominantly resides at late endosomes, which are not typical recycling compartments. Nevertheless, both MHCII-Ii and pMHCII can be transported from late endosomes to the plasma membrane via a route that is physically distinct from early endosomal recycling (Pond and Watts 1997) and with kinetics that are in agreement with other late endosomal recycling pathways (Reid and Watts 1990; Pinet et al. 1995; Lindner 2002). In a recent integrated siRNA screen on MHCII-expressing melanoma cells (Paul et al. 2011), several factors affecting recycling of MHCII to the plasma membrane have been identified, including the small GTPase ARL14/ARF7. This GTPase recruits the effector protein ARF7EP onto MIICs, which acts as a receptor for the actin-based motor protein myosin 1E, putatively driving transport of vesicles containing MHCII from MIIC to the plasma membrane. In maturing DCs, transport of MHCII is mediated via membrane tubules that radiate out from the MIIC toward the plasma membrane, and this pathway depends on an intact microtubule network (Kleijmeer et al. 2001; Boes et al. 2002, 2003; Chow et al. 2002; Vyas et al. 2007; van Nispen tot Panneerden et al. 2010). MHCII, DM, and LAMP1 are not enriched in these tubules relative to the vacuolar endosomes from which they derive, suggesting that this pathway may not require active recruitment but is entered by default (Kleijmeer et al. 2001). This is in agreement with the finding that traffic of MHCII to the cell surface does not rely on sorting information encoded by its cytosolic domains (Théry et al. 1998). Upon arrival at the cell surface, pMHCII complexes can be endocytosed only to recycle again to the plasma membrane via early endocytic compartments (Reid and Watts 1990, 1992). The latter pathway is extremely rapid (Reid and Watts 1990) and potentially may provide opportunities for MHCII to also sample antigens that differ from those found in the late endosomal compartments, thereby contributing to the diversity in antigen presentation



(Pinet et al. 1995; Lindner and Unanue 1996; Griffin et al. 1997; Pathak and Blum 2000). MHCII peptide loading at these alternate locations may differ for the requirements of DM or Ii (Vergelli et al. 1997; Villadangos et al. 2000) and is more prominent for some MHCII isotypes (van Lith et al. 2010).

The mechanism(s) for endocytosis of pMHCII are not well understood. Internalization was negatively affected by truncation of both the cytosolic domains of the α and β chains (Pinet et al. 1995), indicating active recruitment and a role of cytoplasmic domains in endocytic uptake. A conserved di-leucine motif in the cytosolic domain of the β chain was proposed to have a role in pMHCII endocytosis (Zhong et al. 1997; Simonsen et al. 1999), although this motif lacks upstream negatively charged amino acids that are generally thought to be required for interaction with clathrin adaptors (Bonifacino and Traub 2003). We and others proposed that endocytosis of MHCII is facilitated, to a limited extent, by ubiquitination of the cytoplasmic domain of the MHCII β chain (Shin et al. 2006; van Niel et al. 2006). It should be noted, however, that the assays used in these and other studies (Reid and Watts 1990, 1992) on MHCII endocytosis did not discriminate between MHCII–Ii and pMHCII, nor did they correct for recycling of endocytosed MHCII. Other studies could not confirm a role for ubiquitination in endocytosis of pMHCII (Matsuki et al. 2007; Walseng et al. 2010a). Interference with ubiquitination-dependent endosomal sorting may increase recycling, and this can also impact on the readout of endocytosis assays. These experimental problems were tackled in more recent experiments, from which we concluded that ubiquitination has little if any effect on the endocytosis rate of pMHCII (T ten Broeke, R Wubbolts, W Stoorvogel, et al., unpubl.). In contrast to MHCII–Ii complexes, pMHCII is endocytosed via clathrin- and dynamin-independent pathway(s), possibly involving SWAP-70-regulated RhoA/RhoB (Ocana-Morgner et al. 2009) or Arf6- and Rab35-dependent mechanism(s) (Walseng et al. 2008). MHCII is found in detergent-resistant membrane domains (DRMs) (Anderson et al. 2000; Karacsonyi et al. 2004; Rodgers and

Smith 2005), and endocytosis of MHCII was severely hampered after treatment of cells with Filipin, a cholesterol-sequestering agent (Knorr et al. 2009). Perhaps pMHCII uses the clathrin-independent carrier (GLIC)/GPI-AP-enriched early endosomal compartment (GEEC) pathway, an endocytosis route that is also used by lipid raft-associated molecules like GPI-linked proteins and cholera toxin B (Mayor and Pagano 2007; Howes et al. 2010). Furthermore, tetraspanin-enriched microdomains have been implicated in clustering and routing of MHCII from the plasma membrane (Hammond et al. 1998; Engering and Pieters 2001; Kropshofer et al. 2002; Zilber et al. 2005; Poloso et al. 2006; Unternaehrer et al. 2007). Tetraspanins belong to a family of proteins that span the membrane four times and are palmitoylated, a posttranslational modification that stimulates lateral partitioning into protein/lipid domains (Kovalenko et al. 2004; Yáñez-Mó et al. 2009). Most tetraspanins contain motifs for clathrin-mediated endocytosis (Berditchevski and Odintsova 2007) that potentially could assist the internalization of domain-associated molecules such as pMHCII. In conclusion, although several mechanisms for endocytosis of pMHCII have been proposed, the exact pathways and dynamics that apply for different cell systems remain largely undefined.

SORTING OF MHCII AT MVBs

Endosomes generate intraluminal vesicles (ILVs) that progressively increase in number during endosome maturation, giving rise to an eminent multivesicular morphology. Hence, this type of late endosomes is also referred to as a multivesicular endosome or multivesicular body (MVB) (Piper and Katzmann 2007). Membrane proteins that are targeted to ILVs are ultimately transferred to lysosomes for degradation. Transfer to ILVs also terminates signaling of plasma membrane receptors, as exemplified by receptor tyrosine kinases and G-protein-coupled receptors (Sorkin and von Zastrow 2009). As an alternative for a lysosomal destination, MVBs may fuse with the plasma membrane, thereby releasing their ILVs as exosomes in the extracellular space (Raposo and

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Stoorvogel 2013). Whereas one set of MVBs serves as a sorting station for membrane proteins to lysosomes, another set of functionally distinct MVBs is rather destined to release exosomes. In DCs, sorting of MHCII at MVBs into exosomes is, in contrast to lysosomal targeting, independent of MHCII ubiquitination and rather correlates with incorporation into tetraspanin-containing complexes (Buschow et al. 2009). Exosome release was stimulated when DCs engaged antigen-specific CD4⁺ T cells (Buschow et al. 2009). Released exosomes can be recruited by such T cells or bystander DCs in an LFA-1-dependent manner (Segura et al. 2007; Nolte-t Hoen et al. 2009), and in this way, directly or indirectly, contribute to T-cell activation (Théry et al. 2002a,b). Relative to mature DCs, immature DCs release little MHCII with exosomes, and by far most of their MVBs are destined to fuse with lysosomes. At equilibrium, ~60% of all cellular MHCII in immature DCs are located at MVBs, of which ~80% are at ILVs, consistent with active recruitment of MHCII into ILVs (Kleijmeer et al. 2001; ten Broeke et al. 2011). Indeed, the density of MHCII (molecules/micrometers squared) is much higher on ILVs than on the MVB-delimiting membrane (Kleijmeer et al. 2001). We and others reported that MHCII in immature DCs is ubiquitinated at a unique lysine residue at position 225 in the cytoplasmic domain of MHCII β , and this modification was identified as a key regulator of MHCII surface expression and stability (Ohmura-Hoshino et al. 2006; Shin et al. 2006; van Niel et al. 2006; Matsuki et al. 2007). To a lesser extent, MHCII α may also be ubiquitinated, but this was reported only for model cell lines that were cotransfected with MHCII and MARCH ligases (Lapaque et al. 2009b). In immature DCs, surface expression of a K225 mutant of MHCII β was dramatically increased in comparison to wild-type MHCII β (Shin et al. 2006; van Niel et al. 2006; Matsuki et al. 2007), with the major cause being that sorting at MVBs into ILVs was >20-fold less efficient (van Niel et al. 2006). Ubiquitination-driven cargo sorting and ILV formation at endosomes are facilitated by the endosomal sorting complex required for transport

(ESCRT) (Raiborg and Stenmark 2009; Hurley 2010; Henne et al. 2011, 2013; Piper et al. 2013). Consistent with its generic role, it can be expected that the ESCRT machinery also drives sorting of ubiquitinated MHCII into ILVs. Indeed, several ESCRT-associated components, including Tollip, were identified to influence MHCII traffic (Paul et al. 2011). In support for a role for ESCRT, we found that MHCII in DCs is, indeed, enriched in the flat clathrin/ESCRT-0-coated lattices at MIICs (van Niel et al. 2008). ILV formation can proceed only after ESCRT disassembly, and this requires ATP hydrolysis by the class I AAA ATPase Vps4 complex (Hill and Babst 2012). Expression of a dominant-negative mutant of VPS4 interfered with MHCII sorting (T ten Broeke, R Wubbolts, W Stoorvogel, et al., unpubl.). MIICs are composed not only of MVBs but also of multilamellar compartments. The latter contain abundant MHCII-labeled internal membrane sheets, and high-resolution three-dimensional (3D) analysis of these compartments suggests that they represent lysosomal compartments with MHCII trapped on their inner membranes, possibly in the process of being degraded (Murk et al. 2004).

Activation of conventional DCs (cDCs) strongly reduces ubiquitination of MHCII, intervening with its targeting to ILVs (Shin et al. 2006; van Niel et al. 2006; De Gassart et al. 2008; Walseng et al. 2010b). Reduction in ILV targeting leads to rerouting of pMHCII to the cell surface, possibly by default (see above), thereby elevating expression levels at the plasma membrane. In maturing cDCs, pMHCII that had already been sorted to ILVs before cDC activation is still targeted to and degraded in lysosomes, while only newly generated pMHCII is selectively stabilized and transferred to the plasma membrane (ten Broeke et al. 2011). Synthesis of MHCII is shut off in fully matured cDCs. As a consequence, pMHCII that are stably displayed on mature cDCs are predominantly generated directly after pathogen contact. This creates a “snapshot” for presentation of those peptides that are present directly after pathogen contact, therewith optimizing the presentation of “foreign peptides” relative to “self peptides.” Unlike activated cDCs, activated plasmacytoid

DCs (pDCs) carry on synthesizing as well as ubiquitinating MHCII, allowing continuation of the sampling and presentation of acquired endogenous (late) viral antigens in their activated state (Young et al. 2008).

Ubiquitination of MHCII is largely driven by MARCH1 (Matsuki et al. 2007; De Gassart et al. 2008), a member of the family of MARCH E3 ligases (Nathan and Lehner 2009). Besides MHCII, the costimulatory molecule CD86 is also targeted by MARCH1-driven ubiquitination to lysosomes (Baravalle et al. 2011). The molecular mechanism for attenuating MHCII or CD86 ubiquitination in activated cDCs is not entirely clear. Although MARCH1 has a half-life of <30 min (Jabbour et al. 2009), its transcription is turned off only >8 h after DC activation (De Gassart et al. 2008; Young et al. 2008). The relatively late onset of transcription regulation suggests that additional mechanisms are responsible for the rapid interference (<1 h) with MHCII ubiquitination in response to maturation stimuli. One mechanism could involve deubiquitination, although MHCII-specific deubiquitinating enzyme(s) have not yet been identified. Another potential regulatory factor is CD83, which is highly expressed on mature DCs and was shown to bind MARCH1, sequestering it from MHCII (Tze et al. 2011). Yet other undiscovered regulatory mechanisms may provide additional modes to regulate ubiquitination of MHCII. The pivotal role of MARCH1 in regulating MHCII-mediated antigen presentation is further illustrated by observations that the anti-inflammatory cytokine IL-10 enhances MARCH1 expression (Thibodeau et al. 2008; Baravalle et al. 2011; Tze et al. 2011), thereby promoting MHCII degradation at immune-suppressive conditions. The central role of MHC ubiquitination in antigen presentation is also illustrated by reports on how certain pathogens can suppress immunity by targeting this system (Ishido et al. 2009; Lapaque et al. 2009a). The discovery of viral E3-ubiquitin ligases that down-modulate surface expression of MHCII have, in fact, also helped the identification of MARCH ligases that regulate MHCII surface expression (Bartee et al. 2004; Ohmura-Hoshino et al. 2006). In addition to MARCH1, other

MARCH ligases can also ubiquitinate MHCII. In MARCH1 knockout mice, ubiquitination of MHCII was fully suppressed in B cells but only partially in DCs (Matsuki et al. 2007), suggesting some redundant ligase activity in DCs. MARCH8/c-MIR was able to reduce MHCII surface expression when overexpressed (Ohmura-Hoshino et al. 2006), indicating overlapping specificity with MARCH1. Although MARCH1 seems to play a more prominent role in controlling pMHCII traffic in DCs (De Gassart et al. 2008), it cannot be excluded that the two ligases act at distinct sites, for example, at the plasma membrane and at the endosomal membrane, leaving dissimilar sorting signatures. In this regard, it was recently shown that sorting of MHCII is highly dependent on the length of the conjugated ubiquitin chain (Ma et al. 2012). Specificities of MARCH ligases may differ for distinct MHCII isotypes (Jahnke et al. 2012). The biological relevance of ubiquitination-driven MHCII traffic has also been investigated in vivo and has generated some dispute. Knock-in mice for a ubiquitination mutant of MHCII showed dysfunction of DCs similar to that of MARCH1 knockout mice (Ohmura-Hoshino et al. 2009). Others concluded that ubiquitin-dependent control of MHCII localization is dispensable for antigen presentation and antibody production (McGehee et al. 2011). In the latter study, mice in which endogenous MHCII was replaced by GFP-tagged wild-type MHCII were compared with knock-in mice for a K225 ubiquitination mutant of GFP-tagged MHCII. These animals performed equally well in generating CD4⁺ T cells and antibody titers in response to model antigens at DC-activating conditions. However, it should be noted that at DC-activating conditions, ubiquitination of wild-type MHCII is also absent. As explained above, it can be expected that ubiquitination of MHCII rather serves to prevent antigen presentation by nonactivated DCs. In a recent in vivo study, it was found that MARCH1-mediated ubiquitination of MHCII in dendritic cells promotes selection of natural regulatory T cells (Oh et al. 2013), again consistent with a role in the prevention of autoimmune responses.

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MVB DYNAMICS AND MHCII PEPTIDE LOADING

As discussed above, degradation of Ii in the endocytic system liberates MHCII $\alpha\beta$ dimers from the $(\alpha\beta Ii)_3$ nonameric complex and allows MHCII peptide loading. It has been debated whether peptide loading occurs preferentially at the endosomal-delimiting membrane or at ILVs. DM facilitates MHCII peptide loading and is present at the MVB-delimiting membrane and ILVs (Kleijmeer et al. 2001), and both sites provide access to the peptide pool generated in the MVB lumen. In transfected HEK293 cells with experimentally swollen MVBs, MHCII and DM were shown by fluorescence resonance energy transfer (FRET) to interact primarily at ILVs, suggesting that peptide loading onto MHCII may preferentially occur at ILVs (Zwart et al. 2005). In this case, a pathway should exist for pMHCII to travel back from ILVs to the endosomal-delimiting membrane from where it could then reach the plasma membrane. Such a pathway, also referred to as “back-fusion” or retrofusion of ILVs, was also proposed to explain the disappearance of the MVB during DC maturation (Kleijmeer et al. 2001) and to provide a source of membrane needed for the formation of MIIC-associated tubules in maturing DCs (Boes et al. 2002, 2003; Chow et al. 2002; Vyas et al. 2007). More recently, however, it was shown that such membrane tubules can be generated as a consequence of homotypic endosome fusion (Skjeldal et al. 2012), which is accelerated in maturing DCs (van Nispen tot Pannerden et al. 2010). ILV back-fusion has, in fact, never been convincingly recorded in live cells nor in vitro, and therefore remains hypothetical, despite a proposed involvement of lysobisphosphatidic acid, Tsg101, or Alix (Falguières et al. 2009). Several lines of more convincing evidence support the idea that MHCII peptide loading occurs at the endosomal-delimiting membrane, in contrast to ILVs. First, MHCII failed to sort to ILVs in cathepsin-S-deficient intestinal epithelial or professional antigen-presenting cells (Beers et al. 2005; Boes et al. 2005). Cathepsin S is required for the degradation of Ii (p10), and hence also for subse-

quent ubiquitination of MHCII (see above). Thus, Ii is already processed into CLIP and ready for peptide loading at the endosomal-delimiting membrane. Also, DM is abundantly present at MVB-delimiting membranes (Kleijmeer et al. 2001); thus, all essential requirements for peptide loading are at hand at this location. This is consistent with our observation that ubiquitinated MHCII is in a SDS-stable peptide-loaded complex (van Niel et al. 2006). After recruitment by the ESCRT machinery, ubiquitinated cargo is deubiquitinated before incorporation into ILVs (Henne et al. 2011). Thus, MHCII peptide loading occurs before or concomitant with ubiquitination, before ILV incorporation. MHCII peptide loading and ubiquitination are not necessarily mechanistically linked, and the sequence of events could merely be dictated by differences in kinetics. A second indication for MHCII peptide loading at the endosomal-delimiting membrane is that loading of wild-type MHCII and mutant K225 MHCII were equally efficient (Shin et al. 2006; van Niel et al. 2006), and that antigen presentation was unaffected in ubiquitination-deficient MHCII knock-in mice (McGehee et al. 2011). Third, interference with MHCII sorting at MVBs with a dominant-negative mutant of VPS4 also did not affect peptide loading (T ten Broeke, R Wubbolts, W Stoorvogel, et al., unpubl.). Finally, in a recent study, we showed directly that maturing DCs predominantly used newly synthesized MHCII for antigen presentation rather than MHCII that was synthesized before DC activation (ten Broeke et al. 2011). This directly excludes a ubiquitination-dependent cycle of MHCII through ILVs, because ubiquitination is absent (van Niel et al. 2006) and MVB formation diminished upon DC activation (Kleijmeer et al. 2001). These observations also argue against the idea that MHCII at ILV in immature DCs may serve as a “storage pool” that can be recruited in response to DC activation. For maturing DCs, it seems even undesirable to recruit a preexisting pool of pMHCII from ILVs, because this was generated before pathogen contact, and presentation of self peptides loaded into these MHCII molecules at inflammatory conditions may provoke autoimmunity. In this line of thought, and con-

sistent with our data (ten Broeke et al. 2011), it would rather be favorable when all pMHCII at ILVs would be irreversibly destined for degradation in the lysosome, irrespective of DC activation.

AUTONOMOUS PHAGOSOMES

After DC activation, only a minority of MHCII molecules truly display pathogen-derived peptides (Trombetta and Mellman 2005). This poses a conceptual problem that has not yet been fully resolved. How does the immune system prevent the activation of self-reactive T cells by activated DCs that in addition to foreign peptides also display self peptides in the context of MHCII? Part of the answer came with the concept that mature DCs present a “snapshot” of their antigenic environment at the time of activation. In response to DC activation by pathogens, the selectivity of loading microbial-derived antigens over self peptides is achieved through transient up-regulation followed by shutoff of MHCII synthesis in combination with a change in the sorting behavior of newly synthesized MHCII at the endocytic tract (Viladangos et al 2005; ten Broeke et al. 2011). Another way to increase selectivity in antigen presentation is the formation of pMHCII microdomains that are preformed within phagosomes and maintained upon arrival at the DC plasma membrane (Bosch et al. 2013). Further specificity may be achieved through autonomous control by phagosomes in contributing pMHCII for presentation, meaning that antigens are preferentially presented by MHCII when originating from antigen-carrying particles that also stimulate DC-activating receptors from within that same compartment (Blander and Medzhitov 2006; Hoffmann et al. 2012). This idea is supported by the observations that TLR2 (Underhill et al. 1999) and TLR4 (Husebye et al. 2010; Mantegazza et al. 2012) can be recruited to and signal from phagosomes. TLR delivery to phagosomes requires the clathrin adaptor AP-3, and local signaling facilitates pMHCII recruitment from phagosomes for presentation at the plasma membrane (Mantegazza et al. 2012). Phagosome-specific TLR signaling

pathways have not been specified, but besides TLRs may also involve local MHCII molecules (Inaba et al. 1998; Blander and Medzhitov 2006). Recruitment of TLR and/or MHCII to phagosomes may also involve the Arf-like GTPase Arl8b and its downstream effector, the homotypic fusion- and vacuolar protein-sorting (HOPS) complex, analogous to their role in functional targeting of CD1 to phagosomes (Garg et al. 2011). Clearly, pMHCII can also be recruited to the plasma membrane from endocytic compartments other than phagosomes, but recruitment from PAMP-carrying phagosomes may prevail. To achieve immune evasion, pathogens like *Salmonella* (Lapaque et al. 2009a) and *Francisella tularensis* (Wilson et al. 2009) stimulate the host system to ubiquitinate MHCII, stimulating transfer to lysosomes rather than to the plasma membrane. Phagosomes that carry less sophisticated pathogens might stabilize MHCII molecules through local interference with ubiquitination. Maintenance of MHCII ubiquitination in endocytic compartments other than phagosomes within the same cell would enhance selectivity in MHCII presentation of pathogen-derived peptides. Transfer of pMHCII from pathogen-loaded phagosomes to the plasma membrane can occur unidirectionally, for example, toward immunological synapse between DC and T cells (Boes et al. 2002; Keller et al. 2007), applying yet another layer of specificity.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The mechanisms that regulate MHCII antigen presentation by DCs are instrumental in directing specificity in adaptive immune responses. Although MHCII is constitutively synthesized and loaded with peptide, newly formed pMHCII complexes are efficiently displayed only upon DC activation. Surface exposure and lifetime of pMHCII are determined by sorting at MVBs. Ubiquitination drives MHCII into ILVs, resulting in transfer to and degradation in lysosomes. Ubiquitination of MHCII is regulated, possibly differentially depending on the subcellular location, by largely unknown mecha-

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nisms. Clearly, further research is required to understand how selectivity in peptide presentation by MHCII is achieved, particularly with regard to the mechanism(s) of the silencing of MHCII ubiquitination in activated DCs. The potential role of phagosome autonomy herein also needs to be explored. Such knowledge may be instrumental to design more efficient vaccination strategies, for example, through physical coupling of vaccine antigens to immune adjuvants. Disclosure of mechanisms by which some bacteria and viruses avoid immune surveillance by enhancing MHCII traffic to lysosomes may help development of novel medicines toward such pathogens. Finally, whether failure of proper regulation of MHCII ubiquitination can contribute to the development of autoimmune diseases also needs to be investigated.

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MHC Class II Antigen Presentation by Dendritic Cells Regulated through Endosomal Sorting

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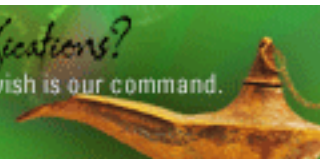


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