ELSEVIER

Class II MHC peptide loading by the professionals

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The loading of class II MHC molecules with antigenic peptides is largely confined to the endocytic vesicles of specialized antigen-presenting cells (APCs), such as B cells, macrophages and dendritic cells. At first glance, the pathway utilized by each of these professional APCs to generate class II–peptide complexes on their surface appears to be indistinguishable. All three types of APC rely on the chaperone Ii for correct class II assembly and transport to the endocytic pathway, they all depend on the action of specific cysteine proteases to remove Ii from the class II–Ii complex, and they all utilize the class II-like molecule DM to facilitate peptide loading. A closer look, however, reveals subtle yet important differences in the class II maturation pathway between each of these APCs, which befit the unique roles these individual cells play in eliciting $CD4^+$ T-cell responses.

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Abbreviations

AEP	asparaginyl endopeptidase
Ag	antigen
APC	antigen-presenting cell
BCR	B-cell receptor
BM-mac	bone-marrow-derived macrophage
Cat	cathepsin
CIIV	multivesicular class II compartment
CLIP	class-II-associated invariant chain peptide
DC	dendritic cell
ER	endoplasmic reticulum
GC	germinal center
GM-CSF	granulocyte-macrophage colony-stimulating factor
IFN	interferon
Ii	invariant chain
IL	interleukin
LPS	lipopolysaccharide
MBP	myelin basic protein
MIIC	for MHC class II compartment
PAMP	pathogen-associated molecular pattern
TLR	Toll-like receptor

Introduction

Antigens internalized by antigen-presenting cells (APCs) are digested into peptides by proteases while traversing the endocytic route [1]. To ensure that the route traveled by MHC class II molecules intersects with the sites at which these peptides are generated, the chaperone invariant chain (Ii) associates with newly synthesized αβ dimers in the endoplasmic reticulum (ER) and delivers them directly to the endocytic pathway. Within the endocytic pathway, aspartic and cysteine proteases progressively degrade Ii, leaving the CLIP (class-II-associated invariant chain peptide) fragment of Ii in the peptide-binding cleft of the MHC class II complex [1,2]. CLIP is dislodged from the αβ dimer and exchanged for resident antigenic peptides in a reaction that is catalyzed by the accessory molecule, H-2/HLA-DM [3,4].

The bulk of newly synthesized MHC class II molecules acquire peptides in late endocytic vesicles, in which epitopes whose generation requires extensive proteolytic processing are generated. Historically, these late endosome/lysosomal-like compartments are referred to as MIICs (for MHC class II compartments; [5]). The multilaminar MIICs are distinct from multivesicular class II compartments (CIIVs), which more closely resemble endosomes [6,7]. Peptide loading can occur in either CIIVs or MIICs [8]. An alternative minor pathway also exists, in which cell surface class II molecules are 'recycled' into endosomes and loaded with resident peptides in an Ii- and DM-independent manner [9•]. Here, we review some of the key factors that dictate where class II molecules acquire peptides, including: the APC type; the maturation and activation state of the APC; the mechanism of antigen (Ag) uptake; and, the active proteases available in the APC to degrade both the antigen and Ii.

Endocytic proteolysis and antigen-presenting cell function

The different types of professional APCs are equipped with similar and distinct intracellular acidic proteases, referred to as cathepsins (e.g. Cat S), most of which contain a cysteine as the attacking nucleophile in the catalytic cleft [1,2]. The function of these endocytic proteases in Ii- and Ag-processing determines the kinetics and intracellular location of class II peptide loading.

Invariant chain proteolysis

The proteolytic removal of class-II-associated Ii in the endocytic route occurs via a series of defined cleavage intermediates, designated αβ–Iip22, αβ–Iip10, and αβ–CLIP [1,2]. The rate-limiting step in Ii degradation — conversion of αβ–Iip10 into αβ–CLIP — is performed most efficiently by Cat S in bone-marrow-derived professional APCs [10–13], and by Cat L in murine cortical

thymic epithelial cells (cTECs; [14]). Although the proteases that cleave Iip10 to CLIP are well defined, those responsible for initiating Ii breakdown to generate the Iip10 substrate are not. The first steps in Ii processing involve cleavage of the carboxy-terminal region (the lumenal domain) of Ii to disrupt the Ii homotrimer (and thus the nonameric αβIi complex) and generate the intermediates αβ–Iip22 and αβ–Iip10. Noncysteine proteases are probably responsible for these early Ii processing events, as the cysteine protease inhibitor, leupeptin, does not prevent elimination of the carboxy-terminal trimerization region [1,2]. Manoury and co-workers [15••] recently identified the leupeptin-insensitive cysteine protease asparaginyl endopeptidase (AEP), as an enzyme present in B cells that is capable of initiating Ii processing to yield the Iip22 (in human cells) and Iip10 (in murine cells) intermediates. It will be important to examine the phenotype of AEP-deficient mice [16•] in this context, and establish their potential defects in class II MHC-restricted antigen presentation.

Antigen degradation

Cat B, Cat S and Cat L also participate in the processing of internalized antigens into class-II-presentable T-cell epitopes [17–19]. When assigning functions to individual proteases in the liberation of epitopes from antigens, however, the destructive nature of these proteases must not be overlooked. In other words, a protease can either generate T-cell epitopes or destroy them [20,21], and every antigen has to be examined as a separate entity. For example, AEP performs the rate-limiting cleavage in the degradation of the tetanus toxin C fragment (TTCF) in B cells [22]. By contrast, the cleavage of myelin basic protein (MBP) by AEP prevents the presentation of the encephalitogenic MBP epitope ($MBP_{85–99}$) by class II molecules in thymic APCs [23••]. This might be a mechanism by which MBP-specific autoreactive T cells escape negative selection.

Class II MHC peptide loading in B cells

B cells function as APCs primarily in secondary lymphoid tissues, where class-II-restricted antigen presentation by these cells enables the cognate T–B cell interactions required to elicit T-cell dependent humoral immunity. Unlike macrophages and dendritic cells (DCs), B cells are not actively phagocytic. However, they effectively use the antigen-specific B-cell receptor (BCR) to capture and concentrate Ag in class II compartments [24], and are equipped with the accessory molecule DO to ensure class II molecules are loaded with these BCR-internalized antigens [4].

Engagement of the BCR directs endocytic peptide loading

The BCR is composed of an Ag-recognition component, membrane Ig (mIg), which is noncovalently associated with an Igα–Igβ heterodimer that contains immunoreceptor tyrosine-based activation motifs (ITAMs), which trigger Ag internalization and a signaling cascade that leads to B-cell activation [24]. BCR–Ag complexes are internalized via clathrin-coated pits into early endosomes, and are then sorted into class II compartments via signals located in the cytoplasmic tails of Igα and Igβ [8,24]. Moreover, signaling through the BCR induces the transient formation of MIICs to which BCR–Ag complexes, class II and DM are recruited, thereby promoting the selective presentation of BCR-internalized antigens [25,26•,27•].

B cells utilize the chaperone DO to regulate DM-mediated peptide loading

In professional APCs, DM not only induces CLIP release from the αβ dimer, but also edits the peptide repertoire by exchanging peptides that interact poorly (high off-rate) for peptides that fit more tightly (low off-rate) in the peptide binding groove [3,4]. Unlike DCs and macrophages, B cells have within their arsenal the accessory molecule DO, which impairs or alters the peptide exchange activity of DM [28–30]. DO is thought to inhibit DM function in all but the most acidic late-endosomal compartments of B cells [28,29,31], to which the majority of BCR-internalized antigens are targeted. Thus, DO might promote the presentation of peptides derived from BCR-internalized antigens [32,33].

Befitting its function in DM-mediated peptide loading, the expression of DO appears to be regulated during both the antigen-dependent and antigen-independent phases of B-cell development [34••]. Whereas the expression of HLA-DR1 and DM was observed at each stage of B-cell development, DO expression was not observed until B-cell maturation was complete. Accordingly, class II molecules in naïve mature B cells were predominantly occupied with CLIP [34••,35••]. The high levels of DO in naïve B cells may thus contribute to their inability to activate Ag-specific T cells during the initiation of an antibody response, a task instead reserved for DCs. When naïve B cells encounter Ag and are activated by Ag-specific T cells, they may seed germinal centers (GCs). Interestingly, DO expression was markedly reduced in GC B cells as compared to naïve and memory B cells [34••,35••,36•]. The reduced levels of DO correlated with a decrease in class II–CLIP complexes and an increase in class II–peptide expression on the cell surface. Down-modulation of DO in GC B cells thus renders these cells 'antigen processing and presentation competent', driving T–B cell cognate interactions and subsequent $CD4^+$ T-cell activation.

Class II peptide loading in macrophages

Macrophages are phagocytic APCs poised in (or recruited to) peripheral tissues where they function as crucial mediators of host defense against pathogens, serving as a link between innate and adaptive immune responses during infection [37]. The phagocytosis of pathogens

activates the antimicrobial killing mechanisms of macrophages, and stimulates the production of inflammatory mediators that initiate acquired T-cell immunity. The latter hinges on the capacity of macrophages to function as APCs. The development of macrophages into potent APCs requires their activation by mediators such as IFN-γ and GM-CSF [38]; before activation, macrophages express only low levels of class II MHC [39] and co-stimulatory molecules [40].

Protease activity in macrophages

Although all bone-marrow-derived APCs express Cat B and Cat S, IFN-γ-activated macrophages express many additional enzymes, including Cat Z, Cat F, Cat L, Cat K and Cat H [1]. In addition to Cat S and Cat L, Cat F [13] and Cat K [41] are capable of digesting Iip10 into CLIP — at least *in vitro*. Accordingly, the delay in Ii breakdown and class II peptide loading seen in the absence of Cat S, although still impaired, is less severe in macrophages than in other class II MHC^{+} cells [13,42]. Nonetheless, the analysis of $S^{-/-}L^{-/-}$ mice clearly demonstrated that Cat L does not cleave Iip10 to CLIP in IFN-γ-activated macrophages — this task is reserved for Cat S [13,42]. Whether or not Cat F contributes to Ii breakdown *in vivo* awaits analysis of Cat $F^{-/-}$ animals.

Delivery of antigens to class II compartments by phagocytosis

The crosslinking of innate receptors on the macrophage surface upon binding pathogens initiates waves of signal transduction events that induce the rearrangement of the actin cytoskeleton as well as extension of pseudopodia, leading to the internalization of the bacteria into the phagosome [43,44]. The ER — not the plasma membrane — is the major reservoir of membrane for phagosome formation, and ER-mediated phagocytosis is viewed as a general mechanism by which pathogens enter the macrophage [44,45]. When formed, the phagosome typically develops into a phagolysosome by fusing with early/late endosomes and lysosomes of the host cell, thereby exposing its contents to lysosomal proteases and class II MHC molecules [46]. In the course of their biogenesis in murine bone-marrow-derived macrophages (BM-macs), phagosomes preferentially fuse with endocytic compartments that contain Cat S [47]. Accordingly, some pathogens have developed mechanisms to subvert phagolysosome biogenesis in an attempt to enhance their survival within the macrophage [37].

Antigens derived from phagocytosed particles or pathogens can be loaded onto newly synthesized class II molecules directly in the phagosome [48,49], as was shown for an epitope derived from *Mycobacterium tuberculosis* Ag85B [50]. Alternatively, bacterial antigens can be released from the phagosome and transferred to classical endocytic compartments for loading onto class II molecules [51–53], as was recently shown for epitopes derived from the M5 protein of *Streptococcus pyogenes* [54]. Furthermore, the uptake of *S. pyogenes* by phagocytosis resulted in the presentation of the $M5_{17–31}$ epitope by recycling class II MHC molecules in early endosomes, whereas macropinocytosis of *S. pyogenes* resulted in presentation of the $M5_{306–319}$ epitope by newly synthesized class II MHC molecules in late endocytic vesicles [55].

Surprisingly, a large number of class-II-bound peptides are derived from cytosolic proteins rather than antigens internalized by the APC [56]. A puzzle that eagerly awaits a solution is the role, if any, of autophagy in delivering these cytosolic antigens to class II peptide loading compartments in the endocytic route. In macroautophagy, a membranous organelle of unknown origin envelops a portion of cytoplasm to form a vacuole called an autophagosome, which fuses with endocytic vesicles [57]. Although we are not yet able to study and manipulate the process of autophagy in mammalian cells as we can in yeast, a recent study demonstrated that the presentation of a cytosolic antigen by class II molecules could be blocked (in B cells) by an autophagy inhibitor [58].

Engagement of TLR2 by bacterial PAMPs influences antigen presentation in macrophages

APCs utilize innate immune receptors, such as the Toll-like receptors (TLRs), mannose receptors, and Fc- and complement-receptors (for opsonized bacteria), to recognize conserved motifs on pathogens, known as PAMPs (pathogen-associated molecular patterns; [59–61]). The recognition of PAMPs by TLRs initially induces the production of pro-inflammatory cytokines, which help the host to combat the infection [62]. Recent reports, however, suggest that prolonged exposure to these PAMPs may eventually lead to inefficient class-II-restricted antigen presentation by the macrophage. Prolonged engagement of TLR2 by the 19 kDa lipoprotein of *M. tuberculosis* interfered with IFN-γ-induced class II expression and Ag presentation in murine BM-macs, as well as impaired phagosome maturation [63,64•–66•]. In addition, the PAMPs lipopolysaccharide (LPS) and CpG DNA, were shown to impair class II MHC antigen presentation in human macrophages by chronic TLR signaling [67]. The membranes of macrophage phagosomes are rich in TLR2 [68] and, upon engagement at the cell surface, TLR2 can target its ligand from the cell surface to the lumen of the phagosome [69••]. The recruitment of TLR2 to the phagosome could enable PAMPs, such as the 19 kDa protein, to continually engage and stimulate the receptor, which may allow intracellular pathogens such as *Mycobacterium* to establish a productive niche within the macrophage.

Class II MHC peptide loading in dendritic cells

DCs survey peripheral tissues for invading pathogens. Immature DCs engulf pathogens, and deliver antigens

derived from these microbes to the nearest lymph node for presentation to T cells [70]. However, although immature DCs are efficient at endocytosing antigens/pathogens, they are incapable of generating class II–peptide complexes. Exposure of DCs to maturation signals (such as LPS) induces class II peptide loading and display at the cell surface [71,72]. In fact, upon maturation, DCs can present peptides derived from antigens internalized hours or even days before maturation [73–76]. Mature DCs stand out as the only APC capable of priming naïve T cells, which is their primary function.

Dendritic cell maturation

The maturation of DCs results in the redistribution of class II molecules and active cathepsins [77•] to peptide-loading compartments, and induces alterations in the architecture of these compartments to favor the deposition of class II–peptide complexes on the cell surface [74–76,78]. Furthermore, the generation of class II–peptide complexes in DCs is facilitated by the induced activation of the vacuolar proton pump upon DC maturation, which results in enhanced lysosomal acidification and protease activity [79••]. In immature DCs, a pool of class II molecules (not loaded with peptides) was observed within the internal vesicles of late endosomal/lysosomal multivesicular bodies (MVBs) resembling MIICs [74]. Upon DC stimulation and maturation, the internal vesicles storing presynthesized class II molecules were transferred to the MVB-limiting membrane rich in DM, and class II–peptide complexes were generated. Moreover, the reorganization of membrane also resulted in the tubular outgrowth of MIICs. The tubular compartments were directed towards the plasma membrane and formed class II carrying vesicles at their tips [74,78].

Visualization of class II molecules during dendritic cell maturation

The trafficking and redistribution of class II molecules upon DC maturation has been visualized in living cells. Class II molecules tagged with green fluorescent protein (GFP) were introduced into DC precursors using either retroviral transduction [76], or a knock-in approach [75]. DCs obtained from cultures supplemented with GM-CSF and IL-4 showed the presence of class II molecules in vesicular compartments, and also in tubular compartments capable of delivering class II molecules to the surface of the DC. In DCs exposed to antigen (ovalbumin or hen egg lysozyme), the addition of T cells specific for the appropriate antigen evoked the formation of long tubules, pointing directly at the interface of the T cell with the DC [75]. The DCs require a signal delivered via a TLR to be able to respond to a T cell in this fashion, as shown by the absence of tubulation upon administration of LPS-free antigen [80]. When DCs are loaded with synthetic peptide instead of whole protein antigen, the T-cell-evoked tubulation response is much reduced. This observation fits well the notion that recruitment of class II molecules from intracellular compartments to the cell surface results in their insertion at the plasma membrane in a discrete domain endowed with unique signaling properties [81].

Conclusions

The recruitment of peptide-loaded class II molecules from endocytic compartments to the cell surface is still the least understood step in the class II antigen presentation pathway. The presence of class II MHC molecules in, and their recruitment to, specialized membrane microdomains — be they sphingolipid-enriched, cholesterol-rich (lipid rafts) or stabilized by tetraspanins such as CD63 and CD81 — is functionally important. Many of the details surrounding the formation of these microdomains, and exactly how they participate in effective class-II-restricted antigen presentation, remain to be clarified. It is likely that class II molecules and the necessary co-stimulatory molecules are organized in the plasma membrane in a non-random manner, and that this process is tightly regulated. The use of genetic interference to examine changes in the architecture of the endocytic pathway (i.e. using siRNA-based approaches to manipulate endosomal complexes required for transport [ESCRT complexes]; [82,83]) or changes in the enzyme content of APCs, will cement further the ties between molecular cell biology and its application to class-II-restricted antigen processing.

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The studies described in [64•,65•] further characterize the mechanism by which MHC class II expression and antigen presentation are inhibited by mycobacterial PAMPs engaging TLR2 on the macrophage (originally described in [63]). Pai *et al.* [64•] demonstrate that the 19 kDa lipoprotein of *M. tuberculosis* inhibits IFN-γ induction of mRNA for the class II transactivator (CIITA), IFN regulatory factor-1 (IRF-1) and MHC class II in murine macrophages. This inhibition of IFN-γ signaling is independent of the suppressor of cytokine signaling (SOCS)1, and does not interfere with the activation of Stat1. Likewise, Gehring *et al.* [65•] demonstrated that prolonged signaling through TLR2 by the 19 kDa protein inhibited IFN-γ induced expression of HLA-DR1 and FcγR1 in THP-1 cells.

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Continuing from the data presented in [64•,65•], the authors further demonstrate that the loading of class I molecules with peptides in endocytic vesicles — a minor yet alternative pathway for class I antigen presentation — is also inhibited by mycobacterial PAMPs engaging TLR2, and that this inhibition is MyD88 dependent. The classical pathway of loading class I molecules in the ER with peptides derived in the cytosol by the proteosome was not impaired. Taken together, the above studies

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