Dendritic Cells Regulate Exposure of MHC Class II at Their Plasma Membrane by Oligoubiquitination

Guillaume van Niel,1,2 Richard Wubbolts,1,2 Toine ten Broeke,1 Sonja I. Buschow,1,2 Ferry A. Ossendorp,3 Cornелиs J. Melief,3 Graca Raposo,4 Bas W. van Balkom,1 and Willem Stoorvogel1,4*  
1Faculty of Veterinary Medicine  
Department of Biochemistry & Cell Biology  
Utrecht University  
P.O. Box 80.176  
NL-3508 TD, Utrecht  
The Netherlands  
2Department of Cell Biology  
University Medical Center  
Utrecht University  
Heidelberglaan 100  
3584 CX, Utrecht  
The Netherlands  
3Department of Immunohematology and Blood Transfusion  
Leiden University Medical Centre  
P.O. Box 9600  
2300 RC, Leiden  
The Netherlands  
4Institut Curie  
Centre National de la Recherche Scientifique-Unité Mixte de Recherche 144  
3584 CX, Utrecht  
France

Introduction

Dendritic cells (DCs) are crucial initiators of adaptive immune responses (Banchereau et al., 2000). Toward this task, they take up pathogens from peripheral tissues by endocytic mechanisms, and internalized proteins are proteolytically processed into peptides that can be loaded onto major histocompatibility complex (MHC) molecules. Pathogen products and other “danger” signals induce a DC maturation program that includes effective loading of MHC class II (MHC II) with pathogen-derived peptides in endosomes and transport of the MHC II-peptide complexes to the plasma membrane. At the same time, maturing DCs migrate from peripheral tissues to secondary lymphoid organs where they can present surface-exposed MHC II-peptide complexes to CD4+ T cells.

Multivesicular bodies (MVB) are specialized endosomal compartments that are composed of a single limiting membrane (LM) surrounding multiple luminal vesicles. The luminal vesicles (LV) are formed from the LM by budding inward, away from the cytosol, and accumulate MHC II in immature DCs (Kleijmeer et al., 2001). MVB fuse with lysosomes, and this pathway is crucial for the downregulation and degradation of many membrane proteins and thus may also explain the abundant presence of MHC II in lysosomes (Barois et al., 2002) and its relative short half-life in immature DCs (Villadangos et al., 2005). Alternatively, the MVB may fuse with the plasma membrane, releasing their LV, now termed exosomes (Thery et al., 2002). DC-derived exosomes have been shown to have immuno-modulatory characteristics, both in vitro and vivo (Thery et al., 2002).

In mature DCs, downregulation of newly synthesized MHC II via the MVB-lysosomal degradation pathway is likely to be inhibited, thereby increasing expression of MHC II at the cell surface. Although crucial, the molecular mechanism(s) of regulation for MHC II traffic within the endocytic tract of DCs has not been resolved (Trombetta and Mellman, 2005).

Both endocytosis and sorting of endocytosed proteins at MVB are often triggered by ubiquitination of their cytoplasmic domain (Staub and Rotin, 2006). It has recently been proposed that oligoubiquitination allows high-avidity interactions with endocytic adaptors (Barriere et al., 2006; Hawryluk et al., 2006) that facilitate clathrin-mediated endocytosis. After uptake, ubiquitinated cargo is sorted at the MVB into LV. This depends on the hepatocyte growth factor-regulated tyrosine kinase substrate Hrs/Vps27 and three protein complexes termed ESCRT-I, II, and III (endosomal sorting complex required for transport) that act sequentially in the sorting of ubiquitinated proteins at MVB (Babst, 2005).

We here report that in immature DCs, MHC II-β is oligoubiquitinated after the degradation of associated invariant chain (II) in endosomes. Ubiquitination of MHC II-β is inhibited in activated DCs, resulting in increased cell-surface expression. These observations indicate that antigen presentation by DCs is efficiently regulated through ubiquitination of MHC II-β.
Results

MHC II-β Is Ubiquitinated in the Endocytic Pathway
To study whether MHC II or MHC II-associated proteins are ubiquitinated, we used both primary bone marrow-derived DCs (BMDC) and D1 cells. The latter are long-term cultured myeloid mouse DCs that have been demonstrated in many aspects to have indistinguishable behavior from freshly isolated DCs (Winzler et al., 1997) and have the advantage that they can be cultured in large quantities without contaminating cell types. MHC II-α complexes were immunoprecipitated from cell lysates with different antibodies. From the eluate, ubiquitinated proteins were selectively reprecipitated with an antibody recognizing both mono- and oligoubiquitinated proteins, P4D1 (Haglund et al., 2003). Subsequent immunoblotting for MHC II-β or ubiquitin (Figure 1A) revealed a characteristic ladder of ubiquitinated protein bands, spaced at ~8 kDa between 50 and 70 kDa. The two major bands at 50 kDa and 58 kDa were also observed in parallel silver-stained gels (Figure 1B) and analyzed by liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS) (data not shown). Both ubiquitin and A2-β were detected with a sequence coverage of >40%, whereas MHC II-α was not detected in these samples. The positive identification of MHC II-β and ubiquitin at the same position by immunoblotting (Figure 1A) and mass spectrometry indicate ubiquitinated MHC II-β. Immunoblot analyses indicate ubiquitinated MHC II-β of total immunoprecipitated MHC II (Figure 1C) did not reveal a clear signal at 50–70 kDa, indicating that only a minor fraction of total MHC II-β is ubiquitinated. Ubiquitinated MHC II-β was detected both in D1 cells and in BMDC but not in BMDC from MHC II-β KO mice (Figure 1C). Together, these results show that MHC II-β is oligoubiquitinated in mouse DCs.

MHC II-β Is Ubiquitinated after β Processing in the Endocytic Pathway
Degradation of the two full-length β splice variants, p31 and p41, is initiated in endosomes at their exoplasmic domain, resulting in the subsequent formation of intermediate degradation products, p18 and p10. Next, processing of p10 results in the formation of the contiguous internal segment of β (CLIP). CLIP remains associated to the peptide binding groove of MHC II, thereby preventing binding of other peptides. Replacement of CLIP on MHC II by antigen-derived peptides is aided by an accessory molecule, DM, and peptide loading of MHC II occurs primarily within the endocytic tract. To narrow down the intracellular location of MHC II-β ubiquitination in relation to processing of associated β, MHC II was immunoprecipitated with either one of three antibodies with distinct specificities: M5/114 recognizes all MHC II, irrespective of associated β, Y3P recognizes MHC II complexes only after p31 or p41 processing, and 15G4 recognizes the CLIP domain of β (Beers et al., 2005). We further exploited the characteristic stability of MHC II complexes in SDS (Sadegh-Nasser and Germain, 1991). In the presence of SDS, MHC II-β dimers dissociated within 30 min at room temperature when associated with p31, p41, or CLIP, whereas prolonged incubations at 20 °C and heating to 100 °C were required to dissociate p10- and peptide-associated MHC II, respectively (Figures 2A and 2B). Collectively, these data suggested that MHC II-β is ubiquitinated only when associated to peptide-loaded MHC II (Figure 2B). This was confirmed by 2D gel analysis (Figure 2C). In the first dimension, ubiquitinated MHC II-β, which was immunoprecipitated from D1 and eluted at 20 °C, migrated as a 75–90 kDa complex. Ubiquitinated MHC II-β dissociated from these complexes at 100 °C as monitored in the second dimension. The fact that MHC II was ubiquitinated only after processing of β by lysosomal proteases excludes the possibility that MHC II-β was ubiquitinated in response to misfolding at the endoplasmic reticulum, for premature retrieval and proteasomal degradation. It also excludes the idea that ubiquitination of MHC II may be related to sorting at the trans-Golgi network. Given that ubiquitinated MHC II has been loaded with peptide, we anticipated that β processing may be required for ubiquitination of MHC II-β. To test this directly, we used the cysteine and serine protease inhibitors leupeptin and E64d to interfere with the processing of MHC II-associated β in endosomes and lysosomes (Neefjes and Ploegh, 1992; Neumann et al., 2001). In addition, we tested the effect of MG132, a proteasome inhibitor that is known to block ubiquitin-dependent sorting of membrane proteins at MVB (Longva et al., 2002), but also inhibits lysosomal proteases (Fuertes et al., 2003). All three reagents interfered with β processing, as indicated by the accumulation of the MHC II-associated β degradation intermediates, p18 and p10, and reduced MHC II peptide loading (Figure 3A). All three reagents also reduced the amount of ubiquitinated MHC II-β. Ubiquitination amounts correlated with the relative efficiencies of interference with β processing, indicating that β processing in the endocytic pathway is required for subsequent ubiquitination of MHC II-β.

Ubiquitination of MHC II-β Diminishes during DC Maturation
To test whether maturing DCs ubiquitinate MHC II-β, D1 were treated with lipopolysaccharide (LPS). Maturation of DCs was indicated by increased cell-surface expression of MHC II and costimulatory molecules (data not shown) and relative increase of SDS-resistant peptide-MHC II complexes (Figure 3B). According to these criteria, maturation was not yet observed after 2 hr of LPS treatment but was clearly evident after 24 hr. In contrast, ubiquitination of MHC II-β was diminished already after 2 hr and partially reappeared from 4 hr onward, whereas the total of MHC II slightly increased during 24 hr (Figure 3B). The amount of total ubiquitinated proteins in cell lysates remained constant up to 4 hr but was reduced at 24 hr (Figure 3B, left). Together, these observations indicate that ubiquitination of MHC II is selectively regulated and demonstrate a correlation between ubiquitination and intracellular retention of MHC II in immature DCs.

Intracellular Retention of MHC II Requires Ubiquitination of MHC II-β on Lysine 225
MHC II-β contains a single lysine residue at position 225, which is highly conserved among MHC II-β haplotypes and mammalian species. To test whether this residue is targeted by the ubiquitination machinery, we
expressed wild-type and mutant \(A_k^-\beta\), in which lysine 225 was replaced by an alanine in D1 cells. \(A_k^-\beta\) efficiently forms haplotype-mismatched heterodimeric complexes with \(A^\beta^-\alpha\), and surface expression of \(A_k^-\beta^-A^\beta^-\alpha\) dimers is as efficient as that of endogenous \(A^\beta^-\alpha^-A^\beta^-\alpha\) dimers (Layet and Germain, 1991). This was confirmed by the formation of SDS-stable \(A^\beta^-\alpha^-A^\beta^-\alpha\)-peptide complexes (data not shown) and a normal subcellular distribution of these complexes in transduced D1 cells (see below). Furthermore, the distribution of \(A^\beta^-\alpha\) in \(A^\beta^-\alpha\)-deficient BMDC expressing \(A_k^-\beta\) was indistinguishable from \(A_k^-\beta\) in wild-type BMDC (Figure 4A), indicating efficient rescue of the distribution of MHC class II in these transduced cells. Expressing \(A^\beta^-\alpha\) in D1 cells allowed us to simultaneously detect two distinct MHC II complexes in the same cells via \(A_k^-\beta\)- and \(A^\beta^-\alpha\)-specific antibodies.

Both transduced wild-type \(A^k^-\beta\) and mutant \(A^k^-\beta^{K225A}\) were detected by immunoblotting immunosolated \(A^\beta^-\alpha^-A^\beta^-\alpha\) complexes (Figure 4B). The signal for \(A^k^-\beta^{K225A}\) was much higher compared to that of wild-type \(A^k^-\beta\), probably as a result of a prolonged half-life of \(A^k^-\beta^{K225A}\) (see below). Transduced wild-type \(A^k^-\beta\) but not mutant \(A^k^-\beta^{K225A}\) was ubiquitinated, demonstrating linkage of oligoubiquitin to lysine 225. Transduced wild-type \(A^k^-\beta\) localized together with endogenous \(A^\beta^-\beta\) to intracellular DM-containing compartments (Figure 4C), again indicating that the exogenous mixed haplotype MHC II were properly assembled and targeted from the ER to endocytic compartments.

In contrast to endogenous \(A^\beta^-\beta\) and wild-type \(A^k^-\beta\), which in immature D1 cells colocalized to the same endocytic compartments, ubiquitination-deficient \(A^k^-\beta^{K225A}\) was primarily found at the plasma membrane (Figure 4C). These experiments also showed that the cells did not mature as a consequence of the transduction procedure, which was confirmed by the lack of the activation marker CD86 (data not shown). After LPS treatment, \(A^\beta^-\beta\), \(A^k^-\beta^{K225A}\), and \(A^k^-\beta^{K225A}\) were primarily present at the plasma membrane along with CD86 (data not shown), indicating that all constructs could be recruited from intracellular MHC II-containing compartments (MIIC) to the plasma membrane during maturation. Premature recruitment of \(A^k^-\beta^{K225A}\) to the plasma membrane was also observed in isolated BMDC from \(A^\beta^-\beta\) KO mice (Figure 4A). Together, these data indicate...
that ubiquitination of MHC II-β occurs at lysine 225 and is required for intracellular retention of MHC II in immature DCs.

**Ubiquitination of MHC II-β Stimulates Targeting to LV**
We anticipated that the lack of intracellular retention of A\(^{β-β}_{β} \text{[K225A]}\) could be due to a sorting deficiency at MVB. To test this directly, we performed immunoelectron microscopy on ultrathin cryosections of immature DCs ( Figure 5). Consistently with earlier observations (Kleijmeer et al., 2001), in nontransduced D1 most of the endogenous MHC II in MVB was associated with LV (74%) rather than with the LM (Figures 5Aa and 5B). In cells transduced for A\(^{β-β}_{β} \text{[WT]}\), similar amounts of A\(^{β-β}_{β} \text{[WT]}\) and endogenous MHC II were associated with LV (Figures 5Ab and 5B). In contrast, in cells transduced with A\(^{β-β}_{β} \text{[K225A]}\), only little (16%) was found in association with LV, whereas the distribution of endogenous A\(^{β-β}_{β}\) was only slightly affected (Figures 5Ac and 5B). Together, these data indicate that ubiquitination of MHC II-β is required for sorting at MVB. Failure of ubiquitination would result in transfer by default to the plasma membrane.

**Ubiquitination of MHC II-β Is Required for Efficient Endocytosis of MHC II**
In immature but not in mature DCs, MHC II is efficiently taken up from the plasma membrane by endocytosis and targeted to MIIC (Chow et al., 2002; Villadangos et al., 2001). Lack of intracellular retention of A\(^{β-β}_{β} \text{[K225A]}\) could thus also result from inefficient endocytosis. To study endocytosis, we took advantage of the possibility to compare simultaneous uptake of endogenous A\(^{β}\) and transduced mutant or wild-type A\(^{β-β}_{β}\) in single cells with fluorescence microscopy. Transduced D1 cells were allowed to internalize labeled monoclonal antibodies that specifically recognize A\(^{β-β}_{β}\) or A\(^{β-β}_{β}\) for 1 hr at 37°C. As expected, in wild-type MHC II-expressing cells, endocytosed antibodies were targeted via early endosomes that labeled for early endosome antigen 1 (EEA1) to DM-positive MIIC. Mutant A\(^{β-β}_{β} \text{[K225A]}\) seemed to be delayed in the transfer from EEA1-positive to DM-positive structures (Figure 6A). To quantify relative uptake of the two haplotypes, the amounts of fluorescence in selected areas representing either plasma membrane or intracellular structures were determined in confocal sections (Figure 6B). The relative efficiencies of uptake were then calculated as a ratio of the relative fluorescence intensities at the plasma membrane (A\(^{β-β}_{β}\)/A\(^{β-β}_{β}\)) with that at intracellular membranes (A\(^{β-β}_{β}\)/A\(^{β-β}_{β}\)). A\(^{β-β}_{β}\) was endocytosed with a similar efficiency as endogenous A\(^{β-β}_{β}\) (ratio 1.1 ± 0.24). In contrast, much less A\(^{β-β}_{β} \text{[K225A]}\) was detected intracellularly as compared to labeled A\(^{β-β}_{β}\) (ratio 5.5 ± 1.4). This may be explained either by a reduced rate of uptake or accelerated recycling of A\(^{β-β}_{β} \text{[K225A]}\). The latter explanation is plausible considering that lack of sorting at MVB of endocytosed mutant MHC II might well result in accelerated recycling to the plasma membrane. To distinguish between these possibilities, antibodies were endocytosed at 20°C, a temperature at which trafficking of endocytosed MHC II to late endosomes and recycling to the plasma membrane are severely hampered (Harding and Unanue, 1990). Indeed, after 1 hr uptake at 20°C, most endocytosed antibodies reached EEA1-positive early endosomes rather than DM-positive MIIC (Figure 6A). Again, wild-type A\(^{β}\) was taken up as efficiently as endogenous A\(^{β}\) (ratio 1.3 ± 0.25). At this temperature, uptake of A\(^{β-β}_{β} \text{[K225A]}\) was less than 50% efficient compared to endogenous A\(^{β-β}_{β}\) (ratio 2.8 ± 0.77; Figures 6B and 6C). Thus, ubiquitination of MHC II-β is not only required for sorting at MVB but also for efficient endocytosis. Together, these processes are responsible for intracellular retention of MHC II in immature DCs.
Discussion

We here demonstrate that in immature DCs a unique and conserved lysine residue within the cytoplasmic domain of MHC II-β is oligoubiquitinated after proteolytic processing of MHC II-associated Ii. Ubiquitination of mature MHC II is required both for efficient uptake from the plasma membrane and sorting at MVB into LV. Together, these two sorting steps result in efficient intracellular retention of peptide-loaded MHC II, thereby preventing premature antigen presentation by immature DCs. In mature DCs, increased expression of MHC II-peptide complexes at the plasma membrane has been attributed to several processes: (1) increased transcription of MHC II, (2) increased Ii processing and MHC II peptide loading (Pierre and Mellman, 1998; Trombetta et al., 2003), and (3) extended half-life of MHC II resulting from decreased endocytosis rates (McCormick et al., 2005; Wilson et al., 2004) and recruitment of MHC II from MIIC toward the plasma membrane (Boes et al., 2002; Chow et al., 2002; Kleijmeer et al., 2001). We here demonstrate that the recruitment of peptide-loaded MHC II to the plasma membrane can be achieved by blocking MHC II-β ubiquitination.

Endocytosis of mature MHC II occurs via clathrin-mediated endocytosis (Dugast et al., 2005; McCormick et al., 2005; Potolicchio et al., 2005), and a conserved dileucine-based signal in the COOH terminus of the cytoplasmic domain of MHC II-β is implicated (Zhong et al., 1997). Elimination of this signal reduced the uptake rate but did not block endocytosis of MHC II. This is consistent with a second endocytosis signal for mature MHC II, which we here identified as an oligoubiquitin tag. Conversely, continued but reduced uptake of MHC II-βK225A in our study may be explained by the dileucine endocytosis motif. In general it is thought that monoubiquitination rather than polyubiquitination is required to downregulate cell-surface receptors by delivering these proteins for lysosome-mediated degradation through the endocytic pathway (Staub and Rotin, 2006). However, it has recently been demonstrated that recruitment by tandem ubiquitin-interacting motifs in clathrin adaptors is much more efficient for oligoubiquitinated than for monoubiquitinated membrane proteins (Barriere et al., 2006; Hawryluk et al., 2006). In addition to peptide-loaded MHC II, DCs may also express Ii-associated MHC II (McCormick et al., 2005; Dugast et al., 2005 and references therein) and empty MHC II (Potolicchio et al., 2005 and references therein) at their plasma membrane. We here demonstrate that Ii-associated MHC II is not ubiquitinated, explaining why surface-exposed MHC II-II complexes are endocytosed predominantly through the interaction of two leucine-based signals encoded on the cytoplasmic domain of Ii with the clathrin adaptor AP-2 (McCormick et al., 2005; Dugast et al., 2005 and references therein). Surface-exposed empty MHC II lacks Ii-encoded internalization signals but can be endocytosed via the dileucine motif on MHC II-β. Although we show that almost all ubiquitinated MHC II was peptide loaded, we cannot exclude the possibility that also empty MHC II can be ubiquitinated, thereby facilitating endocytosis of empty MHC II.
Monoubiquitination of membrane proteins has particularly been implicated in sorting at MVB (Babst, 2005). Our finding that oligoubiquitination can serve as a sorting signal for MHC II-β at MVB is, however, not unprecedented, as illustrated by oligoubiquitination of MHC I in Kaposi’s sarcoma-associated herpes virus-infected cells, which results in efficient endocytosis and endolysosomal degradation (Duncan et al., 2006). After uptake of ubiquitinated cargo, sorting at MVB depends on the function of a group of conserved proteins that were originally identified in Saccharomyces cerevisiae (reviewed in Babst, 2005; Hurley and Emr, 2006; Staub and Rotin, 2006). These include Hrs/Vps27 and the constituents of three heteromeric protein complexes called ESCRT-I, II, and III that act sequentially in sorting at MVB.

Consistent with the observation that endogenous MHC II is ubiquitinated after peptide loading, we observed that also MHC II-βK225A is capable of forming SDS-stable MHC II-peptide complexes (data not shown). This is supported by the observation that B cells expressing MHC II-βK225A were capable of presenting antigens to T cells (Laufer et al., 1997) and is also in concordance with our observation that ubiquitination of MHC II is diminished in response to LPS treatment.

Our finding that LPS and other maturation stimuli (data not shown) rapidly reduce ubiquitination of MHC II suggests strict regulation of this modification and can explain the stable expression and increased half-life of MHC II in maturing DCs. Ubiquitination of MHC II-β in DCs has not been observed previously, which can be explained by the relative small amount of ubiquitinated MHC II-β at steady-state conditions. We favor the idea that cycles of ubiquitination/deubiquitination may occur both at the plasma membrane and endosomes, reminiscent of membrane proteins whose surface expression is regulated either by mono- or polyubiquitination, including several ion channels, tyrosine kinase receptors, cytokine receptors, and T cell and IgE receptors.

Ideally, we would have liked to study whether forced ubiquitination of MHC class II in mature DCs would rescue its endocytosis and sorting at MVB. However, forced expression of ubiquitin ligases or ubiquitin chimeras may target membrane proteins into the MVB pathway, irrespective of the physiological relevance. For example, we considered studying the behavior of transduced MHC II-β-ubiquitin chimeric complexes. In our opinion, however, permanent C-terminal monoubiquitin-tagged MHC II-β can be expected to behave

Figure 4. A<sup>α</sup>-β<sup>K225A</sup> Is Not Ubiquitinated and Targeted to the Plasma Membrane

(A) BMDC from either WT or A<sup>α</sup>-β-deficient mice were transduced for either A<sup>α</sup>-β<sup>WT</sup> or A<sup>α</sup>-β<sup>K225A</sup> as indicated. Fixed cells were immunodouble labeled for DM (red) and A<sup>β</sup> or A<sup>α</sup> (green) as indicated and analyzed by CSLM. The figures are representative for three independent experiments.

(B) Nontransduced D1 cells (control) or D1 transduced for either A<sup>α</sup>-β<sup>WT</sup> or A<sup>α</sup>-β<sup>K225A</sup> were lysed. Endogenous MHC II was immunoprecipitated (IP) with M5/114 and A<sup>β</sup>-α<sup>-</sup>A<sup>α</sup>-β complexes with 10.2.16. Precipitates were eluted as indicated at 100°C and immunoblotted (Odorizzi et al., 1994) for MHC II-β or ubiquitin. The figure is representative for two independent experiments.

(C) D1 cells were transduced for either A<sup>α</sup>-β<sup>WT</sup> (left) or A<sup>α</sup>-β<sup>K225A</sup> (right) and after fixation, immunodouble labeled for A<sup>α</sup> (green) and DM (red) (top) or for A<sup>α</sup> (green) and endogenous A<sup>α</sup> (red) (bottom). The figures are representative for four independent experiments.
differently from transient lysine oligoubiquitin-tagged MHC II-β. First, as indicated above, monoubiquitinated proteins behave differently compared to oligoubiquitinated proteins with respect to endocytosis and MVB sorting. Second, deubiquitination of membrane proteins is crucial for sorting at MVB (Agromayor and Martin-Serrano, 2006). Fusing a tag to the MHC II tail would also bypass the elegant switch of sorting motifs that we disclose in this paper. The endosomal sorting motif present in the II is removed in endosomes but is replaced by the ubiquitin tag (in concert with the double LL motif in the MHC II-β tail) that drives two distinct sorting events, at the plasma membrane and at the MVB LM. Finally, chimeric ubiquitin tagging of membrane proteins that normally recycle, such as the transferrin receptor, results in accelerated MVB sorting and lysosomal degradation irrespective of physiological function (Raiborg et al., 2002). An alternative approach would be to force the expression of a ubiquitin ligase. However, as illustrated in a recent paper (Ohmura-Hoshino et al., 2006), MHC II can be downregulated already after 1–2 hr of LPS treatment, a condition at which II processing was rather accelerated (Figure 3B and data not shown). In our opinion, the machinery responsible for regulating MHC class II ubiquitination can be identified by functional interference only. This complex task is subject to current research.

In conclusion, the described molecular mechanism for regulating surface expression of MHC II is essential for DCs to modulate antigen presentation in response to pathogens. It can be envisioned that artificial interference with MHC II ubiquitination can be a powerful approach to modulate the immune system for treatment of cancer, vaccination, or treatment of autoimmune disease.

Experimental Procedures

Reagents

D1, an immature DC line from C57Bl/6 mice, was cultured as described (Winzler et al., 1997). BMDCs were isolated as described (Lutz et al., 1999) from the bone marrow of WT C57BL/6 mice or B6.129-H2-Ab1tm1Gru -/-N12 (Taconic, Denmark), an Aβ-deficient C57BL/6 mouse (Grusby et al., 1991). LPS (Escherichia coli, serotype 026:B6) was used at 10 μg/ml, Leupeptin at 100 μg/ml, MG132 at 20 μM, and E64 at 100 μM (all from Sigma). Rabbit polyclonal antibodies directed against the cytoplasmic domains of DM (Barois et al., 1998), MHC II-β, and II (Barois et al., 1997) were from N. Barois (University of Oslo, Oslo, Norway). Anti-mouse MHC II M5/114 (Bhattacharya et al., 1981) and 15G4, directed against Aβ/CLIP (Nakagawa et al., 1999) was a gift of A. Rudensky. HRP-conjugated and agarose bead-conjugated anti-ubiquitin P4D1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rat anti-mouse isotype control was ugat.
purchased from Serotec (Raleigh, SC), and anti-tubulin 1A2 was from Sigma.

Protein Isolation and Analyses
For immunoprecipitations, DCs were lysed at 4°C in 1% Triton X100, 1 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8), 10 mM N-ethylmaleimide (Sigma), and complete protease inhibitor mix (Roche Molecular Biochemicals, Almere, The Netherlands), and nuclei were removed by centrifugation. Immunoprecipitations were performed as indicated in the figure legends. Protein G-agarose beads (Sigma Aldrich, Zwijndrecht, The Netherlands) were precoupled to either Y3P, M5/114, 15G4, or IgG2b rat anti-mouse isotype control antibodies in lysis buffer, added to the cell lysates, and gently mixed for 1 hr at 4°C. The beads were then washed, and immunoprecipitated MHC II was eluted either in SDS sample buffer (2% SDS, 62.5 mM Tris-HCl [pH 6.8], 10% Glycerol) for 30 min or 16 hr at room temperature or 5 min at 100°C. When indicated, MHC II was eluted in 1% SDS at 100°C for subsequent immunoprecipitation of ubiquitinated proteins. For this goal, the eluate was diluted 10 times in lysis buffer and precleared for nonspecific binding with nonrelevant IgG2-conjugated agarose beads. Supernatants were collected and incubated with anti-ubiquitin P4D1 conjugated agarose beads or control antibody-conjugated beads, both from Santa Cruz Biotechnology for 1 hr at 4°C. Nonspecifically associated proteins were removed by washing in 4 M Urea, after which ubiquitinated proteins were eluted off the beads by boiling in SDS sample buffer. Western blotting was performed according to standard techniques. For mass spectrometric analyses, protein bands were subjected to in-gel tryptic digestion and subsequent LC-MS/MS analysis as described previously (van Balkom et al., 2005). The data files obtained from LC-MS/MS experiments were converted into peaklist files (*.pkl files) with ProteinLynx Browser version 2.1 software (Micromass) and subsequently submitted to the Mascot search software (Matrix Science, London, UK) (Perkins et al., 1999). The allowed peptide mass tolerance was 0.4 Da, fragment mass tolerance of 0.2 Da, allowing 2 missed cleavages, to search the mouse IPI database version 3.11. (Kersey et al., 2004).

Constructs and Retroviral Transduction of DCs
AKβ WT and AKβ K225A cDNA in pRc/CMV vector were kindly provided by T. Laufer (Harvard School of Public Health, Boston, MA) (Laufer et al., 1997). cDNAs were amplified by PCR and cloned into pQIXN (Clontech, Mountain View, USA), and sequences were verified. Retroviral particles were produced by 293T Phoenix-Eco cells after cotransfection with the pQ vectors and pCL-Eco (provided by H. Rozemuller, Utrecht Medical Centre, Utrecht). Harvested viruses were filtered, mixed with polybrene (4 μg/ml, Sigma), and used to infect either D1 or BMDC by spin occlusion 7 days after isolation. Transduced BMDC were analyzed after 2 days of expression by immunofluorescence or FACS, and transduced D1 were maintained as stable cell cultures.

Microscopy
Cells grown on glass coverslips were fixed and immunolabeled for DM (2E5A, BD Biosciences, San Jose, CA), for Ab-α (Y3P), for AKβ for 1 hour at 37°C or 20°C as indicated. Cells were washed and fixed, and images were acquired by CSLM. Areas representative for plasma membrane (asterisk) or intracellular structures (dot) were encircled (see indicated examples), and the integrated densities of fluorescence within these areas was determined for 10 distinct samples for each condition.

Figure 6. K225 at MHC II-β Is Required for Efficient Internalization of MHC II from the Plasma Membrane
D1 cells were transduced for either AKβ WT or AKβ K225A.


References


Barois, N., Forquet, F., and Davoust, J. (1998). Actin microfilaments are within 20 nm of the LM and with no cross-sectioned LM in the direct vicinity.

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