Ubiquitination by March-I prevents MHC class II recycling and promotes MHC class II turnover in antigen-presenting cells

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MHC class II (MHC-II)-dependent antigen presentation by antigen-presenting cells (APCs) is carefully controlled to achieve specificity of immune responses; the regulated assembly and degradation of antigenic peptide–MHC-II complexes (pMHC-II) is one aspect of such control. In this study, we have examined the role of ubiquitination in regulating pMHC-II biosynthesis, endocytosis, recycling, and turnover in APCs. By using APCs obtained from MHC-II ubiquitination mutant mice, we find that whereas ubiquitination does not affect pMHC-II formation in dendritic cells (DCs), it does promote the subsequent degradation of newly synthesized pMHC-II. Acute activation of DCs or B cells terminates expression of the MHC-II E3 ubiquitin ligase March-I and prevents pMHC-II ubiquitination. Most importantly, this change results in very efficient pMHC-II recycling from the surface of DCs and B cells, thereby preventing targeting of internalized pMHC-II to lysosomes for degradation. Biochemical and functional assays confirmed that pMHC-II turnover is suppressed in MHC-II ubiquitin mutant DCs or by acute activation of wild-type DCs. These studies demonstrate that acute APC activation blocks the ubiquitin-dependent turnover of pMHC-II by promoting efficient pMHC-II recycling and preventing lysosomal targeting of internalized pMHC-II, thereby enhancing pMHC-II stability for efficient antigen presentation to CD4 T cells.

MHC class II | antigen processing and presentation | ubiquitination | recycling | March-I

Major histocompatibility complex class II (MHC-II) molecules are expressed by professional antigen-presenting cells (APCs) and are necessary for the presentation of antigenic peptides to CD4 T cells and the subsequent initiation of an adaptive immune response (1). In their immature state, dendritic cells (DCs) are relatively poor stimulators of naïve T cells. During infection, DCs in the periphery are triggered by exposure to microbial agents or inflammatory mediators to increase their expression of lymph node-homing receptors, MHC-II, and costimulatory molecules including CD80 and CD86 (2). The process of “activation” poises peripheral DCs into a state of readiness needed for efficient recognition by antigen-specific naïve CD4 T cells.

Expression of antigenic peptide–MHC-II complexes (pMHC-II) on the surface of APCs is essential for antigen presentation and the initiation of adaptive immune responses, and it is therefore not surprising that surface expression and transport of these complexes are tightly regulated in APCs (3). Newly synthesized MHC-II binds to a chaperone protein termed the Invariant chain (Ii) that remains associated with MHC-II during transport through the *trans*-Golgi network to the plasma membrane. Once at the plasma membrane, MHC-II–Ii complexes are rapidly internalized by clathrin-mediated endocytosis and traffic from early endosomes to late endosomal multivesicular antigen-processing compartments. It is in these compartments that MHC-II–associated Ii is degraded, immunogenic peptides bind to MHC-II, and newly generated pMHC-II complexes traffic to the plasma membrane (4). Surface-expressed pMHC-II slowly internalizes, and like all internalized integral membrane proteins (5), internalized pMHC-II can (in theory)

either recycle back to the plasma membrane or traffic to lysosomes for degradation. Indeed, pMHC-II has been shown to internalize and recycle in DCs (6) and B cells (7), however the relative contribution of these pathways to the maintenance of surface levels of pMHC-II remains to be determined.

Large amounts of pMHC-II reside in intracellular antigen-processing compartments in immature DCs (6, 8). DC activation largely depletes the internal pool of pMHC-II and dramatically increases surface pMHC-II expression, most likely due to both activation-dependent transport of internal pMHC-II to the plasma membrane (9, 10) and activation-induced neosynthesis of MHC-II (6, 11). More recently, ubiquitination of MHC-II has been identified as an important regulator of MHC-II expression in APCs (12). MHC-II is oligo-ubiquitinated on single lysine residue that is present in the cytoplasmic tail of the MHC-II β -chain (K₂₂₅ on mouse I-A^b). Mutation of this lysine decreased localization of MHC-II to the internal vesicles of multivesicular antigen-processing compartments, and it has been suggested that ubiquitination of MHC-II is involved in several distinct intracellular trafficking steps including internalization, endocytic trafficking, targeting to antigen-processing compartments, and targeting to lysosomes for degradation (13, 14).

The membrane-associated E3 ubiquitin ligase March-I mediates ubiquitination of MHC-II and CD86 in DCs and B cells (15, 16). Activation of DCs using a variety of Toll-like receptor ligands suppresses March-I mRNA expression, suppresses MHC-II ubiquitination, and prolongs MHC-II half-life (6, 8, 17), demonstrating that activation of DCs terminates expression of March-I, which ultimately enhances MHC-II expression. Selective ubiquitination of

Significance

Expression of MHC class II–peptide complexes (pMHC-II) on the surface of antigen-presenting cells (APCs) is required for a wide variety of CD4 T-cell-dependent immunological processes. Regulation of pMHC-II biosynthesis and degradation are, therefore, essential for optimal APC function. We now show that ubiquitination of pMHC-II by the E3 ubiquitin ligase March-I prevents pMHC-II recycling and targets newly synthesized pMHC-II for lysosomal degradation in resting dendritic cells and B cells. Activation of these cells terminates March-I expression, terminates pMHC-II ubiquitination, and prevents pMHC-II degradation by promoting efficient pMHC-II recycling. Our study demonstrates that rapid recycling “spares” internalized pMHC-II from degradation in activated APCs, thereby increasing the probability of efficient pMHC-II interaction with CD4 T cells.

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MHC-II by March-I promotes degradation of internalized pMHC-II in immature DCs, revealing a direct role for ubiquitination in regulating the stability of pMHC-II in DCs (18).

In this study, we have examined the role of ubiquitination in regulation of pMHC-II plasma membrane expression in DCs and B cells. We found no correlation between the capacity for pMHC-II ubiquitination and the overall rate of pMHC-II endocytosis in these cells. However, ubiquitination did prevent the recycling of intracellular, internalized pMHC-II back to the plasma membrane. Furthermore, we found that ubiquitination regulated pMHC-II expression by promoting the rapid degradation of newly synthesized pMHC-II complexes generated in immature DCs. Our data demonstrate that ubiquitination serves as a molecular switch that diverts constitutively recycling pMHC-II to a pathway of lysosomal degradation in both DCs and B cells, thereby providing a mechanism for the selective turnover of pMHC-II complexes in resting but not activated APCs.

Results

pMHC-II Ubiquitination by March-I Is Terminated by Activation of DCs or B Cells. To begin to investigate the relationship between pMHC-II ubiquitination, pMHC-II transport, and surface pMHC-II expression in APCs, we determined the ubiquitination status of pMHC-II in resting and activated DCs or B cells. pMHC-II ubiquitination was almost completely inhibited by LPS-induced activation of spleen B cells, bone marrow-derived DCs (BMDCs), or spleen DCs (Fig. 1 *A* and *B*). When normalized for total expression of MHC-II in each cell type, pMHC-II in freshly isolated spleen B cells and DCs was ubiquitinated to a much greater extent than pMHC-II in BMDCs. As was observed previously (19), pMHC-II isolated from spleen B cells has a short ubiquitin chain length (generally one and two ubiquitin moieties per molecule), whereas ubiquitin chain length of pMHC-II in BMDCs was longer (usually four and five ubiquitin moieties per molecule). We did not observe dramatic differences in ubiquitin chain length in pMHC-II isolated from spleen B cells and spleen DCs. The profound reduction in pMHC-II ubiquitination observed upon spleen B-cell and DC activation mirrored the LPS-induced reduction in March-I mRNA expression in these cells as determined by quantitative RT-PCR (Fig. 1 *C*). By comparing pMHC-II ubiquitination in wild-type BMDCs, March-I knockout (KO) BMDCs, and MHC-II K₂₂₅R ubiquitination mutant BMDCs, we confirmed that K₂₂₅ ubiquitination by March-I was responsible for essentially all pMHC-II ubiquitination (Fig. 1 *D*). These data indicate that pMHC-II is ubiquitinated in resting B cells and DCs by March-I and that activation of these cells reduces March-I expression, resulting in an almost complete block in pMHC-II ubiquitination.

pMHC-II Endocytosis Rates Are Identical in Resting and Activated DCs or B Cells. The dramatic presence of intracellular MHC-II in immature but not in mature DCs has been proposed to be a consequence of selective rapid MHC-II endocytosis in immature DCs (6, 20) and that rapid endocytosis is regulated by ubiquitination of MHC-II by the E3 ubiquitin ligase March-I (13, 14). In contrast to DCs, we are unaware of any study comparing MHC-II endocytosis rates in resting or activated B cells. In most studies, "MHC-II endocytosis" is measured by binding anti-MHC-II mAb to APCs on ice and monitoring the loss of mAb itself (and not MHC-II) from the cell surface. To directly measure transport of the pMHC-II molecule in APCs, we developed biochemical assays in which the fate of covalently tagged surface MHC-II molecules could be followed. Cell surface proteins on APCs were covalently tagged using sulfo-NHS-SS-biotin on ice, the cells were washed, and the APCs were recultured on ice or at 37 °C. At various times, the remaining surface biotin was removed by incubation of cells on ice with membrane-impermeable reduced glutathione. Following cell lysis, immunoprecipitation using the pMHC-II mAb Y3P, and SDS/PAGE/transfer to membranes, blotting with HRP-conjugated

Fig. 1. APC activation inhibits ubiquitination of pMHC-II in DCs and B cells. (*A* and *B*) Spleen B cells, BMDCs, and spleen DCs were left on ice (untreated) or treated with LPS overnight at 37 °C. The cells were lysed and pMHC-II immunoprecipitates were analyzed by blotting for ubiquitinated MHC-II or total MHC-II β -chain. The number of ubiquitin chains attached to the MHC-II β -chain was estimated based on a molecular weight of 7 kDa per ubiquitin moiety. (*B*) The relative amount of pMHC-II ubiquitination in spleen DCs, spleen B cells, and BMDCs was expressed as a percentage of that observed in freshly isolated spleen DCs. The data were normalized to control for the total amount of pMHC-II present in each sample. The data shown are the mean $\pm$ SD obtained from three independent experiments. (*C*) The amount of March-I mRNA present in resting or in vitro-activated spleen DCs and B cells was quantitated by RT-PCR and was expressed relative to the amount present in resting (freshly isolated) spleen DCs. Each data point was normalized to the amount of GAPDH present in the sample. The data shown are the mean $\pm$ SD obtained from three independent experiments; *** P < 0.001. (*D*) The ubiquitination status of pMHC-II isolated from immature BMDCs obtained from wild-type, MHC-II K₂₂₅R ubiquitination mutant, or March-1 KO mice was determined by immunoprecipitation and immunoblot analysis.

avidin revealed the amount of glutathione-resistant (i.e., intracellular) biotinylated pMHC-II present inside the cell.

Quantitative densitometry revealed that this procedure removed >99% of the biotin tag from pMHC-II isolated from cells cultured on ice. We did not find any significant difference in the rate of pMHC-II endocytosis in immature and mature BMDCs (Fig. 2 *A* and *B* and Fig. S14). Analysis of pMHC-II endocytosis in spleen B cells showed that whereas endocytosis of MHC-II was slightly more robust in B cells than in DCs, there was no difference in the rate of pMHC-II endocytosis in resting B cells and activated B cells (Fig. 2 *C* and Fig. S18). To determine whether selective ubiquitination in immature APCs affects pMHC-II endocytosis, we examined pMHC-II internalization in wild-type and MHC-II K₂₂₅R ubiquitination mutant APCs. We found no difference in the fractional amount of surface pMHC-II internalized in either wild-type or K₂₂₅R BMDCs (Fig. 2 *D*) or spleen B cells (Fig. 2 *E*). These data demonstrate that activation of either DCs or B cells does not significantly alter pMHC-II endocytosis and that pMHC-II endocytosis is ubiquitin-independent.

Activation of DCs or B Cells Promotes pMHC-II Recycling. Cell surface labeling studies have shown that surface pMHC-II complexes turn over rapidly in immature DCs and very slowly in mature DCs (6, 18). Because APC activation did not alter pMHC-II endocytosis rates in DCs or B cells, we set out to examine the fate of internalized pMHC-II in these different APC subtypes. Based primarily on data obtained using mAb-tagged MHC-II molecules, it has been proposed that MHC-II recycles efficiently in immature DCs and that DC activation inhibits pMHC-II recycling (6). To follow the recycling of the pMHC-II molecule

Fig. 2. Ubiquitination does not affect pMHC-II endocytosis. Immature or in vitro-activated APCs were reversibly biotinylated on ice, and pMHC-II endocytosis was assayed as described in Materials and Methods. (A) Biotinylated immature BMDCs were cultured on ice ($t = 0$) or at 37°C for various times. The cells were incubated in the absence or presence of glutathione on ice to remove surface-exposed biotin. Cells were lysed, and pMHC-II was isolated by immunoprecipitation and analyzed by SDS/PAGE and blotting for either biotin-labeled pMHC-II or total MHC-II. Only a fraction of the pMHC-II immunoprecipitate from cells incubated in the absence of glutathione on ice ($t = 0$) was loaded on the gel to assist in quantitative analysis of the blots. (B and C) The kinetics of pMHC-II endocytosis in immature (triangles) and in vitro-matured (squares) BMDCs (B) or resting (triangles) or in vitro-activated (squares) spleen B cells (C) was determined. The data shown are the mean $\pm$ SD obtained from three independent experiments. (D and E) The amount of surface pMHC-II internalized after a 15-min chase at 37°C in wild-type or MHC-II K₂₂₅R ubiquitination mutant immature BMDCs (D) or spleen B cells (E) was quantitated using our endocytosis assay. The data shown are the mean $\pm$ SD obtained from three independent experiments.

itself (and not mAb), we once again used our reversible biotinylation assay. Surface-biotinylated proteins on B cells or DCs were allowed to internalize for 30 min at 37°C , after which time the cells were returned to ice and the remaining surface biotin moieties were removed by treatment with reduced glutathione. This procedure left a cohort of biotinylated molecules inside the endosomal system of each cell type. Return of internalized molecules to the plasma membrane was assayed by reculturing the cells at 37°C , and at various times, the cells were once again treated (or not) with reduced glutathione on ice. Under these conditions, blotting of pMHC-II mAb immunoprecipitates with HRP-conjugated avidin revealed a pool of pMHC-II that recycled back to the cell surface (as the difference in signal between untreated cells and reduced glutathione-treated cells).

Using this assay, we found that internalized pMHC-II recycles poorly in immature BMDCs, however pMHC-II recycles efficiently and rapidly in mature BMDCs (Fig. 3A and Fig. S2). A similar result was obtained when we examined pMHC-II recycling in freshly isolated “immature” spleen DCs from mice injected with PBS alone and “mature” DCs isolated from mice injected with CpG DNA (Fig. 3B). Activation-dependent enhancement of pMHC-II recycling was not restricted to DCs, as we found that pMHC-II recycles poorly in resting spleen B cells and activation of these cells with LPS results in very efficient pMHC-II recycling. These data show that internalized pMHC-II does not reappear at the plasma membrane in resting APCs and that APC activation stimulates pMHC-II recycling.

pMHC-II Recycling Is Regulated by Ubiquitination of pMHC-II. The rapid turnover of MHC-II in immature DCs and resting B cells has been attributed in part to selective ubiquitination of K₂₂₅ of

the MHC-II β -chain by March-I (15, 18). We therefore asked whether the failure of internalized pMHC-II to recycle in immature APCs was due to ubiquitination by March-I. In contrast to the results obtained when examining pMHC-II recycling in wild-type BMDCs, pMHC-II expressed in ubiquitination mutant immature BMDCs efficiently recycled to the plasma membrane (Fig. 4A). pMHC-II recycling was identical in both immature and mature ubiquitination mutant BMDCs (Fig. S3), confirming that the alteration in pMHC-II recycling observed upon DC maturation was ubiquitin-dependent. Essentially identical results were obtained when examining pMHC-II recycling in freshly isolated spleen DCs (Fig. 4B) or spleen B cells (Fig. 4C); internalized pMHC-II efficiently recycled back to the plasma membrane only in mature DCs or in immature APCs isolated from MHC-II ubiquitination mutant mice. These results demonstrate that the cellular machinery required for efficient pMHC-II recycling exists in both resting and activated APCs, however selective pMHC-II ubiquitination in immature DCs and resting B cells prevents internalized pMHC-II from recycling.

Ubiquitination Promotes Degradation of Newly Synthesized pMHC-II in Immature DCs.

It has been well established that pMHC-II expressed on the surface of immature DCs has a relatively short half-life, whereas pMHC-II expressed on the surface of mature DCs is long-lived (6, 18). However, it is not known if DC activation alters the turnover of MHC-II molecules synthesized in DCs prior to the activation stimulus. To address this question, immature BMDCs were pulse-labeled with [^{35}S]-methionine, washed, and chased for 2 h to allow MHC-II to traffic to antigen-processing compartments and generate pMHC-II complexes. After this short chase period, either PBS or LPS was added to the cells, and after an additional 18 h of culture, newly synthesized pMHC-II stability was determined by SDS/PAGE and autoradiography. Small amounts of pMHC-II were generated during the pulse-labeling period, and the amount of pMHC-II generated increased during the 2-h chase period (Fig. 5A). Almost all newly synthesized pMHC-II complexes generated in immature DCs were degraded during the 18-h chase period, however the addition of LPS to the culture during the 18-h chase period dramatically increased pMHC-II complex recovery.

Fig. 3. APC activation stimulates pMHC-II recycling in DCs and B cells. (A) Immature or LPS-matured BMDCs were reversibly biotinylated on ice, and pMHC-II recycling was assayed as described in Materials and Methods. BMDCs possessing internalized biotinylated pMHC-II were cultured on ice ($t = 0$) or at 37°C for various times. The cells were placed on ice and incubated in the absence or presence of glutathione to remove surface-exposed biotin. Cells were lysed, and pMHC-II was isolated by immunoprecipitation and analyzed by SDS/PAGE and blotting for either biotin-labeled pMHC-II or total MHC-II. The data shown are the mean $\pm$ SD obtained from three independent experiments; $*P < 0.05$. (B) The reappearance of pMHC-II internalized in immature (I) and LPS-matured (M) BMDCs, freshly isolated (immature, I) and in vitro-activated (mature, M) spleen DCs, or resting (R) and in vitro-activated (A) spleen B cells after a 15-min chase period at 37°C was quantitated using our recycling assay. In each panel, the data shown are the mean $\pm$ SD obtained from three independent experiments; $*P < 0.05$.

Fig. 4. Ubiquitination regulates pMHC-II recycling in DCs and B cells. The extent of reappearance of internalized pMHC-II after a 15-min chase at 37 °C in immature BMDCs (A), freshly isolated spleen DCs (B), or freshly isolated spleen B cells (C) from the indicated mice was quantitated using our recycling assay. In each panel, the data shown are the mean $\pm$ SD obtained from three independent experiments; * P < 0.05, ** P < 0.01.

The fact that DC activation acutely terminates March-I expression and MHC-II ubiquitination in immature DCs (17, 21) suggested to us that the stabilizing effect of LPS on pMHC-II survival was a consequence of LPS-mediated suppression of March-I expression and pMHC-II ubiquitination. To directly address whether ubiquitination regulates pMHC-II synthesis and/or degradation rates, we monitored the survival of newly synthesized [³⁵S]-labeled pMHC-II complexes generated in immature DCs obtained from wild-type, MHC-II K₂₂₅R ubiquitination mutant, and March-I-KO mice. The amount of pMHC-II generated was identical at the 2-h chase time point in wild-type and MHC-II ubiquitination mutant DCs (Fig. 5B and C), demonstrating that the increase in pMHC-II expression in MHC-II ubiquitination mutant DCs was not due to increased rates of pMHC-II synthesis. After 18 h of chase, >90% of newly synthesized pMHC-II was degraded in wild-type immature DCs. By contrast, pMHC-II synthesized in MHC-II K₂₂₅R ubiquitination mutant DCs was far more stable, and less than 50% of pMHC-II generated in these cells was degraded during this same time period (Fig. 5B). Essentially identical results were obtained in pulse-chase studies comparing pMHC-II stability in wild-type and March-I-deficient DCs (Fig. 5C). Taken together with the results above, these data demonstrate that DC activation promotes the survival of pMHC-II complexes generated in immature DCs, that ubiquitination of pMHC-II in immature DCs promotes the degradation of internalizing pMHC-II complexes, and that differences in pMHC-II expression on immature and mature DCs can be explained largely based on ubiquitin-dependent differences in pMHC-II stability.

Efficient Recycling Promotes Prolonged Antigen Presentation by DCs. We next designed experiments to determine whether ubiquitin-mediated alterations in MHC-II recycling affected the ability of DCs to function as APCs. Immature DCs from wild-type or MHC-II K₂₂₅R ubiquitination mutant mice were incubated with OVA_{323–339} peptide on ice to “tag” surface MHC-II with a defined antigenic peptide. The cells were then cultured on ice or at 37 °C for various times and used as APCs to stimulate CD69 expression on OVA-specific OT-II CD4 T cells. Reculture at 37 °C for 6 h resulted in a 50% reduction in the ability of OVA_{323–339} peptide-loaded DCs to stimulate OT-II T cells (Fig. 6A and B). This value is in excellent agreement with biochemical studies showing that surface pMHC-II on immature mouse DCs has a half-life of 4 h (18). By contrast, OVA_{323–339} peptide-loaded MHC-II K₂₂₅R ubiquitination mutant immature DCs were able to stimulate OT-II T cells for a prolonged time; after 6 h of culture at 37 °C, there was only a 15% decrease in their ability to activate OT-II T cells. Together with the data shown above, these data show that ubiquitination of MHC-II promotes the functional turnover of pMHC-II complexes on DCs and that cessation of

MHC-II ubiquitination upon DC activation increases the capacity of mature DCs to stimulate naïve CD4 T cells.

Discussion

Antigen-specific CD4 T cells are stimulated by the binding of their clonotypic T-cell receptor (TCR) to specific pMHC-II on the surface of antigen-bearing APCs. These interactions are important for the ability of DCs to stimulate naïve CD4 T cells and for antigen-loaded B cells to interact with antigen-specific CD4 T cells. Immature DCs express relatively small amounts of pMHC-II on their surface but large amounts of pMHC-II in late endosomes/multivesicular bodies (MVB) (6, 8). Activation of DCs by a variety of inflammatory stimuli dramatically alters the distribution of MHC-II in DCs such that activated (mature) DCs possess large amounts of MHC-II on their surface and very little in intracellular locations (6, 8). The dramatic increase in pMHC-II expression on mature DCs is due to a variety of factors, including (i) activation-induced movement of intracellular pMHC-II to the plasma membrane (8–10, 22), (ii) transient activation-induced stimulation of MHC-II biosynthesis (6, 11), and (iii) enhanced stability of surface pMHC-II in mature DCs (6, 18, 23). It has been proposed that upon maturation, DCs also down-regulate antigen uptake and pMHC-II recycling (6), thereby enhancing antigenic “memory” to those T-cell epitopes generated at the time of APC activation. Ubiquitination has been shown to regulate pMHC-II stability and subcellular distribution in immature DCs (13, 14, 18), however the mechanism by which pMHC-II ubiquitination controls MHC-II trafficking remains unknown. We now show that ubiquitination in immature APCs limits pMHC-II recycling and promotes lysosomal degradation of internalized pMHC-II, thereby directly regulating the cellular localization and fate of pMHC-II.

There has been conflicting data regarding the importance of ubiquitination in regulating MHC-II endocytosis in DCs (13, 14,

Fig. 5. Ubiquitination promotes degradation of newly synthesized pMHC-II in DCs. (A) Immature BMDCs were pulse-labeled with [³⁵S] for 1 h, washed, and chased in complete medium. After 2 h, either PBS or LPS was added to the cultures for an additional 18 h of chase at 37 °C. The recovery of [³⁵S]-labeled pMHC-II was determined by immunoprecipitation, SDS/PAGE, and autoradiography. The abundance of [³⁵S]-MHC-II β-chain present in each sample was quantitated by laser densitometry. The data shown are the mean $\pm$ SD obtained from three independent experiments; * P < 0.05. (B and C) Immature BMDCs isolated from wild-type and MHC-II K₂₂₅R ubiquitination mutant mice (B) or wild-type and March-I KO mice (C) were pulse-labeled with [³⁵S] for 1 h, washed, and chased for the indicated amount of time. The amount of [³⁵S]-MHC-II β-chain present in each sample was determined as described above. The data shown are the mean $\pm$ SD obtained from three independent experiments; * P < 0.05.

Fig. 6. Efficient recycling prolongs antigen presentation by DCs. Immature BMDCs obtained from either wild-type or MHC-II K₂₂₅R ubiquitination mutant mice were treated with OVA_{323–339} peptide for 30 min on ice, washed, and then chased in complete medium at 37 °C for various times. DCs were harvested and cocultured with OVA-specific OT-II CD4 T cells at a 1:3 ratio. (A) After incubation of DCs and T cells for 18 h, cells were stained with CD69 and CD4 mAb, and the percentage of CD4 cells expressing high levels of surface CD69 was determined. (B) The relative extent of T-cell proliferation at each chase point was expressed as a fraction of that obtained by incubating the indicated DC type with OVA_{323–339} peptide-pulsed DCs. The data shown are the mean $\pm$ SD obtained from three independent experiments; ** P < 0.01, *** P < 0.001.

18, 21). Most studies examining MHC-II endocytosis (and recycling) rates have used assays in which plasma membrane proteins are tagged with mAb on ice and the loss (or reappearance) of mAb reactivity with fluorescently conjugated reagents after culture of cells at 37 °C is taken to represent endocytosis (or recycling) (6, 13, 14, 18, 21, 24, 25). In some of these studies, it was reported that DC activation suppressed the kinetics of MHC-II mAb endocytosis (6, 18, 21). To directly follow the fate of the pMHC-II molecule itself (and not pMHC-II mAb), we have developed endocytosis and recycling assays in which all plasma membrane proteins are covalently tagged with sulfo-NHS-SS-biotin, a form of biotin that can be removed from the tagged proteins by incubation with reduced glutathione on ice. Using this technique, we demonstrate that pMHC-II endocytosis rates are essentially identical in immature and mature DCs or in resting and activated B cells. We attribute the discrepancy between our endocytosis results and those obtained by others to technical differences between the two different endocytosis assays used, as even we have noted a slight decrease in the kinetics of pMHC-II endocytosis upon BMDC activation using mAb-based endocytosis assays (18). Using our surface biotinylation assay, we also found that ubiquitination does not alter pMHC-II endocytosis in DCs or B cells, findings that are in excellent agreement with a previous study using a similar biotinylation approach that showed that ubiquitination did not affect the rate of MHC-II endocytosis in spleen B cells (15).

Although we did not observe any effect of APC activation or a role for ubiquitination in regulating pMHC-II endocytosis, our results conclusively demonstrate that internalized pMHC-II efficiently recycles in both mature DCs and activated B cells but does not recycle in immature DCs and resting B cells. Furthermore, our use of MHC-II ubiquitination-defective mice revealed that the inability of pMHC-II to recycle in immature DCs or resting B cells was a consequence of pMHC-II ubiquitination by March-I. These results demonstrate that the failure of pMHC-II to recycle in resting APCs is not due to an inherent defect in the ability of MHC-II to recycle in these cells but is instead a consequence of “diversion” from an efficient recycling pathway to one of lysosomal degradation.

pMHC-II is not the first example of a protein whose endocytic fate is regulated by ubiquitination. Eyster et al. have shown that ubiquitination can alter the itinerary of proteins internalized by clathrin-independent endocytosis from a pathway of recycling to one of degradation (26). Curiously, pMHC-II endocytosis is also clathrin-independent (27), adding yet another protein to the list of those whose endocytic itinerary is altered by ubiquitination. Oligo-ubiquitinated integral membrane proteins, such as MHC-II, are sorted in early endosomes for trafficking to late endosomes, where they interact with endosomal sorting complexes required for transport (ESCRT) proteins for inclusion into MVB (28). Ubiquitination of K₂₂₅ enhances MHC-II sorting into MVB

(13, 14), however some MHC-II is sorted into MVB even in K₂₂₅R MHC-II mutants (13), revealing (presumably) ESCRT-independent sorting of MHC-II into MVB like that observed previously in the melanosomal protein Pmel17 (29).

Because MVBs are intermediates in the lysosomal degradation pathway (28), we propose that ubiquitination of pMHC-II in immature DCs and resting B cells suppresses a “default” pMHC-II recycling pathway and promotes MVB sorting and lysosomal degradation of internalized pMHC-II. Together with constitutive biosynthesis of MHC-II in immature DCs, regulated ubiquitination ensures a reliable mechanism for continual pMHC-II generation and turnover in immature DCs. Upon APC activation, the absence of pMHC-II ubiquitination prevents ESCRT binding and MVB sorting, thereby promoting efficient pMHC-II recycling and suppressing pMHC-II turnover. Together, these processes maintain the high levels of surface pMHC-II necessary for APCs to efficiently stimulate antigen-specific CD4 T cells. Because fully activated DCs express large amounts of MHC-II on their surface but do not synthesize MHC-II protein (6, 20, 30), efficient pMHC-II recycling maintains a large surface pool of pMHC-II and a relatively small pool of intracellular pMHC-II. Most importantly, efficient recycling (and inefficient lysosomal degradation) of pMHC-II would augment the ability of activated APCs to present pMHC-II that are of the most interest to the T-cell repertoire, namely pMHC-II generated immediately following APC activation by pathogens and/or inflammatory stimuli.

Materials and Methods

Mice and Cells. March-I KO mice and MHC-II K₂₂₅R ubiquitination mutant knock-in mice on the C57BL/6 background have been described (15, 31). Mice were bred and maintained in-house at the NCI-Frederick animal facility. All mice were cared for in accordance with National Institutes of Health guidelines with the approval of the National Cancer Institute Animal Care and Use Committee. BMDCs were prepared by differentiating mouse bone marrow cells in medium containing 20 ng/mL GM-CSF for 7 d using standard protocols (32). To generate large numbers of spleen DCs for biochemical studies, mice were injected with 5×10^6 Flt3L-expressing B16 melanoma cells s.c. After 2 wk, mice were injected with either PBS alone or 200 μ g cytosine-phosphate-guanine-rich oligodeoxynucleotide 1668 (CpG) by i.v. injection. After 16 h, spleen cells were incubated with liberase and DCs were isolated by magnetic sorting using a Miltenyi mouse DC isolation kit. Cells were isolated quickly and maintained on ice to limit spontaneous DC activation. Where indicated, immature BMDCs or spleen DCs were activated in vitro by incubation with 1 μ g/mL LPS overnight. Spleen B cells were isolated from spleen cell suspensions using a Miltenyi mouse B-cell isolation kit. Spleen B cells were activated by treatment in medium containing 10 μ g/mL LPS overnight.

Antibodies and Reagents. The following antibodies were used in this study: anti-mouse pMHC-II mAb Y3P (33), biotinylated anti-ubiquitin mAb P4D1 (eBioscience), and anti- λ -A κ β -chain rabbit serum (34). HRP-conjugated reagents were

obtained from Southern Biotech. EZ-Link sulfo-NHS-SS-biotin was obtained from Thermo Scientific, and reduced glutathione was obtained from Calbiochem.

pMHC-II Internalization and Recycling Assays. Analysis of pMHC-II internalization was performed as previously described (27). Briefly, cells were surface-biotinylated with sulfo-NHS-SS-biotin (1 mg/mL) in PBS for 30 min on ice. After washing in complete medium, the cells were chased in complete medium at 37 °C or kept on ice. At various times, the cells were harvested and washed in ice-cold HBSS, and surface-exposed biotin was reduced by incubating the cells with 50 mM reduced glutathione in buffer containing 75 mM NaCl, 10 mM EDTA, and 75 mM NaOH (pH 7.5) for two treatments of 15 min each. To monitor MHC-II recycling, biotinylated cells were incubated at 37 °C for 30 min, and surface-exposed biotin was removed using reduced glutathione as above to generate a pool of intracellular biotinylated proteins. To quantitate recycling, cells were chased again at 37 °C for the indicated times before repeating the reduced glutathione procedure. Additional details for quantitative analyses are described in *SI Materials and Methods*.

SDS/PAGE and Blot Analyses. Cells were lysed in Triton X-100 lysis buffer, and pMHC-II was immunoprecipitated with mAb Y3P and analyzed by SDS/PAGE and immunoblotting using streptavidin-HRP (to detect biotinylated MHC-II) or an MHC-II β -chain antiserum (to detect total MHC-II in the immunoprecipitate) as described previously (35). In experiments examining MHC-II ubiquitination, all buffers contained 25 mM *N*-ethylmaleimide to inhibit de-ubiquitination activity (36). Following SDS/PAGE and transfer to PVDF membranes, ubiquitinated proteins were detected using biotinylated anti-ubiquitin mAb P4D1 and HRP-streptavidin. Quantitative analysis of protein expression in different samples was performed using laser densitometry of immunoblots or autoradiograms as described in *SI Materials and Methods*.

Pulse-Chase Biosynthetic Labeling. BMDCs were incubated in methionine-free media at 37 °C for 10 min before pulsing 10×10^6 cells/mL with 0.5 mCi [35 S] methionine in methionine-free medium. After 1 h, the cells were washed and recultured (chased) in complete RPMI-1640 medium at 37 °C for various

times. In some experiments, LPS (1 μ g/mL) was added during the chase. pMHC-II was immunoprecipitated using mAb Y3P and analyzed by SDS/PAGE on 10% gels. The gels were fixed using 20% methanol/10% (vol/vol) acetic acid, impregnated with Enlightening solution (ICN Biosciences) to enhance signals, dried, and exposed to Kodak X-Omat XAR X-ray film.

Real-Time Quantitative PCR. RNA extractions were carried out with the RNeasy mini kit (QIAGEN), according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using standard procedures and using reagents from Promega. After initial template melting, PCR using 30 cycles of 95 °C for 45 s/65 °C for 45 s/72 °C for 60 s was performed with a 2-min product extension time at 72 °C. The mouse March-I forward primer sequence was AAGAGAGCCCACTCATCACACC (5' to 3'), and the reverse primer sequence was ATCTGGAGCTTTTCCCACTCC (5' to 3'). GAPDH primers were obtained from QIAGEN. Real-time PCR was performed using an ABI Prism 7900HT Sequence detection system and QuantiTect SYBR green PCR kit (QIAGEN) according to the manufacturer's instructions. March-I expression in each sample was normalized using GAPDH as an RNA loading control.

CD4 T-Cell Activation. Immature BMDCs were treated with 1 μ M OVA_{323–339} peptide for 30 min on ice, washed, and cultured in complete medium at 37 °C for various times before harvest. DCs (1×10^5 cells) were cocultured with OVA_{323–339}-specific OT-II CD4 T cells (3×10^5 T cells) for 18 h. After incubation, cells were stained with CD69 and CD4 mAb on ice. The percentage of CD69⁺ cells present in the CD4 cell gate in each condition was determined, and the net percentage CD69⁺ cells in the chase cultures was expressed relative to that elicited by incubating the OT-II T cells with OVA_{323–339} peptide-pulsed DCs that remained on ice (net proliferation = proliferation using peptide-pulsed DCs – proliferation using nonpulsed DCs).

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