

Mature dendritic cells use endocytic receptors to capture and present antigens

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In response to inflammatory stimuli, dendritic cells (DCs) trigger the process of maturation, a terminal differentiation program required to initiate T-lymphocyte responses. A hallmark of maturation is down-regulation of endocytosis, which is widely assumed to restrict the ability of mature DCs to capture and present antigens encountered after the initial stimulus. We found that mature DCs continue to accumulate antigens, especially by receptor-mediated endocytosis and phagocytosis. Internalized antigens are transported normally to late endosomes and lysosomes, loaded onto MHC class II molecules (MHCII), and then presented efficiently to T cells. This occurs despite the fact that maturation results in the general depletion of MHCI from late endocytic compartments, with MHCII enrichment being typically thought to be a required feature of antigen processing and peptide loading compartments. Internalized antigens can also be cross-presented on MHC class I molecules, without any reduction in efficiency relative to immature DCs. Thus, although mature DCs markedly down-regulate their capacity for macropinocytosis, they continue to capture, process, and present antigens internalized via endocytic receptors, suggesting that they may continuously initiate responses to newly encountered antigens during the course of an infection.

antigen presentation | endocytosis | MHC class II molecules | DEC-205 | Fc receptor

Dendritic cells (DCs) are remarkably potent at initiating and directing adaptive immune responses (1, 2). They are localized to both peripheral and lymphatic tissues and sample their surroundings, internalizing, processing, and presenting captured antigens to T cells on MHC class I molecules (MHCI) and MHC class II molecules (MHCII). They distinguish between self- and foreign antigens using receptors of the innate immune system [e.g., Toll-like receptors (TLRs)], inducing immunity when antigen is captured in the presence of microbial products or inflammatory stimuli but tolerance in the absence of these signals (3). DCs exhibit dramatic functional and morphological changes, termed *maturation*, that maximize antigen presentation to T cells in response to such stimuli (1, 2). These changes involve acidification of the lysosomal compartment to optimize antigen processing, up-regulation of costimulatory molecules, and reorganization of MHCII from the late endocytic compartments to the plasma membrane for recognition by T cells (4, 5).

Modulation of endocytosis also occurs during maturation. Immature DCs endocytose avidly through a variety of mechanisms, including “nonspecific” uptake by constitutive macropinocytosis and “specific” uptake via receptor-mediated endocytosis and phagocytosis (2). Macropinocytosis is transiently up-regulated immediately on the receipt of an inflammatory signal (6), but this is followed by its dramatic down-regulation, partly mediated by a reduction of the active form of the Rho GTPase, Cdc42 (7). Most studies of endocytosis in DCs have involved exposing cells to a high concentration of antigens or endocytic tracers. Although such assays mainly measure macropinocytosis, it is generally presumed that all forms of endocytosis are down-regulated in mature DCs. The idea seems to fit well in an immunological context, given that it helps to explain how DCs present pathogens acquired at the time of activation, although

avoiding presentation of self-antigens encountered before or following this event.

Mature DCs continue to form clathrin-coated vesicles (7), however, suggesting that the internalization of surface molecules may still occur. Mature DCs can capture simian immunodeficiency virus in a clathrin-dependent manner (8) and immune complexes (ICs) in vitro (9). They also internalize constitutively ubiquitinated MHCII (10). Together, these findings indicate that the internalization and fate of receptor-bound antigens by mature DCs are in need of direct analysis.

We characterized the ability of mature DCs to internalize antigens using Fcγ receptors (FcγRs) and the decalectin DEC-205 as model endocytic receptors, with each having been documented to enable presentation on MHCI and MHCII (11–13). We found that even as mature bone marrow-derived dendritic cells (BMDCs) completely shut down macropinocytosis, they still internalized these receptors with high efficiency, using receptor-mediated endocytosis and even phagocytosis. Antigens internalized by mature DCs were processed and presented with unexpected efficiency in vitro and apparently in vivo.

Results

Expression and Internalization of Endocytic Receptors by Mature DCs.

We first confirmed the surface expression of DEC-205 and FcγRII/III in mature DCs. We found that FcγRII/III was down-regulated slightly in both BMDCs and splenic DCs (Fig. S1) as previously shown (13, 14). In contrast, DEC-205 was highly up-regulated in mature BMDCs, although its expression on CD8α⁺ DCs did not change on maturation (Fig. S1) (15). We also detected low levels of DEC-205 on CD8α⁻ DCs, which, as with BMDCs, increased on maturation (15) (Fig. S1).

Next, we asked if FcγRs and DEC-205 were internalized in mature BMDCs. We assessed uptake of ICs, which are the physiological ligands for FcγRs. Because no physiological ligands have yet been identified for DEC-205, we used anti-DEC-205 monoclonal antibodies, which lead to the presentation of covalently linked antigens after internalization by DCs (16). ICs and anti-DEC-205 antibodies initially bound to the cell surface at 4 °C were rapidly internalized at 37 °C by both immature and mature DCs, with >60% of the ICs and anti-DEC-205 disappearing from the cell surface within 10 min, although receptor internalization by mature DCs appeared slightly less efficient (Fig. 1*A* and *B*). Most ICs reached H-2M⁺ late endosomal/lysosomal compartments in mature DCs 2 h after internalization (Fig. 1*C*, *Upper*). In contrast, anti-DEC-205

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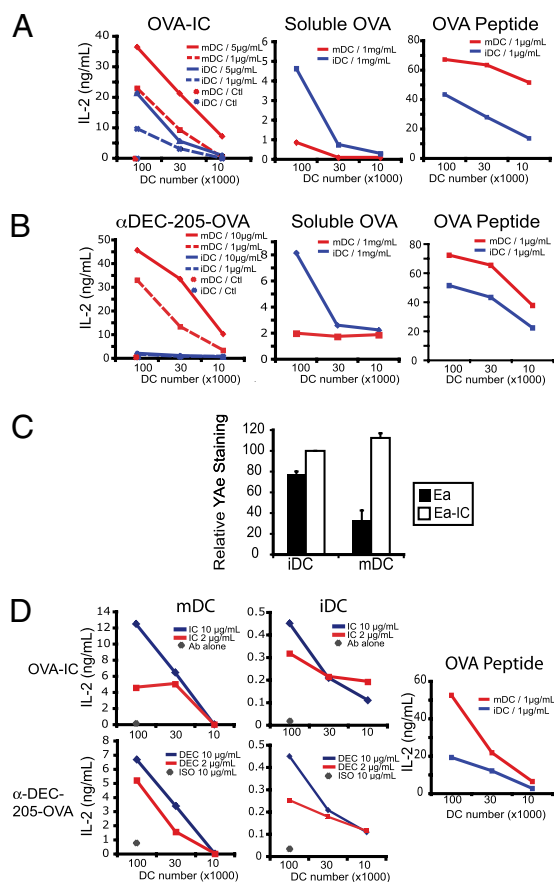


Fig. 3. Fully mature DCs efficiently present antigen internalized by Fc γ R and DEC-205. (A) Untreated and LPS-matured BMDCs were incubated with OVA-Alexa 647 (0.5 μ g/mL) for 10 min at 37 $^{\circ}$ C, stained for CD86 and CD11c, and sorted into highly pure populations of mature and immature cells. Mature DCs were defined as CD86-high OVA-low LPS-treated cells, and immature DCs were defined as CD86-low OVA-intermediate/high untreated cells. Sorted cells were fed with OVA-rabbit anti-OVA ICs or with soluble OVA and LPS for 30 min at 37 $^{\circ}$ C. DCs were cultured with 10^5 OT.2 CD4 $^{+}$ T cells for 24 h on anti-CD28-coated plates. T-cell activation was assessed by measuring IL-2 secretion by ELISA. Averages of triplicates are displayed. (B) Performed as in A but with anti-DEC-205-OVA and isotype control-OVA fusion proteins. (C) Untreated and LPS-matured BMDCs were stained for CD86 and then incubated with E α aggregates or E α -aggregate ICs (30 μ g/mL) and LPS for 7 h or with LPS alone as a control. Cells were then stained with YAc antibody and anti-CD11c and analyzed by flow cytometry. Mature DCs are gated on the CD11c $^{+}$ CD86-high cells, and immature DCs are gated on the CD11c $^{+}$ CD86-low cells. Values are normalized to those of DCs receiving ICs that were immature when first exposed to antigen. Bars indicate SE. Results are averages of two independent experiments. (D) Performed as in A and B but DCs were cultured with CD8 $^{+}$ OT.1 T cells specific for MHCII-OVA peptide complexes to assess presentation on MHCII.

cells (Fig. S1). However, it could not be the explanation for Fc γ R-targeted IC, because Fc γ RII/III surface expression decreases during BMDC maturation (Fig. S1). It is also unlikely that the increased ability of mature DCs to stimulate T cells results from their increased costimulatory capacity, because we provided T cells with artificial costimulation by coating assay plates with anti-CD28 antibodies.

Still, because the mature DCs also presented OVA peptide, which did not require processing, with somewhat higher efficiency (Fig. 3A and B, Right), it was possible that the enhanced ability of mature DCs to stimulate T cells could have masked an inferior antigen processing ability. We thus directly measured MHCII-peptide complex generation using the YAc monoclonal antibody, which recognizes MHCII-E α -peptide (amino acids 52–68) complexes. We compared

the ability of mature and immature DCs to present aggregates of E α protein (taken up nonspecifically by macropinocytosis) or E α ICs (taken up by Fc γ R). Immature DCs formed MHCII-peptide complexes from untargeted E α protein significantly better than the mature DCs, reflecting their different capacities for macropinocytosis (Fig. 3C). However, mature DCs were at least as good as immature DCs at generating MHCII-peptide complexes from E α ICs (Fig. 3C). Similar results were obtained following the phagocytosis of opsonized *Escherichia coli* expressing the E α protein (Fig. S3). DCs matured by other TLR ligands showed similar abilities to present receptor-targeted antigens on MHCII (Fig. S4), suggesting that this is a general property of mature DCs. Thus, although mature DCs lose the ability to present untargeted antigens, their ability to present antigens on MHCII following receptor-mediated uptake by clathrin-coated pits or phagocytosis remains intact.

Because OVA has been reported to bind the mannose receptor (MR) (28), we tested the contribution of this receptor to OVA uptake by immature DCs under our experimental conditions. We found that when MR was blocked by an excess of mannan, uptake was reduced by <20% (Fig. S5). This confirms that immature BMDCs internalize OVA by a combination of receptor-mediated and fluid-phase macropinocytosis, as previously observed (7). MR is markedly down-regulated during maturation (29), however, precluding receptor-mediated OVA uptake in mature DCs. The E α protein was purified from *E. coli*, and is therefore not glycosylated. Thus, lectins could not contribute to the uptake of this antigen.

Previous studies have shown that mature DCs efficiently present endogenous antigen but lose the ability to cross-present exogenous antigen on MHCII (9, 21). This has been attributed to a lack of antigen internalization (21) or to poor translocation of internalized antigen to the cytosol (9). In contrast, we found that mature DCs cross-presented receptor-targeted antigen more efficiently than immature DCs (Fig. 3D). Although the mechanism of cross-presentation on MHCII is still poorly understood, the increased levels of MHCII synthesis in mature DCs may explain the enhanced cross-presentation under conditions in which antigen uptake is not limiting (30). Our studies differ from previous work by making use of receptor-mediated rather than nonspecific endocytosis (21) and by using a more sensitive T-cell assay that more clearly revealed the cross-presentation that was indeed observed earlier (9).

Mature DCs Load MHCII in the Lysosomal Compartment. How MHCII presentation of newly acquired antigens by mature DCs occurs is more difficult to rationalize. The dramatic reorganization of MHCII on maturation suggests that few, if any, acceptor MHCII for antigenic peptides remain in the endocytic compartments of mature DCs (4, 26, 27, 31, 32) (Fig. S6, Upper). Because high levels of surface MHCII might artifactually mask a reduced internal pool of MHCII, we imaged cells after blocking surface MHCII with unlabeled antibody. Indeed, under these conditions, a lysosomal pool of MHCII in mature DCs could be detected (Fig. S6, Lower). Electron microscopy confirmed that ICs internalized via the Fc γ R accumulated in MHCII $^{+}$ lysosomes over time (Fig. S2B). To determine if peptide loading can occur in the MHCII-diminished lysosomal compartment of mature DCs, we incubated mature and immature DCs with E α ICs, and used the YAc antibody to visualize the distribution of the newly formed MHCII-peptide complexes. By 90 min, both mature and immature DCs had the majority of MHCII-peptide complexes localized to the H-2M $^{+}$ late endosomal/lysosomal compartment (Fig. 4A). These complexes were translocated to the plasma membrane with further incubation (Fig. 4A), as found previously for immature DCs then induced to mature (33). This result indicates that like immature DCs, mature DCs load antigenic peptides on MHCII in the lysosomal compartment and then make the MHCII-peptide complexes available on the plasma membrane for T-cell recognition.

It has been suggested that antigenic peptides can be loaded both on newly synthesized or recycling MHCII (2). We first examined

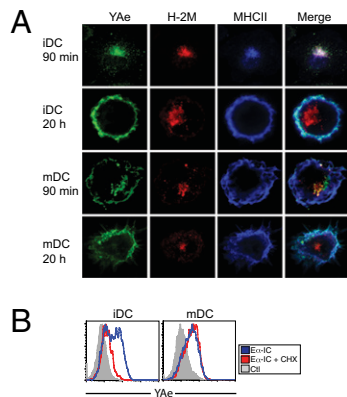


Fig. 4. Fully mature DCs load peptide onto MHCII in the lysosomal compartment. (A) Immature or LPS-matured BMDCs were sorted as in Fig. 3A and incubated at 37 °C for 90 min with E α ICs (30 μ g/mL) and LPS. Cells were washed and then fixed or incubated at 37 °C for an additional 18.5 h before fixation. Cells were stained with YAc, anti-H2M, and anti-MHCII antibodies and then analyzed by confocal microscopy. (B) Immature or LPS-matured BMDCs were stained for CD86 and then incubated with CHX (1 μ g/mL) for 30 min. E α ICs (30 μ g/mL) were added to the DCs for 5 h at 37 °C in presence of CHX. Cells were then stained with YAc and anti-CD11c antibodies and analyzed by flow cytometry. Mature DCs are gated on the CD11c⁺ CD86-high cells, and immature DCs are gated on the CD11c⁺ CD86-low cells.

the rate of MHCII synthesis in immature and mature DCs by metabolic labeling and autoradiography. Like many other studies, we found significant but incomplete down-regulation of MHCII synthesis after maturation (4, 5, 24, 25). MHCII synthesis was decreased by ~90% in mature BMDCs and by 65% in mature splenic DCs when compared with immature cells (Fig. S7).

To determine the contribution of the residual de novo synthesis of MHCII to peptide loading, we treated DCs with cycloheximide (CHX) to arrest protein synthesis 30 min before antigen pulse. As expected, exposure to CHX led to a near-complete blockade of protein synthesis in both mature and immature DCs; the cells remained viable because the protein synthesis block was reversed on CHX removal (Fig. S8). CHX treatment significantly impaired but did not completely abrogate the formation of YAc-reactive MHCII-peptide complexes in immature DCs, demonstrating the contribution of both newly and previously synthesized MHCII to peptide loading (Fig. 4B). However, CHX had little effect in fully mature DCs, indicating that previously synthesized (possibly recycled) MHCII plays a major role in the presentation of newly captured antigens (Fig. 4B).

Altered Access to Antigens Explains Reduced Antigen Presentation by Mature Splenic DCs in Vivo. We next tested whether capture of antigens by mature DCs can occur in vivo. After induction of systemic DC maturation by LPS injection, mice were immunized i.v. with soluble OVA or OVA ICs. T cell activation was assessed by measuring CD69 up-regulation of adoptively transferred CD4⁺ OT.2 T cells. In control mice injected with either antigen formulation (containing LPS as an adjuvant), robust splenic T cell activation was observed as expected. However, in contrast to our in vitro data but consistent with recent results using soluble antigen (21, 24), pretreatment of mice with LPS substantially inhibited activation of T cells even after injection of Fc γ R-targeted OVA ICs (Fig. 5A).

Although surprising, this result did not reflect an inherent inability of mature splenic DCs to capture and present antigens. As found for BMDCs matured in vitro, CD86-high splenic DCs isolated from LPS-treated mice presented both anti-DEC-205-OVA and OVA ICs in vitro (Fig. 5B). The in vivo-matured splenic DCs even presented soluble OVA (Fig. 5B, Upper), suggesting that they retained the capacity to internalize soluble OVA either after

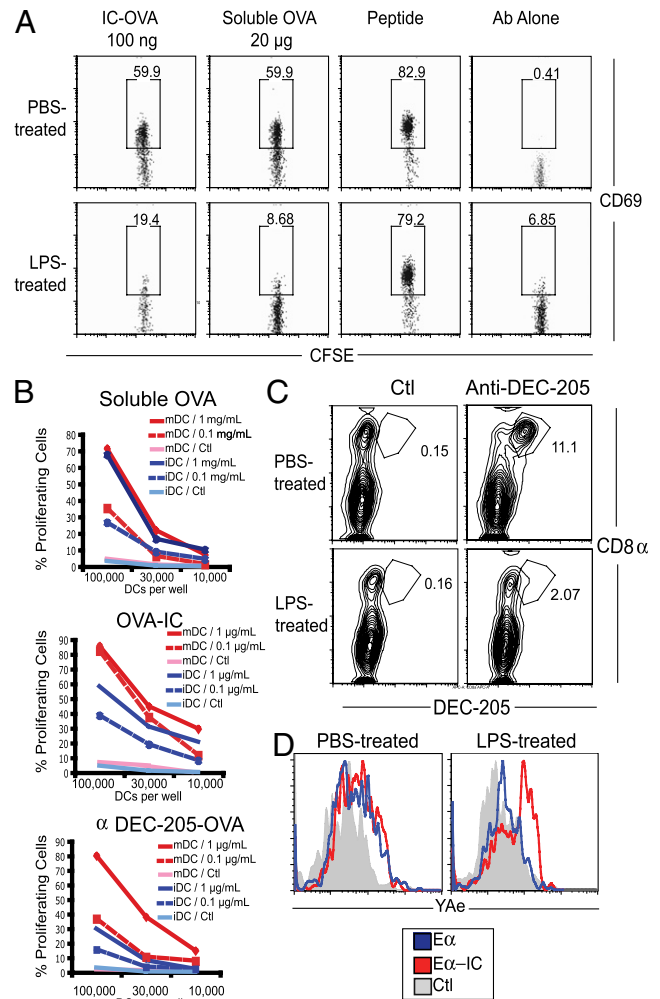


Fig. 5. Mature splenic DCs efficiently present antigen in vitro but have reduced access to antigen in the setting of systemic inflammation. (A) Reduced presentation of antigen by mature splenic DCs in vivo. Twenty-four hours after adoptive transfer of 10^6 carboxyfluorescein succinimidylester (CFSE)-labeled OT.2 CD4⁺ T cells, B6/C57J mice were injected i.p. with 3 μ g of LPS or PBS control. After an additional 5 h, OVA ICs, soluble OVA, SIINFEKL-OVA peptide, or rabbit anti-OVA antibody as a control was i.v. injected. Spleens were harvested 4 h later, and T-cell activation was assessed by CD69 up-regulation. (B) DCs were isolated from the spleens of mice injected i.p. with 3 μ g of LPS or PBS 9 h before harvest. DCs were sorted on CD11c⁺ CD86-high cells; incubated with anti-DEC-205-OVA, OVA ICs, or soluble OVA for 2 h; and cultured with CFSE-labeled CD4⁺ OT.2 T cells. Sixty hours later, the extent of cell division was determined by flow cytometry. Average values of duplicates are displayed. (C) Three micrograms of anti-DEC-205-FITC or isotype control antibody was injected i.v. into mice pretreated for 9 h with 3 μ g of LPS injected i.p. or with PBS control. Spleens were harvested 2 h later, and DCs were analyzed by flow cytometry. Histograms of CD11c⁺ cells are displayed. (D) Mice were injected i.p. with 3 μ g of LPS or with PBS control. Nine hours later, E α aggregates (30 μ g), E α -aggregate ICs (30 μ g), or PBS control was injected s.c. into the hind leg hocks (each injection contained 1 μ g of LPS). Draining popliteal LNs were harvested 4 h later, and cells were stained with the YAc antibody. Histograms are of CD11c⁺ cells.

binding to lectins or through fluid-phase endocytosis, at least when assayed in vitro. This persistence of fluid endocytosis may reflect the fact that splenic DCs activated in vivo are conventionally harvested relatively soon after LPS administration (after approximately 9 h) (24). In addition, splenic DCs down-regulate macropinocytosis with slower kinetics than BMDCs and exhibit residual fluid-phase uptake for at least 24 h (6). In either event, the

failure of splenic DCs to present antigen delivered by receptor-mediated or fluid-phase endocytosis after LPS treatment *in vivo* was unlikely to be attributable to the loss of endocytic capacity.

Instead, the reduced presentation of antigen by mature DCs *in vivo* may reflect reduced access of injected antigen to DCs in the spleen. We tested this hypothesis by directly assessing the ability of *i.v.*-injected fluorescently labeled anti-DEC-205 antibodies to bind mature splenic DCs *in situ*. Compared with immature splenic DCs, mature splenic DCs in LPS-treated mice showed greatly reduced labeling with the antibody (Fig. 5C). Because expression of DEC-205 was unchanged or increased during maturation in CD8 α^+ and CD8 α^- splenic DCs (Fig. S1), this result further suggests that systemic inflammation limited the access of splenic DCs to antigens.

Why does LPS reduce the access of splenic DCs to *i.v.*-injected antigen? One possibility is that LPS (and other microbial stimuli) causes migration of DCs away from the marginal and medullary zones, possibly reducing their ability to compete for incoming antigen (34, 35). We also noted that injection of even low concentrations of LPS changed the overall appearance of the spleen, which became darker and less pliable. As a result, we next asked if there were alterations in splenic blood flow that might interfere with entry of antigen or even DC precursors into the spleen. We measured blood flow to the spleen using microbubble ultrasound perfusion imaging (36, 37) after injecting mice with LPS or PBS as control and observed, on average, a 31% reduction in blood flow in the LPS-treated mice (Fig. S9A and B). This alteration might also contribute to the failure of splenic DCs to access and capture *i.v.*-injected antigen in our study as well as in previous studies (21, 24).

Although *i.v.*-injected antigens poorly access the spleen, we examined whether antigens could access DCs in lymph nodes (LNs) under conditions of systemic administration of TLR agonists. The *i.v.* injection of LPS led to full maturation of LN DCs, as indicated by the uniformly enhanced expression of CD86 (Fig. S10).

We next measured the formation of MHCII-peptide complexes directly *in vivo*. We again used E α as (nonglycosylated) antigen delivered either as soluble protein (which can be internalized only by nonselective macropinocytosis) or Fc γ R-targeted IgG IC. When injected into control mice, as expected, both antigen formulations generated detectable amounts of surface MHCII-E α -peptide complex, even if coinjected with a small quantity of LPS to trigger maturation at the time of antigen encounter (Fig. 5D, *Left*). However, when injected into mice whose DCs had been matured by prior injection of LPS, only the E α IC, which is internalized via Fc γ R, generated MHCII-peptide complexes (Fig. 5D, *Right*). Therefore, mature LN DCs can capture, process, and present receptor-targeted antigens *in vivo*.

Discussion

In this study, we have shown that mature DCs, even as they down-regulate constitutive macropinocytosis and phagocytosis, retain the ability to capture antigens via receptor-mediated endocytosis. In addition, these captured antigens are presented on MHC molecules with high efficiency both *in vitro* and *in vivo*.

Our findings challenge the generally accepted idea that mature DCs have a greatly reduced ability to present newly acquired antigens, a concept strongly implied by the dramatic down-regulation of macropinocytosis and redistribution of MHCII from intracellular compartments to the plasma membrane (1, 38, 39). Although one previous report has suggested that mature DCs retain the capacity for nonspecific phagocytic uptake, the degree of internalization was not well established and LPS-matured DCs were not capable of presenting antigen on MHCI or MHCII (40). Apart from this single study, the ability of mature DCs to capture and process antigens appears to have been overlooked for two primary reasons. First, most *in vitro* work has involved the exposure of DCs to high concentrations of antigens, making macropinocytosis, which is reduced in mature DCs, the main mechanism responsible for antigen

internalization (24, 41). Second, *in vivo* studies have primarily examined the ability of splenic DCs to present antigens following *i.v.* injection into mice treated with TLR agonists (24). Under these conditions, there is greatly reduced access of splenic DCs to antigen, which can be explained, at least in part, by reduced splenic blood flow (Fig. S9). Interestingly, this reduced access did not seem to alter peptide presentation *in vivo* (Fig. 5A). This could perhaps be explained by a size-dependent exclusion of larger antigens from the T-cell zone or could be attributable to the high sensitivity of T cells for this peptide that was provided in saturating amounts.

When antigen was targeted to endocytic receptors under conditions in which antigen access was not disrupted, as was the case with lymphatic drainage after *s.c.* injection, mature DCs efficiently presented newly captured antigens (Fig. 5D). Accordingly, basic presumptions concerning DC biology must be altered substantially. Although immature DCs are specialized for uptake of a broad range of antigens through a variety of endocytic mechanisms and are poor at antigen presentation, mature DCs are capable of both efficient antigen uptake and presentation. However, mature DCs may be primarily restricted to capturing antigens recognized by endocytic receptors, perhaps limiting uptake to pathogens or pathogen-derived antigens that have already been recognized by the immune system (and are complexed with specific IgG) or that bind to specific pathogen receptors (e.g., lectins) on the DC surface (42). Conceivably, antigen receptors used for internalization of self-antigens may be down-regulated. It is known, for instance, that the surface expression of MR, which can bind multiple self-glycoproteins (43), is diminished in mature DCs (14). Alternatively, capture of self-antigen-antibody complexes by mature DCs during pathogen infection could initiate or exacerbate autoimmune disorders associated with autoantibody production. Identifying the immunological consequences of the differential regulation of antigen uptake in immature vs. mature DCs will require a better understanding of the multitude of receptors that may be involved in antigen capture.

The source of MHCII used for presentation by mature DCs will require some additional definition. Although it appears that mature DCs load antigen onto previously synthesized MHCII (Fig. 4B), the fact that surface MHCII is not ubiquitinated and is poorly endocytosed (10) makes the surface MHCII seem an unlikely source. However, ubiquitin may be more important for sorting to lysosomes than for endocytosis *per se*. Thus, surface MHCII internalized in a ubiquitin-independent manner may contribute to maintaining a small but sufficient internal MHCII pool (Fig. S6). B-cell lines are well known to present antigen to CD4 $^+$ T cells in the absence of large internal pools of MHCII (2).

It is clear, however, that a lack of endocytosis can no longer be invoked to indicate that mature DCs are unable to take up and present exogenous antigens. Even after maturation, DCs are likely to remain capable of capturing subsequent rounds of antigen as long as the antigen is targeted to a suitable endocytic receptor. Mature DCs already located in LNs would thus be able to induce T-cell responses to an array of antigens, even when nonsynchronously encountered. Whether a bolus of newly encountered antigen could act to displace previously loaded peptides remains to be determined. However, the fact that mature DCs can take up and present targeted antigens creates opportunities to target DCs using ever more powerful immunization strategies (44).

Methods

Mice. B6/C57J, OT.1, and OT.2 mice were obtained from Jackson Immuno-Research Laboratories. Animal care and experiments were conducted according to the guidelines of Yale University and Genentech, Inc.

Cells. BMDCs were prepared as described (4). Day 5 BMDCs were stimulated with LPS (50 ng/mL; Sigma) for 20 h or left untreated. DCs were isolated from spleen and LN with blendzyme 2 (Roche) and DNaseI (Invitrogen) and purified with a CD11c isolation kit (Miltenyi).

Reagents. The antibodies used were as follows: CD86 (GL1), CD8 α (53-6.7), CD45R (RA3-6B2), CD11c (HL3), Fc γ II/III (2.4G2), CD69 (¹H.2F3), MHCII (KH74 and TIB-120), anti-CD3 (17A2), and anti-CD11c (N418), streptavidine-phycoerythrin (all from BD Biosciences); anti-6 \times His, anti-*E. coli*-FITC and anti-DEC-205 (all from Serotec); YAc (eBiosciences); anti-LAMP-2 (4); anti-H-2M (4); anti-OVA (30); anti-DEC-205-OVA; and isotype control-OVA (R. Steinman, The Rockefeller University, New York, NY) (16). Secondary antibodies and OVA-Alexa647 were from Molecular Probes. *E. coli* expressing 6 \times His-tagged E α protein was a gift from Ruslan Medzhitov (Yale University). ICs were generated as previously described (10).

Endocytosis Assays. To quantify internalization, anti-DEC-205 antibodies or isotype control (5 μ g/mL) or ICs (10 μ g/mL) were surface bound at 4 °C for 20 min, washed, and then allowed to internalize by incubation at 37 °C or 4 °C as a control. Remaining surface antibodies were detected with anti-rat or anti-rabbit antibody, respectively. The percentage of surface antibodies was determined over time [(mean surface fluorescence at 37 °C – mean surface isotype fluorescence at 4 °C)/(mean surface fluorescence at 4 °C – mean surface isotype fluorescence at 4 °C) \times 100]. One-micrometer Fluoresbrite yellow-green carboxylated latex microspheres (Polysciences, Inc.) were coated overnight in 1 mg/mL mouse IgG (Jackson ImmunoResearch Laboratories) or BSA (Sigma) before incubation with DCs at 9.1 \times 10⁷ beads/mL.

In Vitro Antigen Presentation Assays. DCs were incubated for 30 min with antigen and LPS, washed, and cultured with 1 \times 10⁵ CD4⁺ or CD8⁺ T cells purified

by negative selection (Miltenyi) from OT.2 or OT.1 mice, respectively. When indicated, the 96-well plates were coated with anti-CD28 antibody (10 μ g/mL; BD Biosciences). T-cell response was monitored by measuring either IL-2 accumulation in supernatant at 24 h by ELISA or by division of carboxyfluorescein succinimidylester-labeled T cells.

Immunofluorescence Confocal Microscopy. Cells were stained with anti-H-2M, anti-MHCII, or YAc-biotin antibodies as previously described (4). Imaging was performed with a Zeiss LSM 510 or Leica SP5 confocal microscope.

Electron Microscopy. Cells were processed as previously described (4). Sections were labeled with anti-MHCII or anti-LAMP-2 antibody followed by rabbit anti-rat antibody and 5 nm protein A-gold. They were examined with a Tecnai 12 Biotwin electron microscope (FEI).

Flow Cytometry. Data were collected on a BD Canto-II, FACSCalibur, or Aria (all from BD Biosciences), and analyzed with FlowJo software (Tree Star).

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