Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity

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Dendritic cells (DCs) are crucial for priming of naive CD8⁺ T lymphocytes to exogenous antigens, so-called “cross-priming.” We report that exogenous protein antigen can be conserved for several days in mature DCs, coinciding with strong cytotoxic T lymphocyte cross-priming potency *in vivo*. After MHC class I peptide elution, protein antigen-derived peptide presentation is efficiently restored, indicating the presence of an intracellular antigen depot. We characterized this depot as a lysosome-like organelle, distinct from MHC class II compartments and recently described early endosomal compartments that allow acute antigen presentation in MHC class I. The storage compartments we report here facilitate continuous supply of MHC class I ligands. This mechanism ensures sustained cross-presentation by DCs, despite the short-lived expression of MHC class I-peptide complexes at the cell surface.

antigen processing | cross-presentation | endocytosis | Fc receptor | MHC class I

Dendritic cells (DCs) are crucial in the initiation and orchestration of the T cell immune response (1–3). DCs operate as sentinels of an infection in the periphery and subsequently as conductors of the T cell response in the lymph nodes. To exert these functions, DCs are specialized in the ingestion, processing, and presentation of antigens acquired by receptor-independent pinocytosis or by receptor-mediated endocytosis (4–6). To this end, they are equipped with a diverse set of receptors for uptake of antigen, such as scavenger receptors, lectin receptors, or IgG (Fc γ) receptors (7).

Immature DCs have the capacity to efficiently acquire antigen, but a poor capacity to migrate and to stimulate T cells. Proper T cell response initiation requires maturation of the DCs, a process that is triggered by contact with infectious or inflammatory signals. Mature DCs characteristically show enhanced migratory capacity, up-regulation of the MHC class I processing machinery, and enhanced expression of MHC I and II and costimulatory molecules.

DCs present antigenic peptides in either MHC class I to induce CD8 T cell responses and in MHC class II to induce CD4 T cell responses. Whereas MHC class I ligands are commonly derived from breakdown products of endogenous proteins that are degraded by the proteasome (8), DCs also have the unique capacity to present peptides derived from exogenous antigens in MHC class I to CD8⁺ T cells, a process called “cross-presentation.” This process is crucial for induction of effective cytotoxic T lymphocyte (CTL) immunity against tumors, which lack direct priming capacity themselves, but also against microorganisms including viruses (1).

DCs have dedicated organelles to facilitate efficient loading of antigenic peptides in MHC class II molecules. These MHC class II compartments (MIIC) are multivesicular endosomes that express high levels of MHC class II and invariant chain and are abundantly present in immature DCs (9). On maturation of the

DC, rapid reorganization of the MIIC takes place that facilitates transport of MHC class II molecules loaded with peptide to the cell surface for presentation to CD4 T lymphocytes. Cell surface expression of MHC class II molecules loaded with peptide is strongly enhanced (10).

In contrast, the mechanism of MHC class I cross-presentation is less well characterized. In most studies, presentation of exogenous antigen by MHC class I molecules was proteasome and transporter associated with antigen processing (TAP) dependent, indicating that peptides are generated in the cytosol and transported into the endoplasmic reticulum (ER) for MHC class I loading (11, 12). It is not clear, however, how exogenous antigens gain access to the cytosol from the endocytic compartments. Substantial evidence supports involvement of components of the ER-associated degradation system (ERAD) in the translocation from the antigen-containing organelle into the cytosol (13–15). Alternatively, exogenously acquired antigen can also be processed and loaded in the endocytic track (16, 17).

We have now studied the longevity of MHC class I cross-presentation after highly effective receptor-mediated endocytosis of antigen by DCs. We observed storage of antigen for many days in a lysosome-like organelle, distinct from MHC class II compartments and different from the recently described early endosomal loading compartments (18, 19). The storage compartment described here serves as an antigen source for continuous supply of MHC class I ligands to sustain CD8 T cell cross-priming.

Results

Long-Lasting CTL Priming Capacity of DCs After a Short Antigen Pulse.

To study kinetics of cross-presentation, we used an Ab-mediated targeting system to deliver exogenous protein antigen to DCs. Antigen-specific IgG Abs bind antigen with high affinity and are efficiently taken up by Fc γ receptors (20). Uptake of Ab-bound antigen by DCs is Fc γ receptor dependent (Fig. S1A). Endocytosis of Ab-bound ovalbumin (IgG–OVA) by DCs is 1,000-fold more efficient than uptake of free OVA as measured by flow cytometry with Alexa Fluor 488-conjugated OVA (Fig. S1B). Targeting antigen via Fc γ receptors has 2 main advantages: very efficient uptake of the antigen and maturation of the DCs (21). Consequently, robust CTL responses can be induced by DCs loaded with Ab-bound antigen in submicromolar concentrations (20). To study the longevity of antigen presentation and priming capacity by DCs, we pulse loaded DCs with Ab-bound antigen, washed the cells to

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Fig. 1. Long-lasting CTL priming capacity of DCs after a short antigen pulse. (A) Priming of OVA-specific CTL in mice that were injected i.v. with DCs continuously incubated with 1 μ g/mL (20 nM) IgG-OVA for 48 h (c48); or pulse incubated for 1 h with 1 μ g/mL IgG-OVA and cultured for 48 h (p48) or 96 h (p96) in the absence of antigen. Each symbol represents the percentage of tetramer (TM)-specific CD8⁺ T cells per mouse. (B) Priming of OVA-specific CTL in mice with matured DCs 48 h after pulse loading with the following: 20 nM of the minimal MHC class I binding peptide SIINFEKL (OVA8) plus 10 μ g/mL LPS; 20 nM OVA protein (OVA) plus 10 μ g/mL LPS; or 20 nM IgG-OVA. (C) In vivo proliferation of CFSE-labeled OVA-specific TCR transgenic CD8⁺ T cells 2 days after i.v. injection of DCs that were pulse incubated with 1 μ g/mL IgG-OVA. (D) In vivo T cell proliferation (as in C) in spleen (black bars) and lymph nodes (gray bars) at different days after i.v. injection of pulse-incubated DC.

remove free antigen, and analyzed the kinetics of antigen presentation in MHC class I *in vitro* by coculture with OVA-specific B3Z T cells. The optimal incubation time with IgG-OVA was assessed by pulse incubations of different lengths. Pulse incubation of 1–2 h was already sufficient to induce optimal B3Z T cell activation comparable to 48 h of continuous incubation (Fig. S1C). DCs pulse loaded with irrelevant antigen–Ab complexes did not activate B3Z T cells, indicating that activation of B3Z is antigen dependent (Fig. S1D and E).

To analyze the cross-priming potency of pulse-loaded DCs *in vivo*, naive C57BL/6 mice were injected i.v. with DCs at 48 or 96 h after antigen pulse loading. In both groups of mice, high levels of OVA-specific CD8⁺ T cells were observed, similar as in mice injected with DCs continuously incubated with IgG-OVA for 48 h (Fig. 1A). In contrast, very inefficient priming of OVA-specific CTL was found when mice were vaccinated with mature DCs pulse loaded with equimolar amounts of OVA protein or the minimal peptide (OVA8, SIINFEKL) (Fig. 1B). These results show that IgG-OVA-pulsed DCs have significantly prolonged cross-priming capacity *in vivo* compared with minimal peptide-pulsed DCs. As a control, using K^b mutant bm1 and TAP KO-derived bone marrow-derived dendritic cells (BM DCs), we showed that T cells were directly rather than indirectly primed by the injected DCs (Fig. S2).

Furthermore, we examined the longevity of cross-presentation *in vivo* to adoptively transferred 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled naive transgenic T cells that recognize the OVA8 peptide presented in MHC class I. T cell proliferation was analyzed in the spleen and lymph nodes by flow cytometry. Fig. 1C shows strong proliferation of CD8 T cells 48 h after injection of DCs pulse loaded with IgG-OVA. Strikingly, as long as 7 or 14 days after injection of pulse-loaded DCs, significant proliferation was observed (Fig. 1D).

We have previously reported that DCs continuously incubated with IgG-OVA for 48 h fully protect mice in a CTL-dependent fashion against a challenge with the lethal tumor cell line B16-OVA, an OVA-expressing melanoma (22). DCs pulse loaded with IgG-OVA 48 h before injection were fully effective in protecting mice challenged with B16-OVA (Fig. S1F). Importantly, this shows that long-lived antigen presentation by mature DCs results in the induction of highly functional T cells *in vivo*.

Instability of MHC Class I–Peptide Complexes on Cell Surface of Mature DCs. To investigate the mechanism of the sustained cross-priming capacity of DCs, we analyzed the antigen presentation of pulse-loaded DCs *in vitro*. Antigen cross-presentation of Ab-bound OVA remained detectable for at least 3 days, in contrast to presentation of pulse-loaded minimal MHC class I binding peptide, which was almost undetectable after 1 day (Fig. 2A). Similar results were obtained when we analyzed another efficient antigen targeting system: Toll like receptor 2 ligand Pam3CysSK4 conjugated to a long peptide containing the OVA CTL epitope SIINFEKL (23) (TLRL-PEP) (Fig. 2B). Both antigen delivery systems have in common that the antigen needs intracellular processing, in contrast to the minimal MHC class I binding peptide that binds directly to MHC class I molecules at the cell surface. These results indicate that processing-dependent antigen, either protein or long peptide, is presented to CD8 T cells for a prolonged period.

The prolonged antigen presentation could be explained by the stability of the MHC class I–peptide complexes on the maturing DC. MHC class II–peptide complexes have been described as relatively stable at the cell surface of matured DCs (24, 25).

Therefore, we compared the stability of MHC class I and MHC class II–peptide complexes in DCs targeted with IgG-OVA leading to simultaneous maturation of the DC. Twenty-four hours after the pulse loading, all cell surface molecules of the DCs were labeled with biotin and further cultured. Cell samples were lysed at subsequent days, and immune precipitation with MHC-specific Abs was performed to assess the presence of biotinylated MHC class I and class II molecules. Strikingly, the majority of biotinylated MHC class I molecules had disappeared already after 24 h, whereas biotinylated MHC class II molecules were relatively stable; after 48 h, >50% still remained (Fig. 3A).

In parallel, we analyzed antigen presentation capacity of matured DCs pulse loaded with minimal MHC class I and II binding peptides. We observed that antigen presentation to CD4 T cells was sustained significantly longer than antigen presentation to CD8 T cells (Fig. 3B). Together, these data indicate that MHC class I–peptide complexes, in contrast to MHC class II–peptide complexes, are relatively unstable at the cell surface of mature DC and have a high turnover. These data indicate that prolonged cross-presentation of IgG-OVA is not related to the stable presence of MHC class I–peptide complexes at the cell surface.

Sustained MHC Class I Cross-Presentation from an Internal Antigen Source. We further studied the long-lasting cross-presentation by disrupting MHC class I–peptide complexes at the cell surface by elution with mild acid (26). DCs were pulse loaded with IgG-OVA, TLR ligand-long peptide conjugate or the OVA8 minimal peptide. Mild acid elution of the live cells reduced MHC class I presentation to background levels (Fig. 2C–E). Importantly, 16 h after elution, antigen presentation was recovered by IgG-OVA pulse-loaded DCs (Fig. 2C) and TLR ligand-long peptide conjugate-loaded DCs (Fig. 2D), but not by DCs pulsed with the OVA8 minimal peptide (Fig. 2E). The reappearance of MHC class I antigen presentation without renewed uptake of antigen indicates that the MHC class I ligands were derived from an internal antigen source. To examine whether the recovery of MHC class I antigen presentation from the internal antigen source requires processing by the proteasome, DCs were treated with epoxomicin, a specific proteasome inhibitor,

Fig. 2. Sustained MHC class I antigen presentation from an internal antigen source in DCs. (A and B) In vitro CD8⁺ T cell activation of DCs at subsequent days after pulse incubation with 20 nM IgG-OVA (A, black bars) or 0.5 μ M TLR ligand-long peptide conjugate (TLRL-PEP) (B, black bars), compared with equimolar amounts of OVA8 (white bars). Values depicted are relative to day 0. Experiment was repeated twice with similar results in both D1 DCs and BM DCs. (C–E) In vitro CD8⁺ T cell activation by DCs pulse loaded with 10 nM IgG-OVA (C), 0.5 μ M TLRL-PEP (D), or 10 nM OVA8 (E). CD8⁺ T cell activation was assessed 48 h after pulse loading with medium or the different compounds before (black bar) or after treatment with elution buffer (white bar) and after 16 h recovery in the absence of antigen (gray bar). Error bars represent SD of triplicates. Experiments were performed 6 times with similar results in both D1 DCs and BM DCs. (F) CD8⁺ T cell activation by DCs 48 h after pulse loading with medium or after pulse loading with 10 nM IgG-OVA with or without treatment with elution buffer and proteasome inhibitor epoxomicin (epox). CD8⁺ T cell activation was assessed directly after elution (white bar), or after 2 and 4 h of recovery with or without 5 mM epoxomicin. Experiment was performed 3 times with similar results.

before and during recovery after mild acid elution. In untreated cells, CTL recognition was restored for >50% as early as 4 h after elution. In cells treated with epoxomicin, recovery of MHC class I presentation was almost completely inhibited (Fig. 2F). In addition, in cells that lack TAP, as well as in cells that lack both TAP and β_2 -microglobulin (β_2 M), initial presentation as well as recovery from the internal antigen source was not observed (Fig. S3A). Equal uptake of IgG-OVA complexes was verified by using Alexa Fluor 488-conjugated OVA. Exogenous peptide loading of the minimal peptide SIINFEKL shows that the TAP-deficient cells express sufficient MHC class I levels on the cell surface in contrast to cells that also lack the MHC class I light chain β_2 M (Fig. S3B). Taken together, these results indicate that processing of antigen from the newly identified intracellular source involves most likely a cytosolic proteasome and TAP-dependent pathway.

Fig. 3. MHC class I-peptide complexes are short-lived on DCs compared with stable MHC class II-peptide complexes. (A) (Lower) Decrease of cell surface MHC class I (black bars) and β -chain of MHC class II (white bars) 3 consecutive days after biotinylation of D1 DCs that were pulse loaded with IgG-OVA 1 day earlier. (Upper) Immunoprecipitated MHC class I and II molecules detected by Western blot analysis. This experiment was performed 2 times with similar results. (B) Decrease of MHC class I and MHC class II antigen presentation by DCs pulse incubated with minimal peptides. D1 DCs were pretreated for 24 h with 10 μ g/mL LPS and pulse incubated for 2 h with 100 ng/mL MHC class I (OVA8) (black bars) and 20 μ g/mL MHC class II binding peptides (MuLV19) (white bars) at different days before analysis of specific T cell activation. Experiment was performed 2 times with similar results.

Intracellular Conservation of Antigen After Receptor-Mediated Uptake. To analyze the intracellular fate of antigen after receptor-mediated endocytosis, we pulse loaded DCs with Ab-targeted OVA conjugated to Alexa Fluor 488 (IgG-OVA^{AlexaFluor}) and analyzed the cells at several time points by flow cytometry (Fig. 4A). Uptake of fluorescent OVA bound to IgG is very efficient; already, after 1 h, strong fluorescence is detectable as shown previously (20) and in Fig. S1A. After extensive washing, the fluorescence signal still remained for several days. After 2 days, 75% of the fluorescence was detectable and after 4 days the fluorescence diminished to $\approx$ 50% (Fig. 4A).

To analyze the nature of the antigen conserved in DCs, total cell lysates of DCs obtained at several time points after pulse loading with IgG-OVA^{AlexaFluor} were analyzed by SDS/PAGE. Between 0 and 8 h after antigen pulse, the original 45-kDa OVA band and a slightly smaller OVA product ($\approx$ 40 kDa) were visible (Fig. S4). Subsequent days after the antigen pulse, the 40-kDa OVA species remained detectable in the cells that were pulse loaded with

Fig. 4. Intracellular conservation of antigen after receptor-mediated uptake. (A) Persistence of fluorescence in DCs at subsequent days after pulse incubation with IgG-OVA^{AlexaFluor488} as measured by flow cytometry. Experiment was performed 4 times with similar results in both D1 DCs and BM DCs. (B) Persistence of OVA protein fragments in IgG-OVA^{AlexaFluor488} pulse-loaded DCs determined by SDS/PAGE visualized directly in gel. Right lanes, Total cell lysates of 2×10^5 DCs collected at subsequent days after pulse loading. Left lanes, One or 10 ng of OVA^{AlexaFluor488}. Experiment was performed 4 times with similar results in both D1 DCs and BM DCs.

Fig. 6. Antigen storage organelles are electron dense with lysosomal characteristics. (A–C) Immunoelectron microscopy images of D1 DCs at 48 h after pulse loading with IgG–OVA^{AlexaFluor488}. Sections were double ImmunoGold labeled for MHC class II (A), LAMP1 (B), Invariant chain (IC) (C), and Alexa Fluor 488 (A–C) with gold particle sizes as indicated in nanometers in superscript. AD indicate antigen depots (AD); arrows indicate LAMP1. PM, plasma membrane; M, mitochondrion; EE, early endosome; G, Golgi complex. (Scale bars, 200 nm.)

tively and qualitatively different content of lysosomal enzymes than macrophages, relating to efficiency of MHC class II presentation. In addition, it has been reported that endosomes in DCs are actively alkalinized by the NADPH oxidase NOX2, creating an environment unfavorable for lysosomal proteases (28). Thus, these data support our findings that DC can retain antigen for prolonged periods of time in specialized organelles. The crucial finding of our study is that the conservation of protein antigen in DC is related to prolonged MHC class I presentation.

The depot formation described here was studied in 2 different systems of receptor-mediated uptake: IgG-antigen and TLR ligand-long peptide conjugates (Fig. 2). Both modes of presentation combine efficient targeting of antigen to the DC and a DC maturation signal in one compound and are therefore attractive vaccine formulations. This combination leads to a more efficient priming capacity compared with long peptides not conjugated to TLR ligand (23) or OVA not bound to IgG (20). The efficient targeting and maturation in one compound might be a requirement for the depot formation. Indeed, we could not induce depot formation when we pulse loaded DCs with free OVA or OVA peptide even when combined with a separate TLR ligand. Both formulations used in this study contain antigen that requires intracellular processing to release the MHC class I ligand(s). The proteasome is the most important enzyme system responsible for the degradation of protein antigens. We have previously described that the proteasome activator PA28 is strongly up-regulated after stimulation with IgG–OVA, CD40 stimulation, or TLR triggering of DCs (29). This indicates that maturing DCs can increase their antigen-processing mechanisms as well as enhance their costimulatory capacity. Together, these 2 mech-

anisms favor optimal continuous presentation of antigenic peptides derived from the antigen depots to T cells.

We have observed that recovery of cross-presentation after peptide elution was proteasome dependent (Fig. 2F). The antigen may reach the proteasome when it is translocated from the storage compartments into the cytosol as described for Ab-bound antigen (12). After proteasomal degradation, the antigen may follow the classical ER-located MHC class I presentation pathway, because we have observed that Fc γ R-dependent cross-presentation and recovery after elution is TAP dependent (Fig. S3). However, treatment with brefeldin A, an inhibitor of protein secretion, and treatment with cycloheximide, an inhibitor of protein synthesis, only marginally affected recovery after elution. These data would argue against the ER as the site of MHC class I loading and leaves space for an alternative route. Fusion of components of the ERAD with antigen-containing compartment has been described in refs. 13–15. In this study, we did not find evidence in support of such an ER–antigen fusion compartment, because the majority of antigen depots lack the presence of TAP and MHC class I. Therefore, we conclude that the antigen-containing organelles are storage depots but not MHC class I loading compartments. We cannot exclude the involvement of distinct MHC class I loading compartments, possibly containing recycling MHC class I from the cell surface (16, 17). The intracellular MHC class I hotspots we observed in close proximity to the antigen-containing compartments (Fig. 5C and Fig. S5 A and B), and the fast recovery of antigen presentation after elution may support this hypothesis. Therefore, we propose that the antigen-containing organelle described here is an antigen storage compartment rather than an MHC class I processing/loading compartment.

In Fig. 3, we show that cell surface-expressed MHC class I molecules have a significantly shorter half-life than MHC class II molecules on mature DCs. This correlates with different kinetics of presentation of exogenously loaded antigenic peptides to CD8 and CD4 T cells. Whereas MHC class II–peptide complexes are stable for several days, most MHC class I–peptide complexes disappear from the cell surface within 24 h. The kinetics of MHC class I molecule turnover are important for biological function in immune surveillance by CD8 T cells. MHC class I molecules display an up-to-date overview of the internal content of the cell; they continuously present ligands derived from cytosolic proteins, newly synthesized misfolded proteins, or viral proteins (8). However, without a continuous supply route, this high turnover of peptide–MHC complexes does not favor cross-priming of CD8⁺ T cells. Professional antigen-presenting cells such as DCs engulf the antigen in the periphery and travel to the T cell zones in lymphoid organs without encountering a new source of exogenous antigen (30). This migration time has been estimated to last 24–48 h (31, 32). At the time of arrival, the number of cognate peptide–MHC class I complexes in the immunological synapse needs to be above a threshold to ensure effective contacts between DCs and T cells (33). Thus, DCs require the important function of exogenous antigen storage to ensure continuous generation of MHC class I ligands for presentation to CD8 T cells. We have shown that mature DCs have a long-term capacity to prime antigen-specific CD8⁺ T cells in vivo (Fig. 1).

The minimal binding peptide we have used in this study, SIINFEKL, has a very high affinity for MHC class I (34), yet we observed that the T cell-priming capacity of DCs pulsed with this peptide is less effective and less sustained than that of DCs pulsed with Ab-bound protein or long peptide. We propose that this difference is mediated by the depot formation described in the current study. Our data indicate that antigen targeting to internal depots, where the antigen is conserved, may improve the effectiveness of vaccines. Therefore, vaccine formulations composed of targeted protein or long peptides leading to long-lived cross-priming capacity of DCs are to be favored over vaccine formulations composed of minimal peptides that are rapidly lost from MHC class I molecules, which are often used in vaccination and DC-based adoptive transfer trials nowadays.

Materials and Methods

DCs. Both BM DCs and the spleen-derived D1 DC line (20, 35) were used for all experiments except those of Figs. 1, 3, and 6, which were performed with D1 DCs.

Pulse Loading of DCs with IgG-OVA Complexes. IgG-OVA immune complexes (IgG-OVA) were made by incubating OVA (Worthington) or OVA conjugated with Alexa Fluor 488 or Alexa Fluor 647 (Molecular Probes) with polyclonal rabbit anti-OVA IgG (ICN Biomedicals) for 30 min at 37 °C in a mass ratio 1:50 as described in ref. 22. For pulse loading, 10 $\times$ concentrated IgG-OVA was added to medium and incubated for 1 or 2 h at 37 °C. To remove antigen, DCs were washed 3 times with culture medium and subsequently cultured antigen-free for the period indicated.

In Vivo Experiments. Priming of endogenous OVA-specific CTL in vivo was analyzed by using SIINFEKL/K^b-tetramers labeled with allophycocyanin (36).

Cross-presentation of OVA in vivo was determined by using CFSE-labeled lymphocytes of OT-1 mice, transgenic for the T cell antigen receptor (TCR) recognizing the OVA epitope SIINFEKL in H-2K^b.

In Vitro CD8 and CD4 T Cell Activation Assay. CD8 T cell activation by DCs loaded with IgG-OVA, Pam3CysSK4-long peptide conjugate (23), or synthetic MHC class I-binding peptide OVA₂₅₇₋₂₆₄ (OVA8, SIINFEKL) in vitro was determined by using B3Z T cell hybridoma (37). CD4 T cell activation by DCs loaded with murine leukemia virus (MuLV) helper peptide (EPLTSLTPRCNTAWNRLKL) was determined by using 3A12-Z hybridoma (generated in our laboratory), specific for MuLV-derived peptide in I-A^b (38).

Mild Acid Elution of DCs. DCs pulse loaded with antigen were incubated for 90 s with mild acid citrate/phosphate buffer (pH 3.3) at room temperature to disrupt MHC class I-peptide complexes (26). Cells were either fixed in 0.2% paraformal-

dehyde directly after elution or incubated in culture medium for recovery at 37 °C and then fixed. In experiments with the proteasome inhibitor, cells were treated for 1 h with 5 μ M epoxomicin before elution and during recovery.

Confocal Scanning Laser Microscopy. DCs were transferred to glass-bottom dishes (MatTek) 48 h after pulse incubation with IgG-OVA^{AlexaFluor488}, fixed with 3.7% formaldehyde (Merck), and permeabilized with 0.5% saponin. DCs were subsequently incubated with Abs (see *SI Methods*) for 30 min at 37 °C in medium containing 0.1% saponin. In Fig. 5E, live BM DCs of the MHC class II EGFP knockin mouse were cultured on glass-bottom dishes, pulse incubated with IgG-OVA^{AlexaFluor647}, and imaged after 48 h. Optical zoom was 63 $\times$.

Immunoelectron Microscopy. Cryosections of D1 DCs were prepared as described in ref. 39. Forty-eight hours after pulse loading with IgG-OVA^{AlexaFluor488}, sections were ImmunoGold double-labeled by using specific Abs against Alexa Fluor 488, LAMP1, LAMP2, MHC class II, or IC with 10- and 15-nm gold particles as indicated.

Turnover of MHC Class I and MHC Class II. Twenty-four hours after pulse loading with IgG-OVA, DCs were cell surface biotinylated. At indicated time points, MHC class I and MHC class II molecules were immunoprecipitated (B8.3.24 and M5/114) from Triton X-100 lysates. Subsequently, biotinylated MHC was visualized by Western blot analysis using streptavidin-HRP.

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