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Dendritic Cells Continue To Capture and Present Antigens after Maturation In Vivo

Scott B. Drutman and E. Sergio Trombetta

Dendritic (DC) maturation is critical for the regulation of T cell responses. The downregulation of endocytosis on maturation is considered a key adaptation that dissociates prior Ag capture by DCs from subsequent T cell engagement. To study the dynamics of Ag capture and presentation in situ, we studied the capacity for Ag uptake by DCs matured in their natural tissue environment. We found that after maturation in vivo, mouse DCs retained a robust capacity to capture soluble Ags. Furthermore, Ags internalized by mature DCs were efficiently presented on MHC class II and cross-presented on MHC class I. These results suggest that under inflammatory conditions, mature DCs may contribute to T cell stimulation without exclusively relying on prior exposure to Ags as immature DC precursors. *The Journal of Immunology*, 2010, 185: 2140–2146.

**Materials and Methods**

**Mice**
C57BL/6 (B6). OT-I/RAG1 (OT-I), OT-II2.a/RAG1 (OT-II), CHHeN (C3H), B6.SJL (CD45.1), and Abb (MHC class II knockout [MHC-II-KO]) mice (29) were from Taconic Farms (Germantown, NY). B6, C57BL/6 (B6), OT-I/RAG1 KO or OT-II/RAG1 KO mice by disruption through a 40-μm cell strainer, treated with ACK Buffer (Lonza, Walkersville, MD) to remove red cells, and resuspended in PBE. DCs were subsequently sorted on a Dako MoFlo.

**Reagents**
PBE is PBS, 0.5% BSA (endotoxin free, Equitech-Bio, Kerrville, TX), 1 mM EDTA, pH 7.4. Complete RPMI 1640 is RPMI (Life Technologies, Rockville, MD), 10% heat-inactivated FBS (endotoxin free, Invitrogen, San Diego, CA), nonessential amino acids, 110 μg/ml sodium-pyruvate, 2 mM t-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 100 μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). For preparation of bacterial lysate (BL), DH5 K-12 Escherichia coli (Invitrogen) were grown overnight in LB media, washed three times in PBS, resuspended at 3 × 10⁶ bacteria/ml, heated at 80°C for 45 min, subjected to five freeze/thaw cycles and finally passed through a fine gauge needle three times to disrupt clumps. The lysate contained ~6 × 10⁵ EU/ml (~10 μg/ml) endotoxin by LAL test (Cambrex, East Rutherford, NJ).

**Cells**
For isolation of DCs, spleens were digested with Liberase Blendzyme 2 (Roche Diagnostics Systems, Somerville, NJ) for 15 min in PBS at 21°C, passed through a 40-μm cell strainer, treated with ACK Buffer (Lonza, Walkersville, MD) to remove red cells, and resuspended in PBE. DCs were first enriched to 30–50% by magnetic negative depletion by incubating splenocytes with biotinylated CD19 (MB19.1), CD3 (145-2C11), NK1.1 (PK136), Ly-6G/Gr-1 (RB6-8C5), and erythroid cell marker (TER-119) Abs (eBioscience, San Diego, CA), followed by enrichment using the EasySep biotin selection kit (StemCell Technologies, Vancouver, British Columbia, Canada). DCs were subsequently sorted on a Dako MoFlo. Postsort analysis confirmed purity of 99% and viability of 95%. OT-I or OT-II cells were isolated from the lymph nodes and spleens of OT-I/RAG1 KO or OT-II/RAG1 KO mice by disruption through a 40-μm cell strainer, followed by negative selection using mouse CD8 T cell or mouse CD4 T cell enrichment kit, respectively (StemCell Technologies). Enriched T cells were pulsed with 0.5 mM CFSE (Invitrogen) for 5 min, washed twice, and resuspended in PBS for adoptive transfers or complete RPMI for T cell stimulation assays.

**Preparation of GFP-OT**
GFP-OT construct (Supplemental Fig. 4) in pET-28 vector (Novagen, Madison, WI) was transformed into BL21 E. coli (Novagen). Bacteria

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The online version of this article contains supplemental material.

Abbreviations used in this paper: BL, bacterial lysate; DC, dendritic cell; KO, knockout; MHC-I, MHC class I; MHC-II, MHC class II; NTx, nontreated; VSV, vesicular stomatitis virus.

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The Journal of Immunology

[2010] Dendritic cells (DCs) are critical APCs characterized by their efficient conversion of internalized Ags into peptide–MHC complexes required to orchestrate T cell responses (1–3). Critical DC functions, such as Ag presentation, expression of costimulatory molecules, and migration, are tightly controlled by a maturation process, where stimuli, such as TLR ligands or inflammatory cytokines, convert immature DCs into mature DCs specialized for T cell stimulation (3, 4). Manipulation of DC maturation shows high therapeutic potential, especially in inflammatory conditions, mature DCs may contribute to T cell stimulation without exclusively relying on prior exposure to Ags as immature DC precursors.
were grown in TB media (Invitrogen) at 37˚C until they reached ~0.1 absorbance units at 600 nm, then at 24˚C for 16 h with 1 mM IPTG (Sigma-Aldrich), spun down at 8000 × g for 15 min, resuspended in 50 mM Tris pH 8.0, 500 mM NaCl, 50 µg/ml lysozyme (Sigma-Aldrich), incubated for 30 min, and then PMSF and benzamidine were added to 10 µg/ml (Sigma-Aldrich). After three rounds of ~80˚C freeze/37˚C thaw and sonication, the lysate was centrifuged at 20,000 × g for 30 min, and the supernatant 0.22 µm filtered. Imidazole was added to 20 mM, GFP-OT was affinity purified on Ni-Sepharose (GE Healthcare, Piscataway, NJ), and eluted in 50 mM Tris pH 8.0, 200 mM NaCl, 500 mM imidazole. The eluate was diluted 10-fold with H2O, adjusted to pH 9.5 with NaOH, bound to Q-Sepharose (GE Healthcare), washed with 0.5% NP-40, 50 mM Tris pH 9.5 to remove endotoxin, washed with 50 mM Tris pH 9.5 to remove all detergent, eluted with 500 mM NaCl, 50 mM Tris pH 9.5, and dialyzed against PBS. The resulting GFP-OT protein had <1.26 EU/mg (~<1.25 pg/mg) endotoxin by LAL test (Cambrex).

Maturation of DCs

Maturation protocols were chosen to provide the maximum level of inflammation achievable with these reagents as judged by upregulation of maturation markers on DCs. Sterile, endotoxin-free PBS was used for control injections. For maturation of DCs with TLR agonists, 1 µg LPS (Salmonella enterica serotype typhimurium, Sigma-Aldrich) or 20 nmol of CpG-B (ODN 1668 5'-TCCATGACGTTCCTGATGCT-3' with phosphorothioate bonds, Invitrogen) were injected i.p. 16 h prior to experiments. In vivo BL mediated maturation, 200 µl E. coli lysate (~6 × 108 bacteria, containing ~2 µg LPS) was injected i.p. 16 h prior to experiments.

Endocytosis assays

For in vitro endocytosis assays, 1 × 107 cells/ml were incubated with GFP-OT (100 µg/ml) at 37˚C (or kept on ice for negative controls) for 30 min in complete RPMI 1640. All cells were washed three times in PBE, before analysis by flow cytometry. For in vivo endocytosis assays, mice were injected i.p. with 0.5–2 µg of GFP-OT and 30 min later, splenocytes were collected and analyzed for Ag capture as compared with a similarly treated mouse not injected with GFP-OT.

In vitro Ag presentation assays

To assay presentation of Ag captured by DCs in vivo, 0.5–2.0 µg GFP-OT or OVA was injected i.v. into mice, and 30 min later DCs were purified as described. Various numbers of DCs were coincubated in U-bottom 96-well plates with 50,000 CFSE-labeled OT-I CD8+ T cells or OT-II CD4+ T cells in complete RPMI 1640 to allow for a variety of T cell to DC ratios. Sixty hours later, T cell proliferation was assessed by dilution of CFSE using flow cytometry. In addition, supernatants from these incubations were harvested to measure IFN-γ expression achievable with these reagents as judged by upregulation of maturation markers on DCs.

In vivo Ag presentation assays

Mice were injected i.p. with maturation stimuli or vehicle control, and i.v. with 1 × 108 CFSE-labeled OT-I and/or OT-II cells. After 16 h, mice were injected i.v. with 200 µg OVA. Sixty hours later, splenocytes were isolated and T cell proliferation was analyzed by flow cytometry. To measure serum anti-OVA IgG, Costar type 2592 plates coated with OVA were incubated with sera, and IgG was detected with HRP-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA). ELISAs were developed with 1-Step TurboTMB-ELISA substrate (Pierce, Rockville, IL), and absorbance at 450 nm was read with a Tecan Sunrise plate reader.

DC maturation in culture

For in vitro maturation, splenocytes were cultured overnight in complete RPMI at ~8 × 106 cells/ml. In some experiments the 10% FBS in the complete RPMI was replaced with either 10% mouse serum from a non-treated mouse, or 10% mouse serum from a mouse inflamed with BL for 16 h. For adoptive transfer experiments, splenocytes cultured overnight to mature the DCs were i.e. injected into CD45.1 mice that we either non-treated or inflamed with BL for 16 h. (1.0 × 107 splenocytes per mouse). After various amounts of time, 42 mg GFP-OT was injected, and 30 min

![FIGURE 1. DCs matured in vivo with LPS or CpG present subsequently encountered soluble Ag. A, Analysis by flow cytometry of surface expression of maturation markers on spleen DCs from non-treated mice (solid line), mice treated with LPS 16 h prior (dotted line), and mice treated with CpG-B 16 h prior (dashed line). Shaded histograms show staining obtained with isotype controls. B, Experimental scheme for Ag presentation assays. C, Immature DCs from NTx mice, mature DCs from CpG inflamed mice, or mature DCs from LPS inflamed mice were purified (as described in Supplemental Fig. 1) 30 min after injection with 1 mg OVA, and cocultured with CFSE-labeled OT-I or OT-II T cells over a range of T cell/DC ratios. After 60 h, Ag presentation was assessed by flow cytometric analysis of CFSE dilution to measure T cell proliferation. Flow cytometry data shown is T cell/DC ratio of 1:1. DCs purified from mice not injected with Ag were similarly analyzed. D, Quantification of T cell proliferation as described in B induced by immature DCs from non-treated mice (triangles), mature DCs from LPS-treated mice (squares), or mature DCs from CpG-treated mice (circles).](http://www.jimmunol.org/Download?uri=10.1181/00221767.2011.605269)
later, splenocytes were harvested and GFP capture by the DCs was analyzed by flow cytometry as compared with a similarly treated mouse, not injected with GFP. The endogenous DCs were discriminated from the transferred DCs by CD45.1 and CD45.2 staining.

**Flow cytometry**

Cells were preincubated with 10 μg/ml 2.4G2 mAb (Bio X Cell, West Lebanon, NH) for 15 min at 4°C in PBE, incubated with mAb conjugates for 30 min at 4°C and resuspended in PBE with 0.5 μg/ml 7-aminoactinomycin-D (7AAD) (Invitrogen) 10 min before analysis. All samples were gated on live cells by scatter and 7AAD exclusion. Data were collected on a FACSCanto (BD) and analyzed with FlowJo software (Tree Star, Ashland, OR). Abs: PE or allophycocyanin-Alexa750-CD8α (53-6.7), PE-Cy7 CD11c (N418), allophycocyanin-Alexa750-CD45.1 (A20), Pacific Blue or allophycocyanin-CD45.2 (104), allophycocyanin-CD4 (GK1.5), FITC-Ly-6C (HK1.4), Alexa488, allophycocyanin, or PE or allophycocyanin-CD8α (16-10A1) and corresponding isotype control (Armenian Hamster IgM), Alexa488-CD40 (HM40-3) and Alexa488-isotype control (Armenian Hamster IgG), Alexa488-CD40 (HM40-3) and Alexa488-isotype control (Armenian Hamster IgM), Alexa488 or allophycocyanin-CCR7 (4B12) and corresponding-isotype control (Rat IgG2a), PE or allophycocyanin-CD80 (16-10A1) and corresponding isotype control (Armenian Hamster IgG), Alexa488-CD45.1 (A20) conjugated by immature DCs from nontreated mice or mature DCs from BL-treated mice (squares). Similar to experiments in A and B, analysis as in A, but on lymph node DCs. C, Immature DCs from NTx mice or mature DCs from BL inflamed mice were purified (as described in Supplemental Fig. 1) 30 min after injection with 2.0 mg OVA, and analyzed for Ag presentation as described in Fig. 1C. D, Quantification of T cell proliferation as described in C induced by immature DCs from nontreated mice (triangles), or mature DCs from BL-treated mice (squares). E, Similar to experiments in B and C, except using 0.5 mg OVA injections. Additional flow cytometry data are shown in Supplemental Fig. 3A. F, Similar to experiments in D and E but after 60 h the supernatants were harvested and used to measure IFN-γ released by the OT-I T cells stimulated by immature DCs from nontreated mice (triangles) or mature DCs from BL-treated mice (squares). G, Similar to experiments in D and E but after 60 h, the OT-I T cells stimulated by immature DCs from nontreated mice (triangles), or mature DCs from BL-treated mice (squares) were assessed for cytolytic function as described in Materials and Methods.

**Results**

DCs matured in vivo continue to capture and present soluble Ags

Systemic DC maturation was induced in mice with the TLR agonists LPS or CpG DNA. Injection of either inflammatory stimuli (LPS or CpG) resulted in systemic DC maturation evidenced by the uniform upregulation of surface markers characteristic of maturation on spleen DCs (Fig. 1A). We next compared the capacity of immature DCs (in control, noninflamed mice) and DCs matured in vivo (in mice treated with either LPS or CpG) to internalize and present soluble Ags. Control mice or mice that had been previously inflamed (in which mature DCs had been generated and accumulated for 16 h as described in Fig. 1B) were injected i.v. with OVA. The Ag (OVA) was then allowed to circulate in the mice for 30 min, a period much shorter than the time needed for conversion of immature into mature DCs, assuring that the phenotype of the DCs did not change during their brief exposure to the injected Ag. After this 30 min Ag pulse, spleens were harvested and immature DCs from control mice or mature DCs from inflamed mice were isolated by cell sorting. The method used to isolate mature DCs removed residual immature or incompletely mature DCs present in the sample (Supplemental Fig. 1). The isolated DCs were then assayed for their capacity to present the Ag they may have captured in vivo (Fig. 1B).

As expected, immature DCs isolated from control mice (nontreated [NTx]-immature) were able to stimulate OT-I CD8+ or OT-II CD4+ T cells after injection of OVA, indicating that the DCs had captured Ag in vivo and presented the internalized Ag to T cells (Fig. 1C, 1D). Surprisingly, mature DCs isolated from OVA injected mice that had been previously inflamed with LPS or CpG were also able to stimulate both CD8+ OT-I and CD4+ OT-II T cells similarly to immature DCs isolated from control
The maintenance of Ag capture by DCs matured in vivo is not limited to a specific TLR ligand

To determine whether our findings were specific to the individual TLR ligands used, we decided to use a whole cell lysate of E. coli, providing a natural mix of microbial products that stimulate multiple TLRs mimicking the exposure to microbes (30, 31). Injection of mice with this BL also induced systemic maturation of both spleen DCs (Fig. 2A) and lymph node DCs (Fig. 2B). These changes were also accompanied by the characteristic redistribution of MHC-II to the cell surface of mature DCs (Supplemental Fig. 2). Similar to our results obtained with LPS- and CpG-matured DCs, mature DCs from mice inflamed with BL continued to present OVA captured briefly after i.v. injection (Fig. 2C, 2D, Supplemental Fig. 3A). A similar result was observed after injection of a lower dose of OVA (Fig. 2E, Supplemental Fig. 3A). We also verified that OT-I T cells were stimulated with similar efficacy to produce IFN-γ (Fig. 4F) and develop cytotoxic effector activity (Fig. 4G) by spleen DCs that were either immature or mature at the time of Ag capture.

Given that the mannose receptor has been reported to contribute to the internalization of OVA (32, 33), we extended our experiments by using a soluble chimeric GFP (referred to as GFP-OT, Supplemental Fig. 4) containing MHC-I and MHC-II restricted epitopes recognized by CD8+ OT-I and CD4+ OT-II transgenic T cells. This GFP-OT protein is devoid of carbohydrates and therefore unlikely to be internalized by lectin-like receptors. Injection of GFP-OT gave results similar to injection of OVA at two different Ag doses (Fig. 3, Supplemental Fig. 3B), further supporting the retention of endocytic activity by DCs matured in vivo.

We verified that the maturation status of the DCs did not change during the isolation procedure and that the quantity and types of DCs present in the samples did not differ or reflect the contribution of monocyte-derived inflammatory DCs (Supplemental Fig. 5). We also wanted to verify that there was no carry-over of Ag from the DC isolation procedures or release of Ag from these purified DCs that might have been subsequently recaptured during the assay and presented to T cells. For this, we used DCs purified from C3H mice, which cannot directly stimulate OT-I or OT-II T cell proliferation due to MHC haplotype mismatch. DCs purified from C3H mice injected with OVA did not induce T cell proliferation, even in the presence of B6 DCs that would have been able to present any released Ag (data not shown), indicating Ag is not released from the isolated DCs during the assay, and therefore all T cell stimulation
observed (Figs. 1–3, Supplemental Fig. 3) was due to presentation of Ag internalized in vivo.

**Presentation of Ags encountered in vivo by mature DCs is due to maintenance of their capacity to internalize soluble Ag**

The results of the Ag presentation experiments (Figs. 1–3) are compatible with different scenarios. First, these results might be due to the extracellular loading of peptides on immature and/or mature DCs, bypassing the need for internalization and processing. We ruled this out by assaying the presentation of the injected Ag by B cells, which capture and present OVA protein very inefficiently, but can present peptides loaded extracellularly. B cells isolated from mice injected i.v. with OVA peptide were able to load it extracellularly and subsequently induce OT-I T cell proliferation in vitro, whereas B cells from OVA injected mice did not (Supplemental Fig. 6). These results indicate that in the experiments described previously (Figs. 1–3), OVA injections did not result in detectable release of peptides that could be loaded extracellularly on MHC molecules.

Alternatively, our Ag presentation results could reflect extremely efficient processing and presentation of trace amounts of Ag that might still have been captured by most of the DCs matured in vivo after a significant but incomplete downregulation of Ag uptake. Finally, and in contrast to prevailing views, the DCs matured in vivo could maintain the capacity to internalize Ags at a level similar to immature DCs. Because these two possibilities imply a different pattern of Ag capture by mature DCs, we evaluated directly the endocytic capacity of in vivo matured DCs by assessing...
their internalization in situ of i.v. injected soluble GFP-OT (Fig. 4). We found that BL-mature DCs showed a similar capacity to capture i.v. administered GFP-OT Ag as immature DCs (Fig. 4B), indicating that DCs matured in vivo maintain a robust capacity to internalize soluble Ags. The internalization of GFP-OT in vivo was further confirmed by microscopy. In immature DCs purified from control mice, internalized GFP-OT was present in lysosomal compartments that also showed the accumulation of MHC-II (Fig. 4C) characteristic of immature DCs (18, 19). Mature DCs purified from mice treated with BL also exhibited intracellular GFP-OT, but MHC-II accumulated at the cell surface (Fig. 4C), as expected for mature DCs (34–36).

**Comparison of DCs matured in vivo to DCs matured in vitro**

Because it has been widely demonstrated that DCs shut down Ag capture upon maturation in vitro (2, 3, 9–19), we wanted to directly compare DCs matured in vivo to DCs matured in culture. Spleen DCs matured in vitro showed upregulation of maturation markers similar to that of DCs matured in vivo (Fig. 5A). In the same assays where in vivo matured DCs were able to capture Ag, spleen DCs matured in vitro did not (Fig. 5B). This difference was not due to factors (such as cytokines) present in the serum of inflamed mice, because the addition of mouse serum to the maturation cultures did not rescue Ag capture (Fig. 5C). Furthermore, the shut down of Ag capture could not be reversed if DCs matured in culture were adoptively transferred back into a mouse and directly compared with the endogenous DCs that had been matured in vivo (Fig. 5D). These results emphasize the differences in endocytic capacity between DCs matured in vivo and those matured in culture.

**DCs matured in vivo can capture and present Ag to T cells in situ**

We sought further direct in vivo evidence that DCs matured in vivo are able to internalize, process, and present Ag to T cells in situ. As a first test, we adoptively transferred CD8+ OT-I and CD4+ OT-II T cells into mice, and 16 h after the induction of DC maturation with BL, mice were injected with soluble Ag (Fig. 6A). We observed a similar extent of Ag-specific OT-I and OT-II T cell proliferation regardless of whether most of the DCs were immature (control mice) or mature (BL inflamed mice) at the time of Ag delivery (Fig. 6A). Such Ag presentation observed when most of the DCs were either immature or mature at the time of Ag capture resulted in a similar adaptive immune response as indicated by anti-OVA IgG titers (Supplemental Fig. 7).

To rule out the possibility that these results (Fig. 6A) are exclusively due to residual immature DCs still present in the inflamed mice, we adoptively transferred OT-I T cells into BM1 mice, which due to point mutations in H2-Kb are unable to present OVA-derived peptides to OT-I T cells (25, 26). As expected, when these mice were injected with OVA, no proliferation of the transferred OT-I T cell was detected (Fig. 6B). However, when purified immature DCs from a control (nontreated) wild-type B6 mouse were adoptively transferred into BM1 mice prior to the injection of OVA, proliferation of the transferred OT-I T cells was observed (Fig. 6B). The same result was obtained when we transferred mature DCs isolated from inflamed wild-type B6 mice into BM1 recipients (Fig. 6B). Because the endogenous DCs in the BM1 mouse cannot present Ag to OT-I T cells, these results confirm that the transferred BL-mature DCs were able to capture, process, and present soluble Ag in situ. We conducted a similar experiment to assess presentation on MHC-II. We found that OT-II T cells that had been adoptively transferred into Abb (MHC-II KO) mice responded similarly when mature or immature DCs were adoptively transferred (Fig. 6C), confirming that spleen DCs matured in vivo can capture and present Ag on both MHC-I and MHC-II.

**Discussion**

Our findings that mature DCs can capture and present Ags to CD8+ and CD4+ T cells provide interesting new perspectives on Ag sampling during inflammation. Our results indicate that Ag capture is sustained by mature DCs. Such capacity for Ag uptake by mature DCs may be important during infections, when the continuous capture and presentation of Ags by all the available DCs (regardless of their maturation status) that have access to infected tissues and/or to microbial pathogens might offer a greater opportunity to contribute to the stimulation of adaptive immunity. Although our studies relied on a experimentally synchronized populations of mature DCs from mouse spleen, large numbers of mature DCs have been described during viral infections (37–40), bacterial infections (41, 42), and autoimmune disorders (43, 44). Our results suggest that populations of mature DCs present under various inflammatory conditions may play a role in Ag capture and presentation without necessarily relying on the generation of additional immature DC precursors.

Previous studies have described impaired (9) or enhanced (45) internalization and presentation of cell-associated Ags by DCs in mice exposed to TLR ligands. It will be important to elucidate the effects of natural infections or inflammatory process on the uptake of soluble and cell-associated Ags by DCs. It will also be crucial to discern the type(s) of T cell priming and immunomodulatory response(s) to Ags captured by mature DCs, given the increasingly appreciated capacity to mature DCs to stimulate regulatory T cells (46). Also, mature DCs may be abundant in an environment rich in infected apoptotic cells where their capacity to internalize and present Ags can contribute to induction of Th17 T cell development (47).

Our findings may also relate to the mechanism of action of adjuvants that induce DC maturation. Mature DCs may be present at sites of vaccination, in an environment where inflammation and TLR-ligand stimulation may be prevalent. Therefore, Ag capture by mature DCs at (or near) vaccination sites may contribute to T cell stimulation without relying on a constant supply of immature DCs that need to be subsequently activated by the vaccine formulations (21). The continued Ag capture by DCs seems to be in line with the behavior of macrophages, which also maintain or enhance Ag capture on activation by inflammatory stimuli (48).

Additional questions remain regarding the potentially different pathways of processing and presentation of Ags captured by mature or immature DCs. Although immature DCs load Ag onto MHC-II in specialized intracellular compartments, the redistribution of MHC-II to the cell surface on maturation (Fig. 4C) (34–36, 49, 50) suggests that Ags internalized by mature DCs are likely to be loaded onto MHC-II that is recycled from the plasma membrane, as recently proposed for Ags internalized by receptor-mediated uptake by bone marrow-derived DCs (28). This process may benefit from the enhanced Ag processing observed on TLR engagement and DC maturation (51, 52). It will also be interesting to evaluate the role of different routes of cross-presentation (7, 53–55) and their modulation by signals that also induce DC maturation (56, 57). A more detailed understanding of the contribution of small GTPases (11, 12, 58), cytoskeletal rearrangements (10), and the expression of different endocytic receptors in the capture, processing, and presentation of Ag by DCs at different stages of maturation will provide a better understanding of the role of DCs in regulating T cell responses under resting and inflammatory settings.

**Acknowledgments**

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**Disclosures**

The authors have no financial conflicts of interest.
References


SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Method for isolation of mature or immature DCs from mouse spleen. A. Harvested spleens were magnetically enriched by depletion to ~30% DCs before cell sorting. DCs were gated as CD11c(high). B. To collect mature DCs from mice that had been inflamed, only the CD86(high) DCs were collected. To collect immature DCs from control non-treated mice, DCs were not gated on CD86. The resulting DCs were >95% live by 7AAD exclusion, and were >98% pure as assessed by surface phenotype post-sort.

Supplementary Figure 2. Maturation of DCs induces characteristic redistribution of MHC-II from intracellular stores to the surface. Immature DCs from non-treated mice, or mature DCs from mice injected 16 hours earlier with bacterial lyate (3x10⁹ lysed E. coli) were purified and analyzed by confocal microscopy.

Supplementary Figure 3. Soluble antigen presentation over a range of T-cell/DC ratios as quantified in figures 2 and 3. A. Briefly, Mice were injected with 2.0 mg or 0.5mg of OVA 30 minutes before DC isolation, and then co-cultured at the indicated ratios with CFSE-labeled OT-I and OT-II T-cells for 60 hours before analysis. B. Similar to experiments in A, but using GFP-OT protein.
Supplementary Figure 4. Diagram of the GFP-OT protein. GFP-OT contains antigens that when processed intracellularly and presented on MHC-I or MHC-II are recognized by OT-I or OT-II T-cells, as well as a GFP moiety for endocytosis studies.

Supplementary Figure 5. A. Spleens were harvested from control mice injected with PBS (source of immature DC, left panel “NTx DCs”) or from mice injected 16 hours earlier with bacterial lyate (3x10⁹ lysed E. coli, source of mature DC, right panel “BL-DCs”). The spleens were incubated with Liberase Blendzyme 2 on ice, or at 37°C for the indicated times (up to one hour). The cells where then harvested, stained and analyzed by flow cytometry, gating on live cells, 7AADneg, CD11chigh, MHC-IIhigh. B. Enumeration of the total number of DCs in the spleens of inflamed and control mice. Mice were either mock treated with PBS as a control, or injected intra-peritoneally 16 hours before analysis with bacterial lyate (3x10⁹ lysed E. coli). Splenocytes were harvested and total numbers of live cells were quantified by microscopy using trypan blue exclusion. The percentage of the total live splenocytes identified as resting DCs (7AADneg, CD11chigh, MHC-IIhigh, CD86low, CD80low) or as mature DCs (7AADneg, CD11chigh, MHC-IIhigh, CD86high, CD80high) were estimated by flow cytometry. These values were used to determine the number of total DCs or mature DCs. Data from five mice in each condition are shown as individual dots. C. Minimal contribution of monocyte-derived cells in the DC fractions isolated from spleens. As in B, but cells were also analyzed for expression of Ly-6C, as indicated in the corresponding dot plots.
Supplementary Figure 6. Extra-cellular peptide loading does not contribute to antigen presentation. Mice were either non-treated (NTx) or inflamed with bacterial lysate (BL), and then injected with 2 mg OVA, 1ug OVA257-264 peptide, or 10 μg OVA257-264 peptide. 30 minutes later, splenocytes were harvested and B-cells (CD19(+), CD11c(-), CD11b(-)) were sorted to >99% purity. These B-cells were co-cultured with CFSE-labeled OT-I T-cells for 60 hours at a T-cell:B-cell ratio of 8:1. B-cells were able to load extra-cellularly the small amounts of injected peptide and thereby stimulate OT-I T-cells. B-cells from mice injected with OVA did not stimulate T-cells, indicating that there is little or no in vivo release of peptide available for extra-cellular loading following injection of OVA.

Supplementary Figure 7. Induction of antigen-specific IgG responses. A. Lots of three mice were injected with 500μg of OVA, either simultaneously or 16 hours after the administration of 3x10⁹ lysed E. coli. A set of control mice were injected with 500 μg of OVA and PBS (without lysed E. coli) as a no-adjuvant control. After 7 days, sera was collected and analyzed for OVA-specific IgG by ELISA. B. Simultaneous analysis of sera taken from the same mice 2 days prior to immunization to demonstrate that the pre-immune sera were negative for OVA-specific IgG.
Supplementary Figure 1

A

magnetic negative enrichment of DCs

DCs gate shown in B

# Cells

CD11c

CD11c

B

bacterial lysate inflamed

non-treated

presort (gated on DCs)

not gated on CD86

Sort CD86 hi DCs

Sort all DCs

postsort analysis

postsort overlay

Green: NTx
Blue: BL mature

CD86

CD86
Supplementary Figure 2

NTx immature

BL mature

MHC-II

CD11c

merge

5 μm
Supplementary Figure 3A
Supplementary Figure 3B

<table>
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<tr>
<th>T-cell/DC ratio</th>
<th>2.0mg GFP-OT OT-II</th>
<th>2.0mg GFP-OT OT-I</th>
<th>0.5mg GFP-OT OT-II</th>
<th>0.5mg GFP-OT OT-I</th>
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<td>18.5, 5.0</td>
<td>8.8, 8.2</td>
<td>4.1, 1.7</td>
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</tbody>
</table>

# Cells

CFSE
Supplementary Figure 4

HIS-tag

OT-I Epitope
OVA(257-254)

OT-II Epitope
OVA(323-339)

EGFP (237aa)
Supplementary Figure 5

A

Supplementary Figure 5 A shows the expression of CD86 in NTx DCs and BL DCs. The graphs depict the percentage of cells with varying CD86 expression levels at different time points: 0 min, 20 min, 40 min, and 60 min at 37°C.

B

Supplementary Figure 5 B illustrates the spleen DC counts per mouse for NTx and BL DCs, both for all DCs and mature DCs. The data points indicate that BL DCs have a higher count compared to NTx DCs.

C

Supplementary Figure 5 C displays the gating strategy for NTx and BL DCs. The panels show the progression from 7-AAD and SSC to CD11c and Ly-6C, with the corresponding percentage values indicated for each gate.
Supplementary Figure 6
Supplementary Figure 7

closed symbols: BL at T= 0, OVA at T= 0
open symbols: BL at T= -12, OVA at T= 0
no symbols: OVA at T= 0 only

A  post-immune anti-OVA antibody titers

B  pre-immune anti-OVA antibody titers