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

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Dendritic cell (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.

**Materials and Methods**

**Mice**

C57BL/6 (B6), OT-I/RAG1 (OT-I), OT-IIa/RAG1 (OT-II), C3H/HeN (C3H), B6.SJL (CD45.1), and Abb (MHC class II knockout [MHC-II-KO]) mice (29) were from Taconic Farms (Germantown, NY). B6, C-H2^moro/Bj (B1) were from The Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific pathogen-free conditions and maintained in compliance with institutional and federal regulatory guidelines.

**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 × 10^8 bacteria/ml, heat killed 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 × 10^15 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...
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 (∼<125 pg/mg) endotoxin by LAL test (Cambrex).

Maturation of DCs

Maturation protocols were chosen to provide the maximum possible 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′-TCCATGAGCTTCCGTAGCT-3′ with phosphorothioate bonds, Invitrogen) were injected i.p. 16 h prior to experiments. For in vivo BL mediated maturation, 200 µl E. coli lysate (∼6 × 10^10 bacteria, containing ∼2 µg LPS) was injected i.p. 16 h prior to experiments.

Endocytosis assays

For in vitro endocytosis assays, 1 × 10^7 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 PBS, before analysis by flow cytometry. For in vivo endocytosis assays, mice were injected i.p. with 0.5–2 mg of GFP-OT and 30 min later, spleenocytes 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 variable 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 for 16 h (or kept on ice for negative controls) for 30 min in complete RPMI 1640. All cells were washed three times in PBS, before analysis by flow cytometry. For in vitro maturation, splenocytes were cultured overnight in complete RPMI at ∼8 × 10^6 cells/ml. In some experiments the 10% FBS in the complete RPMI was replaced with either 10% mouse serum from a non-treated mice, 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.v. injected into CD45.1 mice that we either non-treated or inflamed with BL for 16 h. (1.0 × 10^7 splenocytes per mouse). After various amounts of time, 42 mg GFP-OT was injected, and 30 min

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, spleenocytes were cultured overnight in complete RPMI at ∼8 × 10^6 cells/ml. In some experiments the 10% FBS in the complete RPMI was replaced with either 10% mouse serum from a non-treated mice, 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.v. injected into CD45.1 mice that we either non-treated or inflamed with BL for 16 h. (1.0 × 10^7 splenocytes per mouse). After various amounts of time, 42 mg GFP-OT was injected, and 30 min
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 (TreeStar, Ashland, OR). Abs: PE or allophycocyanin-Alexa750-CD8a (53-6.7), PE-Cy7 CD11c (N418), allogeneic/CD8a-CD45.1(A20), Pacific Blue or allophycocyanin-CD45.2 (104), allophycocyanin-CD86 (GL-1) and the corresponding isotype control (Rat IgG2a), PE or allophycocyanin-CD80 (16-10A1) and corresponding isotype control (Armenian Hamster IgG), Alexa488, allogeneic/CD40 (HM40-3) and Alexa488-isotype control (Armenian Hamster IgM), Alexa488 or allophycocyanin-CCR7 (4B12) and corresponding-isotype control (Rat IgG2a), were purchased from eBioscience or BioLegend (San Diego, CA).

**Confocal microscopy**

Sorted DCs were attached to Alcian Blue 8GX (Sigma-Aldrich) coated cover slips by centrifugation at 100 × g for 1 min and incubation at 37°C for 5 min. All subsequent steps were at room temperature. Cells were fixed in 2% paraformaldehyde in PBS for 30 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and blocked with 5% goat 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 (TreeStar, Ashland, OR). Abs: PE or allophycocyanin-Alexa750-CD8a (53-6.7), PE-Cy7 CD11c (N418), allogeneic/CD8a-CD45.1(A20), Pacific Blue or allophycocyanin-CD45.2 (104), allophycocyanin-CD86 (GL-1) and the corresponding isotype control (Rat IgG2a), PE or allophycocyanin-CD80 (16-10A1) and corresponding 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), were purchased from eBioscience or BioLegend (San Diego, CA).

**FIGURE 2.** DCs matured in vivo with BL 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) or mice treated with BL 16 h prior (dashed line). Shaded histograms show staining obtained with isotype controls. 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 non-treated 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 non-treated 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 non-treated mice (triangles), or mature DCs from BL-treated mice (squares) were assessed for cytolytic function as described in Materials and Methods.
mice (Fig. 1C, 1D). These results suggested that DCs matured in vivo retained their capacity for Ag capture and its subsequent presentation to T cells.

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 be 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, most 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

**FIGURE 6.** DCs matured in vivo capture soluble Ag and present it to T cells in situ. A, Analysis by flow cytometry of surface expression of the maturation marker CD86 on spleen DCs from nontreated mice (solid line), spleen DCs from mice treated with BL for 16 h (dotted line), or spleen DCs from untreated mice that were cultured for 16 h (dashed line). Shaded histograms show staining obtained with isotype control. B, Top, Experimental scheme. Bottom, Immature DCs from NTx, mature DCs from BL inflamed mice, or DCs matured in culture (in vitro mature) were incubated with GFP-OT for 30 min in vitro at 37°C (solid line), or at 0°C (shaded; to measure background) before analysis of Ag internalization by flow cytometry. C, Similar to experiments in B, except with DCs matured in vitro in media made with mouse serum from either nontreated or BL-inflamed mice, or FBS. D, Top, Experimental scheme. Bottom, CD45.2 DCs were matured in vitro, and then adoptively transferred back into either nontreated or BL-inflamed CD45.1 mice. After a 2-h period to allow the in vitro matured DCs to reacclimate with the in vivo environment, GFP-OT was injected i.v., and 30 min later Ag capture was assessed by the endogenous immature DCs in the nontreated mice and the endogenous mature DCs in the BL mice, and compared with the transferred in vitro matured DCs in the same mouse.
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 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 vitro due to residual immature DCs still present in the inflamed wild-type B6 mice into BM1 recipients (Fig. 6A). In the same assays where in vivo matured DCs were able to capture Ag, spleen DCs matured in vitro did not (Fig. 6B). 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.

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.
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 (3x10^9 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 (3×10⁹ 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 (3×10⁹ 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**
- **# Cells**
  - CD11c
- magnetic negative enrichment of DCs
  - CD11c
- DCs gate shown in B

**B**
- bacterial lysate inflamed
  - presort (gated on DCs)
  - CD86
- Sort CD86 hi DCs
- postsort analysis
  - CD86
- non-treated
  - not gated on CD86
  - Sort all DCs
- postsort overlay
  - Green: NTx
  - Blue: BL mature

**Legend**
- Green: NTx
- Blue: BL mature
Supplementary Figure 2

NTx immature

MHC-II  CD11c  merge

BL mature

5um
Supplementary Figure 3A

A

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# Cells

CFSE
Supplementary Figure 3B

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<th>NTx immature</th>
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<th>NTx immature</th>
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<th>NTx immature</th>
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CFSE
Supplementary Figure 4

HIS-tag
OT-I Epitope OVA(257-254)
OT-II Epitope OVA(323-339)
EGFP (237aa)
Supplementary Figure 5

A

NTx DCs

BL DCs

% of Cells

CD86

0 10^2 10^3 10^4 10^5

% of Max

CD86

0 10^2 10^3 10^4 10^5

60 min @37°C

40 min @37°C

20 min @37°C

0 min @37°C

B

Spleen DCs

per mouse (x10^4)

NTx DCs BL DCs

all DCs all DCs mature DCs

0 25 50 75 100 125

C

NTx DCs

BL DCs

SSC

7-AAD

CD11c

Ly-6C

CD11c

SSC

7-AAD

CD11c

Ly-6C

CD11c

0 20 40 60 80 100

% of Cells

0 20 40 60 80 100

% of Max

2.38

2.29
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