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Internalization of Chlamydia by Dendritic Cells and Stimulation of Chlamydia-Specific T Cells

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Chlamydia species are the causative agents of trachoma, various forms of pneumonia, and the most common sexually transmitted diseases. Although the infection cycle has been extensively characterized in epithelial cells, where the Chlamydia entry-vacuoles avoid fusion with host-cell lysosomes, the cellular immune response has received less attention. Moreover, despite the abundant presence of dendritic cells (DC) in the sites of infection, the interaction between Chlamydia and DC has never been studied. We observe that DC kill Chlamydia trachomatis and Chlamydia psittaci. The chlamydiae are internalized by the DC in a nonspecific manner through macropinocytosis, and the macropinosomes fuse subsequently with DC lysosomes expressing MHC class II molecules. The interaction induces maturation of the DC, since presentation of an exogenous Ag is severely inhibited after a 1-day incubation, although chlamydial Ags are still presented and recognized by Chlamydia-specific CD4+ T cells. Thus, DC most likely play a role in initiating the T cell response in vivo and could potentially be used in adoptive transfer therapies to vaccinate against Chlamydia.


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The abbreviations used in this paper: EB, elementary body; CCD, cytochalasin D; DC, dendritic cell; HEL, hen egg lysozyme; RB, reticulate body; GPIC, guinea pig inclusion conjunctivitis; MoPn, mouse pneumonitis; RITC, rhodamine isothiocyanate; ILN, iliac lymph nodes; LAMP, lysosome associated membrane protein; DAMP, 3-(2,4-dinitroanilino)-3-methylpropylamine; TIR, transferrin receptor; m.o.i., multiplicity of infection.

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vivo should be considered as part of future immunization strategies against *Chlamydia* infections.

**Materials and Methods**

**Cells and materials**

The two *Chlamydia* species used here, the guinea pig inclusion conjunctivitis (*GpC*) serovar of *C. psittaci* and the *C. trachomatis* agent of mouse pneumonia (*MoPn*), have been described elsewhere (2). The DC line, D25C1/1 (13), was generously provided by Dr. P. Ricciardi-Castagnoli (University of Milan, Italy). Primary dendritic cells were isolated from the bone marrow of BALB/c mice as described (14).

The following have been previously described (15): FITC-labeled anti-Chlamydia mAb; the anti-Chlamydia LPS mAb; the mAb against human LAMP-1, HA3; the mAb against the human transferrin receptor (TIR), 5E9; anti-human CD11b (clone M1/70) and anti-CD8 (53-6.7) from PharMingen. The cells were incubated with bacteria and the indicated final concentrations of heparin (Sigma, St. Louis, MO). For infections with *C. psittaci* and *C. trachomatis*, MoPn, and the *agent of mouse pneumonitis* (MoPn), have been described elsewhere (2). The DC line, D2SC/1 (13), was generously provided by Dr. P. Ricciardi-Castagnoli (University of Milan, Italy).

**Cell culture conditions and infection with Chlamydia***

Chlamydiae were obtained from infected HeLa cells as described (16). A partially purified suspension was aliquoted and stored at −80°C until ready for use. For heat pretreatment experiments, the bacteria were incubated at 55°C for 30 min before incubating with HeLa or DC. Typically, the DC line was prestimulated for 2 days with 100 U/ml IFN-γ, then incubated with bacteria in RPMI 1640 for the indicated times at 37°C in 5% CO2. Unless indicated otherwise, the *Chlamydia* concentration gave a multiplicity of infection (m.o.i.) of 1:3 in HeLa cells. HeLa cells, L cells, and primary DC were similarly incubated with bacteria at an m.o.i. of 1:3. The primary DC were used for experiments with *Chlamydia* after 4 days of culture with 10 mg/ml recombinant granulocyte/macrophage-CSF (PharMingen).

**Confocal microscopy**

Samples for confocal microscopy were fixed with paraformaldehyde, incubated with antibodies, and mounted as previously described (17). After fixation, DC were first incubated with blocking Abs for Fc receptors (rabbit or human serum) in permeabilization buffer. Next, to determine the distribution of the TfR, LAMP, or MHC class II for both DC and L cells, the following have been previously described (15): FITC-labeled anti-MHC class II mAb recognizing IAb,d was from PharMingen. Rhodamine LAMP, anti-CD107a, was from PharMingen (San Diego, CA). The following have been previously described (15): FITC-labeled anti-Chlamydia mAb; the anti-Chlamydia LPS mAb; the mAb against human LAMP-1, H4A3; the mAb against the human transferrin receptor (TIR), 5E9; anti-human CD11b (clone M1/70) and anti-CD8 (53-6.7) from PharMingen. 2.5% glutaraldehyde for at least 2 h at room temperature. The fixed cells were then prepared for electron microscopy as previously described (16). Thin sections were poststained with uranyl acetate and lead citrate for examination on a Zeiss electron microscope at an accelerating voltage of 50 kV.

**Measurement of TNF-α secretion**

Secretion of TNF-α from DC was measured using the Genzyme mouse TNF-α ELISA kit following the manufacturer’s instructions. DC were stimulated in 96-well plates with 100 U/ml IFN-γ or MoPn for 16 h at 37°C. The supernatant was collected, centrifuged in Eppendorf tubes to remove cellular debris, and kept frozen at −80°C until ready for use. Supernatants were diluted in the ELISA kit wash buffer, and the absolute concentrations of TNF-α were obtained by calibrating the ELISA kit with a known concentration of TNF-α provided by the manufacturer.

**HEL Ag presentation assays**

DC were plated at a concentration of 3 × 105 cells/ml and stimulated with 100 U/ml murine IFN-γ (Genzyme) and 2 ng/ml murine rIFN-α (Genzyme) for 2 days. The DC were incubated with bacteria and the indicated final concentrations of heparin (Sigma, St. Louis, MO). The cells were then incubated with microbeads conjugated to goat anti-mouse polyclonal Abs. The bacteria were detected by incubating the cells with FITC-coupled anti-Chlamydia mAbs.

Acidic compartments were detected with DAPM as previously described (15). Macropinosomes were visualized by incubating cells growing on coverslips with 1 mg/ml of rhodamine B-labeled dextran at 37°C during the last 15 min of infection with bacteria, which were revealed with FITC-labeled anti-Chlamydia mAb.

**Measurement of bacteria internalization**

To distinguish between intracellular bacteria and those still on the host cell surface, cells were infected with chlamydiae, and at indicated times the host cells and associated bacteria were fixed with paraformaldehyde. However, instead of permeabilizing the cells after fixation, they were first incubated with the unlabeled anti-Chlamydia mAb, followed by the second RITC-labeled mAb. After rinsing, the samples were then permeabilized and incubated with the FITC-labeled anti-Chlamydia mAb. Photographs were taken with both the RTFC and FITC filters on the same photograph frame. Internalized bacteria appeared green, while bacteria on the surface appeared red or yellow. When host cells were fixed with the fixation buffer before incubating with bacteria, all bacteria appeared red or yellow.

The effects of 10 µg/ml CDD or 10 µM dimethyl amiloride were measured by incubating the host cells with inhibitor for 30 min at 37°C before adding the chlamydiae. The inhibitors were then maintained at the same concentration throughout the internalization experiment.

Inhibition of adherence and early internalization steps of *Chlamydia* by heparin was measured with either HeLa or DC that had been incubated with bacteria and the indicated final concentrations of heparin (Sigma, St. Louis, MO). The bacteria were incubated in the heparin solutions in PBS for 1 h at 4°C before adding them to an equivalent volume of HeLa cells or DC in culture medium. After an hour, unbound bacteria were removed by washing once with PBS and replacing the supernatant with culture medium. After an additional 3 h, the cells were fixed, permeabilized, and incubated with FITC-labeled anti-Chlamydia mAb. Bacteria were then counted with a 63× lens using a Zeiss Axioskop fluorescence microscope.

**Electron microscopy**

DC or HeLa cells infected with chlamydiae for 0, 4, or 24 h were fixed with 5% glutaraldehyde for at least 2 h at room temperature. The fixed cells were then prepared for electron microscopy as previously described (16). Thin sections were poststained with uranyl acetate and lead citrate for examination on a Zeiss electron microscope at an accelerating voltage of 50 kV.
Results

Differential internalization mechanisms of Chlamydia in epithelial and dendritic cells

The internalization rate of chlamydiae in epithelial cells has not been rigorously characterized before, and it was not known whether chlamydiae could infect DC. The bacteria were therefore incubated with HeLa cells or DC for different times, the unbound bacteria were removed, and the host cells with associated bacteria were fixed. Bacteria that had not entered the host cells were then revealed by incubating the cells with an anti-Chlamydia mAb, followed by incubation with a RITC-labeled second Ab. Internalized bacteria were then revealed by permeabilizing the cells and incubating them with a FITC-labeled anti-Chlamydia mAb, which would bind to both internalized bacteria and external bacteria. Thus, internalized bacteria appeared green, while external bacteria were red or yellow. Figure 1A shows the kinetics for bacterial entry into HeLa cells and DC. The epithelial cells, representing the preferential physiologic target for Chlamydia (1), were infected inefficiently, with only 20 to 30% of the associated bacteria having entered within 6 h. The DC not only internalized the bacteria faster, but to a greater extent, with >60% of the bacteria entering within 6 h. Cytoskeleton-dependent phagocytosis or macropinocytosis (21) appeared to be involved in the entry in both cell types, however, since CCD inhibited entry by 76 ± 12% in HeLa cells and 87 ± 13% in DC (not shown). Macropinocytosis was further implicated by the observation that dimethyl amiloride, which inhibits macropinocytosis in primary DC and bone marrow macrophages (22, 23), decreased chlamydial internalization by the DC by 75 ± 7% (not shown).

Heparin has previously been reported to inhibit the adherence of C. trachomatis (24, 25) and C. psittaci (16) to epithelial cells. To consider, additionally, the possible effects of heparin on early stages of entry, we measured the number of bacteria associated with host cells after a 4-h incubation with chlamydiae. Heparin efficiently inhibited bacterial attachment by HeLa cells, with >90% inhibition by 100 μg/ml heparin (Fig. 1B). On the other hand, heparin did not have a significant effect on internalization by DC lines (Fig. 1B) or primary DC (not shown). In addition, heat inactivation, which inhibits C. psittaci attachment to HeLa cells (16), had no effect on the kinetics of internalization by DC (not shown). These data suggest that different receptors and/or entry mechanisms may be used by Chlamydia in DC.

Differential intracellular fate of Chlamydia in epithelial cells and dendritic cells

To investigate the possibility that the Chlamydia vacuoles may be routed differently in epithelial cells and DC, host cells were incubated with bacteria for 4 or 24 h, and the interaction between the entry vacuoles and lysosomes was analyzed by confocal microscopy using LAMP as a marker for lysosomes (26). Most Chlamydia vacuoles in epithelial cells avoided fusion with lysosomes at early stages of entry (Fig. 2A), and within 24 h the vacuoles had developed into the expected inclusions, having diameters of approximately 10 μm, which also excluded the lysosomal marker (Fig. 2B). However, the Chlamydia vacuoles displayed a very different behavior in DC, as essentially all vacuoles fused with lysosomes within a few hours. Figure 2C shows one vacuole that had fused with lysosomes in the DC line; the same results were obtained with a 20-fold increase in the bacterial concentration. Fusion between Chlamydia vacuoles and lysosomes was also observed in primary DC (not shown). No inclusions were ever detected at 24 h. To exclude the possibility that the intracellular fate of Chlamydia in DC may be due to prestimulation of the DC.
line with IFN-γ, the same experiments were repeated in the absence of IFN-γ prestimulation. No differences were observed in vacuole fusion with lysosomes or survival of bacteria in either stimulated or unstimulated DC (not shown).

Interaction with other host cell compartments was analyzed using Abs against the TR, a marker for early and recycling endosomes (27, 28), and DAMP, a weakly basic amine that concentrates in acidic compartments and is revealed with fluorescently labeled Abs (15). Three cell types were studied: HeLa cells, L cells (fibroblasts), and DC. The L cells behave the same as HeLa cells, in the sense that infection gives rise to large inclusions after 24 h and the vacuoles avoid fusion with lysosomes (not shown). The vacuoles in DC display lysosomal markers and are acidic, as expected for phagolysosomes (29). In addition, consistent with the observation that CCD inhibits internalization by HeLa cells and DC, the TR was not detected in most of the Chlamydia vacuoles in either of these two cell types (not shown).

Extracellular material that is phagocytosed and sorted to phagolysosomes is normally destined for degradation, although microbes that survive in phagolysosomes have also been identified (30, 31). The Chlamydia epitope recognized by the mAb LPS was sometimes detected in DC 24 h after infection, but as LPS from both Shigella and Chlamydia can detach from the bacteria and may be present even when the bacteria are degraded (18, 32), we evaluated the state of the chlamydiae in HeLa cells and DC by electron microscopy. Chlamydia EBs in tight-fitting vacuoles were observed in HeLa cells within 4 h of infection (Fig. 3A), and at 24 h most of the bacteria had differentiated into RBs and were proliferating within large inclusions (Fig. 3B). In DC, many EBs were observed in large, transparent vacuoles at 4 h (Fig. 3C), and many bacteria that appeared to be severely damaged were also observed (not shown), although in most cases it was difficult to distinguish them from other cellular debris. No intact bacteria were ever found after a 24-h infection.

Effect of Chlamydia on presentation of exogenous Ags
Pathogen-induced maturation of DC implies that, among other changes, the DC lose the capacity to present new exogenous Ag (9, 10). We therefore determined whether a 24-h incubation with chlamydiae had any effect on the ability to subsequently present HEL to a HEL-specific CD4+ T cell hybridoma (19). DC were able to present HEL efficiently to the hybridoma, but the ability to stimulate the hybridoma decreased dramatically following incubation with either C. psittaci (Fig. 4) or C. trachomatis (not shown). The inhibition of HEL presentation was not due to loss of surface expression of MHC class II, since the DC incubated with either of the two Chlamydia species could still present an HEL-derived peptide added exogenously, which can presumably bind to surface MHC class II without being internalized (33).

Interaction between Chlamydia vacuoles and dendritic cell macrophages and MHC class II compartments
Given the ability of chlamydiae to inhibit presentation of exogenous HEL, we examined the interaction between Chlamydia vacuoles and compartments involved in Ag presentation by MHC class II. The DC line displayed numerous macropinosomes with diameters of 2 μm to over 10 μm, as determined by incubating DC with rhodamine-labeled dextran. At early stages of Chlamydia entry, bacteria also colocalized with the dextran (Fig. 5A), suggesting that many if not all bacteria are internalized through macropinocytosis. Since entry of bacteria into DC is slow, whereas the fluorescence signal from macropinosomes loaded with dextran is intense and saturates within 30 min, the cells were incubated with chlamydiae for 2 h before incubating with dextran for an additional

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Electron micrographs of HeLa cells infected with C. psittaci for 4 h (A) or 24 h (B) and DC incubated for 4 h (C). Vacuoles containing Chlamydia EB were observed in both HeLa cells and DC at 4 h, but only HeLa cells had RB-laden inclusions at 24 h. Scale bars: 0.25 μm (A), 1 μm (B), 0.5 μm (C).

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Effect of Chlamydia infection on presentation of exogenous Ags. Control DC presented either HEL or an HEL-derived peptide recognized by an HEL-specific hybridoma (black left-hand columns). DC-fed C. psittaci for 24 h (gray right-hand columns) were unable to present whole HEL, but could still stimulate the hybridoma after incubation with the HEL-derived peptide.
15 min. Thus, not all internalized bacteria colocalized with dextran, probably because some of the bacteria entered before the incubation with dextran. No dextran was accumulated in HeLa cells under the same conditions (not shown). The entry vacuoles in DC were also acidic (not shown), consistent with their expression of lysosomal markers, and expressed MHC class II molecules (Fig. 5B), suggesting that chlamydiae could be processed for Ag presentation in these compartments.

Fewer macropinosomes and acidic compartments expressing LAMP were present in DC after a 24-h infection with chlamydiae, and heat-inactivated bacteria induced the same changes as live bacteria (not shown). Thus, these results may partially account for the observation that incubation of DC with Chlamydia for 24 h inhibits presentation of exogenous HEL.

Chlamydia-induced TNF-α secretion by dendritic cells

In addition to bacterial products, TNF-α was shown to induce differentiation of primary DC into a phenotype with few macropinosomes and low ability to present exogenous Ags (22, 34). To ascertain whether chlamydiae might induce differentiation of the DC line independently of bacterial internalization and degradation, we measured whether incubation with Chlamydia would cause secretion of TNF-α by the DC. As seen in Figure 6, both C. trachomatis and C. psittaci induced secretion of high levels of TNF-α. Significant levels of TNF-α in the supernatant were already detected within 2 h, at which time few bacteria have been internalized, suggesting that simple contact between bacterial surface epitopes and the DC may contribute to TNF-α secretion.

Presentation of Chlamydia-derived Ags by DC

To determine whether the DC were capable of presenting Ag to sensitized T cells, mice were infected intravaginally with C. trachomatis, and ILN were removed 13 days later. CD4+ T cells were obtained and incubated with C. trachomatis and DC. When the proliferative response was assessed, DC or CD4+ T cells incubated separately with Ag displayed little if any proliferation, while a large increase in proliferation was observed when CD4+ T cells were incubated in the presence of DC and Chlamydia Ag (Fig. 7). These data demonstrate that the DC not only express Chlamydia Ag-MHC complexes on the cell surface, but that these complexes are capable of providing a stimulatory signal for sensitized CD4+ T cells.

Discussion

The urogenital tract is an important site of contact with pathogens, and local immune responses to a variety of pathogens have been previously reported. Among the cells first encountered by Chlamydia in the cervix and vagina, DC are likely to play a major role in initiating the immune response. DC have been identified in the stratified squamous epithelium of the vagina and ectocervix and within the columnar epithelium of the endocervix in primates and mice (6, 7, 35–37). While many DC are present in the vaginal and cervical stroma in mice, the scarcity or absence of T and B lymphocytes in the stroma (8) suggests that DC may process Ags from this site but present the Ags to lymphocytes at other sites. That vaginal DC could process exogenous Ags was suggested by the observation that intravaginally administered fluorescent protein tracers are internalized by cells with DC morphology in the vaginal epithelium and stroma (5). Finally, primary T cell responses to recombinant fragments of the major outer membrane protein of C. trachomatis have been shown to be dependent on DC (38).

The general outlines of the development and migration of DC have been previously described (9, 10). Bone marrow-derived DC

![FIGURE 5. Entry of Chlamydia via macropinosomes and interaction with MHC class II compartments in DC. A, DC were incubated for 2 h with C. psittaci and then for 5 min with dextran. Chlamydiae (green) are found in large vacuoles that accumulate dextran (red). B, After a 2-h infection with C. psittaci (green), the cells were revealed with an anti-MHC class II mAb (red); similar results were obtained with C. trachomatis. Scale bars, 5 μm.](image)

![FIGURE 6. Incubation of DC with Chlamydia results in secretion of TNF-α from the DC. DC were incubated with C. psittaci (○) or C. trachomatis (■) for the indicated times, and the supernatant concentration of TNF-α was measured in three separate experiments.](image)

![FIGURE 7. DC incubated with C. trachomatis present Ags to C. trachomatis-specific CD4+ T cells. There is little proliferation of DC or T cells incubated separately with Chlamydia or control buffer (left and middle columns); there is a large increase in proliferation when T cells are incubated with DC and C. trachomatis (right columns).](image)
precursors translocate to nonlymphoid tissue, including epithelia of the genital tract, where they develop into “immature DC” capable of internalizing and processing Ag and expressing high levels of MHC molecules. Pathogens and inflammatory cytokines can induce their differentiation into “mature DC” with a decreased capacity for Ag processing but stable presentation of previously acquired Ags by the MHC molecules. The mature DC migrate into secondary lymphoid tissues, where they initiate T cell-dependent immune responses.

We have characterized the interaction between two species of Chlamydia, C. trachomatis and C. psittaci, and DC using a fully functional DC line (13). In parallel, key features of internalization, namely the effects of heparin and fusion of Chlamydia vacuoles with lysosomes, were confirmed with primary DC. These results were compared with the intracellular behavior of the chlamydial in an epithelial cell line, HeLa, representing the preferred physiologic target cell of the bacteria. The most striking difference between the epithelial cells and the DC is that the entry vacuoles avoid fusion with lysosomes in the epithelial cells, evolving within a day into large inclusions, while in DC there is rapid fusion between the Chlamydia vacuoles and host cell lysosomes. By electron microscopy, chlamydial, mostly at the metabolically active RB developmental stage, were found proliferating in large numbers in the epithelial cells after 1 day. In DC, on the other hand, no intact bacteria were detected in DC after a 1-day incubation, suggesting that the bacteria were killed by the DC.

At early time points, chlamydiae were detected in large vacuoles in the DC, consistent with macroinclusions, and the internalization was inhibited by dimethyl amiloride. This internalization appears to be nonspecific or at least different from that used in epithelial cells, since heparin, which inhibits invasion of epithelial cells (16, 24, 25), has no effect on DC internalization. Thus, it is conceivable that DC could also internalize other bacteria through macroinocytosis.

Interaction of the DC with Chlamydia leads to maturation of the DC, as defined by down-regulation of macroinocytosis and loss of the ability to present new exogenous Ag. The decrease in macroinocytosis was observed by fluorescence microscopy, which revealed a large drop in the number of acidic vesicles expressing LAMP and capable of accumulating dextran. The intracellular distribution of MHC class II in the DC line suggests that the DC line behaves as immature primary DC (14), while the disappearance of intracellular class II molecules after incubation with Chlamydia is consistent with the phenotype of mature primary DC (14). In addition, after a 1-day incubation with Chlamydia, the DC were no longer able to present exogenous HEL to HEL-specific T cells, but they still presented chlamydial Ags efficiently to T cells, in line with the role of mature DC to present Ags from the pathogen that induced the maturation.

As both infectious and inactivated chlamydiae were used in our experiments, it is likely that some of the stimulatory activity of C. trachomatis and C. psittaci may be due to TNF-α secretion induced by simple contact with Chlamydia surface epitopes. Assuming that surface epitopes (e.g., LPS) from other bacteria may also induce differentiation of DC and that other bacteria may also be internalized through macroinocytosis, the only requirement for subsequent presentation of bacterial Ags by the DC is that the macroinosomes fuse with lysosomes (39), where the bacteria could be degraded for subsequent presentation by MHC class II. Although this appears to be the case for Chlamydia, this argument cannot be generalized to all microbes, as seen in the example of L. monocytogenes, which escapes from DC phagolysosomes (40).

The observation that infection with Chlamydia gives rise to short-lived CD4+ -dependent immunity against subsequent infections (2) implies that the frequency of T cells recognizing Chlamydia Ags is low. The frequency of active, Chlamydia-specific T cells may thus be augmented by transferring Chlamydia-fed DC into animals, as has recently been done with DC to induce protective immunity to lethal challenge by tumors (12). This strategy could be extended to microbes that nominally survive within DC by incubating DC with previously inactivated microbes.

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