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Amphisomal Route of MHC Class I Cross-Presentation in Bacteria-Infected Dendritic Cells

Dorothee Fiegl,*† Danny Kägebein,*† Elisabeth M. Liebler-Tenorio,† Tanja Weisser,*,‡ Mareen Sens,* Melanie Gutjahr,* and Michael R. Knittler*

Dendritic cells (DCs) are among the first professional APCs encountered by the obligate intracellular bacterium Chlamydia during infection. Using an established mouse bone marrow–derived DC line, we show that DCs control chlamydial infection in multiple small inclusions characterized by restricted epithelial mucosa, impaired cytosolic export of the virulence factor chlamydial protease–like activity factor, and interaction with guanylate-binding protein 1, a host cell factor involved in the initiation of autophagy. During maturation of infected DCs, chlamydia-containing inclusions disintegrate, likely because they lack chlamydial protease–like activity factor–mediated protection. Released cytosolic Chlamydia are taken up by autophagosomes and colocalize with cathepsin-positive amphisomal vacuoles, to which peptide transporter TAP and upregulated MHC class I (MHC I) are recruited. Chlamydial Ags are subsequently generated through routes involving preprocessing in amphisomes via cathepsins and entry into the cytosol for further processing by the proteasome. Finally, bacterial peptides are reimported into the endosomal pathway for loading onto recycling MHC I. Thus, we unravel a novel pathway of MHC I–mediated cross-presentation that is initiated with a host cellular attack physically disrupting the parasitophorous vacuole, involves autophagy to collect cytosolic organisms into autophagosomes, and concludes with complex multistep antigenic processing in separate cellular compartments. The Journal of Immunology, 2013, 190: 2791–2806.

Chlamydia are Gram-negative obligate intracellular bacteria that infect mainly epithelial mucosae and have been implicated in a broad spectrum of diseases in humans, other mammals, and birds (1). For instance, Chlamydia psittaci is the causative agent of psittacosis, a widespread infection in psittacine birds and domestic poultry (2). Transmission of C. psittaci to humans and the zoonotic potential of this infection were documented by different studies (3, 4). Furthermore, C. psittaci is regularly detected in nonavian domestic animals (5, 6). Serological data indicated chlamydioses in domestic animals as a relevant source of infection for humans (7). In natural hosts, clinical outcomes of C. psittaci infection range from clinical silence to severe or even mortal illness (8). After transmission of avian strains of C. psittaci from birds to human, life-threatening pneumonia with systemic bacterial spread, including myocarditis, hepatitis, and encephalitis, occurs (9). Nonavian strains are known to cause abortion and chronic obstructive pulmonary disease in various animals (10).

Upon entry into eukaryotic cells, Chlamydia replicate within membrane-bound vacuoles, termed inclusions (11). Within these inclusions Chlamydia undergo a biphasic developmental cycle that alternates between infectious metabolically inactive elementary bodies (EBs) and noninfectious metabolically active reticulate bodies (RBs) (12). Infection is initiated by the attachment and subsequent parasite-mediated endocytosis of EBs (12). After internalization, EBs differentiate into RBs, which start to replicate. As the developmental cycle progresses, the inclusion enlarges to accommodate increasing numbers of organisms (12). At the end of the cycle RBs differentiate back into EBs (12), which are released from the host cell, allowing propagation of infection.

Consistent with the intracellular localization of Chlamydia, cell-mediated immune responses against Chlamydia have been observed in infected humans and mice (13). Furthermore, transfer of either CD4+ and/or CD8+ Chlamydia-specific T cells into naive mice was shown to protect against challenge with Chlamydia (14), and studies with mice deficient in MHC confirm the importance of T cell–dependent responses (15).

Dendritic cells (DCs) are equipped with specialized machinery that promotes effective display of MHC/peptide complexes, rendering them the most potent stimulators of T cells (16). DCs are present in most tissues and are organized as a specialized “network” that enables them to sample Ags from their environment (17). They are thought to perform a kind of “sentinel” function because of their ability to process internalized Ags before migration to secondary lymphoid organs where they stimulate CD4+ and CD8+ T cells (16).

The classical MHC class I (MHC I) pathway of Ag presentation is understood in detail (18). Cytosolic and nuclear Ags are degraded into peptides by the proteasome and transported from the cytosol into the endoplasmic reticulum (ER) lumen by the peptide transporter TAP (18). Peptides are then loaded onto newly synthesized MHC I, and these complexes are released from the ER and transported to the cell surface via the Golgi (18). However,
DCs have developed the ability to efficiently present present peptides derived from endo- and exogenous Ags on MHC I via a process called cross-presentation (17). Ag cross-presentation to CD8\(^+\) T cells is an important mechanism for the development of CTL responses against extracellular or vacuolar pathogens (19). Different routes of MHC I cross-presentation have been proposed: in the cytosolic cross-presentation pathway, internalized Ags are translocated from phagoendosomes into the cytosol where their further processing requires active proteasome and TAP function but apparently not lysosomal proteases (19). Cytosolic cross-presentation is an alternative pathway that is independent of the proteasome and TAP and requires endocytic cathepsins (20, 21). A variation of this latter pathway has been proposed in which Ags undergo cathepsin-dependent pre-processing in late endosomal compartments before they are transported to the cytosol where they are further processed by the proteasome (22). Thus, it seems that multiple pathways coexist and that they functionally cooperate with each other (23).

DCs are among the first professional APCs (pAPCs) that are encountered by \textit{Chlamydia} during infection (24), and cytotoxic CD8\(^+\) T cells, primed by infected DCs, likely play an important role in the effective antichlamydial immune response (24). However, despite the crucial role of pAPCs and CD8\(^+\) T cells during chlamydial infection, the mechanisms by which chlamydial Ags are processed for MHC I presentation are not fully understood. In fact, \textit{Chlamydia} replicate within membrane-enclosed compartments, which do not obviously intersect with compartments of the classical MHC I pathway in the host (13). Consequently, only a few chlamydial proteins appear to reside in the host cytosol (25), whereas a large family of proteins is present within inclusions and/or inserts into the inclusion membrane (13). Thus, one important open question is how MHC I gains access to chlamydial Ags. In the last years, several \textit{Chlamydia} T cell Ags were identified from murine models of infection with different chlamydial strains (13). One of the most interesting observations from these studies was that none of the identified \textit{Chlamydia}-specific CD8\(^+\) T cell Ags appears to be secreted into the host cell cytosol (13). Hence, classical MHC I presentation is unlikely to be the mechanism by which these Ags are processed in the cell. Although cross-presentation offers a possible scenario of how CD8\(^+\) T cells may be stimulated against chlamydial infection (26), it should be noted that intact chlamydial inclusions normally escape cell fusion with cellular endolysosomal structures and, thus, should also escape from the described cross-presentation pathways (27).

Using an established mouse bone marrow–derived DC (BMDC) line (28), to our knowledge we demonstrate for the first time a crucial role for autophagy in the generation of chlamydial Ags. The processing of these Ags occurs via amphibosomal degradation following the release of \textit{Chlamydia} from disintegrated inclusions into the cytosol. We propose a new alternative cross-presentation model in which autophagy constitutes an important pathway promoting proteasome/TAP-dependent MHC I processing of intracellular bacterial Ags. Our findings provide new insights into cellular mechanisms of endocavular processing of facultative intracellular microbes, which might help in the development of DC-based vaccines, as well as novel strategies to generate optimal CD8\(^+\) T cell responses against chlamydial Ags.

Materials and Methods

\textbf{Cell culture}

JAWS II, a myeloid DC line established from bone marrow cells of a p53-knockout C57BL/6 mouse (28), was purchased from the American Type Culture Collection (CRL-1194; Manassas, VA). Immortalized epithelial cells from newborn mice were obtained from the cell culture collection of the Friedrich-Löffler-Institut, Isle of Riems (Collection of Cell Lines in Veterinary Medicine [CCLV], order number: CCLV-RIE #282). The epithelial African green monkey kidney cell line BGM (29) was obtained from the National Reference Laboratory for Chlamydiosis of the Friedrich-Löffler-Institut, Jena (order number: CCLV-RIE #136). Cells were grown at 37°C and 7.5% CO\(_2\) in cell culture medium consisting of IMDM with 5–20% FCS. A concentration of 5 ng/ml murine GM-CSF was used for culturing JAWS II cells.

\textbf{Abs}

Polyclonal antisera against cathepsins and mouse granzyme-b-like protein (GBP1) were purchased from Santa Cruz. Mouse monoclonal anti-CD8\(\alpha\) (mAb 53-6.7), anti-CD8\(\beta\) (mAb GL-1), and anti–MHC I (anti-H-2K\(^\alpha\), mAb AF6-88.5, anti-H-2\(^D\), mAb KH95) Abs were from Becton Dickinson. Abs against \textit{Chlamydia} (mAb BD1618, polyclonal antisera ab21211 and ab13943), \textit{Chlamydia}-LPS (mAb ACF-FITC), Rab11, mouse MHC I (H-2\(^K\)), and H-2\(^D\)) (30, 31) were kindly provided by Tim Elliott (University of Bristol, Bristol, U.K.), recognizes mouse MHC I (H-2\(^D\)) (32). The rabbit antisera D90 recognizes the C terminus of murine TAP1 (32). The mouse monoclonal anti-KDEL Ab (MAC256) (33) was obtained from Geoff Butcher (Babraham Institute, Cambridge, U.K.). The rabbit polyclonal antisera R.SinE against tapasin (34) was a gift from Peter Cresswell (Yale University, New Haven, CT). The mouse polyclonal antisera against chlamydial protease–like activity factor (CPAF) was generously supplied by Andreas Essig (Uniklinik Ulm, Ulm, Germany). All secondary Abs and mAbs for isotype-control staining were purchased from DiaNovo and BioLegend, respectively.

\textbf{Western blotting}

Cells were washed twice in ice-cold PBS (pH 7.5) prior to solubilization in lysis buffer (PBS [pH 7.5] containing 1% Triton X-100; Sigma-Aldrich) containing Complete Protease Inhibitor mixture (Roche). After 30 min of incubation on ice, lysed cells were centrifuged at 1300 x \(g\) for 15 min before postnuclear supernatants were analyzed in Western blots treated with appropriateAbs. Bands were visualized with ECL (Sigma-Aldrich). Fluorographs were quantified using GelEval 1.32 software (FrogDance Software).

\textbf{Quantitative PCR and RT-PCR}

For SYBR Green–based quantitative real-time PCR, mRNA was isolated from infected cells using the TRizol method (peqGOLD Trifast from PELAB). The analysis was performed on a Cepheid SmartCycler (Cepheid) in two triplicates using the primer pairs 5\(^\prime\)-TTGTTCCCTGCCGTCCCTACCAAGGTTCTGACG-3\(^\prime\) and 5\(^\prime\)-CAACACGTGAGCAATGCCTGGA-3\(^\prime\) (\textit{Chlamydia} strain DC15) (35) was grown in BGM cells. Chlamydial inclusions were quantitated by densitometric scanning.

\textbf{Chlamydia}

Nonavian \textit{C. psittaci} strain DC15 (35) was grown in BGM cells. Chlamydial EBs were purified by discontinuous density-gradient ultracentrifugation (36) using Visipaque (Nycomed). Purified EBs were stored in sucrose-phosphate-glutamic acid buffer at \(-80^\circ\)C (37). EBs for the analysis of IFN-\(\gamma\)-mRNA expression in lungs of infected mice (5 \times 10\(^5\) inclusion-forming unit [IFU], 11 d postinfection [dpi]) were obtained from infected mice. Total RNA was extracted from lung tissues and assayed by RT-PCR using specific primers for IFN-\(\gamma\)-5\(^\prime\)-AAGCCTTACACACTGCATCT-3\(^\prime\) and 5\(^\prime\)-TGCTCATTGTAATGCTTGG-3\(^\prime\) for \textit{C. psittaci} (DC15) were applied to the nasal orifice and inhaled. Controls received droplets of sterile PBS. Mice were weighed daily and examined for clinical signs until...
14 dpi. At 2.4, and 11 dpi, mice were sacrificed, pulmonary lesions were characterized, and numbers of Chlamydia were semiquantitatively evaluated in paraffin sections. All animal procedures were approved by the local District Governments and carried out according to the guidelines of the German law for the protection of animal life.

**Analysis of JAWS II cells** was performed on a FACS Calibur (BD Bioscience) and MACSQuant (Miltenyi Biotec) using CellQuest (BD Bioscience) and MACSQuantify (Miltenyi Biotec) software. Cells were incubated with medium alone and/or with live EBs. After being detached and washed with FACS buffer (2% FCS, 0.1% NaCl in PBS), cells were stained on ice for 30 min using FITC-conjugated anti-mouse Abs against CD86 (GL1), CD40 (HM40-3), and MHC I (AF6-88.5) and were subjected to flow cytometry. JAWS II cells were gated on live cells. For the analysis of endocytosis, cell infection, and cell survival, all events were analyzed and displayed.

**Dextran-uptake assay**

Endocytic activity of DCs was measured by uptake of dextran (40,000 kDa) conjugated with FITC (Sigma-Aldrich). Briefly, DCs were suspended in IMDM supplemented with 10% FBS (pH 7.4), incubated with 1 mg/ml Dextran-FITC for 1 h at 37°C, and analyzed by flow cytometry.

**Cytokine profile analysis**

At the end of 24 h, supernatants from C. psittaci-infected, as well as noninfected, JAWS II cells were analyzed using a Bio-Plex cytokine reagent kit with Bio-Plex mouse cytokine 23-plex Panel in the Bio-Plex 200 system (Bio-Rad), as directed by the manufacturer. Chemokine/cytokine levels in the serum from noninfected and infected mice (4 dpi) were analyzed by Mouse Cytokine Array (Panel A Array Kit), according to the manufacturer’s instructions (R&D Systems). Signals were visualized by ECL (Sigma-Aldrich). Obtained fluorographs were analyzed by densitometric scanning using GelEval 1.32 software (FrogDance Software).

**Immunofluorescence**

For fluorescence microscopy, cells were grown on cover slips and fixed for 20 min in 2% paraformaldehyde, quenched with 3% BSA, permeabilized with 0.1% saponin (Sigma-Aldrich), and incubated serially with the indicated primary and corresponding secondary Abs. Images were captured using an Axiovert 200M/ApoTome microscope (Zeiss) and/or an LSM 5 EXCITER laser scanning microscope (Zeiss). Colocalization was measured using AxioVision colocalization software (Zeiss), ImageJ (National Institutes of Health) Color Profiler plug-in, and CoLocalizer Express 1.7 (Colocalization Research Software).

**DC/T cell cocultivation assay and IFN-γ ELISA**

JAWS II cells (treated with specific inhibitor, small interfering RNA [siRNA] silenced for tapasin, TAP1, or MHC I) and nontreated control cells were plated into 96-well plates at 1 × 10^5 cells in 200 μl complete IMDM well and incubated with medium alone or live EBs for 24 h. In parallel DC cultures, viability of the pretreated and infected cells was checked by trypan blue exclusion. Dead cells and cell debris were removed from the DC cultures by extensive washing. For DC/T cell cocultivation, pretreated and washed DCs were cocultured with 5 × 10^5 Chlamydia-sensitized CD8+ T cells in 200 μl fresh medium/well for 48 h. The culture supernatants were collected, and IFN-γ secreted by CD8+ T cells was assayed by Mouse IFN-γ Platinum ELISA (E Bioscience). Reactions were read at 405 nm with a Microplate ELISA Reader (Sunrise Remote; Tecxan). Following the protocol of Jiang et al. (38), Chlamydia-specific CD8+ T cells were generated by immunizing mice i.p. with 5 × 10^7 IFU live EBs and boosting 2 and 3 wk later. After 4 wk, mice were challenged intranasally with 5 × 10^4 IFU. T cells were purified from murine spleens by negative selection with the CD8+ T Cell Isolation Kit II (Miltenyi Biotec). As a control, CD8+ T cells from naïve mice were purified in parallel. CD8+ T cells ≥90% purity were obtained, as measured by flow cytometry (data not shown). CD8+ T cells cultured with anti-CD3/CD28-conjugated beads (Dynabeads, mouse CD3/CD28; Invitrogen) were used as positive control.

**siRNA-mediated gene silencing**

For siRNA silencing of tapasin, TAP1 and MHC I-annaeled double-stranded siRNA with high knockdown efficiency were designed and purchased from QiAGEN. To knock down the expression of these genes, 4 × 5 μg oligo duplex RNAs specific for tapasin (target sequences; 5'-CCG-GTCTGCTGGAGCTAATAA-3', 5'-CTGTTGGTTCTCGGCCCTTTA-3', 5'-AACGTTCTCTGATCACTTTA-3', 5'-CACCCTCTGAGCTCAA-3', 5'-CACACTCTCTGTCAGA-3', 5'-CTGCGTCTGTAGTACAC-3', 5'-CTGAGATCGAGACTA-3', 5'-TGGAGAAATGAGACTCTA-3', QiAGEN), TAP1 (target sequences; 5'-CACACTCTCTGTCAGA-3', 5'-CTGCGTCTGTAGTACAC-3', 5'-CTGAGATCGAGACTA-3', 5'-TGGAGAAATGAGACTCTA-3', QiAGEN), and MHC I (H-2K^d^ target sequences; 5'-CACAGCAGCCTGAAGA-3', 5'-TAGATGAATACCTCCAGA-3', 5'-GGCGTGATCACA-3', 5'-TCCGAACTTGTTGGTACTAA-3', and H-2D^d^ target sequences; 5'-CAGAGGTGTGGTCCAGAC-3', 5'-TCCGAGATGGACTGACGT-3', 5'-CCTCAGTCTTCTTACAAA-3', 5'-CCGGTACATCTTCGTCGCTA-3') were transfected into 2 × 10^5 cells using the nucleofector system and the Mouse Dendritic Cell Nucleofector kit (Lonza). As a negative control, AllStars siRNA (QiAGEN) was used. Cells were maintained for up to 48 h and screened for gene silencing by Western blot.

**Transmission electron microscopy**

For electron microscopy, cell culture supernatants of JAWS II cells were replaced by 2.5% glutaraldehyde in cacodylate buffer (0.1 M [pH 7.2]) at 48 h postinfection (hpi). After 2 h of fixation at 4°C, cells were gently scraped from the cell culture dish and centrifuged for 5 min at 1500 × g. The pellet was embedded in 2% agarose and sectioned into 1 mm^3 cubes. Cubes were postfixed in 2% osmium tetroxide and embedded in Araldite Cu212. Ultrathin sections (85 nm) were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (TEM; Tecnai 12; FEI).

**Histological and immunohistological examination**

Lung tissues were fixed in neutral-buffered formalin and embedded in paraffin. Lesions were examined in H&E-stained paraffin sections. Chlamydia were labeled by the indirect immune-peroxidase method in paraffin sections using an anti-chlamydial LPS Ab (AC1-FITC; Progen).

**Results**

**Mice susceptible to C. psittaci (DC15) infection show induction of antichlamydial CD8+ T cell immunity**

Despite the crucial role of CD8+ T cells in chlamydial infection, the mechanisms by which chlamydial Ags are processed for presentation by MHC I are only poorly understood. Although DCs present a spectrum of chlamydial Ags expressed at different stages of the chlamydial development cycle, it is not clear how Ags from chlamydial proteins, which are located within the parasitophorous vacuole, become accessible to MHC I processing (13). Before these pathways were investigated in murine DCs (JAWS II cells) (28), we first examined whether C57BL/6 mice are susceptible to infection with C. psittaci (DC15) and whether infected DCs are able to stimulate antichlamydial CD8+ T cells.

During the first 4 dpi, a progressive loss of body weight, apathy, rapid breathing, and rough fur occurred in Chlamydia-infected mice but not in noninfected controls (Fig. 1A). Infected mice reached the maximum weight loss (20%) at day 4 postinfection and then began to recover. They had almost completely recovered with respect to clinical signs and weight loss at 14 dpi. At 2 dpi, an acute multifocal bronchopneumonia with alveolar infiltrates predominated by neutrophils was seen (Fig. 1B1), and few chlamydial inclusions were present in alveolar epithelial cells (Fig. 1B1). At the height of sickness, at 4 dpi, pulmonary tissue was more extensively affected, and inflammatory infiltrates consisted of neutrophils and mononuclear phagocytes (Fig. 1B1). The transmigration of inflammatory cells through vascular walls was accompanied by exudation. There was a marked increase in chlamydial inclusions compared with 2 dpi, and they were predominantly associated with neutrophils and mononuclear phagocytes (Fig. 1B1V). Chemokines/cytokines and their receptors are important mediators of leukocyte trafficking and act as modulators of T cell responses during infections and inflammation. Therefore, we analyzed the expression of multiple chemokines/cytokines, which are associated with the initiation of Th1 cell immune responses. Indeed, we found elevated expression levels of different Th1 chemokines/cytokines,
such as IP-10, MIG, TIMP-1, IL-1α, TREM-1, and MCP-5, at day 4 compared with noninfected control mice (Fig. 1C). In addition, increased levels of Th2 chemokines/cytokines, such as BLC, IL-1α, G-CSF, and I-309, were seen (Fig. 1C). The observed chemokine/cytokine profile is consistent with the histopathological findings at 2 and 4 dpi, because several of the detected chemokines/cytokines are known to play a role in chemoattraction, as well as activation/stimulation of neutrophils, monocytes, and lymphocytes. At 11 dpi, lesions had regressed, but marked peribronchial and perivascular infiltrates of lymphocytes, plasma cells, and mononuclear phagocytes (positive control). Bronchopneumonia (positive control) and numerous Chlamydia (negative control) were seen (Fig. 1BV). Very few, weakly labeled chlamydial inclusions were found in mononuclear phagocytes (positive control) and few, large chlamydial inclusions in mononuclear phagocytes are present (right panel). Inclusions are indicated by arrows. (C) Relative protein levels of chemokines/cytokines analyzed by using plasma samples from noninfected and infected mice at 4 dpi. (D) IFN-γ mRNA expression in lungs of infected mice (11 dpi) assayed by RT-PCR. Analysis was performed on agarose gels, and results were quantitated by densitometric scanning. A representative result of three independent PCR experiments is shown. (E) Infected JAWS II cells (24 hpi) were cocultured for 48 h with CD8+ T cells isolated from noninfected and infected mice. IFN-γ secreted by stimulated CD8+ T cells was assayed by ELISA. Purified CD8+ T cells (from noninfected and infected mice), cultured with anti-CD3/CD28-conjugated beads, were used as positive control. Br, Bronchiolus; Bv, blood vessel; Exempl., exemplarily.

whether Chlamydia-specific CD8+ T cells are generated and whether JAWS II cells present chlamydial Ags via MHC I, we tested the ability of CD8+ T cells from Chlamydia-infected C57BL/6 mice to recognize chlamydial Ags presented by JAWS II cells. To this end, JAWS II cells were pulsed with C. psittaci for 24 h, followed by coculture with Chlamydia-specific purified CD8+ T cells for 48 h at a ratio of 1:5 (DC/CD8+ T cells). Coculture media were analyzed by ELISA for IFN-γ as an indicator of functional T cell stimulation (Fig. 1E). Successful chlamydial infection of DCs was confirmed by flow cytometry and immunofluorescence (data not shown). Chlamydia-pulsed JAWS II cells induced CD8+ T cells from infected, but not from uninfected, mice to produce high levels of IFN-γ, whereas all T cells could be activated via CD3/CD28 (Fig. 1E). In contrast, Chlamydia-specific CD8+ T cells failed to produce IFN-γ when cocultured with noninfected DCs (Fig. 1E). Thus, the observed secretion of IFN-γ was due to Ag-specific DC recognition. Collectively, these results show that C. psittaci-infected mice generate a Chlamydia-specific CD8+ T cell response and that JAWS II cells present chlamydial Ags efficiently via MHC I.

Taken together, our findings depicted in Fig. 1 suggest that the host–pathogen interaction between C57BL/6 mice and nonavian
C. psittaci provides an excellent experimental infection model to investigate molecular and cellular processes involved in MHC I-mediated processing of chlamydial Ags by pAPCs.

Morphological and functional maturation of C. psittaci–infected DCs

It was reported that different chlamydial strains can infect and undergo limited replication in murine and human primary BMDCs and/or DC lines (26, 38, 40). However, to our knowledge, MHC I processing and presentation of chlamydial Ags have not been studied in these cells. BMDCs exhibit variable heterogeneity in the developmental response, which makes it difficult to interpret and reproduce results from cellular infection studies. These limitations could be overcome with the immortal DC line JAWS II, which has stable characteristics, provides standardized quality, and was shown to be a convenient substitute for primary BMDCs (38). Immunofluorescence analysis revealed that C. psittaci forms multiple inclusions in infected JAWS II cells associated with the formation of numerous dendritic extensions (Fig. 2A). Most interestingly, this cellular development of DCs appeared to be accompanied by a detectable reduction in Chlamydia-specific immunolabeling, indicating that the intracellular growth of Chlamydia is affected during DC maturation. An important attribute of immature DCs is their capacity to take up Ag through endocytosis. This property enables DCs to sample their surroundings for potential pathogens, but it is downregulated upon cell maturation (41). Chlamydia-infected DCs show a significant reduction in their endocytic activity compared with noninfected

![FIGURE 2](http://www.jimmunol.org/)

FIGURE 2. Morphological and functional maturation of C. psittaci (DC15)-infected DCs. (A) JAWS II cells (24 and 48 hpi) were immunostained for Chlamydia (green) using an IMAGEN kit and examined with a Zeiss ApoTome microscope. (B) Endocytosis properties of noninfected, infected, and LPS-matured JAWS II cells (left panel). JAWS II cells (72 hpi or treated for 72 h with 10 μg/ml LPS) were cocultured with FITC-labeled dextran. After 6 h, uptake of dextran was determined by flow cytometry. The white graph shows background staining in the absence of dextran. JAWS II cells were pulsed with EBs for 24, 48, or 72 h or were treated with LPS (10 μg/ml) for 72 h, and the activation marker CD86 was analyzed by flow cytometry (middle panel). The gray graphs without outlining represent isotype control stainings with suitable IgG2a, κ mAb (BioLegend). Percentages of CD40-positive cells (JAWS II, 72 hpi, and LPS treated) are shown from pooled data of three independent experiments (right panel). (C) JAWS II cell cultures were analyzed for levels of different chemokines/cytokines. Supernatants of noninfected and infected DCs (24 hpi) were analyzed by ELISA. Values obtained for noninfected control cells were set to 1 arbitrary unit.
control cells (Fig. 2B, left panel). Similar results were obtained with LPS-matured DCs when compared with untreated cells (Fig. 2B, left panel). Thus, the change in the endocytic behavior of infected JAWS II cells seems to be a characteristic of DC maturation. Indeed, no such changes in endocytic uptake were seen for infected epithelial cells (data not shown). Additionally, chlamydial infection caused the upregulation of DC activation marker CD86 (Fig. 2B, middle panel) and an increase in the number of JAWS II cells expressing CD40 (Fig. 2B, right panel). These findings are comparable to results that we obtained with LPS-matured DCs (Fig. 2B, middle and right panels) and are in agreement with previous observations (38).

Also, the surface expression of MHC I molecules (H-2Kb and H-2Dk) was augmented, as described later. These findings are consistent with a functional maturation of the infected DCs. Further, C. psittaci significantly induced IL-12, IL-1α, IL-1β, IL-6, MIP-1α, MIP-1β, G-CSF, MCP-1, and RANTES secretion by the infected JAWS II cells, whereas the release of TNF-α, a cytokine known to induce surface expression of MHC I (42), also was enhanced (Fig. 2C). Altogether, these data indicate that, upon infection, JAWS II cells undergo a functional maturation typical of myeloid-type DCs.

### Cellular characteristics of chlamydial inclusions within infected DCs

To investigate whether chlamydial vacuoles develop differently in DCs and epithelial cells, JAWS II cells and mouse epithelial cells were incubated with C. psittaci for 48 h before the formation of inclusions was analyzed by immunofluorescence microscopy. As can be seen from Fig. 3A (lower panel), in epithelial cells chlamydial vacuoles developed into extended inclusions, with characteristic juxtanuclear localization and diameters in the range of 40–60 μm. In contrast, in Chlamydia-infected DCs the bacterial inclusions displayed a very different phenotype, featuring multiple peripheral small vacuoles with diameters <10 μm at 48 hpi (Fig. 3A, upper panel). When assessed by TEM, both EBs and RBs, but no aberrant forms, were found in C. psittaci–infected DCs. However, most small inclusions mainly contained the larger RB form, and these compartments were scattered over the entire cell (Fig. 3B). Strikingly, many of the chlamydial vacuoles were incompletely surrounded by the inclusion membranes or totally disrupted (Fig. 3B), and Chlamydia were observed freely residing in the cytosol (Fig. 3C, Chlamydia indicated by green color). Areas of disintegration were mainly found at the central sides of inclusions (Fig. 3C). Free cytosolic bacteria had characteristic chlamydial features and were surrounded by a plasma membrane (PM) and an outer undulant layer characteristic of Gram-negative bacteria, suggesting that the cytosolically released pathogens were still physically intact. Moreover, the structural integrity of these cytosolic forms was confirmed by cellular-fractionation experiments (Fig. 3D). The majority of Chlamydia within the inclusion membranes seems to be smaller than those outside the membranes. Enlargement of released bacteria was also observed for other intracellular bacterial pathogens (43) and might occur in response to the different biochemical conditions within the cytosol. Most interestingly, the vesicular compartments (Fig. 3C, indicated by orange color) surrounding the disintegrated inclusions were observed freely residing in the cytosol of host nucleus and intracellular Chlamydia are visualized by DAPI (blue).

### FIGURE 3.

Characteristics of chlamydial inclusions within infected DCs. (A) Formation of inclusions in epithelial cells and DCs. Cells (48 hpi) were immunostained for chlamydial LPS (green). DNA of host nucleus and intracellular Chlamydia are visualized by DAPI (blue). (B) Electron photomicrographs of infected JAWS II cells (MOI 10, 72 hpi; left panel). (C) Structural disintegration of inclusions in infected JAWS II cells (MOI 10, 72 hpi). Enlarged photographs (lower panels) show the disrupted inclusion membranes (a partially disintegrated inclusion membrane is indicated by arrowheads) and release of Chlamydia into the cytosol. In (B) and (C), Chlamydia are colored green; mitochondria are colored blue; trans-Golgi network, Golgi, and exocytic compartments are colored yellow; and endolysosomal compartments are colored orange (right panels). (D) Infected JAWS II cells (MOI 10, 72 hpi) were homogenized with a Dounce homogenizer. After removal of the nuclear fraction by low-speed centrifugation (<1,000 × g), the remaining cell extract was separated into a soluble fraction (first lane) and a high-speed organelle/membrane pellet (100,000 × g, second lane) by ultracentrifugation. Fractions were analyzed by Western blots stained with anti-chlamydial HSP60 (recognizing chlamydial HSP60-1 and HSP60-2) and anti-GAPDH (cytosolic fraction marker).
ressembled, with regard to their phenotypic features, amphisomes, endosome–autophagosome fusion compartments that are characterized by multivesicular morphology (44).

Our studies indicate that chlamydial growth and replication in DCs differ from in epithelial cells. To analyze the disparate behavior of *C. psittaci* in DCs and epithelial cells, we examined mRNA expression of chlamydial genes associated with cell division (ftsW) and protein synthesis (groEL-1). The PCR analysis demonstrated that the time-dependent accumulation of chlamydial mRNA in infected DCs was much lower than that seen in infected epithelial cells (Fig. 4A). Moreover, DCs harbored and released much lower numbers of infectious *Chlamydia* compared with epithelial cells (Fig. 4B). Thus, DCs appear to be less permissive to the developmental cycle of *C. psittaci*, resulting in impaired growth of the bacteria and eventually leading to a reduced production of infectious EBs.

CPAF is a virulence factor that appears to modulate immune responses against chlamydial infections. To this end, CPAF is normally secreted from the chlamydial inclusion into the cytosol of the host cell (45), where it is essential to preserve the integrity of the inclusion (46). In view of our findings in Figs. 3A, 3B, and 3D, we were interested to see whether and to what extent infected epithelial cells and DCs differed in the intracellular localization of CPAF. Thus, we performed immunostaining of CPAF of *Chlamydia*-infected cells (Fig. 4C). In agreement with our findings in Fig. 3D, we observed noticeable CPAF staining inside, but not outside, chlamydial structures in infected DCs. In contrast, CPAF was detected predominantly in the cytosol in infected epithelial cells, indicative of its functional translocation out of the inclusion. This was confirmed by corresponding cell-fractionation experiments (Fig. 4C, right panels).

**Chlamydial structures colocalize with autophagosomal and amphisomal compartments in infected DCs**

Recent reports provided evidence that GBPs are involved in autophagy pathways of phagocytes and act as cell-autonomous effector molecules against intracellular bacteria (47, 48). GBP1, which can be induced by IFN-γ, TNF-α, and/or LPS (49, 50), was found to target to chlamydial inclusion membranes (51) and to polymerize around free cytosolic bacteria for recruitment of specific antimicrobial and autophagic effector proteins (48). After chlamydial infection, JAWS II cells significantly upregulated GBP1 in a time-dependent manner (Fig. 4D, upper left panel) comparable to GBP1 induction in the presence of LPS (Fig. 4D, lower left panel). In addition, corresponding immunofluorescence studies demonstrated that GBP1 colocalized with the dispersed chlamydial structures in infected DCs, whereas noninfected JAWS II cells on the same slide showed no or very low immunofluorescence staining (Fig. 4D, right panel, indicated by asterisk).

**FIGURE 4.** Chlamydial growth and cellular characteristics of host–pathogen interaction in infected DCs. (A) Survival of *Chlamydia* in JAWS II and epithelial cells. For quantitative real-time PCR analysis, mRNA was isolated from infected cells (24 and 48 hpi). Experiments were performed in two triplicates using specific primer pairs for chlamydial ftsW (left panel) and groEL-1 (right panel). (B) JAWS II or epithelial cells were infected with *Chlamydia* for 48 h. Homogenates from cells and corresponding culture supernatants were collected and titrated on BGM cells to determine the number of recoverable IFU. IFU obtained for infected JAWS II cell cultures were set to 1 arbitrary unit. Pooled data from three separate experiments are shown. (C) Localization of CPAF in infected epithelial cells and DCs. Infected cells (72 hpi) were stained using a polyclonal Ab against CPAF. DNA of the host nucleus and inclusions (indicated by arrowheads) is visualized by DAPI (blue). A total of 200 cells was counted from eight random fields of view (using a 63× objective) to determine the number of infected DCs exhibiting retention of CPAF in chlamydial structures. The analysis revealed that ≥96% of the counted infected cells had the phenotype depicted in the upper panels. Additionally, cells were fractioned as described in Fig. 3D and analyzed by Western blots (far right panels) stained with anti-CPAF and anti-GAPDH (cytosolic fraction). (D) Expression of GBP1 during DC infection (left panels). JAWS II cells were infected for 24, 48, or 72 h or treated with LPS (10 μg/ml, 72 h), and GBP1 was detected by Western blot. Anti-β-actin staining was used as loading control. Interaction between inclusions and GBP1 in infected DCs (48 hpi) was analyzed by confocal microscopy (right panels).
Thus, cytosolic *Chlamydia* may become targets for GBP1-mediated autophagy after their release from disrupted vacuoles (48). In support of this scenario, TEM showed that disintegrated chlamydial inclusions and free cytosolic bacteria were often located in close proximity to autophagosomal vacuoles (Fig. 5A, *left panel*). Strikingly, in addition to electron lucent cytosolic material, the latter seemed to contain morphologically intact *Chlamydia* (black arrowheads in Fig. 5A). Autophagosomes normally fuse with endosomes to become mature single-membrane amphisomes (52). Indeed, many of these multivesicular vacuoles (containing spherical structures the size of EBs and/or RBs) were found close to free cytosolic *Chlamydia* (Fig. 5A, *right panel*).

Further supporting a scenario in which bacteria are sequestered into autophagosomes, fluorescence microscopy showed that chlamydial structures displayed a high level of colocalization with autophagy markers LC3 and Beclin-1 at 72 hpi (Fig. 5B1–VI). Moreover, we observed significant colocalization between *C. psittaci* and compartments containing cathepsin D, a critical proteolytic enzyme in autophagic catabolism (Fig. 5B VII–IX). Furthermore, cathepsin D was directly associated with LC3-positive autophagosomal structures colocalizing with *C. psittaci* (Fig. 5B–XIII). Thus, it seems that in infected DCs, free *Chlamydia* are engulfed by autophagosomes, which subsequently fuse with cathepsin D-positive endovacuoles. The association of endosomal and autophagosomal marker proteins with *Chlamydia* indicates that many of these intracellular structures are amphisomes (53). Another characteristic feature was the colocalization of *Chlamydia*-associated autophagosomes with Rab11, a marker of endosomal recycling and multivesicular endosomes (53) (Fig. 5B XIV–XVII).

Autophagy can be inhibited by the presence of adenine analogs (54). Thus, we investigated the effect of 3-methyladenine (3-MA) on chlamydial growth in DCs. Interestingly, blocking autophagy resulted in a drastic increase in the number of intracellular bacteria and *Chlamydia*-positive DCs at 48 and 72 hpi (Fig. 6A). Moreover, administration of 3-MA diminished the colocalization between *Chlamydia* and the autophagosomal/amphisomal markers Beclin-1,
FIGURE 6. Inhibition of autophagy by 3-MA enhances chlamydial growth in infected JAWS II cells. (A) Infected JAWS II cells (MOI 1, 48 and 72 hpi) were analyzed by flow cytometry using the IMAGEN kit (Oxoid). We measured the amount of Chlamydia-positive cells (left panel), as well as the increase in mean fluorescence intensity (MFI; right panel), reflecting the bacterial load of infected cells. The MFI values obtained for noninfected cell cultures were set to 1 arbitrary unit. (B) DCs were infected for 48 h in the presence of 3-MA and immunostained for Beclin-1, LC3, and cathepsin D, as well as chlamydial LPS. DNA of host nucleus and inclusions was visualized by DAPI (blue). (C) Effect of autophagy inhibition on the viability of infected DCs. JAWS II cells were infected or not for 48 h in the presence or absence of 3-MA. Cell death was determined by propidium iodide staining using flow cytometry. One representative experiment of three independent experiments with similar results is shown.

Characteristics of MHC I presentation in Chlamydia-infected DCs

MHC I expression increased up to 4-fold in JAWS II cells during chlamydial infection (Fig. 7A, upper right panel), and this induction was largely dependent on the activity of TNF-α (Fig. 7A, lower right panel), one of the cytokines secreted by DCs in response to the infection (Fig. 2C). This was also reflected by significantly elevated levels of H-2Kb and H-2Db at the cell surface of infected cells (Fig. 7B), which is comparable to the induced MHC I surface expression observed in the presence of LPS or TNF-α (Fig. 7B, right panel). Most interestingly, this upregulation was accompanied by an enhanced colocalization between H-2Db/H-2Kb and the endosomal recycling marker Rab11 (Fig. 7C, left panel). To assess the rate of MHC I recycling in infected, noninfected, and LPS-matured DCs, we analyzed MHC I surface expression in the presence of primaiquake, which selectively inhibits endosomal recycling of cell surface molecules. By using this approach, we observed a substantial increase in the recycling rates for both H-2Kb and H-2Dd in infected and LPS-treated JAWS II cells (Fig. 7C, right panel).

Our experiments in Fig. 5 show that Chlamydia from disintegrated inclusions are targeted via autophagy to LC3/cathepsin D-positive amphisomal cell structures. Thus, we were interested to see whether MHC I gains access to these compartments. Indeed, immunofluorescence analysis of H-2Kb/cathepsin D double-labeled cells (Fig. 8AIX–XI), as well as a careful evaluation of corresponding scattergrams (CoLocalizer Express) (Fig. 8BXIII–XV), showed a time-dependent increase in colocalization between 48 and 72 hpi, suggesting that MHC I gets enriched in cathepsin D–positive compartments during maturation of Chlamydia-infected DCs. The same phenomenon could be seen in LPS-matured JAWS II cells (Fig. 8AXII, 8BXVI). Moreover, similar results were obtained for cathepsin D/H-2Dd and for cathepsin S/H-2Db or S/H-2Kb double-labeled cells (data not shown), whereas no enrichment of MHC I in cathepsin-positive structures was seen in uninfected DCs (Fig. 8AXIX, 8BXIII). The observed increase in colocalization between MHC I and cathepsin D apparently did not occur at the expense of the MHC I fraction displaying perinuclear Golgi/Trans-Golgi network staining, suggesting that MHC I translocated to cathepsin D–positive compartments is not directly recruited from the biosynthetic pathway. Indeed, the continuous presence of H89, a drug blocking the entire exocytic pathway, during DC infection diminished the dominant perinuclear appearance of MHC I, but it had no detectable effect on the colocalization between MHC I and cathepsin D (Fig. 8C). Moreover, we observed that MHC I also colocalized to some extent with LC3-positive compartments in Chlamydia-infected DCs (Fig. 8D), providing evidence that MHC I gains access to autophagosomes in infected DCs.

Different studies demonstrated the essential role of different TLRs (e.g., TLR2 and TLR4) and TLR adapter molecule MyD88 in the generation of immune responses to chlamydial infection (55). Most recently, TLR4/MyD88-dependent relocation of TAP (56), which is normally found within the ER, to endosomal structures was shown to enhance the processes of MHC I cross-presentation (56). Thus, we were interested to see whether and to what extent intracellular TAP localization changed during maturation of Chlamydia-infected DCs. As expected, TAP was present in the ER, but not in cathepsin D-containing compartments, in noninfected cells (Fig. 9AVI, 9BXIX). In sharp contrast, in Chlamydia-infected and LPS-treated DCs, TAP showed, in addition to ER staining, a notable presence within cathepsin D–positive vacuoles, which were in close proximity to and/or overlapping with chlamydial structures (Fig. 9AXII, 9AXVIII, 9BXX, 9BXXI). Thus, it is tempting to speculate that, in Chlamydia-infected DCs, the ER, as well as endocytotic compartments, contain TAP for the transport and delivery of antigenic peptides.

To identify factors in infected DCs that are functionally involved in chlamydial MHC I Ag presentation, we analyzed the stimulation
of Chlamydia-specific CD8+ T cells by infected JAWS II cells that were blocked for the expression or function of different components of the classical and vacuolar MHC I pathway (Fig. 9C). As expected, siRNAs specific for H-2Kb or H-2Db dampened JAWS II cell–mediated stimulation of Chlamydia-specific CD8+ T cells by up to 60% (Fig. 9C), demonstrating that CD8+ T cell stimulation is due to MHC I–mediated Ag presentation and that both allelic MHC I products are equally involved in the processing of chlamydial Ags. Most interestingly, siRNA-mediated silencing of TAP1, but not of tapasin, reduced the IFN-γ secretion of coul-
tured CD8+ T cells by 80–90% (Fig. 9C). This suggests that, in infected DCs, MHC I presentation of chlamydial Ags occurs via a tapasin-independent mechanism that requires the proper function of TAP. Moreover, Chlamydia-specific CD8+ T cells showed a significantly reduced production of IFN-γ when cocultured with primaquine-treated DCs, whereas only mild effects were seen for H89-treated DCs (Fig. 9C). This suggests that endosomal MHC I recycling takes over a central function in the surface presentation of chlamydial Ags and that the classical biosynthetic route of MHC I loading and surface presentation plays a minor role in chlamydial Ag processing.

Our results in Fig. 6C show that inhibition of autophagy by 3-MA had detrimental effects on the survival of infected DCs. Thus, we decided to focus on a possible requirement for amphisomal hydrolases cathepsin D and S. Inhibition of either cathepsin in Chlamydia-infected DCs had a dramatic influence on the stimulation of Chlamydia-specific CD8+ T cells, suggesting that both hydrolases are required for efficient processing of chlamydial Ags (Fig. 9C). Also, proteasomal inhibition of infected DCs strongly affected the stimulation of cocultured CD8+ T cells (Fig. 9C).

In summary, our findings suggest a scenario in which chlamydial Ags are generated through an autophagosomal/amphisomal route that involves preprocessing in endovacuolar compartments via different cathepsins and entry into the cytosol for further downstream processing by the proteasome (Fig. 10). The finding that neither tapasin nor biosynthetic MHC I export is required indicates that, after proteasomal action, chlamydial peptide Ags are retranslocated into the endosomal pathway via TAP and are loaded on MHC I derived from endosomal recycling.

Discussion

In the current study, we investigated the requirements of MHC I presentation during Chlamydia infection using C57BL/6 mice, the mouse DC line JAWS II, and the nonavian C. psittaci (DC15) as an infection model system. The most intriguing finding of our work is that autophagy constitutes a critical pathway in MHC I processing of chlamydial Ags in infected DCs.

Our data demonstrate that the disease in C. psittaci–infected C57BL/6 mice is comparable to what was recently described for infected cattle (37), the natural host of C. psittaci strain DC15. In
particular, the onset of the most pronounced clinical signs due to bronchopneumonia, the pathological features, and the course of disease are remarkably similar. Chlamydia-infected DCs show morphological, as well as functional, maturation, which is characterized by elevated expression of distinct activation/maturation markers and secretion of chemokines/cytokines known to be associated with optimal Ag presentation and clearance of bacterial infections (13). These infected matured DCs allow only a restricted developmental cycle of Chlamydia, which is consistent with previous reports (26, 40). Multiple small inclusions forming...
in infected DCs contain predominantly RBs, suggesting that the growth of *Chlamydia* surviving in JAWS II cells is substantially impaired. Consistent with this, significantly lower numbers of infectious units are recovered from DCs than from infected epithelial cells. Nevertheless, bacteria remain metabolically active in either environment. The controlled survival of *Chlamydia* in DCs might be a critical feature to ensure a broad Ag reservoir allowing sustained and efficient chlamydial Ag presentation, which might be more difficult in a scenario of stringent chlamydial “persistence” with arrested growth and strongly reduced biosynthetic activity (57).

Strikingly, the limited development of *C. psittaci* in infected DCs is accompanied by retention of CPAF within chlamydial structures, as well as structural disintegration of the inclusion membranes and cytoplasmic release of bacteria. CPAF is known to be a key factor protecting the integrity of the chlamydial vacuole (46). In this context, it was suggested that cytosolic CPAF is required for the chlamydial integral inclusion membrane protein turnover and/or processing of intermediate filaments at the inclusion (46). Heuer et al. (45) found that, in situations of restricted chlamydial growth, cytosolic CPAF translocation and its virulence function are impaired. Restricted chlamydial growth in DCs seems to be controlled by TNF-α (58), which is released by *Chlamydia*-infected DCs and stimulates bactericidal activity by IDO (59). Thus, it is tempting to speculate that, in infected DCs, TNF-α-mediated suppression of chlamydial development results in CPAF retention and, in turn, disintegration of bacterial compartments. Further, it seems that DCs use specific cellular mechanisms to initiate autophagosomal degradation of *Chlamydia*. In support of this, our experiments revealed that GBP1 is upregulated in *Chlamydia*-infected matured DCs and localizes to chlamydial inclusions. Members of the GBP family detect bacterial pathogens in the cytosol and mark them for disruption (48). It was speculated that GBP1, together with GBP5 and GBP7, recruits autophagy proteins, which drive the extension of autophagic isolation membranes around bacteria within damaged bacterial compartments. This targets the organism to autolysosomes and, thus, confers cell-autonomous host defense (48). Most interestingly, GBP5 functionally cooperate with different members of the p47 GTPase family (60, 61), which are thought to induce interaction of chlamydial structures with autophagosomes to reroute the pathogen to autolysosomes (62). For cargo degradation by cathepsins (63, 64), autophagosomes fuse with endosomes to form multivesicular amphisomes (44). It was recently proposed that amphisomes are involved in pathogen degradation and Ag presentation in DCs (65). There is increasing evidence that this pathway is structurally and functionally connected with endocytic and exocytic systems (66). For example, Rab11-containing vesicular compartments, which have a critical role at the recycling endosomes, mediate amphisome formation (53). Our results suggest that *Chlamydia* from disintegrated inclusions are targeted to autophagosomal vacuoles, as well as multivesicular amphisomes, which are in close proximity to the collapsed chlamydial structures. These compartments are clearly different in morphology, shape, and size compared with the disrupted bacterial inclusions. Moreover, our findings demonstrate that chlamydial structures localize to Beclin-1–, LC3–, cathepsin D–, and Rab11-positive compartments, supporting the presence of *Chlamydia* in amphisomes, where they can be subjected to proteolytic degradation. Because this chlamydial colocalization is nearly quantitative and depends on proper function of autophagy, it is tempting to speculate that the degradation of cytoplasmic *Chlamydia* is associated with the generation of bacterial epitopes for Ag presentation in infected DCs.

In line with other findings (67), we observed that *Chlamydia* apparently does not interfere with the ability of DCs to present Ag by MHC I. Thus, infected and mature DCs are characterized by an increased expression and surface presentation of MHC I, which is mainly controlled by TNF-α. Moreover, our experiments showed that MHC I, as well as TAP, is concentrated in cathepsin D–endosomal compartments. These intracellular changes are most likely due to TLR4-stimulated rearrangement of endosomal compartments (68), MHC I mobilization (69), and relocation of TAP to endosomal structures (56). In infected DCs, an MHC I subfraction colocalizes with LC3-positive compartments. This is reminiscent of the involvement of MHC class II (MHC II) in autophagosomal/amphisomal Ag processing (70). In this context, it is interesting to note that studies by Tiwari et al. (21) showed that internalized surface MHC I molecules intersect with endosomal compartments of the MHC II–presentation pathway in which low pH favors peptide exchange on recycling MHC I (71).

Our experiments revealed that H-2Kb and H-2Db are equally involved in the Ag-specific functional presentation of chlamydial Ags to CD8+ T cells. This is in agreement with studies that identified chlamydial CD8+ T cell Ags with characteristics of H-2Kb– or H-2Db–binding peptides (13). Inhibition of cathepsin D and S in infected DCs had a dramatic influence on the MHC I–mediated stimulation of *Chlamydia*-specific CD8+ T cells. Cathepsin D and S are two well-characterized endocaval aspartic- and cysteine-type hydrolases, respectively, which are expressed in many cells of the immune system (72). Both hydrolases are known to function as important downstream proteases in autolysosomal/amphisomal degradation (73, 74) in the endocaval MHC II–presentation pathway (75–78) and are thought to be functionally involved in DCs in the alternative generation of antigenic MHC I peptides (20–22). In this context, it is interesting to note that autophagosomal processes regulate the expression and activity of amphisomal cathepsins (79). Thus, autophagy leads to a time-dependent decrease in hydrolases and, in turn, controlled Ag processing (79). It was hypothesized that modulated cathepsin levels might favor the generation of MHC-peptides as the result of a less efficient lysosomal protein digestion. This hypothesis was suggested to explain the superiority of DCs over macrophages as pAPCs. It was previously shown that different combinations of cathepsins act sequentially and/or at the same time on substrates (80). This might explain the observation that both cathepsins are critically involved in MHC I processing of chlamydial Ags (Fig. 9C).

*Chlamydia*-specific CD8+ T cells displayed a significantly reduced stimulation when cocultured with primaqueine-treated infected DCs, demonstrating that endosomal Ag processing and MHC I recycling take over a critical function in the surface presentation of chlamydial Ags. In contrast, blocking the ER-to-PM transport by H89 had only a limited effect on T cell recognition. Although cathepsins are able to produce peptide products with suitable MHC-anchor residues (20), it was suggested that cathepsin-generated precursor peptides require further downstream trimming by the proteasome to generate mature peptide Ags of permissible length (22). In accordance with this idea, we found that silencing of TAP1 or inhibition of the proteasome caused a strong reduction in the T cell stimulation, suggesting that cytosolic processing is also required for functional MHC I processing of chlamydial Ags.

Our experiments suggest that the presence of the MHC I–loading chaperone tapasin is not required for functional MHC I presentation of chlamydial Ags. It is plausible to assume that the acidic environment in endocaval-loading compartments (71) substitutes for the peptide-editing function of tapasin by conferring high stringency on MHC I loading and allowing only optimal peptide ligands to bind stably. Interestingly, a recent study showed that, following autophagy, some viral Ags could be processed and...
directly loaded on MHC I within autophagolysosomal compartments (81). Thus, autophagy appears to be a possible route to take up cytosolic Ags and deliver them to endovacuolar compartments (23). However, our findings suggest a more complex Ag trafficking between endovacuolar compartments and the cytosol. It seems that Ags are first processed in amphisomal structures, where cathepsins cleave pathogenic proteins into large peptides. After the retrotranslocation of antigenic precursors into the cytoplasm, the predigested peptides are further handled by the proteasome, auto-phagosomal compartments might be able to acquire the MHC I machinery, including TAP (82). Thus, mature Ags might finally be imported back into the amphisome for loading onto MHC I.

It is known that DC-mediated cross-presentation of pathogen-associated Ags obtained from phagocytosis of infected dead cells is an important step in T cell activation (83). However, as mentioned above (Materials and Methods), the viability of infected and noninfected JAWS II cells was checked before DC/T cell cocultivation, and dead cells and cell debris were removed from the DC cultures by extensive washing during chlamydial infection. Thus, in the experiment depicted in Fig. 9C, the number of dead cells was reduced to <1%, suggesting that phagocytosis of infected dead cells might make only a minor contribution, if any, to the DC/T cell cocultivation results. However, further studies are required to clarify the functional involvement of different Ag-uptake receptors and pathways.

On the basis of our data, we propose a working model (Fig. 10) in which Chlamydia infects DCs and forms parasitophorous vacuoles. These inclusions contain predominantly RBs and are characterized by a relatively small size (Fig. 3), but no aberrant forms are present (Fig. 10, step 1). These bacterial structures are characterized by impaired CPAF release (Fig. 4C), and the absence of this critical vacuole-protecting factor from the cytosol results in the disintegration of the chlamydial compartment. Deteriorating vacuoles and/or released Chlamydia are then targeted by GBP1 (Fig. 4D), most likely in cooperation with other GBPs, which recruit additional antimicrobial partners, including autophagy effectors (Fig. 10, step 2). Hence, cytosolic Chlamydia are engulfed by autophagosomes (Figs. 5, 6) containing Beclin-1 and LC3 (Fig. 10, step 3), which subsequently fuse with MHC I-containing endosomes to form amphisomes. A characteristic feature of these hybrid organelles is the presence of the hydrolase cathepsin D and the endosomal recycling marker Rab11 (Fig. 10, step 4). We found strong evidence that chlamydial Ags are preprocessed in these amphisomes via cathepsins (Fig. 9). Based on these findings and the observation of other investigators (84), we propose that preprocessed Ags transfer across the endoamphisomal membranes via Sec61 or another retrotranslocation channel for final processing by the proteasome (Fig. 10, step 5). Antigenic peptides generated by the proteasome could be reimported into the amphisomal compartments by TAP (Fig. 9). After this step, chlamydial peptides are loaded primarily on MHC I derived from endosomal recycling (Fig. 9) of surface molecules (Fig. 10, step 6), which gain direct access to endovacuolar compartments. MHC I loading with Chlamydia-derived peptides most likely involves pH-controlled peptide exchange of preloaded MHC I complexes. Finally, MHC I, successfully loaded with chlamydial Ags, is presented on the cell surface (Fig. 10, step 7), from which they can recycle back to amphisomal “loading compartments” for further

FIGURE 10. Hypothetical working model of MHC I presentation of chlamydial Ags by infected DCs. Details are provided in the Discussion. Postulated steps are indicated by numbers 1–7. Steps 1 and 2 describe disintegration of inclusions. Steps 2–4 depict the autophagosomal processes involved in the generation of chlamydial Ags. Steps 5–7 illustrate postulated cellular routes involved in MHC I loading of chlamydial Ags.
rounds of Ag loading and presentation. We are currently interested in the identification of distinct chlamydial peptide Ags loaded on MHC I of the infected DCs. In future studies, this and the use of CD8+ T cells, which are specific for single Ags, will allow us to discriminate immune reactions to chlamydial inclusion proteins from those that are directed against bacterial polypeptides actively secreted into the cytosol.

The advantage of autophagosomal cross-presentation is that poly-peptides from intracellular bacteria, which reside in parasitophorous vacuoles, can still be processed for MHC I loading. Another important benefit might be the efficiency with which autophagosomal cross-presentation occurs. Thus, autophagosomal Ags would not have to compete with abundant endogenous Ags in the ER.

Clearance of Chlamydia depends on the ability of CD8+ T cells to recognize epithelial cells, in which the bacteria predominantly replicate (13). One could imagine that, in Chlamydia-infected epithelial cells, IFN-γ from activated T cells creates a situation that reflects the above-described scenario for infected DCs (Fig. 9). Indeed, in infected epithelial cells, IFN-γ restricts chlamydial growth (85), affects cytoplasmic CPAF translocation (45), rescues surface presentation of MHC I (86), and induces autophagic degradation of Chlamydia (62).

Our findings in Chlamydia-infected DCs provide new insights into the cellular mechanisms of MHC I presentation of intracellular bacterial pathogens and might have important implications for the future design of vaccination strategies based on Ag targeting to DCs. Because previous studies describe autophagosomal clearance of intracellular bacterial pathogens (87), it will be interesting to see whether autophagosomal MHC I presentation is also involved in the processing of intracellular pathogens other than Chlamydia.

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Disclosures

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