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Interaction of *Mycobacterium avium*-Containing Phagosomes with the Antigen Presentation Pathway

Heinz-Joachim Ullrich,‡ Wandy L. Beatty,† and David G. Russell*

Pathogenic mycobacteria infect macrophages where they replicate in phagosomes that minimize contact with late endosomal/lysosomal compartments. Loading of Ags to MHC class II molecules occurs in specialized compartments with late endosomal characteristics. This points to a sequestration of mycobacteria-containing phagosomes from the sites where Ags meet MHC class II molecules. Indeed, in resting macrophages MHC class II levels decreased strongly in phagosomes containing *M. avium* during a 4-day infection. Phagosomal MHC class II of early (4 h) infections was partly surface-derived and associated with peptide. Activation of host macrophages led to the appearance of H2-M, a chaperon of Ag loading, and to a strong increase in MHC class II molecules in phagosomes of acute (1 day) infections. Comparison with the kinetics of MHC class II acquisition by IgG-coated bead-containing phagosomes suggests that the arrest in phagosome maturation by mycobacteria limits the intersection of mycobacteria-containing phagosomes with the intracellular trafficking pathways of Ag-presenting molecules. The Journal of Immunology, 2000, 165: 6073–6080.

Antigen presentation is a multistep process that involves the uptake of complex forms of Ag, its processing into small peptides or lipids, the binding to Ag-presenting molecules, and subsequent transport of the newly formed complex to the plasma membrane. To achieve this elaborate task the distinct steps in Ag presentation are highly compartmentalized, and specific sites exist where the molecular rendezvous of Ags and Ag-presenting molecules occurs. Cytosolic proteins like viral proteins, or bacterial proteins with access to the host’s cytosol, are degraded by the proteasome and bind to MHC class I molecules in the endoplasmic reticulum, leading to cytolytic CD8⁺ T cell responses. Ags sequestered from the cytosol in endocytic or phagocytic compartments encounter their partners through fusion with endocytic vesicles containing MHC class II molecules, leading to CD4⁺ T cell responses (1–3). CD1-mediated presentation is a notable exception to this rule because CD1 meets Ags in endocytic compartments, but leads to the activation of CD1-specific cytolytic CD8⁺ T cells and CD4⁺ CD8⁻ T cells (4, 5).

The colocalization of Ags and MHC class II molecules alone is not enough for efficient Ag presentation. Proteins must be degraded to peptides of permissible length, and MHC class II molecules must undergo compartment-specific maturation processes such as glycosylation and the proteolytic removal of an associated chaperon, the invariant chain, before Ags can bind (6). Furthermore, loading of Ags requires the removal of a class II-associated invariant chain-derived peptide (CLIP), from the binding groove, a process that occurs optimally at low pH and is catalyzed by another chaperon, HLA-DM (H2-M in mice) (7, 8). Moreover, after binding of the Ag to the heterodimer, the complex must reach the plasma membrane via a poorly defined exocytic process (9, 10). APCs have evolved specialized compartments that optimize the complex requirements for Ag presentation. They are connected either to the early (MHC class II-containing vesicles) or late (MHC class II-containing compartments (MIICs)) stages in the endocytic continuum (reviewed in Ref. 11).

Pathogens or particles that reside in phagosomes that remain in communication with the endocytic pathway, such as *Leishmania* (12, 13), *Coxiella* (14), heat-killed *Listeria* (15), or bead particles (16), acquire MHC class II molecules. In contrast, pathogens such as *Legionella*, *Toxoplasma*, or *Chlamydia*, which reside in “non-communicative” phagosomes, avoid acquisition (14, 17, 18). Between these poles, pathogenic *Salmonella*- or mycobacteria-containing phagosomes show a highly selective interaction with the host’s endocytic network (19–22). The consequence of the restricted interaction with respect to the acquisition of the Ag presentation “machinery” was investigated for phagosomes containing the opportunistic pathogen *Mycobacterium avium*.

Materials and Methods

**Cells and Abs**

Macrophages were derived from bone marrow cells of BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) and maintained in DMEM supplemented with 10% FCS, 5% horse serum, 2 mM l-glutamine, 1 mM sodium pyruvate, and 20% L cell-conditioned medium. After 5 days in bacteriological petri dishes, macrophages were transferred to tissue culture-treated T160 flasks (Costar, Cambridge, MA) at a density of 1 × 10⁶ and left for 24 h to establish a monolayer. Human monocytes were isolated as described (23) and were cultured for 5 days in RPMI 1640 medium, 10% FCS, and 1% human serum (Sigma, St. Louis, MO) in petri dishes. The J774 murine macrophage cell line was acquired from American Type Culture Collection (ATCC, Manassas, VA) and cultivated in DMEM containing 10% FCS. *M. avium* 101 is a highly mouse-virulent, clinical isolate that...

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*Abbreviations used in this paper: CLIP, class II-associated invariant chain peptide; 1D-IEF, one-dimensional isoelectric focusing; MIIC, MHC class II-containing compartment; p.i., postinfection.*
was used after passage through a mouse to maintain virulence. Fresh aliquots had a smooth colony morphology (>90%) and were >90% viable before infection. For activation, macrophages were treated with rIFN-γ (Genetics Institute, Cambridge, MA).

Monoclonal Abs against HLA-DRα (1B5) and HLA-DMA (5C1) were a gift from Prof. J. Trowsdale (Cambridge University, Cambridge, U.K.). KL 295, an Ab against the β-chains of I-Aα and I-Eα, was obtained from ATCC. 1D4B and H3A4 are Abs against LAMP-1 from mice and humans respectively, and were purchased from the Developmental Studies Hybridoma Bank (National Institute of Child Health and Human Development, Iowa City, IA). H2-M was detected with a polyclonal rabbit antisemur obtained from Dr. C. Nelson (Washington University, St. Louis, MO). Cathepsin D was detected by a polyclonal rabbit antisemur from Prof. S. Kornfeld (Washington University). The Ab against the E11 subunit of the v-ATPase (30 kDa) had been described (24).

**Phagosome isolation**

Human IgG coupling to magnetic beads and the isolation of IgG-coupled bead-containing phagosomes have been described in detail elsewhere (25). In short, IgG-coated beads were added to cells at 4°C for 5 min and pulsed through the cells for 10 min at 37°C. After the indicated times, cells were lysed by disruption through a 25-gauge syringe in homogenization buffer (250 mM sucrose, 20 mM HEPES (pH 7.0), 0.5 mM EDTA, 0.5 mM EGTA, 0.05% gelatin, 50 μg/ml pepstatin, 100 μg/ml Ne-p-tosyl-L-lysinechloromethylketone, and 25 μg/ml E64), and phagosomes were purified through multiple passages in 15% Ficoll 400,000 (Sigma) and subsequent passages through 30% sucrose in homogenization buffer using a magnet. Phagosomes containing beads were counted, and equal amounts of phagosomes (10^3) were loaded on 12% SDS-polyacrylamide gels by SDS-PAGE. For the isolation of *M. avium*-containing phagosomes, macrophages were infected for 2 h with a multiplicity of infection of 20 leading to over 95% of cells being infected. After the indicated times, phagosomes were isolated from infected cells by lysis of cells through multiple passages through a 25-gauge needle in homogenization buffer (according to the protocol for IgG-coated bead-containing phagosome purification).

**Lysates were centrifuged for 4 min at 200 × g to remove nuclei. The nuclei-free supernatant was layered on a step gradient consisting of 2 ml of 12% Ficoll 400,000 in 5% sucrose, 0.05% gelatin, 0.5 mM EDTA, 0.5 mM EGTA, and 20 mM HEPES (pH 7.0) and centrifuged at 2000 × g for 45 min. The pellet containing phagosomes was resuspended in homogenization buffer and again passed through the 12% Ficoll 400,000 cushion. Phagosomes were analyzed for purity visually on a 3.5–10 MIPES buffer (pH 3.5–10). The stacker gel contained 6 M urea, 7% acrylamide, 2% ampholines (pH 3.5–10). The purified phagosomes were taken up in 50 μL PBS, 1 mM g/ml pepstatin A, 0.05 mg/ml bromphenol blue. MHC class II heterodimer dissociation was enforced by adding 10 μg/ml Texas red fluorescein diacetate (Molecular Probes, Eugene, OR) at 37°C for 20 min.

*Phagosomes containing live mycobacteria showed green fluorescence due to cellular esterase activity. Greater than 95% of all particles visible under a 63X Axioscope lens (Zeiss, Oberkochen, Germany) contained live bacteria.*

**To measure contamination with nonphagosomal proteins, macrophages were cultivated in four T25 flasks (Costar), and half of the flasks were labeled with 1 μCi of [35S]methionine for 1 h. One radioactive macrophage culture and one nonradioactive culture were incubated with particles (IgG-coated bead-containing phagosome purification) for 4 h. Cells from the particle-containing flasks and from two control flasks, one of which was labeled with [35S]methionine and the other with [35S]methionine and bloting of identical amounts of radioactivity on Western blots followed by probing with an anti-IgG-Ab. In short, mouse macrophages were infected with *M. avium* for 4 h and 4 days, labeled with 1 μCi of [35S]methionine for 2 h, and chased for 1 h in the absence of label. Phagosomes were purified, and identical amounts of radioactivity were loaded per lane of a SDS-PAGE gel. Labeled control lanes were loaded with 10% cold proteinase K-digested mouse proteins as applied for each lane as determined by LAMP-1 staining. The LAMP-1 signal of phagosomes was found to be an indicator of total phagosomal protein content by radioactive labeling of phagosomes from different infection time points with [35S]methionine and blotting of identical amounts of radioactivity on Western blots followed by probing with an anti-IgG-Ab. In short, mouse macrophages were infected with *M. avium* for 4 h and 4 days, labeled with 1 μCi of [35S]methionine for 2 h, and chased for 1 h in the absence of label. Phagosomes were purified, and identical amounts of radioactivity were loaded per lane of a SDS-PAGE gel. Labeled control lanes were loaded with 10% cold proteinase K-digested mouse proteins as applied for each lane as determined by LAMP-1 staining. The LAMP-1 signal intensity derived from 4-day phagosomes was slightly less (<10%) than that of 4-h phagosomes as determined by densitometry.

**Immunoelectron microscopy**

Murine bone marrow-derived macrophages were activated with 20 μM of rIFN-γ for 16 h before infection to increase the level of MHC class II expression. Cells were infected for 4 h or 4 days with *M. avium* in the absence of rIFN-γ and fixed in 4% paraformaldehyde in Pipes buffer (200 mM Pipes (pH 7.0), 0.5 mM MgCl2) at 4°C. Fixed cells were embedded in gelatin and infiltrated with 2.3 M sucrose/20% polyvinyl pyrrolidone in Pipes buffer. The blocks were frozen and sectioned in an RMC MT7/CR21 cryoultramicrotome (Ventana Medical Systems, Tucson, AZ). KL 295 Ab (against MHC class II) was incubated with sections in block buffer (5% goat serum and 5% FCS in Pipes buffer). Parallel labeling of sections using an IgG control Ab as the primary Ab did not reveal any background labeling. The secondary Ab was a goat anti-mouse IgG conjugated to 18 nm gold (Amersham, Arlington Heights, IL). Immunolabeling was scored by counting the number of gold particles in 100 mycobacteria-containing phagosomes per time point. Variation from cell to cell appeared greater than variation between phagosomes inside a single cell; therefore, no more than three vacuoles were scored per individual cell.

**One-dimensional isoelectric focusing (1D-IEF) and Western blotting**

Mouse macrophage monolayers in T150 flasks (Costar) were placed at 37°C for 10 min and subsequently treated with 0.5 U/ml neuraminidase (type VI, EC 3.2.1.24, Calbiochem, Rockford, IL). Cells were washed and chased for an additional 2 h and then processed for phagosome purification. Control cells were first infected with *M. avium*, chased for 2 h, and then treated with neuraminidase at 4°C for 90 min. The purified phagosomes were taken up in 50 μL of 1% Nonidet P-40, resuspended, left on ice for 15 min, and centrifuged for 10 min at 2500 × g to pellet bacteria. The supernatant containing mycobacterial proteins was taken up in 100 μL of 8 M urea, 2% Nonidet P-40, 2% ampholines (pH 3.5–10), (Pharmacia Biotech, Uppsala, Sweden), and 0.05 mg/ml bromphenol blue. MHC class II heterodimer dissociation was enforced by adding 10 μL of 0.5 N HCl for 1 min followed by neutralization with 0.5 N NaOH. Proteins were loaded on a 1-D IEF gel containing 6 M urea, 2% octylglycoside, 7% acrylamide, 1.6% ampholines (pH 5–7), and 0.4% ampholines (pH 3.5–10). The stacker gel contained 6 M urea, 7% acrylamide, 2% Nonidet P-40, 1.6% ampholines (pH 5–7), and 0.4% ampholines (pH 3.5–10). Gels (20 cm × 16 cm) were run at 400 V for 18 h followed by 800 V for 2 h in 50 mM NaOH (cathode) and 0.2% H3PO4 (anode). Gels were soaked four times for 10 min in 50% MeOH, 1% SDS, and 5 mM Tris-Cl (pH 8.0) to remove the nonionic detergent (26) and transferred on Immobilon-P membranes (Millipore, Bedford, MA) by Western-blotting according to the manufacturer’s protocol.

Western blots from 1D-IEF gels and SDS-polyacrylamide gels were incubated overnight in blocking buffer containing the primary Ab, washed, probed with HRP-conjugated secondary Ab (Jackson ImmunoResearch, West Grove, PA), and developed by Luminol chemiluminescence (Pierce, Rockford, IL). For cellular (total) MHC class II and H2-M quantification, equal amounts of proteins from whole-cell lysates were applied.

**Confocal microscopy**

Macrophages were plated on glass coverslips in 24-well plates (Costar) at 1 × 10^5 cells per well. *M. avium* was labeled with 1 μg/ml Texas red succinimidyl ester (Molecular Probes) according to the manufacturer’s protocol. The labeling did not affect the viability of the bacteria. Cells were infected with labeled *M. avium* for 2 h or left uninfected, and all cells were subsequently activated with rIFN-γ at 100 U/ml. After a 20 h incubation in the presence of rIFN-γ, the cells were washed, fixed in 95% ethanol/formaldehyde (27), permeabilized with 1 mg/ml Zwittergent 3–12 (Calbiochem, La Jolla, CA), and incubated with Ab KL 295 (MHC class II) followed by incubation with FITC-conjugated goat anti-mouse secondary

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6074 ACQUISITION OF MHC CLASS II AND HLA-DM BY PHAGOSOMES CONTAINING MYCOBACTERIA
Results

MHC class II acquisition along the phagocytic pathway

Engulfment of a particle by a phagocytic cell leads to the formation of a new compartment, the phagosome, which undergoes complex maturation steps leading to the formation of a hydrolyase-rich, acidic compartment, the phagolysosome (28). Maturation occurs through fusion with endosomal and biosynthetic vesicles, causing profound changes in the protein profile of phagosomes during the first few hours postinternalization (29). Mycobacteria are able to interfere with the maturation process, causing the phagosome to “freeze” at a developmental stage reminiscent of early phagosomes containing inert particles such as IgG-coated beads (20, 22, 25, 30).

To analyze whether the block in maturation has any impact on the acquisition of molecules involved in Ag presentation, we first determined how MHC class II molecules, HLA-DM, and proteases are delivered to phagosomes formed around inert particles that do not arrest phagosome biogenesis. A 4 h kinetic of phagosomes containing IgG-coated beads in human blood monocytes revealed a highly dynamic profile for MHC class II (HLA-DR) and HLA-DM (Fig. 1). Appreciable amounts of phagosomal HLA-DR at early time points were followed by a drop at 20 min, followed by an increase at 50 min postinternalization. Subsequently, phagosomal HLA-DR levels decreased again, followed by a strong increase at very late time points (170 min postinternalization). The kinetic suggested that MHC class II molecules entered nascent phagosomes from the plasma membrane, and at later time points from MIICs (50 min postinternalization). Fusion with MIICs was indicated by an increase in HLA-DM and lysosomal cathepsin D.

The impact of the arrested state of mycobacteria-containing phagosomes on MHC class II acquisition

Although mycobacteria arrest phagosome maturation, the compartment is not excluded from the host cell’s membrane-trafficking pathways, but remains highly dynamic, communicating with the host’s early endocytic, recycling, and biosynthetic pathways (21, 22, 25, 30, 34). MHC class II molecules enter the biosynthetic and endocytic system and therefore could have access to phagosomes containing mycobacteria. Indeed, early phagosomes, which harbored M. avium isolated at 4 h postinfection (p.i.) from human blood monocytes, contained MHC class II (HLA-DR), but the content of HLA-DR decreased sharply as the infection continued to 4 days (Fig. 2A). The decline in phagosomal HLA-DR content was accompanied by a much less pronounced decline in total cellular HLA-DR levels. Low amounts of the lysosomal form of cathepsin D (30 kDa) and the v-ATPase, a proton pump mediating the acidicification of vesicles, were detected in early (4 h p.i.) and acute (1 day p.i.) but not late (4 days p.i.) phagosomes. The acquisition of these late endocytic/lysosomal markers likely reflects the transition of a small fraction of phagosomes into phagolysosomes, possibly the ones harboring dead mycobacteria present in the inoculum.

These experiments were extended using murine bone marrow-derived macrophages to avoid the variability in MHC class II haplotypes and in expression levels of the various human donors. Murine macrophage-derived early and late phagosomes containing M. avium also differed substantially in the content of MHC class II molecules (Fig. 2B). The phagosomes contained the 46-kDa intermediate form, but not the 30-kDa lysosomal form, of cathepsin D, indicating that they did not develop into phagolysosomes. A small reduction in the cathepsin D content of late phagosomes was observed, which was not due to the lower amount of total phagosome proteins analyzed (see Material and Methods). The time-dependent decline of MHC class II in mycobacteria-containing phagosomes was also observed by immunoelectron microscopy of murine bone marrow-derived macrophages, supporting the kinetics derived from Western blot analysis of purified phagosomes (Fig. 2C).

The kinetics of MHC class II acquisition by M. avium-containing phagosomes was notably different from IgG-coated, beads-containing phagosomes in that the initial decline in MHC class II during the infection was not followed by a subsequent increase, pointing to a sequestration of mycobacteria-harboring phagosomes from intracellular pools of MHC class II molecules.

MHC class II enters M. avium-containing phagosomes from the plasma membrane

MHC class II heterodimers are stable when complexed to peptides. The CLIP region of the invariant chain offers this stabilizing effect during transport of MHC class II molecules through the biosynthetic pathway to MIIC compartments, where peptides with higher affinity than CLIP are selected for binding, and stable complexes are shuttled to the surface (1, 9, 10). Consequently, the selection
for high affinity peptides in the MIIC makes surface MHC class II molecules less likely to exchange peptides than intracellular MHC class II molecules complexed to CLIP. To determine whether MHC class II molecules in early phagosomes containing \textit{M. avium} are surface-derived, we treated murine bone marrow-derived macrophages with neuraminidase at 4°C before infection. This ensures the desialylation of surface molecules and leaves intracellular pools unaffected (35). Removal of sialic acid from phagosomal MHC class II \(\beta\)-chains was indicated by a basic shift in the isoelectric points by 1D-IEF (Fig. 3, arrows). MHC class II \(\beta\)-chains from phagosomes of macrophages that were first infected with \textit{M. avium} and then treated with neuraminidase did not show the shift, demonstrating that the enzyme did not have access to intracellular pools of MHC class II molecules.

Complex formation of MHC class II heterodimers with a peptide of higher affinity than CLIP is indicated by the ability of the \(\alpha\beta\) strands to withstand dissociation in SDS at room temperature (36). Indeed, a majority of \(\alpha\beta\)-chains in early phagosomes containing \textit{M. avium} withstood SDS dissociation, supporting the view that the bulk of MHC class II molecules was taken up from the plasma membrane during phagocytosis (Fig. 4).

\textbf{Conversion of phagosomes to MIIC-like compartments by activation}

\(\text{rIFN-}\gamma\) strongly induces the expression of MHC class II molecules and other proteins involved in Ag presentation, like HLA-DM (37, 38). This could have an impact on the levels of MHC class II in phagosomes containing mycobacteria. Because mycobacteria are known to interfere with \(\text{rIFN-}\gamma\) induction of MHC class II expression (39–41), and because \textit{Mycobacterium tuberculosis} recently has been shown to alter MHC class II trafficking, leading to an accumulation of MHC class II molecules in the perinuclear region (42), we first determined whether an infection with \textit{M. avium} would render macrophages anergic to \(\text{rIFN-}\gamma\) activation or re-organize the distribution of MHC class II molecules. Both infected and noninfected cells strongly increased MHC class II expression after a 20-h treatment with \(\text{rIFN-}\gamma\), and no discernable effect on the distribution of MHC class II molecules in infected cells was observed by confocal microscopy (Fig. 5).
Activation led to a strong increase in phagosomal MHC class II content from acute (1 day) infections when compared with phagosomes of resting macrophages (Fig. 6). rIFN-γ was added to the macrophages after the phagosomes had formed to insure equal incorporation of surface MHC class II during phagocytosis. A moderate decrease in cellular MHC class II expression in infected macrophages compared with noninfected cells was detected by Western blotting. This was not a result of cell death as the Mannose-6-phosphate receptor, the v-ATPase, lysosomal cathepsin D, and the relatively high pH in phagosomes harboring mycobacteria are able to arrest maturation at an early time point during phagosome biogenesis, a process that has been linked to the acquisition of the cell cortex protein TACO (also known as coro-nin 1) (55). A consequence of this block in phagosome biogenesis is the avoidance of a late endosomal-like phagosomal environment also led to the appearance of H2-M, the mouse homologue of MHC class II decreased steadily either by recycling or degradation. This was not a result of cell death as indicated by the absence of late endocytic markers such as the mannose-6-phosphate receptor, the v-ATPase, lysosomal cathepsin D, and the relatively high pH in phagosomes harboring mycobacteria for 4 h in medium that lacked neuraminidase. Proteins of purified phagosomes were separated by 1D-IEF, blotted, and probed with an Ab against the β-chains of I-A^d and I-E^d. Control phagosomes were prepared from cells that were first infected and then treated with neuraminidase. Arrows point to desialylated phagosomal MHC class II β-chains, which run closer to the basic pole (top) of the gel. Equal amounts of phagosomes were loaded. A pseudophagosome preparation of noninfected cells did not show any background signal (not shown).

Peptide occupation of phagosomal MHC class II. Preactivated (50 U rIFN-γ/mL, 16 h) murine bone marrow-derived macrophages were infected with M. avium for 2 h, washed, and chased for 1 h in the absence of rIFN-γ. Activation was necessary to increase the amount of surface MHC class II. Purified phagosomes were taken up in SDS sample buffer and either boiled (b.) for 10 min or left at room temperature for 30 min (n.b.). Proteins were separated, blotted, and probed with an Ab against the MHC class II β-chains. The double bands reflect differences in the glycosylation of the β-chains of I-A^d and I-E^d. The control lane contained boiled material from a pseudophagosome purification of noninfected cells.

FIGURE 3. Early phagosomes contain surface-derived MHC class II. Murine bone marrow-derived macrophages were treated with neuraminidase at 4°C to desialylate surface MHC class II and were subsequently infected with M. avium for 4 h in medium that lacked neuraminidase. Proteins of purified phagosomes were separated by 1D-IEF, blotted, and probed with an Ab against the β-chains of I-A^d and I-E^d. Control phagosomes were prepared from cells that were first infected and then treated with neuraminidase. Arrows point to desialylated phagosomal MHC class II β-chains, which run closer to the basic pole (top) of the gel. Equal amounts of phagosomes were loaded. A pseudophagosome preparation of noninfected cells did not show any background signal (not shown).

FIGURE 4. Peptide occupation of phagosomal MHC class II. Preactivated (50 U rIFN-γ/mL, 16 h) murine bone marrow-derived macrophages were infected with M. avium for 2 h, washed, and chased for 1 h in the absence of rIFN-γ. Activation was necessary to increase the amount of surface MHC class II. Purified phagosomes were taken up in SDS sample buffer and either boiled (b.) for 10 min or left at room temperature for 30 min (n.b.). Proteins were separated, blotted, and probed with an Ab against the MHC class II β-chains. The double bands reflect differences in the glycosylation of the β-chains of I-A^d and I-E^d. The control lane contained boiled material from a pseudophagosome purification of noninfected cells.

Discussion

CD4^+ T cell recognition of mycobacterial peptide Ags in the context of MHC class II molecules and subsequent activation of macrophage effector mechanisms by rIFN-γ are essential for M. tuberculosis and M. avium containment and clearance in humans and mice (43–48). Mycobacteria have evolved strategies to compromise the activation of T cells by the induction of immunosuppressive cytokines such as IL-6, IL-10, and TGF-β (49–51), and by down-regulation of the surface expression of MHC class II, CD1, and costimulatory molecules (39–41, 52–54). We observed a moderate effect on cellular MHC class II and H2-M expression levels in M. avium-infected cells. Unlike Hmama et al. (42), who observed an accumulation of perinuclear MHC class II in M. tuberculosis-infected human macrophages, we did not detect any major changes in the distribution of MHC class II molecules by confocal microscopy of M. avium-infected murine macrophages. It is likely that the inhibitory effects are delicately balanced between the actual bacterial load and the strength of the activating signal and are also influenced by the type of host cell and mycobacterial strain, explaining the differences observed between laboratories.

Recently, Ramachandra et al. (16) have shown that phagosomes containing beads constitute functional Ag-loading compartments and acquire MHC class II and H2-M molecules. Because mycobacteria-containing phagosomes differ with respect to bead-harbor- ing phagosomes in their interaction with the host’s endocytic network, we asked whether or not this difference influences the delivery of the Ag-presentation “machinery” to phagosomes containing M. avium.

During phagocytosis, nascent M. avium-harbor- ing phagosomes acquired neu- ramimidase-sensitive MHC class II molecules from the plasma membrane, and an appreciable amount of heterodimers were complexed with peptide as indicated by SDS-stability. Our data do not clarify whether all phagosomal MHC class II were surface-derived. Indeed, SDS stability is not definite proof of surface location, and a substantial fraction of phagosomal MHC class II remained neuraminidase resistant (Fig. 3). This could point to an intracellular route of MHC class II delivery; alternatively, the neuraminidase-resistant forms could reflect incomplete desialylation of surface MHC class II during neuraminidase treatment at 4°C.

As the infection proceeded, the levels of phagosomal MHC class II decreased steadily either by recycling or degradation. This was different from phagosomes containing IgG-coated beads, which acquired MHC class II and HLA-DM intracellularly after much of the surface MHC class II had left the phagosomes. What is the cell-biological basis for this difference? Phagosomes containing mycobacteria are able to arrest maturation at an early time point during phagosome biogenesis, a process that has been linked to the acquisition of the cell cortex protein TACO (also known as coro-nin 1) (55). A consequence of this block in phagosome biogenesis is the avoidance of a late endosomal-like phagosomal environment, as indicated by the absence of late endocytic markers such as the mannose-6-phosphate receptor, the v-ATPase, lysosomal cathepsin D, and the relatively high pH in phagosomes harboring...
mycobacteria (20, 24, 56, 57). It was at a late stage during phagosome development when IgG-bead-containing phagosomes acquired MHC class II and HLA-DM, either by fusion with MIIC vesicles or by intersection with MHC class II or HLA-DM-containing transport vesicles on their route from the trans-Golgi to MIIC compartments. Consequently, the paucity of MHC class II in late phagosomes containing mycobacteria is a result of their ability to avoid these later stages during phagosome development and to either degrade or recycle plasma membrane-derived MHC class II that was taken up during phagocytosis.

Phagosomes containing IgG-coated beads developed a lysosome-like environment by 110 min postinternalization. This is indicated by the presence of high amounts of the lysosomal proteins LAMP-1 and cathepsin D and low amounts of MHC class II. The latter is consistent with the paucity of MHC class II molecules in dense lysosomes (32). The rebinding of phagosomal MHC class II after the decline at 110 min in human macrophages was not observed in phagosomes from J774 murine macrophages. The difference may be related to the great variability in MHC class II expression of human macrophages or to the previously observed alterations in postlysosomal trafficking of bead-containing phagosomes (33).

The sequestration of M. avium-containing phagosomes from MHC class II trafficking pathways was no longer maintained after activation, which led to an increase in phagosomal MHC class II and to the appearance of H2-M when compared with phagosomes of resting cells. This agrees with earlier reports detailing the detection of MHC class II in phagosomes containing M. tuberculosis of activated macrophages (20, 42). It remains to be determined which pathway leads to the delivery of MHC class II and H2-M to mycobacteria-containing phagosomes after IFN-γ treatment. Activation lifts the block in phagosome maturation (58), causing the enclosed bacterium to descend “down” the same pathway, like IgG-coated bead-containing phagosomes. Thus, fusion of phagosomes with MIICs or intersection with post-trans-Golgi carrier vesicles on their route to MIICs could have caused the increase in MHC class II and H2-M. Alternatively, other pathways of MHC class II delivery cannot be excluded, such as fusion with biosynthetic vesicles carrying MHC class II or H2-M.

The presence of surface MHC class II in phagosomes of resting macrophages that were partially complexed to high affinity (self) peptides, and the absence of H2-M, suggests that these phagosomes will not be efficient sites of peptide loading. Furthermore, mycobacteria-harboring phagosomes have a pH of 6.2–6.4 (24), which is about 1 U higher than the pH at which Ag loading is most efficient (7). Another impairment for Ag presentation is the lower hydrolytic content of live mycobacteria-containing phagosomes when compared with phagosomes harboring dead mycobacteria (25). Despite these less than optimal conditions for Ag presentation, Pancholi et al. (59) noticed a strong T cell response when resting human monocytes were infected with Mycobacterium bovis bacillus Calmette-Guérin for 2 days. This suggests that surface-derived MHC class II in mycobacteria-harboring phagosomes participates in Ag presentation in the absence of H2-M, likely via the alternate recycling pathway, which is independent of HLA-DM (60). Presentation of mycobacterial Ags through an alternate pathway at early time points is also indicated by the report that monocytes infected for 12 h with M. tuberculosis present Ags in the presence of brefeldin A (61). Brefeldin A blocks the transport of newly synthesized MHC class II through the Golgi complex and thereby blocks Ag presentation via the “classical” pathway. However, the maturation of a fraction of mycobacteria-containing phagosomes to acidic, hydrolase-rich phagolysosomes at early time points (Fig. 2A) could also explain such a result.

The paucity of MHC class II molecules in late phagosomes points to an impairment of long-term infected macrophages in presenting mycobacterial Ags. Indeed, in the study by Pancholi et al. (59), a strong decrease in the ability of chronically infected monocytes to stimulate mycobacteria-specific T cells was observed. The effect was specifically targeted to the mycobacteria-containing phagosome as soluble Ags were presented normally and MHC class II expression was unchanged. However, T cell activation by
long-term infected monocytes was not completely abolished, argu-
ging for a low transit of MHC class II molecules through myco-
bacteria-containing phagosomes. Because mycobacteria-contain-
ing phagosomes intersect with the transferrin recycling pathway
(22, 34) and acquire plasma membrane constituents (62), low lev-
els of surface MHC class II could enter the phagosomes via recy-
cling from the plasma membrane and participate in T cell activa-
tion at late stages during an infection.

Taken together, the ability of mycobacteria to arrest phagosome maturation in resting macrophages not only helps them to avoid
the harsh conditions of a phagolysosome, but also leads to the sequestration from intracellular pools of MHC class II and H2-M
molecules en route to the surface. Such immunological silencing could play a significant role in immune evasion and the persistence of infection.

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