

Class II MHC Molecules Are Present in Macrophage Lysosomes and Phagolysosomes That Function in the Phagocytic Processing of *Listeria Monocytogenes* for Presentation to T Cells

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Abstract. Phagocytic processing of heat-killed *Listeria monocytogenes* by peritoneal macrophages resulted in degradation of these bacteria in phagolysosomal compartments and processing of bacterial antigens for presentation to T cells by class II MHC molecules. Within 20 min of uptake by macrophages, *Listeria* peptide antigens were expressed on surface class II MHC molecules, capable of stimulating *Listeria*-specific T cells. Within this period, degradation of labeled bacteria to acid-soluble low molecular weight catabolites also commenced. Immunoelectron microscopy was used to evaluate the compartments involved in this processing. Upon uptake of the bacteria, phagosomes containing *Listeria* fused rapidly with both lysosomes and endosomes. Class II MHC molecules were present in a tubulo-vesicular lysosome compartment, which appeared to fuse with phagosomes, as well as in the resulting phagolysosomes

containing internalized *Listeria*; these compartments were all positive for Lamp 1 and cathepsin D and lacked 46-kD mannose-6-phosphate receptors. In addition, class II MHC and Lamp 1 were co-localized in vesicles of the trans Golgi reticulum, where they were segregated from 46-kD mannose-6-phosphate receptors. Vesicles containing both *Listeria*-derived components and class II MHC molecules were also observed; some of these may represent vesicles recycling from phagolysosomes, potentially bearing processed immunogenic peptides complexed with class II MHC. These results support a central role for lysosomes and phagolysosomes in the processing of bacterial antigens for presentation to T cells. Tubulo-vesicular lysosomes appear to represent an important convergence of endocytic, phagocytic and biosynthetic pathways, where antigens may be processed to allow binding to class II MHC molecules and recycling to the cell surface.

AFTER internalization by antigen presenting cells, exogenous antigens are processed within vesicular compartments by subtotal degradation to produce antigenic peptides that can bind to class II MHC molecules (MHC-II)¹ (Brodsky and Guagliardi, 1991; Harding and Unanue, 1990a). The resulting peptide-MHC-II complexes are then displayed on the plasma membrane to stimulate specific CD4 T cells, which mediate or control both cellular and humoral immunity. The intracellular mechanisms of antigen processing remain somewhat unclear, including the site where the peptide-MHC-II complexes are formed. MHC-II molecules are distributed largely on the plasma membrane, with a significant minority present within endocytic compartments (Harding et al., 1990; Peters et al., 1991; Guagliardi et al., 1990; Harding and Unanue, 1989; Pieters et al.,

1991). Dense lysosomes or lysosome-associated late endocytic compartments appear to play important roles in the processing of soluble antigens (Harding et al., 1991a, b; Collins et al., 1991; Peters et al., 1991). However, our understanding is still complicated by a persisting lack of definition of the trafficking pattern of late endosomal and lysosomal compartments, and their communication with vesicles associated with the *trans*-Golgi reticulum (TGR).

Whereas a number of previous studies have dealt with the processing of soluble antigens internalized by endocytosis, there is little information about the roles of phagocytic compartments during processing of microbial antigens for presentation to T cells. The immune response to *Listeria monocytogenes*, a human pathogen (Farber and Peterkin, 1991), has been extensively studied in murine systems. Previous reports have described the phagocytic processing of *Listeria monocytogenes* for presentation to T cells (Ziegler and Unanue, 1979; Ziegler and Unanue, 1981; Allen et al., 1984; Kaufmann et al., 1987; Brunt et al., 1990; Saffley et al., 1991). Viable *Listeria monocytogenes* also express listeriolysin O, which mediates penetration of the organism

1. *Abbreviations used in this paper:* CF-HKLM, cationized ferritin-HKLM; HKLM, heat-killed listeria monocytogenes; LA, *Listeria* antigen; MHC-I and -II, class I and II major histocompatibility complex molecule; MPR, mannose-6-phosphate receptor; TGR, *trans*-Golgi reticulum; IL-2, interleukin-2.

through phagosomal membranes into the cytosol (Gaillard et al., 1987; Portnoy et al., 1988; Tilney and Portnoy, 1989). Hence, these bacteria are processed by the class I MHC processing pathway (Brunt et al., 1990; De Libero and Kaufmann, 1986; Brown et al., 1992; Pamer et al., 1991), which processes cytosolic antigens for presentation to CD8 T cells by class I MHC (MHC-I) molecules. However, heat killed *Listeria monocytogenes* (HKLM) or mutants deficient in the listeriolysin remain within the phagocytic vacuolar system, are primarily processed for presentation by MHC-II, are not pathogenic, and do not evoke protective immunity for pathogenic strains (Wirsing von Koenig and Finger, 1982; Gaillard et al., 1986; Berche et al., 1987; Kathariou et al., 1987; Portnoy et al., 1988; Brunt et al., 1990). We have used HKLM as a model to study the roles of phagosomal and phagolysosomal compartments involved in the processing of bacterial antigens for MHC-II-mediated presentation to T lymphocytes.

Materials and Methods

Cells

Peritoneal macrophages were elicited from CBA/J mice (The Jackson Laboratory, Bar Harbor, ME) by sequential i.p. inoculation with viable *Listeria monocytogenes* and peptone. In brief, 2×10^4 viable *Listeria* were injected i.p. into female, retired breeder CBA/J mice. After 7–14 d these mice received an additional i.p. injection of 1 ml of 10% peptone (Difco Laboratories, Detroit, MI). Peritoneal macrophages were harvested 3–5 d later. In some cases, macrophages were harvested 7–14 d after *Listeria* injection without peptone injection. The macrophages were adhered to plastic plates for 2 h and washed to remove non-adherent cells. This protocol produces activated macrophages with induced MHC-II expression. When macrophages were cultured overnight (below), MHC-II expression was maintained by the addition of recombinant murine interferon- γ (30 ng/ml, generous gift of Dr. Robert Schreiber, Washington University, St. Louis, MO) and 10^{-6} M indomethacin (Sigma Chemical Co., St. Louis, MO).

The oligoclonal *Listeria*-specific T lymphocyte line, CD4A, was a generous gift from Dr. L. M. Brunt (Washington University); it was prepared from *Listeria*-immunized CBA/J mice as described (Brunt et al., 1990). In brief, CBA/J mice were injected i.p. with 10^5 -viable *Listeria* monocytogenes. 6 d later, splenocytes were prepared and negatively selected by panning with the anti-CD8 mAb 6.72 and rabbit anti-mouse immunoglobulin coated plates to remove B cells and CD8 T cells. The remaining cells were cultured in the presence of irradiated (3,000 rad) splenocytes (10^7 /ml) plus HKLM (10^7 /ml), with biweekly passage. The resulting CD4A cells are uniformly Thy-1 and CD4 positive (by flow cytometry of cells stained with GK1.5 anti-CD4 and AT83A anti-Thy-1) and CD8 negative (using 6.72 anti-CD8). They are MHC-II (I-E^k and I-A^k) restricted (their response is inhibited by 10.3.6.2 anti-I-A^k and 14.4.4 anti-I-E^k). They respond to unidentified epitope(s) present in viable *Listeria* monocytogenes and HKLM by secreting interleukin-2 (IL-2) and interferon- γ . CD4A secrete these cytokines in response to *Listeria* antigen presented by viable macrophages or macrophages fixed with paraformaldehyde after exposure to *Listeria* (although they proliferate only in response to presentation by viable cells, consistent with the co-stimulator requirement of primary T cell clones). Heat killed and viable stocks of *Listeria* monocytogenes were prepared as described from a virulent strain expressing listeriolysin O (serotype 1/4) (Brunt et al., 1990). Unless specified, all incubations were performed in standard medium: DME (Gibco Laboratories, Grant Island, NY) with 10% FCS (Hyclone, Logan, UT), 5×10^{-5} M 2-mercaptoethanol, antibiotics, and the following supplements: L-arginine HCl (116 mg/l), L-asparagine (36 mg/l), NaHCO₃ (2 g/l), and sodium pyruvate (1 mM).

Antigen Catabolism

HKLM were iodinated using the chloramine T method (Allen et al., 1984); a specific activity of ~ 10 cpm per bacterium was achieved. Catabolism of bacteria was studied as previously described for soluble antigen (Harding

and Unanue, 1990b). In brief, 1.25×10^6 ¹²⁵I-HKLM were added at 4°C to 2×10^6 adherent macrophages in 24-well plates, and the plates were centrifuged at 1,300 g for 5 min. They were next warmed in a water bath at 37°C for 5 min to allow phagocytosis of bacteria, and the macrophages were then washed. After further incubation at 37°C for varying periods of time, the medium was removed and the cells were solubilized in 1% Triton X-100. High molecular weight proteins were immediately precipitated from the medium or solubilized cells in the presence of 10% TCA on ice with 5% bovine serum as carrier.

Antigen Presentation to T Cells

Antigen presentation assays were performed as previously described (Harding et al., 1991b) with the following modifications. HKLM were added to adherent macrophages (2×10^5 /well in 96-well plates), and the plates were centrifuged as above for 5 min. The cells were next incubated at 37°C for various periods and then fixed in 1% paraformaldehyde in PBS, followed by thorough washing. CD4A cells were added (10^5 /well) and incubated with the fixed macrophages for 24 h. Resulting IL-2 levels in the medium served as a measure of presentation of processed *Listeria* antigen and resulting CD4A activation; IL-2 levels were assessed by proliferation and [³H]thymidine incorporation by IL-2-dependent CTLL cells.

Electron Microscopy

To functionally label lysosomes, adherent peritoneal macrophages were incubated with 5- or 10-nm diam colloidal gold conjugated with BSA (BSA-gold) for 2–4 h, washed thoroughly, and incubated for 12–14 h in the presence of interferon- γ and indomethacin (above). They were then exposed to HKLM for various periods under the same conditions used for antigen presentation studies and finally fixed in 1% acrolein, 2% paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.4, for 2–4 h at 4°C. The cells were then scraped loose and transferred to 2% paraformaldehyde in the same buffer pending shipping and further processing. Some studies used HKLM that were derivitized with cationized ferritin (CF-HKLM). These were prepared by incubating HKLM with 1.5 mg/ml cationized ferritin (Sigma Chemical Co.) in saline for 20 min at room temperature. The CF-HKLM were then pelleted by centrifugation for 5 min at 1,300 g and washed thoroughly in PBS.

For immuno-electron microscopy, fixed cells were washed three times in 0.1 M sodium phosphate buffer, pH 7.4. They were then placed in 10% gelatin in phosphate buffer, which was solidified on ice. Subsequent blocks were immersed in 2.3 M sucrose in phosphate buffer for 2 h at 4°C, and ultrathin cryosections were single, double, or triple immuno-labeled as described (Slot et al., 1991) with 5, 10, and 15 nm protein A-conjugated colloidal gold probes (Slot and Geuze, 1985). In the multiple labeling procedure we modified the original double labeling method (Geuze et al., 1981) by inserting 1% glutaraldehyde stabilization steps between the first gold labeling and the second antibody treatment, and between the second gold labeling and the third antibody step. This prevented any interference between the different antibody gold complexes on the sections (Slot et al., 1991). The antibodies used were rabbit anti-human cathepsin D (Geuze et al., 1988), affinity purified rabbit anti-human 46-kD mannose-6-phosphate receptor (MPR) tail, MSCI (kind gift of Dr. K. Von Figura, Göttingen, Germany; Schulze-Garg, A., and A. Hille, manuscript in preparation), rabbit anti-mouse Lamp 1 (kind gift of Dr. L. Arterburn, Johns Hopkins University, School of Medicine, Baltimore; Chen et al., 1985), rabbit anti-*Listeria* Antigen (Difco Laboratories), and protein A-affinity-purified mouse monoclonal 10.2.16 (American Type Culture Collection, Rockville, MD) against the murine I-A^k MHC-II molecule.

MHC-II distribution was quantitated in macrophages that were stimulated with interferon- γ for 36 h without exposure to HKLM or BSA-gold. Ultrathin cryosections of these cells were double immuno-labeled with 15- and 5-nm protein A/gold particles for the demonstration of MHC-II and Lamp 1, respectively. From two blocks with cells, 20 cell profiles were randomly selected at low magnification for gold counting. A total of 5,042 MHC-II immuno-gold particles were counted and assigned to compartments as defined below. As controls, sections from the same blocks were labeled with anti-rat pancreatic amylase (Geuze et al., 1979) instead of anti-MHC-II, and gold particles were counted over 20 random cell profiles encompassing the same cell surface area. Background counts for amylase were subtracted from the MHC-II counts to reveal specific labeling over each compartment listed in Table II. The overall background was 6%; the nucleus, mitochondria, ER, and Golgi showed no labeling above background levels. The counting was done at 11,000 \times magnification directly from the screen in the microscope.

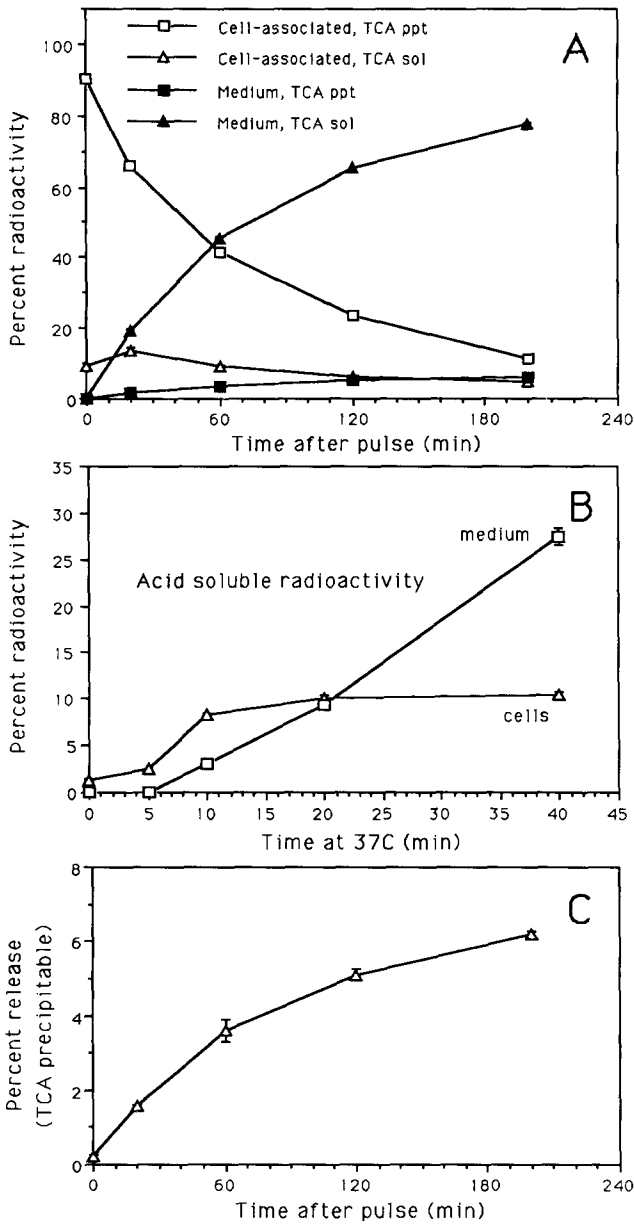


Figure 1. Catabolism of ^{125}I -HKLM by macrophages. (A) Labeled HKLM were added to adherent macrophages, centrifuged for 5 min (see Materials and Methods), and incubated at 37°C for 10 min to allow phagocytosis. The macrophages were then washed and incubated for the indicated periods (after the 10-min pulse) at 37°C . The medium was removed and the cells were solubilized in 1% Triton X-100. TCA precipitation was used to separate intact bacterial proteins (*TCA ppt*) and low molecular weight catabolites (*TCA sol*) from the cells and medium. (B) A similar experiment with emphasis on early time points. HKLM were pelleted on macrophages for 5 min at 4°C , and the cells were then warmed to 37°C for 5 min before washing. The indicated periods include time at 37°C both before and after the washing. (C) Data from the experiment shown in A indicating the amount of high molecular weight radioactive protein (precipitated by TCA) that was released into the medium.

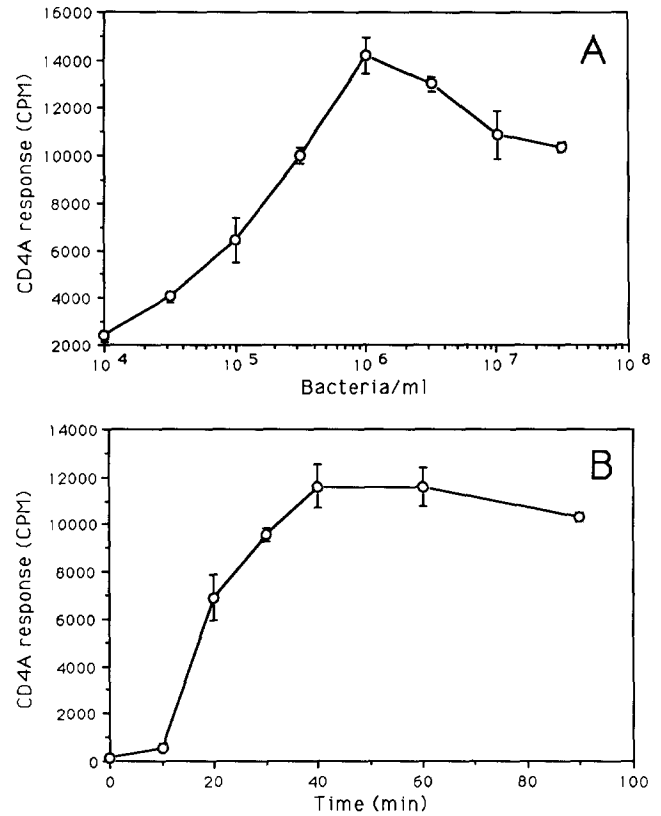
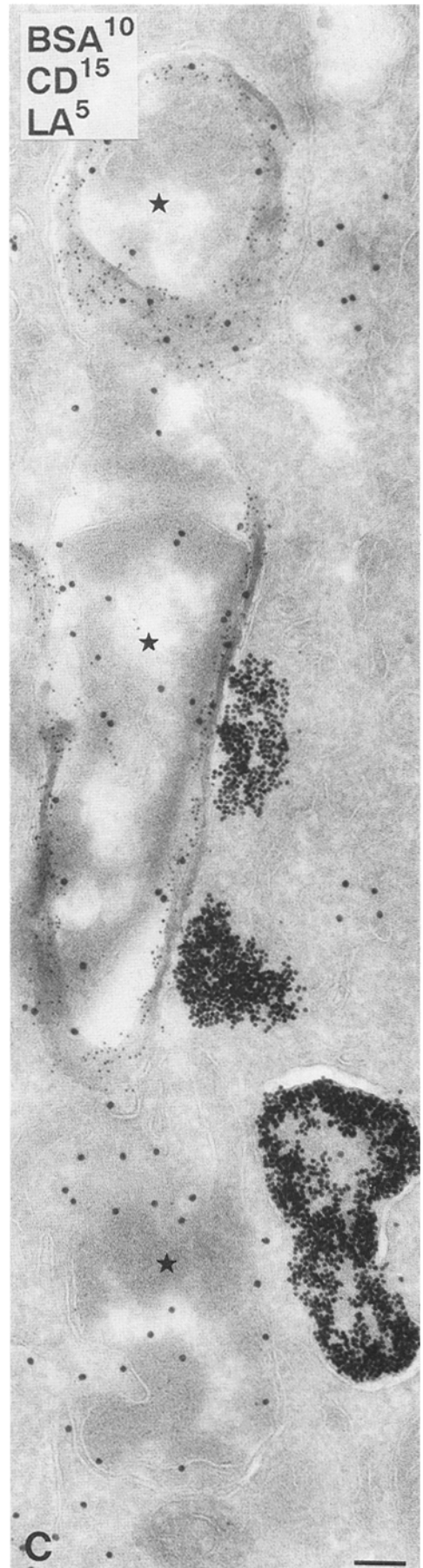
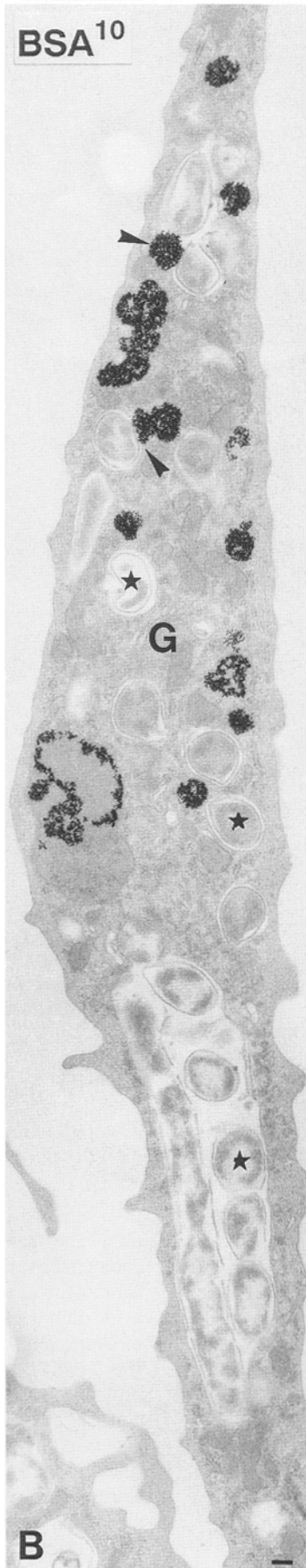
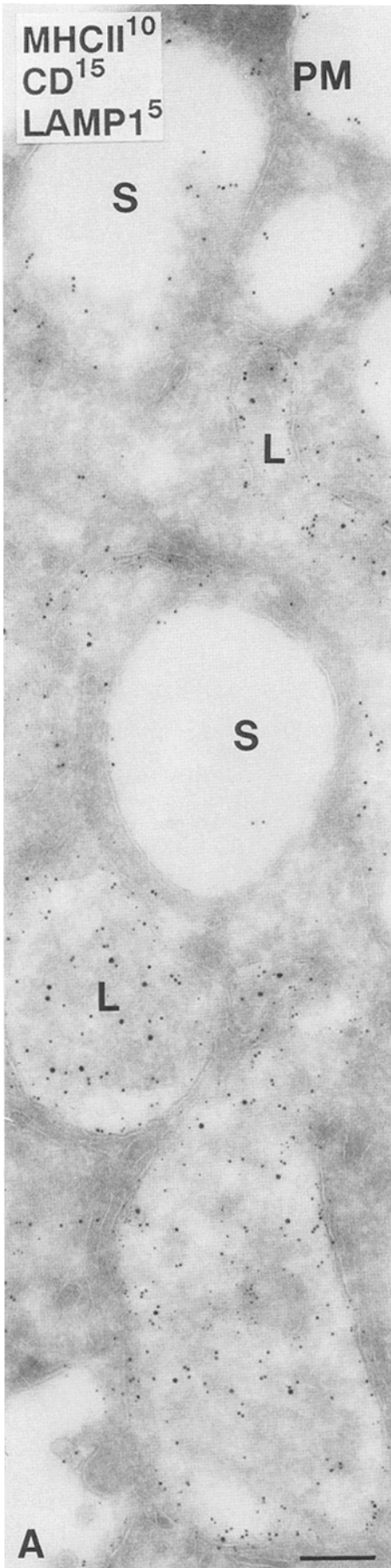


Figure 2. Presentation of HKLM to Listeria specific CD4 T lymphocytes. HKLM were pelleted on adherent macrophages and incubated at 37°C to allow processing of HKLM antigens. The macrophages were then washed and fixed with paraformaldehyde. CD4 T cells were incubated with the fixed macrophages and their response (IL-2 secretion) to presentation of HKLM peptides bound to MHC-II molecules was measured by IL-2 dependent ^3H thymidine incorporation by CTLL cells. The plateau response represents the plateau of the CTLL bioassay for IL-2 production, not the actual maximal presentation or T cell response. (A) HKLM dose response with incubation at 37°C for 90 min. The indicated concentrations were present in 0.1-ml incubation volume. (B) Kinetics of HKLM processing at 10^8 HKLM/ml in 0.1 ml.

Results

Kinetics of Listeria Catabolism and Processing for Presentation to T Lymphocytes

^{125}I -HKLM were added to peritoneal macrophages, centrifuged, and incubated at 37°C to allow their internalization. The macrophages were then washed and further incubated for various periods to evaluate the kinetics of HKLM catabolism after phagocytosis, assessed by the production of acid soluble low molecular weight catabolites in the cells or medium. HKLM catabolism commenced within 10 min and was largely complete by 200 min (Fig. 1). This indicates that proteolytic enzymes were rapidly delivered to phagosomes, consistent with fusion of phagosomes with lysosomes to generate phagolysosomes within 10 min of uptake (below). In addition, a small amount of high molecular weight radioactivity was released by the cells into the medium with kinetics that were similar to intracellular catabolism (Fig. 1c), suggesting the existence of recycling pathways by which high molecular weight catabolites could be released from phago-



lysosomal compartments (although the release of some surface-adherent bacteria could have also contributed).

We also investigated the kinetics of HKLM processing to generate peptide-MHC-II complexes for presentation to T lymphocytes. Macrophages were exposed to HKLM, washed, and then fixed with paraformaldehyde. CD4A cells, an oligoclonal HKLM-specific T lymphocyte line, were then used to determine the level of *Listeria* antigen presentation by MHC-II. These cells responded to macrophages (2×10^3 /well, 0.1 ml/well) exposed to as few as 3,000 bacteria/well (Fig. 2 *a*). HKLM peptide-MHC-II complexes were expressed on the plasma membrane of macrophages within 20 min of incubation with HKLM (Fig. 2 *b*). HKLM processing required viable macrophages; presentation to T cells did not occur when macrophages were fixed before their exposure to HKLM.

Endocytic, Phagocytic, and Lysosomal Structures of Macrophages Processing HKLM

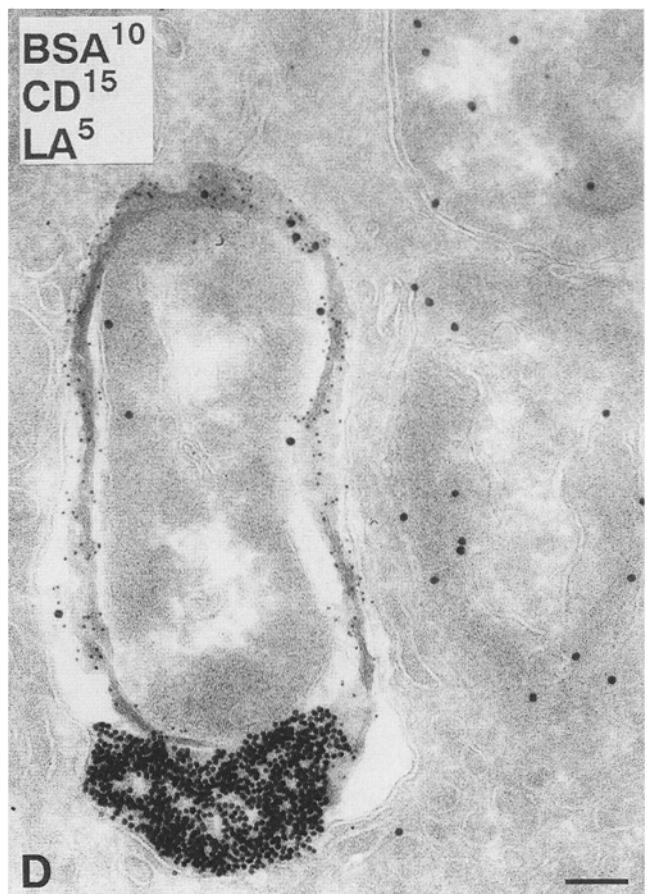
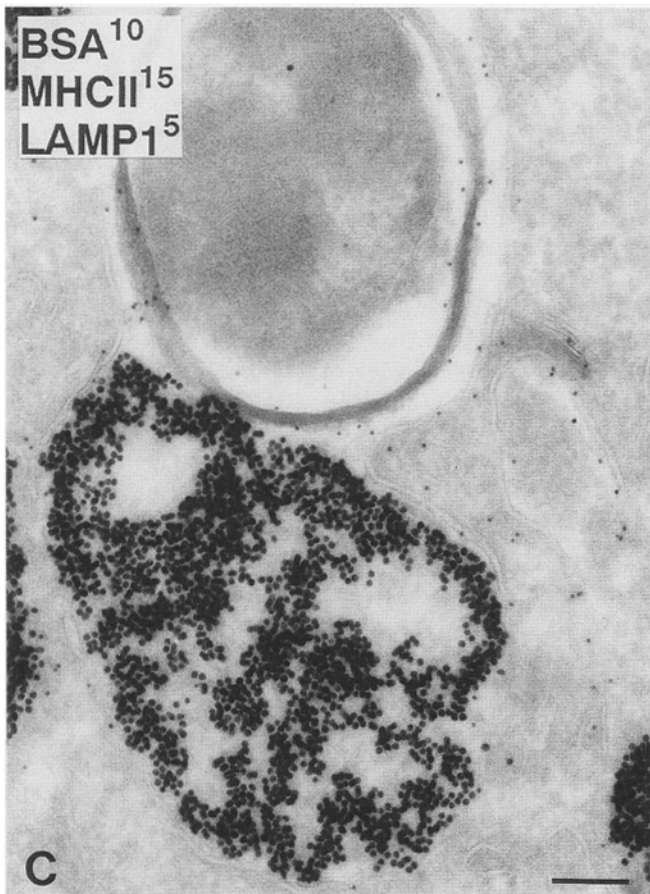
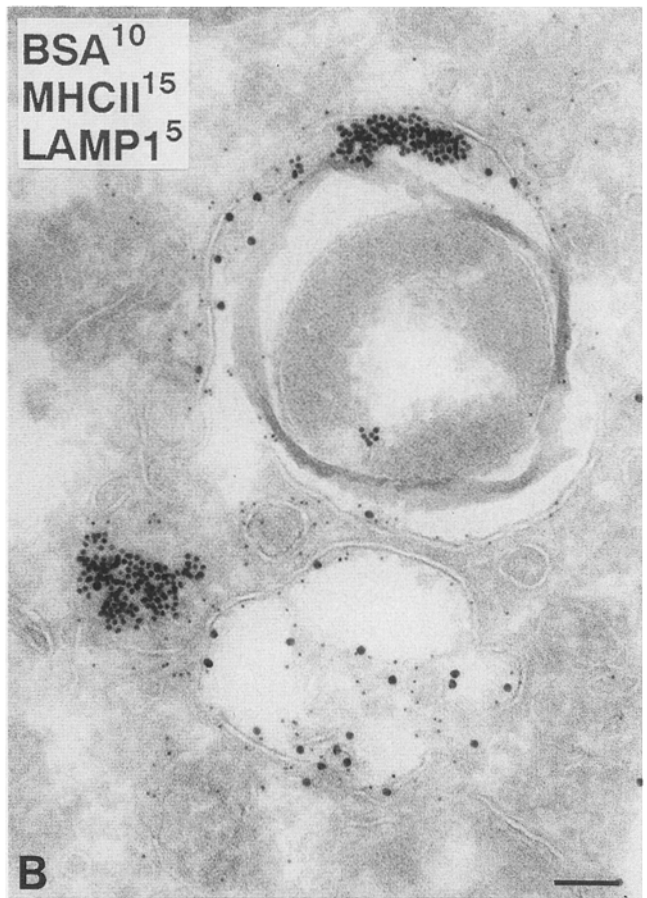
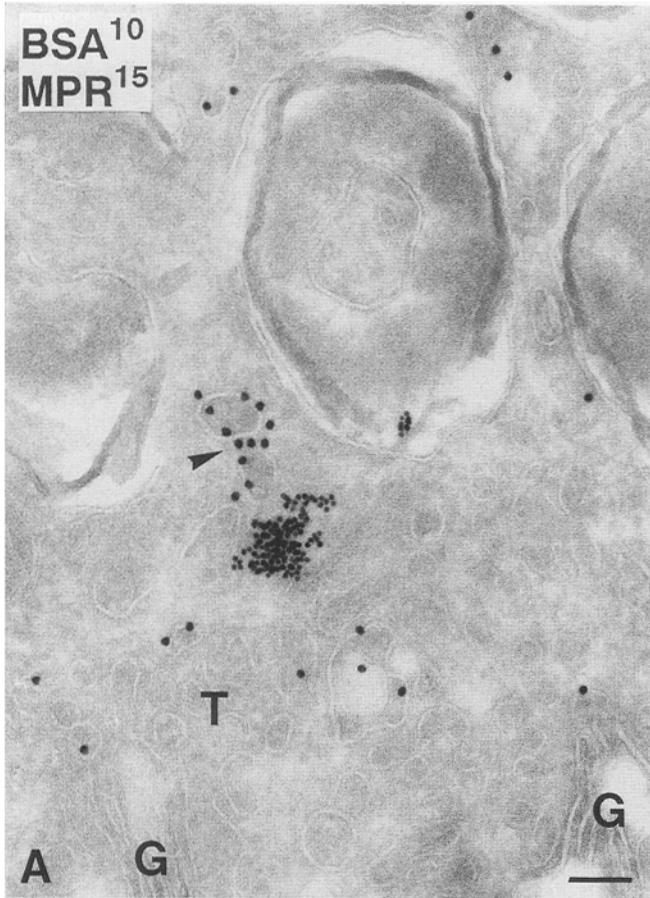
We have previously described the presence of characteristic sac-like vacuoles in the periphery of peritoneal macrophages and have postulated that these sacs play a role in phagocytosis and endocytosis. These sacs may be equivalent to the "macropinosomes" described by others (Brunk et al., 1976; Racoosin and Swanson, 1989). The membranes of the sacs contain MHC-II, similar in density to the plasma membrane, and share with the latter the presence of coated pits (Harding et al., 1990). Close to the sacs we observed an elaborate system of tubules, cisternae, and vacuoles containing floccular material (Fig. 3 *a*). This appeared to be a tubulovesicular lysosomal compartment for the following reasons. Immunogold labeling revealed that this compartment contained the endosomal/lysosomal markers cathepsin D and Lamp 1 (Fig. 3 *a*). In studies of the endocytic pathway the presence or absence of MPR is often used to discriminate between endosomes and lysosomes, respectively (Geuze et al., 1984, 1985, 1988; Brown et al., 1986; Griffiths et al., 1988, 1990; Sahagian and Neufeld, 1983; Von Figura et al., 1984). With four different anti-215-kD MPR antibodies we were unable to demonstrate any significant labeling in these macrophages. In contrast, the 46-kD MPR was abundant and was found in subdomains of the TGR and in other dense vesicles and tubules nearby endocytic and phagocytic vacuoles (Figs. 4 *a*, and 5 *a*). However, 46-kD MPR labeling occurred only rarely in the membranes of the tubulo-vesicular lysosomes themselves. Thus, the absence of MPR and the presence of cathepsin D and Lamp 1 led us to consider this system lysosomal. It is probably equivalent to previously described tubular lysosomes (Swanson et al., 1987; Heuser,

1989; Phaire-Washington et al., 1980; and Robinson et al., 1986), or tubulo-reticular compartments described by others in macrophages (Essner and Haimes, 1977; Rabinowitz et al., 1992). Typical examples of these lysosomes are shown in Figs. 3 *a* and 5 *c*. In addition to the tubulo-vesicular lysosomes, macrophages contained other, more dense, vacuolar lysosomes. These appeared to be very late in the endocytic route, since they were heavily labeled by BSA-gold tracer after an overnight chase (Fig. 3 *b*). The tubulo-vesicular lysosomes retained large amounts of BSA-gold after 1–2 h of chase, but with overnight chase they retained little of the tracer.

To study the phagocytic pathway, macrophages were allowed to phagocytose HKLM for 10, 20, or 40 min, and the appearance of the bacteria in intracellular compartments was evaluated. Phagocytosis of HKLM resulted in a loss of empty sacs and the appearance of numerous phagocytic vacuoles containing single or multiple bacteria (Fig. 3 *b*). This suggests that the sac membranes directly or indirectly contributed to the formation of phagosomes. After 10 min of uptake, a majority of internalized bacteria were present in phagosomes that were negative for cathepsin D and Lamp 1, but about 30% had already fused with tubular lysosomes, i.e., were in phagolysosomes, positive for cathepsin D and Lamp 1 (Table I). Some phagolysosomes contained the tracer BSA-gold that was previously targeted to lysosomes, also indicating that fusions between lysosomal and phagocytic compartments had occurred (see fusion events in Figs. 3, *b* and *c*; 4, *b* and *c*). After BSA-gold exposure with shorter chase periods, some phagolysosomes contained both BSA-gold and small vesicles typical of multivesicular endosomes (Fig. 4 *b*). Thus, within 10 min phagosomes appeared to fuse extensively with both endosomal and lysosomal compartments.

To quantitate the appearance of HKLM in phagosomes and phagolysosomes during the first 20–60 min of phagocytosis, which is a sufficient period to initiate antigen presentation (Fig. 2 *b*), HKLM profiles were counted in sections that were double-labeled for cathepsin D and Lamp 1 to identify phagosomes and phagolysosomes. We defined type I phagolysosomes as Lamp 1 positive and cathepsin D positive, but devoid of BSA-gold that was internalized with an overnight chase. These appeared to result from the fusion of phagosomes with the tubulo-vesicular lysosomal compartment (see Discussion). Type II phagolysosomes were defined as having similar immunolabeling but also containing lysosomal BSA-gold tracer. After 10 min, HKLM appeared largely in phagosomes (devoid of Lamp 1 and cathepsin D) or type I phagolysosomes, with increasing presence in type II phagolysosomes at later time points (Table I).

Figure 3. HKLM and MHC-II in phagocytic and lysosomal compartments of macrophages. (*A*) Control cell (no BSA-gold or HKLM exposure) showing sacs (*S*) just beneath the plasma membrane (*PM*), both with MHC-II labeling. Adjacent to the sacs are tubulo-vesicular lysosomes (*L*) containing floccular material and label for MHC-II, cathepsin D, and Lamp 1. Note that the MHC-II labeling in the contents of the lysosomes is not always membrane associated. In the lower left is part of a multivesicular endosome containing MHC-II. (*B*) Macrophages were exposed to BSA-gold for 100 min and then chased for 8 h before uptake of HKLM for 40 min. This is a survey micrograph showing numerous phagocytosed HKLM particles (*asterisks*). Two phagosomes have fused with lysosomes containing BSA-gold to form phagolysosomes (*arrowheads*). *G*, Golgi complex. (*C*) Cells were treated as in *B* except that HKLM uptake was for 10 min only. A tubular phagolysosome contains 3 HKLM profiles (*asterisks*), BSA gold, and cathepsin D (*CD*). *Listeria* antigen (*LA*) is present in the bacterial capsule. The cathepsin D is present throughout the bacteria. The lower HKLM profile does not show a capsule. A few LA gold particles can be seen in nearby cytoplasmic vesicles. Bars, 0.2 μ m.



Localization of MHC-II Molecules in Macrophages

MHC-II expression was found on the plasma membrane (Fig. 3 *a*), in the membranes of the sacs, lysosomes (Fig. 3 *a*) and phagolysosomes (Figs. 4 *b*, and 6), and in the TGR (Fig. 5). MHC-II was very abundant in phagolysosomes and occurred at their limiting membrane and in internal membrane sheets (Fig. 5 *c*). The Golgi stacks were not significantly labeled, but MHC-II was detected in TGR tubules and in vacuoles that appeared to be formed from the TGR tubules (Fig. 5 *b*). In both the TGR tubules and forming vacuoles MHC-II was co-localized with Lamp 1 (Fig. 5 *b*) and segregated from MPR (Fig. 5 *a*). The forming vacuoles at the TGR did not contain detectable MPR (not shown). Table II shows the distribution of MHC-II in interferon- γ stimulated macrophages (without exposure to BSA-gold or HKLM). Almost 60% of MHC-II occurred at the cell surface, which is similar to macrophages without in vitro stimulation described by us previously (Harding et al., 1990). Tubulo-vesicular lysosomes contained 13% and dense lysosomes 4% of the MHC-II labeling. Thus, a significant proportion of MHC-II molecules were contained in lysosomes.

The presentation of bacterial antigens to T lymphocytes must include the recycling of immunogenic peptides from phagolysosomal compartments. To detect the vesicles by which MHC-II and *Listeria* antigen might escape the phagolysosomes to be presented at the cell surface, we labeled sections with anti-*Listeria* antigen (LA) antibodies. Only low levels of LA labeling could be found outside the phagolysosomes (Figs. 3 *c*, and 4 *d*), probably due to low labeling efficiency. We therefore let macrophages phagocytose HKLM that was derivatized with cationized ferritin (CF-HKLM) to more easily trace recycling pathways. Fig. 6 shows that vesicles and tubules derived from phagolysosomes contained both MHC-II and free ferritin particles derived from CF-HKLM, suggesting a role for these structures in transporting MHC-II together with HKLM-derived components.

Discussion

We have characterized the kinetics of HKLM catabolism and processing by activated peritoneal macrophages and correlated these functional data with an ultrastructural analysis of the subcellular compartments involved in antigen processing. This is the first report to characterize phagocytic compartments involved in bacterial processing with regard to both targeting kinetics and the distribution of MHC-II and

other key marker proteins by immuno-electron microscopy. HKLM catabolism and presentation of HKLM antigens to T cells commenced within 20 min of uptake by macrophages. EM studies revealed that within this period HKLM were internalized into phagosomes and these rapidly fused with lysosomes to form phagolysosomes, consistent with other observations (Pfeifer et al., 1992; Pryzwanski et al., 1979; Kielian and Cohn, 1981; Jaconi et al., 1990). Extensive fusion with lysosomes was evident by 10 min.

Two related populations of macrophage lysosomes were observed. One was the tubulo-vesicular lysosome compartment, which was distributed throughout the macrophage cytoplasm and was typically characterized by its extended cisternal and saccular appearance. It contained floccular material, membrane sheets, and vesicles with extensive labeling for cathepsin D, Lamp 1, and MHC-II; it was generally without MPR staining. A second lysosome compartment was characterized by dense vacuolar lysosomes which contained the majority of BSA-gold after an overnight chase. These structures were positive for cathepsin D and Lamp 1, had lower levels of MHC-II, and were devoid of MPR. At early times after the uptake of bacteria, phagosomes were seen to fuse frequently with the tubulo-vesicular lysosomes, while extensive fusion with the vacuolar lysosomes was apparent with longer incubations. The kinetics of fusion with phagosomes and the BSA-gold targeting data both suggest that the tubulo-vesicular lysosomes might also be termed "early lysosomes," while the vacuolar examples might be "late lysosomes." However, these patterns may not reflect an absolute sequence, since late lysosomes were occasionally observed to fuse with phago(lyso)somes at early time points. The late lysosomes may simply be less fusogenic, leading to a lower frequency of fusion events, while retaining capacity to fuse with the same spectrum of compartments as the early lysosomes.

Our definition of these compartments as lysosomal rests upon their expression of typical lysosomal markers (cathepsin D and Lamp 1), deficiency in 46-kD MPR, and late position in the endocytic pathway for BSA-gold (see below; Rabinowitz et al., 1992). In addition, subcellular fractionation of these macrophages has revealed that the tubulo-vesicular lysosomes are isolated in the high density fractions from 27% Percoll gradients, characteristic for lysosomes (Harding C., and H. Geuze, manuscript submitted for publication). The tubulo-vesicular lysosomes appear to represent the tubular lysosomes previously described by several laboratories (Swanson et al., 1987; Knapp and Swanson, 1990; Heuser, 1989; Phaire-Washington et al., 1980; Robin-

Figure 4. Fusion of HKLM phagosomes with lysosomes and endosomes containing BSA-gold. (A) Macrophages were exposed simultaneously to HKLM and BSA-gold for 10 min. The BSA-gold is present in an endosomal structure and in the phago(lyso)some. The 46-kD MPR is present in the TGR (T) and in dense vesicles (arrowhead) adjacent to the phagocytic structures. G, Golgi stacks. (B) Macrophages were incubated with BSA-gold for 20 min followed by a 20 min chase. They were then exposed to HKLM for 10 min. Two phagolysosomes can be seen; the upper one has probably fused with a multivesicular endosome because of the presence of small vesicles and BSA-gold. The lower one is cut tangentially. Both show label for Lamp 1 and MHC-II. Note the Lamp 1 positive tubules and vesicles surrounding the phagolysosomes. (C) Macrophages were pulsed with BSA-gold for 100 min and chased for 8 h before uptake of HKLM for 10 min. A phagolysosome with a bacterium in it is fusing with a large lysosome containing BSA-gold. These structures and the surrounding tubules contain Lamp 1. (D) Macrophages were sequentially exposed to BSA-gold and HKLM exactly as in C, except HKLM uptake was for 40 min. The phagolysosome at the left contains a HKLM profile with a capsule that labels for *Listeria* antigen (LA). The long phagolysosome at the right shows HKLM without capsule. Cathepsin D (CD) is present throughout the HKLM content. Bars: (A, C, and D) 0.2 μ m; (B) 0.1 μ m.

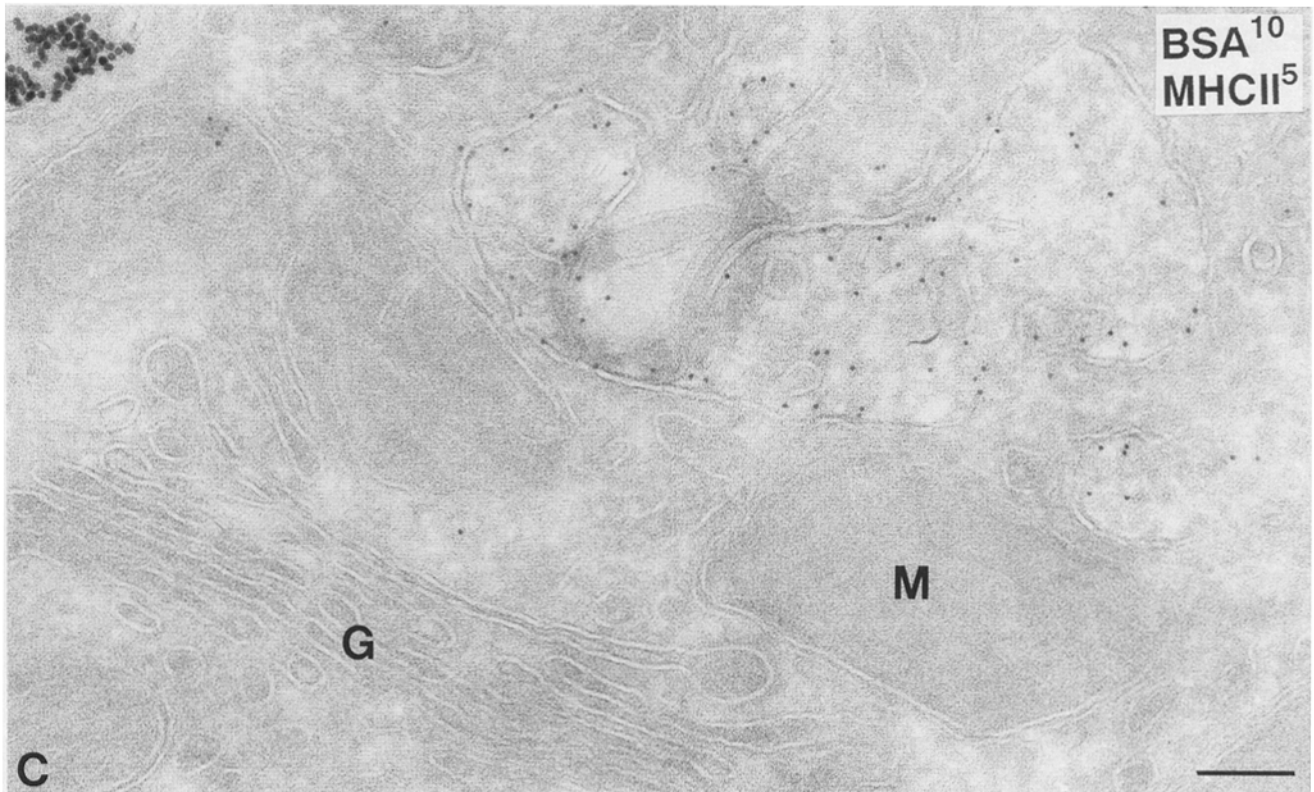
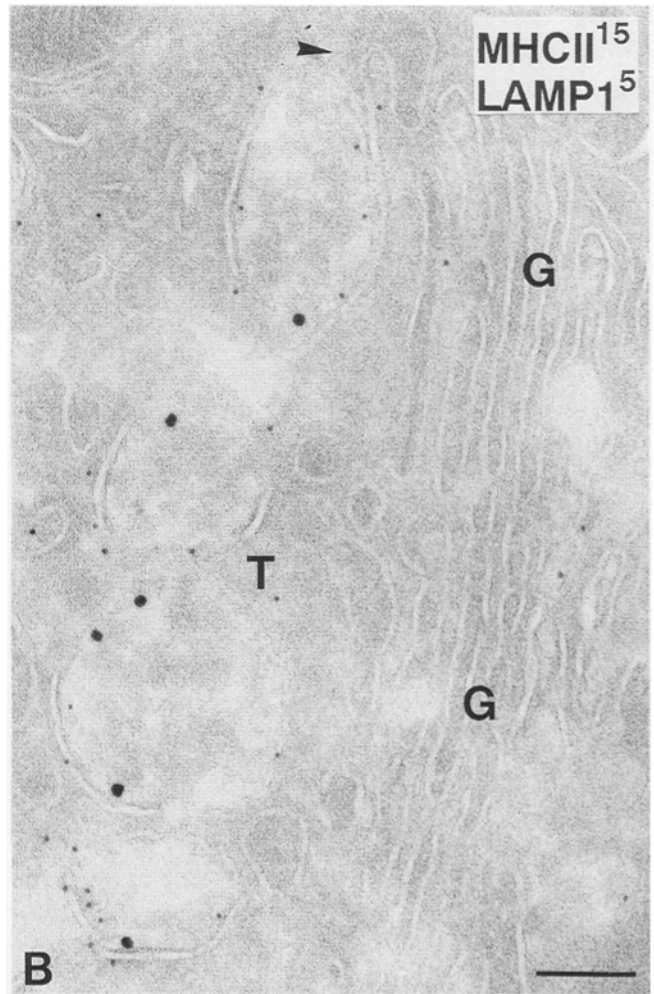
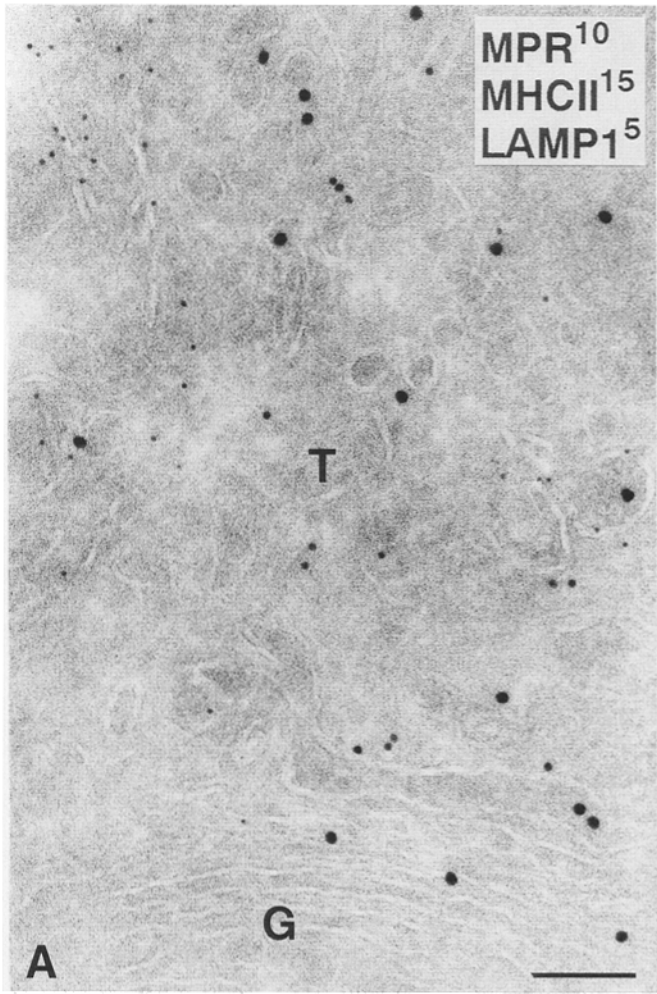


Table I. Distribution of HKLM in Phagocytic Compartments of Macrophages*

Incubation		Percent of HKLM in phagocytic compartments		
HKLM Pulse	Chase	Phagosomes [‡]	Phagolysosomes I [§]	Phagolysosomes II
10'	0'	72	27	1**
10'	30'	28	57	15
10'	60'	8	52	40

* Lysosomes were labeled by uptake of BSA-gold followed by overnight chase.

[‡] Phagosomes: -lamp 1, -Cath. D, -BSA-gold.

[§] Phagolysosomes I: +lamp 1, +Cath. D, -BSA-gold.

^{||} Phagolysosomes II: +lamp 1, +Cath. D, +BSA-gold.

** Examples of early phagosome-lysosome fusion are illustrated in Figs. 3 C and 4 C. In cells with labeling of lysosomes by BSA-gold uptake followed by a shorter chase, e.g., 20-40 min before bacterial uptake, phagocytic structures containing BSA-gold were more common at early time points.

son et al., 1986). Knapp and Swanson (1990) demonstrated that tubular lysosomes fuse with phagosomes. Lang et al. (1988b) and Tassin et al. (1990) have described distinct sets of lysosomes and phagolysosomes defined by different targeting patterns and kinetics using endocytic and phagocytic tracers; these may also correspond to the two lysosome populations we have observed. Rabinowitz et al. (1992) defined a tubular compartment in macrophages which they termed late endosomal or "prelysosomal," but they also demonstrated the continuity of this compartment with phagolysosomes and equated it with tubular lysosomes. In their studies, this compartment retained BSA-gold after chase incubations of 60 min to overnight. Our results taken together with all of the above studies suggest that the tubulo-vesicular compartment in question is best termed a lysosomal compartment.

The results of Rabinowitz et al. (1992), although not dealing with bacterial phagocytosis and antigen processing, are generally consistent with our own findings. One contrast, however, is their detection of the 215-kD MPR in the tubular compartment (although not in all portions). We failed to detect 215-kD MPR anywhere within the peritoneal macrophages using four different antisera, but we were able to detect 46-kD MPR. The two types of MPR have been observed to have a similar, overlapping distribution in other macrophages (Bleekemolen et al., 1988). We observed 46-kD MPR in vesicles distributed throughout the macrophage cytoplasm, consistent with earlier data (Bleekemolen et al., 1988), but not in the tubular lysosomal elements. Several possible explanations exist to reconcile these observations. First, Rabinowitz et al. (1992) found that the 215-kD MPR was "restricted to juxta-Golgi elements of the (tubular compartment)" (consistent with the data of Tassin et al., 1990). With this localization it is possible that the tubular elements

Table II. Distribution of MHC II in Interferon- γ Stimulated Macrophages

Percent Immunogold over Subcellular Compartments	
Plasma membrane	58
Sacs*	21
Tubulo-vesicular lysosomes [‡]	13
Dense lysosomes [§]	4
Endosomes	3
TGR	1
Golgi complex	-

* Lamp 1, negative.

[‡] Lamp 1, positive, cisternal, electron-lucent, internal membranes.

[§] Lamp 1, positive, vacuolar, electron-dense.

^{||} Lamp 1, positive, multivesicular bodies with adjacent vesicles and tubules.

containing MPR were, in fact, parts of the TGR (where we did detect 46-kD MPR), distinct from tubulo-vesicular lysosomes. Second, it should be noted that the macrophages used by Rabinowitz et al. (1992) were elicited with a different protocol and were probably not activated for antigen processing as those in the current study (MHC-II was not evaluated in their study). The discrepancy in 215-kD MPR results may reflect varying MPR expression due to macrophage differentiation in different states of activation, or it may simply reflect technical differences in the sensitivity for the detection of 215-kD MPR. Third, although the 46- and 215-kD MPR have been reported to have similar distributions in other macrophages, differences in the localization of the 46-kD MPR (localized in this study) and the 215-kD MPR (localized by Rabinowitz et al., 1992) cannot be excluded. Finally, the exclusion of MPR from lysosomal compartments may not be complete in all cell types in all states of differentiation or activation. MPR positive "prelysosomes" which retain endocytic markers after an overnight chase (Pieters et al., 1991; Rabinowitz et al., 1992) may correspond to "lysosomes" defined in other studies.

Our previous immuno-electron microscopy studies of peritoneal macrophages that were not stimulated with interferon- γ demonstrated that 40% of total cellular MHC-II labeling was in intracellular compartments. Most of this was located in sacs (Lamp 1 and cathepsin D negative, probably equivalent to "macropinosomes") (Brunk et al., 1976; Racoosin and Swanson, 1989) or endosomes (Lamp 1 and cathepsin D positive), while 2% of total cellular staining was located in typical homogeneous, electron-dense lysosomes (corresponding to late lysosomes). In the present study, macrophages stimulated *in vitro* with interferon- γ again exhibited ~40% of the MHC-II labeling in intracellular compartments, with 13% associated with tubulo-vesicular lysosomes and 4% associated with dense lysosomes. Thus, despite an increase in MHC-II expression, *in vitro* treatment with inter-

Figure 5. MHC-II in the TGR and related structures. *A* and *B* show *trans*-Golgi regions of control cells without exposure to HKLM or BSA-gold. The TGR (*T*) in *A* shows labeling for the 46-kD MPR, Lamp 1, and MHC-II. The labeling for MHC-II and Lamp 1 shows slight overlap (see *b*) but MPR seems to reside in a different TGR subdomain. The Golgi stack (*G*) is only lightly labeled for MHC-II. In *B*, the TGR (*T*) contains vacuoles labeled for MHC-II and Lamp 1. Note the continuity between a TGR tubule and vacuole (arrowhead). *G*, Golgi stack. *C*. Macrophages were exposed to BSA-gold for 100 min and then chased for 8 h. The typical tubular lysosome contains many membrane sheets and vesicles and is abundantly labeled for MHC-II but does not contain BSA-gold. It is located nearby the Golgi complex (*G*). *M*, mitochondria. Bars, 0.1 μ m.



Figure 6. Tubules and vesicles with phagolysosome-derived content and MHC-II provide a potential recycling pathway. This figure shows parts of two phagolysosomes (lower right and top) in a macrophage that was exposed to CF-HKLM for 40 min. Note that the tubules extending from the phagolysosomes (arrowheads) contain abundant ferritin particles together with labeling for MHC class II. Bar, 0.1 μ m.

feron- γ had little effect on the overall distribution of MHC-II relative to elicited macrophages that were activated *in vivo*. While the current study confirms that MHC-II is included within endocytic structures of varying stages, including sacs and endosomes (Table II), it is likely that some of the structures classified previously as endosomes actually correspond to the currently defined tubulo-vesicular lysosomes. The tubulo-vesicular lysosomes are much more apparent when *Listeria*-elicited macrophages are additionally stimulated *in vitro* with interferon- γ (as done in this study, not done in the earlier study). This highlights the important dependence of

the morphology and function of the lysosomal system in macrophages on the physiological state of these cells. This can be seen by the variation in tubular lysosome morphology in various states in earlier studies (Heuser, 1989; Phaire-Washington et al., 1980; Knapp and Swanson, 1990) and by the effects of macrophage activation on phagosome-lysosome fusion (Kielian and Cohn, 1981). The tubulo-vesicular lysosomes in macrophages show some similarities to the late endocytic compartment in B cells containing MHC-II observed by Peters et al., (1991) (e.g., both compartments contain characteristic internal membrane sheets and have similar immuno-labeling characteristics), although this type of compartment was not reported in B cells by Guagliardi et al. (1990).

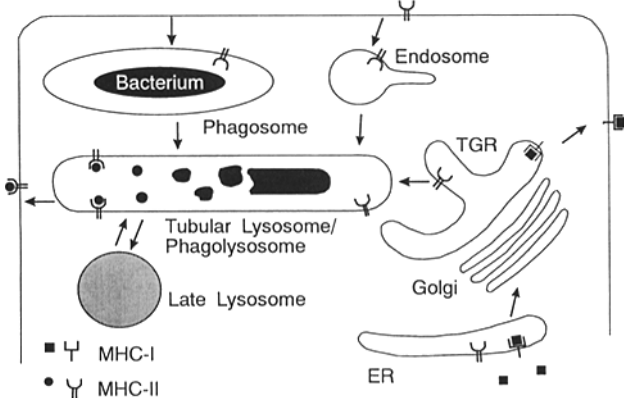


Figure 7. A model for lysosomal and phagolysosomal functions in antigen processing. Microbes and soluble antigens are internalized via phagosomes and endosomes, respectively, both of which can fuse with the tubulo-vesicular lysosomal compartment. The latter compartment contains lysosomal enzymes and MHC-II transported from the TGR (some may also be delivered by fusion with other endosomes or lysosomes). The tubulo-vesicular lysosomes or phagolysosomes mediate antigen catabolism, including the production of immunogenic peptides, which may bind to MHC-II molecules present in the same compartment. Peptide-MHC-II complexes are then transported to the plasma membrane via recycling vesicles for presentation to T cells. MHC-I molecules, which may be "loaded" with peptides in the ER, follow a separate pathway and diverge from MHC-II molecules in the TGR, where the two molecules are sorted (Peters et al., 1991).

While our studies have focussed on the rapid fusion of lysosomes with phagosomes, other groups have demonstrated endosome-phagosome fusion as well (Rabinowitz et al., 1992; Mayorga et al., 1991; Pitt et al., 1992; Lang et al., 1988a; Hart and Young, 1991). We also observed fusion of multivesicular endosomes with phagolysosomes (Fig. 4 b). Together, these observations suggest that the tubulo-vesicular phagolysosomal compartment represents an important intersection of the endosomal and phagosomal pathways. In addition, recycling pathways may retrieve material from phagolysosomes, which were observed to generate small vesicles containing HKLM-derived material as well as MHC-II. Other studies have also demonstrated structures emanating from phagolysosomes in macrophages (Pfeifer et al., 1992; Lang et al., 1988a). These vesicles may recycle processed immunogenic peptides for presentation to T cells.

The tubular lysosomal compartment may also receive materials transported from the TGR. Earlier observations established that MHC-II was segregated from MHC-I and MPR in the TGR (Peters et al., 1991). Our data demonstrate that MHC-II and Lamp 1 are co-localized in TGR-derived vacuoles, that are devoid of MPR. This suggests that MHC-II and Lamp 1 use the same vesicular pathway for post-Golgi transport, which segregates them from MPR and MHC-I. This transport may lead from the TGR either directly to tubulo-vesicular lysosomes, or first to endosomes, which could then deliver materials to the lysosomal compartments.

Transport of MHC-II via this specific pathway may be dependent on signals conveyed by the invariant chain, which associates with nascent MHC-II in the ER. Recent studies have demonstrated the importance of invariant chain for directing transport of MHC-II to vesicular compartments in transfected non-lymphoid cells (Lotteau et al., 1990; Bakke and Dobbstein, 1990). While the dynamic function and morphology of such compartments may vary greatly between cell types, the compartment defined by invariant chain targeting in these cells may be analogous to tubulo-vesicular lysosomes in macrophages.

The exact site where peptide-MHC-II complexes are formed is still undetermined, but the tubulo-vesicular lysosome represents a good candidate for this "loading" compartment. It is clear that tubulo-vesicular lysosomes represent an important intersection of the endocytic and phagocytic pathways, from which material can be recycled. They may receive nascent MHC-II, transported from the TGR, as well as any MHC-II internalized during endocytosis or phagocytosis. They may represent the dense lysosomal compartment in which antigens are processed and then recycled for presentation to T cells (Harding et al., 1991a). Thus, both soluble and particulate antigens could be delivered to this lysosomal compartment for catabolism, and immunogenic peptides generated thereby could then be recycled after binding to MHC-II molecules, perhaps within tubulo-vesicular lysosomes, for presentation to T cells (Fig. 7).

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