23

Class II MHC Antigen Processing in Phagosomes

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Summary

Phagocytic antigen-presenting cells (APCs) are involved in innate and adaptive immune responses to bacteria. Adaptive responses to bacteria involve processing of bacterial antigens for presentation by class II major histocompatibility complex (MHC II) molecules and class I MHC (MHC I) molecules to stimulate $CD4^+$ and $CD8^+$ T cells, respectively. To examine the role of phagosomes in processing of antigens for presentation by MHC II molecules to $CD4^+$ T cells, phagosomes have been biochemically and functionally analyzed by a variety of techniques that include flow analysis (flow organellometry), SDS-PAGE/Western blotting, and an antigen-presenting organelle assay. Using these techniques, we have demonstrated that phagosomes containing latex beads or *Mycobacterium tuberculosis* (MTB) contain components of the MHC II processing pathway and support the formation of peptide–MHC II complexes.

Key Words: Phagosome; antigen processing; class II MHC; *Mycobacterium tuberculosis*; macrophage.

1. Introduction

Macrophages and immature dendritic cells are phagocytic cells that internalize particulate antigens (Ags, such as microbes or latex beads conjugated to Ag) and present peptides derived from these Ags on MHC I and MHC II molecules for T-cell recognition ***(1–3)***. During the processing of particulate Ags, peptide–MHC II complexes may be formed within phagosomes or within endocytic compartments (e.g., MHC II compartment [MIIC]) that receive Ag fragments from phagosomes.

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To assess the role of phagosomes in the formation of peptide–MHC II complexes, we have developed and modified a variety of techniques to biochemically and functionally evaluate phagosomes. Phagosomes were analyzed for the presence of components of the MHC II processing pathway by flow organellometry ***(4–7)*** and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)/Western analysis of purified phagosomes ***(5–7)***. Degradation of phagosome-associated Ag was analyzed by flow organellometry ***(4–6)***. Finally, presence of peptide–MHC II complexes in phagosomes was functionally evaluated by the Ag-presenting organelle assay ***(5–8)***.

Analysis of phagosomes by these techniques has to be done with the exclusion of other organelles. This can be achieved either by analytic isolation by flow organellometry or by physical isolation. Latex bead phagosomes can be analytically isolated by flow organellometry from other components of the cells without the need for extensive prior physical purification ***(4–6)***. Latex bead phagosomes can also be physically isolated on sucrose density gradients or Percoll density gradients, and magnetic latex bead phagosomes can be isolated using a magnet. Phagosomes containing *Mycobacterium tuberculosis* (MTB) can be purified on Percoll density gradients.

By analyzing purified phagosomes by the techniques described, we have demonstrated that latex bead phagosomes rapidly degrade Ag (not evaluated for MTB phagsomes ***(4–6)***) . Phagosomes containing either latex beads or MTB acquire lysosomal-associated membrane protein-1 (LAMP-1) and MHC II as well as some of the other components of the MHC II–processing pathway, e.g., H2-DM and invariant chain ***(5,7,9)***. Both latex bead and MTB phagosomes also support the formation of peptide–MHC II complexes, which are subsequently transported to the cell surface and presented to T cells ***(5,7)***.

2. Materials

2.1. Cell Culture

Macrophages can be obtained directly from the peritoneal cavity of mice or from progenitor cells in the bone marrow. Generation of peritoneal exudate cells (PECs) for experiments such as subcellular fractionation requires a large number of mice (1 million PECs is usually obtained per mouse). Significantly fewer mice (10-fold less) are required to generate the same number of bone marrow–derived macrophages (BMMs). For experiments described in the Methods section, we have found interferon (IFN)-γ-activated BMMs to be functionally equivalent to PECs. For studies using human macrophages/monocytes, activated human monocytic cell lines (e.g., THP-1) or human monocyte-derived macrophages (MDMs) can be used.

To analyze either phagocytic Ag processing and presentation in intact cells or the presence of specific peptide–MHC II complexes in subcellular fractions (including phagosomes), Ag-specific, MHC II–restricted T cells have been used. Therefore, for these types of studies, it is essential to use macrophages expressing the MHC II alleles recognized by the T cells. The MHC II alleles expressed by murine macrophages can be determined by the strain of mice being used. The MHC II alleles expressed by human macrophages can be determined by HLA typing of DNA extracted from cells.

1. For murine macrophages: Mice expressing known MHC II alleles for generation of PECs or BMMs. Sterile scissors and forceps for dissection.
2. For human macrophages: Human monocytic cell line THP-1 (ATTC, Manassas, VA) and/or human MDMs expressing known MHC II alleles.
3. T cells: Murine T hybridoma cells with appropriate Ag specificity and MHC II restriction. Murine T-cell hybridomas derived from HLA-transgenic mice can be used to detect peptide:MHC complexes presented by human APCs ***(10)***. Alternatively, human T cell lines may be used, but they may not respond to fixed APCs or subcellular organelles due to requirements for (fixation-sensitive) accessory molecules.
4. CTLL-2 cells (ATTC) or IL-2 ELISA (eBioscience, San Diego, CA).

2.2. Media

1. Standard medium for murine cells: Dulbecco's modified Eagle's medium (DMEM) with glutamine (Hyclone, Logan, UT) supplemented with 10% heat-inactivated fetal calf serum (FCS, Hyclone), 50 μ*M* 2-mercaptoethanol (2-ME, Sigma, St. Louis, MO), 1 m*M* sodium pyruvate, 10 m*M* hydroxyethyl piperazine ethane sulfonate (HEPES) buffer, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen Corporation, Carlsbad, CA).
2. Medium for generation of BMMs: Standard medium supplemented with 20% LADMAC cell-conditioned media ***(11)***. For generation of LADMAC cell-conditioned media, grow LADMAC cells in Alpha medium (Eagle's minimal essential medium [EMEM, Cambrex, Walkersville, MD], supplemented with 10% FCS [Hyclone] and 4m*M* L-glutamine [Hyclone]) for 7 d and harvest supernatants. Store supernatants at –80°C for up to 1 yr.
3. Standard medium for human cells: For THP-1 cells use RPMI 1640 (BioWhitaker, Walkersville, MD) supplemented with 10% heat-inactivated FCS (Hyclone), 50 μ*M* 2-mercaptoethanol (2-ME, Sigma), 1 m*M* sodium pyruvate, 2 m*M* L-glutamine, 10 m*M* HEPES buffer, nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). For human monocytes, use standard medium with 10% pooled human serum (PHS) instead of FBS.

2.3. Reagents for Activation/Maintenance of Cells

1. For activation of murine BMMs: Murine IFN-γ (Genzyme, Cambridge, MA). Working concentration is 50–100 U/mL.
2. For activation of THP-1 cells: Phorbol 12-myristate 13-acetate (PMA, Sigma) and human IFN-γ (Endogen, Woburn, MA). Working concentration is 10 ng/mL for PMA and 50 U/mL for IFN-γ.
3. For maintanence of CTLL-2 cells: Rat interleukin (IL)-2 (BD Pharmingen, San Diego, CA) ***(12)***.

2.4. Preparation of Particulate Ag (Ag-Conjugated Latex Beads and Bacteria)

1. Latex beads: 2-μm latex beads (fluorescent or nonfluorescent; Polysciences, Warrington, PA) or 1- to 2-μm magnetic latex beads (Polysciences). 1-μm latex beads will not readily pellet in a typical tabletop centrifuge and should not be used in experiments described in this chapter.
2. Preparation of antigen for conjugation to latex beads: The antigen to be used for conjugation to latex beads is determined by the antigen specificity of the T-cell hybridomas available for the project. For example, DOBW T hybridoma cells recognize ovalbumin (OVA) peptide 323-339 bound to MHC II molecules I-A^d or I-A^b ***(5)***. Therefore, latex beads have to be conjugated to OVA in experiments using DOBW T hybridoma cells.

   Polysciences provides instructions for conjugation of proteins to beads (covalent or noncovalent). Noncovalent conjugation (passive adsorption) is sufficient for most purposes. The pH of the conjugation buffer may require adjusting for each protein. For example, prepare solution of OVA (Sigma, St. Louis, MO) or hen egg lysosyme (HEL, Sigma) at 5 mg/mL in citrate buffer (pH 4.2) or phosphate-buffered saline (PBS) (pH 7.4), respectively.
3. Bacteria such as MTB, *E. coli*, *Salmonella*, or *Streptococcus*.
4. For declumping MTB: 18- and 22-gauge needles.
5. For fluorescent labeling of MTB or other bacteria: FLUOS (Boehringer), prepared when needed in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/mL, and PBS (pH 9.0).

2.5. Inhibitors

1. Cytochalasin D (Sigma) is dissolved in DMSO at a concentration of 1 mg/mL and stored in aliquots at –20°F. Working concentration is 10 μg/mL.
2. Chloroquine (Sigma) is prepared when needed in water at a concentration of 10 m*M*. Working concentration is 100 μ*M*.
3. Brefeldin A (BFA, Sigma) is dissolved in ethanol at a concentration of 1 mg/mL and stored in aliquots at –20°F. Working concentration is 1 μg/mL.

2.6. Sucrose Density Gradients

1. Versene (Gibco-BRL).
2. Homogenization buffer: 0.25 *M* sucrose (Mallinckrodt, St. Louis, MO) in 10 m*M* HEPES buffer solution, pH 7.5 (100 m*M* stock from Gibco BRL). Homogenization buffer should be sterile filtered and stored at 4°C. On the day of the experiment, supplement a small portion of the homogenization buffer with the following protease inhibitors: 1.0 m*M* phenylmethysulfonyl fluoride (PMSF, Sigma), 1 μg/mL pepstatin (Sigma), 20 μg/mL leupeptin (Sigma). Protease inhibitors: PMSF is dissolved in 100% isopropanol at a concentration of 100 m*M* and stored in the dark at room temperature. Pepstatin is dissolved in ethanol (with heat up to 60°C) at 1 mg/mL and stored in aliquots at –20°C. Leupeptin is dissolved in Millipore water at 2 mg/mL and stored in aliquots at –20°C (*see* **Note 1**).
3. Sucrose solutions of different percentages (62, 32, 26, 21, and 10%) are made by dissolving sucrose in 10 m*M* HEPES buffer solution (pH 7.5, Gibco-BRL). Sucrose solutions should be sterile filtered through a 0.22-μm filter and stored at 4°C (*see* **Note 2**).
4. Cell lifters (Corning).
5. Dounce homogenizer (7 mL capacity, Kontes, Vineland, NJ), autoclaved prior to use.

2.7. Percoll Density Gradients

1. Versene (Gibco-BRL).
2. Homogenization buffer (*see* **Subheading 2.6., step 2**).
3. Percoll solutions: Made by diluting Percoll (Amersham) to 20, 23, 27, or 40% in homogenization buffer lacking protease inhibitors.
4. Cell lifters (Corning).
5. Dounce homogenizer (7 mL capacity, Kontes, Vineland, NJ), autoclaved prior to use.
6. Polycarbonate centrifuge tubes (for ultracentrifuge), autoclaved prior to use.

2.8. Biochemical Analysis of Percoll Density Gradient Fractions

1. β-Hexosaminidase assay: Assay buffer is 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid (MES, Sigma, pH 6.5) in 0.2% Triton X-100 (Sigma). The substrate is *p*-nitrophenyl-acetyl-β-D-glucosaminide and is made just before addition at a concentration of 1.36 mg/mL in Millipore water.
2. For identification of plasma membrane: ^{125}I-labeled anti-MHC II Abs or sulfo-NHS-biotin (Pierce) at 0.5 mg/mL in PBS (pH 8.0).
3. For identification of phagosomes: Fluorescent latex beads (2 μm, Polysciences) or FLUOS-labeled MTB (*see* **Subheadings 2.4., step 5** and **3.2.2.**).

2.9. Flow Analysis of Phagosomes

1. Homogenization buffer (*see* **Subheading 2.6., step 2**).
2. 2% paraformaldehyde (Polysciences) is made in PBS (pH 7.4). Solution should be stirred on a heating plate maintained at 56°C until paraformaldehyde is dissolved and then vacuum-filtered through 0.22-µm filter and stored at 4°C (Caution: *see* **Note 3**).
3. 0.4 *M* lysine (DL-lysine, Sigma) is made in Millipore water. Solution should be stirred on a heating plate maintained at 56°C until lysine is dissolved and then sterile filtered through 0.22-µm filter and stored at 4°C.
4. Cell lifters (Corning).
5. 96-Well round bottom plates.
6. FACS buffer is made by adding 1% FCS, 1% bovine serum albumin (BSA), and 0.1% saponin to PBS (pH 7.4). A 1% stock solution of saponin should be made in PBS and added to FACS buffer.
7. Antibodies (Abs) against MHC II, LAMP-1 and against Ags that have been conjugated to latex beads (anti-OVA and anti-HEL).

2.10. Western Blot Analysis of Phagosomes

Lysis buffer: PBS (pH 7.4) containing 1% Nonidet P-40 (Sigma) and the protease inhibitors 1 m*M* PMSF, 20 µg/mL leupeptin, and 2 µg/mL pepstatin (*see* **Subheading 2.6., step 2** on how to make and store protease inhibitors and **Note 4**).

2.11. Ag Processing and Presentation Assay (T-Cell Assay)

1. 96-Well flat-bottom plates.
2. Murine PECs or BMMs, human monocytes, or monocyte-derived macrophages (*see* **Subheading 2.1.**) expressing known MHC II molecules.
3. Ag-specific, MHC II-restricted T hybridoma cells (*see* **Subheading 2.1., step 3**).
4. Ag: Latex-beads conjugated to Ag or bacteria (*see* **Subheadings 2.4.** and **3.2.**).
5. 2% paraformaldehyde and 0.4 *M* lysine (*see* **Subheading 2.9., step 2** and **2.9., step 3**).
6. For detection of IL-2 by the CTLL-2 proliferation assay: CTLL-2 cells (ATCC, Manassas, VA ***(12)***), culture supernatants containing IL-2 and Alamar Blue (Trek Diagnostics, Cleveland, OH). Alternatively, an IL-2 ELISA may be used (eBioscience).

2.12. Ag-Presenting Organelle Assay

1. 96-Well flat-bottom plates.
2. Percoll density gradient fractions or purified phagosomes derived from macrophages expressing known MHC II molecules.
3. MHC II-restricted T hybridoma cells (*see* **Subheading 2.1., step 3**).
4. Percoll solution (*see* **Subheading 2.7., step 3**).
5. Reagents for the detection of IL-2 by the CTLL-2 proliferation assay or IL-2 ELISA (*see* **Subheading 2.11., step 6**).

3. Methods

3.1. Isolation/Preparation and Maintenance of Cells

3.1.1. Murine Macrophages

3.1.1.1. PECs

1. For generation of PECs, inject mice intraperitonally with 1 mL of 1×10^4 live *Listeria monocytogenes* (LM) in PBS (pH 7.4; *see* **Note 5**) ***(13)***. Alternatively, inject mice intraperitonally with 100 µg of concanavalin A (Con A) in 500 µL of PBS (*see* **Note 6**).
2. After 4 d (for Con A) or 10–14 d (for LM), harvest cells from the peritoneal cavity of mice ***(13)***.
3. Plate cells in 96-well (2×10^5 cells/well) or 6-well plates (3×10^6 cells/well) for 2 h to allow macrophages to adhere to plastic. Rinse off nonadhered cells with DMEM.

3.1.1.2. Murine BMMs

1. For generation of BMMs, isolate both femurs from mice. Cut ends of femurs with sterile scissors and flush bone with DMEM using a 23-gauge needle.
2. Spin and resuspend cells in 12 mL of LADMAC-supplemented media. Plate cells in 6-well plates (2 mL per well). Grow cells at 37°C in 5% CO_2. Replace media after 3 d with 3 mL of LADMAC-supplemented media and subsequently replace after every 2 d. BMMs can be used after 7–14 d. Cells have to be activated with 50–100 U recombinant murine IFN-γ for 24 h prior to use in Ag-processing assays to upregulate MHC II.

3.1.2. Human Macrophages

3.1.2.1. Activation of THP-1 Cells

To induce differentiation of THP-1 cells into macrophages, activate cells in media supplemented with 10 ng/mL PMA. After 24 h, remove media and incubate cells for an additional 24 h with new medium containing 50 U/mL recombinant human IFN-γ to upregulate MHC II.

3.1.2.2. Human Monocyte-Derived Macrophages

For generation of human monocyte-derived macrophages, isolate peripheral blood mononuclear cells (PBMCs) and enrich monocytes by allowing them to adhere to tissue culture plates (plastic adherence) for 1 h at 37°C. Alternatively, purify $CD14^+$ monocytes by immunomagnetic cell separation according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Culture monocytes for 5–7 d to differentiate them into MDMs.

3.1.2.3. Maintenance of CTLL-2 Cells

Seed CTLL-2 cells at a concentration of 1×10^5 cells/10 mL for a 2-d passage, 5×10^4 cell/10mL for a 3-d passage, or 2.5×10^4 for a 4-d passage. Add 2 U/mL of rat IL-2 (BD Pharmingen). Use only 3- or 4-d passage for IL-2 assay; 2-, 3-, or 4-d passages may be used to set up additional passages ***(12)***.

3.2. Preparation of Particulate Ags

3.2.1. Conjugation of Ag to Latex Beads

1. Ag is conjugated to latex beads by passive adsorption. For conjugation of OVA to latex beads, wash 100 µL of latex beads in 1 mL of citrate buffer (pH 4.2). Resuspend beads in 1 mL of 5 mg/mL OVA solution. For conjugation of HEL to latex beads, wash 100 µL of latex beads in 1 mL of PBS (pH 7.4). Resuspend beads in 1 mL of 5 mg/mL HEL solution.
2. Rotate beads with Ag for 2 h at room temperature or at 4°C overnight. Wash beads a minimum of three times in 1 mL of DMEM and resuspend in DMEM corresponding to the original volume of beads. Samples should be stored at 4°C and can used for up to 3 wk. Yield obtained is approximately 10^{12} beads/mL and 100 µg OVA/mL or 400 µg HEL/mL.

3.2.2. Preparation of MTB

1. MTB has to be declumped prior to use in experiments. For declumping, passage MTB twice through an 18-gauge needle and thrice through a 22-gauge needle. Centrifuge sample at 150*g* for 5 min to remove clumps. Estimate concentration of viable MTB by analyzing colony forming units (CFUs) on Middlebrook 7H11 plates (Difco, Detroit, MI).
2. For labeling MTB with fluorescein, pellet 10^9 bacteria in an Eppendorf tube and resuspend in 1 mL of PBS (pH 9.1). Combine with 25 µL of 20 mg/mL FLUOS in DMSO for 5 min at room temperature. Wash labeled MTB twice in DMEM and declump before use.

3.3. Ag-Presenting Assay (T-Cell Assay Using Intact Cells)

Prior to addition of Ag to wells, warm up centrifuge to 30–37°C (*see* **Note 7**). An example of results from a T-cell assay is shown in **Fig. 1**.

3.3.1. Standard T-Cell Assay

Plate macrophages at a concentration of 2×10^5 cells/well in 96-well flat-bottom plates. Leave overnight for BMM. In the case of PECs remove nonadherent cells after 2 h by washing the wells once with 100 µL of medium. Certain cells may have to be activated with IFN-γ prior to use (*see* **Subheading 3.1.**).

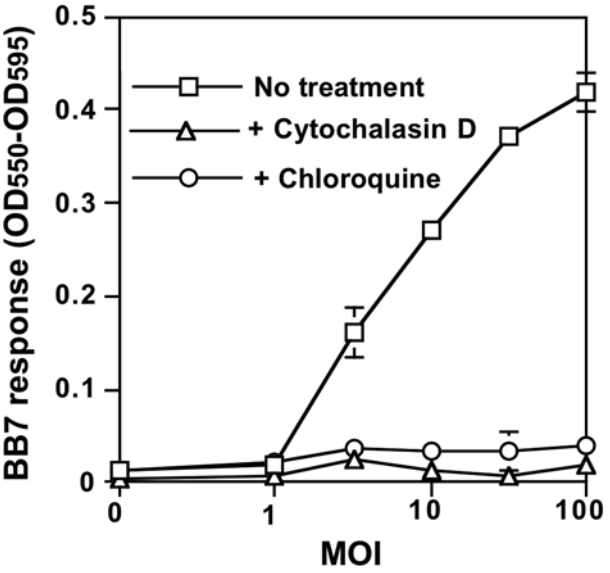

Fig. 1. Requirements for processing of MTB bacilli for presentation of MTB Ag 85B to T cells. Macrophages were pretreated with or without 10 µg/mL cytochalasin D (to inhibit phagocytic uptake) or 100 µ*M* chloroquine (to inhibit acidification of vacuolar compartments) beginning 15 min before addition (pulse) of HK bacteria for 20 min. Samples were washed to remove extracelluar bacteria, chased for 10 min at 37°C and fixed. BB7 T hybridoma cells were used to detect Ag 85B(241-256):I-A^b complexes. MOI = multiplicity of infection. (Adapted from Ramachandra et al., 2001, with permission from the Journal of Experimental Medicine, The Rockefeller University Press.)

1. Add particulate Ag (*see* **Subheadings 2.4.** and **3.2.**) to the wells in a final volume of 100 µL. Spin the plate at 900*g* for 5 min (10 min for MTB) to pellet the Ag onto the cells. Incubate the cells at 37°C for an additional 5 min (10 min for MTB) to provide a total pulse time of 10 min (20 min for MTB).
2. Place the plate on ice and wash the wells twice in 100 µL ice-cold DMEM to remove extracellular particulates (observe cells under the microscope and repeat wash if necessary).
3. Add prewarmed media to the cells and incubate cells at 37°C to achieve the desired chase incubation.
4. Fix the cells as follows to prevent any additional processing. Prepare a 1:1 solution of DMEM (no FCS) and 2% paraformaldehyde (fixative solution) as well as a 1:1 solution of 0.4 *M* lysine and DMEM (Barf solution). Wash wells with 150 µL of DMEM. Add 120 µL of fixative solution to the wells and incubate for 15 min at room temperature. Wash wells with 150 µL of DMEM and then incubate cells in 175 µL of Barf solution for 30 min at room temperature. Wash wells four times with 200 µL of DMEM and then resuspend cells in 100 µL of media.

5. Add T hybridoma cells (100 µL at 10^6 cells/mL) to achieve 10^5 T hybridoma cells/well in a total volume of 200 µL. Incubate cells for 24 h (*see* **Note 8**).
6. Harvest supernatants (100 µL) and assess for IL-2 content using the CTLL-2 proliferation assay or IL-2 ELISA (*see* **Note 9**).
7. For the CTLL-2 assay, add CTLL-2 cells (50 µL at 5×10^3/mL) to 100 µL of supernatant. After 24 h add 15 µL of the indicator dye Alamar blue to each well and measure the difference between absorbance at 550 and 595 nm (or 570 and 600 nm) after another 24 h. Blanks for spectrophotometry are provided by wells containing medium alone (added at the initiation of the CTLL-2 assay) and Alamar blue (added at the same time as for the other wells).

3.3.2. Antigen-Presenting Assay with Inhibitors

The following inhibitors can be used in T-cell assays to functionally characterize phagocytic Ag processing and should be present throughout the duration of the Ag-processing step (added prior to addition of Ag and present until fixation of APCs, but not present during the T-cell assay) **(Fig. 1)**.

1. Cytochalasin D inhibits phagocytic uptake and should be added at a concentration of 10 µg/mL to cells, 15 min prior to addition of particulate Ag.
2. Chloroquine inhibits acidification of vacuolar compartments and hence formation of peptide–MHC II complexes and should be added at a concentration of 100 µ*M* to cells 15 min prior to addition of particulate Ag.
3. BFA is a fungal metabolite that inhibits anterograde transport through the endoplasmic reticulum (ER) and Golgi complexes. BFA blocks the supply of nascent MHC II molecules to the endocytic/phagocytic pathway and inhibits the formation of most peptide:MHC II complexes in the MIIC compartment as well as in phagosomes ***(6)***. Add BFA at a concentration of 1 µg/mL to cells 3 h prior to the addition of particulate Ag. BFA blocks ER-Golgi transport rapidly, but a period of 3 h is required to deplete the post-Golgi reservoir of nascent MHC II.

3.4. Isolation of Latex Bead Phagosomes by Sucrose Density Gradients

Phagosomes are isolated by sucrose density gradients essentially as described by Desjardins et al., with minor modifications ***(14)***. Use fluorescent latex beads to easily identify phagosomes in the sucrose density gradient. This technique produces highly purified phagosomes as judged by electron microscopy and the low degree of contamination by plasma membrane marker (<0.4%) ***(5,6)***. Isolated phagosomes can be analyzed by SDS-PAGE/Western blot analysis.

1. Incubate two 6-well plates containing macrophages (3×10^6 cells/well) with fluorescent latex beads (10 µL in 4 mL media/well) as described in **Subheading 3.3.1**, **steps 1–3,** to achieve a 10-min pulse and a 0 to several hour chase

incubation. The length of the pulse and chase incubations should be determined for your specific needs.

2. Transfer the plates immediately onto ice and wash each well with 3 mL of cold DMEM followed by 3 mL of cold PBS (*see* **Note 10**). Remove PBS and add 1 mL of Versene into each well. Immediately scrap the cells gently off the well using a cell lifter. Rinse each well with an additional 0.5 mL of Versene.
3. Transfer the cells to a 50-mL conical tube and spin at 300*g* for 7 min at 4°C. Wash the cells with 10 mL of homogenization buffer and resuspend cells in 1 mL of homogenization buffer with protease inhibitors.
4. Homogenize the cells very gently to obtain 80–85% lysis (*see* **Note 11**).
5. Transfer homogenate to 15-mL tube and remove intact cells and nuclei by centrifugation at 200*g* for 10 min at 4°C. Transfer the supernatant to a 5-mL polystyrene round-bottom tube with cap (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) and centrifuge at 1900*g* for 10 min at 4°C (tabletop centrifuge) to pellet the crude phagosome preparation.
6. Resuspend the crude phagosome preparation in 2 mL of homogenization buffer and combine with 2 mL of 62% sucrose solution (resulting sucrose concentration = 40%).
7. Split sample into two equal parts (2 mL each) and gently load into two ultracentifuge tubes containing a 1-mL cushion of 62% sucrose solution. Layer the following solutions on top: 2 mL of 32% sucrose, 2 mL of 26% sucrose, 2 mL of 21% sucrose, and 2.5 mL of 10% sucrose.
8. Centrifuge the tubes in a swinging bucket rotor for 1 h at 100,000*g* (e.g., SW-41 rotor, Beckman Coulter, 30,000 rpm) at 4°C.
9. Collect the phagosomes from both gradients at the interface of the 10 and 21% sucrose solutions. Dilute the sample at least threefold in PBS. Pellet by centrifugation at 10,000 rpm for 5 min in an Eppendorf centrifuge (*see* **Note 12**).
10. Remove all liquid and immediately freeze phagosome pellet on dry ice and store sample at –80°C.
11. Frozen phagosome pellets can be analyzed by SDS-PAGE/Western blot analysis as described in **Subheading 3.9**.

3.5. Isolation of Magnetic Latex Bead Phagosomes

Phagosomes containing magnetic latex beads are easy to physically isolate and also have the highest purity (<0.003% plasma membrane contamination) ***(5,6)***. These phagosomes can be analyzed by flow organellometry (*see* **Subheading 3.8.1.**), SDS-PAGE/Western blotting (*see* **Subheading 3.9.**) or by the Ag-presenting organelle assay (*see* **Subheading 3.10.**). However, functional analysis of other subcellular organelles by the Ag presenting organelle assay will require Percoll density gradient fractionation.

1. Incubate cells with magnetic latex beads conjugated to Ag to achieve the desired pulse and chase incubation.

2. Detach and homogenize cells as described in **Subheading 3.4., steps 2–4,** to obtain 80–85% lysis (*see* **Note 11**).
3. Isolate phagosomes containing magnetic latex beads with a magnetic particle concentrator (Dynal, Great Neck, NY). Wash the phagosomes three times in 2 mL of homogenization buffer. Examine the phagosomes under the microscope to ensure that there are no intact cells contaminating the magnetic phagosome preparation (*see* **Note 13**).

3.6. Percoll Density Gradient Fractionation for Ag-Presenting Organelle Assays and Biochemical Analysis

Percoll density gradient fractionation maybe a preferred method to isolate phagosomes for some applications, since sucrose gradient fractions introduce deleterious amounts of sucrose into subsequent T-cell assay steps. The percentage of Percoll used in this technique determines the degree of separation of the different organelles. For example, subcellular fractionation of murine macrophages containing latex bead phagosomes on a 23% Percoll gradient ensures better separation of latex bead phagosomes from MIIC compartment, while a 20% Percoll gradient ensures better separation of latex bead phagosomes from plasma membrane ***(5)***. This technique has been used for the isolation of MTB phagosomes for analysis by flow organellometry or SDS-PAGE/Western blotting ***(7)***.

3.6.1. Fractionation of Latex Bead Phagosomes for Ag-Presenting Organelle Assay

Three 6-well plates containing confluent macrophage cultures (3×10^6 cells/well) are needed for each fractionation.

1. Incubate cells with Ag-conjugated latex beads to achieve the desired pulse and chase incubation.
2. Detach and homogenize cells as described in **Subheading 3.4., steps 2–4**, to obtain 80–85% lysis (*see* **Note 11**).
3. Remove intact cells and nuclei by centrifugation at 200*g* for 10 min at 4°C and collect supernatant in a 6-mL capped tube.
4. Pellet phagosomes by centrifugation at 850*g* for 10 min at 4°C (save supernatant containing nonphagosomal membranes for the next step). Wash phagosomal pellet three times in 2 mL homogenization buffer and resuspend in 1 mL of homgenization buffer to produce the crude phagosome preparation.
5. From the supernatant from **step 3**, pellet the nonphagosomal membranes by centrifugation at 36,000*g* for 35 min in a Ti50 fixed-angle rotor (Beckman Coulter). Resuspend pellet in 1 mL of homogenization buffer using a Dounce homogenizer (20 strokes) to produce the nonphagosomal membrane sample.

6. Meanwhile, place 9 mL of 20 and 23% Percoll solution in two separate ultracentrifuge tubes (e.g., Beckman Coulter polycarbonate centrifuge tube).
7. Gently layer the phagosomes on top of the 20% Percoll solution and the nonphagosomal membranes on top of the 23% Percoll solution.
8. Centrifuge at 4°C for 60 min at 36,000*g* in a Ti50 fixed-angle rotor (Beckman Coulter) with minimum speed for acceleration and no or minimum brake on for deceleration.
9. Take out 330 µL (two 165-µL aliquots) and place into individual wells in a 96-well flat-bottom plate. You should obtain 30–31 fractions from this entire gradient (*see* **Note 14**).
10. If samples are to be used in a T cell assay, 10-, 30-, or 50-µl aliquots of the fractions should be plated in a separate 96-well flat-bottom plate.
11. If samples are to be analyzed for β-hexoseaminidase, 50-µL aliquots of the fractions should be plated in separate 96-well flat-bottom plate.
12. All of the samples should be wrapped in plastic wrap and stored at –80°C for further analysis.

3.6.2. Fractionation of MTB Phagosomes for Ag-Presenting Organelle Assay

Subcellular fractionation of murine macrophages containing MTB phagosomes on a 40% Percoll gradient ensures better separation of MTB phagosomes from MIIC compartment, while the distribution of phagosomes and MIIC do overlap on 27% Percoll gradients. However, the use of 40% Percoll complicates some procedures, such as fixation and pelleting of phagosomes for flow organellometry. Therefore, for isolation of MTB phagosomes for flow organellometry, differential centrifugation is used to separate phagosomes from smaller membrane structures (e.g., MIIC), followed by fractionation on 27% Percoll gradients (see next section). Both of these approaches provide extremely pure preparations of MTB phagosomes (<0.015% plasma membrane contamination *(7)*). Three 6-well plates containing confluent macrophage cultures (3×10^6 cells/well) are needed for each fractionation.

1. Incubate cells with MTB (MOI = 40) to achieve the desired pulse and chase incubations.
2. Detach and homogenize cells as described in **Subheading 3.4., steps 2–4**, to obtain 80–85% lysis.
3. Transfer homogenate to a 15-mL tube and remove intact cells and nuclei with three consecutive spins at 200*g* for 5 min at 4°C. Collect supernatant containing phagosomes.
4. Meanwhile, place 9 mL of 40% Percoll solution in an ultracentrifuge tube (e.g., Beckman Coulter polycarbonate centrifuge tube, Beckman Coulter Inc, Fullerton, CA).
5. Gently layer supernatant containing phagosomes on top of the Percoll solution.

6. Centrifuge at 4°C for 60 min at 36,000*g* in a Ti50 fixed-angle rotor (Beckman Coulter) with minimum speed for acceleration and no brake on for deceleration.
7. Fractionate the gradient as described in **Subheading 3.6.1., steps 9–12**, and freeze aliquots of the samples at –80°C for further analysis.

3.6.3. Modification of Percoll Density Gradient Factionation Protocol for Isolation of MTB Phagosomes for Flow Organellometry or Western Blot Analysis

Two 6-well plates containing confluent macrophage cultures are needed for each fractionation.

1. Incubate cells with FLUOS-labeled (for flow organellometry) or unlabeled MTB (MOI = 40) and homogenize as described in **Subheading 3.6.2.**, **steps 1–3**.
2. Pellet phagosomes from the supernatant at 500*g* for 15 min at 4°C, and resuspend in 1 mL of homogenization buffer.
3. Meanwhile, place 9 mL of 27% Percoll solution in ultracentrifuge tube.
4. Gently layer supernatant containing pelleted phagosomes on top of the Percoll solution. Centrifuge sample and fractionate gradient as described in **Subheading 3.6.2.**, **steps 6–7**. Phagosomes will appear near the bottom of the gradient. Fix and analyze phagsoomes by flow organellometry (*see* **Subheading 3.8.2.**) or dilute the sample at least threefold in PBS and pellet by centrifugation at 10,000 rpm for 5 min in an Eppendorf centrifuge for 5 min (*see* **Note 12**). Store sample at –80°C for analysis by Western blotting (**Subheading 3.9.**).

3.7. Biochemical Analyses of Subcellular Fractions

Percoll density gradient fractions (generated in **Subheading 3.6.**) can be biochemically analyzed to identify fractions containing plasma membrane, phagosomes, lysosomes (β-hexoseaminidase), or the MHC class II compartment (MIIC). An example of biochemical analyses of subcellular fractions is shown in **Fig. 2**.

3.7.1. Plasma Membrane

1. To identify fractions containing plasma membrane, the plasma membrane has to be marked prior to homogenization of cells. Label cells for 60 min at 4°C with ^{125}I-labeled Ab that specifically recognizes molecules expressed on the cell surface (e.g., anti-MHC II Abs). Wash wells extensively (four to five times) prior to detachment and homogenization. Analyze 50-µL aliquots of each subcellular fraction for radioactivity.
2. Alternatively, label plasma membrane with 0.5 µg/mL sulfoavidin-biotin at 4°C for 30 min. Wash wells (three times) and incubate with 10 µg/mL streptavidin-FITC at 4°C for 40 min prior to detachment and homogenization. Transfer 50-µL

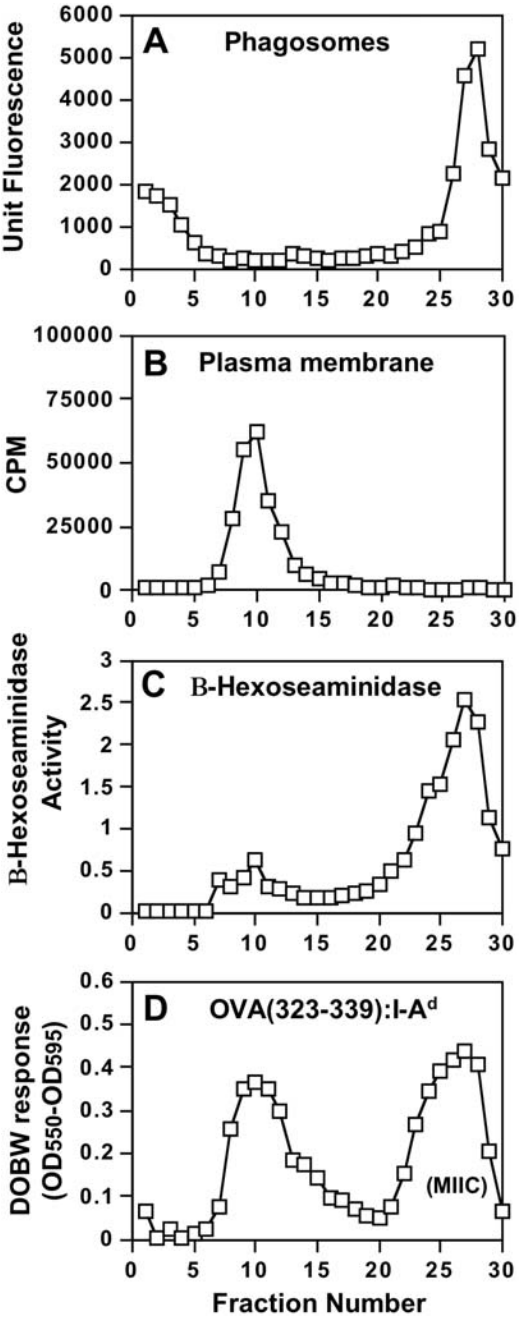

Fig. 2. Characterization of subcellular organelles (inluding MTB phagosomes) isolated on 27% Percoll density gradients. Macrophages were incubated with soluble

aliquots of each fraction to a 96-well, clear-bottom black plate (Costar, Cambridge, MA) and analyze for fluorescence (*see* **Note 15** for additional alternative).

3.7.2. Phagosomes

1. Phagosomes can be identified by incubating cells with fluorescent latex beads or FLUOS-labeled MTB.
2. Transfer 50-µL aliquots of each subcellular fraction to a 96-well, clear-bottom black plate (Costar) and analyze for fluorescence with a fluorimeter (e.g., Spectra Fluor Plus fluorimeter, Tecan, UK).

3.7.3. β-Hexoseaminidase

β-Hexoseaminidase activity is measured as a representation of lysosomal enzyme distribution.

1. To 20- or 45-µL aliquots of each subcellular fraction add 150 µL of assay buffer and 50 µL of substrate and incubate for 90 min at 37°C. Transfer 100 µL of the reaction samples to a replicate plate containing 100 µL of stop solution.
2. Determine optical density of the samples at 405 nm.

3.7.4. MIIC

To identify the MIIC compartment where peptide:MHC II complexes (derived from the processing of soluble Ag) are formed, macrophages should be incubated with a soluble Ag for 1 h prior to the addition of beads. The soluble Ag to be used in these experiments should be distinct from the particulate Ag and should be determined by the Ag specificity of the T-cell hybridoma available for the project. For example, murine macrophages expressing the MHC II molecules I-A^d or I-A^b can be incubated with OVA, and OVA peptide:I-A^d or I-A^b complexes can be subsequently detected using DOBW T hybridoma cells ***(5)***. This approach reveals all membranes bearing peptide–MHC II complexes,

Fig. 2. *(Continued)* OVA (3 mg/mL) for 1 h, incubated with fluorescein-labeled heat-killed MTB and OVA for 20 min, washed, chased for 30 min at 37°C in the presence of soluble OVA, homogenized and fractionated on 27% Percoll gradients. **(A)** Distribution of MTB phagosomes detected by fluorimetry. **(B)** Distribution of plasma membrane radioactivity when macrophage plasma membranes were labeled with ^{125}I-Y-3P (anti-I-A^b) at 4(C prior to fractionation. **(C)** β-Hexosaminidase activity (a marker of lysosomal enzyme distribution). **(D)** Distribution of OVA(323-339):I-A^d complexes assessed by DOBW T hybridoma assay. (Adapted from Ramachandra et al., 2001, with permission from the Journal of Experimental Medicine, The Rockefeller University Press.)

including plasma membrane as well as MIIC (depending on the kinetics of the experiment), but should not label phagosomes. Dense fractions labeled by this approach represent MIIC.

1. Incubate cells in media containing OVA (3 mg/mL, 2 mL/well) for 1 h. Wash cells two times with 5 mL of PBS prior to the addition of particulate Ag or homogenization of cells.
2. Analyze fractions for OVA peptide: I-A^d or I-A^b complexes by the antigen presenting organelle assay (*see* **Subheading 3.10.**).

3.8. Analysis of Phagosomes by Flow Organellometry

3.8.1. Latex Bead Phagosomes

Macrophages (10^7 cells/well) growing in three wells of a 6-well plate are sufficient to generate enough phagosomes for flow organellometry as shown in **Fig. 3A**.

1. Incubate cells with nonfluorescent latex beads conjugated to Ag (10 µL in 4 mL media/well) to achieve the desired pulse and chase incubation.
2. Detach and homogenize cells and follow **steps 2–5** in **Subheading 3.4.** to generate a crude phagosome preparation. Pellet the phagosomes at 1900*g* for 10 min at 4°C and resuspend pelleted phagosomes in 100 µL PBS (pH 7.4).
3. Fix the phagosomes by adding 100 µL of 2% paraformaldehyde and incubating for 10 min at room temperature. Add equivalent volume of 0.4 *M* lysine (200 µL) and immediately pellet the phagosomes at 1900*g* for 10 min at 4°C. Wash the phagosomes once in 1 mL of PBS and resuspend in 1 mL of PBS.
4. Transfer 80–100 µL of the fixed phagosome preparation into 96-well round-bottomed plates and pellet phagosomes at 1900*g* for 10 min.
5. Gently resuspend phagosomes in 50 µL of FACS buffer containing the desired Ab or isotype control Ab (e.g., anti-MHC II or anti-LAMP-1 or LAMP-2 Abs). FACS buffer contains saponin to permeabilize the phagosome and allow access to luminal epitopes. Incubate plate on ice in the dark for 30 min (*see* **Note 16**).
6. Add 200 µL of FACS buffer to each well to dilute the Abs. Spin plate, remove supernatant, and wash the wells two more times with 250 µL of FACS buffer.
7. Gently resuspend phagosomes in 50 µL of FACS buffer containing the desired secondary Ab and incubate plate on ice in the dark for 30 min.
8. Wash phagosomes three times as described above and resuspend phagosomes in 100 µL of PBS. Add 100 µL of 2% paraformaldehyde into each well and store samples at 4°C until ready to analyze (*see* **Note 17**).
9. Distinct optical properties of the latex bead phagosomes allow the phagosomes to be easily identified by flow analysis using a narrow gate based on scatter properties ***(4)***.

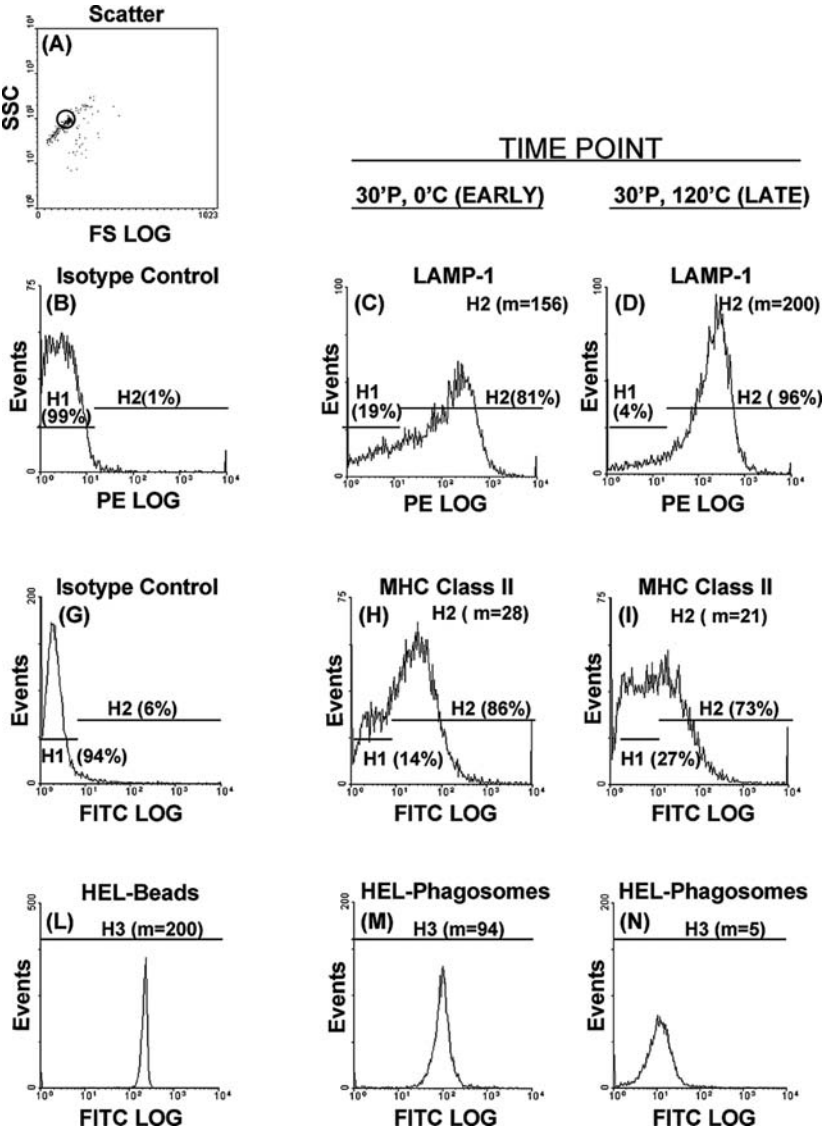

Fig. 3. Flow organellometry. **(A)** Analysis of latex bead phagosomes: BMM were pulsed with latex-HEL beads for 30 min at 37°(C, followed by no chase (30'P, 0'C) or a chase incubation of 120 min (30'P, 120'C) at 37°C. Phagosomes were isolated, fixed with paraformaldehyde, permeabilized with saponin, stained for LAMP-1, MHC-II, or HEL, and analyzed by flow organellometry. Gating by optical scatter parameters was used to select single-bead phagosomes (gate indicated in **A**) for immunolabeling analysis. **(B–D)** Staining of phagosomes with rat IgG2a isotype control Ab or with rat MAb ID4B, specific for LAMP-1. **(E–G)** Staining of phagosomes with murine

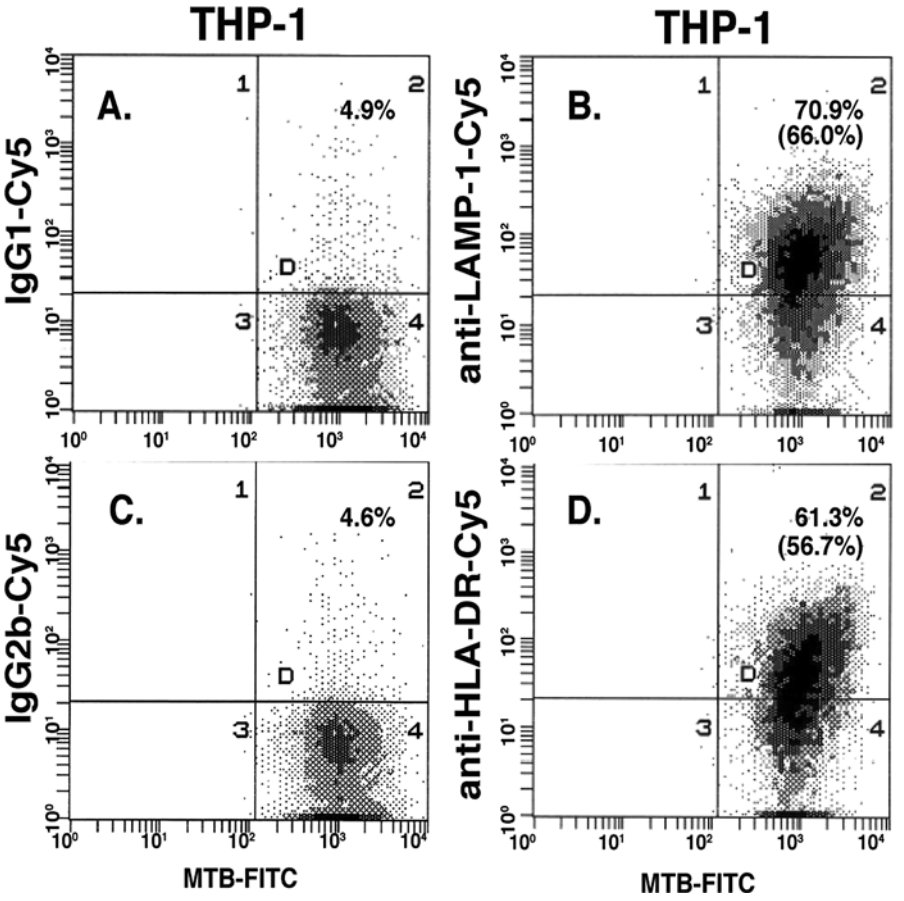

Fig. 3. *(Continued)* IgG2a and IgG2b isotype control Abs or with murine MAbs 10.3.6.2 and H116-32, against I-A^k. **(H)** Staining of latex-HEL beads with murine MAb 1B12, against HEL. **(I, J)** Staining phagosomes with murine MAb 1B12. H1 and H2 are gates that were identified following staining with control Ab (H1: positive staining; H2: negative staining). H3 represents all gated events (adapted from Ramachandra et al., 1998, with permission from Elsevier). **(B)** Analysis of MTB phagosomes: THP-1 cells were incubated with fluorescein-labeled MTB for 20 min and chased for an additional 30 min at 37°C. Phagosomes were purified on 27% Percoll gradients, fixed with paraformaldehyde, permeabilized with saponin, and stained for MHC-II (Cy5-labeled anti HLA-DR) or LAMP-1 and analyzed by flow organellometry. Gating FITC-positive events was used to select for MTB phagosomes. MHC-II and LAMP-1 were detected using MAb, and negative-control staining with isotype-matched control Abs was used to define the quadrants. (Adapted from Torres et al., 2006, with permission from Infection and Immunity, American Society of Microbiology.)

3.8.2. MTB Phagosome

FLUOS-labeled MTB phagosomes are used for flow organellometry. An example of flow analysis of MTB phagosomes is shown in **Fig. 3B**.

1. FLUOS-labeled MTB phagosomes are isolated as described in **Subheading 3.6.3.** Transfer the fractions containing phagosomes (can be identified visually) to a clear 6-mL tube with cap. Determine the volume of the phagosome sample.
2. Fix the phagosomes by adding an equivalent volume (x) of 2% paraformaldehyde and incubating for 10 min at room temperature. Add 2x volume of 0.4 *M* lysine and immediately pellet the phagosomes at 1900g for 10 min at 4°C. Wash the phagosomes twice in 1 mL of cold PBS and resuspend in 0.5 mL of PBS.
3. Stain MTB phagosomes with desired Abs exactly as described above for latex bead phagosomes **(Subheading 3.8.1., steps 4–9)**.
4. FLUOS-labeled MTB phagosomes can be easily identified by flow analysis by gating for FITC-positive events *(7)*.

3.9. Analysis of Phagosmes by SDS-PAGE/Western Blotting

Latex bead phagosomes isolated by sucrose density gradients **(Subheading 3.4.)** or MTB phagosome isolated on Percoll density gradients **(Subheading 3.6.2.)** can also be analyzed by SDS-PAGE/Western blotting. An example of a Western blot analysis of latex bead phagosomes is shown in **Fig. 4**.

1. To the phagosome pellet, add 20–50 µL of lysis buffer.
2. Keep the sample on ice for 30 min. Occasionally tap the tube to disperse contents.
3. Spin the sample in an eppendorf centrifuge at 10,000 rpm for 10 min at 4°C and transfer supernatant to a fresh tube.
4. Add SDS-PAGE buffer and boil supernatants. Analyze by SDS-PAGE/Western blotting. Immediately freeze aliquots of unused samples at –80°C. When comparing samples by SDS-PAGE, known concentrations of protein (e.g., 10 µg) or proteins extracted from the same number of phagosomes or cells can be loaded in the wells.

3.10. Ag-Presenting Organelle Assay

Ag-presenting organelle assay is used to analyze presence of peptide:MHC II complexes in Percoll density gradient fractions isolated and aliquoted as described in **Subheadings 3.6.1.** and **3.6.2.**, or in magnetic latex bead phagosomes isolated as described in **Subheading 3.5**. To disrupt organelle membranes and allow T cells access to the luminal MHC II Ag-presenting domains, freeze and thaw fractions prior to assay. An example of an assay with Percoll density gradient fractions containing MTB phagosomes is shown in **Fig. 5**.

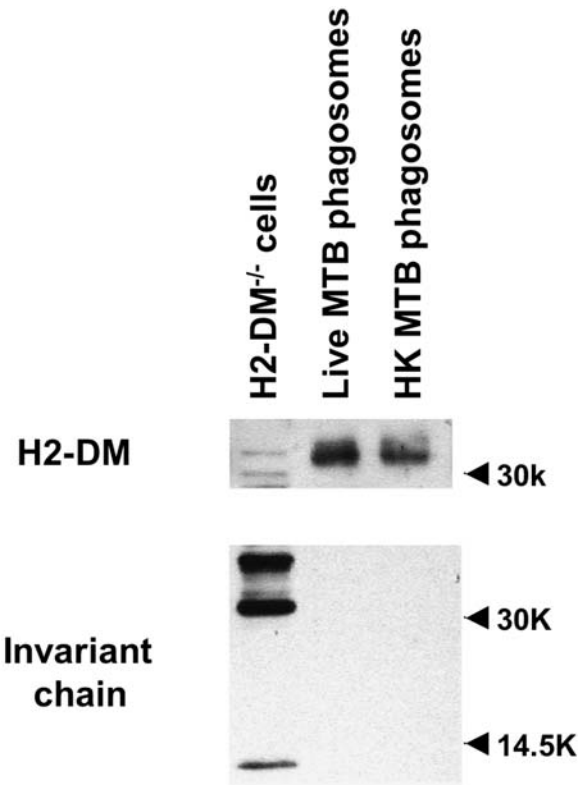

Fig. 4. Western blot analysis of MTB phagosomes for components of the MHC-II processing pathway, H-2M, and invariant chain. MTB phagosomes (20 min pulse, 10 min chase) were purified by differential centrifugation and isolated on 40% Percoll density gradients. Purified phagosomes or whole cells were solubilized, and the resulting samples were boiled under reducing conditions, subjected to SDS-PAGE and blotted onto membranes. The blots were probed with antiserum, specific for the alpha chain of H-2M, stripped and reprobed with the MAb (In-1), specific for invariant chain. Abs were detected by chemiluminescence.

1. Freeze and thaw aliquots of the fractions or magnetic latex bead phagosomes three times in a –80°C freezer, prior to starting the assay.
2. Add medium and T hybridoma cells (10^5/well) to a final volume of 200 µL (*see* **Note 8**).
3. Incubate plate at 37°C for 24 h. Harvest supernatants (100 µL) and assay for IL-2 by the CTLL assay **(Subheading 3.3.1.)** or by using a commercially available IL-2 ELISA kit. Generate control supernatants for the IL-2 assays by adding only Percoll, T hybridoma cells, and medium to wells.

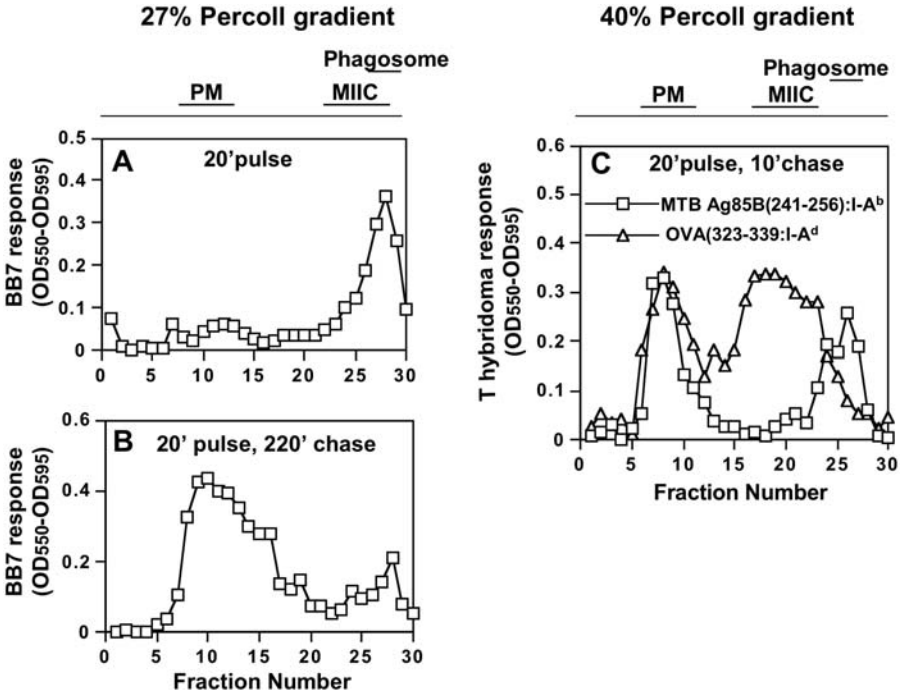

Fig. 5. Ag 85B(241-256), I-A^b complexes are initially found in MTB phagosomes and later appear on the plasma membrane. **(A, B)** Macrophages were pulsed with HK MTB (MOI = 40) for 20 min, washed, chased for various periods, and fractionated on 27% Percoll density gradients. **(C)** Macrophages were incubated with soluble OVA for 1 h and then pulsed with HK MTB and OVA for 20 min, washed, chased for 10 min at 37°C in the presence of OVA, and fractionated on 40% Percoll density gradients. Aliquots (50 μL) of each fraction were frozen, thawed, and analyzed for Ag 85B(241-256):I-A^b and OVA(323-339):I-A^d complexes using BB7 and DOBW T hybridoma cells, respectively. Diagrams at the top summarize the positions of different compartments in the Percoll gradients. PM, plasma membrane. (Adapted from Ramachandra et al., 2001, with permission from the Journal of Experimental Medicine, The Rockefeller University Press.)

4. Notes

1. Cocktails of protease inhibitors are available (e.g., from Sigma) and can be used in the homogenization buffer. Additional protease inhibitors can also be added than the ones suggested in the protocol. PMSF is rapidly inactivated in aqueous buffers. Most other protease inhibitors are stable for only a few hours. Therefore, add protease inhibitors to the homogenization buffer just before homogenization.
2. It is easier to dissolve high concentrations of sucrose in 10 m*M* HEPES buffer by warming the solution in a 37–50°C water bath. This also decreases the viscosity

of the sucrose solutions and significantly decreases the time required to filter sterilize them.

3. Procedure must be done in a fume hood using nitrile gloves and respirator. When dissolving paraformaldehye in PBS it is important to ensure that the temperature of the solution does not exceed 56°C.
4. Nonidet P-40 and other detergents are usually viscous. To pipet accurate volumes of any viscous detergent, cut off the ends of the tip and withdraw the detergent slowly to obtain the desired volume.
5. *Listeria monocytogenes* (LM) is a human pathogen and should be treated with caution. LM should not be handled by pregnant women. The dose of LM suggested in the protocol is for CBA/J and C57Bl/6 strains of mice. The amount of LM that should be administered to other strains of mice should be determined by individual investigators.
6. PECs generated with Con A are not as activated as those generated using LM. PECs can also be elicited with other reagents, e.g., thioglycolate. However, thioglycolate-elicited PECs do not express high levels of MHC II.
7. To warm up a centrifuge lacking a heating device, keep rotor spinning at the maximum speed allowed for at least 25–30 min.
8. T-cell lines can be used instead of T hybridoma cells. When using T-cell lines in an Ag-presenting organelle assay, fractions should be supplemented with anti-CD28 Ab at a concentration of 2 μg/mL.
9. If no IL-2 is detected in supernatants (or in some of the wells) in T-cell assays using the fixative paraformaldehyde, it is highly probable that not all the paraformaldehyde was successfully removed from the wells. In subsequent experiments plates can be briefly spun in the centrifuge after the fixation step and washed a couple of additional times to remove excess paraformaldehyde.
10. Use a 5-mL pipet to wash wells. Do not use a 1-mL pipet tip as this will result in high loss of cells.
11. Homogenization of cells is a crucial step in the isolation of intact phagosomes. Homogenization needs to be done gently to prevent loss of phagosomal membranes. Number of strokes needed to achieve 80–85% lysis will vary from person to person and has to be carefully standardized. Individual who need less than 25 strokes to achieve this percentage of lysis may be applying too much force and may isolate phagosomes lacking phagosomal membranes.
12. It is essential to maximize yields by taking the following precautions. Repeatedly pellet phagosomes in a single Eppendorf tube to minimize loss. Remember to place Eppendorf tube in the same orientation in centrifuge during each spin to ensure phagosomes pellet in the same region of the centrifuge tube.
13. If intact cells repeatedly appear in the phagosome preparation, consider homogenizing the cells to achieve a greater percentage of lysis. However, this may result in loss of phagosomal membrane.
14. Use density marker beads (Pharmacia, Uppsala, Sweden) and manufacturer's instructions to check Percoll gradient.

15. An additional alternative is to use an endogenous plasma membrane marker (e.g., 5′-nucleotidase) ***(15)***.
16. Directly conjugated Abs can be used for flow analysis of phagosomes. However, since the signal can be low, use of primary and secondary Abs may help amplify the signal.
17. Phagosomes should be analyzed by flow organellometry within 48 h.

Acknowledgments

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