# Quantitative analysis of phagolysosome fusion in intact cells: inhibition by mycobacterial lipoarabinomannan and rescue by an 1α,25-dihydroxyvitamin D3–phosphoinositide 3-kinase pathway

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## Summary

**Macrophage cell membranes were labeled with PKH26 and subsequently incubated with latex beads to generate phagosomes surrounded by a red-fluorescent membrane suitable for flow cytometry. Following cell disruption and partial purification of phagosomes, these vesicles were readily distinguished from both cell debris and free beads released from disrupted vacuoles. Flow cytometry analysis of phagosomes stained with specific mAbs and FITC-labeled secondary antibodies showed progressive acquisition of both Rab7 and LAMP-1 consistent with movement along the endocytic pathway. Alternatively, macrophages were preloaded with the lysosomal tracer FITC-dextran before membrane labeling with PKH and incubation with latex beads. Phagosome-lysosome fusion was then quantified on the basis of the colocalization of red and green signals. Using these flow cytometry-based systems, we showed that co-internalization of beads with lysates of *Mycobacterium tuberculosis*, but not lysates from the nonpathogenic organism *Mycobacterium smegmatis*, markedly decreased phagosome acquisition of Rab7 and LAMP-1 and vesicle fusion with FITC-dextran-loaded lysosomes. Inhibition of phagolysosome fusion could be attributed, at least in part, to the mycobacterial cell wall glycolipid lipoarabinomannan, and further analysis showed complete rescue of phagosome maturation when cells were pretreated with vitamin D3 before exposure to lipoarabinomannan. Moreover, the ability of vitamin D3 to reverse the phenotype of phagosomes in the presence of the glycolipid was completely abrogated by LY-294002, suggesting that vitamin D3 promotes phagolysosome fusion via a phosphoinositide 3-kinase signaling pathway.**

**These findings establish a robust platform technology based on labeling of phagocyte cell membranes and flow cytometry capable of supporting broad-based screens to identify microbial and other bioactive compounds that influence phagosome biology.**

Key words: Flow cytometry, THP-1, PKH26, Phagosome maturation, Mycobacteria, Lipoarabinomannan

## Introduction

Particle internalization by macrophages and other phagocytic cells results in the generation of phagocytic vacuoles. The usual fate of such vacuoles is progressive maturation along the endocytic pathway leading to fusion with late endosomes and ultimately lysosomes where ingested material is degraded (Desjardins et al., 1994; Russell, 1995). In contrast to this normal sequence of events, phagosome maturation arrest leading to the failure of vacuoles to fuse with lysosomes is a frequent finding following the internalization of pathogenic mycobacteria (Via et al., 1997; Clemens et al., 2000; Pieters, 2001).

Seminal studies on the biogenesis of the mycobacterial phagosome have shown that vacuoles containing viable mycobacteria lie within the sorting/recycling endosomal pathway and are resistant to fusion with late endocytic compartments (Armstrong and D'Arcy Hart, 1971; Frehel et al., 1986; Xu et al., 1994; Clemens and Horwitz, 1995). Mycobacterial phagosomes display the small GTPase Rab5, a marker of fusion with early endosomes, but fail to acquire Rab7 and cathepsin D, markers of fusion with late endosomes and lysosomes (Via et al., 1997; Clemens and Horwitz, 1995). The mycobacterial vacuole also excludes the vacuolar proton-ATPase that is essential for acidification along the endocytic pathway (Sturgill-Koszycki et al., 1994). Inhibition of phagosomal maturation appears to be an active process given that killed mycobacteria are readily transported to endosomal/lysosomal organelles (Hasan et al., 1997; Ferrari et al., 1999; Tailleux et al., 2003).

The molecular mechanisms involved in inhibition of phagosome maturation by mycobacteria are not completely understood, but probably involve both host and bacterial factors. It appears that viable, pathogenic mycobacteria transfer various proteins and glycolipids into the endocytic network of the host cell where they may interfere with phagosome maturation (Beatty and Russell, 2000; Neyrolles et al., 2001; Beatty et al., 2001). Some of these factors are induced in response to stress, perhaps related to conditions of relatively low pH and oxidative stress within the vacuole, and are potential virulence factors (Lee and Horwitz, 1995; Fisher et al., 2002). Further insight into the mechanisms responsible for phagosome maturation arrest during intracellular infection would be facilitated by more robust approaches to study phagosome trafficking.

Historically, two principal approaches have been used to analyze phagosome composition. One approach consists of cell disruption and differential gradient centrifugation to yield highly purified phagosomes for subsequent western blot analysis (Chakraborty et al., 1994; Burkhardt, 1998). This approach requires a large number of phagocytic cells (up to $10^7$-$10^8$ per sample) and is labor intensive, requiring vacuole purification using percoll or sucrose gradient centrifugation. The second approach involves examining intact cells by either fluorescence or electron microscopy after phagocytic challenge. Direct examination of intact cells has several advantages, including the potential for high resolution and the need for relatively small amounts of starting material (Clemens and Horwitz, 1995; Vitelli et al., 1997; Vieira et al., 2001; Tailleux et al., 2003). However, only limited numbers of cells and phagosomes can be examined. Flow cytometry-based methods offer the potential for important advances in the rapid and quantitative assessment of the composition and maturation of phagosomes (Meresse et al., 1997; Steele-Mortimer et al., 1999; Scianimanico et al., 1999; Ramachandra and Harding, 2000). Here, we report the development of a new approach to prepare labeled phagosomes using a red fluorescent cell membrane linker PKH26 that is incorporated in the cell's lipid bilayer. This results in the generation of phagosomes surrounded by a red fluorescent membrane. Analysis of labeled phagosomes by flow cytometry provided a rapid approach to examine directly whether subcellular fractions of mycobacteria could influence phagosome maturation and the mechanisms involved. Using this novel approach, we present findings below showing that cell wall preparations from pathogenic mycobacteria are capable of attenuating phagosome maturation and fusion with lysosomes. Moreover, analysis of labeled phagosomes by flow cytometry proved to be a sensitive and quantitative approach to evaluate the abilities of anti-mycobacterial agents such as 1α,25-dihydroxyvitamin $D_3$ (VitD3) and interferon (IFN)-γ to promote phagosome maturation.

## Materials and Methods

### Reagents and chemicals

RPMI 1640, HBSS, PMA, PKH26, PMSF, protease inhibitor mixture and trypsin-EDTA were obtained from Sigma-Aldrich (Oakville, ON, Canada). Latex beads (1, 2 and 4 μm diameter) were from Interfacial Dynamics (Portland, OR). Rabbit anti-Rab5 and rabbit anti-Rab7 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human LAMP-1 mAb (H4A3) was from the Hybridoma Bank of the University of Iowa (Iowa city, IA) and anti-pleckstrin mAb was from BD Biosciences (San Diego, CA). FITC-conjugated dextran (F-DXT), FITC-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were from Sigma. Human rIFN-γ was a generous gift from Genentech (South San Francisco, CA). VitD3 and LY-294002 were from Calbiochem (San Diego, CA).

### Differentiation and PKH staining of THP-1 cells

The monocytic cell line THP-1 (ATCC, Rockville, MA) was cultured in RPMI 1640 supplemented with 5% FCS (Life Technologies, Burlington, ON, Canada), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were seeded at a density of $10^5$ per $cm^2$ in 6 cm diameter culture dishes (Corning, NY) and allowed to adhere and differentiate in the presence of PMA (20 ng/ml) at 37°C in a humidified atmosphere of 5% $CO_2$ for 24 hours. Cells were then washed three times with HBSS and adherent monolayers were incubated with 20 μM PKH26 for 5 minutes at room temperature. Cells were washed and immediately used for assays.

### Phagosome preparation and staining

Latex beads were added to PKH26-labeled THP-1 cells in complete RPMI (ratio of beads to cells of 5:1) and plates were incubated 30 minutes at 4°C to allow attachment of the beads to the cell surface and to synchronize phagocytosis. Cells were then washed and transferred to 37°C in a humidified, 5% $CO_2$ atmosphere for various time periods. Partially attached, noningested beads were removed by a 5 minute treatment with trypsin-EDTA and extensive washing with HBSS. This resulted in a phagocytosis rate of ~90% with an approximate range of one to five particles per cell. Cells were scraped in a hypotonic homogenization buffer consisting of 20 mM HEPES, 0.5 mM EGTA, 0.25 M sucrose and 0.1% gelatin (Chakraborty et al., 1994) and lysed by repeated passage (5-10 times) through a 1 ml syringe fitted with a 23-gauge needle. Cell lysis was monitored by light microscopy to the point where at least 70% of the cells were disrupted. Homogenates were centrifuged at 150 ***g*** for 5 minutes to pellet intact cells and nuclei and supernatants were centrifuged 5 minutes at 13,000 ***g*** to pellet phagosomes. Phagosomes were permeabilized in RPMI containing 0.2% saponin and 10% normal goat serum for 5 minutes and incubated with specific antibodies to endosomal/lysosomal markers in the same buffer for 30 minutes at room temperature. Cells were then washed and stained with FITC-labeled goat secondary antibodies in the same buffer.

### Flow cytometry

Phagosome fluorescence was analyzed using a Coulter Elite flow cytometer (Hialeah, FL) equipped with an argon laser emitting at 488 nm for the excitation of PKH and FITC (emission at 551 nm and 525 nm, respectively). Flow was adjusted to ~250-300 events per second, thereby limiting the generation of coincidental events. Phagosomes containing latex beads were identified by SS (side scatter), FS (forward scatter) and FL2 (red fluorescence) parameters. Relative fluorescence intensities of 5000 phagosomes were recorded as single SS, FS, FL2 and FL1 (green fluorescence) histograms (log scale, 1024 channels and 4 decades). Results were expressed as dual parameter dot blots (SS/FS or FL1/FL2) and as single FL1 fluorescence histograms. Mean fluorescence intensities (MFI) were determined for single fluorescence histograms.

### Fluorescence microscopy

PMA-treated THP-1 cells were adhered to cell culture-treated coverslips (Fisher Scientific, Nepean, ON, Canada) in 24-well plates, labeled with PKH26 and exposed to latex beads. After phagocytosis,

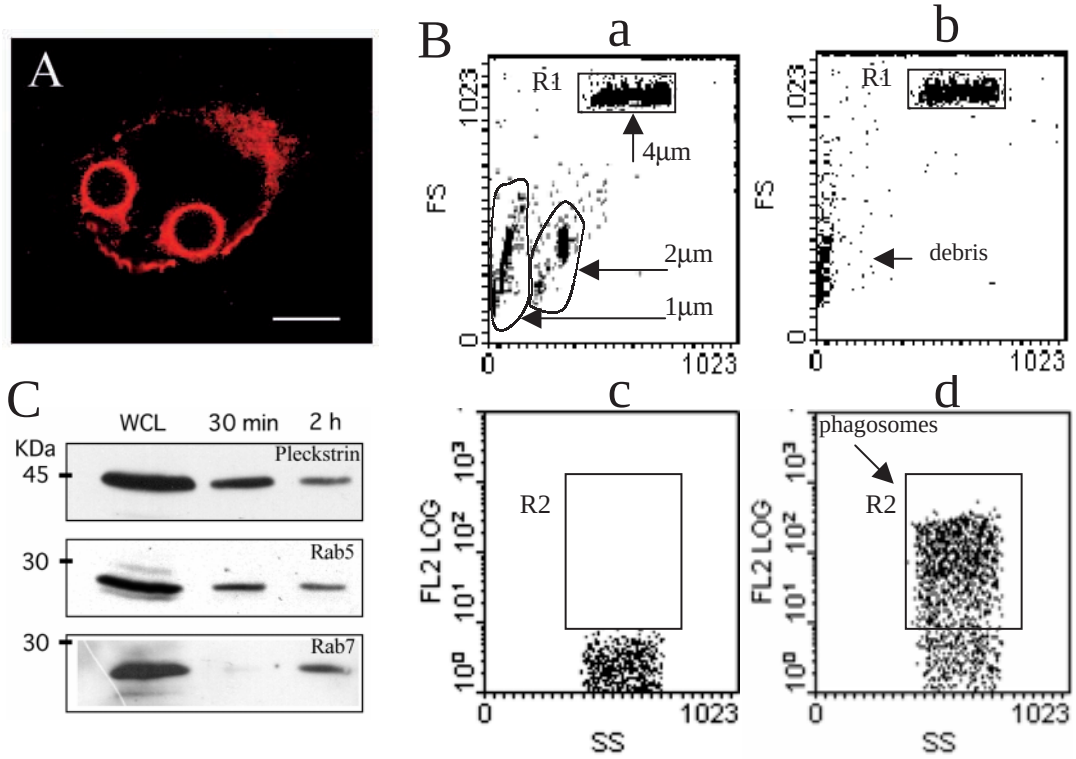



**Fig. 1.** Flow cytometry analysis of latex bead phagosome. (A) PKH-labeled THP-1 cells adherent to coverslips and ingesting latex beads (4 μm) were fixed and mounted on microscopy slides. Samples were examined at the optimal wavelengths for PKH26 (551 excitation, 567 nm emission) using a PL-Apochromat 63×/1.4 oil immersion objective on a Zeiss Axioplan II microscope. Latex beads are surrounded by a red fluorescent membrane. Bar, 5 μm. (B) In (a), equal volumes of 1, 2 and 4 μm latex beads were mixed and analyzed by flow cytometry. Beads of different sizes were localized by plotting forward (FS) versus side (SS) scatter channels on a linear scale. (b) Postnuclear supernatant from THP-1 homogenate mixed with 4 μm beads and analyzed by flow cytometry using similar SS and FS parameter settings as in (a). Large latex beads (R1 window) were readily discriminated from cell debris. (d) Red fluorescence (FL2) of partially purified phagosomes from PKH labeled cells that have ingested 4 μm latex beads. Events were recorded from the R1 window, which is the predicted position of phagosomes based on the localization of free beads in (b). (c) Fluorescence of latex beads alone (background autofluorescence), allowing for positioning of window R2 in (d) to include signals from true phagosomes only. A comparison of (c) and (d) shows that a significant fraction of latex beads were released from phagosomes during homogenization and centrifugation. (C) Phagosomes were sorted using a Becton Dickinson FACStar plus flow cytometer on the basis of positive red fluorescence (gate R2 shown in Bd); ~5 million sorted early (30 minutes) and late (2 hours) phagosomes were pelleted and phagosome membrane proteins were solubilized in 1×Laemmli buffer. Samples were separated by SDS-PAGE along with 100 μg of total cell lysate (WCL). Proteins were transferred to nitrocellulose membranes and probed with antibodies against pleckstrin, Rab5 and Rab7.

cells were treated with trypsin-EDTA and washed with HBSS to remove noningested beads. Cells were fixed for 15 minutes at 37°C with 2.5% paraformaldehyde/HBSS then washed three times with HBSS and once with distilled water. Coverslips were then mounted in FluorSaveTM (Calbiochem-Novabiochem, La Jolla, CA) to minimize photobleaching. Slides were examined using an epifluorescence microscope (Zeiss Axioplan II) and images were recorded using a CCD camera and Empix software.

### Western blotting

Whole-cell lysates and sorted phagosomes were analyzed by SDS-PAGE and immunoblotting with anti-Rab5 and anti-pleckstrin (early endosomes) and anti-Rab7 (late endosomes). Membrane blots were probed with appropriate HRPO-conjugated secondary antibodies and developed by ECL as described previously (Hmama et al., 1998).

### Mycobacterial preparations

Whole-cell lysates (WCL), cell wall fractions (CW), lipoarabinomannan (LAM) and its precursor phosphatidylinositol mannoside (PIM) from *Mycobacterium tuberculosis* (Mtb) ($H_{37}$Rv strain) were provided by John Belisles [Colorado State University, Ft Collins, CO, through TB Research Materials and Vaccine Testing contract (NIH, NIAID NO1-A1-75320)].

## Results

### Flow cytometry discriminates between latex bead-phagosomes, free beads and cell debris

PKH reagents are lipophilic, nontoxic dyes used widely to label membranes of living mammalian cells, including leukocytes (Horan and Slezak, 1989; Albertine and Gee, 1996; Batard et al., 2000; Hammer et al., 2001; Krause et al., 2001). As shown by fluorescence microscopy (Fig. 1A), PMA-differentiated THP-1 cells displayed surface fluorescence after incubation with PKH, and ingestion of beads by labeled cells resulted in the appearance of phagosomes surrounded by a red fluorescent membrane. Fig. 1B summarizes the degree to which flow cytometry could discriminate among free latex beads and cell debris, both of which were expected to be present as contaminants in the phagosome preparations. SS/FS dot blots ‘a’ and ‘b’ show the positions of latex beads of different sizes relative to each other and to cell debris, indicating that beads of larger size (4 μm compared with 1 and 2 μm diameter) could easily be distinguished from cell debris. Red fluorescent signals (FL2) corresponding to phagosomes isolated from PKH-labeled cells ingesting 4 μm beads were measured in window R1 based on the localization of free latex beads in ‘b’. To subtract beads released from phagosomes during cell disruption, red signals from beads alone

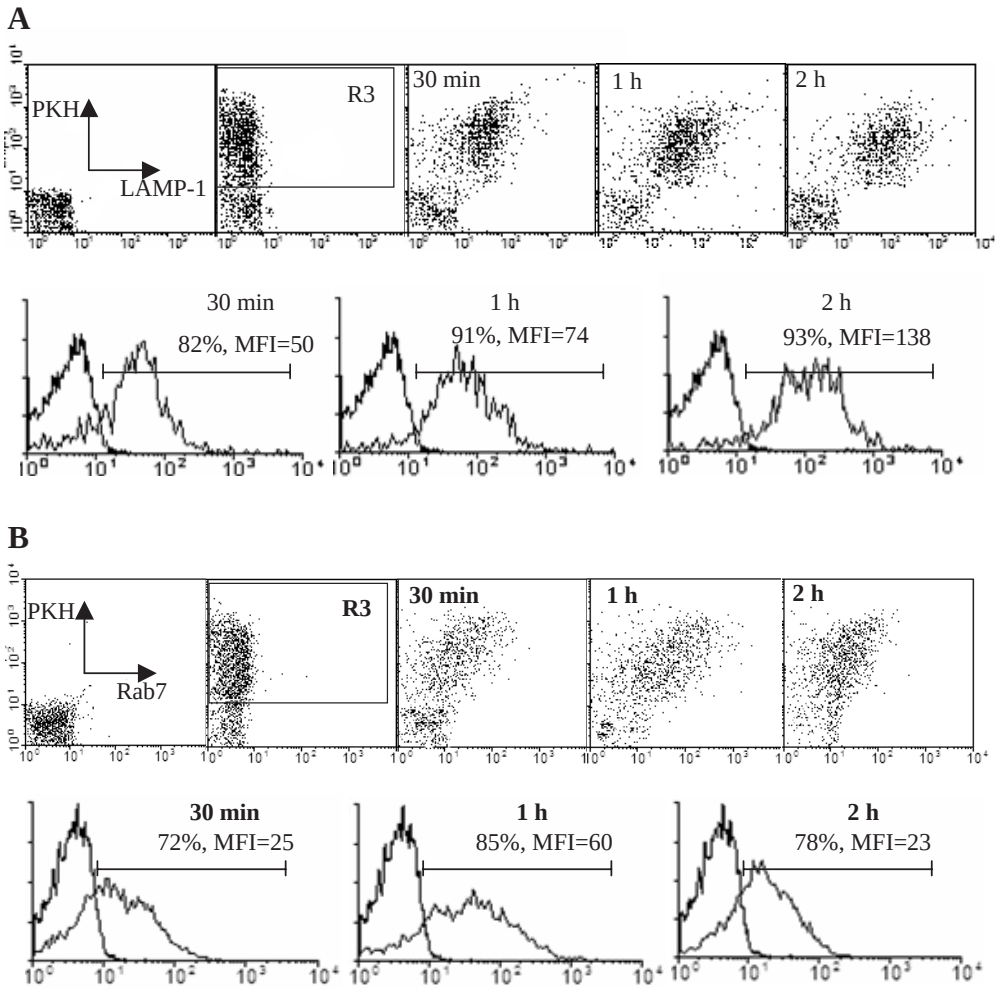


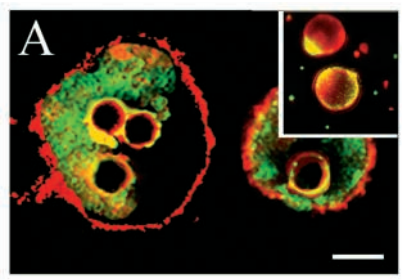

**Fig. 2.** Flow cytometry analysis allows the quantitative evaluation of phagosome maturation. PKH-stained cells were allowed to ingest latex particles, and phagosomes were prepared after 30 minutes, 1 hour and 2 hours. Phagosome preparations were fixed/permeabilized and stained with irrelevant antibodies or specific antibodies to LAMP-1 (A) or Rab7 (B). Preparations were washed and stained with FITC-labeled secondary antibodies. After adequate green/red compensations, green fluorescence was analyzed on positive red fluorescent events, which correspond to particles surrounded by a red phagosomal membrane (gate R3), and the results were expressed as green fluorescence histograms. In each panel, the histogram on the left represents phagosomes stained with irrelevant antibodies, and the histogram displaced to the right represents phagosomes stained with specific antibodies. The proportion of phagosomes expressing LAMP-1 (A) and Rab7 (B) increases as a function of maturation. Values represent the averages of two independent experiments.

(autofluorescence) were recorded in dot plot 'c', allowing for the positioning of window R2 in 'd' to include signals from true phagosomes only. Thus, the results in Fig. 1B show that the specific optical properties (SS, FS and FL2) of PKH-labeled phagosomes allow their identification without the need for extensive purification and isolation from other organelles and debris. The events within the R2 window were further characterized by sorting and western blotting using antibodies to markers of the endocytic pathway. Fig. 1C shows that pleckstrin and Rab 5, markers of early endosomes (Gorvel et al., 1991; Brumell et al., 1999), were present on phagosomes at 30 minutes post ingestion and were significantly decreased in abundance by 2 hours. By contrast, Rab7, a marker of late endosomes (Feng et al., 1995), was absent from early phagosomes but was detected at 2 hours post phagocytosis.

## Quantitative analysis of phagsome maturation and fusion with lysosomes

Specific antibodies to several markers of phagosome maturation are available and this opens up the potential to accurately analyze phagosome maturation by flow cytometry. To achieve this, PKH-labeled cells were allowed to ingest latex particles for 1 hour at 37°C, and phagosomes were prepared at 30 minutes, 1 hour or 2 hours post phagocytosis. Phagosome preparations were fixed and permeabilized to allow access to intraluminal epitopes and stained with specific antibodies to LAMP-1 (mouse IgG) or Rab7 (rabbit IgG) or with irrelevant, control antibodies (normal mouse or rabbit IgG). Preparations were washed and stained with FITC-labeled secondary antibodies. The dot blots in Fig. 2 show red (FL2) and green (FL1) fluorescence intensities for phagosomes gated based on the light scatter parameters as described in Fig. 1 (1B, gate R1). After adequate green/red compensations, green fluorescence was analyzed on positive red fluorescent events, the latter corresponding to particles surrounded by a phagosomal membrane (gate R3). The results are also expressed as green fluorescence histograms. These show that the frequencies of phagosomes expressing LAMP-1 (Fig. 2A) and Rab7 (Fig. 2B) increased with time. Also, the MFI values indicated that the level of LAMP-1 expressed by phagosomes increased progressively through 2 hours of chase, whereas maximal acquisition of Rab7 was observed at 1 hour and decreased thereafter.

To evaluate phagolysosome fusion in intact cells, THP-1 cells were differentiated in 1% FCS/RPMI with PMA for 18 hours in the presence of 0.1 mg/ml FITC-dextran (F-DXT), then washed and chased for 3-4 hours. F-DXT is a nondegradable, cell-permeable probe that accumulates in lysosomes thereby allowing colocalization of green fluorescent



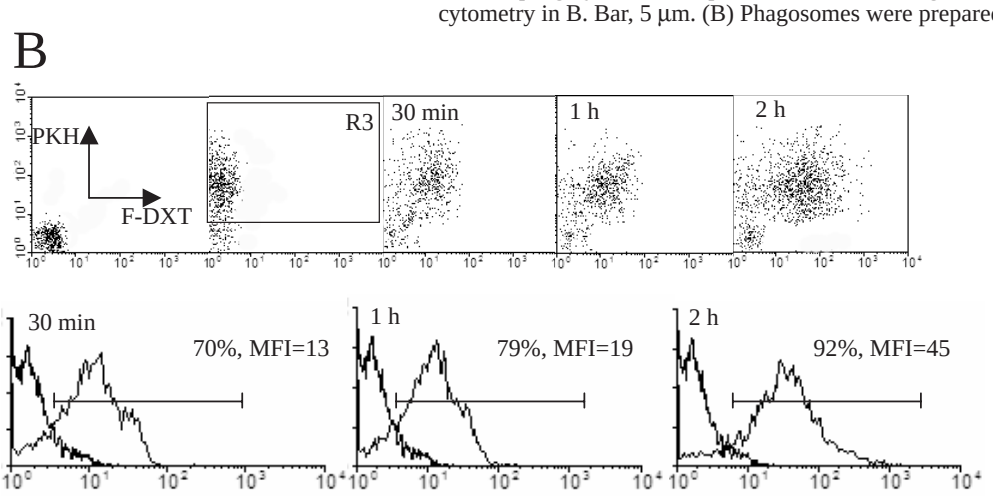




**Fig. 3.** Quantitative analysis of phagosome-lysosome fusion by flow cytometry. THP-1 cells were differentiated with PMA in the presence of 1 mg/ml F-DXT then washed and chased for 4-5 hours. F-DXT-loaded cells were stained with PKH and allowed to ingest latex beads for the indicated times. (A) Adherent cells on coverslips were then examined by fluorescence microscopy at 2 hours post phagocytosis, as described in Fig. 1A. Color images of merged green (F-DXT) and red (PKH) fluorescence signals show colocalization (yellow), which indicates substantial fusion between phagosomes containing latex beads and F-DXT-loaded lysosomes. The inset shows isolated phagolysosomes in post-nuclear cell homogenates, which were examined by flow cytometry in B. Bar, 5 μm. (B) Phagosomes were prepared 30 minutes, 1 hour and 2 hours after phagocytosis and analyzed by flow cytometry. Green fluorescence was analyzed on positive red fluorescent events, which corresponded to particles surrounded by a phagosomal membrane (gate R3), and the results were expressed as green fluorescence histograms. In each panel, histograms on the left represent PKH-labeled phagosomes isolated from cells without F-DXT loading, and histograms on the right represent phagosomes from cells loaded with F-DXT. The frequencies of phagosomes colocalizing with F-DXT and MFIs increased as a function of time. Values represent the averages of two independent

dextran with red fluorescent phagosomes as a measure of phagolysosomal fusion. In initial experiments, F-DXT-loaded cells on coverslips were stained with PKH and allowed to ingest latex beads. Cells were then examined by fluorescence microscopy. Color images of merged green (F-DXT) and red (PKH) fluorescence signals (Fig. 3A) showed significant colocalization (yellow), indicating substantial fusion between phagosomes containing latex beads and F-DXT-loaded lysosomes. Subsequent quantitative analysis of colocalization between PKH-labeled phagosome and F-DXT was carried out by flow cytometry using partially purified phagosomes (Fig. 3B). The results show that phagosome-lysosome fusion increased as a function of phagosome age. After two hours post phagocytosis, ~90% of phagosomes showed colocalization with F-DXT, with a 3.5-fold increase in green fluorescence over the signal measured at 30 minutes post phagocytosis.

## Identification of mycobacterial strains that inhibit phagosome maturation and fusion with lysosomes

Taken together, the findings presented above suggest that quantitative analysis of phagosomal markers and fusion with lysosomes by flow cytometry may provide a basis for investigating factors that regulate phagosome maturation. Therefore, we applied this approach to examine the capacity of mycobacterial factors to influence phagosome development. The important intracellular pathogen *M. tuberculosis* causes phagosomal maturation arrest (Sturgill-Koszycki et al., 1994; Clemens et al., 2000) and has emerged as a tool for dissection of events regulating phagosome biogenesis. To examine whether subcellular factors from *M. tuberculosis* have the capacity to influence phagosome development, PKH-labeled cells were incubated with latex beads in the presence of either BSA (control) or with soluble fractions prepared from whole-cell lysates of either *M. tuberculosis* or the nonpathogenic mycobacterium, *Mycobacterium smegmatis*. This allowed for fluid phase co-internalization of particles and bacterial factors. Phagosomes were isolated at 2 hours post phagocytosis, permeabilized and incubated with antibodies to Rab7 and LAMP-1. Samples were analyzed by flow cytometry, and green fluorescent histograms reflecting labeling with anti-Rab7 and anti-LAMP-1 were constructed with gating on red fluorescent phagosomes. As shown in Fig. 4, co-internalization of *M. tuberculosis* lysates with latex beads substantially impaired the acquisition of late endosome and lysosome markers consistent with phagosome maturation arrest. Two independent experiments showed that treatment with *M. tuberculosis* soluble lysate decreased the MFI values for staining with Rab7 and LAMP-1, respectively, by 47% and 63%. Similar observations were made when using the *M. tuberculosis* variant *Mycobacterium bovis* BCG (data not shown). By contrast, lysate from *M. smegmatis* caused only a slight reduction in the acquisition of Rab7 (8%) and LAMP-1 (15%). A reduction in the accumulation of Rab7 and LAMP-1 by phagosomes generated in the presence of *M. tuberculosis* lysates correlated with a dramatic decrease of fusion with lysosomes. Indeed, the MFI value corresponding to colocalization with F-DXT decreased by 84% in phagosomes prepared from cells incubated with *M. tuberculosis* lysates. By contrast, treatment with *M. smegmatis* extracts decreased acquisition of F-DXT by only 25%, which is consistent with a previous report showing that about 80% of *M. smegmatis* phagosomes are fusogenic towards lysosomes (Via et al., 1998). These findings suggest that factors expressed by pathogenic mycobacteria decelerate phagosome fusion with lysosomes. This is in agreement with studies showing that

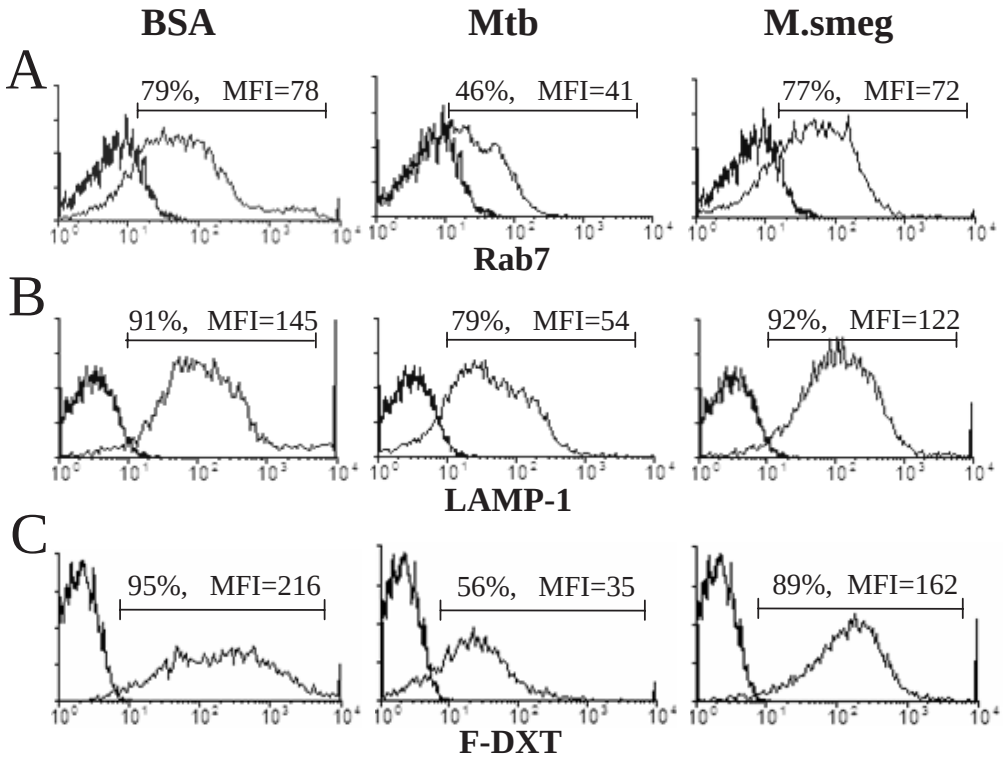



**Fig. 4.** Mycobacterial lysate inhibits phagosome maturation and fusion with lysosomes. (A,B) PKH-labeled cells were exposed to latex beads in the presence of 100 μg/ml of BSA (control) or a soluble fraction derived from whole-cell lysates of either *M. tuberculosis* (Mtb) or *M. smegmatis* (M.smeg). Phagosomes were isolated at 1 hour (A) and 2 hours (B) post phagocytosis, permeabilized and incubated with antibodies to Rab7 and LAMP-1, respectively, and samples were analyzed by flow cytometry as described in Fig. 2. (C) F-DXT-loaded THP-1 cells were stained with PKH and allowed to ingest latex beads in the presence of BSA, or a soluble fraction derived from whole-cell lysates of either *M. tuberculosis* or *M. smegmatis*. Phagosomes were prepared at 2 hours post phagocytosis and analyzed by FACS as described in Fig. 3. Values represent the averages of two independent experiments.

viable pathogenic mycobacteria block phagosome maturation, including fusion with lysosomes (Ferrari et al., 1999; Gatfield and Pieters, 2000).

### Mycobacterial cell wall components attenuate phagosome maturation

The mycobacterial cell wall, which is in direct contact with host cell membrane during phagosome biogenesis, contains numerous bioactive substances including importantly modified phosphoinositides such as the heavily glycosylated LAM and its mono- and di-mannosylated precursor PIM (Brennan and Nikaido, 1995). LAM and PIM molecules, which have been reported to translocate to the membranes of endosomal vesicles (Beatty et al., 2000), have the potential to disrupt phagosome maturation. Flow cytometry analysis showed that the acquisition of F-DXT by PKH26-labeled phagosomes decreased as a function of the concentration of a crude, soluble cell wall fraction added to the cells before the uptake of latex beads (Fig. 5). This marked attenuation of fusion between bead-containing phagosomes with lysosomes appeared to be accounted for, at least in part, by the action of purified LAM (~53% inhibition at 5 μg/ml), but not by similar concentrations of PIM. These findings are consistent with the observation that phagosomes containing latex beads coated with LAM have a diminished capacity to recruit the early endosome autoantigen-1, a Rab5 effector (Fratti et al., 2001), and suggest that the abundance of mannose residues on mycobacterial LAM confers specific properties that are absent from species of phosphoinositides that are less extensively glycosylated.

### VitD3 antagonizes mycobacterial inhibition of phagolysosome fusion

Both VitD3 (Rook et al., 1986; Crowle et al., 1987) and IFN-

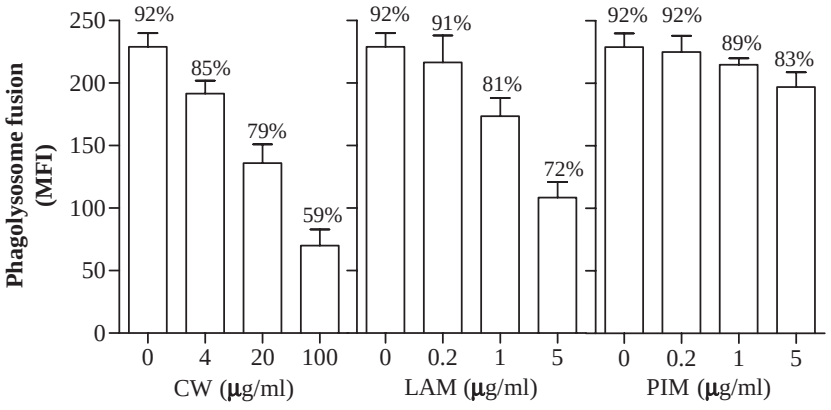



**Fig. 5.** Mannose capped mycobacterial glycolipid inhibits lysosomal delivery to phagosomes. F-DXT-loaded THP-1 cells were stained with PKH and allowed to ingest latex beads in the presence of BSA (control) or various concentrations of soluble cell wall fraction (CW), LAM or PIM. Phagosomes were prepared at 2 hours post phagocytosis and analyzed by flow cytometry as described in Fig. 3. The data shown are means±s.d. of three separate experiments. The value on the top of each bar represent the proportion of phagosomes fusing with lysosomes.

**Table 1. VitD3 abrogates LAM-induced attenuation of phagosome-lysosome fusion**

| | | Acquisition of F-DXT | |
|---|---|---|---|
| Pretreatment | LAM | % Positive | MFI |
| – | – | 79.0±1.6 | 94.7±6.1 |
| – | + | 49.8±5.5 | 39.9±9.7 |
| IFN-γ | + | 56.6±3.1 | 44.2±8.0 |
| IFN-γ+LPS | + | 62.2±3.9 | 57.9±7.5 |
| VitD3 | + | 76.4±4.0 | 90.5±8.5 |

F-DXT-loaded THP-1 cells were untreated or stimulated with IFN-γ (200 U/ml) or LPS (100 ng/ml), or both, or VitD3 (1 μM) for 24 hours. Cells were then stained with PKH and allowed to ingest latex beads in the presence or absence of LAM (5 μg/ml). Phagosomes were prepared at 2 hours post phagocytosis and analyzed by flow cytometry as described in Fig. 3. Values represent means±s.d. of three independent experiments.

γ (Cooper et al., 1993; Flynn et al., 1993; Zhang et al., 1995) are known to activate the anti-mycobacterial activity of mononuclear phagocytes. To investigate whether the anti-mycobacterial properties of these agents involve their ability to influence phagosome maturation, differentiated THP-1 cells were loaded with F-DXT and stimulated with VitD3 (1 μM) or IFN-γ (200 U/ml) for 24 hours. Cells were then stained with PKH before exposure to LAM and latex beads. Phagosome preparations were analyzed by flow cytometry and green fluorescence histograms were generated as described above. LAM reduced the proportion of phagosomes that colocalizes with F-DXT from 79% to 50% and IFN-γ pretreatment was unable to mitigate this effect (Table 1). However, simultaneous treatment of cells with both IFN-γ and LPS partially reversed the inhibitory effect of LAM by increasing the proportion of phagolysosomes to 62.2%. In contrast to IFN-γ alone, treatment with VitD3 was highly effective in preventing phagosome maturation arrest induced by LAM (Table 1).

### Rescue of phagosome maturation by VitD3 is phosphoinositide 3-kinase dependent

We have previously shown that VitD3 initiates a signaling pathway in human mononuclear phagocytes involving activation of phosphoinositide 3-kinase (PI3K) (Hmama et al., 1999). Subsequently, this lipid kinase was also shown to be a regulator of the anti-mycobacterial action of VitD3, which was dependent on activation and recruitment of NADPH oxidase to early phagosomes (Sly et al., 2001). To examine the role of PI3K in VitD3-induced phagolysosome fusion, the acquisition of F-DXT by phagosomes was studied in VitD3-treated cells in the presence and absence of the PI3K inhibitor LY-294002. F-DXT-loaded THP-1 cells were treated with LY-294002 for 30 minutes and washed before VitD3 stimulation. Twenty-four hours later, macrophages were labeled with PKH and exposed to latex beads in the presence or absence of LAM. The results shown in Table 2 indicate that in the absence of PI3K inhibitor, the ability of LAM to inhibit phagolysosome fusion was completely prevented by VitD3, whereas the addition of LY-294002 abrogated nearly completely the rescue of phagosome-lysosome fusion by VitD3. These findings are consistent with the conclusion that the anti-mycobacterial action of VitD3 also involves a PI3K-regulated phagosome fusion with lysosomes.

**Table 2. Rescue of phagosome-lysosome fusion by VitD3 is dependent on a phosphoinositide 3-kinase signaling pathway**

| | | Acquisition of F-DXT | |
|---|---|---|---|
| Pretreatment | LAM | % Positive | MFI |
| – | – | 77.9±1.3 | 87.8±4.5 |
| – | + | 52.0±3.8 | 34.7±7.2 |
| VitD3 | + | 76.1±1.8 | 84.3±3.7 |
| LY | – | 72.7±4.9 | 75.3±8.4 |
| LY/VitD3 | + | 56.8±3.5 | 43.8±7.9 |

THP-1 cells were loaded with F-DXT in the absence of FCS, washed and treated with 25 μM LY-294002 (LY) for 30 minutes and washed again. Control cells were treated similarly in the absence of LY-294002. After 24 hours of stimulation with VitD3 (1 μM), cells were stained with PKH and allowed to ingest latex beads in the presence or absence of LAM (5 μg/ml). Phagosomes were prepared at 2 hours post phagocytosis and analyzed by flow cytometry as described in Fig. 3. Values represent mean±s.d. of three independent experiments.

## Discussion

In this study, THP-1 cells, which represent a versatile human macrophage model in which to study phagosome biology (Ghigo et al., 2002; Zheng and Jones, 2003), were used along with latex beads to develop a flow cytometry-based approach to analyze phagosome maturation. Latex beads have been used extensively to examine phagosome biology and continue to provide important insights into phagosome biogenesis (Fratti et al., 2001; Vieira et al., 2001; Gagnon et al., 2002). Labeling of cell membranes with PKH26 and the gating strategies combining light scatter and fluorescence produced a distinctive pattern allowing latex bead phagosomes to be readily distinguished from cell debris and free beads in post nuclear homogenates (Fig. 1). The results presented above show that staining of only partially purified, PKH-labeled (red fluorescent) phagosomes with antibodies to endocytic markers combined with FITC-labeled (green fluorescent) secondary antibodies allowed for quantitative measurement of phagosome maturation by flow cytometry (Fig. 2). Direct evidence confirming that this approach correctly detected phagosome maturation was provided by the finding that the lysosomal marker (F-DXT) colocalized with PKH26-labeled phagosomes after a lag period of about 2 hours, which is typical for fusion with lysosomes (Fig. 3).

Given the robustness of this system, we examined whether it could be used to assess the influence of biologically active materials on phagosome biology. *M. tuberculosis* is of considerable interest in this context because it is an organism that causes phagosome maturation arrest (Pieters, 2001; Russell, 2001). Several mycobacterial components, including glycolipids, have been shown to be released from phagosomes into subcellular compartments (Beatty and Russell, 2000; Beatty et al., 2000), suggesting that mycobacterial products that are exported intracellularly have the potential to affect trafficking in the endosomal system and might influence phagosome maturation. This possibility was examined by flow cytometry, and the results showed clearly that factors

associated with or released by *M. tuberculosis*, such as LAM, which is a major component of the mycobacterial cell wall, were able to inhibit fusion of latex bead-containing phagosomes with both late endosomes (Rab7, LAMP-1) and lysosomes (fusion with F-DXT vesicles) (Figs 4, 5). These observations are consistent with recent data from other laboratories showing that LAM interferes with phagosomal recruitment of syntaxin 6 and Rab 9, proteins that are markers of fusion with vesicles of the trans-Golgi network (Fratti et al., 2003).

Taking advantage of the power of this flow cytometry approach, we investigated whether and how the observed inhibitory effect of mycobacterial factors may be affected by cell stimulation with anti-mycobacterial agents. On the basis of the intracellular colocalization of red-labeled latex bead phagosomes and lysosomal F-DXT, it was possible to show that IFN-γ had only a marginal effect on phagosome maturation in the presence of LAM. However costimulation with IFN-γ and LPS significantly improved phagolysosome fusion in the presence of LAM, which is consistent with the observation that LPS (perhaps through TNF-α) potentiates IFN-γ action, and leads to mycobacterial phagosome acidification and fusion with lysosomes (Shaible et al., 1998). In contrast to the negligible effect of IFN-γ alone, pre-treatment of cells with VitD3 alone promoted phagolysosome fusion, despite the presence of LAM, suggesting the presence of pathways in these cells to promote phagosome maturation independently of IFN-γ (Table 1). Other evidence for the role of VitD3 in host resistance to tuberculosis has emerged recently from this laboratory. It suggests that monocyte anti-mycobacterial activity induced by VitD3 is mediated by PI3K-regulated activation and recruitment of the NADPH-dependent phagocyte oxidase to early endosomes (Sly et al., 2001). PI3K has been shown to regulate a wide range of physiological processes including cytoskeletal rearrangement, movement of organelle membranes and phagosome maturation (reviewed by Toker, 2002). For example, PI3K was shown to be essential for the formation of the phagosomal cup (type IA PI3K) and for fusion with lysosomes (type III PI3K, hVPS34) in normal cells (Vieira et al., 2001; Fratti et al., 2001). In this study, on the basis of the effects of LY-294002, inhibitor of both type I and type III PI3K, and accurate measurement by flow cytometry, a role for this enzyme in regulating the enhanced phagosome fusogenicity in response to VitD3 was shown (Table 2). In these experiments LY-294002 was washed away 30 minutes after treatment and VitD3 was added for 24 hours. Under these conditions, acquisition of F-DXT by phagosomes was only minimally reduced in control cells, but rescue of phagolysosome fusion by VitD3 in the presence of LAM was completely abrogated. These findings highlight a differential functional role of PI3K involved specifically in VitD3-induced phagosome fusion with lysosomes, possibly by antagonizing an inhibitory effect of LAM on the phagosome regulator type III PI3K, Vps34. A recent report, which is consistent with this hypothesis, suggested that LAM blocked a cytosolic $Ca^{2+}$/calmodulin hVPS34 cascade along with the inhibition of the recruitment of the phagosome maturation marker EEA1 to latex bead vacuoles (Vergne et al., 2003). It is also conceivable that changes in the distribution of key proteins, other than PI3K, on the phagosomal surface are part of the mechanisms of VitD3-dependent phagosome maturation. Further investigations based on phenotyping the phagosome membrane using flow cytometry will address this hypothesis.

Taken together, the results presented above suggest that this flow cytometry-based system will be a useful tool to identify specific factors from mycobateria and other organisms with the properties of regulating phagosome trafficking. This will allow more precise and mechanistic studies of the molecular interactions that determine the intracellular fate of pathogen-containing phagosomes. Understanding these key interactions may contribute to current efforts to develop improved targeted therapeutics and vaccines for tuberculosis and other chronic intracellular infections.

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