Macrophages are key to the pathogenesis of atherosclerosis. They take up and store excessive amounts of cholesterol associated with oxidized low density lipoprotein, through scavenger receptors, eventually becoming foam cells that display altered immune responsiveness. We studied the effects of cholesterol accumulation on phagosome formation and maturation, using lipid transport antagonists and cholesterol transport-deficient mutants. In macrophages treated with U18666A, a transport antagonist that prevents cholesterol exit from endosomes/lysosomes, the early stages of maturation proceeded normally; phagosomes acquired Rab5, phosphatidylinositol 3-phosphate, and EEA1 and merged with LAMP-containing vesicles. However, fusion with lysosomes was impaired. Rab7, which is required for phagolysosome formation, was acquired by phagosomes but remained inactive. Maturation was also studied in fibroblasts from Niemann-Pick type C individuals that have defective cholesterol transport. Transfection of FcγIIA receptors was used to confer phagocytic capability to these fibroblasts. Niemann-Pick type C phagosomes failed to fuse with lysosomes, whereas wild type fibroblasts formed normal phagolysosomes. These findings indicate that cholesterol accumulation can have a detrimental effect on phagosome maturation by impairing the activation of Rab7, sequestering it and its effectors in cholesterol-enriched multilamellar compartments.

Macrophages are key to the pathogenesis of atherosclerosis. They take up and store excessive amounts of cholesterol associated with oxidized low density lipoproteins through scavenger receptors, eventually becoming foam cells that display altered immune responsiveness. Circulating blood monocytes are attracted and activated by a variety of proinflammatory cytokines, including interleukin-1, -6, -8, -10, and -12 and tumor necrosis factor-α, produced by subendothelial cells in response to infiltrating lipoproteins (1). In the arterial intima, monocytes differentiate into macrophages (2), at least in part in response to macrophage colony-stimulating factor present in atherosclerotic lesions (3, 4). These subendothelial macrophages proceed to ingest oxidized low density lipoprotein and other modified forms of low density lipoprotein by either endocytosis or phagocytosis, depending on the size of the particles. Scavenger receptors feature prominently in the uptake of modified LDL particles, but other receptors also contribute (1, 5).

A key event during atherogenesis is the conversion of blood-derived macrophages into foam cells, which are characterized by a massive accumulation of cholesterol esters in cytoplasmic lipid droplets (6). Lipid-laden foam cells are found within the subendothelial space of arteries in the fatty streaks of early lesions as well as in more advanced atherosclerotic plaques (7). Excessive uptake of oxidized low density lipoprotein is thought to be the primary cause of the accumulation of free and esterified cholesterol in foam cells. Normally, internalized lipids are delivered to lysosomes, where cholesterol esters are hydrolyzed. The released free cholesterol is then transported out of the lysosomes and, if present in excess, it is re-esterified in the endoplasmic reticulum for storage in lipid droplets (8). During the early fatty streak phase of atherosclerotic disease, the order amount of accumulated lipid generates the large cytoplasmic inclusions characteristic of foam cells. As the disease advances from a fatty streak to a fibrous plaque, lipid accumulates also in the lysosomes of macrophages.

Lysosomes play a critical role in the innate immune function of macrophages by fusing with phagosomes. Insertion of lysosomal proton pumps is required for effective phagosomal acidification that, together with the delivery of microbicidal lysosomal contents, is key for the elimination of pathogens. Luminal acidification and protein degradation are also central to antigen presentation. These functions could in principle be altered by the excessive accumulation of cholesterol in the lysosomes of late stage foam cells.

Remarkably little is known about the effects of excess lysosomal lipid accumulation on the immune function of macrophages. This paucity of information is most likely the result of technical limitations; it is virtually impossible to isolate and analyze primary late stage foam cells, and generation of foam cells in vitro by exposure to synthetically modified LDL is a time-consuming and expensive enterprise that has yielded varying results in different laboratories. In this report, we used pharmacological and genetic means to induce a pronounced and reliable accumulation of cholesterol in lysosomes and were able to study its consequences on the formation, acidification, and maturation of phagosomes.
Reagents and Antibodies—Fetal bovine serum, Dulbecco’s modified Eagle’s medium, α-minimum Eagle’s medium, Hepes-buffered solution RPMI 1640, and phosphate-buffered saline were from Wisent (St. Bruno, Canada). 3-β-[2-(Diethylamino)-ethoxy]-androst-5-en-17-one (compound U18666A), o-phe

human IgG for 1.5 h at room temperature or overnight at 4 °C. Sheep RBC were opsonized with donkey anti-sheep IgG at 1:50 for 1.5 h at room temperature or overnight at 4 °C. RAW cells grown on glass coverslips were changed to prewarmed serum-free Dulbecco’s modified Eagle’s medium and were overlaid with 50–100 μl of opsonized beads or RBC. In most experiments, phagocytosis was synchronized by centrifugation of the cells after the addition of beads or RBCs at 300 × g for 1 min. The samples were incubated with the beads or RBC for 3–15 min at 37 °C, as specified. RBCs that were not internalized were lysed by hypotonic shock with water. External beads were distinguished from ingested beads by labeling with Cy5-, Cy3-, or Cy2-conjugated donkey anti-human IgG antibodies. The cells were then placed back at 37 °C to allow maturation to proceed for the indicated time. The phagocytic index was defined as the number of phagosomes per 100 macrophages. This value was estimated by differential interference contrast microscopy, and the external adherent beads were discounted based on their fluorescence as a result of secondary labeling with fluorophore-conjugated antibodies against human IgG or rabbit IgG. In the case of NPC fibroblasts, phagocytosis was permitted for 20 min, followed by secondary antibody labeling of external beads and then maturation for 40 min.

Fluid Phase Endocytosis—To label the lysosomal compartment, RAW cells were incubated overnight in serum-free medium with 0.2 mg/ml rhodamine dextran (M, 10,000) in the presence or absence of U18666A. The dextran-containing medium was then removed, and the cells were chased in serum-free medium in the presence or absence of U18666A for 2 h to ensure delivery of contents to lysosomal compartments. A similar pulse-chase protocol was applied to NPC and control fibroblasts.

Immunostaining, Fluorescence Microscopy, and Image Analysis—Detection of LAMP-1 or LAMP-2 by immunofluorescence was performed after fixing and permeabilizing the cells in methanol, blocking in 5% donkey serum, and then probing with rat anti-mouse LAMP-1 or rat anti-mouse LAMP-2 at 1:200 for 1 h at room temperature. After washing with phosphate-buffered saline, samples were incubated with Cy3-labeled donkey anti-rat antibody (1:1000) for 1 h at room temperature and then washed and mounted on slides using Dako. A similar procedure was used to detect LAMPA-2 and EE1A. Detection of cholesterol by fluorescence microscopy was performed after fixing cells in 4% paraformaldehyde, followed by quenching with 100 mM glycine and then staining for 16 h at 4 °C with 0.5 mg/ml filipin. Analysis of the distribution and density of the membrane markers was performed using either an LSM510 laser-scanning confocal microscope (Zeiss) or a spinning-disk laser confocal microscope (Quorum) with a ×63 oil immersion objective. Image analysis was performed using Volocity version 3.2 (Improvision). Fluorescence Recovery after Photobleaching—Cells grown on coverslips were transfected with wild-type GFP-Rab7 or YFP-Rab7(Q67L), and after 24–48 h, individual coverslips were placed into Attofluor chambers that were mounted on the heated stage of the confocal laser-scanning microscope. After acquiring two base-line fluorescence measurements, one of the selected areas was irreversibly photobleached, and then the fluorescence of both areas was measured over time. The fractional
fluorescence recovery of the bleached area was determined relative to the average of the two prebleach measurements. The unbleached area was used to estimate and correct for possible bleaching incurred during image acquisition.

Electron Microscopy—Cells were fixed in 2% glutaraldehyde in 0.1 M Sorensen’s phosphate buffer, pH 7.2, for 5 min before being scraped off of the coverslip and centrifuged. Fixation was continued at room temperature for at least a further 1 h. Cells were then postfixed in 1% OsO4 in phosphate buffer at room temperature for 2 h, stained en bloc for 1 h with 1% uranyl acetate in H2O, and then dehydrated and embedded in Epon resin. Sections 80 nm in thickness were collected on copper grids and stained with uranyl acetate and lead citrate. Sections were viewed using an FEI Tecnai 20 electron microscope, and images were captured using a Gatan Duelview camera.

Immunoblotting—Whole cell lysates were prepared in 1× Laemmli buffer. Lysates were analyzed by SDS-PAGE on 7% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, blocked for 1 h with 5% bovine serum albumin in phosphate-buffered saline (v/v) with 0.1% Tween 20 (v/v), and then probed for 1 h at room temperature with the primary antibody in blocking solution. After washing with phosphate-buffered saline/Tween, blots were incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature, washed vigorously, and then visualized by enhanced chemiluminescence (Amersham Biosciences).

Spectroscopic Determination of LAMP Content—RAW cells were grown on 6-well plates and treated with or without U18666A. Following treatment with the drug, the cells were fixed in methanol and exposed to LAMP-1 or LAMP-2 antibodies, followed by a secondary, horseradish peroxidase-conjugated antibody. The amount of horseradish peroxidase associated with the cells was then quantified using the o-phenylenediamine dihydrochloride reagent kit, followed by spectrophotometric measurement at 492 nm. Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control and was quantified similarly.

Statistics—All experiments were performed at least in triplicate. For comparisons of means, paired t-tests or analysis of variance were used, as appropriate. A probability of <0.05 was deemed significant. Signals were deemed “strong” if the marker was visible all around the phagosome with intensity comparable with that of the punctate signal from endosomes or lysosomes. Signals were scored as “weak” when the marker was visible all around the phagosome with intensity lower than that of the endosomes or lysosomes. Significance was determined combining the strong and weak signals as positive. A signal was considered negative if the marker was absent from the vicinity of the phagosome or if its distribution around the phagosome was discontinuous, indicative of vesicle apposition without fusion.

RESULTS

Loading of Lysosomes with Cholesterol

To mimic the effects of oxidized low density lipoprotein on lysosomal cholesterol accumulation, we treated cultured RAW macrophages with compound U18666A, a commercially available cholesterol transport antagonist. This compound has been previously shown to reduce cholesterol efflux from lysosomes (13–15). It specifically inhibits the movement of low density lipoprotein-derived cholesterol to the plasma membrane, resulting in the accumulation of free cholesterol in the lumen of late endosomes/lysosomes. The advantages of this cholesterol-loading method include its rapidity, homogeneous effectiveness, and low cost. As reported earlier for other cells, macrophages incubated 16 h with 0.75 or 1.0 µg/ml U18666A displayed a marked intracellular accumulation of unesterified cholesterol, visualized by staining with filipin (Fig. 1, A and B). Consistent with previous reports (16), the excess cholesterol was found in comparatively large intracellular structures, probably late endosomes/lysosomes. Accordingly, ultrastructural analysis of U18666A-treated cells revealed a marked increase in the number, size, and electron density of multivesicular and multilamellar structures that are typical of the late stages of the endocytic pathway (Fig. 1, C and D). Two other lines of evidence suggest that the excess cholesterol accumulated in enlarged lysosomes. LAMP-1 and LAMP-2, both resident membrane proteins of lysosomes, concentrated in large punctate structures in cells treated with U18666A (Fig. 1, E and F). The enlarged appearance of the LAMP-containing compartments was due in part to a net accumulation of LAMP-1 and LAMP-2 in the cholesterol-laden cells, as revealed by immunoblotting and by the quantitative spectroscopic immunoassay described under “Experimental Procedures” (Fig. 1). Second, the enlargement of the lysosomes was validated when this compartment was labeled selectively using a pulse-chase protocol with a fluorescent fluid-phase marker (Fig. 1, G and H).

We proceeded to study the effect of lysosomal cholesterol accumulation on the immune responsiveness of macrophages. Specifically, we compared the ability of control and cholesterol-laden macrophages (as a result of U18666A treatment) to engulf IgG-opsonized particles by phagocytosis. The phagocytic index, defined as the number of particles ingested per 100 cells, was quantified in cells treated without or with U18666A. The phagocytic index was depressed by ~40 and 55% after treatment with 0.75 and 1.0 µg/ml U18666A, respectively (supplemental Fig. 1). Although the degree of inhibition was significant, the remaining phagocytic activity enabled us to assess whether excess cholesterol affects the ability of lysosomes to fuse with formed phagosomes.

Effect of Lysosomal Cholesterol Accumulation on Phagosome Maturation

Early Stages—Phagosome maturation entails sequential fusion of distinct endocytic compartments with the formed phagosome. Nascent phagosomes merge initially with early and recycling endosomes, followed by fusion with late endosomes and ultimately with lysosomes in a unidirectional and time-dependent manner (17). We analyzed the effects of cholesterol loading on the individual stages of the maturation sequence. Rab5, a hallmark of early endosomes (18), is the first endosomal marker detectable on sealed phagosomes (19). Because the endogenous GTPase cannot be detected by immunostaining with available antibodies, we transfected macrophages with a GFP-tagged construct of wild-type Rab5. As illustrated in Fig.
Cholesterol Accumulation Impairs Phagosome Maturation

2A, Rab5 was detectable in the majority of control phagosomes within 5 min of completion of phagocytosis. Similarly, Rab5 was present on the membranes of phagosomes formed by U18666A-treated cells (Fig. 2B). Under the conditions studied, the extent of acquisition of Rab5 was indistinguishable between normal and cholesterol-loaded cells (p = 0.494 and p = 0.587 for cells exposed to 0.75 and 1.0 µg/µl U18666A, respectively) (Fig. 2G).

Rab5 is involved in the recruitment of EEA1 and Vps34 to phagosomes. We next analyzed these subsequent stages of the maturation process. Immunostaining was used to visualize the endogenous EEA1 on the phagosomal membrane and was reinforced with transfection of cDNA encoding EEA1-GFP. As found for Rab5, most of the early (10 min) phagosomes were positively stained for EEA1, and a similar degree of staining was found for control and cholesterol-loaded macrophages (Fig. 2, C and D; insets in H show endogenous EEA1).

Recruitment and activation of Vps34, the class III phosphatidylinositol 3-kinase results in the accumulation of phosphatidylinositol 3-phosphate (PI(3)P) on the phagosomal membrane (20). The formation of this phosphoinositide can be monitored in live cells using genetically encoded biosensors consisting of a protein domain fused to a fluorescent protein that binds to the headgroup of PI(3)P with high specificity and affinity. We transfected RAW cells with one such construct, the PX-GFP chimera, and observed its distribution by confocal microscopy (Fig. 2, E and F). As reported, PI(3)P was detectable on the phagosomal membrane shortly after sealing (Fig. 2, E and I). The accumulation of PI(3)P persisted for ~10–15 min, waning abruptly thereafter. When analyzed 30 min after the initiation of phagocytosis, virtually all phagosomes were again devoid of the phosphoinositide (Fig. 2, E and F, insets). Neither the extent nor the kinetics of PI(3)P acquisition were significantly affected in cells treated with U18666A (Fig. 2, F and I, and supplemental Fig. 2). Together, these findings indicate that although phagocytic efficiency is partially depressed, the early stages of phagosomal maturation are unaffected by the excessive accumulation of cholesterol in late endosomes/lysosomes.

Intermediate Stages—Following the recruitment of PI(3)P and EEA1, phagosomes acquire Rab7 and LAMP, which serve as indicators of the intermediate stages of maturation. The effects of cholesterol loading on these markers were investigated next. Control and U18666A-treated macrophages were allowed to internalize particles for 15 min, and maturation was allowed to proceed for a further 45 min. When analyzed by immunostaining, virtually all of the phagosomes in both control and drug-treated cells were positive for LAMP-1 (Fig. 3, A, B, and E). As for Rab5, we resorted to transfection of a fluorescent dextran pulse-chase protocol detailed under “Experimental Procedures.” The data, which are the means of two experiments, each performed in duplicate, represent the amount of LAMP-1 and LAMP-2 normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase.

Cholesterol Accumulation Impairs Phagolysosome Formation

The final step in the maturation sequence is the fusion of the phagosome with lysosomes. Although LAMP isoforms and
Rab7 is present in both late endosomes and lysosomes, the latter can be unambiguously identified by preloading them with fluid phase markers. This involves a well defined protocol consisting of a prolonged (>16-h) pulse to reach the lysosomal compartment, followed by a long (≥2-h) chase period to clear the marker from all of the earlier compartments of the endocytic pathway. We used this protocol to load lysosomes with fluorescent dextrans in order to assess the effect of cholesterol accumulation on phagolysosome fusion. The cells were then induced to internalize opsonized beads, and the resulting phagosomes were allowed to undergo maturation for 45 min. As illustrated in Fig. 4A, the otherwise untreated cells displayed a clear ring of fluorescence surrounding the ingested beads, indicative of phagolysosome fusion. Nearly 90% of the control phagosomes were dextran-positive (Fig. 4E). In stark contrast, most of the phagosomes in U18666A-treated cells were devoid of fluorescence (Fig. 4, B and E), implying that loading with cholesterol impaired the fusion of lysosomes with phagosomes.

The lumen of late phagosomes is highly acidic, due largely to the high density of vacuolar ATPases. This extreme acidification promotes the accumulation of weak bases like LysoTracker, a lysosomotropic fluorescent probe. We took advantage of this feature of phagolysosomes to analyze by an alternate method the effects of cholesterol accumulation on lysosome fusion. As reported by others, at times ≥1 h after ingestion, virtually all of the phagosomes of control cells were stained by LysoTracker (Fig. 4C). In contrast, the phagosomes of U18666A-treated cells failed to accumulate the probe (Fig. 4D). However, the enlarged cholesterol-loaded lysosomes did accumulate LysoTracker, indicating that they possessed active vacuolar ATPases. The ability of the cholesterol-laden lysosomes to maintain a low pH implies that their membrane remains intact, capable of retaining the concentrated protons.

**Rab7 Is Present but Is Not Active on Phagosomes from Cholesterol-loaded Cells**

Impairment of traffic of intracellular cholesterol has been reported to impact Rab GTPase function (21–24). Importantly, fusion between phagosomes and lysosomes is thought to be directed by Rab7 in its active GTP-loaded conformation (25). This GTPase was found earlier to be recruited to the phagosomal membrane (Fig. 3), although its activation state was not defined. In principle, the state of activation of Rab7 can be deduced from its subcellular localization, since the inactive, GDP-bound form normally associates with a soluble guanine nucleotide dissociation inhibitor and therefore resides largely in the cytosol, whereas the active, GTP-bound state is membrane-anchored. However, the ability of guanine nucleotide dissociation inhibitor to extract GDP-Rab7 may depend on the lipid composition of the membrane (23), which is likely to be altered in U18666A-treated lysosomes and phagosomes.

To assess the state of activation of Rab7 on phagosomes, we expressed RFP-tagged Rab7 along with GFP-tagged RILP. RILP is a Rab7 effector that interacts exclusively with the active, GTP-bound state of Rab7 and is therefore a reliable index of its activation state (26). As reported (26), RILP was present...
allowed to undergo phagosomal maturation for an additional 45 min. Incompletely internalized adherent beads were labeled with fluorophore-conjugated anti-IgG, and the cells were then allowed to undergo phagosomal maturation for an additional 45 min. A and B, macrophages were fixed and permeabilized with methanol and probed with LAMP-1 antibodies, followed by Cy3-conjugated secondary antibodies. C and D, macrophages expressing GFP-Rab7 were exposed to IgG-opsonized beads and allowed to mature as outlined above. Representative images of control and U18666A-treated macrophages expressing GFP-Rab7 were exposed to IgG-opsonized beads and allowed to mature for 45 min. C and D, control and U18666A-treated macrophages were allowed to internalize IgG-opsonized as described above. After 45 min of maturation, the cells were stained with LysoTracker Green to identify acidic compartments. Fluorescence images of representative control (A and C) and U18666A-treated macrophages (B and D) labeled with LAMP-1 antibodies (A and B) or LysoTracker Green (C and D) are illustrated, and the corresponding differential interference contrast images are shown as insets in B and D. E, quantitation of the proportion of phagosomes that acquired dex- tran. Data are means ± S.E. of at least 100 phagosomes for each condition. The difference between control and 0.75 or 1.0 µg/ml U18666A-treated cells was significant (p = 0.032 and p = 0.041, respectively). Images are representative of three separate experiments. The filled white arrowheads point to positive phagosomes, and the empty arrowheads point to negative phagosomes for the indicated marker. Size bars, 10 µm.

FIGURE 4. Effect of U18666A on phagosome and lysosome fusion. A, B, and E, macrophages were exposed overnight to 0.2 mg/ml rhodamine-conju-gated dextran in the presence or absence of either 0.75 or 1.0 µg/ml U18666A. The following day, the cells were chased for 2 h in dextran-free medium and then exposed to IgG-opsonized beads for 15 min. After labeling incompletely internalized or adherent beads, the formed phagosomes were allowed to mature for 45 min. C and D, control and U18666A-treated macrophages were allowed to internalize IgG-opsonized as described above. After 45 min of maturation, the cells were stained with LysoTracker Green to identify acidic compartments. Fluorescence images of representative control (A and C) and U18666A-treated macrophages (B and D) labeled with LAMP-1 antibodies (A and B) or LysoTracker Green (C and D) are illustrated, and the corresponding differential interference contrast images are shown as insets in B and D. E, quantitation of the proportion of phagosomes that acquired dex- tran. Data are means ± S.E. of at least 100 phagosomes for each condition. The difference between control and 0.75 or 1.0 µg/ml U18666A-treated cells was significant (p = 0.032 and p = 0.041, respectively). Images are representative of three separate experiments. The filled white arrowheads point to positive phagosomes, and the empty arrowheads point to negative phagosomes for the indicated marker. Size bars, 10 µm.

FIGURE 3. Effect of U18666A on acquisition of late endosomal markers by phagosomes. Macrophages were allowed to perform phagocytosis of IgG-opsonized beads for 15 min. Incompletely internalized adherent beads were labeled with fluorophore-conjugated anti-IgG, and the cells were then allowed to undergo phagosomal maturation for an additional 45 min. A and B, macrophages were fixed and permeabilized with methanol and probed with LAMP-1 antibodies, followed by Cy3-conjugated secondary antibodies. C and D, macrophages expressing GFP-Rab7 were exposed to IgG-opsonized beads and allowed to mature as outlined above. Representative images of control and U18666A-treated macrophages are shown in A and C and in B and D, respectively. The filled arrowheads refer to LAMP-1 or Rab7-positive phagosomes. In E, the proportion of phagosomes that acquired LAMP-1 after 60 min was quantified. The difference between control and 0.75 or 1.0 µg/ml U18666A-treated cells was not significant (p = 0.867 and p = 0.878, respectively). In F, the proportion of phagosomes that had acquired Rab7 was quantified. The difference between control and 0.75 or 1.0 µg/ml U18666A-treated cells was not significant (p = 0.358 and p = 0.696, respectively). Representative images of control and U18666A-treated macrophages immunostained for LAMP-1 or expressing GFP-Rab7 are shown in A and C and in B and D, respectively. The filled arrowheads point to LAMP-1- or GFP-Rab7-positive phagosomes. Images are representative of three separate experiments. Data are means ± S.E. of at least 100 phagosomes for each condition. Size bars, 10 µm.

together with Rab7 in late endosomes/lysosomes, and its ectopic (over)expression induced the juxtanuclear clustering of these organelles (Fig. 5, A and B). In control cells, Rab7 and RILP co-localized also in phagosomes (Fig. 5, A and B). In contrast, although U18666A-treated phagosomes acquired Rab7, they were markedly devoid of RILP, suggesting that most of the Rab7 present on phagosomes was inactive (Fig. 5, C and D).

In addition to its Rab7-interacting site, RILP contains other protein interaction domains. It was therefore conceivable that recruitment of the protein to normal phagosomes was dictated by determinants other than active Rab7. To rule out this possi-
Cholesterol Accumulation Impairs Phagosome Maturation

Rab9, a GTPase that also resides on late endosomes, has recently been shown to be sequestered on cholesterol-rich membranes (22). It therefore seemed possible that the pool of active Rab7 may be similarly immobilized on cholesterol-laden organelles. To investigate this notion, we assessed whether RILP-GFP, an indicator of GTP-loaded Rab7, colocalized with cholesterol-rich structures induced by U18666A and Rab7. As illustrated in Fig. 6, A and B, RFP-Rab7 was largely associated with the enlarged cholesterol-laden vesicles, implying that active Rab7 binds preferentially to organelles containing cholesterol. Furthermore, RILP-GFP was also concentrated on enlarged cholesterol-laden vesicles (Fig. 6, B (inset), C, and D).

Accumulation of Rab7 in cholesterol-enriched vesicles is due, at least in part, to its reduced rate of dissociation from the membrane. This was concluded from the experiments of Fig. 6, E–G, where the rate of exchange of GFP-Rab7 between the vesicular and soluble pools was measured. In this setting, fluorescence recovery after photobleaching of an entire organelle can only result from exchange with the cytosolic pool of unbleached Rab7. Entire Rab7-positive endosomes of similar size were photobleached in both control and U1866A-treated cells, and the rates of fluorescence recovery were monitored as shown in Fig. 6, E–E3 and F–F3. As illustrated in Fig. 6G, the rate of recovery as well as the fraction of the total fluorescence that was recovered over the observation period were considerably lower in cholesterol-laden cells than in the corresponding controls. The reduced rate of dissociation is predicted to retain and accumulate active Rab7 on cholesterol-loaded endosomes/lysosomes. This conclusion is consistent with the findings of Gruenberg and co-workers (23), who noted that the fraction of membrane-associated Rab7 was greater in cholesterol-loaded than in untreated cells.

The propensity of active Rab7 to associate strongly with cholesterol-enriched membranes, together with its paucity on phagosomes, suggests that the cholesterol content of phagosomes may be abnormal in U18666A-treated cells. This possibility was analyzed by staining the cells with filipin. As shown in Fig. 6, H and I, cholesterol was readily apparent in the membranes of virtually all control phagosomes. In stark contrast, phagosomes in U18666A-treated cells were markedly devoid of filipin staining, despite the fact that the overall cellular content of cholesterol was greatly increased (Fig. 6, I and J). Together, these data imply that Rab7 is abundant in its active form on cholesterol-rich organelles yet is largely absent from phagosomes, perhaps because they lack cholesterol.
FIGURE 6. Characterization of Rab7 and RILP in U18666A-treated cells. Macrophages were co-transfected with cDNA encoding RFP-Rab7 (B) and RILP-GFP (B, inset) and then treated with 1 µg/ml U18666A and finally stained with filipin to visualize unesterified cholesterol (A). An untransfected cell is identified with an arrow, and transfected cells are demarcated with a dashed line. C and D, cells transfected with RILP-GFP (D) were treated with 1 µg/ml U18666A, and then stained with filipin (C). Note that Rab7 and filipin-positive organelles, which are dispersed throughout the cell in untransfected cells (e.g. arrow in A), are clustered near the nucleus when RILP is overexpressed. E–G, fluorescence recovery after photobleaching of GFP-Rab7 on endosomes before and after U18666A treatment. Macrophages were transfected with cDNA encoding GFP-Rab7 and incubated without (E) or with U18666A (F) and then used for determination of GFP-Rab7 fluorescence recovery after photobleaching. The regions photobleached are indicated by a circle, and the nucleus is denoted by N. Representative images acquired before (E, E1, F, and F1) immediately after photobleaching (E2 and F2) and 160 s thereafter (E3 and F3) are illustrated. E1–E3 and F1–F3 are enlargements of the regions of E and F where bleaching was performed. G, macrophages transfected with GFP-Rab7 were treated without (control; circles) or with either 0.75 µg/ml (squares) or 1.0 µg/ml U18666A (triangles). The cells were then used for determination of fluorescence recovery after photobleaching. Data are representative of three separate experiments. H–J, control (H) and U18666A-treated (I) macrophages were exposed to IgG-opsonized zymosan and allowed to mature as described in the legend to Fig. 3 and then stained with filipin. The inset in I illustrates the location of zymosan particles. The filled white arrowheads denote filipin-positive phagosomes, and the empty arrowheads denote negative phagosomes. J, quantitation of filipin fluorescence around phagosomes. Data are means ± S.E. of at least 10 phagosomes for each condition and three separate experiments. Size bars, 10 µm.
Phagosomal Maturation Is Also Arrested in Niemann-Pick Type C Cells

U18666A is thought to act primarily by impairing cholesterol export, causing accumulation of the lipid in late endosomes/lysosomes (13–15). Although this primary effect is likely responsible for the observed arrest in phagosome maturation, a separate mode of action, which is unrelated to cholesterol accumulation, is nevertheless conceivable. We therefore sought alternative experimental models to analyze the role of cholesterol in phagolysosome formation.

NPC is an autosomal recessive lipid storage disorder associated with mutations in two genes, NPC1 and NPC2. Although their exact function is still unknown, the proteins encoded by NPC1 and NPC2 appear to be involved in late endosomal/lysosomal transport of cholesterol and glycolipids. Loss-of-function mutants cause an accumulation of cholesterol that resembles that seen in U18666A-treated cells. Indeed, the drug has been used extensively to replicate the NPC phenotype in cell cultures (16, 23, 27). To circumvent possible secondary effects of U18666A, we studied phagosome maturation also in NPC cells. Because primary phagocytes are refractory to transfection by both normal and NPC fibroblasts expressing FcγRIIA-GFP receptors avidly bound and internalized IgG-opsonized particles. Moreover, phagosomes of both control and NPC fibroblasts acquired LAMP-1 (Fig. 8, C–E) during the course of maturation. However, only the control fibroblasts merged with lysosomes (Fig. 7, C–E) during the course of maturation.
with cholesterol using U18666A and also studied primary fibroblasts with a genetic predisposition to accumulate cholesterol. Our findings, which were observed consistently in both systems, support a role for cholesterol as a critical determinant of phagosome maturation. Specifically, we found that accretion of free cholesterol impairs the ability of lysosomes to fuse with phagosomes.

The early stages of the maturation process are seemingly unaltered; Rab5 and PI(3)P are acquired and subsequently lost with normal kinetics. This implies that the impairment of maturation results from interference with a specific stage and is not caused by whole scale damage to the endocytic pathway. In fact, LAMP-1 was also acquired normally by phagosomes of cholesterol-laden cells. LAMP-1 was formerly thought to be a marker of lysosomes but is now known to be present also in late endosomes and, to a lesser extent, in other compartments of the endocytic and secretory pathways. In the case of cholesterol-laden cells, LAMP-1 found on phagosomes must derive from late endosomes or other subcompartments yet not from lysosomes, which fail to fuse. It is noteworthy that the sequential fusion of separate pools of LAMP-1 with phagosomes had been inferred earlier from experiments using inhibitors of phosphatidylinositol 3-kinase (20, 29). Indeed, the LAMP proteins present in these earlier compartments seem to be critical determinants of the phagolysosome fusion event, since phagosome maturation is arrested at an early stage in cells from LAMP-1 and LAMP-2 double-deficient mice (30).

Like LAMP, Rab7 was also recruited to phagosomes in both normal and cholesterol-loaded cells. However, a significant difference was noted; although (at least a fraction of) the Rab7 associated with normal phagosomes was active (i.e. GTP-bound and capable of associating with effectors), no active Rab7 was detectable on phagosomes from cholesterol-laden cells. Remarkably, active Rab7 was readily detectable elsewhere in these cells, especially in association with cholesterol-enriched organelles (Fig. 5). Others had reported earlier that, like Rab7 (23), upon cholesterol loading, Rab4 (21), Rab11 (24), Rab5, and Rab9 (22) are also enriched in cholesterol-enriched membranes, where they are protected from extraction by guanine nucleotide dissociation inhibitor. This raises the possibility that most of the active Rab7 is sequestered in these structures, becoming unavailable to mediate fusion with the phagosome. Conversely, we found that the phagosomes of U18666A cells were depleted of cholesterol, despite the overabundance of the lipid in the cells. Depletion of cholesterol may have diminished the ability of the phagosomes to retain active Rab7 or to permit efficient turnover, possibly contributing to their defective fusion. However, the cause-effect relationship between these events remains unclear.

Since Rab7 is required for continued phagosome maturation (25), it is conceivable that confinement of active Rab7 to endosomes prevents maturation. It is well established that in both NPC- and U18666A-treated cells, late endosomes/lysosomes acquire a multilamellar structure, trapping vast amounts of membranes in their lumen. Sequestration of critical fusogenic molecules other than Rab7 in such an inaccessible compartment may be the cause of impaired phagolysosome formation.
By preventing the normal cycling of lipids from endosomes to other organelles, U18666A induces depletion of plasmalemmal cholesterol. Cholesterol is a key component of plasmalemmal detergent-resistant microdomains known as “rafts.” Although disagreement still exists, some authors believe that rafts are critical for signaling normal phagocytosis (31–33). However, a recent report by Sobo et al. (16) found no difference in the prevalence of rafts (raft/nonraft ratio) of cells exposed to U18666A, despite observed changes in overall lipid composition. We therefore suggest that the main cause of the impairment seen in both U18666A-treated and NPC cells is the intracellular accumulation of cholesterol.

In conclusion, accumulation of unesterified cholesterol in endomembranes can cause marked alterations in the fate of phagosomes. By preventing their fusion with lysosomes, excessive cholesterol is anticipated to interfere with the microbialidal role of phagosomes. In this regard, there is precedent for abnormal immune function in macrophages with abnormal handling of lipids in their endocytic pathway, typified in patients suffering from Gaucher disease. Of particular interest is the accumulation of cholesterol in lysosomes of late stage foam cells and of macrophages incubated in vitro with modified low density lipoprotein. In these cases, defective phagolysosome formation can be anticipated. This may in turn compromise the antimicrobial capacity of macrophages, perhaps accounting for the well documented association of *Chlamydia pneumoniae* with atherosclerotic plaques.

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