

Pivotal Advance: Macrophages become resistant to cholesterol-induced death after phagocytosis of apoptotic cells

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Abstract: One of the most important functions of macrophages is the phagocytosis of apoptotic cells (ACs). ACs deliver large amounts membrane-derived cholesterol to phagocytes, which, if not handled properly, can be cytotoxic. In atherosclerosis, where the ACs are cholesterol-loaded, this situation is exaggerated, because the ACs deliver both endogenous membrane cholesterol and stored lipoprotein-derived cholesterol. To examine how phagocytes handle this very large amount of cholesterol, we incubated macrophage phagocytes with cholesterol-loaded ACs. Our results show that the phagocytes call into play a number of cellular responses to protect them from cholesterol-induced cytotoxicity. First, through efficient trafficking of the internalized AC-derived cholesterol to acyl-CoA:cholesterol acyltransferase (ACAT) in the endoplasmic reticulum, phagocytes efficiently esterify the cholesterol and thus prevent its toxic effects. However, the phagocytes show no signs of cytotoxicity even when ACAT is rendered dysfunctional, as might occur in advanced atherosclerotic lesions. Under these conditions, the phagocytes remain viable through massive efflux of AC-derived cholesterol. Remarkably, these phagocytes still show a survival response even when high cholesterol levels are maintained in the post-phagocytosis period by subsequent incubation with atherogenic lipoproteins, as also may occur in atheromata. In this case, death in phagocytes is prevented by activation of survival pathways involving PI-3 kinase/Akt and NF- κ B. Thus, macrophages that have ingested ACs successfully employ three survival mechanisms—cholesterol esterification, massive cholesterol efflux, and cell-survival signaling. These findings have implications for macrophage physiology in both AC clearance and atherosclerotic plaque progression. *J. Leukoc. Biol.* 82: 1040–1050; 2007.

Key words: apoptosis · atherosclerosis · cell survival · efferocytosis

INTRODUCTION

A major function of macrophages is to engulf apoptotic cells (ACs) [1, 2]. ACs deliver to the phagocytes large amounts of membrane cholesterol, which can be cytotoxic if not handled properly [3–5]. In particular, without proper esterification to fatty acids and efflux, cholesterol accumulates in the endoplasmic reticulum (ER) membrane, which, in turn, triggers ER dysfunction and the activation of an ER stress-related apoptotic pathway [6]. In atherosclerotic lesions, macrophages also have to handle ingested cholesterol, which is acquired through the endocytosis or phagocytosis of atherogenic lipoproteins [7–9]. The majority of lesional macrophages survive this stress by the mechanisms outlined above, that is, esterification and efflux. Indeed, the cellular processes that keep most cholesterol-loaded macrophages alive throughout atherogenesis, a pathophysiological process of aging and lifestyle that is not under evolutionary pressure, likely evolved to protect macrophages from the cholesterol burden of AC ingestion, a fundamentally important physiological process.

Although, as mentioned above, most cholesterol-loaded macrophages in atherosclerotic lesions survive, a significant number lose the aforementioned adaptive responses and undergo cholesterol-induced apoptosis [10]. This observation raises two important and related questions: first, are there physiological or pathophysiological conditions in which phagocytes that have ingested ACs also undergo cholesterol-induced apoptosis; and second, what happens to the neighboring macrophages (“phagocytes”) in atherosclerotic lesions that engulf these cholesterol-loaded ACs (Chol-ACs)? In this situation, the phagocytes are confronted with a tremendous cholesterol burden, that is, endogenous membrane cholesterol plus stored lipoprotein-derived cholesterol from the ingested Chol-ACs [11]. We reasoned that addressing these questions would give insight into both the physiologic process of how phagocytes handle cholesterol as well as the pathophysiologic process of how macrophages in advanced atherosclerotic lesions handle cholesterol-loaded apoptotic cells. For example, if phagocytes

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were to succumb to this extreme stress and undergo cholesterol-induced apoptosis, it would define a limit to the physiological process of phagocyte survival during AC ingestion. Such a finding might also provide a mechanism for the acceleration of macrophage death in advanced atherosclerosis, an important step in the clinical progression of atherothrombotic vascular disease [12–19].

In this context, we set up an experimental model of phagocytic clearance of Chol-ACs. We show that macrophages ingesting these Chol-ACs have a remarkable set of survival responses, some of which are not present during the loading of macrophages with lipoprotein-derived cholesterol. These responses include massive cholesterol efflux and the triggering of cell survival signal transduction pathways. Thus, the ability of macrophage phagocytes to handle AC-derived cholesterol persists even under extreme conditions of cholesterol loading, suggesting that the threshold for this process is very high. Moreover, given the likely overall benefit of apoptotic cell clearance in advanced atherosclerosis, our data suggest that therapeutic strategies that promote this process may not be compromised by the potential problem of phagocyte death.

MATERIALS AND METHODS

Materials

The Falcon tissue culture plasticware or nontreated bacterial plastic dishes were purchased from Fisher Scientific (Pittsburgh, PA, USA). Cell culture media, reagents, and heat-inactivated FBS (GIBCO BRL, Gaithersburg, MD, USA) were from Invitrogen (Carlsbad, CA, USA). DiI-C18 (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), Alexa Fluor 488 annexin V, Alexa Fluor 594 annexin V, Calcein Green™-acetoxymethyl ester (AM) were obtained from Invitrogen-Molecular Probes. [³H]cholesterol and [¹⁴C]oleate were purchased from Perkin-Elmer Life Sciences (Waltham, MA, USA). All other chemicals and reagents were from Sigma (St. Louis, MO, USA), and HPLC-grade organic solvents were from Fisher Scientific (Pittsburgh, PA, USA). Low-density lipoprotein (LDL; *d* 1.020–1.063 g/ml) and high-density lipoprotein (HDL; *d* 1.063–1.21) was isolated from fresh human plasma by ultracentrifugation [20]. Acetyl-LDL was prepared by reaction of LDL with acetic anhydride as described previously [21] and labeled with DiI-C18 by the method of Pitas et al. [22]. Human apoA-I was purchased from Biodesign International (Saco, ME, USA). Compound 58035 (3-[decyldimethylsilyl]-*N*-[2-(4-methylphenyl)-1-phenylethyl] propanamide), an inhibitor of acyl-CoA:cholesterol *O*-acyltransferase (ACAT), was obtained from Dr. John Heider, formerly of Sandoz, Inc. (East Hanover, NJ, USA) [23]. PS1145, an inhibitor of Iκ kinase-β (IKKβ), was a gift from Millennium Pharmaceuticals [24, 25]. Anti-phospho-AKT antibody was from Cell Signaling Technology (Danvers, MA, USA), and monoclonal anti-β-actin antibody was from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated donkey anti-mouse and anti-rabbit IgG antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Peritoneal macrophages

For routine experiments, peritoneal macrophages were collected from 8–10 wk-old female C57BL/6J mice that had been injected intraperitoneally with concanavalin A or methyl-BSA, as described previously [26]. Cells were cultured in medium containing Dulbecco's modified Eagle's medium (DMEM), 10% FBS, 100 units/ml penicillin/streptomycin, and 20% L-cell-conditioned medium for at least 48 h. The medium was replaced every 24 h until the macrophages were confluent. For a few experiments as indicated, peritoneal macrophages were obtained from *Acat1*^{-/-} (*Soat1*^{-/-}) mice on the C57BL/6J background [27].

Generation of cholesterol-loaded apoptotic cells

Macrophages cultured as described above were incubated for 16–20 h with medium containing 100 μg/ml of acetyl-LDL and 10 μg/ml of the ACAT inhibitor 58035 to induce early apoptosis (cholesterol-loaded apoptotic cells, or “Chol-ACs”). In some experiments, *Acat1*^{-/-} macrophages were used instead of the ACAT inhibitor. Typically, 30–40% of macrophages were apoptotic, and less than 5% were late apoptotic or necrotic, as assessed by annexin V and propidium iodide staining, respectively.

Phagocytosis

Chol-ACs were prepared on less-adherent, nontreated bacterial dishes to facilitate detachment, and they were removed from the dishes using enzyme-free cell dissociation buffer in PBS (Invitrogen-GIBCO; catalog #13151-014). The cells were then added to a monolayer of fresh macrophages (“phagocytes”) at an approximate ratio of 5:1 Chol-ACs:phagocytes. In certain experiments, the Chol-ACs were labeled with Alexa Fluor 488 annexin V or Calcein Green-AM for 20 min before addition to the phagocytes in order to mark those phagocytes that had ingested the Chol-ACs (“ingesting phagocytes,” or IPs). After 30 min of incubation, the noningested apoptotic cells were removed by thorough rinsing, as described previously [26], and the phagocytes were incubated in fresh medium for the indicated times. In some experiments, the phagocytes were incubated in medium containing acetyl-LDL and 58035 and/or signaling pathway inhibitors during the postingestion incubation. To assay apoptosis in the phagocytes, the cells were stained with Alexa Fluor 594 annexin V and viewed by fluorescence microscopy. For quantification, 4–6 representative fields for each condition were counted for the number of apoptotic phagocytes and total phagocytes.

Whole-cell cholesterol esterification assay

For this experiment, Chol-ACs were made by incubating macrophages from *Acat1*^{-/-} mice with acetyl-LDL to ensure that the phagocytes would not be exposed to residual ACAT inhibitor in the Chol-ACs. Phagocytes were incubated with these Chol-ACs as above and then, after removals of noningested cells, were incubated in fresh medium containing [¹⁴C]oleate for the indicated times. The cells were washed twice with PBS, air-dried, and then extracted twice with 500 μl of hexane/isopropyl alcohol (3:2, v/v) for 30 min at room temperature. Cholesterol esterification activity was then determined in lipid extracts of the cells by measuring the cellular content of cholesteryl [¹⁴C]oleate by thin-layer chromatography [28]. The lipid-extracted cells were dissolved in 1 ml of 0.1 N NaOH and assayed for protein by the method of Lowry [29].

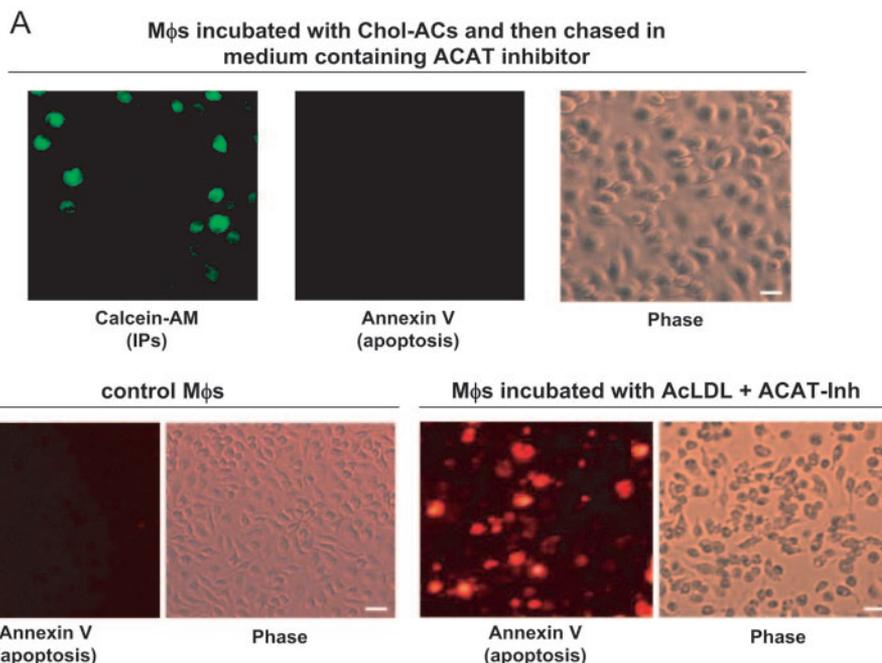
[³H]cholesterol efflux assay

[³H]cholesterol-labeled Chol-ACs were prepared using acetyl-LDL that had been labeled with [³H]cholesterol. Specifically, 1 mg acetyl-LDL was incubated with 10 μCi [³H]-cholesterol for 30 min at 37°C and then added to a 100-mm dish of macrophages in 10 ml of medium containing 10 μg/ml 58035. After 18–20 h of incubation to induce apoptosis, the monolayer was rinsed thoroughly with PBS. The labeled Chol-ACs were then added to a fresh monolayer of phagocytes for 30 min. Noningested apoptotic cells were then removed by intensive washing, and the phagocytes were further incubated in fresh medium for the indicated times. An aliquot of medium was collected at the indicated time points, and the radioactivity was quantified by liquid scintillation counting. The cells were dissolved in 1 ml of 0.1 N NaOH at room temperature for 5 h, and the radioactivity in the cell lysates was quantified. Cholesterol efflux was calculated as [(media cpm) ÷ (cell + media cpm)] × 100.

Fluorescent-activated cell sorting and cellular-free cholesterol mass assay in phagocytes

After exposure to Chol-ACs and postingestion incubation, phagocytes were washed two times with cold PBS and then extracted twice with 0.5 ml of hexane/isopropyl alcohol (3:2, v/v) for 30 min at room temperature. To isolate IPs from non-IPs, Chol-ACs were labeled with Alexa Fluor 488 annexin V before exposure to phagocytes. After incubation with these fluorescently labeled IPs, the phagocytes were dissociated from the plates using GIBCO enzyme-free Cell Dissociation Buffer (above) and then subjected to fluorescent-activated cell sorting (FACS) to separate IPs (green) from non-IP macro-

Fig. 1. Ingestion of Chol-ACs by ACAT-inhibited phagocytes does not induce apoptosis. (A) Chol-ACs were labeled with Calcein GreenTM-AM (green) and then briefly exposed to phagocytes. After noningested Chol-ACs were removed by stringent rinsing of the cells, the phagocytes were incubated in fresh medium containing the ACAT inhibitor 58035 for 24 h. The phagocytes were then stained with Alexa Fluor 594-annexin V (red) to detect apoptosis. The left panel shows the green-filter image (ingesting phagocytes, or IPs), the middle panel shows the red-filter image (apoptosis), and the right panel is the phase image. (B) Macrophages were incubated for 18 h with medium alone (control) or with medium containing 100 $\mu\text{g/ml}$ acetyl-LDL plus 10 $\mu\text{g/ml}$ ACAT inhibitor 58035 (AcLDL + ACAT-Inh) to effect cholesterol-induced apoptosis (Chol-ACs). The macrophages were then assayed for apoptosis by staining with Alexa Fluor 594-annexin V (red). Scale bar, 10 μm .



phages (non-green). FACS was conducted using a BD FACS-Aria sorting instrument at an excitation of 488 nm. The free cholesterol mass was determined by gas-liquid chromatography as described previously [30]. The cell monolayers were dissolved in 1 ml of 0.1 N NaOH, and aliquots were assayed for protein by the method of Lowry et al. [29].

Western blot analysis

Whole-cell lysates were prepared by homogenizing cells with Sample Loading buffer from Bio-Rad, as described previously [26]. These lysates were fractionated on 4-20% gradient SDS-polyacrylamide gels (Invitrogen) and then transferred to nitrocellulose membranes. After blocking the membranes with 5% (w/v) nonfat milk in Tris-buffered saline, 0.1% Tween-20 (TBST) at room temperature for 1 h, they were incubated overnight at 4°C with primary antibody. The membranes were then incubated with HRP-conjugated secondary antibody, and the immunoreactive protein bands were detected by enhanced chemiluminescence (ECL; Pierce, Rockford, IL, USA).

Statistics

Data are presented as mean \pm SEM of triplicate experiments. Absent error bars in the bar graphs signify SEM values smaller than the graphic symbols. Statistical significance was determined using the Student's *t* test with unequal variance or one-way ANOVA.

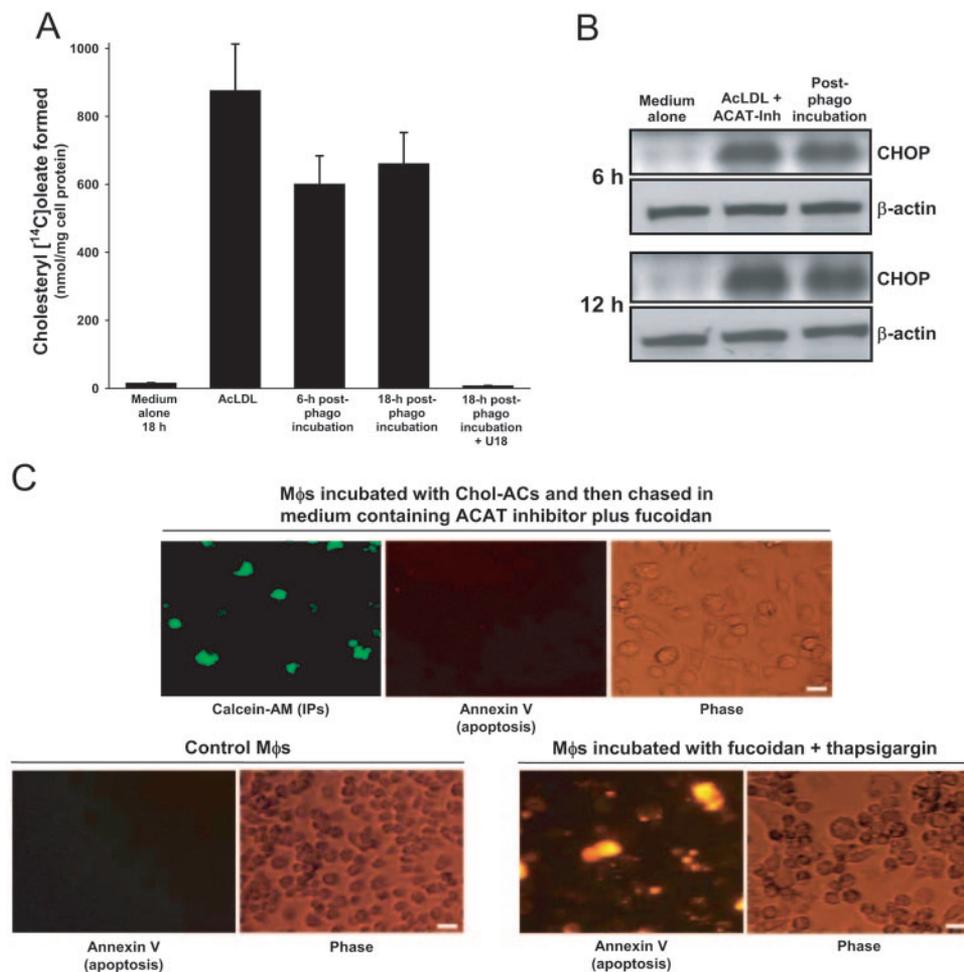
RESULTS

Ingestion of Chol-ACs by phagocytes does not induce apoptosis even when cholesterol esterification is blocked

To accomplish the goals stated in the introduction, we chose a particular model of Chol-ACs that represents a good tool to test our hypothesis and is relevant to advanced atheromata. Specifically, macrophages were rendered apoptotic by incubating them under conditions that favor the intracellular accumulation of unesterified cholesterol. This is accomplished by incubating ACAT-inhibited or *Acat1*^{-/-} macrophages with acetyl-LDL, a commonly used model of an atherogenic lipoprotein. These conditions mimic the proposed dysfunction of cholesterol es-

terification and the presence of apoptotic, unesterified cholesterol-loaded macrophages in advanced lesions [6, 11, 16, 31–39]. These Chol-ACs were added briefly to a fresh monolayer of untreated macrophages (phagocytes) to allow internalization [26]. Thirty minutes after Chol-AC addition, the phagocyte monolayer was rinsed thoroughly to remove noningested apoptotic cells and then incubated in fresh serum-containing medium for various periods of time. To detect the subpopulation of phagocytes that actually ingested Chol-ACs, the Chol-ACs were labeled with the green vital fluorescent dye, Calcein GreenTM-AM. We refer to the subpopulation of phagocytes that ingest Calcein GreenTM-AM-labeled Chol-ACs as “ingesting phagocytes,” or “IPs.” Previous studies documented that the labeled IPs represent phagocytes that have fully ingested Chol-ACs [26].

Pilot experiments showed that phagocytes do not die after ingestion of Chol-ACs and that this event is associated with efficient ACAT-mediated esterification of Chol-AC-derived cholesterol (below). However, as alluded to above, cholesterol is most toxic to cells when it accumulates in the unesterified form [3–5]. Therefore, to cholesterol-stress the IPs further in a manner that may be relevant to advanced atheromata (above), we used ACAT-inhibited phagocytes. Remarkably, there were no signs of cytotoxicity and no nuclear condensation by visual inspection of the IPs even after 24 h under these normally proapoptotic conditions. To look for more subtle signs of cytotoxicity, the phagocytes were stained with Alexa Fluor 594-conjugated annexin V (red) to detect externalized phosphatidylserine, a sign of early to midstage apoptosis. As shown in **Fig. 1A**, a subpopulation of phagocytes were labeled green, indicating uptake of the Calcein Green-AM-labeled Chol-ACs. Consistent with our initial morphological observations, the IPs were not labeled by annexin V. As a positive control, macrophages that were loaded directly with unesterified cholesterol by incubation with acetyl-LDL plus an ACAT inhibitor stained



phagocytes were incubated in fresh medium containing the ACAT inhibitor 58035 and 25 $\mu\text{g}/\text{ml}$ fucoidan for 24 h. The phagocytes were then stained with Alexa Fluor 594-annexin V (red) to detect apoptosis. The left panel shows the green-filter image (IPs), the middle panel shows the red-filter image (apoptosis), and the right panel shows the phase image. Bottom: Macrophages were incubated for 24 h with medium alone (left pair of panels) or with medium containing 25 $\mu\text{g}/\text{ml}$ fucoidan plus 0.5 μM thapsigargin to effect apoptosis (right pair of panels). The cells were then assayed for apoptosis by staining with Alexa Fluor 594-annexin V (red). Scale bar, 10 μm .

intensely with annexin V, as expected (Fig. 1B). In other experiments not displayed, we showed that IPs remain resistant to cholesterol-induced apoptosis when cholesterol-loading is initiated 6 h after Chol-AC ingestion and, remarkably, some apoptosis resistance persists even when the interval between AC ingestion and cholesterol loading was 70 h. Thus, phagocytes that have ingested Chol-ACs, a very rich source of cholesterol, are markedly and persistently resistant to apoptosis, even under conditions of compromised cholesterol esterification.

Neither a cholesterol trafficking defect nor deficient engagement of the type A scavenger receptor can explain the lack of Chol-AC-induced apoptosis in ACAT-inhibited ingesting phagocytes

In order for atherogenic lipoprotein-derived cholesterol to trigger apoptosis in ACAT-inhibited macrophages, a number of proapoptotic “hits” must be effected in concert [37, 38]. Two of these hits require the trafficking of ingested cholesterol to the ER: 1) activation of the proapoptotic CHOP branch of the ER stress pathway known as the unfolded protein response (UPR)

Fig. 2. Suppressed apoptosis in IPs cannot be explained by defects in cholesterol trafficking to the ER, CHOP induction, or SRA engagement. (A) The first two bars represent macrophages incubated for 18 h in medium alone or medium containing 100 $\mu\text{g}/\text{ml}$ acetyl-LDL. The last three bars represent macrophages exposed to Chol-ACs for 30 min to enable phagocytosis and then, after removal of ACs, chased in medium for 6 or 18 h. In the experiment represented by the last bar, the 18-h post-phagocytosis chase medium contained 1 μM U18666A to block cholesterol trafficking to the ER. For all of the phagocytosis experiments, Chol-ACs were made using ACAT1-deficient macrophages rather than ACAT inhibitor. In all of the experiments, the 6 h or 18 h of incubation media contained [¹⁴C]oleate. After the indicated time points, the macrophages were assayed for cholesteryl [¹⁴C]oleate as an indicator of cholesterol trafficking to ACAT in the ER. (B) Lanes 1 and 2 refer to macrophages were incubated for 6 or 12 h with medium alone or with medium containing acetyl-LDL plus 58035 (AcLDL+ACAT-Inh) as a positive control for CHOP induction. Lane 3 refers to macrophages incubated with Chol-ACs for 30 min followed by removal of the Chol-ACs and then incubation in medium containing ACAT inhibitor for 6 or 12 h. Cell lysates were subjected to SDS-PAGE and immunoblotted for CHOP and β -actin. (C) Top 3 images: Chol-ACs were labeled with Calcein Green-AM (green) and then briefly exposed to phagocytes. After noningested Chol-ACs were removed, the

[6]; and 2) release of ER calcium stores into the cytoplasm, which triggers calcium-dependent apoptotic signaling [38]. Therefore, one possible mechanism for the lack of apoptosis in IPs is that Chol-AC-derived cholesterol cannot traffic to the ER. This might occur, for example, if the cholesterol were trapped in phagolysosomes. To evaluate this possibility, we took advantage of the fact that ACAT-mediated esterification, an ER-localized process, is a reliable marker of cholesterol trafficking to the ER [40]. Thus, Chol-ACs were incubated with phagocytes in the presence of [¹⁴C]oleate, and the formation of cholesteryl [¹⁴C]oleate was assayed. To make sure that the phagocytes would not be exposed to residual ACAT inhibitor in the Chol-ACs, the Chol-ACs for this experiment were generated by incubating macrophages from *Acat1*^{-/-} mice with acetyl-LDL without ACAT inhibitor. As shown in **Fig. 2A**, exposure of the phagocytes to Chol-ACs resulted in a marked increase in cholesterol esterification. Note that the level of esterification is similar to that in macrophages incubated with acetyl-LDL, even though acetyl-LDL is taken up by all of the macrophages on the dish, whereas Chol-ACs is taken up by only ~25% of the macrophages. Moreover, the increase in Chol-AC-induced cholesterol esterification was completely

blocked by compound U18666A, which inhibits cholesterol trafficking from degradative organelles to peripheral sites like the ER [6]. Consistent with the conclusion that Chol-AC-derived cholesterol is efficiently trafficked to the ER, we found that CHOP was induced in ACAT-inhibited IPs to a similar level as that in ACAT-inhibited macrophages incubated with acetyl-LDL (Fig. 2B). These data indicate that cholesterol-derived from the ingestion of Chol-ACs traffics efficiently to the ER and activates the UPR/CHOP pathway, and so the explanation for the lack of apoptosis in IPs must lie elsewhere.

Our studies with macrophages loaded with lipoprotein-derived cholesterol revealed that apoptosis in this scenario requires the combination of UPR/CHOP activation (above) plus proapoptotic signaling triggered by ligands of the atherogenic lipoprotein receptor SRA (type A scavenger receptor) [37, 38]. Whereas atherogenic lipoproteins activate both of these hits, that is, through cholesterol delivery to the cell (UPR/CHOP) and binding to the SRA, apoptosis can also be induced by triggering each hit separately. For example, incubation of macrophages with a UPR activator like thapsigargin plus an SRA ligand like the polysaccharide fucoidan triggers apoptosis, whereas apoptosis does not occur with each reagent alone [37]. With this background, one possible explanation for the lack of apoptosis in IPs could be the absence of SRA engagement. To test this important possibility, we incubated ACAT-inhibited IPs with the SRA ligand fucoidan. Somewhat to our surprise, we found that apoptosis was still not induced under these conditions (Fig. 2C, top 3 images). As mentioned above and as shown in the pair of images in the bottom right of Fig. 2C, apoptosis is marked in macrophages that have been incubated with the UPR activator thapsigargin and the SRA ligand fucoidan. Therefore, lack of SRA engagement cannot explain the resistance to apoptosis in IPs.

IPs demonstrate massive cholesterol efflux after ingestion of Chol-ACs

We next considered the possibility that, after the initial burst of cholesterol delivery and UPR/CHOP activation by engulfment of Chol-ACs, cholesterol rapidly left the phagocyte, which might reverse the apoptotic program. We therefore carefully monitored unesterified cholesterol mass in ACAT-compromised phagocytes as a function of time after ingestion of Chol-ACs. For this set of experiments, ACAT-inhibited phagocytes were incubated with Chol-ACs labeled with fluorescent annexin V to distinguish IPs from non-IPs. After a 3-h post-ingestion incubation, the IPs and non-IPs were separated by FACS and assayed for cholesterol mass by gas-liquid chromatography. As originally predicted, the IPs accumulated a substantial amount of unesterified cholesterol compared with non-IPs (**Fig. 3A**). We next directly compared the fold increase in unesterified cholesterol accumulation in ACAT-inhibited IPs with the fold increase in Chol-ACs themselves, because the latter represents a level of cholesterol known to induce apoptosis. As shown in Fig. 3B, the fold increase in cholesterol at 10 h was similar under each condition. In addition, the absolute level of intracellular cholesterol in 7 h IPs was even greater than that in 10 h Chol-ACs (Fig. 3C, 2nd and 3rd bars). Thus, the initial amount of unesterified cholesterol accumulating in the IPs should be adequate to induce apoptosis. How-

ever, as shown in Fig. 3C (4th bar), intracellular cholesterol in IPs drops substantially at 20 h post-ingestion. Moreover, this was associated with marked efflux of ingested cholesterol during the 20 h post-ingestion period (Fig. 3D). These data support the aforementioned hypothesis that apoptosis resistance in Chol-AC-loaded IPs was due, at least in part, to efflux of cholesterol before irreversible death signaling occurred. This hypothesis was supported further by the finding that ACAT-inhibited macrophages loaded with lipoprotein-cholesterol survive if the lipoproteins are removed at 8-10 h (data not shown), which mimics the decrease of cholesterol levels that naturally occurs in Chol-AC-loaded IPs (Fig. 3C above). In contrast, when ACAT-inhibited macrophages are incubated with lipoprotein-cholesterol for 20 h, which are the conditions for inducing apoptosis in Chol-ACs, cholesterol levels remain high (data not shown).

To determine the role of cholesterol acceptors in cholesterol efflux from IPs, IPs were first chased in serum-free medium containing 0.2% BSA. The data in Fig. 3E show that under these conditions, there was no decrease in intracellular cholesterol in IPs between 4 and 20 h, indicating no efflux. In contrast, when the IPs were incubated in serum-free medium containing the cholesterol acceptors apolipoprotein A1 or HDL [41], the 20-h cellular cholesterol level decreased to the level seen with serum-containing medium (Fig. 3E). These data indicate that either apolipoprotein A1 alone or HDL alone can mediate cholesterol efflux from IPs.

IPs are partially resistant to apoptosis even when intracellular cholesterol levels are maintained at a high level

If the efflux of intracellular cholesterol were the sole mechanism of survival in ACAT-inhibited IPs, then we should be able to induce apoptosis by maintaining their cholesterol levels over the course of the 20-h post-ingestion period. As a tool to maintain the unesterified cholesterol levels in IPs, we incubated IPs with acetyl-LDL plus ACAT inhibitor during the 20-h post-ingestion chase period. **Figure 4A** shows that IPs are able to internalize acetyl-LDL, and the unesterified cholesterol levels in these cells were maintained for 20 h at a level that was 4- or 5-fold higher than when cholesterol loading was not conducted during the 20-h period (Fig. 4B). Indeed, this level of unesterified cholesterol is even greater than that seen with Chol-ACs (refer to Fig. 3C). To determine the susceptibility to apoptosis of IPs treated under these persistently high-cholesterol conditions, phagocytes were incubated with Calcein Green-AM-labeled Chol-ACs (green) to distinguish IPs from non-IPs. After the 20-h post-ingestion cholesterol-loading period, the phagocytes were stained with fluorescent annexin V (red) to detect apoptosis. Although some of these cholesterol-loaded IPs became apoptotic, apoptosis was approximately twofold more prevalent in non-IPs (red only) than in IPs (red and green) (Fig. 4C). Thus, the process of phagocytosis of Chol-ACs appears to partially protect the phagocytes from apoptosis even when intracellular unesterified cholesterol levels are maintained at a very high level.

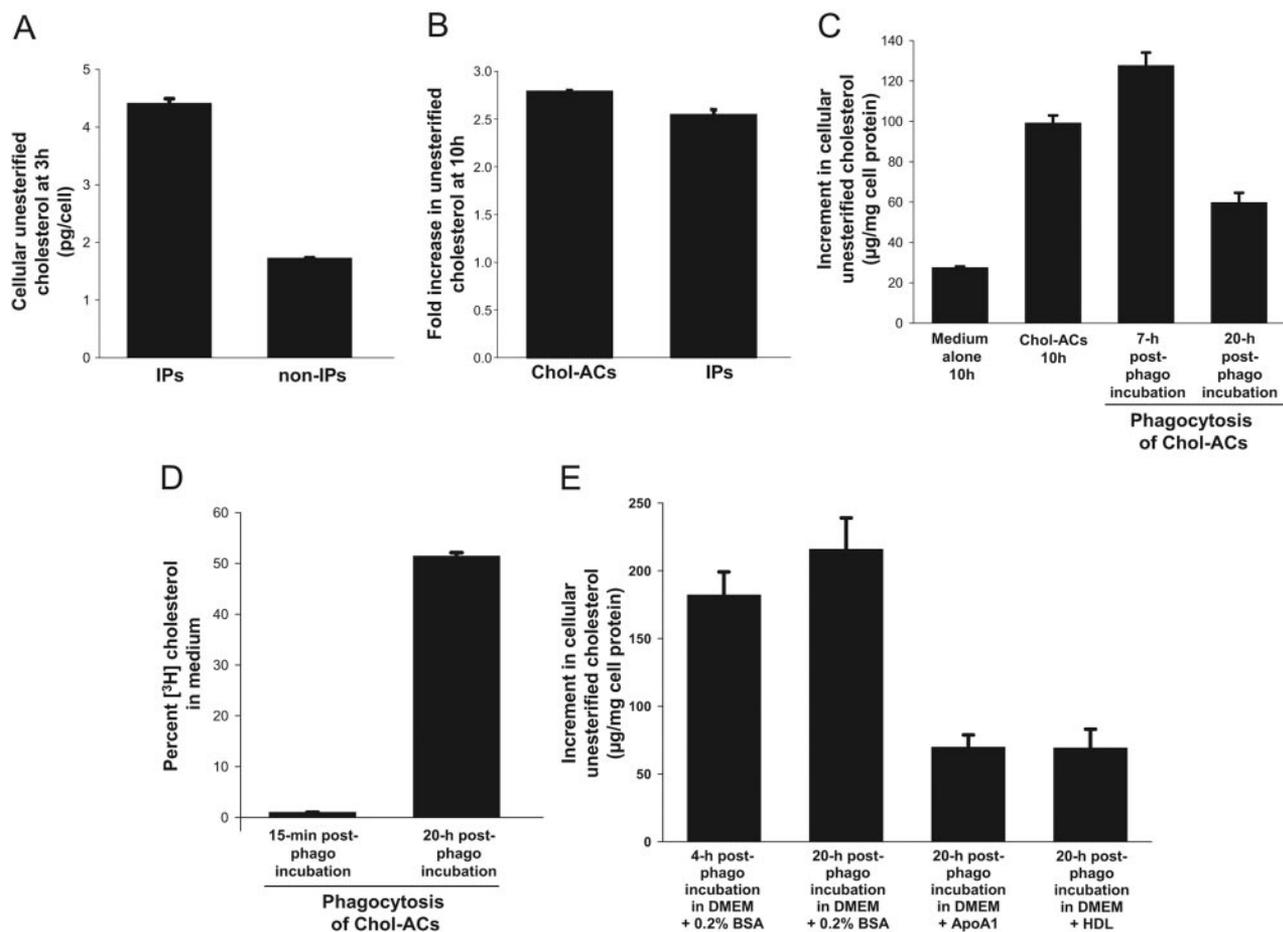


Fig. 3. IPs massively efflux cholesterol post-ingestion of Chol-ACs. (A) Chol-ACs were labeled with Alexa Fluor 488-annexin V (green) and then added to phagocytes for 30 min. The phagocytes were washed to remove noningested Chol-ACs and incubated in fresh medium containing ACAT inhibitor for 3 h. The phagocytes were then subjected to FACS sorting to separate IPs (green) from non-IP macrophages (non-green). Lipids were extracted from the IPs or non-IP macrophages, and cellular unesterified cholesterol mass measured by gas-liquid chromatography. The differences between the two values were statistically significant ($P < 0.005$). (B) The first bar involves macrophages that were incubated for 10 h in medium alone or medium containing acetyl-LDL + 58035 to effect cholesterol loading. The unesterified cholesterol mass ratio in cholesterol-loaded macrophages (Chol-ACs) to nonloaded macrophages is shown. The second bar involves a protocol similar to that in (A), except a 10-h incubation period was used. The bar shows the cholesterol mass ratio in IPs to non-IP macrophages. (C) The first two bars involve macrophages that were incubated for 10 h in medium alone or medium containing acetyl-LDL + 58035 to effect cholesterol loading (Chol-ACs). The last two bars involve macrophages that were incubated for either 7 h or 20 h postingestion of Chol-ACs. Cellular unesterified cholesterol mass was measured. The results for the last two bars were normalized using the basal level of cholesterol in control macrophages, and the final values were adjusted to reflect the percentage of phagocytes ingesting Chol-ACs (22%). The differences between the value represented by the fourth bar and those represented by the second and third bars were statistically significant ($P < 0.005$). (D) Chol-ACs were induced by incubation with [^3H]-acetyl-LDL+58035. Phagocytes were then exposed to these Chol-ACs and then, after noningested Chol-ACs were removed, chased for 15 min or 20 h in fresh media containing ACAT inhibitor. The media were then collected assayed for tritium radioactivity. The results are expressed as the percentage of total tritium (i.e., cells+medium tritium) that was in the medium. (E) Phagocytes were then exposed to these Chol-ACs and then, after noningested Chol-ACs were removed, chased for 4 or 20 h in DMEM/0.2% BSA alone or containing 15 $\mu\text{g/ml}$ apolipoprotein A1 or 50 $\mu\text{g/ml}$ HDL. Cellular-free cholesterol mass was measured. The results were normalized using the basal level of cholesterol in macrophages not exposed to Chol-ACs, and the final values were adjusted to reflect the percentage of phagocytes ingesting Chol-ACs (25%). The differences between the values represented by the third and fourth bar and those represented by the first and second bars were statistically significant ($P < 0.005$).

NF- κB and PI-3 kinase/AKT signaling pathways are required for the survival response of IPs

A recent study from our laboratory showed that exposure to Chol-ACs activates NF- κB signaling pathway in IPs [26]. Because NF- κB is known to signal survival responses in cells [42, 43], we investigated the functional significance of NF- κB signaling in the survival response of IPs against cholesterol-induced apoptosis. Compound PS1145, a specific inhibitor of IKK β that efficiently inhibits NF- κB signaling in IPs [24–26], was added to the IPs immediately after Chol-AC ingestion had occurred, that is, during the 20-h cholesterol-loading period.

As shown in **Fig. 5A**, the IKK β inhibitor led to an approximately threefold increase in apoptosis in cholesterol-loaded IPs. We next considered the possible role of another well-known survival pathway, namely, that triggered by activation of Akt [44]. We found that phosphorylation of Akt, a marker of Akt activation, was prominent throughout the postingestion period and much greater than that in macrophages incubated under identical conditions but without exposure to Chol-ACs (Fig. 5B). To test the role of Akt in survival, Chol-AC-loaded IPs were incubated in the absence or presence of compound LY294002, which blocks Akt signaling through its ability to

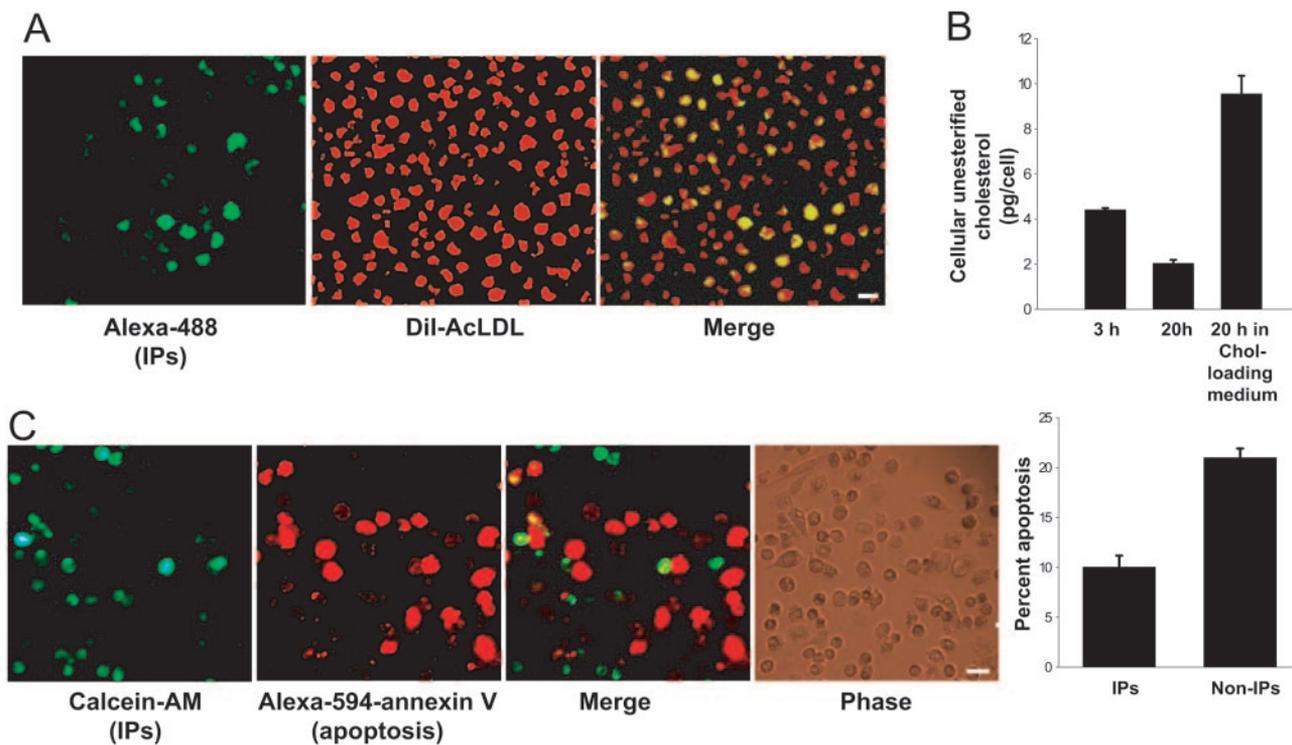


Fig. 4. IPs are resistant to cholesterol-induced apoptosis. (A) To show that IPs are able to internalize acetyl-LDL, macrophages were exposed briefly to Chol-ACs that had been labeled with Alexa Fluor 488-annexin V (green) and then, after removal of the non-ingested Chol-ACs, incubated for 1 h in fresh medium containing DiI-labeled acetyl-LDL (red). The cells were then viewed for green fluorescence to identify IPs (left panel) and red fluorescence to identify acetyl-LDL uptake (middle panel); the merged image is shown in the right panel. (B) The first two bars show IPs that were incubated in medium containing ACAT inhibitor alone for 3 or 20 h post-Chol-AC ingestion. The third bar shows IPs incubated for 20 h post-ingestion in medium containing acetyl-LDL + 58035 to effect additional cholesterol loading. The IPs were isolated by FACS as in Fig. 3A and assayed for cellular unesterified cholesterol mass. The differences between the values represented by the second bar and the other two bars and between the third bar and the other two bars were statistically significant ($P < 0.005$). (C) Macrophages were exposed briefly to Chol-ACs that had been labeled with Calcein Green-AM (green) and then, after removal of the noningested Chol-ACs, incubated for 20 h in fresh medium containing acetyl LDL + 58035. The cells were then assayed for apoptosis using Alexa Fluor 594-annexin V (red). Panel 1 shows green fluorescence to identify IPs, and panel 2 shows red fluorescence to identify apoptosis. Panel 3 shows the merged image, and panel 4 shows the phase image. Panel 5 shows the quantified data for the percent of IPs (green cells) vs. non-IPs (non-green cells) that were labeled with red annexin V. Bar, 10 μ m. The differences between the two values were statistically significant ($P < 0.005$).

inhibit the Akt activator PI-3 kinase [45]. As shown in Fig. 5C, this treatment increased apoptosis in IPs by approximately threefold. Finally, to determine whether NF- κ B and Akt represented independent and complementary survival pathways vs. pathways signaling through a common mechanism, we inhibited both NF- κ B and Akt signaling in cholesterol-loaded IPs. We predicted that pathways would be complementary, because Akt is activated at relatively early time points post-phagocytosis (Fig. 5B), while NF- κ B is activated at later time points (i.e., ≥ 6 h after phagocytosis; cf. Ref. [26]). Indeed, the combination of both inhibitors led to an additive survival response that resulted in $\sim 80\%$ apoptosis of the cholesterol-loaded IPs (Fig. 5D). Thus, NF- κ B and Akt survival pathways play independent and complementary roles in the ability of IPs to remain viable despite high levels of cholesterol loading.

IPs are also resistant to UV-induced apoptosis through a mechanism that relies primarily on Akt signaling

To determine whether the resistance of IPs to apoptosis might extend beyond cholesterol loading, we subjected post-ingestion IPs to a dose of UV irradiation that is known to induce

apoptosis in macrophages [26]. As shown in Fig. 6A, IPs were partially resistant to UV-induced apoptosis. This survival response was almost completely abrogated by inhibition of PI-3 kinase/Akt signaling (Fig. 6B) but not by inhibition of NF- κ B (Fig. 6C). Thus the ability of IPs to survive a death insult extends beyond cholesterol-induced apoptosis, although the relative importance of specific survival pathways appears to differ depending upon the nature of the insult.

DISCUSSION

The data in this report address the general issue of how phagocytes deal with the large amount of cholesterol they ingest during the uptake of ACs, even under rather extreme conditions, as well as the more specific issue of the fate of phagocytes confronted with cholesterol-loaded ACs, as likely occurs in advanced atheromata. The overall conclusion is that phagocytes acquire a remarkable ability to survive the stress of excess cholesterol delivery. This survival capability is greater than that seen with macrophages loaded with lipoprotein-cholesterol, suggesting that some unique aspect of AC-phago-

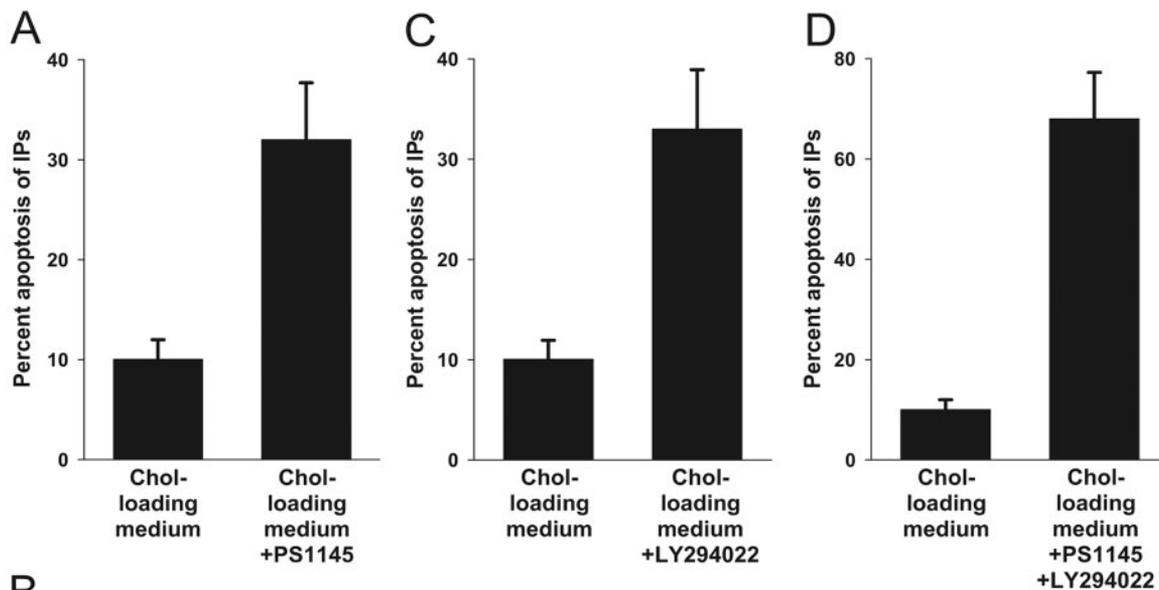


Fig. 5. NF- κ B and AKT signaling pathways are required for the survival response of IPs against cholesterol-induced apoptosis. (A, C, and D) Phagocytes were exposed to Chol-ACs for 30 min and then non-ingested Chol-ACs were removed by vigorous rinsing. The phagocytes were then

(i.e., after ingestion) incubated for an additional 20 h in cholesterol-loading medium either in the absence or presence of 10 μ M of the IKK inhibitor PS1145 (A), 10 μ M of the PI-3 kinase/Akt inhibitor LY294022 (C), or both compounds (D). The percent apoptosis in IPs was determined using the protocol described in Fig. 4C. (B) Macrophages were either exposed or not exposed to Chol-ACs and then incubated for the indicated time in medium containing ACAT inhibitor; “c” refers to control macrophages not exposed to Chol-ACs and “p” (phagocytosis) refers to macrophages exposed to Chol-ACs. Cell lysates were subjected to SDS-PAGE and immunoblotted for phosphorylated AKT and total AKT. The differences between the two values in (A, C, and D) were statistically significant ($P < 0.005$). The values for the bar data in (A and C) appear indistinguishable; the actual values are 10.02 ± 1.98 and 32.0 ± 5.69 in (A) and 10.04 ± 1.90 and 33.0 ± 5.92 for (C).

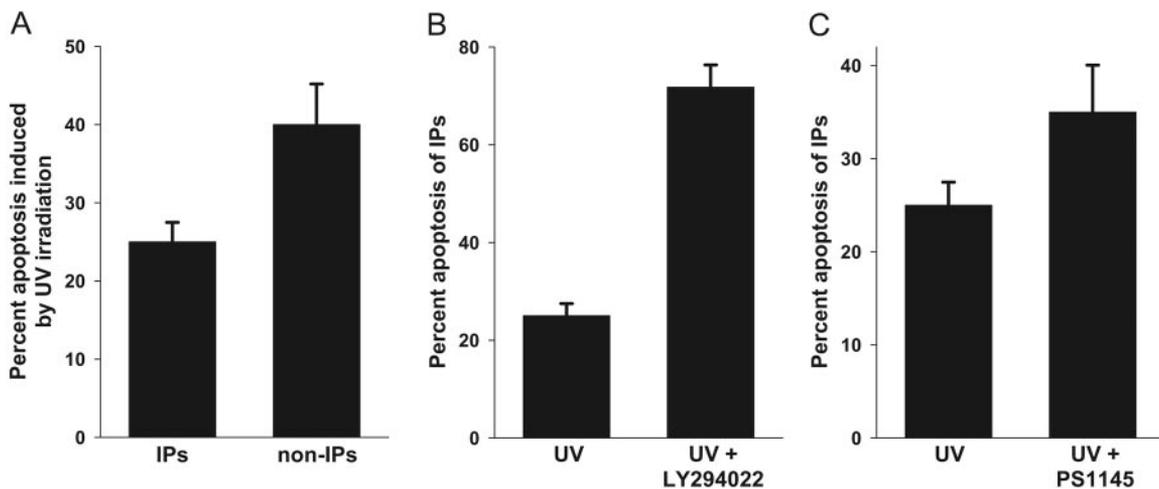


Fig. 6. IPs are resistant to UV-induced apoptosis through a mechanism that relies primarily on Akt signaling. (A) Chol-ACs were labeled with Calcein Green-AM (green) and then added to phagocytes for 30 min. The phagocytes were washed to remove non-ingested Chol-ACs, incubated in fresh medium for 10 min, and then subjected to UV irradiation for 20 min. After an additional 8 h incubation, the cells were assayed for apoptosis using Alexa Fluor 594-annexin V (red). The graph shows the quantified data for the percent of IPs (green cells) vs. non-IPs (non-green cells) that were labeled with red annexin V. (B and C) Percent apoptosis was determined in IPs that were incubated as in panel A (1st bar) except that some of the cells were treated with 10 μ M LY294022 (B) or 10 μ M PS1145 (C) during the post-ingestion incubation period. The differences between the two values on all three panels were statistically significant ($P < 0.005$ for (A and B) and $P < 0.01$ for (C)).

cyte contact or AC uptake triggers the cell-survival responses described herein, namely, massive efflux of cholesterol and activation of NF- κ B and Akt.

Future studies will be needed to elucidate the upstream and downstream mechanisms that mediate these effects. Upstream triggering could be mediated by engagement of a phagocyte receptor by ACs, by secretion of a paracrine factor by ACs, or by the engulfment process itself. Regarding the downstream process of cholesterol efflux, we found that both HDL and apolipoprotein A-I can mediate cholesterol efflux from Chol-AC-loaded IPs (Fig. 3E), suggesting that cholesterol efflux transporters ABCA1 and ABCG1 may be involved [41]. In this regard, we previously showed that ABCA1 mRNA and protein were markedly induced in phagocytes exposed to Chol-ACs [46], and Kiss et al. [47] showed a similar phenomenon with the uptake of apoptotic T cells. Interestingly, loading of macrophages with lipoprotein-cholesterol leads to proteasome-mediated degradation of ABCA1 and defective cholesterol efflux [48]. Thus, it is possible that some downstream effect of phagocytosis of apoptotic cells prevents cholesterol-induced degradation of ABCA1. Such a mechanism might explain why cholesterol-loaded IPs, but not Chol-ACs themselves, have a survival mechanism involving robust cholesterol efflux.

The upstream triggers and downstream cell-survival effectors of NF- κ B and Akt signaling in IPs will also be the subject of future studies. Our previous work showed that NF- κ B activation in IPs requires Chol-AC-phagocyte contact but not Chol-AC ingestion [26]. Exactly how this cell-cell contact triggers I κ B kinase activation remains to be investigated. Moreover, there may be a second stimulus for NF- κ B activation in IPs that are loaded with additional cholesterol during the post-ingestion period, because cholesterol trafficking to the ER in cholesterol-loaded macrophages was shown to activate the NF- κ B pathway [49]. Regarding induction of the PI 3-kinase/Akt pathway in IPs, we initially considered the possibility that the phagocytes secreted one or more autocrine/paracrine factors, e.g., growth factors that could trigger Akt signaling. However, the addition of conditioned medium from phagocyte cultures to cholesterol-loaded nonphagocytic macrophages did not elicit a survival response (data not shown). Therefore, the activation of PI-3 kinase/Akt signaling may be related to the phagocytosis process itself [50]. Finally, future work will be needed to determine the cell-survival effectors downstream of NF- κ B and PI-3 kinase/Akt, of which many have been identified in previous studies using other systems [42–44]. Notable examples include the inhibitor-of-apoptosis (IAP) proteins such as c-IAP-2 and the antiapoptotic members of the Bcl family, especially Bcl-2. In the specific case of IPs ingesting Chol-ACs, additional NF- κ B survival mechanism may be induction of ABCA1 and cholesterol efflux [46], thus linking two layers of survival responses identified in this study. In addition, liver X receptor (LXR), which is activated in cholesterol-loaded macrophages, may play a role in the survival of IPs exposed to Chol-ACs, because LXR induces both the cholesterol efflux transporter ABCA1 and the macrophage survival protein SP α (AIM, A β i6) [51, 52].

The fact that both NF- κ B and Akt are involved in the survival response of Chol-AC-loaded IPs may be important to cover a broad temporal range, because activation of Akt occurs

within 15–30 min after ingestion, while activation of NF- κ B occurs 6–8 h after ingestion. Interestingly, when Chol-AC-exposed phagocytes were subsequently exposed to UV irradiation instead of additional cholesterol loading, survival also occurred, but here the PI-3 kinase/Akt pathway was much more important than the NF- κ B pathway. Two studies investigating the uptake of apoptotic T cells by macrophages showed evidence of Akt-mediated cell survival signaling plus other cell survival pathways but not NF- κ B [53, 54]. Thus, apoptotic cell phagocytosis may trigger a “universal” Akt survival pathway, plus additional survival pathways, depending upon the specific nature of the secondary apoptotic insult.

We have used an experimental model that contains several key features of advanced atherosclerotic lesions to address the fate of phagocytic macrophage exposed to very large loads of cholesterol. Whether or not this model reflects the complexity of advanced atheromata, however, remains to be determined in future in vivo experiments. If so, the new findings from this study may explain a feature of advanced lesions that was heretofore a mystery, namely, the presence of unesterified cholesterol-loaded macrophages that appear to be viable. It is possible that these cells represent phagocytic macrophages that have ingested cholesterol-loaded macrophages in the setting of inefficient cholesterol esterification and efflux. Whether the remarkable survival ability of phagocytic macrophages is beneficial or detrimental in terms of plaque progression is a critical question. Addressing this question will require the creation of in vivo models based on the types of mechanistic studies described in this study. Either answer has the potential to increase our knowledge of how lesions progress and may suggest novel therapeutic strategies.

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