Free Cholesterol Loading of Macrophages Is Associated with Widespread Mitochondrial Dysfunction and Activation of the Mitochondrial Apoptosis Pathway*

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Macrophage death in advanced atherosclerotic lesions leads to lesional necrosis and possibly plaque rupture and acute vascular occlusion. Among the likely causes of lesional macrophage death is intracellular accumulation of excess free cholesterol (FC), which is known to occur in vivo. We recently showed that FC loading of macrophages causes apoptosis, ~50% of which is mediated by activation of cell-surface FasL and triggering of the Fas pathway (Yao, P. M., and Tabas, I. (2000) J. Biol. Chem. 275, 23807–23813). To elucidate other pathways of death in FC-loaded macrophages, we investigated mitochondrial transmembrane potential (ΔΨm) and the mitochondrial apoptosis pathway in FC-loaded mouse peritoneal macrophages. Starting between 3 and 6 h of FC loading, ΔΨm was markedly decreased in the majority of macrophages and was independent of the Fas pathway. The decrease in ΔΨm by FC loading was not prevented by GSH, thus distinguishing it from 7-ketocholesterol-induced mitochondrial dysfunction. Cytochrome c release into the cytosol was noted by 4 h of FC loading, and activation of caspase-9 and effector caspases was observed at 6 h. Finally, we found that both cellular and mitochondrial levels of the pro-apoptotic protein Bax were increased severalfold as early as 4 h after FC loading. Thus, FC loading, perhaps via increased levels of Bax and/or cholesterol overloading of mitochondria, triggers cytochrome c release and activation of caspase-9 and the effector caspases, leading to macrophage apoptosis. These findings and our previous data support a model in which FC loading of macrophages promotes a dual program of caspase-mediated death.

Macrophage death is a prominent feature of atherosclerotic lesions (1–4) and may affect lesion progression and/or complications. For example, death of macrophages may contribute to the release of plaque-destabilizing and thrombogenic molecules in more advanced lesions. In support of this idea, “necrotic” cores of advanced atheroma, which contain the debris of dead macrophages (2, 3), are located in areas predisposed to plaque rupture and acute thrombosis (5). Moreover, fragments of plasma membraneshed by apoptotic lesional cells are rich in thrombogenic tissue factor activity (6). More directly, apoptotic macrophages, but not apoptotic smooth muscle cells or T cells, are greatly increased in ruptured plaques versus stable plaques (7), and atherectomy specimens from patients with unstable angina have approximately twice the number of dead intimal cells compared with specimens from patients with stable angina (4).

To elucidate the roles of macrophage death in atherosclerosis, it is necessary to gain a thorough understanding of the inducers and cellular death pathways involved. Although many molecules and processes have been proposed to cause macrophage death in lesions, intracellular accumulation of excess unesterified or free cholesterol (FC)† has been the focus of several investigators. Macrophages in advanced lesions are known to accumulate excess FC (8–11), and excess FC is a potent inducer of macrophage death (12, 13). In this regard, recent work from our laboratory has demonstrated that FC loading of macrophages leads to caspase-dependent externalization of phosphatidylserine and to DNA fragmentation, consistent with an apoptotic process (14). Most interestingly, approximately half of the apoptosis could be blocked by mutations in or inhibitors of the Fas pathway of cell death, implying a partial role for the Fas pathway in FC-induced apoptosis; the mechanism involves FC-induced activation of cell-surface FasL (Fas ligand) (14).

In this study, our goal was to determine the pathway(s) leading to caspase-dependent death in the substantial portion of FC-loaded macrophages that die independently of the Fas pathway. The data reported herein show that there is widespread, Fas-independent mitochondrial dysfunction in FC-loaded macrophages, as well as cytochrome c release, activation of caspase-9, and Fas-independent activation of effector caspases. Interestingly, levels of cellular and mitochondrial Bax, which is known to induce cytochrome c release and mitochondrion-dependent caspase activation (15), are increased in FC-loaded macrophages. These findings and our previous data support a model in which FC loading of macrophages promotes a dual program of caspase-mediated death.

EXPERIMENTAL PROCEDURES

Materials—The Falcon tissue culture plasticware used in these studies was purchased from Fisher. Tissue culture media and other tissue

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† The abbreviations used are: FC, free cholesterol; FBS, fetal bovine serum; AFC, 7-amino-4-trifluoromethylcoumarin; Z-DEVD-fmk, benzyl-oxycarbonyl-Leu-Glu-His-Asp fluoromethyl ketone; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; LDL, low density lipoprotein; PS, phosphatidylserine; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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culture reagents were obtained from Life Technologies, Inc. Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT) and was heat-inactivated for 1 h at 65 °C. Compound 58035 (3-[de-cylidimethylisilyl]-N-2-[4-(methylphenyl)-1-phenylethyl]propanamide) (16), an inhibitor of acyl-CoA:cholesterol acyltransferase, was generously provided by John Hefti (Novo Nordisk, Hanover, NJ); a 10 mg/ml stock solution was prepared in dimethyl sulfoxide, and the final dimethyl sulfoxide concentration in both treated and control cells was 0.05%. Rhodamine 123, MitoTracker Red CMXRos, JC-1 (5',5',6',6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide), anti-cytochrome oxidase subunit IV IgG, and Alexa 488-labeled goat anti-rabbit IgG (1:2000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-cytochrome c IgG was obtained from Pharmingen (San Diego, CA), and anti- caspase-9 IgG was obtained from Nu-Chek Prep, Inc. (Elysian, MN). All other chemicals and reagents, including 7-ketocholesterol, phosphatidylserine, and GSH, were from Sigma, and all organic solvents were from Fisher.

Harvesting, Culturing, and Lipoprotein Incubations of Mouse Peritoneal Macrophages—Macrophages were harvested from BALB/c mice (Jackson ImmunoResearch Laboratories, Inc.) 3 days after the intraperitoneal injection of 40 μg of concanavalin A in 0.5 ml of PBS and then cultured as described previously (17). On the day of the experiment, the cells were washed three times with warm PBS and incubated for the indicated times in DMEM and 1% (w/v) FBS alone or containing 100 μg/ml acetyl-LDL plus 10 μg/ml compound 58035 (FC-loading conditions) as previously described (18). At the end of the incubation period, the cells were assayed for the end points described below.

Incubation of Macrophages with Non-lipoprotein Sterols—Cholesterol or/PS liposomes were made by mixing 2.8 mg of PS and 1.5 mg of cholesterol (1:1 molar ratio) in chloroform. The solvent was completely removed by evaporation under nitrogen, followed by lyophilization. Three ml of FBS was added to the dried lipids. To prevent lipid oxidation during liposome preparation, butylated hydroxytoluene (10 μM final concentration) and diethyl enethi rminepentaacetic acid (100 μM final concentration) were added. The suspension was sonicated under argon for 20 s at 3-min bursts at 4 °C using a tapered micropipet on a Branson 450 sonicator (setting 5). For the experiment displayed in Fig. 4, a 800 μg/ml stock solution of 7-ketocholesterol was prepared as described by Lizard et al. (19). Briefly, 800 μg of 7-ketocholesterol was dissolved in 50 μl of absolute ethanol, and 950 μl of DMEM containing 1% FBS was added. Fifty μl of this solution was then added to 1 ml of culture medium to obtain a final concentration of 40 μg/ml. A 100 μM stock solution of GSH was prepared in DMEM and 1% FBS and then diluted 1:5 in medium to obtain a final concentration of 20 μM GSH.

RESULTS—Macrophages were incubated under control or FC-loading conditions for various times and then stained with 5 μM rhodamine 123, 100 nM MitoTracker Red CMXRos, or 1 μg/ml JC-1 (1.9 μM) in PBS at 37 °C for 15–30 min. The fluorescent images were collected with a laser scanning confocal microscope (Model LSM 410 with a 100× numerical aperture and 1.4 Plan Apo objective; Carl Zeiss, Thornwood, NY). Confocal optical sections were 0.4 μm thick. To quantify the staining of rhodamine 123, we used the NIH Image Version 1.62f program. Intensity values represent the mean integrated fluorescence intensity per cell. All images were obtained within the linear range of the assay.

Apop totosis Assay—Macrophages were incubated under control or FC-loading conditions for 8.5 h, stained with Alexa 488-labeled annexin V, and viewed by fluorescence microscopy as previously described (14). For quantification, three to five fields of cells were counted for the number of annexin-positive cells and total cells.

Statistics—Results are given as means ± S.E. (n = 3 unless noted otherwise).

RESULTS

FC Loading of Macrophages Leads to Mitochondrial Dysfunction—As an initial assessment of mitochondrial function in FC-loaded macrophages, peritoneal macrophages from C57BL/6 mice were incubated for 3, 6, or 9 h in medium alone or containing acetyl-LDL plus the acyl-CoA:cholesterol acyltransferase inhibitor compound 58035 to effect FC loading (13, 20).
The cells were then stained with rhodamine 123, which is a cationic fluorescent dye that is sequestered in mitochondria only if there is an intact transmembrane potential ($\Delta \Psi_m$) across the inner mitochondrial membrane (21). As shown in Fig. 1 (A–C), macrophages incubated under control conditions demonstrated widespread cytoplasmic staining consistent with a mitochondrial pattern of multiple punctate structures (see below). The overall intensity and pattern of the staining were similar at all three time points. Macrophages loaded with FC for 3 h demonstrated a staining similar to that seen with unloaded cells (panel D). In marked contrast, however, the staining of macrophages loaded with FC for 6 or 9 h was substantially decreased (panels E–F). The staining pattern of macrophages incubated with compound 58035 alone was indistinguishable from that of the control macrophages (panels A–C). Quantification of the data in panels A–F using fluorescence intensity measurements of individual cells (average of 100 cells/group) is shown in panel G.

**Fig. 1. Rhodamine 123 staining of control and FC-loaded macrophages.** Macrophages were incubated in DMEM and 1% FBS alone (control (Con); A–C) or containing 100 µg/ml acetyl-LDL plus 10 µg/ml compound 58035 (FC; D–F) for 3 h (A and D), 6 h (B and E), or 9 h (C and F). The live cells were then stained with rhodamine 123 and viewed by confocal microscopy. Shown in G are quantitative data from five fields of cell (100 cells total) for each condition.
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More detailed images of mitochondrial staining with rhodamine 123 are shown in Fig. 2 (A and B). The punctate staining pattern of unloaded macrophages is clearly seen in panel A. In macrophages loaded with FC for 6 h (panel B), there were less numerous punctate structures per cell, and the cytoplasm was diffusely stained with the dye (arrow). This cytoplasmic staining indicates that the FC-loaded cells do not have a defect in the cellular uptake of rhodamine 123 due to a defect in the plasma membrane potential; rather, it is the ability of mitochondria to sequester the dye that is compromised under FC-loading conditions. To confirm these results, macrophages incubated under control or FC-loading conditions for 6 h were stained with MitoTracker Red CMXRos, another fluorescent dye that accumulates in mitochondria only if $\Delta\psi_{m}$ is intact (22). As shown in panel C, control macrophages demonstrated bright punctate fluorescence throughout the cytoplasm. In contrast, macrophages loaded with FC for 6 h (panel D) showed markedly less fluorescence. Finally, we investigated mitochondrial membrane potential with JC-1, a dye that has been reported to be a particularly reliable probe of $\Delta\psi_{m}$ (23). With normal $\Delta\psi_{m}$, JC-1 concentrates in mitochondria, leading to aggregate formation and a high red/green fluorescence ratio. With decreasing $\Delta\psi_{m}$, there is less aggregate formation and thus a decrease in the red/green fluorescence ratio. As shown in panels E–H, FC loading was associated with a marked decrease in red fluorescence (panel F versus panel E) and an increase in green fluorescence (panel H versus panel G), indicative of a decrease in $\Delta\psi_{m}$. In summary, the data in Figs. 1 and 2 indicate that by 6 h of FC loading, macrophages show markedly defective $\Delta\psi_{m}$, indicating widespread mitochondrial dysfunction.

Mitochondrial Dysfunction and Apoptosis Can Be Induced by Non-lipoprotein Cholesterol and Are Not Prevented by Glutathione Treatment—It is possible that macrophages incubated with acetyl-LDL plus an acyl-CoA:cholesterol acyltransferase inhibitor could be exposed to toxic oxysterols or other toxic components in acetyl-LDL or that the cholesterol derived from acetyl-LDL could be converted intracellularly to toxic oxysterols (24, 25). To address these issues, we conducted two experiments. First, we determined whether mitochondrial dysfunction could be caused by incubating macrophages with cholesterol, but not the other components of acetyl-LDL. To accomplish this goal, we made cholesterol/PS liposomes in the presence of antioxidants; PS-containing liposomes enter macrophages by the type A scavenger receptor (26). Macrophages were stained with rhodamine 123 following incubation for 6 h with medium alone (control), with cholesterol/PS liposomes plus compound 58035, or with PS liposomes without cholesterol plus compound 58035. As shown in Fig. 3 (A–C), mitochondrial staining in macrophages incubated with cholesterol/PS liposomes was markedly less than that observed in control macrophages or macrophages incubated with PS liposomes without cholesterol; quantitative data are shown in panel D. Thus, a non-lipoprotein cholesterol-carrying particle can induce mitochondrial dysfunction in macrophages; if acetyl-LDL contains other cytotoxic components, they are not necessary for mitochondrial dysfunction. Regarding the end point of apoptosis itself, we could not use the annexin assay due to the presence of PS in the liposomes. However, cell detachment correlates with apoptosis in FC-loaded macrophages, and percent cell loss was the same in macrophages treated with acetyl-LDL plus compound 58038 (42.3 ± 2.2%) versus cholesterol/PS liposomes plus compound 58035 (41.9 ± 0.6%).

Next, to address the issue that cytotoxicity might be induced by toxic oxysterols, we took advantage of the observation by Lizard et al. (19) that 7-ketocholesterol-induced death in human monocytes is partially prevented by co-incubation with the antioxidant GSH. First, we determined whether 7-ketocholesterol could cause mitochondrial dysfunction in mouse peritoneal macrophages and, if so, whether it could be prevented by GSH. As shown in Fig. 4C, macrophages exposed to 7-ketocholesterol plus compound 58035 displayed two distinct rhoda-
mine 123 staining patterns: overall paucity of staining (arrow) and intense diffuse staining not reflective of a normal mitochondrial pattern (arrowhead). Note that the latter pattern, which was predominant in these cells, was never observed with acetyl-LDL plus compound 58035 (see above and panel H). Importantly, the mitochondrial staining pattern was mostly normal when GSH was included during the incubation (panel D; quantification in panel E). In contrast, the decreased mitochondrial staining of macrophages incubated with acetyl-LDL plus compound 58035 was not prevented at all by co-incubation with GSH (compare panel I with panel H; quantification in panel J). Thus, mitochondrial dysfunction caused by acetyl-LDL plus compound 58035 can be distinguished from that caused by a toxic oxysterol both by the pattern of mitochondrial staining and by the lack of prevention by co-incubation with GSH. We also examined the effect of GSH on apoptosis itself because GSH blocks 7-ketocholesterol-induced apoptosis as well as mitochondrial dysfunction (27). In an 8.5-h incubation, the percent apoptotic cells in macrophages incubated with acetyl-LDL plus compound 58035 in the presence of 20 mM GSH was 28.1 ± 0.8, compared with 18.9 ± 1.2 in the absence of GSH and 1.0 ± 0.3 in macrophages that were not FC-loaded at all. Thus, consistent with the rhodamine 123 data, GSH treatment did not block macrophage apoptosis induced by acetyl-LDL plus an acyl-CoA:cholesterol acyltransferase inhibitor.

Mitochondrial Dysfunction Induced by FC Loading Is Independent of the Fas Pathway—We have previously shown that a portion of macrophages loaded with FC for 9 h show signs of apoptosis, including externalization of phosphatidylserine and DNA fragmentation (14). These events are decreased by ~40–60% in macrophages with defective Fas (lpr (lymphoproliferative mutation)) or FasL (gld (generalized lymphoproliferative mutation)) or in wild-type macrophages in the presence of an anti-FasL antibody (14). Thus, the Fas pathway of apoptosis is activated in a portion of FC-loaded macrophages. Given that activation of the Fas pathway can directly lead to mitochondrial dysfunction in certain types of cells (28), we determined whether FC-induced mitochondrial dysfunction was dependent upon an intact Fas pathway. Macrophages from wild-type mice or from mice with the lpr Fas mutation (29) were incubated under control or FC-loading conditions for 6 h and then stained with MitoTracker Red CMXRos (see above). As shown in Fig. 5, the decrease in mitochondrial staining in FC-loaded versus unloaded lpr macrophages (panel D versus panel C) was similar to that observed in wild-type macrophages (panel B versus panel A). Similar results were obtained using rhodamine 123 staining (data not shown). Thus, in FC-loaded macrophages, mitochondrial dysfunction does not depend upon Fas activation.

Cytochrome c Is Released from Mitochondria and Caspase-9, and Effector Caspases Are Activated in FC-loaded Macrophages—Mitochondrial dysfunction can be associated with release of cytochrome c and activation of the proximal caspase (caspase-9), followed by effector caspase activation (e.g. caspase-3, -6, and -7) (15). To examine this pathway, we first subjected mitochondrially enriched fractions from control and FC-loaded macrophages to cytochrome c immunoblot analysis. As shown in Fig. 6 (A and B), mitochondrially associated cytochrome c was decreased in macrophages loaded with FC for 4 or 5.5 h. To verify that these data were not simply due to less mitochondrial protein in the FC lane, we showed that the mitochondrial preparations from these control and FC-loaded macrophages contained approximately equal amounts of another mitochondrial protein, cytochrome oxidase (panels C and D). Finally, cytochrome c was detectable in the cytosol of FC-
loaded macrophages, but not in the cytosol of control macrophages (panel E). In summary, FC loading of macrophages leads to a decrease in mitochondrial cytochrome c and an increase in cytosolic cytochrome c.

We used two approaches to detect caspase-9 activation. First, homogenates of control and FC-loaded macrophages were incubated with a caspase-9 substrate (LEHD-AFC) that fluoresces when cleaved (30). As shown in Fig. 7A (cross-hatched bars), LEHDase activity was increased in FC-loaded macrophages. The absolute level of activity was similar to that observed in staurosporine-treated Jurkat cells (3.47 units/60 min), a system in which caspase-9 activation is known to occur (31). However, it was necessary to show that the LEHDase activity observed in FC-loaded macrophages was not due to nonspecific cleavage by effector caspases. To approach this issue, we first assayed effector caspase activity using the substrate DEVD-AFC (30). As shown in panel B (cross-hatched bars), DEVDase activity was increased in FC-loaded macrophages, which indicates effector caspase activation. To determine whether the mitochondrial pathway might be important in this increase in DEVDase activity, we conducted experiments in which the two other major pathways of effector caspase activation, the Fas and tumor necrosis factor-α pathways, were blocked. In these experiments (data not shown), we showed that DEVDase activity was decreased by only 20% in FC-loaded Fas-deficient macrophages (i.e. from lpr mice), and blocking the tumor necrosis factor receptor antibody had no effect at all on DEVDase activity. These data are consistent with a major role for the mitochondrial pathway in effector caspase activation.

We then used the competitive inhibitor of LEHDase activity, Z-LEHD-fmk (32). As shown in Fig. 7B (solid bars), Z-LEHD-fmk did not inhibit effector caspase activity at all under the conditions of our assay. In marked contrast, the inhibitor decreased LEHDase activity to the basal level seen in unloaded macrophages (panel A, solid bars). Although the absolute values for LEHDase activity varied somewhat among repeat experiments, we always found a substantial level of Z-LEHD-fmk-inhibitable LEHDase activity under conditions in which DEVDase activity was not inhibited by the peptide. For example, in one of our repeat experiments, the absolute level of LEHDase activity in FC-loaded macrophages was ~3-fold

2 In our previous study, we showed that 40–60% of apoptosis at 9 h of FC loading could be blocked by mutations in Fas or FasL, or by blocking FasL with an antibody (14). Our finding here that DEVDase activity in homogenates of lpr macrophages was only 20% less than that in wild-type macrophages is probably due to differences between assaying caspases in vitro versus assaying apoptotic changes in intact cells.
In Figs. 6 and 7 strongly support the conclusion that FC loading leads to cytochrome c release, caspase-9 activation, and effector caspase activation.

**Bax Is Increased in FC-loaded Macrophages**—We next considered how FC loading might lead to the release of cytochrome c from mitochondria. Because the pro-apoptotic protein Bax is a known inducer of cytochrome c release, presumably via the direct interaction of Bax with mitochondrial membranes (15), we investigated the effect of FC loading on mitochondrial Bax. Thus, mitochondrial fractions from macrophages incubated under control or FC-loading conditions for 6 h were probed for Bax by immunoblot analysis. As shown in Fig. 8A, Bax was increased moderately in the mitochondrial fraction from FC-loaded macrophages. To distinguish between nonspecific adherence of Bax, which is sensitive to alkali (34), versus true mitochondrial association, we homogenized the samples in a pH 11.5 buffer prior to mitochondrial isolation. Under these conditions, a striking increase in mitochondrial Bax in FC-loaded macrophages was evident (panel B). To determine whether increased mitochondrial Bax reflected an increase in total cellular Bax or was due entirely to translocation from the cytosol, as has been reported in other systems (35, 36), we probed Bax in the cytosolic fraction from the 6-h experiment described above as well as from macrophages incubated under control or FC-loading conditions for 4 and 8 h. At all time points, cytosolic Bax was increased (panels C–E). Thus, FC loading of macrophages is associated with a total increase in cellular Bax, and a portion of this increased Bax is tightly associated with mitochondria.

Further demonstration that mitochondrial Bax is increased in FC-loaded macrophages is shown by the anti-Bax immunofluorescence images in Fig. 9. In control macrophages, there was a basal level of diffuse Bax staining above that seen with nonimmune IgG (panel A versus panel C), but the staining of FC-loaded macrophages was more intense and punctate (panel B). More detailed images of FC-loaded macrophages double-stained with MitoTracker CMXRs are shown in panels E and F. The punctate pattern of Bax staining (green) is demonstrated in panel E, and the MitoTracker image (red) in panel F shows a similar pattern. The merged image in panel G clearly shows partial colocalization of Bax and mitochondria (yellow). In summary, the data in Figs. 8 and 9 demonstrate that FC loading of macrophages is associated with a substantial increase in mitochondrially associated Bax. This finding raises the possibility of a molecular link between FC loading of macrophages and the release of cytochrome c and subsequent mitochondrion-dependent caspase activation.

**DISCUSSION**

Macrophage death occurs during atherogenesis and is likely to influence disease progression (2–7). Among the inducers of macrophage death that are present in lesions is excess intracellular FC accumulation (8–13). For these reasons, our laboratory has devoted effort to understanding cellular pathways of FC-induced macrophage death. Our first study in this area demonstrated that after 9 h of FC loading in our cell culture model, ~15–25% of the cells had externalization of phosphatidylserine (13). The current study was initiated to identify potential cellular pathways contributing to FC-independent death in these cells. We focused on mitochondrially induced caspase activation and apoptosis because of the general importance of this pathway in cellular death events (15). In this context, the data herein demonstrate that FC loading of macrophages causes widespread, FC-independent mitochondrial dysfunction (first evident between 3 and 6 h after the start of FC loading); an increase in cellular Bax (first evident at 4 h) and in mitochondrially associated Bax; activation of caspase-9 and ef-

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2 In control and treated macrophages and Jurkat cells, the anti-caspase-9 antibody also recognized uncleaved pro-caspase-9, which appeared as a heavily stained band at 42 kDa (not shown in the blots in Fig. 7, C–E).
factor caspases (6 h); and, from our previous study (14), externalization of phosphatidylserine and DNA cleavage (7–9 h). From these data, we hypothesize that at least a portion of the Fas-independent apoptosis of FC-loaded macrophages is caused by a mitochondrial pathway involving release of cytochrome c, possibly induced by Bax, and subsequent caspase activation.

In the context of this hypothesis, an important issue concerns the possible mechanistic links between increased levels of mitochondrial Bax, decreased mitochondrial membrane potential \( \Delta \Psi_m \), and mitochondrial cytochrome c release in FC-loaded macrophages. As alluded to above, it is tempting to speculate that increased mitochondrial Bax plays a role in the release of cytochrome c and possibly in the drop in \( \Delta \Psi_m \). However, proof of this idea would require inactivating Bax during FC loading of macrophages, which we have not yet been able to accomplish. Furthermore, cytochrome c release can occur in the absence of mitochondrial depolarization (37), and mitochondrial depolarization can occur in the absence of cytochrome c release (38). An alternative idea is that FC enrichment of mitochondrial membranes, leading to their structural alteration, directly results in a drop in \( \Delta \Psi_m \) and/or release of cytochrome c. Cholesterol is known to be trafficked to mitochondria in macrophages (39), and mitochondrial ATPase has been shown to be affected by a high cholesterol environment (40–43). Future work will be needed to fully elucidate the role of FC in mitochondrial apoptosis.

**Fig. 7.** LEHDase and DEVDase activities and caspase-9 cleavage in control and FC-loaded macrophages. In A and B, macrophages were incubated under control (Con) or FC-loading (FC) conditions \( \pm 40 \mu M \) Z-LEHD-fmk for 6 h, and then extracts were assayed for LEHDase (A) or DEVDase (B) activity for 60 min. Forty \( \mu g \) of extract protein was used for the LEHDase assay, and 10 \( \mu g \) was used for the DEVDase assay. In C and D, whole cell lysates from these macrophages (Mφ) were subjected to polyacrylamide gel electrophoresis and immunoblot analysis using an antibody that recognizes the cleavage forms of murine caspase-9 that result from proteolytic activation. A positive control for this immunoblot assay is shown in E, which compares control and staurosporine (STS)-treated Jurkat cells. In the blots shown in C–E, uncleaved pro-caspase-9 appeared as a heavily stained band at 45 kDa (not shown). k, kilodaltons; Mφ, macrophage.

**Fig. 8.** FC loading of macrophages is associated with increased levels of Bax. Shown are anti-Bax immunoblots of mitochondrial (Mitoch.) and cytosolic fractions of macrophages incubated under control (Con) or FC-loading (FC) conditions. A, mitochondrial fraction (6 h); B, mitochondrial fraction extracted under alkaline conditions (6 h); C–E, cytosolic fractions (4, 6, and 8 h, respectively).

**Fig. 9.** Anti-Bax immunofluorescence confocal microscopy in control and FC-loaded macrophages. Macrophages incubated under control (A and C) or FC-loading (B and D–G) conditions for 4 h were incubated with MitoTracker Red and then immunostained using an anti-rabbit Bax antibody (A, B, and E–G) or nonimmune rabbit IgG (C and D). In A–D, the green signal from the anti-rabbit IgG secondary antibody is shown. In E–G, a single enlarged field of FC-loaded macrophages was viewed for Bax immunostaining (E), MitoTracker staining (F), or a merge of the two images (G). The yellow color in G represents overlap of the two signals. Bars in A (for A–D) and in E (for E–G) = 10 \( \mu m \).
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