# Oxidized low density lipoprotein inhibits macrophage apoptosis through activation of the PI 3-kinase/PKB pathway

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**Abstract** **Oxidized LDL (oxLDL) is known to induce endothelial adhesion molecule and monocyte chemoattractant protein 1 expression and this is thought to be involved in monocyte recruitment into atherosclerotic lesions. oxLDL has also been found to induce macrophage proliferation. The purpose of the present study was to determine whether oxLDL might also have the ability to increase macrophage populations by inhibiting apoptosis. We found that oxLDL caused a dose-dependent inhibition of the apoptosis that occurs in cultured bone marrow-derived macrophages after macrophage colony-stimulating factor (M-CSF) withdrawal without inducing proliferation. Incubation of macrophages with either native LDL or acetylated LDL had no effect on apoptosis. The prosurvival effect of oxLDL was not inhibited by neutralizing antibodies to granulocyte-macrophage colony-stimulating factor, was maintained in mice homozygous for a mutation in the M-CSF gene, and was not due to other secreted cytokines or growth factors. oxLDL caused activation of the mitogen-activated protein kinases ERK1/2 (extracellular signal-regulated kinases 1 and 2) as well as protein kinase B (PKB), a target of phosphatidylinositol 3-kinase (PI 3-kinase). Furthermore, there was phosphorylation of two important prosurvival PKB targets, I-κBα(Ser-32) and Bad(Ser-136). The MEK inhibitors PD 98059 and U0126 blocked ERK1/2 activation but did not diminish survival. Conversely, the PI 3-kinase inhibitors LY 294002 and wortmannin blocked PKB activation, and the ability of oxidized LDL to promote macrophage survival. Taken together, these results indicate that oxLDL can directly activate a PI 3-kinase/PKB-dependent pathway that permits macrophage survival in the absence of growth factors.**—Hundal, R. S., B. S. Salh, J. W. Schrader, A. Gómez-Muñoz, V. Duronio, and U. P. Steinbrecher. **Oxidized low density lipoprotein inhibits macrophage apoptosis through activation of the PI 3-kinase/PKB pathway.** ***J. Lipid Res.*** **2001.** 42: **1483–1491.**

**Supplementary key words** atherosclerosis • oxidized lipoproteins • M-CSF • signal transduction

Macrophages are the main precursors of foam cells, particularly in the early atherosclerotic lesions termed fatty streaks (1). More recently, evidence has implicated macrophages in the evolution of advanced lesions, which are characterized by intimal proliferation of smooth muscle cells, matrix deposition, and lipid-rich "necrotic cores." Macrophages facilitate lesion progression by the elaboration of various growth factors and cytokines that promote vascular smooth muscle migration, metalloproteinase-mediated extracellular matrix dissolution, and creation of an oxidative environment that promotes LDL modification (2, 3). Furthermore, macrophages have been shown to be key determinants of plaque instability, in that macrophage-rich lesions are much more likely to undergo plaque rupture and thrombosis. Such events are responsible for most of the acute clinical complications of atherosclerosis, such as myocardial infarction and stroke (4–6). Therefore, a comprehensive understanding of the factors that influence macrophage numbers and function in atherosclerotic lesions is of obvious importance and could lead to new targets for therapeutic intervention.

Oxidized LDL (oxLDL) has been implicated as an important pathogenetic factor in atherosclerosis, and has been identified as a potential cause of macrophage recruitment and retention in lesions (7–9). oxLDL has also been shown to increase macrophage cell numbers through growth induction. It has been demonstrated that macrophages are the predominant proliferating cell type in atherosclerotic lesions (10, 11), and that oxLDL induces the proliferation of mouse peritoneal macrophages (12–16) as well as human monocyte-derived macrophages in vitro (17). Another potential mechanism for increasing

Abbreviations: ac-LDL, acetylated LDL; BMDM, bone marrow-derived macrophages; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; n-LDL, native LDL; oxLDL, oxidized LDL; PARP, poly(ADP-ribose) polymerase; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B.

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macrophage cell numbers is enhanced macrophage survival or inhibition of apoptotic cell death. To date, however, this has received only limited attention (18) and the mechanism of this effect as well as its relationship to the growth-inducing effect of oxLDL remain unclear.

The objective of the present study was to characterize the effect of oxLDL on the survival of bone marrow-derived macrophages (BMDM). BMDM differ from resident macrophages in that they have an absolute requirement for a macrophage survival cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF) or macrophage colony-stimulating factor (M-CSF), to maintain their viability in culture (19). Similarly, human monocyte-derived macrophages have a similar requirement for growth factors to maintain viability in culture (20). We found that oxLDL prevented BMDM apoptosis induced by M-CSF withdrawal through a mechanism that did not involve cytokine elaboration, and therefore differs from the reported mechanism for growth induction by oxLDL in peritoneal macrophages (15). However, oxLDL activated intracellular survival signaling pathways similar to those activated by GM-CSF and M-CSF, namely, the mitogen-activated protein (MAP) kinases ERK1/2 (extracellular signal-regulated kinases 1 and 2) and the phosphatidylinositol 3-kinase (PI 3-kinase) target protein kinase B (PKB). The antiapoptotic effect required activation of the PI 3-kinase/PKB pathway, but was independent of the activation of ERK1/2.

## MATERIALS AND METHODS

### Materials

DMEM and RPMI 1640 medium were purchased from Canadian Life Technologies (Burlington, ON, Canada). HyClone defined FBS was purchased from HyClone (Logan, UT). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS), was purchased from Promega (Madison, WI). Propidium iodide, RNase, and phenazine methosulfate (PMS) were purchased from Sigma (St. Louis, MO). Caspase 3, PKB, cyclin D1, and ERK1 antibodies were purchased from Stressgen (Victoria, BC, Canada). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Advanced ImmunoChemical (Long Beach, CA). Poly(ADP-ribose) polymerase (PARP) was purchased from BD PharMingen (Carlsbad, CA). Centricon microconcentrators were purchased from Amicon (Beverly, MA). Phospho-PKB(Ser-473), phospho-ERK1/2(Thr-202/Tyr-204), phospho-I-κBα(Ser-32), phospho-Bad-(Ser-136), Bad, and I-κBα were purchased from New England BioLabs (Beverly, MA). Goat anti-rabbit IgG and goat anti-mouse IgG, horseradish peroxidase-conjugated secondary antibodies, MEK inhibitors PD 98059 and U0126, and PI 3-kinase inhibitors LY 294002 and wortmannin were purchased from Calbiochem (La Jolla, CA). Nitrocellulose membrane and low molecular weight protein standards for immunoblotting were purchased from Bio-Rad (Hercules, CA). Reagents for enhanced chemiluminescence were purchased from Amersham International (Little Chalfont, Buckinghamshire, UK). GM-CSF was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Anti-GM-CSF antibodies were provided by J. Schrader (Biomedical Research Centre, Vancouver, BC, Canada). Osteopetrotic mice homozygous for a mutation in M-CSF (B6C3Fe-a/a-Csf1$^{op}$) (*op/op*) were purchased from the Jackson Laboratory (Bar Harbor, ME).

### Lipoprotein isolation and oxidation

LDL (d = 1.019–1.063) was isolated by sequential ultracentrifugation of EDTA-anticoagulated fasting plasma obtained from healthy normolipidemic volunteers (21). The concentrations of EDTA in LDL preparations were reduced before oxidation by dialysis against Dulbecco's phosphate-buffered saline containing 10 μM EDTA. Oxidation was performed with LDL at 200 μg/ml in Dulbecco's phosphate-buffered saline containing 5 μM $CuSO_4$, incubated at 37°C for 24 h (22, 23). Acetylation of LDL was performed by the sequential addition of acetic anhydride (24).

### Cell culture

Bone marrow cells were isolated from the femurs of female CD-1 mice or female *op/op* (B6C3Fe-a/a-Csf1$^{op}$) mice as described (18). Cells were plated for 24 h in RPMI 1640 containing 10% FBS and 10% L-cell conditioned medium; a crude source of M-CSF, kindly provided by J. Schrader (Biomedical Research Centre). The nonadherent cells were removed and cultured in the above-described medium until confluence was reached (5–7 days). Thereafter, the cells were harvested with a Teflon cell lifter and seeded at $10 \times 10^3$ cells/well in 96-well plates, $1 \times 10^6$ cells/well in 6-well plates, or $5 \times 10^6$ cells/100-mm dish in RPMI 1640 with 10% FBS, but without M-CSF for 24 h before use to render the cells quiescent. BMDM are a relatively pure and homogeneous population with more than 95% of the adherent cells binding M-CSF (25). RAW 264.7 cells were purchased from the American Type Culture Collection (Manassas, VA). RAW 264.7 cells were cultured in DMEM containing 10% FBS and starved of FBS for 24 h to render the cells quiescent.

### MTS cell viability assay

Macrophage survival was determined by the MTS-formazan method. This assay is based on the cellular bioreduction of MTS by mitochondrial dehydrogenase enzymes in metabolically active cells. The quantity of formazan product formed was measured by the amount of absorbance at 490 nm and is directly proportional to the number of viable cells in culture. MTS-PMS solution (20 μl/well) was added to wells containing 100 μl of culture medium in 96-well plates 3 h before terminating the experiment. This resulted in final MTS and PMS concentrations of 333 μg/ml and 25 μM, respectively. After 3 h at 37°C in a humidified 5% $CO_2$ atmosphere, the absorbance at 490 nm was recorded with an ELISA plate reader. For conditioned medium studies, control or oxLDL-treated medium was removed and filtered with 100-kDa microconcentrators before reapplication. For inhibitor studies, the cells were preincubated with the indicated concentrations of either the MEK inhibitors (PD 98059 or U0126) or PI 3-kinase inhibitors (LY 294002 or wortmannin) for 1 h before the addition of oxLDL.

### DNA fragmentation

Macrophages were harvested by scraping and were then washed twice in 4°C PBS. Cells were fixed in ice-cold 70% ethanol for 1 h at −20°C, washed three times with 4°C PBS, and resuspended in hypotonic fluorochrome buffer consisting of 0.1% Triton X-100, 0.1% sodium citrate, RNase (25 μg/ml), and propidium iodide (50 μg/ml). After 24 h of incubation at 4°C, fluorescence was measured with an Epics XL-MCL fluorescence-activated cell sorter (Beckman Coulter, Fullerton, CA). Subdiploid DNA content analysis was performed on singlet populations using WinMDI 2.8 (J. Trotter, Scripps Research Institute, La Jolla, CA). At least $10 \times 10^3$ cellular events were counted.

### Western blotting

Macrophages were harvested as described above and lysed in ice-cold homogenization buffer [20 mM morpholinepropane-

sulfonic acid (MOPS, pH 7.2), 1% Triton X-100, 50 mM β-glycerol phosphate, 5 mM EGTA, 2 mM EDTA, 1 mM sodium vanadate, 25 μM β-methyl aspartic acid, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, aprotinin (10 μg/ml), and leupeptin (10 μg/ml)]. Lysates were centrifuged at 14,000 rpm for 10 min and the protein content of supernatants was quantified by the Bradford protein assay. Fifty micrograms of protein from each sample was loaded and separated by SDS-PAGE, using a 10% separating gel. Gels were calibrated with prestained SDS-PAGE low molecular weight standards (Bio-Rad). Proteins were then transferred to nitrocellulose paper and blocked for 1 h with 4% skim milk, 0.01% $NaN_3$ in TBS-0.1% Tween 20 followed by incubation with the primary antibody overnight in TBS-0.1% Tween 20 at room temperature. After three 10-min washes with TBS-0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at a 1:5,000 dilution for 1 h. Thereafter, the proteins were visualized by enhanced chemiluminescence. For inhibitor studies, the cells were preincubated with the indicated concentrations of MEK inhibitors or PI 3-kinase inhibitors for 1 h before the addition of oxLDL.

### Statistical analysis

Results were expressed as means ± SEM. Statistical analysis was done by ANOVA or Student's *t*-test as appropriate. A *P* value of less than 0.05 was taken as significant.

## RESULTS

### oxLDL promotes macrophage survival by inhibiting apoptosis

Incubation of BMDM in the absence of M-CSF caused a progressive decline in cell viability induced over a 96-h time course as illustrated in **Fig. 1**. This decline was completely inhibited by oxLDL at 25 μg/ml, whereas native LDL (n-LDL) and acetylated LDL (ac-LDL) were without effect. To determine whether this effect of oxLDL involved induction of cell growth, cyclin D1 levels were measured in CSF-deprived macrophages before and after incubation with oxLDL (25 μg/ml) or M-CSF (5,000 U/ml) for 24 h. As shown in **Fig. 2**, there was a decrease in cyclin D1 levels in both control and oxLDL-treated cells, whereas M-CSF treatment caused an increase in cyclin D1 levels as expected. These results indicate that the enhanced viability of macrophages conferred by oxLDL was

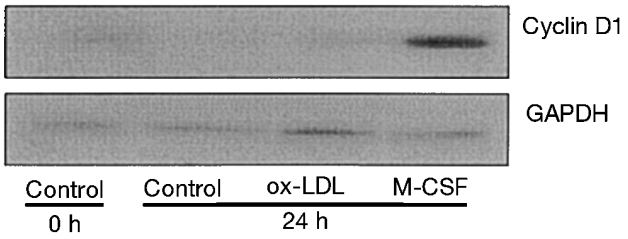



**Fig. 2.** OxLDL does not lead to an increase in cyclin D1. BMDM were seeded at $1 \times 10^6$ cells/well in 6-well plates and preincubated in RPMI 1640 with 10% FBS, but without M-CSF, for 24 h (0 h). Macrophages were then incubated for an additional 24 h with this medium alone (Control), with the addition of oxLDL (oxLDL, 25 μg/ml) or M-CSF (M-CSF, 5,000 U/ml). Immunoblotting for cyclin D1 was performed as described in Materials and Methods. GAPDH was used as a control to monitor protein loading. Data are representative of two separate experiments.

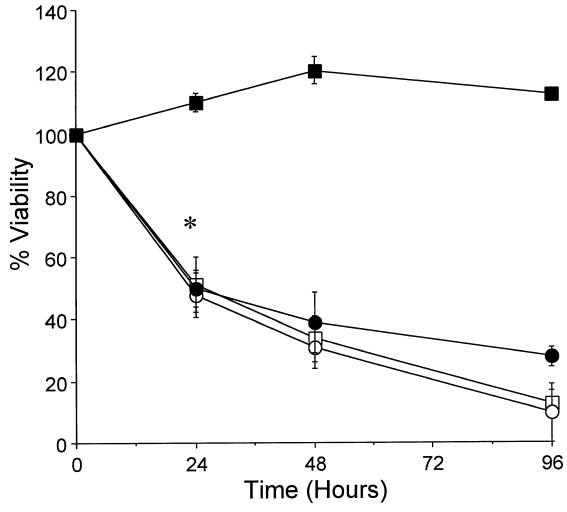



**Fig. 1.** Time-dependent inhibition of apoptosis in BMDM by oxLDL. BMDM were seeded at $10 \times 10^3$ cells/well in 96-well plates and preincubated in RPMI 1640 with 10% FBS, but without M-CSF, for 24 h. Macrophages were then incubated for 0–96 h with this medium alone (open squares), or with addition of a 25-μg/ml concentration of oxLDL (closed squares), n-LDL (open circles,) or ac-LDL (closed circles). Macrophage viability was measured by the bioreduction of the soluble tetrazolium salt MTS as described in Materials and Methods. Results are expressed relative to control cells treated without oxLDL at 0 h. Data represent means ± SEM of quadruplicate samples. Similar results were obtained in each of two replicate experiments (* $P < 0.05$ vs. 0 h).

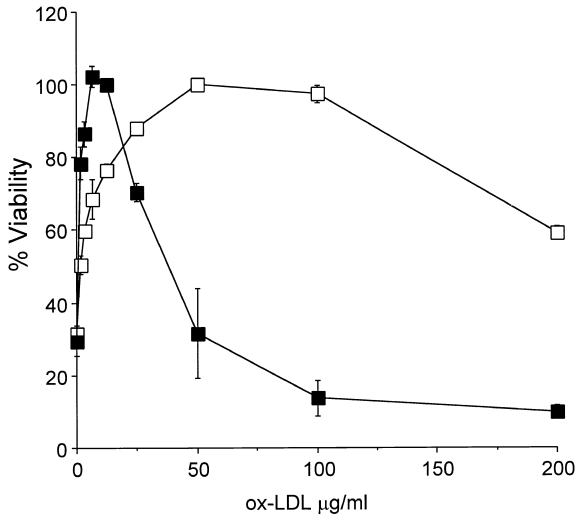



**Fig. 3.** Dose-dependent inhibition of apoptosis in BMDM by oxLDL. BMDM were seeded at $10 \times 10^3$ cells/well in 96-well plates and preincubated in RPMI 1640 with 10% FBS, but without M-CSF, for 24 h. BMDM were treated for 24 h with increasing concentrations of oxLDL (1.56–200 μg/ml) in RPMI 1640 containing 10% FBS (open squares) or no FBS (closed squares). Macrophage viability was measured by the bioreduction of the soluble tetrazolium salt MTS as described in Materials and Methods. Results are expressed relative to control cells treated without oxLDL at 0 h. Data represent means ± SEM of quadruplicate samples. Similar results were obtained in each of two replicate experiments.

not due to stimulation of cell proliferation. The cyclin D1 results were further confirmed by cell cycle analysis for the S phase, as well as by absolute cell counts (data not shown). The concentration curve for oxLDL shown in **Fig. 3** demonstrates a biphasic effect on viability, whereby the prosurvival effect was overcome at high concentrations presumably because of cytotoxicity, as previously reported by others (13, 26). Figure 3 also shows that incubation in the absence of FBS increased the apparent susceptibility of the cells to cytotoxic effects of oxLDL. Furthermore, in contrast to our previous finding that extensive oxidation of LDL was required to induce macrophage proliferation (16), even mild oxidation of LDL (2–4 h) was able to induce macrophage survival (**Fig. 4**). To determine whether the prosurvival effect of oxLDL was due to the inhibition of apoptosis, cells were assayed for two hallmark features of apoptosis: DNA fragmentation and caspase 3 activation. Withdrawal of M-CSF caused extensive DNA fragmentation (53.6% subdiploid DNA) and this was completely blocked by a 25-μg/ml concentration of oxLDL (4.4% subdiploid DNA), whereas n-LDL (54.3% subdiploid DNA) and ac-LDL (52.1% subdiploid DNA) were ineffective (**Fig. 5**). oxLDL prevented both the activation of caspase 3 as detected by the disappearance of the proenzyme form of caspase 3 in control, n-LDL-, and ac-LDL-treated cells, and degradation of the 116-kDa PARP substrate to its 85-kDa cleaved fragment by active caspase 3 (**Fig. 6**). Taken together, these results represent compelling evidence that oxLDL prevents apoptosis of cytokine-deprived BMDM, independent of cell proliferation.

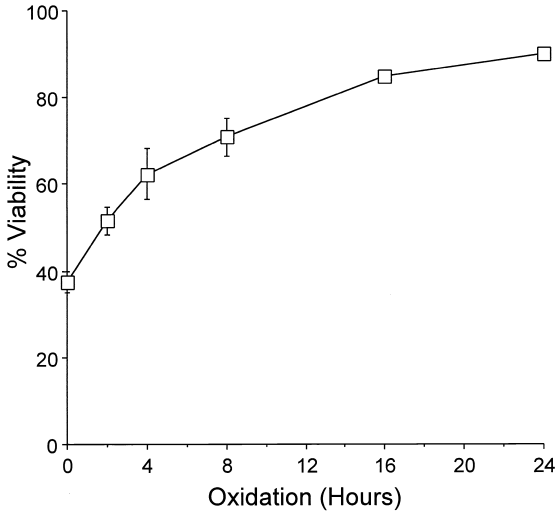



**Fig. 4.** Oxidation-dependent inhibition of cell death in BMDM by oxLDL. BMDM were seeded at $10 \times 10^3$ cells/well in 96-well plates and preincubated in RPMI 1640 with 10% FBS, but without M-CSF, for 24 h. Macrophages were then incubated for 24 h with this medium alone or with the addition of a 25-μg/ml concentration of LDL oxidized for 2–24 h. Macrophage viability was measured by the MTS assay. Results are expressed relative to cells treated without oxLDL at 0 h. Data represent means ± SEM of quadruplicate samples. Similar results were obtained in each of two replicate experiments. LDL oxidation from 2 to 24 h was statistically significant compared with n-LDL (0 h of oxidation) ($P < 0.05$).

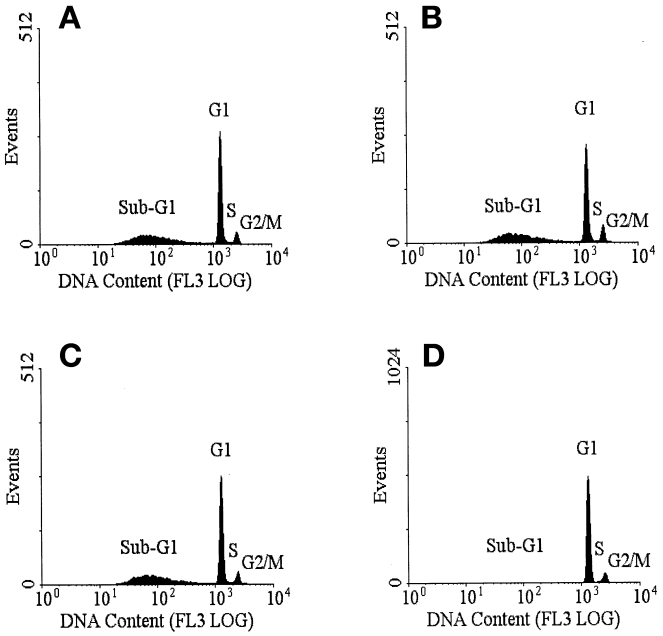



**Fig. 5.** OxLDL inhibits DNA fragmentation in BMDM. BMDM were seeded at $1 \times 10^6$ cells/well in 6-well plates and preincubated in RPMI 1640 with 10% FBS, but without M-CSF, for 24 h followed by 24 h in RPMI 1640-10% FBS alone (A), or with a 25-μg/ml concentration of n-LDL (B), ac-LDL (C), or oxLDL (D). DNA fragmentation was analyzed by flow cytometry, using propidium iodide-stained cells as described in Materials and Methods. Similar results were obtained in each of two replicate experiments.

### Role of cytokine release in oxLDL-mediated macrophage survival

It has been reported that GM-CSF plays an essential role in the stimulation of macrophage growth induced by oxLDL (15). On the other hand, others have failed to confirm a role for either GM-CSF or M-CSF in this process (18). To clarify whether the prosurvival effect of oxLDL required the secretion of soluble mediators or cytokines, medium from cells treated with oxLDL for various time intervals was removed, filtered to remove noninternalized oxLDL, and tested for its effect on macrophage viability. As shown in **Fig. 7A**, conditioned medium from oxLDL-treated cells was unable to prevent cell death at any time point, suggesting that stable soluble mediators were not sufficient for the antiapoptotic effect. A positive control with M-CSF at 5,000 U/ml excluded the possibility that this cytokine was removed by the filtration process (data not shown). To confirm that release of GM-CSF was not necessary for this effect, we added neutralizing antibodies against GM-CSF together with oxLDL. A 1:20 dilution of anti-GM-CSF failed to block the antiapoptotic effect of oxLDL, but completely blocked the effect of GM-CSF on macrophage survival (Fig. 7B). A requirement for M-CSF was ruled out by the experiment shown in Fig. 7C, in that oxLDL promoted survival of BMDM from osteopetrotic B6C3Fe-a/a-Csf1$^{op}$ mice, which carry a homozygous null mutation in the M-CSF gene. Similar results were reported previously by Hamilton and colleagues (18). These experiments show that GM-CSF and M-CSF are not necessary for the antiapoptotic effect

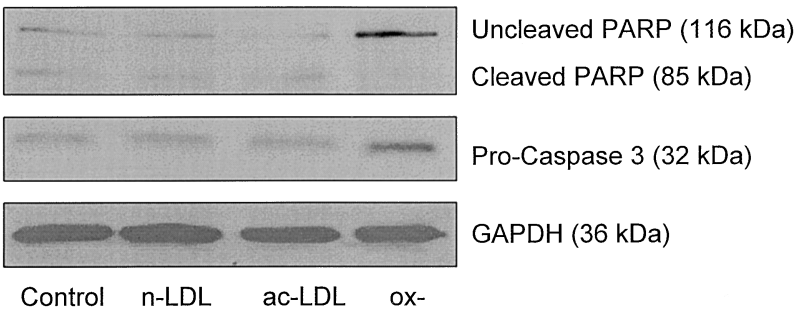



**Fig. 6.** OxLDL inhibits caspase 3 activation in BMDM. BMDM were seeded at $1 \times 10^6$ cell/well in 6-well plates and preincubated in RPMI 1640 with 10% FBS, but without M-CSF, for 24 h followed by 24 h in RPMI 1640-10% FBS alone, or with n-LDL, ac-LDL, or oxLDL (25 μg/ml). Immunoblotting for procaspase 3 and poly(ADP-ribose) polymerase (PARP) was done as described in Materials and Methods. GAPDH was used as a control to monitor protein loading. Similar results were obtained in each of two replicate experiments.

of oxLDL, but this involves a direct action of oxLDL on macrophages rather than an indirect effect mediated by cytokine release.

### Activation of ERK1/2 MAP kinases and PKB by oxLDL

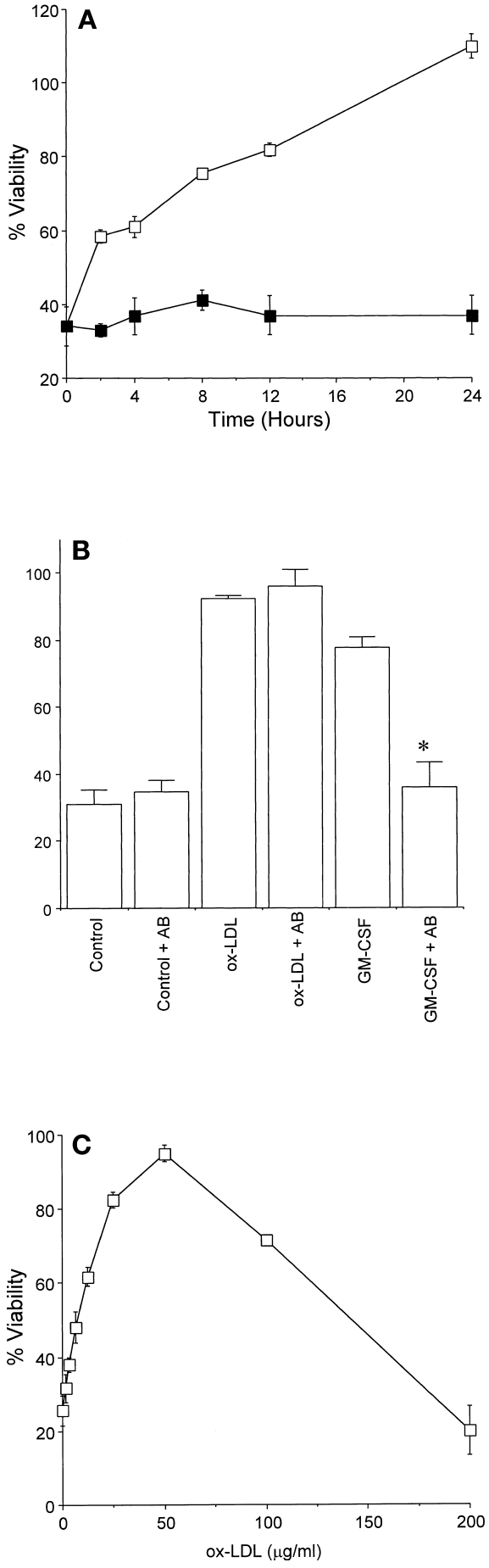
Although the receptors for GM-CSF and M-CSF are different (27, 28), both cytokines lead to the activation of the intracellular survival signaling pathways involving ERK1/2 MAP kinases and the PI 3-kinase downstream target, PKB. Therefore, we hypothesized that oxLDL might cause cytokine-independent survival by directly activating one or both of these pathways. Immunoblotting with antibodies that detect only the phosphorylated and active forms of these kinases indicated that oxLDL activated both ERK1/2 and PKB in BMDM whereas n-LDL or ac-LDL had no effect (**Fig. 8A**). The activation of PKB by oxLDL was confirmed by in-gel kinase assays (data not shown). As expected, the MEK inhibitors PD 98059 and U0126 inhibited ERK1/2 activation, and the PI 3-kinase inhibitors LY 294002 and wortmannin blocked PKB activation by oxLDL (Fig. 8B).

### OxLDL promotes the phosphorylation of I-κBα and Bad

Several targets of the PI 3-kinase/PKB signaling cascade have been identified that may underlie the ability of this pathway to promote survival (29). These substrates may include components of the apoptotic machinery, including Bad (Bcl-$X_L$/Bcl-2-associated death promoter; the proapoptotic member of the Bcl-2 family of proteins); transcription factors of the forkhead family; and two kinases, glycogen synthase kinase-3β (GSK-3β) and I-κB kinase. The latter kinase leads to the phosphorylation and degradation of I-κBα, thereby liberating NF-κB and allowing it to translocate to the nucleus and activate target survival genes (30). Given the importance of caspase 3 in apoptosis, we examined the phosphorylation of Bad and I-κBα, both of which act at the level of Bcl-$X_L$ to prevent caspase 3 activation and apoptosis. As shown in **Fig. 9**, oxLDL induced phosphorylation of I-κBα(Ser-32) and Bad(Ser-136). As expected, I-κBα levels declined after phosphorylation, reflecting subsequent proteasome-mediated degradation. GSK-3β and FKHR (forkhead in rhabdomyosarcoma) were also phosphorylated in response to oxLDL, but the importance of these two factors in mediating macrophage survival has not yet been ascertained. Interestingly, oxLDL also activated PKB in the RAW 264.7 murine macrophage cell line, but increased phosphorylation was demonstrable only in I-κBα and not in GSK-3β, FKHR, or Bad (data not shown). These results in RAW 264.7 cells indicate that there may be intrinsic alteration of signal transduction pathways in immortalized cell lines and that the role of any given pathway or target should be confirmed in primary cell cultures. Last, to determine whether activation of the PI 3-kinase/PKB pathway or the MAP kinase pathway was essential for the inhibition of apoptosis, we tested the effect of inhibitors of each pathway on cell survival in the presence of oxLDL. As shown in **Fig. 10**, the PI 3-kinase inhibitors LY 294002 and wortmannin blocked the effect of oxLDL on macrophage survival, whereas the MAP kinase inhibitors PD 98059 and U0126 did not affect survival. These results suggest that activation of the PI 3-kinase/PKB signaling cascade plays a selective and pivotal role in oxLDL-mediated cytokine-independent macrophage survival.

## DISCUSSION

Reports from several groups, including our own, have previously demonstrated that oxLDL can induce proliferation of macrophages (12–17). The objective of the present study was to determine whether oxLDL also affected macrophage survival. The experimental conditions were different from those previously used for evaluating effects on macrophage growth, so that we were able to selectively measure a prosurvival effect. We found that oxLDL can prevent the cell death induced by the removal of the macrophage survival factor M-CSF in BMDM. To demonstrate that there were no effects on cell proliferation under these conditions, the expression of cyclin D1 was examined. D-type cyclins are considered to be good markers for cell proliferation because they are essential for $G_1$ progression. D-type cyclins are essential cofactors for the cyclin-dependent kinases (CDK) CDK4 and CDK6, and a key substrate for the cyclin D/CDK holoenzyme is the retinoblastoma protein (pRB). Phosphorylation of pRB releases the transcriptional repression of a variety of genes required for proliferation (31). Cyclin D1 levels declined after oxLDL treatment, thereby excluding a role for macrophage proliferation in the observed effect with oxLDL. Moreover, we showed that this prosurvival effect was due to inhibition of apoptotic cell death, as reflected by three independent indices of apoptotic cell death, including mitochondrial dysfunction, internucleosomal DNA




fragmentation, and activation of effector caspases (32, 33). Neither n-LDL nor ac-LDL had an effect on these markers of apoptosis, indicating that the enhanced survival was not due simply to delivery of cholesterol or phospholipids to cells. Other investigators found that oxLDL was cytotoxic and caused apoptotic cell death in macrophages (13, 26), vascular smooth muscle cells (34, 35), and endothelial cells (36, 37). We confirmed that oxLDL could have a toxic effect on macrophages, but this occurred only at concentrations of oxLDL usually exceeding 100 μg/ml. We speculate that the cytotoxic effect of oxLDL is mediated by components other than the prosurvival effect and probably affects different intracellular signaling pathways (38).

Biwa and coworkers (15) reported that oxLDL caused the release of GM-CSF by mouse peritoneal macrophages and that this release was essential for the proliferation of macrophages. However, Hamilton and colleagues (18) reported that neither GM-CSF nor M-CSF plays an important role in macrophage survival induced by oxLDL. The present study supports the conclusions of this latter group regarding the role of CSF, and extends their findings in three ways. First, our results exclude a role for other prosurvival factors secreted by macrophages in response to oxLDL. Second, we demonstrate that the survival effect is specific for oxLDL, and third, we have defined the key signal transduction pathways involved. The activation of ERK1/2 by oxLDL has been previously described in macrophages and smooth muscle cells (39), but we show that these kinases were not essential for the antiapoptotic effect of oxLDL. Our studies provide the first evidence of the activation of PKB by oxLDL, and demonstrate a direct role for the activation of the PI 3-kinase/PKB pathway in the enhancement of macrophage survival. It is possible that, in vivo, oxLDL rescues macrophages from programmed cell death and permits their subsequent proliferation in response to cytokines such as GM-CSF or M-CSF

**Fig. 7.** OxLDL promotes cytokine-independent survival. A: BMDM were seeded at $10 \times 10^3$ cells/well in 96-well plates and preincubated in RPMI 1640 with 10% FBS, but without M-CSF, for 24 h. One set of macrophages was incubated with oxLDL (25 μg/ml) for the indicated periods of time. The conditioned medium was then removed, filtered through a 100-kDa microconcentrator to remove oxLDL, and then transferred for the remainder of the 24-h incubation to a second set of macrophages that previously had been incubated in the absence of oxLDL (solid squares). To verify that prosurvival activity was present, the cells that originally received oxLDL were given RPMI 1640-10% FBS alone for the remainder of the 24-h incubation (open squares). A positive control with M-CSF (5,000 U/ml) was used as a control to eliminate the possibility of membrane absorption (data not shown). B: BMDM were incubated with control medium, oxLDL (25 μg/ml), or GM-CSF (5 units) in the presence or absence of a 1:20 dilution of a neutralizing anti-GM-CSF antibody (AB) (* $P < 0.05$ compared with no AB). C: BMDM from *op/op* mice were treated for 24 h with increasing concentrations of oxLDL (1.56–200 μg/ml). Macrophage viability was measured by the MTS assay. Results are expressed relative to cells treated without oxLDL at 0 h. Data represent means ± SEM of quadruplicate samples. Similar results were obtained in each of two replicate experiments.

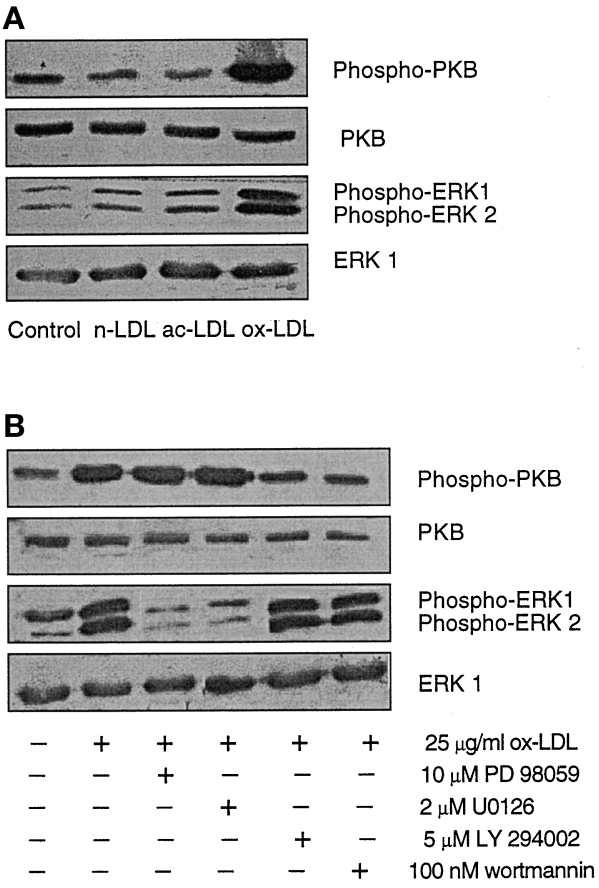



**Fig. 8.** OxLDL activates ERK1/2 and PKB. A: BMDM were seeded at $5 \times 10^6$ cells/100-mm dish in RPMI 1640-10% FBS and were incubated with control medium, n-LDL, ac-LDL, or oxLDL (25 μg/ml) for 10 min and the activation of the intracellular survival signaling pathways, ERK1/2 and PKB examined by phosphospecific immunoblotting as described in Materials and Methods. ERK1 and PKB served as controls to monitor protein loading. B: BMDM were preincubated with the MEK inhibitors (PD 98059 or U0126) and the PI 3-kinase inhibitors (LY 294002 or wortmannin) for 1 h before treatment with oxLDL and were then treated and processed as described above. Similar results were obtained in each of two replicate experiments.

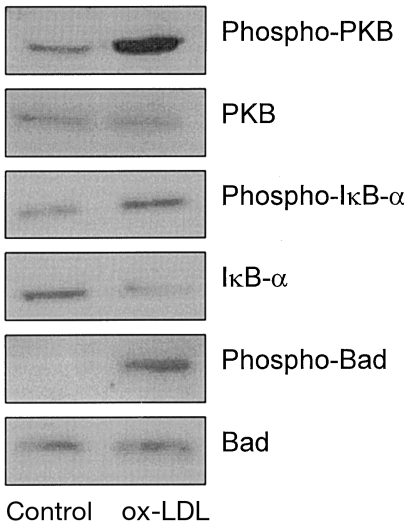



**Fig. 9.** OxLDL phosphorylates the PKB targets, IκB-α and Bad. BMDM were seeded at $5 \times 10^6$ cells/100-mm dish and preincubated in RPMI 1640 with 10% FBS, but without M-CSF, for 24 h. Cells were then incubated for 1 h with medium alone (control) or with oxLDL (25 μg/ml) and the phosphorylation of two putative PKB downstream targets, IκB-α and Bad, was examined by phosphospecific immunoblotting as described in Materials and Methods. Similar results were obtained in each of two replicate experiments.

that have been shown to be expressed in atherosclerotic lesions (40).

The PI 3-kinase target PKB has been shown to mediate macrophage survival by M-CSF (41). PKB might promote survival by directly phosphorylating components of the apoptotic machinery, such as forkhead transcription factors, I-κBα, or Bad, or indirectly by changing the level of expression of the genes that encode components of cell death, such as the Bcl-2 family members (29). In the present study, oxLDL caused activation of PKB and phosphorylation of two important prosurvival downstream targets, I-κBα and Bad. Phosphorylation of I-κBα targets it for ubiquitination and proteasome-mediated degradation and its phosphorylation state is therefore considered to be a good marker of NF-κB activation (42). Previous reports have reached conflicting conclusions about the role of oxLDL in the activation of NF-κB (43, 44). However, active NF-κB has been shown to be present in atherosclerotic lesions, suggesting that NF-κB could play a role in atherosclerosis (45, 46). The mechanism by which NF-κB promotes cell survival may involve the upregulation of antiapoptotic genes. In particular, the Bcl-2 family of proteins includes some of the most important cellular regulators of apoptosis (47). Although *bcl-2* itself does not appear to be a target for NF-κB, the antiapoptotic Bcl-2-like protein A1/Bfl1 (48, 49) and Bcl-$X_L$ (50, 51) contain functional upstream NF-κB-binding sites. Moreover, activation of the PI 3-kinase/PKB pathway has been shown to lead to the upregulation of Bcl-$X_L$ by activation of NF-κB (52, 53). Bad phosphorylation also acts at the level of Bcl-$X_L$ and sequesters it in the cytoplasm with 14-3-3 protein and prevents heterodimerization and inactivation of the antiapoptotic members of the Bcl-2 family of proteins, such as Bcl-$X_L$ (54). Preliminary results indicate that treatment with oxLDL prevents the decline in Bcl-$X_L$ levels after M-CSF withdrawal, and also prevents the generation of ceramide (R. Hundal, unpublished data). As ceramide is known to inhibit PKB, we are currently exploring the possibility that the effects of oxLDL on PKB are mediated by changes in ceramide levels.

In summary, oxLDL promotes the survival of cultured BMDM after M-CSF withdrawal. oxLDL was shown to activate both ERK1/2 MAP kinases and PKB, but only the activation of the PI 3-kinase/PKB pathway was essential for the prosurvival effect. Moreover, we demonstrate that oxLDL promotes the phosphorylation of two important antiapoptotic PKB downstream targets, I-κBα(Ser-32), thereby releasing NF-κB, as well as Bad(Ser-136). Both NF-κB and Bad can act at the level of Bcl-$X_L$ to promote

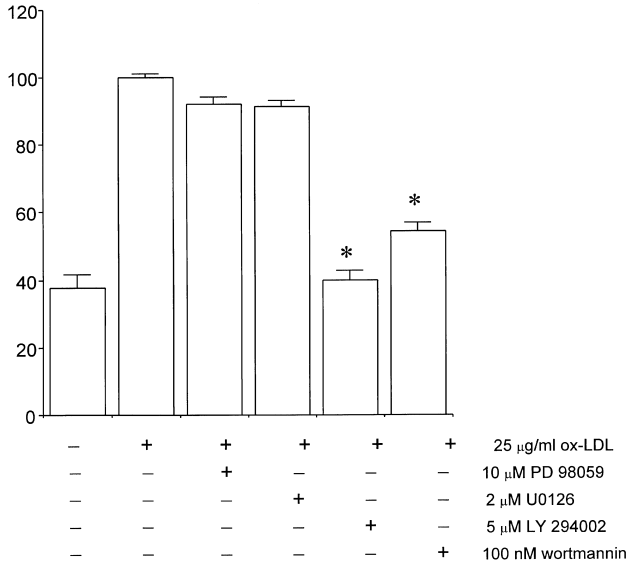



**Fig. 10.** PI-3 kinase inhibitors block oxLDL mediated macrophage survival. BMDM were seeded at $10 \times 10^3$ cells/well in 96-well plates and preincubated in RPMI 1640 with 10% FBS, but without M-CSF, for 24 h. Macrophages were then preincubated with MEK inhibitors (PD 98059 or U0126) or PI-3 kinase inhibitors (LY 294002 or wortmannin) for 1 h before treatment with oxLDL. Macrophage viability was measured after 24 h by the MTS assay. Results are expressed relative to control cells treated without oxLDL for 0 h. Data represent means ± SEM of quadruplicate samples. Similar results were obtained in each of two replicate experiments (* $P < 0.05$ vs. oxLDL).

survival. This intracellular survival signaling pathway is similar to that activated by GM-CSF and M-CSF, and its direct activation by oxLDL could lead to "inappropriate" survival of macrophages in the absence of these cytokines.

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