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Cholesterol-induced Apoptotic Macrophages Elicit an Inflammatory Response in Phagocytes, Which Is Partially Attenuated by the Mer Receptor*

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Macrophage apoptosis and the ability of phagocytes to clear these apoptotic cells are important processes in advanced atherosclerosis. Phagocytic clearance not only disposes of dead cells but usually elicits an anti-inflammatory response. To study this process in a model of advanced lesional macrophage death, macrophages rendered apoptotic by free cholesterol loading (FC-AMs) were incubated briefly with fresh macrophages ("phagocytes"). FC-AMs were promptly ingested by the phagocytes, which was dependent upon actin polymerization and the phagocyte Mer receptor. Surprisingly, this brief exposure to FC-AMs triggered a modest proinflammatory response in the phagocytes: tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β were induced, whereas the levels of transforming growth factor-β and IL-10 were not increased. This response required cell contact between the FC-AMs and phagocytes but not FC-AM ingestion. TNF-α and IL-1β induction required one or more proteins on the FC-AM surface and was dependent on signaling through extracellular signal-regulated kinase-1/2 mitogen-activated protein kinase and nuclear factor-κB in the phagocytes. TNF-α production was markedly greater when Mer-defective phagocytes were used, indicating that Mer attenuated the inflammatory response. Interestingly, a more typical anti-inflammatory response was elicited when phagocytes were exposed to macrophages rendered apoptotic by oxidized low density lipoprotein or UV radiation. Thus, the proinflammatory milieu of advanced atherosclerotic lesions may be promoted, or at least not dampened, by contact between FC-induced apoptotic macrophages and neighboring phagocytes prior to apoptotic cell ingestion.

Macrophages in advanced atherosclerotic lesions accumulate excess unesterified, or "free," cholesterol (FC)[2] (1–3). FC accumulation is a potent inducer of inflammatory cytokines and apoptosis in macrophages (4–6), which represent two important features of advanced, unstable plaques (6, 7). The molecular mechanisms of FC-induced inflammation and apoptosis in cultured macrophages and the consequences of inflammation in atherogenesis have been studied in depth (4, 8–11). However, the significance of macrophage apoptosis in atherosclerosis development remains unclear. During the initial or intermediate stages of atherosclerosis, accelerated apoptosis and rapid removal of the apoptotic cells by neighboring macrophage phagocytes appear to reduce the number of atherogenic and inflammation-prone macrophages within the plaque and therefore decelerate lesion progression (12–14). On the other hand, macrophage apoptosis in advanced lesions has been implicated in the generation of necrotic cores, a "graveyard of dead macrophages" that is thought to promote plaque instability (6, 15–17). The difference may lie not only in accelerated apoptosis in late lesions, but perhaps even more so in defective phagocytic clearance of apoptotic cells (14, 18–21). When apoptotic cells are not rapidly cleared, they undergo postapoptotic necrosis, which could contribute to lesional necrosis.

Although defective clearance of apoptotic macrophages by a subpopulation of incompetent phagocytes in advanced atheromata likely contributes to necrotic core formation, it is almost certain that other phagocytes in advanced lesions are competent and can ingest apoptotic macrophages. According to the literature, this event should at least partially counterbalance the effect of the incompetent phagocytes for two reasons: postapoptotic necrosis is prevented, and the competent phagocytes should elicit an anti-inflammatory response upon exposure to apoptotic cells (18, 19). The latter point is based upon a number of studies showing that apoptotic cells induce in phagocytes the secretion of TGF-β and IL-10, which have anti-inflammatory roles in atherosclerosis (22–27) and suppress the secretion of proinflammatory cytokines, such as TNF-α (28, 29).

To model the latter scenario, we set up an experimental system in which FC-induced apoptotic macrophages were exposed briefly to competent phagocytes to enable ingestion, and then the noningested apoptotic cells were removed. We found that this brief exposure to FC-induced apoptotic cells did not lead to the typical anti-inflammatory response in the phagocytes but rather induced the production of the proinflammatory cytokines TNF-α and IL-1β. Thus, even in the case where FC-induced apoptotic macrophages are ingested rapidly by competent phagocytes, as likely occurs to some degree in advanced atherosclerotic lesions, inflammation is not suppressed.

EXPERIMENTAL PROCEDURES

Materials—Chemical reagents were from Sigma unless specified below. Low density lipoprotein (LDL; d 1.020–1.063 g/ml) was isolated

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[2] The abbreviations used are: FC, free cholesterol; ACAT, acyl-CoA:cholesterol *O*-acyltransferase; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; ERK1/2, extracellular signal-regulated kinase 1/2; FC-AM, free cholesterol-induced apoptotic macrophage; IκB, inhibitor of NF-κB; IL, interleukin; JNK, c-Jun N-terminal kinase; LDL, low density lipoprotein; LRP, LDL receptor-related protein; MAP kinase, mitogen-activated protein kinase; NF-κB, nuclear factor κB; PS, phosphatidylserine; RT-QPCR, reverse transcription-quantitative PCR; TGF-β, transforming growth factor-β; TLR, toll-like receptor; TNF-α, tumor necrosis factor-α.

from fresh human plasma by preparative ultracentrifugation as described previously (30). Acetyl-LDL was prepared by reaction of LDL with acetic anhydride (31). Oxidized LDL was from Biomedical Technologies, Inc. Compound 58035 (3-[decyldimethylsilyl]-*N*-[2-(4-methylphenyl)-1-phenylethyl] propanamide), an inhibitor of acyl-CoA:cholesterol *O*-acyltransferase (ACAT), was generously provided by Dr. John Heider, formerly of Sandoz, Inc. (East Hanover, NJ) (32). U18666A was from Biomol Research Laboratories, Inc. SB203580, SB202474, and PD98059 were from EMD Biosciences. PS1145 was a generous gift from Millennium Pharmaceuticals (33). Antibodies against p38, phospho-p38, ERK1/2, phospho-ERK1/2, and IκB-α were from Cell Signaling Technology. Anti-nucleophosmin was from Zymed Laboratories, Inc. Anti-α-tubulin and anti-β-actin were from Santa Cruz Biotechnologies, Inc.

Mice—Female C57BL6/J mice from Jackson Laboratories, 8–10 weeks of age, were used in this study. *Mertk* mice on the C57BL6/J background were created as described previous (34). *Myd88*−/− mice on the C57BL6/J background were generously provided by Drs. Douglas Golenbock (University of Massachusetts) and Mason Freeman (Harvard Medical School) (35). *Cd36*−/− mice on the C57BL6/J background were generously provided by Dr. Kathryn J. Moore (Harvard Medical School) (36). Mice processing the loxP-flanked LRP/CD91 were provided by Dr. Joachim Herz (University of Texas Southwestern Medical Center) (37). The mice were crossed to LysM-Cre mice, and the offspring were bred to homozygosity at the loxP-flanked LRP locus.

Elicitation and Culturing of Mouse Peritoneal Macrophages—Methyl-BSA (mBSA)- or concanavalin A-elicited macrophages (4, 11) were used in most of the studies and yielded similar results. mBSA-elicited macrophages were generated by intraperitoneal injection of mBSA in mice previously immunized with this antigen. 2 mg/ml mBSA in 0.9% saline was emulsified in an equal volume of complete Freund's adjuvant (CFA, DIFCO). Mice were immunized intradermally with 100 μl emulsion. 14 days later, the immunization protocol was repeated except incomplete Freund's adjuvant was used instead of complete Freund's adjuvant. 7 days later, the mice were injected intraperitoneal with 0.5 ml of phosphate-buffered saline containing 100 μg mBSA. 4 days later, macrophages were harvested by peritoneal lavage. Concanavalin A-elicited macrophages were obtained from mice that were injected intraperitoneal with 0.5 ml of phosphate-buffered saline containing 40 μg of concanavalin A. Macrophages were harvested 3 days later by peritoneal lavage. Thioglycollate-elicited or resident macrophages were used as phagocytes in some experiments. Thioglycollate-elicited macrophages were obtained from mice that were injected intraperitoneally with 1 ml of 3.8% thioglycollate broth. Macrophages were harvested 4 days later by peritoneal lavage (38). Resident macrophages were harvested by peritoneal lavage without any previous stimulation. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin/streptomycin, and 20% L-cell-conditioned medium for 24–48 h until confluence.

Generation of Apoptotic Cells—FC-induced apoptotic macrophages (FC-AMs) were generated by incubating the cells with 50 μg/ml acetyl-LDL and 10 μg/ml of the ACAT inhibitor 58035 for 16–20 h. Oxidized LDL-induced apoptotic macrophages were generated by incubating the cells with 100 μg/ml oxidized LDL for 24–30 h. UV-induced apoptotic macrophages were generated by radiating the cells for 10–15 min (254 nm, 20 J/cm^2), followed by culturing the cells for an additional 2–3 h. Apoptosis was confirmed by annexin V staining. In a typical experiment, a population of macrophages with 30–40% apoptosis (annexin V-positive) and less than 5% necrosis (PI-positive) were collected and exposed to phagocytes for 30 min. There was no significant increase in necrosis (*i.e.* PI-positivity) during this 30-min period. To generate FC-AM-conditioned medium, the FC-loading medium was removed, and the FC-AMs were gently washed twice and then incubated in fresh medium for 1 h. The medium was then centrifuged at 14,000 rpm for 5 min before use. To generate membranes, FC-AMs or control macrophages were subjected to three cycles of rapid freezing and thawing. Limited proteolysis was done by treating FC-AMs with 0.1–1.0 mg/ml trypsin in DMEM at 37 °C for 20 min and then washing the cells with DMEM containing 10% fetal bovine serum six times.

Phagocytosis—Apoptotic cells were added to a monolayer of fresh macrophages ("phagocytes") at a ratio of 5:1 (apoptotic cells:phagocytes). In a typical phagocytosis experiment, the apoptotic cells were labeled with Alexa Fluor 488- or 594-conjugated annexin V (Molecular Probes). After incubation for 15–30 min with the phagocytes, noningested apoptotic cells were removed by vigorous washing. The phagocytes were then fixed with 4% paraformaldehyde and viewed by confocal fluorescence microscopy. Additional experiments to confirm internalization of the apoptotic cells are presented under "Results." To examine the cellular responses in the phagocytes following contact with apoptotic cells, noningested apoptotic cells were removed by vigorous washing after a 30-min incubation as above, and the phagocytes were incubated in fresh media for the indicated times.

Cytokine Production—Phagocytes were incubated with apoptotic cells for 30 min. Noningested apoptotic cells were removed, and phagocytes were cultured in fresh media for various times. At the end of incubation, the media were centrifuged at 14,000 rpm for 5 min, and the supernatant fractions were stored in aliquots at −80 °C. Cytokine levels in the media were determined by high sensitivity ELISA analysis, which was conducted by the Cytokine Core Laboratory (Baltimore, MD).

RT-QPCR—Total RNA was extracted from macrophages using the RNeasy kit from Qiagen. cDNA was synthesized from 4 μg of total RNA using oligo(dT) and Superscript II (Invitrogen). 0.5 μl of cDNA was subjected to QPCR amplification using Taqman universal PCR master mix (Applied Biosystems). The forward and reverse primers for TGF-β were TGACGTCACTGGAGTTGTACGG and GGTTCATGTCATGGATGGTGC, respectively, and the probe was 6FAM-TTCAGCGCTCACTGCTCTTGTGACAG. The forward and reverse primers for TNF-α were CGGAGTCCGGGCAGGT and GCTGGGTAGAGAATGGATGAACA, respectively, and the probe was 6FAM-CTTTGGAGTCATTGCTCTGTGAAGGGAATG. The forward and reverse primers for IL-1β were CAACCAACAAGTGATATTCTCCATG and GATCCACACTCTCCAGCTGCA, respectively, and the probe was 6FAM-CTGTGTAATGAAAGACGGCACACCCACC. 36B4 was used as the internal control. The forward and reverse primers for 36B4 were AGATGCAGCAGATCCGCAT and GTTCTTGCCCATCAGCACC, respectively, and the probe was CAL-CGCTCCGAGGGAAGGCCG. The reactions were run on an MX4000 multiplex quantitative PCR system (Stratagene). The thermal profile settings were 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and then 60 °C for 1 min.

Immunoblotting—Whole cell lysates were prepared by homogenizing the cells into 1× sample loading buffer from Bio-Rad. Nuclear extracts were prepared using the Nuclear Extraction kit from Panomics, Inc. Cell extracts were electrophoresed on 4–20% gradient SDS-polyacrylamide gels and transferred to 0.22-μm nitrocellulose membranes. The membranes were blocked in Tris-buffered saline, 0.1% Tween 20 (TBST) containing 5% (w/v) nonfat milk at room temperature for 1 h. The membranes were then incubated with the primary antibody in TBST containing 5% (w/v) nonfat milk or 5% BSA at 4 °C overnight, followed by incubation with the appropriate secondary antibody coupled to horseradish peroxidase. Proteins were detected by ECL chemiluminescence (Pierce).

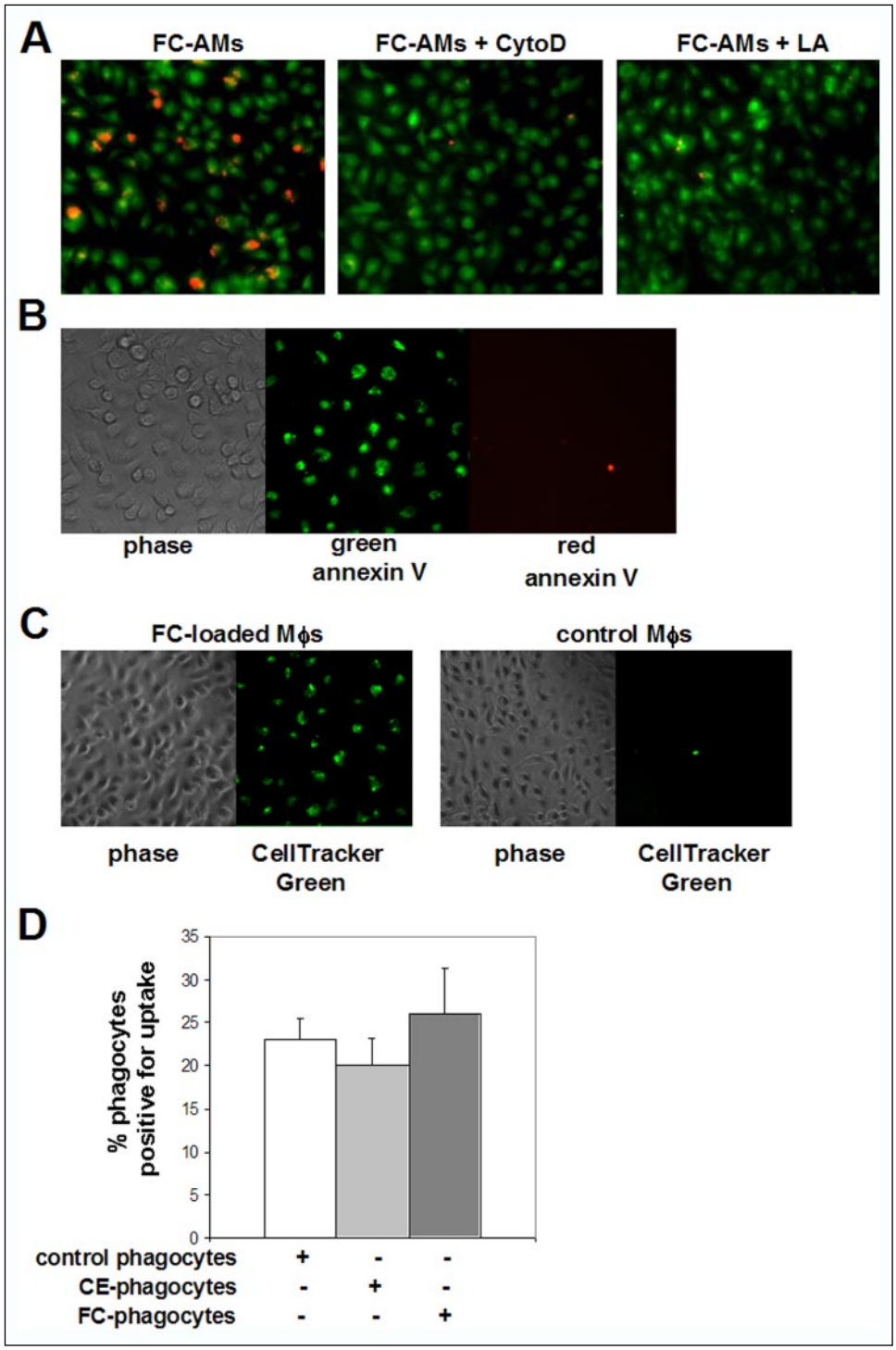

FIGURE 1. **FC-AMs are ingested by control or cholesterol-loaded phagocytic macrophages via a process dependent on actin polymerization.** *A*, CellTracker Green-labeled phagocytes (*green*) were preincubated with control medium, 1 μM cytochalasin D (*cytoD*), or 100 nM latrunculin A (*LA*) for 30 min. The phagocytes were then incubated for an additional 30 min in the presence of the aforementioned reagents with FC-AMs that had been prelabeled with Alexa Fluor 594-annexin V (*red*). Noningested cells were removed by thorough washing, and the phagocytes were fixed and viewed by confocal fluorescence microscopy. *B*, FC-AMs were prelabeled with Alexa Fluor 488-annexin V (*green*) and then added to unlabeled phagocytes for 30 min. Noningested FC-AMs were removed by washing as above. The phagocytes were then incubated in fresh medium for 30 min, followed by staining with Alexa Fluor 594-annexin V (*red*). The *left panel* is the phase image, the *middle panel* is the green-filter image, and the *right panel* is the red-filter image. *C*, FC-loaded macrophages (*left pair* of *images*) or non-FC-loaded control macrophages (*right pair* of *images*) were stained with CellTracker Green and then incubated with unlabeled phagocytes for 30 min. Noningested cells were removed as above. In each *pair* of *images*, the *left image* is phase, and the *right image* is fluorescence. *D*, phagocytes were preincubated with control medium (*control phagocytes*), 50 μg/ml acetyl-LDL (*CE-phagocytes*), or 50 μg/ml acetyl-LDL plus 10 μg/ml ACAT inhibitor 58035 (*FC-phagocytes*) for 12 h. The phagocytes were then incubated with FC-AMs for 30 min and washed as above. The percentage of phagocytes that ingested FC-AMs was quantified.

Statistics—Data are presented as the mean ± S.E. To quantify the percentage of phagocytes positive for uptake, $n = 10$ fields of cells at a magnification of ×20 were studied. For RT-PCR and ELISA data, $n = 3$ separate samples for each condition were examined. Statistical significance was determined using Student's t test with unequal variance.

RESULTS

FC-induced Apoptotic Macrophages Are Removed Promptly by Phagocytes—To examine how phagocytes ingest and respond to FC-induced apoptotic macrophages, we added these cells to a monolayer of fresh macrophages for 15–60 min, followed by removal of noningested cells by vigorous rinsing. To avoid confusion, we refer to the FC-induced apoptotic macrophages as *FC-AMs* and the phagocytic macrophages as *phagocytes*. To begin, we examined whether FC-AMs were substrates for phagocytic ingestion by adding Alexa Fluor 594-conjugated annexin V-labeled FC-AMs (*red*) to a monolayer of CellTracker Green-labeled phagocytes (*green*). Using confocal microscopy, we found that ingestion of FC-AMs by the phagocytes, indicated by red inclusions in green cells, occurred as early as 15 min after incubation (Fig. 1*A*, *left panel*). The typical percentage of phagocytes positive for ingesting FC-AMs cells was 20–30%, compared with 40–50% for UV-irradiated Jurkat cells, which are often used in phagocytosis studies of

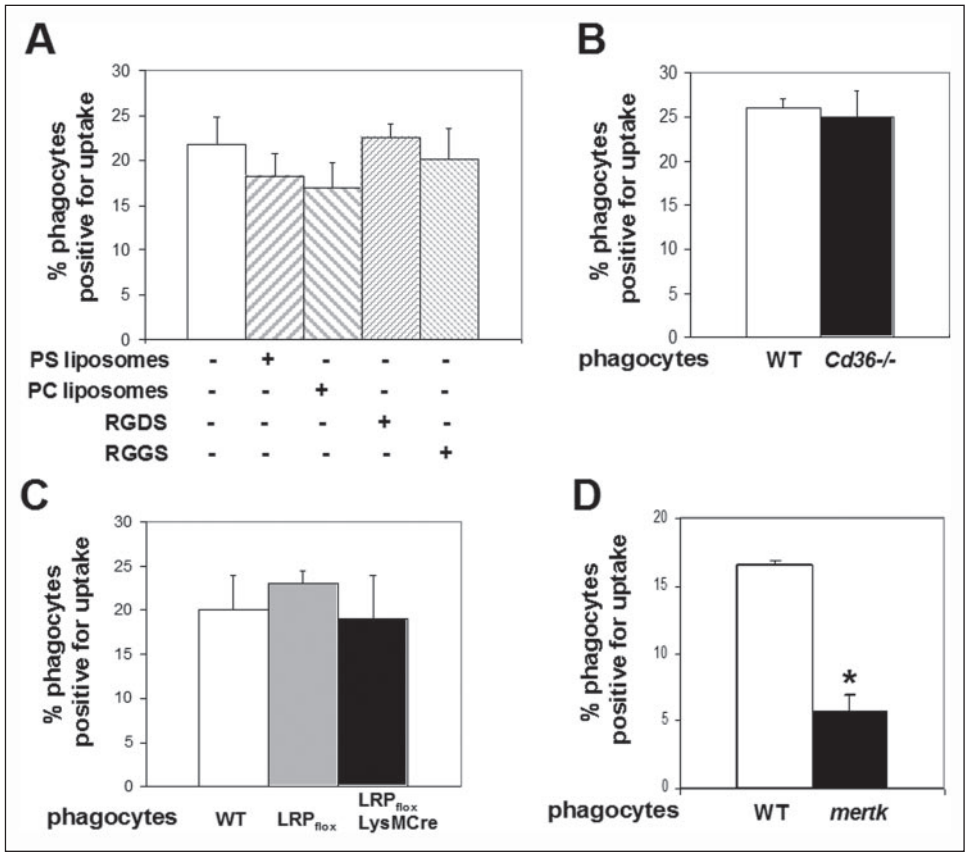

FIGURE 2. **Internalization of FC-AMs by phagocytes is highly dependent on the phagocyte Mer receptor.** Phagocytosis of FC-AMs was quantified under the following experimental conditions of phagocyte alteration. *A*, phagocytes were preincubated with control medium, PS- or phosphatidylcholine (PC)-containing liposomes (100 μM), the integrin-specific blocking peptide RGDS, or the inactive form RGGS (1 mg/ml) for 30 min. The phagocytes were then incubated for an additional 30 min with FC-AMs in the presence of the aforementioned reagents. *B*, wild-type (*WT*) or *Cd36−/−* phagocytes were incubated with FC-AMs for 30 min. *C*, wild-type, LRP_{flox}, or LRP_{flox}-LysMCre phagocytes were incubated with FC-AMs for 30 min. *D*, wild-type or *mertk* phagocytes were incubated with FC-AMs for 30 min. *Asterisk*, $p < 0.005$.

this nature. Careful examination of the confocal micrographs showed that the rinsing protocol removed bound but noningested FC-AMs, and so only ingestion is being assayed in this protocol. Preincubation of the phagocytes with cytochalasin D or latrunculin A, compounds that block phagocytosis by disrupting actin polymerization, completely inhibited apoptotic cell uptake (Fig. 1*A*, *middle* and *right panels*).

Ingestion of FC-AMs, rather than simple binding of the cells to the phagocytes, was also demonstrated by a sequential labeling protocol: FC-AMs labeled with Alexa Fluor 488-conjugated annexin V (*green*) were added to phagocytes as above. After noningested cells were removed, phagocytes were incubated in fresh media for 30 min and then stained with Alexa Fluor 594-conjugated annexin V (*red*). The second stain would label any FC-AMs that were not ingested, as demonstrated by a control experiment in which FC-AMs that were not exposed to phagocytes were labeled with both green and red annexin V using this sequential procedure (data not displayed). As shown in Fig. 1*B*, very little red staining was observed, indicating that almost all of the FC-AMs were completely internalized by the phagocytes.

The FC-loaded macrophages added to the phagocytes contained a proportion of viable macrophages as well as FC-AMs (*i.e.* not all of the macrophages are rendered apoptotic by FC loading (8)). To confirm that the viable cells were removed efficiently from the phagocyte monolayer using our rinsing protocol, we labeled populations of FC-loaded and non-FC-loaded macrophages (*i.e.* 100% viable) with CellTracker Green. These cells were then added to unlabeled phagocytes and subjected to the incubation and rinsing protocol described above. In the FC-loaded population, there was abundant association of green-labeled cells with phagocytes (Fig. 1*C*, *left pair of images*). In contrast, the unloaded ("control") population of macrophages did not label the phagocytes (Fig. 1*C*, *right pair of images*). Thus, viable macrophages are removed efficiently by the rinsing protocol used in our phagocytosis assay. The data also show that labeling FC-loaded macrophages with CellTracker Green yields phagocytosis results similar to those observed when the FC-AMs are labeled with fluorescent annexin V.

As mentioned above, the experimental system used herein was designed as a model of those phagocytes in advanced lesions which remain competent to clear apoptotic macrophages. However, there is evidence that some of the phagocytes in advanced atherosclerotic lesions are relatively inefficient at clearing apoptotic macrophages (14, 20). In this context, we asked whether two types of alterations of macrophages that occur in advanced lesions, namely, cholesteryl ester loading ("foam cells") and predeath FC loading, would alter the ability of phagocytes to ingest FC-AMs. To test this point, phagocytes were first incubated for 12 h in the absence or presence of acetyl-LDL or acetyl-LDL plus the ACAT inhibitor 58035 to generate control phagocytes, foam cell phagocytes, or preapoptotic FC-loaded phagocytes, respectively. The phagocytes were then exposed to FC-AMs as above and assayed for apoptotic cell ingestion. Interestingly, these lipid-laden phagocytes were able to ingest FC-AMs as efficiently as control phagocytes that did not contain excess cholesterol (Fig. 1*D*). Thus, to the extent that certain phagocytes in advanced lesions are unable to clear apoptotic cells, cholesteryl ester accumulation or predeath FC loading does not appear to be the cause of the phagocytic defect.

Mer Plays a Critical Role in the Uptake of FC-AMs—A number of phagocyte receptors have been implicated in apoptotic cell recognition and uptake, including the membrane tyrosine kinase Mer, CD36, $\alpha_v\beta_3$ integrin, LRP, and perhaps a heretofore unidentified receptor that recognizes phosphatidylserine (PS) (39, 40). Apoptotic cell removal *in vivo* is defective in Mer-deficient (*mertk*) mice (41) but appears to be normal in other receptor knock-out mouse models. We explored the potential role of these receptors in phagocytosis of FC-AMs by using the following manipulations: (*a*) preincubation of phagocytes with PS-liposomes

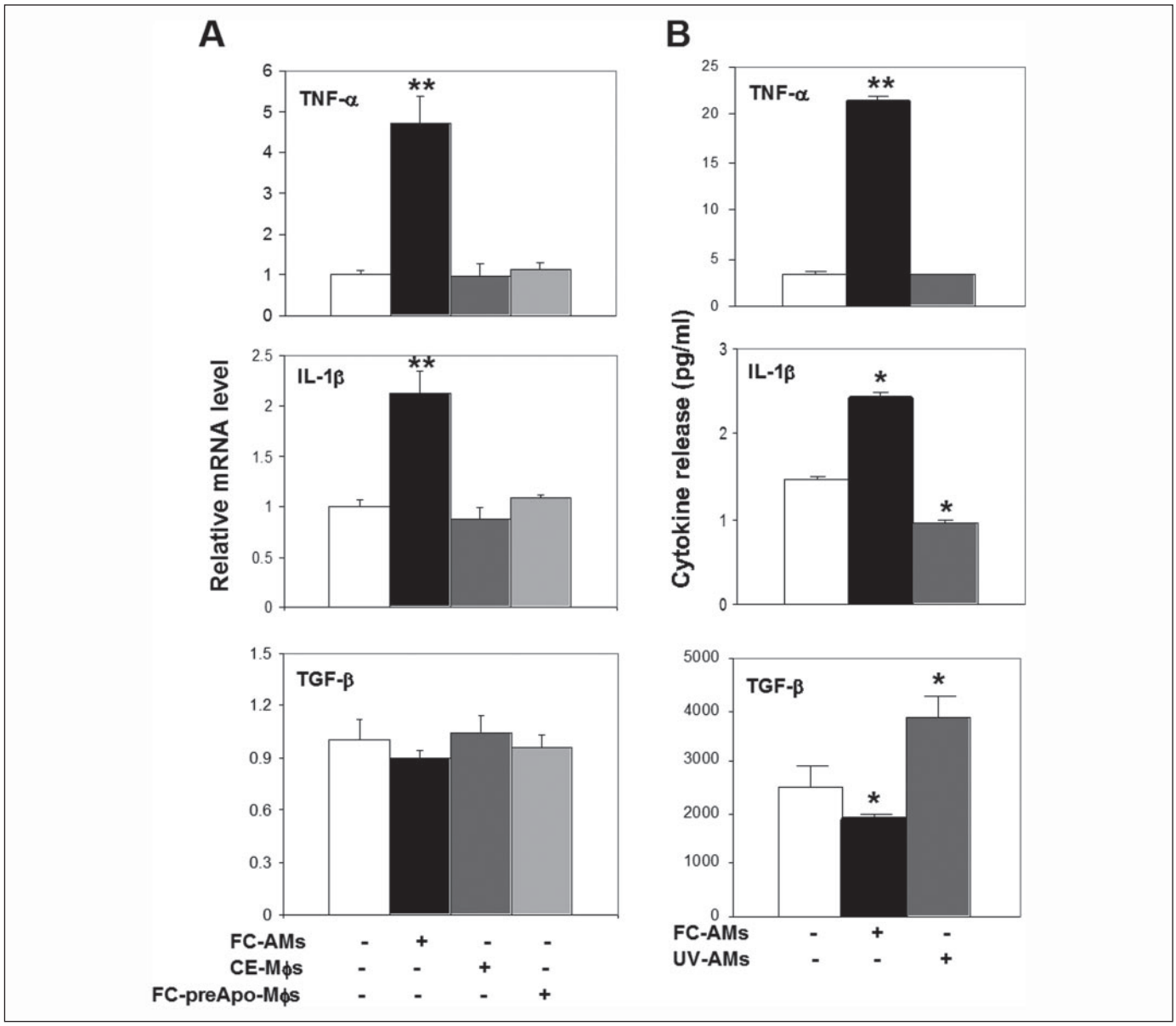

FIGURE 3. **Induction of TNF-α and IL-1β mRNA and protein in phagocytes after brief contact with FC-AMs.** *A*, phagocytes were incubated for 30 min with FC-AMs, cholesteryl ester-loaded macrophages (*CE-Mϕs*), or preapoptotic FC-loaded macrophages (*FC-preApo Mϕs*). The phagocytes were washed thoroughly and then incubated in fresh medium for 8 h. Total RNA was extracted and processed by RT-PCR for the indicated cytokine mRNAs. *B*, phagocytes were incubated with FC-AMs or UV radiation-induced apoptotic macrophages (*UV-AMs*) for 30 min. The phagocytes were washed thoroughly and then incubated in fresh medium for 18 h. The culture medium was collected and subjected to ELISA analysis for the indicated cytokines. *Asterisks*, $p < 0.01$; *double asterisks*, $p < 0.005$ compared with control phagocytes (*white bar*).

or with the integrin-specific blocking peptide RGDS (Fig. 2*A*); or (*b*) using phagocytes from *Cd36*−/− mice, macrophage-specific LRP-deficient mice, or *mertk* mice, which have a mutation in Mer that renders the receptor dysfunctional (34) (Fig. 2, *B–D*). The data show that interrupting interactions through PS, RGDS-integrins, LRP, and CD36 had minimal effects on uptake of FC-AMs, even though we were able to reproduce the inhibitory effect of the above manipulations using phagocytosis systems reported previously (42, 43 and data not displayed). On the other hand, when the phagocytes were from *mertk* mice, uptake of FC-AMs was suppressed by ~60% (Fig. 2*D*). Thus, Mer plays an important role in the uptake of FC-AMs by phagocytes.

Phagocytes Exposed to FC-AMs Produce a Number of Proinflammatory Cytokines and Fail to Induce TGF-β and IL-10—Phagocytosis of apoptotic T cells and neutrophils by macrophages induces the production of the anti-inflammatory cytokine TGF-β, which activates signaling pathways leading to transcriptional suppression of proinflammatory cytokines (28, 29). We examined whether FC-AMs would also elicit anti-inflammatory responses in phagocytes. Surprisingly, we found modest increases in the mRNA and protein levels of the proinflammatory cytokines TNF-α and IL-1β, whereas TGF-β mRNA and protein were not increased (Fig. 3, *black bar* in each *graph*). IL-10 mRNA was also unchanged, and IL-10 protein was below the detection limit of the ELISA (data not shown). Time course studies indicated that the increase in TNF-α and IL-1β mRNA was initiated at ~4 h postphagocytosis and was maximal at ~6–10 h postphagocytosis. Accumulation of TNF-α and IL-1β protein in the media was observed at ~10 h postphagocytosis and reached a plateau at 18–20 h. Incubation of the phagocytes with viable cholesteryl ester-loaded macrophages or FC-loaded preapoptotic macrophages did not affect the cytokine profile (Fig. 3*A*, *last two bars* in each *graph*).

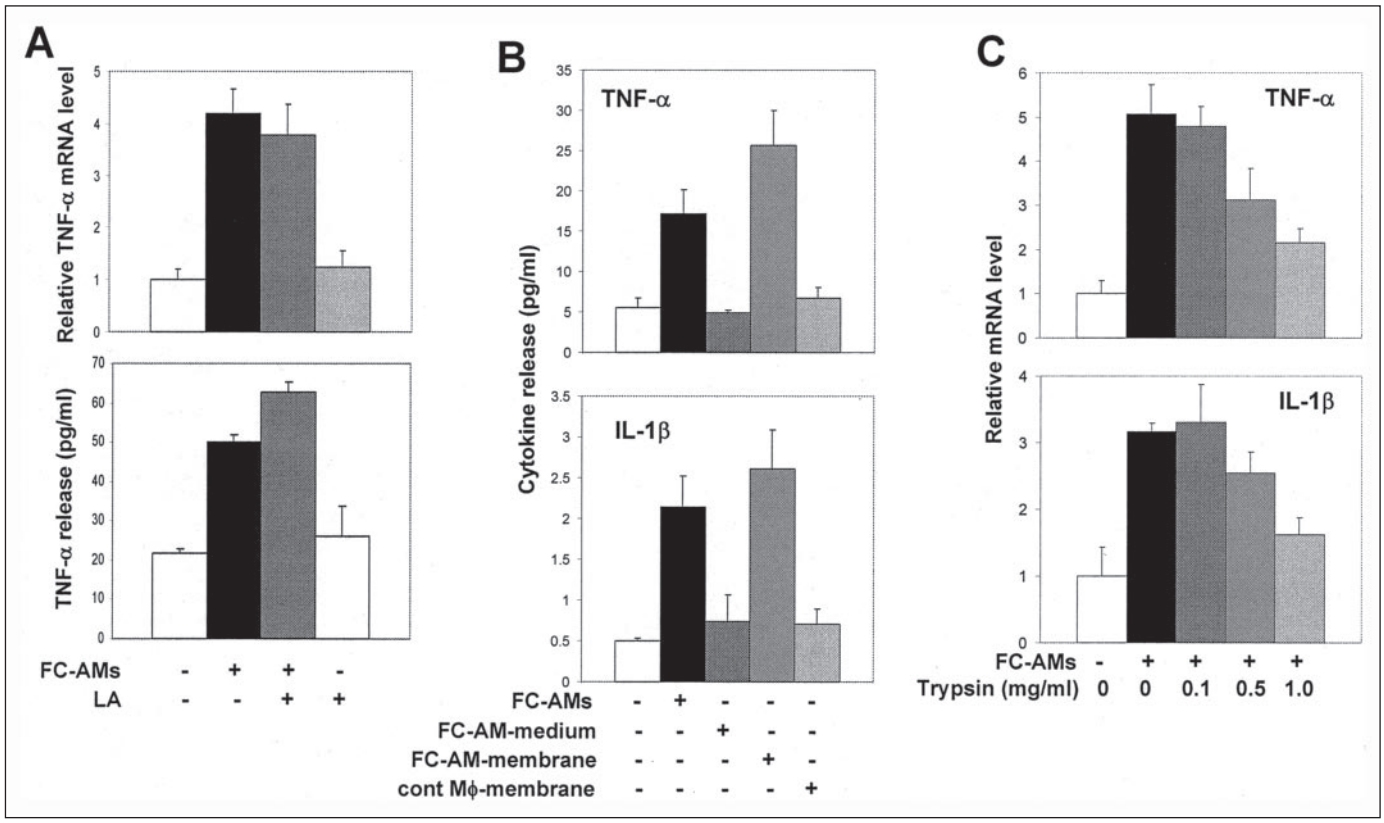

FIGURE 4. **Internalization of FC-AMs is not required for the induction of TNF-α and IL-1β in phagocytes.** *A*, phagocytes were preincubated for 30 min with medium alone or medium containing 100 nM latrunculin A (*LA*) and then incubated for an additional 30 min with FC-AMs in the absence or presence of latrunculin A. The phagocytes were washed thoroughly and then incubated for 8 h in fresh medium. Total RNA was extracted and processed by RT-PCR for TNF-α mRNA. In another set of experiments, the culture medium was collected 18 h later and subjected to ELISA analysis for TNF-α protein. *B*, phagocytes were incubated for 30 min FC-AMs, FC-AM-conditioned medium, membranes from FC-AMs, or membranes from control macrophages (*cont Mϕ-membrane*). The membranes were prepared by three cycles of freezing-thawing the cells. The phagocytes were washed thoroughly and then incubated for 18 h in fresh medium. The culture medium was collected and subjected to ELISA analysis for the indicated cytokines. *C*, FC-AMs were incubated with the indicated concentrations of trypsin in DMEM at 37 °C for 20 min and then rinsed thoroughly with DMEM and 10% fetal bovine serum. The trypsin-treated FC-AMs were then incubated with phagocytes for 30 min. The phagocytes were washed thoroughly and then incubated in fresh medium for 8 h. Total RNA was extracted and processed by RT-QPCR for the indicated cytokine mRNAs.

FC-AMs altered a number of other inflammatory responses in phagocytes that, based on *in vivo* studies, predict a net increase in inflammation (44–46). For example, there was a 2-fold elevation in prostaglandin E_2 and IL-6. Moreover, granulocyte macrophage colony-stimulating factor, a molecule that can induce inflammation as well as leukocyte differentiation (46), was markedly suppressed by exposure of phagocytes to apoptotic Jurkat cells but not at all by FC-AMs. These findings, together with the data on TNF-α, IL-1β, TGF-β, and IL-10, indicate a global inflammatory response in phagocytes exposed to FC-AMs.

To test specificity, we asked whether ingestion of macrophages rendered apoptotic by either oxidized LDL or UV radiation would also trigger a proinflammatory response in the phagocytes. We carefully adjusted the conditions of oxidized LDL or UV treatment so that the degree of apoptosis by these two methods was similar to that of FC-AMs, *i.e.* 30–40% as indicated by annexin V positivity. We found that the phagocytes were able to ingest all three types of apoptotic macrophages with the same efficiency. However, phagocytosis of oxidized LDL-induced apoptotic macrophages (not shown) or UV radiation-induced apoptotic macrophages (Fig. 3*B*, *gray bar* in each *graph*) resulted in a typical anti-inflammatory response. These data suggest that the proinflammatory response seen with FC-AMs is unique to this type of apoptotic macrophage. We next considered the possibility that the proinflammatory response to FC-AMs was related to the state of activation of the phagocytic macrophages, which were elicited by either intraperitoneal injection of concanavalin A or by immunization with methyl-BSA (see "Experimental Procedures"). However, similar results were found using resident macrophages or thioglycollate-elicited macrophages as the phagocytes (data not shown). Therefore, the proinflammatory response in phagocytes after contact with FC-AMs is independent of the activation state of the phagocytes.

FC-AM Membranes, through One or More Cell Surface Proteins, Can Trigger the Induction of TNF-α and IL-1β in Phagocytes without the Need for FC-AM Ingestion—To test whether the induction of TNF-α and IL-1β described here can be triggered without engulfment of FC-AMs, phagocytes were treated with latrunculin A prior to incubation with the FC-AMs to block apoptotic cell engulfment (see Fig. 1*A*). As shown in Fig. 4*A*, TNF-α induction was not diminished in the presence of latrunculin A, indicating that internalization of the FC-AMs is not necessary for induction of the inflammatory response. The induction of IL-1β by FC-AMs was also not affected by latrunculin A (data not shown).

Because ingestion of FC-AMs is not required, the induction of TNF-α and IL-1β in the phagocytes could be mediated by a soluble factor secreted from the FC-AMs or by a molecule on the surface of the FC-AMs. To distinguish between these two possibilities, we incubated phagocytes with FC-AM-conditioned media or with membranes derived from FC-AMs. For the latter purpose, FC-AMs were rapidly frozen and thawed for three cycles to lyse the cells without disrupting the overall orientation of cell surface molecules. As shown in Fig. 4*B*, FC-AM-derived membranes were able to elicit the same cytokine

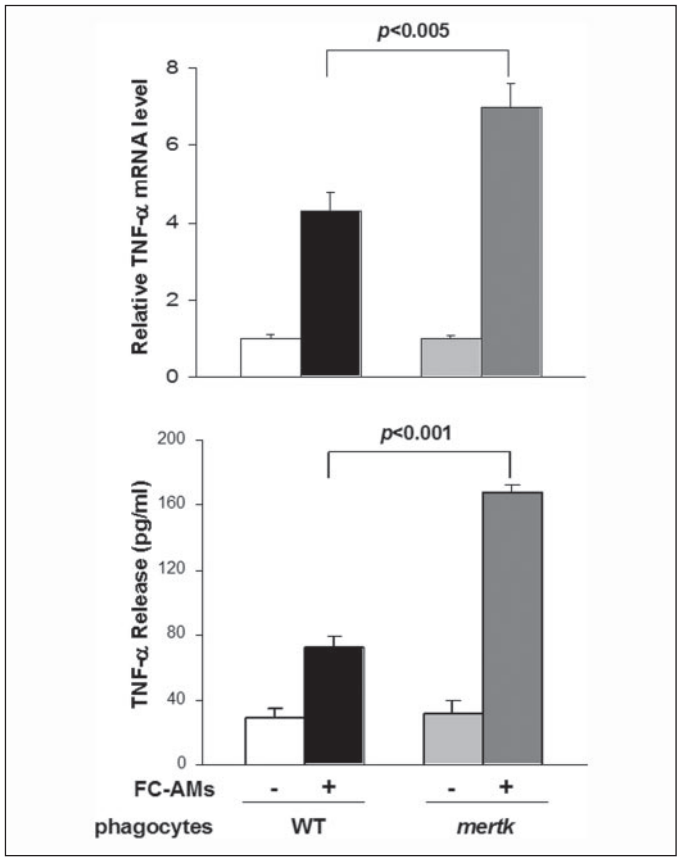

FIGURE 5. **Induction of TNF-α in phagocytes exposed to FC-AMs is attenuated by Mer.** Wild-type (*WT*) and *mertk* phagocytes were incubated for 30 min with FC-AMs. The phagocytes were washed thoroughly and then incubated for 8 or 18 h in fresh medium. The 8-h cells were processed for TNF-α RT-QPCR, and the medium from the 18-h cells were subjected to TNF-α ELISA.

response as intact FC-AMs, whereas FC-AM-conditioned media had no effect. In contrast, membranes generated from viable macrophages failed to trigger the cytokine response. Furthermore, brief treatment of FC-AMs with trypsin decreased their ability to induce the cytokine response in phagocytes in a dose-dependent manner (Fig. 4*C*). These important data suggest that one or more proteins on the surface of FC-AMs are necessary for triggering the induction of TNF-α and IL-1β in the phagocytes.

The Induction of TNF-α and IL-1β in Phagocytes Exposed to FC-AMs Requires neither LRP nor the TLR Adaptor Myd88 but Is Attenuated by Mer—LRP, despite its lack of a role in FC-AM internalization (above), or Toll-like receptors (TLR) 2 and 4 might mediate the cytokine response in phagocytes exposed to FC-AMs (47–49). However, we found that the TNF-α response to FC-AMs was not inhibited in phagocytes from either macrophage-specific LRP-deficient mice or from mice deficient in MyD88, an adaptor necessary for a number of proinflammatory TLR2 and TLR4 signaling pathways (50) (data not shown). Because proinflammatory signaling by necrotic cells and endotoxin signaling require MyD88 (49), the negative *Myd88−/−* data indicate that the cytokine response elicited by FC-AMs is not the result of either a small percentage of necrotic cells in the FC-AM population or endotoxin contamination in our experimental system.

Although we have emphasized that phagocytes exposed to FC-AMs do not undergo a typical anti-inflammatory response, the actual level of induction of TNF-α and IL-1β was modest. In this context, previous reports have shown that the engagement of Mer attenuates LPS-stimulated TNF-α production in macrophages (34). We therefore considered the possibility that Mer, in addition to mediating FC-AM uptake, may also play a role in dampening the TNF-α response in phagocytes exposed to FC-AMs. As shown in Fig. 5, TNF-α mRNA and protein production in *mertk* phagocytes exposed to FC-AMs were substantially higher than those from wild-type phagocytes, indicating that Mer lessens the phagocyte inflammatory response. Because Mer-mediated internalization of FC-AMs is not needed for the cytokine response (above), these data suggest that the highest level of inflammation would occur under conditions in which Mer function is suboptimal, which might occur in advanced atherosclerotic lesions (see "Discussion").

Intracellular Signaling Pathways Implicated in Triggering the Induction of TNF-α and IL-1β in Phagocytes Exposed to FC-AMs—We demonstrated recently that FC loading of macrophages induces the production of two proinflammatory cytokines, TNF-α and IL-6, but not IL-1β, prior to the onset of apoptosis (4). Mechanistic studies revealed that cholesterol trafficking to the endoplasmic reticulum (ER) is an essential event in FC-induced inflammation. The fact that phagocytes do not need to internalize FC-AMs for the TNF-α and IL-1β response suggested that the FC-to-ER pathway was not involved. Nonetheless, we tested this point by using two strategies to specifically block cholesterol trafficking to the ER in the phagocytes: the use of phagocytes from mice with a heterozygous mutation in the cholesterol-trafficking protein NPC1, and incubation of wild-type phagocytes with nanomolar concentrations of the amphipathic amine U18666A (10, 51). As shown in Fig. 6*A*, incubating the phagocytes with 70 nM U18666A did not affect TNF-α and IL-1β production, and similar results were obtained when *Npc1±* phagocytes were used (data not shown). These data indicate that the TNF-α and IL-1β response is independent of intracellular cholesterol trafficking to the ER in the phagocytes, which is consistent with the finding that FC-AM uptake is not needed for the response to occur.

Sustained activation of p38, ERK1/2 and JNK1/2 MAP kinases are necessary for the induction of TNF-α and/or IL-6 in FC-loaded macrophages (4). To test the role of these MAP kinases in the current model, we assayed their activation and causative roles in TNF-α and IL-1β production in phagocytes following exposure to FC-AMs. As shown in Fig. 6*B*, p38 and ERK1/2 were transiently activated ~4 h after phagocytosis. In contrast, JNK1/2 phosphorylation was not detected (data not shown). The role of p38 and ERK1/2 signaling in mediating TNF-α and IL-1β production in the phagocytes was evaluated using specific inhibitors of these two MAP kinases. As shown in Fig. 6*C*, at concentrations known to block their target kinase activities completely, the ERK1/2 inhibitor PD98059 completely suppressed FC-AM-induced TNF-α and IL-1β production in the phagocytes. In contrast, the p38 inhibitor SB203580 or its inactive form SB202474 had no effect. A more detailed examination of PD98059 dose response showed that the inhibitory effect of this drug on cytokine induction took place at concentrations that blocked ERK1/2 phosphorylation (Fig. 6*D*). Thus, the ERK1/2 signaling pathway is involved in the induction of TNF-α and IL-1β in phagocytes after their contact with FC-AMs.

Activation of the transcription factor NF-κB has been implicated in the transcriptional regulation of cytokines in a wide variety of systems, including FC-loading of macrophages (4). We therefore explored the possible involvement of the NF-κB pathway in FC-AM-induced TNF-α and IL-1β production in phagocytes. Both degradation of the NF-κB inhibitory protein IκB-α and accumulation of p65 NF-κB in the nucleus were detected at ~6 h postphagocytosis (Fig. 7, *A* and *B*), indicating that NF-κB is activated in phagocytes exposed to

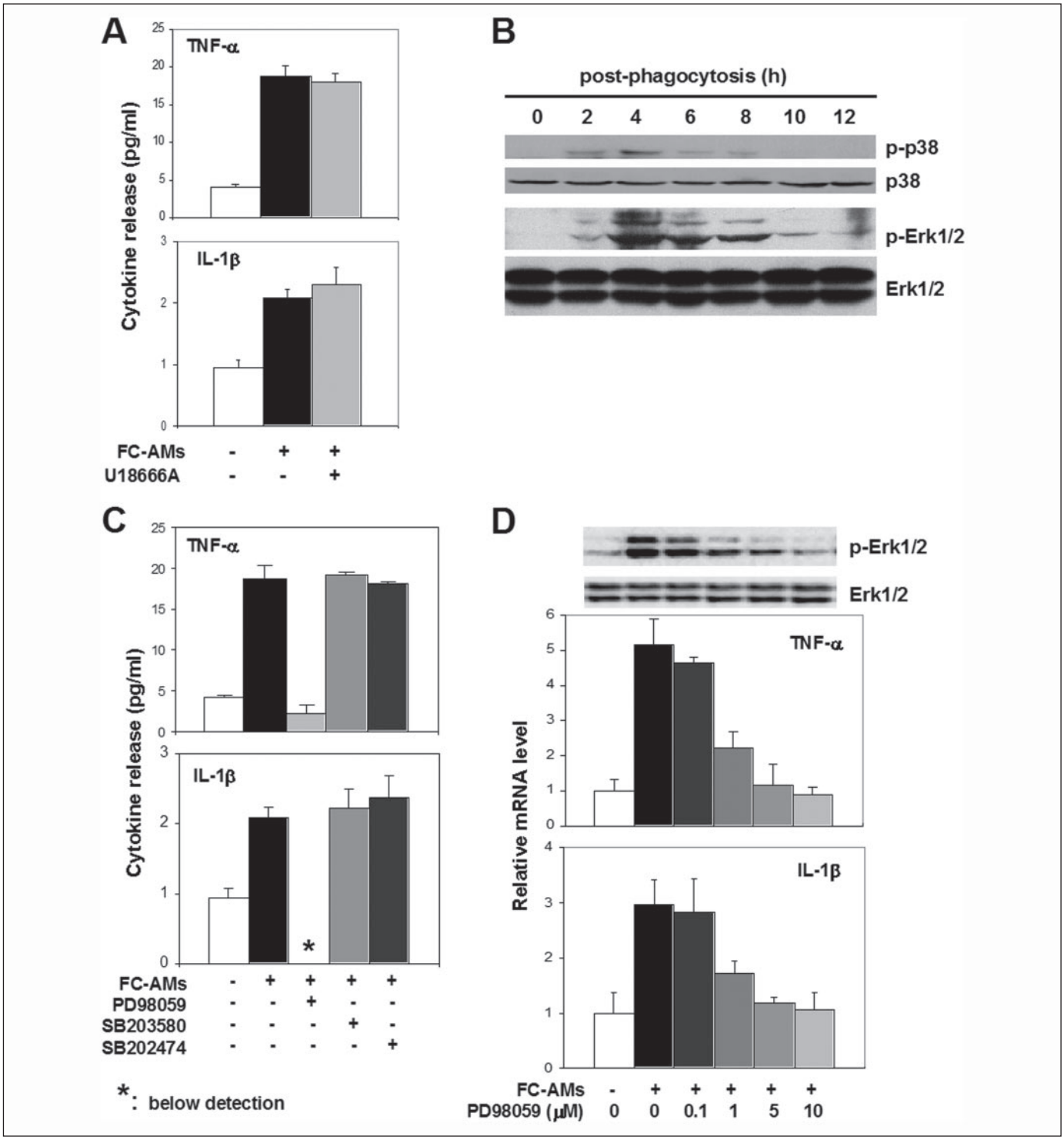

FIGURE 6. **TNF-α and IL-1β production in phagocytes exposed to FC-AMs requires ERK1/2 MAP kinase signaling but not cholesterol trafficking to the ER.** *A*, phagocytes were incubated for 30 min with FC-AMs in the absence or presence of 70 nM U18666A. The phagocytes were washed thoroughly and then incubated in fresh medium in the absence or presence of U18666A for 18 h. The culture medium was collected and subjected to ELISA analysis for the indicated cytokines. *B*, phagocytes exposed to FC-AMs for 30 min as above were incubated in fresh medium for the indicated times. Total cell lysates were subjected to SDS-PAGE followed by immunoblotting for phosphorylated and total p38 and ERK1/2. *C*, phagocytes were incubated for 30 min with the FC-AMs in the absence or presence of the ERK inhibitor PD98059 (10 μM), the p38 inhibitor SB203580, or its inactive analog SB202474 (10 μM). The phagocytes were washed thoroughly and then incubated in fresh medium in the absence or presence of the above inhibitors for 18 h. The culture medium was collected and subjected to ELISA analysis for the indicated cytokines. *D*, phagocytes were incubated 30 min with FC-AMs in the presence of the indicated concentration of PD98059. The phagocytes were washed thoroughly and then incubated in fresh medium in the presence of the inhibitor. One set of phagocytes was incubated for 5 h, and the cells were lysed and subjected to SDS-PAGE followed by immunoblotting for phosphorylated and total ERK1/2. Another set of phagocytes was incubated for 8 h, and total RNA was extracted and processed by RT-QPCR for the indicated cytokine mRNAs.

FC-AMs. Most importantly, a specific IκB kinase inhibitor, PS1145 (33), significantly suppressed FC-AM-induced TNF-α and IL-1β mRNA. Cytokine suppression by PS1145 occurred in a dose-dependent manner that paralleled the decrease in nuclear p65 (Fig. 7*C*). Thus, NF-κB activation plays a role in the transcriptional regulation of TNF-α and IL-1β induced by contact of phagocytes with FC-AMs.

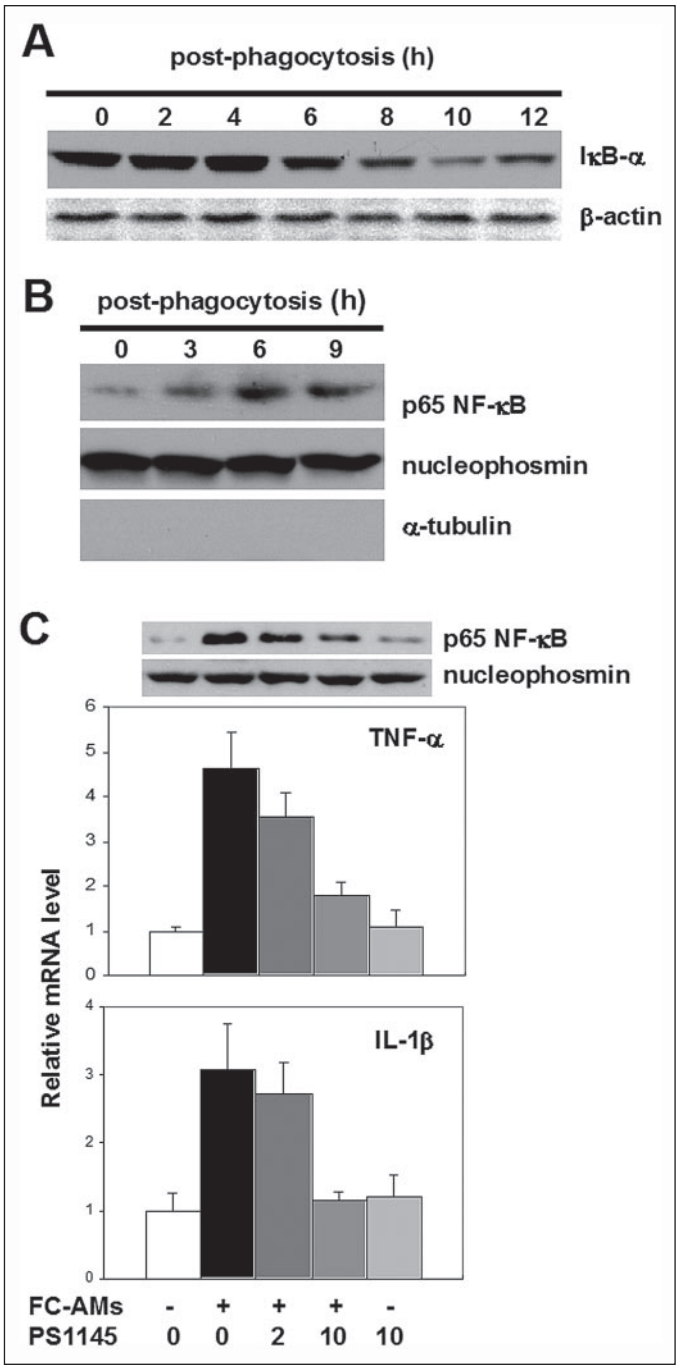

FIGURE 7. **TNF-α and IL-1β production in phagocytes exposed to FC-AMs requires NF-κB activation.** *A*, phagocytes exposed to FC-AMs for 30 min were incubated in fresh medium for the indicated times. Total cell lysates were subjected to SDS-PAGE followed by immunoblotting for IκB-α. β-Actin was used as loading control. *B*, phagocytes exposed to FC-AMs for 30 min were incubated in fresh medium for the indicated times. Nuclear extracts were prepared and subjected to SDS-PAGE followed by immunoblotting for p65 NF-κB, the nuclei protein nucleophosmin, and the cytosolic protein α-tubulin (absence of α-tubulin signal is an indication of the purity of the nuclear extract). *C*, phagocytes were incubated for 30 min with the FC-AMs in the absence or presence of the IκB kinase inhibitor PS1145 (2 or 10 μM). The phagocytes were washed thoroughly and then incubated in fresh medium in the absence or presence of the inhibitor for 8 h. Nuclear extracts were prepared from one set of cells and subjected to SDS-PAGE followed by immunoblotting for p65 NF-κB and nucleophosmin. Another set of cells was subjected to total RNA extraction and processed by RT-QPCR for the indicated cytokine mRNAs.

TABLE 1

Summary of interactions of FC-AMs with phagocytes

The data in this report suggest two types of interactions between FC-AMs and phagocytes: cell-cell contact mediated by one or more cell surface proteins on the FC-AMs, and engagement of the phagocyte Mer receptor by FC-AMs. The former interaction is responsible for the induction of TNF-α and IL-1β through ERK1/2 and NF-κB activation and is not dependent upon ingestion of the FC-AMs. Further evidence that this interaction results in an overall proinflammatory response is that prostaglandin E_2 and IL-6 are also induced, granulocyte macrophage colony-stimulating factor is not suppressed, and TGF-β and IL-10 are not increased. The interaction through Mer has two consequences: actin-dependent ingestion of FC-AMs and attenuation of the TNF-α response. The effect of Mer engagement on the other cytokines mentioned above was not examined. For details, see text.

	Cell-cell contact	Interaction through Mer
Ligand on FC-AM	Cell-surface protein(s)	? Mer ligand
Receptor on phagocyte	?	Mer
Responses of phagocyte	• Erk1/2 and NF-κB activation • ↑ TNF-α and IL-1β • No increase in TGF-β or IL-10	• Actin-dependent ingestion of FC-AMs • Attenuation of TNF-α response

DISCUSSION

Although macrophage apoptosis occurs throughout the course of atherogenesis, recent *in vivo* studies suggest that the consequences of macrophage apoptosis are different in early and advanced lesions (14). In early lesions, where macrophage apoptosis is almost certainly not induced by FC loading, the net effect of this event appears to be a decrease in lesion cellularity and no evidence of increased inflammation (12–14). The most likely explanation is that phagocytic clearance of early lesional apoptotic macrophages is efficient and results in an anti-inflammatory response that is typical when phagocytes ingest apoptotic cells. Indeed, we found that macrophages rendered apoptotic by one possible inducer of cell death in early lesions, oxidized LDL, does not elicit a proinflammatory response in phagocytes. In late lesions, however, *in vitro* and *in vivo* data support a model in which defective phagocytosis of apoptotic macrophages results in postapoptotic macrophage necrosis, the precursor of the necrotic core, and a heightened state of inflammation (14, 20, 52–54).

Nonetheless, given the large number of macrophage phagocytes in late lesions and the fact that cholesterol loading does not impair their ability to clear apoptotic cells, it is likely that ingestion of apoptotic macrophages does occur to some degree in these advanced plaques. We therefore wondered to what extent the competent subpopulation of phagocytes in advanced lesions could counterbalance the effect of the defective subpopulation of phagocytes. In theory, a potent anti-inflammatory response by these competent phagocytes, as predicted by previous studies examining phagocytic uptake of apoptotic cells (28, 29), might partially negate the proinflammatory effect of necrosis and other processes in late lesions. However, the data in this report, which are summarized in Table 1, refute this idea: the typical anti-inflammatory response does not result when phagocytes are exposed to macrophages rendered apoptotic by a mechanism that is likely to occur in advanced plaques.

The consequences of the proinflammatory response observed after contact between FC-AMs and phagocytes raise important mechanistic issues. We initially considered the possibility that the cytokines were simply left over from the FC-AMs themselves, which secrete abundant amounts of TNF-α and IL-6 (see below and Ref 4). However, our mechanistic data indicate that this is not the case. First, IL-1β was produced only after FC-AMs were added to the phagocytes, not by the FC-AMs themselves. Second, mRNA as well as protein levels for TNF-α and IL-1β were elevated well after the phagocytes had ingested the FC-AMs and the noningested FC-AMs had been removed. Third, induction of TNF-α and IL-1β in the phagocytes was mediated by intracellular signaling pathways distinct from those in FC-loaded macrophages (4). For example, blocking p38 signaling abolished TNF-α production in FC-loaded macrophages but had no effect in the phagocytes exposed to

FC-AMs. For these reasons, we conclude that TNF-α and IL-1β are induced in the phagocytes themselves.

Although we do not yet know the detailed signaling mechanism, cytokine induction is NF-κB- and ERK1/2-dependent and requires cell contact between the FC-AMs and the phagocytes but not ingestion of FC-AMs. The fact that limited proteolysis of FC-AM membranes diminishes their ability to elicit the proinflammatory response in the phagocytes indicates that one or more cell surface proteins from the FC-AMs are necessary for the signaling pathways in phagocytes (Table 1). Because the activation of NF-κB and ERK1/2 and the induction of TNF-α and IL-1β occurred several hours after the initial FC-AM-phagocyte contact, it is likely that these events are induced secondarily by an as-yet unidentified intermediary signaling cascade. Such an intermediary signaling cascade might occur entirely intracellularly in a delayed manner or involve paracrine/autocrine factors secreted by the phagocytes in response to the initial FC-AM-phagocyte contact. In the context of this latter possibility, we collected medium from phagocytes that had been briefly challenged by FC-AMs and added it to fresh macrophages, but no inflammatory response was induced. However, a putative inducing factor might be difficult to identify in conditioned medium if it is very labile or rapidly sequestered (*i.e.* taken out of solution) by cells.

The one receptor that we have shown to be involved in the interaction of FC-AMs with phagocytes is the cell surface tyrosine kinase Mer. Mer plays at least two roles in our system: ingestion of FC-AMs and modulation of the proinflammatory response (Table 1). Both roles have been demonstrated previously in other cell culture models and, most importantly, *in vivo*. For example, mice with deficient Mer activity have evidence of defective phagocytic clearance of apoptotic thymocytes and a heightened TNF-α response to endotoxin challenge (34, 41). The mechanisms of these effects are not completely understood, but Mer activation elicits changes in the cytoskeleton which are likely to be important in phagocytosis (55, 56), and Mer engagement inhibits the NF-κB pathway (34). Another interesting question is how Mer recognizes apoptotic cells. Previous studies have suggested that the Mer ligand Gas6 binds to externalized PS on apoptotic cells and bridges these cells to the Mer receptor on phagocytes (57). If this process were occurring in our system, it is unclear why PS-containing vesicles did not compete for Gas6-apoptotic cell interaction and thereby inhibit phagocyte ingestion of FC-AMs. Nonetheless, to the extent that Gas6 may be necessary for proper Mer function, our data suggest that a relative deficiency of Gas6 in advanced atherosclerotic lesions could further heighten the inflammatory response. In this context, vascular smooth muscle cells appear to be the major source of Gas6 in lesions (58), and vulnerable plaques have a deficiency of smooth muscles in areas of plaque disruption (59). Finally, another possible consequence of Mer engagement is protection of phagocytes from apoptosis (60), which may be important in maintaining the viability of phagocytes in the face a large and potentially cytotoxic FC load.

The work herein has emphasized a specific scenario relevant to advanced atherosclerosis in which apoptotic cells elicit a proinflammatory response in phagocytes. Are there other scenarios in late lesions which may be anti-inflammatory? Although the role of oxidized LDL-induced macrophage apoptosis in late lesions is not known, our data would suggest that phagocytosis of macrophages rendered apoptotic by this means may elicit the typical anti-inflammatory response. Likewise, it is possible that phagocytosis of macrophages made apoptotic by other means, as well as ingestion of apoptotic smooth muscle cells, could elicit an anti-inflammatory response in phagocytes. Another important consideration is related to our previous finding that FC-loaded macrophages secrete large amounts of TNF-α and IL-6 over time. In one scenario, these cytokines might further enhance an inflammatory response in the phagocytes. However, it is also possible that neighboring macrophages might actually dampen the TNF-α/IL-6 response by FC-loaded macrophages. In the experimental system used in the current study, which was designed to model rapid and efficient clearance of apoptotic cells, the FC-AMs were removed rapidly by either phagocytic ingestion or, for those FC-AMs not ingested, by our postingestion rinsing protocol. Therefore, there was not enough time for accumulation of cytokines secreted by the FC-loaded macrophages, and so the experimental system used here would not address the consequences or fate of cytokines secreted over time by FC-loaded macrophages. In this regard, future studies will be needed to model another likely scenario in advanced lesions, namely, prolonged exposure of macrophages to non-ingested FC-loaded macrophages. In all likelihood, the situation *in vivo* is likely to be complex, with several scenarios occurring simultaneously. Therefore, the net effect of late lesional macrophage apoptosis and phagocytosis *in vivo* will require genetic manipulations that alter these events in advanced plaques of experimental animals.

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