THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 23, Issue of June 10, pp. 21763–21772, 2005

Printed in U.S.A.

Free Cholesterol-loaded Macrophages Are an Abundant Source of Tumor Necrosis Factor-α and Interleukin-6

MODEL OF NF-κB- AND MAP KINASE-DEPENDENT INFLAMMATION IN ADVANCED ATHEROSCLEROSIS*

Received for publication, February 15, 2005, and in revised form, April 11, 2005
Published, JBC Papers in Press, April 11, 2005, DOI 10.1074/jbc.M501759200

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Two key features of atherosclerotic plaques that precipitate acute atherothrombotic vascular occlusion ("vulnerable plaques") are abundant inflammatory mediators and macrophages with excess unesterified, or "free," cholesterol (FC). Herein we show that FC accumulation in macrophages leads to the induction and secretion of two inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). The increases in TNF-α and IL-6 mRNA and protein were mediated by FC-induced activation of the IκB kinase/NF-κB pathway as well as activation of MKK3/p38, Erk1/2, and JNK1/2 mitogen-activated protein kinases (MAPK). Activation of IκB kinase and JNK1/2 was needed for the induction of both cytokines. However, MKK3/p38 signaling was specifically involved in TNF-α induction, and Erk1/2 signaling was required for IL-6. Most interestingly, activation of all of the signaling pathways and induction of both cytokines required cholesterol trafficking to the endoplasmic reticulum (ER). The CHOP branch of the unfolded protein response, an ER stress pathway, was required for Erk1/2 activation and IL-6 induction. In contrast, one or more other ER-related pathways were responsible for activation of p38, JNK1/2, and IκB kinase/NF-κB and for the induction of TNF-α. These data suggest a novel scenario in which cytokines are induced in macrophages by endogenous cellular events triggered by excess ER cholesterol rather than by exogenous immune cell mediators. Moreover, this model may help explain the relationship between FC accumulation and inflammation in vulnerable plaques.

Macrophages are the most prominent cell type in atherosclerotic lesions and are associated with two hallmarks of the disease, lipid deposition and inflammation (1–3). Recruitment of circulating monocytes to the subendothelial space is one of the earliest events in atherogenesis. Monocytes differentiate into macrophages, which subsequently internalize large amount of atherogenic, cholesterol-rich lipoprotein particles (4). In the early stages of atherosclerosis, most of the internalized cholesterol is stored as cholesteryl-fatty acid esters, resulting in "foam cell" formation. As a lesion becomes more advanced, however, there is a progressive decrease in the cholesteryl ester content and a reciprocal increase in the unesterified, or "free" cholesterol (FC)[1] content (5–7). FC accumulation is a potent inducer of macrophage apoptosis and secondary necrosis, which is thought to contribute to lesional necrosis and plaque disruption, leading to acute atherothrombotic cardiovascular events (8, 9). Of interest, the mechanism of FC-induced macrophage apoptosis involves overloading the normally cholesterol poor and fluid endoplasmic reticulum (ER) membrane (10). This event, which leads to an abnormal "stiffening" of the ER membrane, activates the ER stress pathway known as the unfolded protein response (UPR) (11, 12). A particular branch of the UPR pathway involving the effector protein CHOP then triggers apoptosis (13).

Another key characteristic of advanced, or "vulnerable," atherosclerotic lesions is the presence of a variety of inflammatory cytokines, many of which are thought to be secreted by macrophages (14–17). However, it remains largely unknown how the inflammation is triggered and what inflammatory pathways are involved. The typical model, which is based upon processes in non-atherosclerotic inflammatory lesions, envisions immune cell mediators triggering cytokine induction in lesional macrophages. Herein we present evidence for a new model in which endogenous, FC-mediated signaling events in macrophages are sufficient to induce the synthesis of two inflammatory cytokines, TNF-α and IL-6. As with FC-induced apoptosis, cytokine induction requires FC-mediated ER stress pathways that lead to the activation of NF-κB and MAPK inflammatory signaling pathways. This new model may help explain the relationship between FC accumulation and the inflammatory component of plaque vulnerability in advanced atherosclerosis.

* This work was supported by National Institutes of Health Grants HL54591 and HL75662 (to I. T.), American Heart Association Scientist Development Grant 0435364T (to Y. L.), American Heart Association Post-doctoral Training Grant 04255805T (to T. D.-S.), and a Research Scholar Award from the American Gastroenterological Association and Procter & Gamble (to R. F. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: FC, free cholesterol; NF-κB, nuclear factor kappa B; IκB, inhibitor of NF-κB; MAPK, mitogen-activated protein kinases; Erk, extracellular signal-related kinase; JNK, c-Jun NH_2-terminal kinase; IL-6, interleukin-6; ER, endoplasmic reticulum; MEK, MAPK/Erk kinase; UPR, unfolded protein response; LDL, low density lipoprotein; IKK, IκB kinase; TNF-α, tumor necrosis factor-α; BSA, bovine serum albumin; RT-PCR, reverse transcription-PCR.

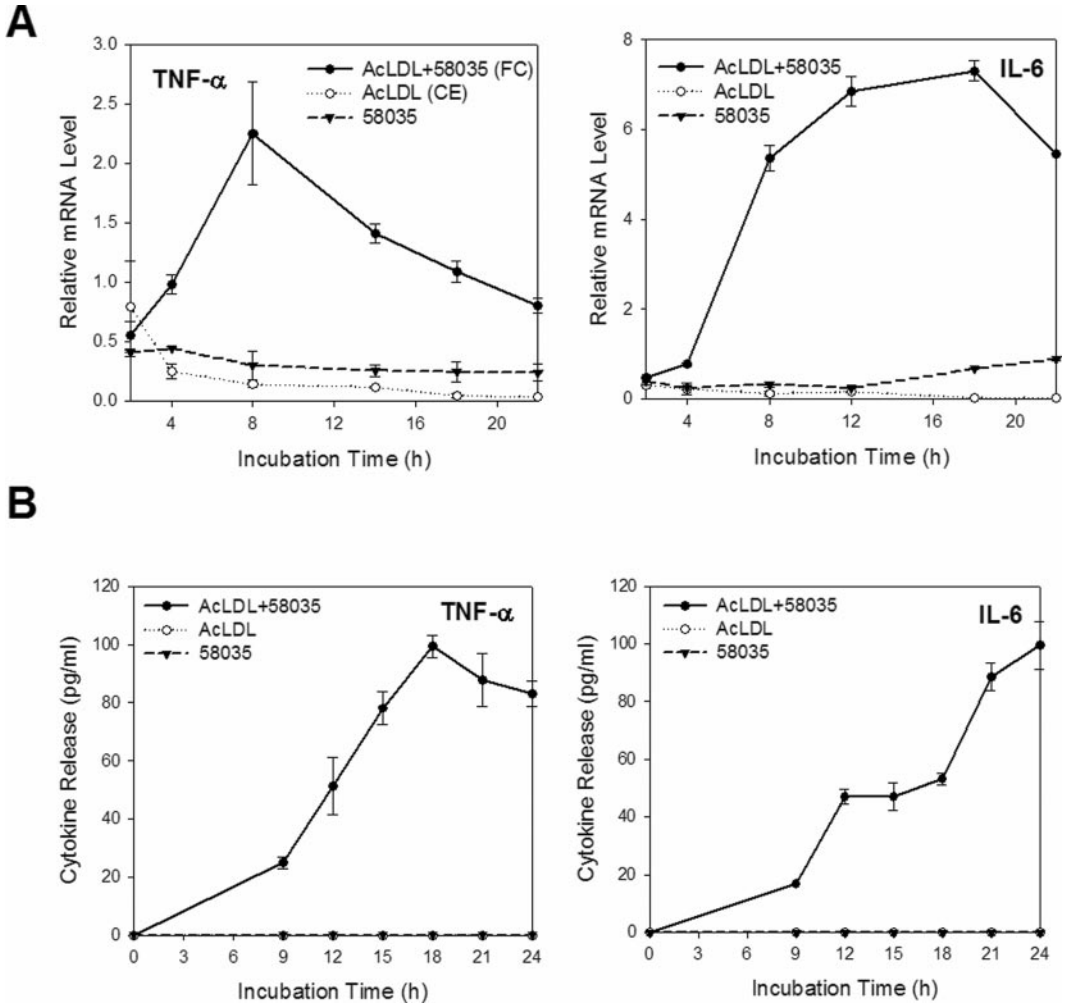

FIG. 1. **FC loading of macrophages induces the synthesis and secretion of TNF-α and IL-6.** Macrophages were incubated for various times with 100 μg/ml acetyl-LDL (*AcLDL*), 10 μg/ml compound 58035, or acetyl-LDL plus 58035 (FC). At the end of the incubation, total RNA was extracted from the cells and subjected to quantitative RT-PCR amplification (*A*). The mRNA level of TNF-α and IL-6 was normalized with that of the control gene 36B4. The culture media were collected, and the amounts of TNF-α and IL-6 released to the media were analyzed by enzyme-linked immunosorbent assay (*B*).

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and reagents were from Invitrogen. HPLC grade organic solvents were from Fisher Scientific. Chemical reagents were from Sigma unless specified below. Low density lipoprotein (LDL; *d* 1.020–1.063 g/ml) was isolated from fresh human plasma by preparative ultracentrifugation as described previously (18). Acetyl-LDL was prepared by reaction of LDL with acetic anhydride (19). Compound 58035 (3-[decyldimethylsilyl]-*N*-[2-(4-methylphenyl)-1-phenylethyl] propanamide), an inhibitor of acyl-CoA:cholesterol *O*-acyltransferase, was generously provided by Dr. John Heider, formerly of Sandoz, Inc. (East Hanover, NJ) (20). A stock solution of 10 mg/ml was prepared in dimethyl sulfoxide (Me_2SO) and stored at −20 °C. U18666A (3-β-[2-diethylaminoethoxy]androst-5-en-17-one hydrochloride) was from Biomol Research laboratories, Inc. PD98059 (2′-amino-3′-methoxyflavone) and U0126 (1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene) were from EMD Biosciences. SP600125 (anthra[1,9-cd]pyrazol-6(2*H*)-one) was from BioSource International, Inc. PS1145 was a generous gift from Millennium Pharmaceuticals (21).

Antibodies against p38, phosphor-p38, Erk1/2, phosphor-Erk1/2, JNK1/2, phosphor-JNK1/2, and p65 NF-κB were from Cell Signaling Technology. Anti-nucleophosmin was from Zymed Laboratories Inc.. Anti-IKKγ, anti-α-tubulin, and anti-β-actin were from Santa Cruz Biotechnologies, Inc. TNF-α blocking antibody was from R&D Systems.

Mice—Female mice, 8–10 weeks of age, were used in this study. C57BL6/J mice, *Tnfa*−/− mice on the B6129S background, and B6129SF2 control mice were from Jackson Laboratories. *Chop*−/− on the C57BL/6 background were from Drs. David Ron (New York University) and Robert Burke (Columbia University). *Mkk3*−/− on the C57BL/6 background mice were created as previously described (22). *Npc1*+/− BALB/C mice were backcrossed into the C57BL/6 background for four generations as described (23).

Eliciting, Culturing, and Incubations of Mouse Peritoneal Macrophage—Peritoneal macrophages were elicited by intraperitoneal injection of methyl-BSA in mice previously immunized with this antigen (24). 2 mg/ml methyl-BSA in 0.9% saline was emulsified in an equal volume of complete Freund's adjuvant (Difco). Mice were immunized intradermally with 100 μl of 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 intraperitoneally with 0.5 ml of phosphate-buffered saline containing 100 μg of methyl-BSA. 4 days later, macrophages were harvested by peritoneal lavage. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin/streptomycin, and 20% L-cell-conditioned medium for 24–48 h to reach confluence. FC loading was effected by incubating the cells with acetyl-LDL (100 μg/ml) and compound 58035 (10 μg/ml) in the aforementioned medium for various times. Note that most of the experiments shown in this study were also conducted in macrophages elicited by the intraperitoneal concanavalin A method (10), and the data were always similar in the two models.

Quantitative RT-PCR—Total RNA was extracted from macrophages using the RNeasy kit (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 quantitative RT-PCR amplification using Taqman universal PCR master mix (Applied Biosystems). The forward and reverse primers for TNF-α were CGGAGTCCGGGCAGGT and GCTGGGTAGAGAATGGATGAACA, respectively, and the probe was 6FAM-CTTTGGAGTCATTGCTCTGTGAAGGGAATG. The forward and reverse

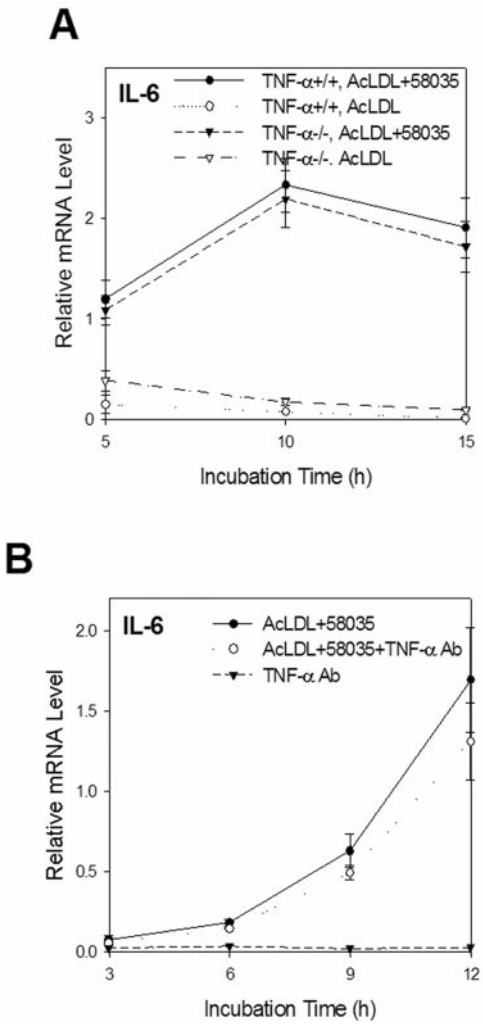

FIG. 2. **FC-induced IL-6 mRNA increase is independent of TNF-α secretion.** *A*, *Tnfa*+/+ and *Tnfa*−/−macrophages were incubated for various times with acetyl-LDL (*AcLDL*) or acetyl-LDL plus 58035 (FC), and IL-6 mRNA levels were measured. *B*, wild-type macrophages were incubated for various times under FC-loading conditions ± blocking TNF-α antibody (5 μg/ml) or with TNF-α antibody alone, and IL-6 mRNA levels were measured.

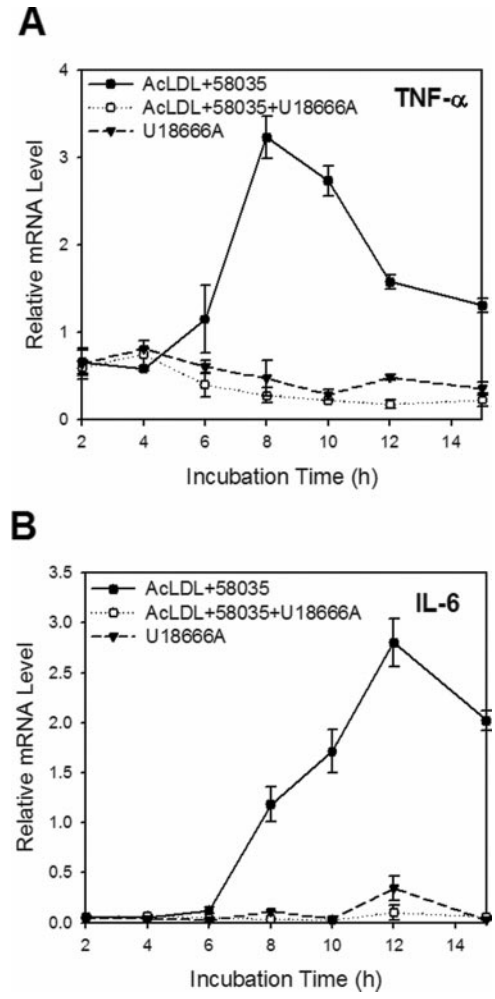

FIG. 3. **Cholesterol trafficking to the ER is required for induction of TNF-α and IL-6 by FC loading of macrophages.** Macrophages were incubated for the indicated times under FC-loading conditions ± 70 nM U18666A or with U18666A alone. Total RNA was extracted from the cells, and the mRNA levels of TNF-α (*A*) and IL-6 (*B*) were determined.

primers for IL-6 were GAGGATACCACTCCCAACAGACC and AAGTGCATCATCGTTGTTCATACA, respectively, and the probe was 6FAM-CAGAATTGCCATTGCACAACTCTTTTCTCA. 36B4 was used as the internal control, the forward and reverse primers for 36B4 were AGATGCAGCAGATCCGCAT and GTTCTTGCCCATCAGCACC, respectively, the probe was CAL-CGCTCCGAGGGAAGGCCG. The reactions were run on a MX4000 multiplex quantitative PCR system (Stratagene), the thermal profile settings were 50 °C for 2 min, 95 °C for 10 min, then 45 cycles at 95 °C for 15 s, and 60 °C for 1 min.

Nuclear Fractionation—Cells on 35-mm plates were washed with cold phosphate-buffered saline twice. 100 μl of Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 10 mM EDTA, 1 mM dithiothreitol, 0.4% (octylphenoxy)polyethoxyethanol, plus protease inhibitors) was added, and the plate was put on a rocking platform at 4 °C for 10 min. Cells were scraped from the plates, and cell clumps were disrupted by repetitive pipetting. The suspension was centrifuged at 14,000 rpm at 4 °C for 3 min, and the supernatant was collected (cytoplasmic fraction). The pellet was resuspended into 20 μl of Buffer B (20 mM HEPES, pH 7.9, 200 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol; plus protease inhibitors) by vortexing at the highest setting for 5 min and then at the medium setting for 30 min at 4 °C. The suspension was centrifuged at 14,000 rpm at 4 °C for 5 min, and the supernatant was collected (nuclear fraction).

Cytokine Enzyme-linked Immunosorbent Assays—Cells on 12-well plates were incubated with acetyl-LDL, 58035, or acetyl-LDL plus 58035 for various times. In certain experiments, cells were incubated as above for 9 h, the incubation media were removed, and then the cells were washed twice and incubated with fresh media for various times. At the end of the incubation period, the media were collected and centrifuged at 14,000 rpm for 5 min. The supernatants were stored in aliquots at −80 °C. TNF-α and IL-6 levels in the media were determined by enzyme-linked immunosorbent assay. The assays were performed in the Cytokine Core Laboratory (Baltimore, MD).

Immunoblotting—Cell extracts were electrophoresed on 4–20% gradient SDS-PAGE gels and transferred to 0.22-μm nitrocellulose membranes. The membrane was blocked in Tris-buffered saline, 0.1% Tween 20 (TBST) containing 5% (w/v) nonfat milk at room temperature for 1 h and 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).

IKK Activity Assay—IKK assays were performed as described previously (25), with slight modifications. Briefly, cells were lysed in the Triton lysis buffer containing protease inhibitors and phosphatase inhibitors. 200 μg of protein was immunoprecipitated with 2 μl of anti-IKKγ for 2 h, followed by the addition of 20 μl of protein A/G-agarose (Santa Cruz Biotechnology) for 1 h. The kinase reaction was performed for 30 min at 30 °C using glutathione *S*-transferase-IκB 1-54. The supernatant from the kinase reactions was analyzed on 10% SDS-PAGE. Equal substrate loading was confirmed by Coomassie Blue staining. Equal immunoprecipitate of IKK was shown by loading the beads onto SDS-PAGE gel and Western blotting for IKKγ.

Whole-cell Cholesterol Esterification Assay—Macrophages were incubated in Dulbecco's modified Eagle's medium and 0.2% BSA containing 0.1 mM [^{14}C]oleate complexed with albumin and 100 μg/ml acetyl-LDL for 5 h. The cells were washed twice with phosphate-buffered saline,

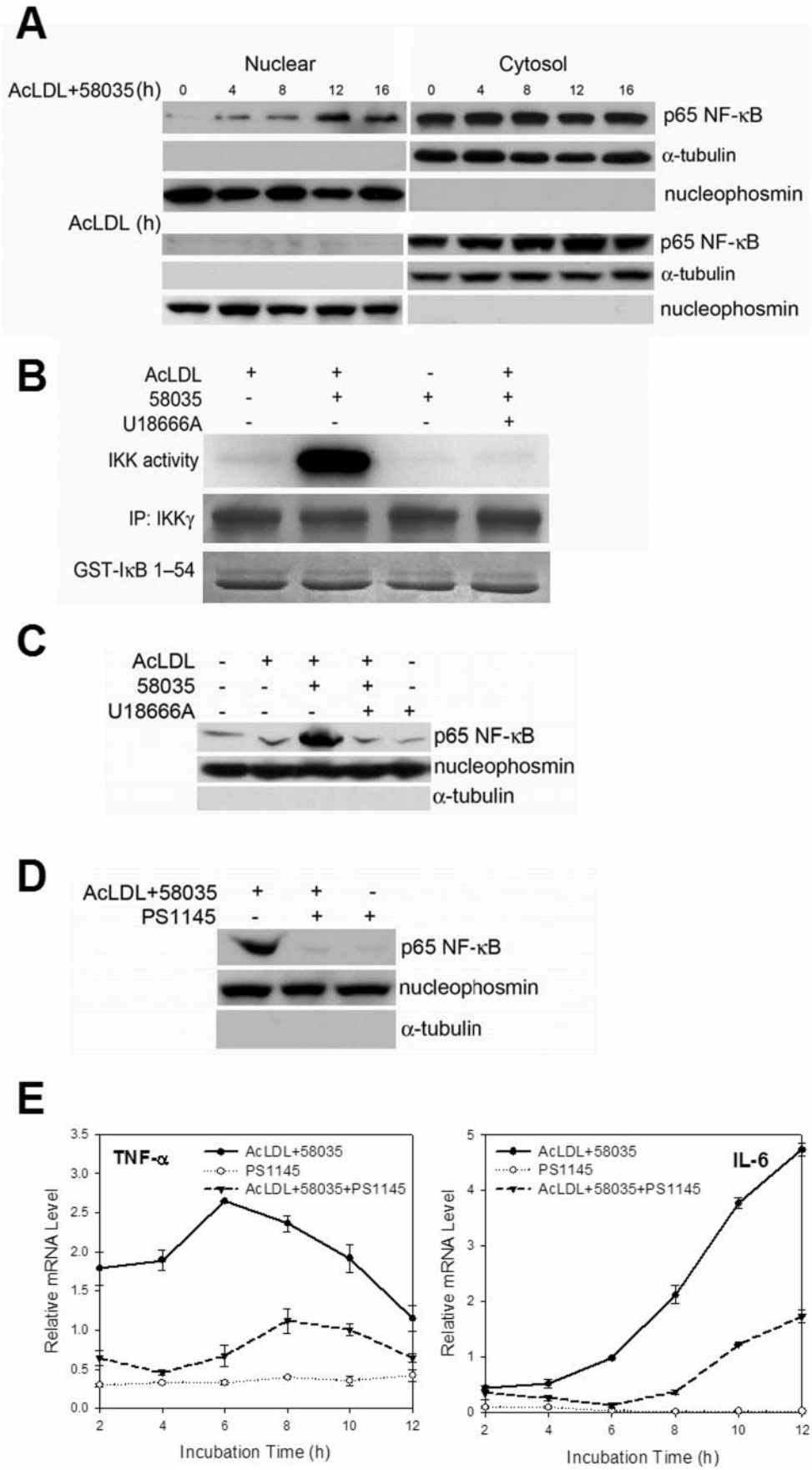

FIG. 4. **FC loading of macrophages activates the IKK/NF-κB pathway, which is required for the full induction of TNF-α and IL-6.** *A*, FC-induced p65 NF-κB nuclear translocation. Macrophages were incubated for various times with acetyl-LDL (*AcLDL*) plus 58035 (FC) or with acetyl-LDL alone. The cells were lysed and subjected to nuclear fractionation. Nuclear (15 μg) and cytosolic (50 μg) fractions were analyzed by SDS-PAGE and immunoblotting for p65 NF-κB, the nuclear marker nucleophasmin, and the cytosolic marker α-tubulin. *B*, FC-induced IKK

air-dried, and then extracted twice with 500 μl of hexane/isopropyl alcohol (3:2, v/v) for 30 min at room temperature. Cholesterol esterification activity was determined by measuring the cellular content of cholesteryl [^{14}C]oleate by thin-layer chromatography. The cells were dissolved in 1 ml of 0.1 N NaOH and assayed for protein by the method of Lowry (26).

Statistics—Data are presented as mean ± S.E. of triplicate experiments unless stated otherwise. Absent error bars in the bar graphs signify S.E. values smaller than the graphic symbols.

RESULTS

FC Loading of Macrophages Induces the Synthesis and Secretion of TNF-α and IL-6—Our recent *in vivo* study showed that macrophages incubated with acetyl-LDL plus the acyl-CoA:cholesterol acyltransferase inhibitor 58035 are a good model of FC-loaded macrophages in advanced atherosclerotic lesions (23). Moreover, the data described below were confirmed using acetyl-LDL-incubated macrophages from acyl-CoA:cholesterol acyltransferase-1-deficient mice (not shown), which was also shown to be relevant for advanced atherosclerosis *in vivo* (27). Using the acetyl-LDL/58035 model, we assayed the mRNA level of a number of cytokines, including TNF-α, IL-1β, IL-4, IL-6, IL-10, IL-12, TGF-β, and MIP-2 in macrophages. FC loading with acetyl-LDL plus 58035 led to a marked increase of TNF-α and IL-6 mRNA (Fig. 1*A*) but did not affect the other cytokines mentioned above. Acetyl-LDL (cholesteryl ester loading) or 58035 alone did not alter the transcription of any of the cytokines. Using enzyme-linked immunosorbent assays, we showed that both TNF-α and IL-6 were detected in the medium of FC-loaded cells but not in the medium of macrophages incubated with either acetyl-LDL or 58035 alone (Fig. 1*B*). This latter observation emphasizes the requirement for FC loading and rules out endotoxin contamination of the reagents as an explanation for cytokine induction. Moreover, in a related study, we demonstrated the biological activity of the secreted TNF-α by showing its ability to induce ABCA1 in neighboring macrophages.[2]

We noticed that FC-induced TNF-α mRNA and protein secretion peaked prior to that of IL-6. Because TNF-α is a known inducer of a number of cytokines including IL-6, we considered the possibility that IL-6 induction was a secondary effect of FC-induced TNF-α release. However, as shown in Fig. 2*A*, IL-6 mRNA was elevated to the same degree in FC-loaded *Tnfa*+/+ and *Tnfa*−/− macrophages. We also showed that IL-6 induction was not diminished by a TNF-α-blocking antibody (Fig. 2*B*). Thus, IL-6 induction is independent of TNF-α secretion in FC-loaded macrophages.

Cholesterol Trafficking to the Endoplasmic Reticulum Is Required for TNF-α and IL-6 Induction—FC-induced macrophage apoptosis is dependent on cholesterol trafficking to the ER, which triggers the ER-based stress pathway known as the UPR (13). Macrophages from mice with a heterozygous mutation in the cholesterol-trafficking protein NPC1 have a specific defect in transporting lipoprotein-derived cholesterol to the ER, and *Npc1*+/− macrophages are resistant to FC-induced UPR and apoptosis (23). Similar results were obtained when FC-loaded macrophages were treated with nanomolar concentrations of the amphipathic amine U18666A, which also selectively blocks cholesterol trafficking to the ER (13). Accordingly, we investigated whether cholesterol trafficking to the ER was also required for TNF-α and IL-6 induction. As shown in Fig. 3, the presence of 70 nM U18666A completely suppressed FC-induced TNF-α and IL-6 mRNA elevations. Similarly, TNF-α and IL-6 mRNA in *Npc1*+/− macrophages remained at basal levels upon FC loading (data not shown). Thus, cholesterol trafficking to the ER is a key event in the induction of TNF-α and IL-6 by FC loading.

FC Loading of Macrophages Activates the NF-κB Pathway, Which Is Required for the Full Induction of TNF-α and IL-6—Activation of the transcription factor NF-κB has been implicated in the transcriptional regulation of TNF-α and IL-6 in a wide variety of systems (28, 29). We therefore determined whether FC loading of macrophages leads to activation of the NF-κB pathway. As shown in Fig. 4*A*, a gradual accumulation of p65 NF-κB in the nuclei was detected in FC-loaded, but not acetyl-LDL-loaded, macrophages over a prolonged period of time. In addition, robust IKK kinase activity was detected (Fig. 4*B*). 70 nM U18666A completely blocked FC-induced IKK activation and p65 NF-κB translocation (Fig. 4, *B* and *C*), indicating that NF-κB activation depended on cholesterol trafficking to the ER. To examine the role of NF-κB activation in TNF-α and IL-6 induction, macrophages were loaded with FC in the absence and presence of a specific IKK inhibitor, PS1145. 10 μM PS1145 completely blocked FC-induced p65 translocation (Fig. 4*D*), and it markedly suppressed FC-induced elevation of TNF-α and IL-6 (Fig. 4*E*). Importantly, cellular cholesterol uptake and trafficking to the ER were not affected by PS1145 (data not shown). Therefore, FC loading of macrophages activates the IKK/NF-κB pathway, which is required for the full induction of TNF-α and IL-6.

FC Loading of Macrophages Leads to Sustained Activation of p38, Erk1/2, and JNK1/2 MAP Kinases, Which Differentially Regulate TNF-α and IL-6 Induction—MAP kinase signaling pathways have been reported to mediate TNF-α and IL-6 in a variety of biological systems, and the transcription factors resulting from these pathways often act in concert with NF-κB transcription factors to induce inflammatory cytokines (30, 31). To test the hypothesis that MAP kinases are involved in TNF-α and/or IL-6 induction in FC-loaded macrophages, we first examined the activation of the three MAP kinase signaling pathways. As shown in Fig. 5*A*, robust and sustained phosphorylation of all three MAP kinases, p38, Erk1/2, and JNK1/2, was observed upon FC loading. There are a number of upstream kinases that may activate p38 kinase, notably MKK3 and MKK6. Fig. 5*B* demonstrates that FC-induced p38 phosphorylation was abolished in MKK3-deficient macrophages, indicating FC-induced p38 activation is mediated through MKK3. 70 nM U18666A completely blocked the phosphorylation of all three MAP kinases (Fig. 5*C*), implying that MAP kinase activation, like apoptosis and NF-κB activation, is dependent on cholesterol trafficking to the ER.

To test the functional significance of p38 activation in cytokine induction, we assayed TNF-α and IL-6 mRNA in *Mkk3*+/+ *versus Mkk3*−/− macrophages. As shown in Fig. 6*A*, the FC-induced TNF-α mRNA level was abolished in *Mkk3*−/−

[2] M. Gerbod-Giannone, Y. Li, A. Holleboom, D. Mangelsdorf, I. Tabas, and A. R. Tall, submitted manuscript.

activation. Macrophages were incubated for 10 h with acetyl-LDL, 58035, or both (FC) ± 70 nM U18666A. Cells were lysed, and IKK kinase activity was measured using GST-IκB-(1–54) as the substrate. *IP*, immunoprecipitate. *C*, FC-induced p65 NF-κB nuclear translocation requires cholesterol trafficking to the ER. Macrophages were incubated for 10 h with acetyl-LDL or acetyl-LDL plus 58035 (FC) ± 70 nM U18666A. The cells were lysed, and nuclear fractions were immunoblotted for p65 NF-κB, nucleophasmin, and α-tubulin. *D*, macrophages were incubated for 10 h under FC-loading conditions ± the IKK inhibitor PS1145 (10 μM) or with the inhibitor alone. The cells were lysed, and nuclear fractions were immunoblotted for p65 NF-κB, nucleophasmin, and α-tubulin. *E*, IKK/NF-κB activation is required for the full induction of TNF-α and IL-6 in FC-loaded macrophages. Macrophages were incubated for various times under FC-loading conditions ± the IKK inhibitor PS1145 (10 μM) or with the inhibitor alone. Total RNA was extracted from the cells, and the mRNA levels of TNF-α and IL-6 were determined by quantitative RT-PCR.

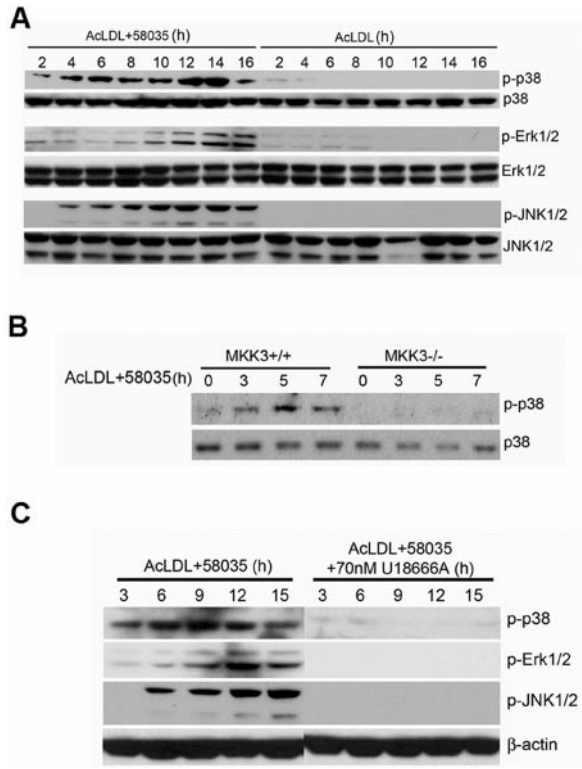

FIG. 5. **FC loading of macrophages leads to sustained activation of p38, Erk1/2, and JNK1/2 MAP kinases.** *A*, FC loading of macrophages induces p38, Erk1/2, and JNK1/2 MAP kinase activation. Macrophages were incubated for various times with acetyl-LDL (*AcLDL*) plus 58035 (FC) or with acetyl-LDL alone. Cell lysates (50 μg) were analyzed by SDS-PAGE and immunoblotting for p38, phosphorylated (*p*-) p38, Erk1/2, phosphorylated Erk1/2, JNK1/2, and phosphorylated JNK1/2. *B*, FC-induced p38 activation requires MKK3. *Mkk3*+/+ and *Mkk3*−/− macrophages were incubated under FC-loading conditions for various times. Cell lysates were analyzed by SDS-PAGE and immunoblotting for p38 and phosphorylated p38. *C*, FC-induced p38, Erk1/2, and JNK1/2 MAP kinase activation depends on cholesterol trafficking to the ER. Macrophages were incubated for various times under FC-loading conditions ± 70 nM U18666As. Cell lysates were analyzed by SDS-PAGE and immunoblotting for phosphorylated p38, phosphorylated Erk1/2, phosphorylated JNK1/2, and β-actin.

macrophages, whereas IL-6 mRNA levels were not affected by MKK3 deficiency. The role of Erk1/2 MAP kinase signaling was assessed using PD98058 and U0126, which are two specific inhibitors of the upstream MAPK kinase activators of Erk1/2 and MEK1/2. As shown in Fig. 6*B*, when the cells were loaded with FC in the presence of 10 μM PD98058 or 2.5 μM U0126, IL-6 induction was abolished, whereas TNF-α was not affected. The role of JNK1/2 MAP kinase signaling in the induction of TNF-α and IL-6 was assessed using a specific inhibitor against JNK1/2, SP600125. Fig. 6*C* shows that when the macrophages were loaded with FC in the presence of 10 μM SP600125, TNF-α induction was suppressed completely, and IL-6 was inhibited by ~50%. At the concentrations used, the MAP kinase inhibitors did not affect cholesterol uptake or trafficking to the ER. To summarize the findings in this and the preceding sections, activation of IKK/NF-κB and JNK1/2 is needed for the full induction of both TNF-α and IL-6, whereas MKK3/p38 signaling is specifically involved in TNF-α induction and Erk1/2 signaling is specifically required for IL-6.

The CHOP Branch of the UPR Pathway Is Required for FC-induced IL-6 Induction and for Full Activation of Erk1/2 MAPK—As mentioned above, FC loading activates the UPR ER stress pathway, resulting in induction of the cell death effector CHOP (13). Because all of the cellular events we observed in this study, the induction of TNF-α and IL-6, IKK/NF-κB activation, and p38, Erk1/2, and JNK1/2 MAP kinase activation, were dependent of cholesterol trafficking to the ER, we questioned whether the CHOP branch of the UPR was implicated in any of these events. Using macrophages from *Chop*−/− mice as the instrument to address this question, we found that TNF-α induction, IKK/NF-κB activation, nor p38 or JNK1/2 activation required CHOP (Fig. 7). On the other hand, IL-6 induction and Erk1/2 activation were diminished in *Chop*−/− macrophages. These results indicate that CHOP induction is upstream of Erk1/2 activation, which, as described above, is required for IL-6 induction. In contrast, activation of IKK/NF-κB, p38, and JNK1/2, which are upstream of TNF-α induction, are not CHOP-dependent despite requiring cholesterol trafficking of the ER. Thus, these pathways are likely activated by a non-CHOP branch of the UPR and/or by a non-UPR ER stress pathway.

DISCUSSION

Previous studies on the biology of FC-loaded macrophages have focused on FC-induced apoptosis in these cells, an event that is likely to be important in the generation of the rupture-prone and clinically important vulnerable plaque (13, 32, 33). In the present study, we show that another important series of events associated with plaque vulnerability, namely, inflammation, is also induced by FC loading prior to apoptosis. Our data show that multiple inflammatory signaling pathways are triggered by FC accumulation and implicated in the induction of TNF-α and/or IL-6. Among the three MAP kinase signaling pathways, JNK1/2 cooperates with MKK3/p38 to induce TNF-α (Fig. 8, *left panel*) and with UPR/Erk1/2 to induce IL-6 (Fig. 8, *right panel*). The IKK/NF-κB pathway is required for both TNF-α and IL-6 induction (Fig. 8).

From a mechanistic viewpoint, these findings raise a number of important issues that will be the subject of future studies. For example, we wish to understand how CHOP leads to Erk1/2 activation in FC-loaded macrophages. One possibility is that CHOP directly activates the Erk1/2 pathway via upstream molecules such as Raf, c-Mos, MEKK1/2, or other Erk1/2 activators. For example, Hu *et al.* (34) showed that the UPR activators thapsigargin or tunicamycin activate Erk1/2 through a pathway involving phosphatidylinositol 3-kinase. In another scenario, which is suggested by the data of Nguyen *et al.* (35), ER stress may promote the repression of an Erk1/2 inhibitor. In that study, ER stress induced by protein-misfolding agents led to the dissociation of the ER-based Erk1/2 inhibitor Nck from the ER and presumably from ER-associated Erk1/2, leading to Erk1/2 activation. However, these studies did not report a direct link between CHOP expression and either activation of phosphatidylinositol 3-kinase or dissociation of Nck.

Another area of interest is the question of why activation of IKK/NF-κB, p38, and JNK1/2 requires cholesterol trafficking to the ER but is normal in *Chop*−/− macrophages. The most likely explanation is that either a non-CHOP branch of the UPR (*e.g.* IRE1-XBP-1) (36) or a non-UPR ER stress pathway is involved. For example, the accumulation of normal or misfolded proteins in the ER triggers the ER-overload response, which signals NF-κB activation (37, 38). There is evidence that the mechanism of this activation involves release of ER calcium stores, which we have shown occurs with FC loading and/or by the generation of reactive oxygen intermediates. Whether this mechanism or others is applicable to FC-loaded macrophages will be the subject of future studies.

In contemplating the results of this study and our prior apoptosis studies, it is intriguing to imagine how the ER stress-related pathways identified herein might be related to ER stress-induced apoptosis in FC-loaded macrophages. For example, Erk activation generally promotes cell survival, even when activated as part of an ER stress response (34). Cell survival is

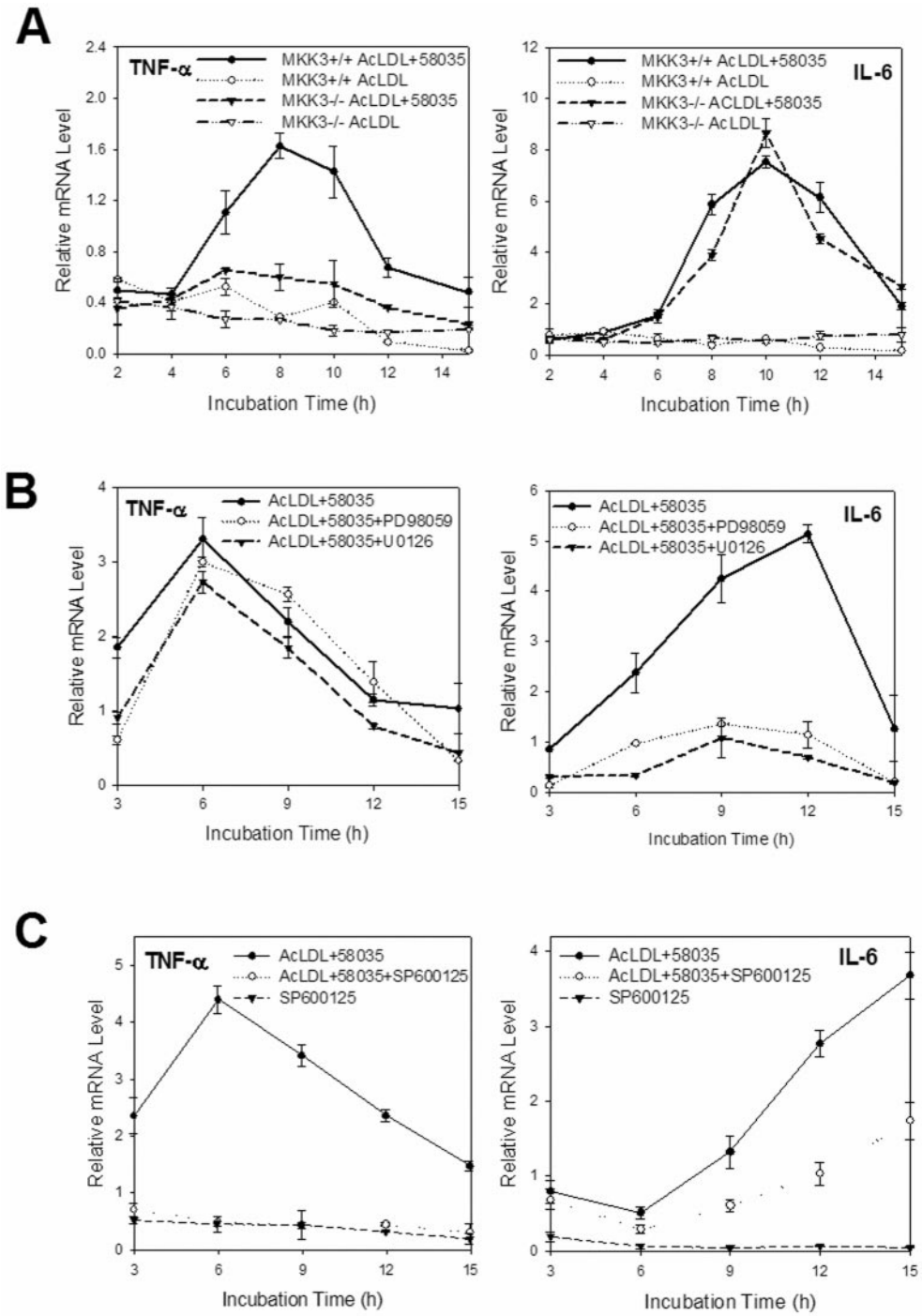

FIG. 6. **MAP kinase signaling pathways differentially regulate TNF-α and IL-6 induction in FC-loaded macrophages.** *A*, MKK3/p38 signaling is required for the FC-induced increase of TNF-α mRNA. *Mkk3*+/+ or *Mkk3*−/− macrophages were incubated for various times with acetyl-LDL (*AcLDL*) plus 58035 (FC) or with acetyl-LDL alone. The mRNA levels of TNF-α and IL-6 were determined by quantitative RT-PCR. *B*, Erk1/2 signaling is required for the FC-induced increase of IL-6 mRNA. Macrophages were incubated under FC-loading conditions ± the Erk1/2 inhibitors PD98059 (10 μM) or U0126 (2.5 μM). The mRNA levels of TNF-α and IL-6 were determined by quantitative RT-PCR. *C*, JNK1/2 signaling is required for the full increase of both TNF-α and IL-6 mRNA. Macrophages were incubated under FC-loading conditions ± the JNK1/2 inhibitor SP600125 (10 μM) or with the inhibitor alone. The mRNA levels of TNF-α and IL-6 were determined by quantitative RT-PCR.

an integral part of UPR induction, presumably to provide an opportunity for repair before UPR-induced apoptosis is triggered (12, 39). Thus, ER-induced Erk activation might tip the balance in favor of survival during this critical period. In a similar manner, NF-κB activation can mediate a potent survival response in cells (40–42). On the other hand, Futami *et al.* (43) recently reported that thapsigargin-induced Erk activation was actually *required* for apoptosis in human colon carcinoma HCT116 cells.

The impetus for this study is the well recognized association between plaque vulnerability and inflammation (14, 15, 17). Indeed, TNF-α and IL-6 are the main proinflammatory cytokines associated with atherosclerosis and other inflammatory diseases (44, 45), and these cytokines are abundantly expressed in atherosclerotic lesions (46, 47). Moreover, Maier *et al.* (48) found that levels of IL-6 in the coronary circulation at the site of plaque rupture in humans were increased the most among a number of other molecules implicated in late lesional

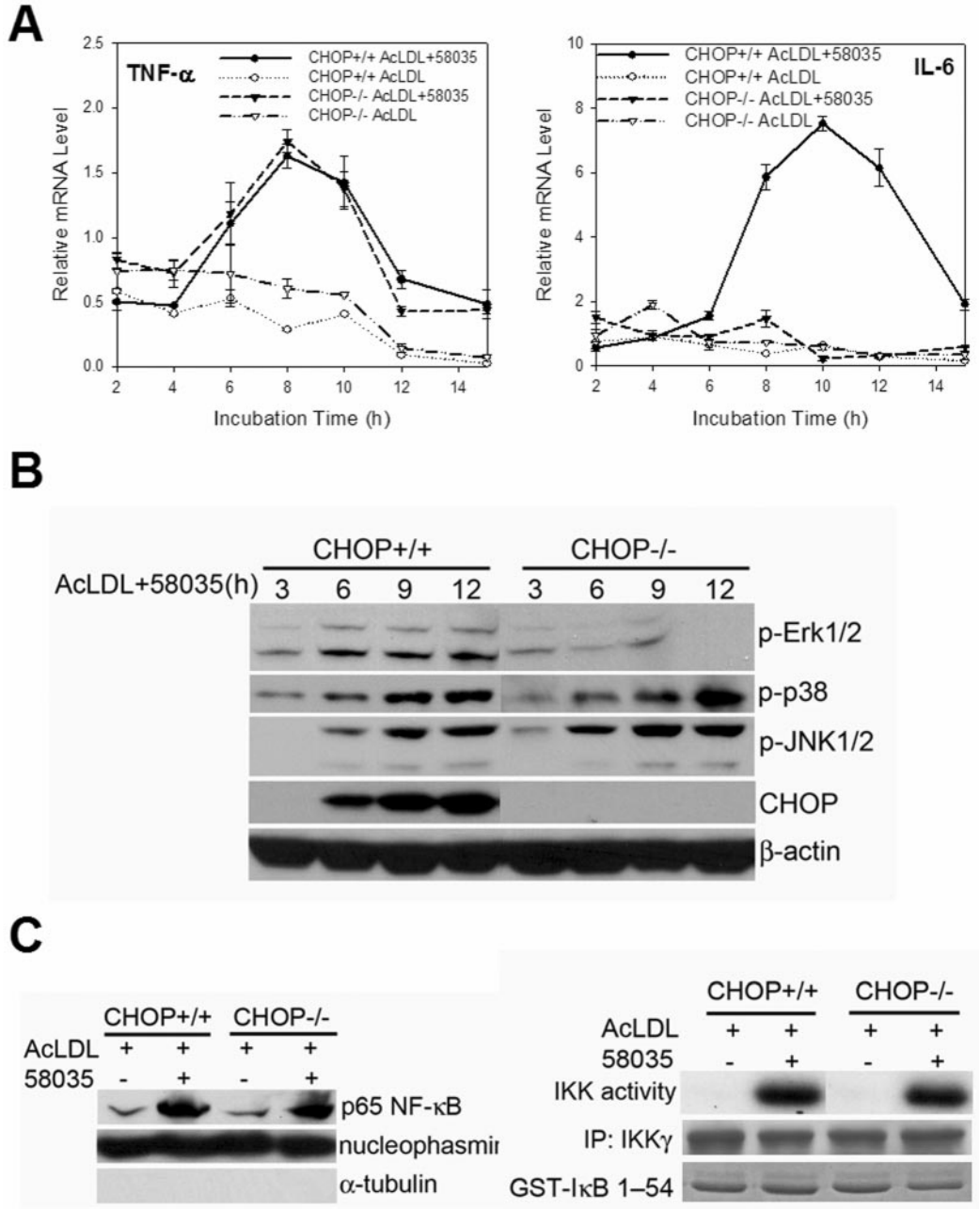

FIG. 7. **The CHOP branch of the UPR pathway is required for FC-induced elevation of IL-6 and for full activation of Erk1/2 MAPK.** *A*, CHOP is required for the FC-induced increase in IL-6 mRNA. *Chop*+/+ or *Chop*−/− macrophages were incubated for various times with acetyl-LDL (*AcLDL*) plus 58035 (FC) or with acetyl-LDL alone. The mRNA level of TNF-α and IL-6 was determined by RT-PCR. *B*, CHOP signaling is involved in FC-induced activation of Erk1/2 but not p38 or JNK1/2. *Chop*+/+ or *Chop*−/− macrophages were incubated for various times under FC-loading conditions, and cell lysates were analyzed by SDS-PAGE and immunoblotting for phosphorylated (*p*-) p38, phosphorylated Erk1/2, phosphorylated JNK1/2, CHOP, and β-actin. *C*, CHOP signaling is not required for FC-induced NF-κB activation. *Chop*+/+ or *Chop*−/− macrophages were incubated for various times with acetyl-LDL plus 58035 (FC) or with acetyl-LDL alone. Nuclear fractions were immunoblotted for p65 NF-κB, nucleophasmin, and α-tubulin. In a parallel experiment, *Chop*+/+ or *Chop*−/− macrophages were incubated with acetyl-LDL plus 58035 (FC) or with acetyl-LDL alone for 10 h, and cell lysates were assayed for IKK activity.

events. TNF-α plays a major role in the recruitment and activation of inflammatory cells and stimulation of matrix metalloproteinase production, which promotes plaque rupture (49, 50). Circulating IL-6 is the central mediator of the acute phase response (51) and the primary inducer of C-reactive protein production in liver, which, along with TNF-α and IL-6, has been associated with coronary artery disease risk in a number of studies (52–55). Thus, TNF-α and IL-6 have been speculated to be proatherogenic based on their inflammatory activities. In favor of this hypothesis is the finding that *Apoe*−/− mice in which TNF-α was inhibited by chronic administration of soluble TNF-α receptor showed a dramatic reduction in lesion size (56). In another study with more specific relevance to the data herein, TNF-α deficiency in APOE*3-Leiden mice was associated specifically with less advanced lesions (57). Regarding IL-6, administration of supraphysiologic concentrations of exogenous IL-6 in *Apoe*−/− mice greatly exacerbated atherosclerotic lesion formation (58). However, other *in vivo* studies have reported results suggestive of a protective effect or no effect on aortic root lesion size in *Apoe*−/− or *Ldlr*−/− mice (51, 56, 59–61). The difficulty in interpreting these studies *vis à vis* the role of macrophage-secreted cytokines in advanced atherosclerotic lesions lies in several areas. First, TNF-α and IL-6 are secreted by a number of immunocompetent cell types in addi-

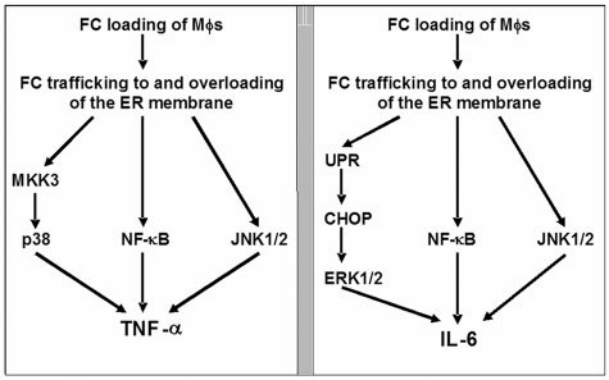

FIG. 8. **Model of inflammatory cascades induced by FC loading of macrophages.** The key upstream event for increases in TNF-α and IL-6 mRNA and secreted protein is FC loading and trafficking to the ER. This event leads to the activation of four signaling pathways. Two of these pathways, IKK/NF-κB and JNK1/2, are involved in the increase in mRNA levels of both cytokines (*both panels*). One the four pathways, MKK3 → p38, is specifically involved in the increase in TNF-α mRNA (*left panel*), and another of the pathways, UPR → CHOP → Erk1/2, is specifically involved in the increase in IL-6 mRNA (*right panel*). See "Results" for details. Preliminary data indicate that NF-κB and MAPK-mediated regulation of TNF-α and IL-6 mRNA levels in FC-loaded macrophages is accompanied by the predicted changes in secreted cytokine levels, but Erk1/2 may have an additional role in FC-induced secretion of TNF-α via a translational or posttranslational mechanism (see "Discussion").

tion to macrophages (62, 63), and therefore the effects of globally absent cytokine secretion in knock-out models may overshadow macrophage-specific and stimuli-specific functions. Second, elimination of a multifunctional gene at the beginning of embryonic development may trigger adaptive and compensatory responses in the animals. Third, with the exception of the study of Boesten *et al.* (57), very few of the studies have conducted a careful analysis of advanced atherosclerotic plaques. Indeed, if TNF-α is protective specifically in early foam cell lesions, it may be related to our recent finding that the TNF-α secreted from FC-loaded macrophages can induce ABCA1 and ABCA1-mediated cholesterol efflux in neighboring macrophages.[2] Thus, the most informative studies on the roles of TNF-α and IL-6 on plaque vulnerability will come from future models in which macrophage-specific cytokines can be targeted in an inducible manner, and the analysis of advanced lesions is included in the study.

Although FC loading is associated with an increase in both mRNA and secreted protein levels of TNF-α and IL-6 (Fig. 1), the mechanistic studies in this paper have focused only on the steady-state mRNA levels of the two cytokines. In future studies, the mechanism of mRNA regulation, *i.e.* transcriptional *versus* mRNA stability, and the possible roles of translational and posttranslational control will be considered. For example, a number of studies have shown that the p38/MAPKAP kinase 2 (MK2) pathway regulates the AU-rich element-dependent degradation of TNF-α and IL-6 transcripts (64–66). Regarding translational control, MHC class II ligands have been shown to increase the translation of TNF-α transcripts in primary human monocytes (67). Finally, the secretion of soluble TNF-α is dependent on ectodomain cleavage of transmembrane TNF-α, a "shedding" process controlled by TNF-α converting enzyme. Signaling through the ERK pathway has been shown to regulate the activation of the TNF-α converting enzyme (68–70). In preliminary studies in our laboratory, we found that most of the manipulations that inhibit TNF-α and/or IL-6 transcripts, including U18666A, the IKK inhibitor PS1145, and the JNK inhibitor SP600125, also inhibit cytokine protein secretion. However, although secreted TNF-α and IL-6 protein levels were both inhibited by the ERK inhibitors PD98059 and U0126, these inhibitors did not block the FC-induced increase in TNF-α mRNA. These preliminary data suggest the ERK signaling pathway is involved in the posttranscriptional regulation of TNF-α, which might occur through the ERK-TNF-α converting enzyme pathway mentioned above (68–70). Elucidating the role of posttranscriptional regulation of proinflammatory cytokines in FC-loaded macrophages will be an important goal of future investigations.

In summary, the data herein and in our previous studies provide new molecularly based mechanistic links between three important properties of vulnerable plaques, FC accumulation, inflammation, and macrophage death. Most other models of inflammation and apoptosis involve *exogenous* mediators acting on cell surface receptors (1, 17, 71). In contrast, the models developed here and in our previous studies provide a model in which an *endogenous* biophysical perturbation, FC-induced stiffening of the normally fluid ER membrane, is the common precipitating event (10). What is most important about this concept is that it implies specificity for the unique environment of the advanced atherosclerotic plaque, *i.e.* FC overload. This specificity affords the opportunity for therapeutic strategies that have a high benefit:risk ratio, because general inhibition of inflammation and apoptosis would have detrimental effects on host defense and other physiologic processes. In this context, we have previously shown that inhibition of cholesterol trafficking to the ER markedly and selectively diminishes macrophage apoptosis and lesional necrosis in advanced atherosclerotic plaques. In view of the data in the current report, future work will extend these studies to advanced lesional inflammation.

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