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

Yankun Li†, Robert F. Schwabe‡, Tracie DeVries-Seimon†, Pin Mei Yao‡, Marie-Christine Gerbod-Giannone‡, Alan R. Tall‡, Roger J. Davis§, Richard Flavell¶, David A. Brenner¶, and Ira Tabas‡**

From the Departments of †Medicine and ‡Anatomy and Cell Biology and Physiology and Cellular Biophysics, Columbia University, New York, New York 10032, the §Howard Hughes Medical Institute and Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605, and the ¶Section of Immunobiology, Yale University School of Medicine and Howard Hughes Medical Institute, New Haven, Connecticut 06520

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) 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 1744 solely to indicate this fact.
** To whom correspondence should be addressed: Dept. of Medicine, Columbia University, 630 West 168th St., New York, NY 10032. Tel.: 212-305-9430; Fax: 212-305-4834; E-mail: iat1@columbia.edu.

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 NH2-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.
**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-[decyl(dimethyl)silyl]-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 (Me2SO) and stored at −20 °C. U18666A ([3β-[2-diethylaminoethoxy]androst-5-en-17-one hydrochloride) was from BioSource International, Inc. PS1145 (3-[decyldimethylsilyl]-N-(3-carboxy-2-thienyl)propylaminothio)butadiene) were from EMD Biosciences. SP600125 (anthra[1,9-b:2,3-b]pyrazol-6(2H)-one) was from BioSource International, Inc. PD98059 (2-(2-aminoethoxy) androst-5-en-17-one hydrochloride) was from Millennium Pharmaceuticals (21).

**Antibodies**—Antibodies against p38, phosphor-p38, Erk1/2, phosphor-Erk1/2, Nck1/2, phosphor-Nck1/2, and p65 NF-κB were from Cell Signaling Technology. Anti-nucleophosmin was from Zymed Laboratories Inc., and anti-β-tubulin, and anti-β-actin were from Santa Cruz Biotechnologies, Inc.

**Mice**—Female mice, 8–10 weeks of age, were used in this study. C57BL/6J mice, Tphα−/− mice on the B6129S background, and B6129SF2 control mice were from Jackson Laboratories. Chop−/− mice on the C57BL/6 background were from Drs. David Ron (New York University) and Robert Burke (Columbia University). Mkh3−/− 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 Freunds adjuvant (Difco). Mice were immunized intradermally with 100 μl of emulsion. 14 days later, the immunization protocol was repeated except incomplete Freunds adjuvant was used instead of complete Freunds adjuvant. 7 days later, the mice were injected intraperitoneal 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 μl 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 CGGAGTGCGGCGAGGT and GCTGGGTAGAATGATGAAACA, respectively, and the probe was 6FAM-CTTTCGAGATGGATGAAATG. The forward and reverse primers for Npc1−/− were CGGAGTCCGGCGAGGT and GCTGGGTAGAATGATGAAACA, respectively, and the probe was 6FAM-CTTTCGAGATGGATGAAATG.
phages were incubated for various times under FC-loading conditions 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.

primers for IL-6 were GAGGATACCCTCCCAACAGACC and AAGTGCATCATCGTTGTCATCA, respectively, and the probe was 6FAM-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, and 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 5 min, and the supernatant was collected (cytoplasmic fraction). 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 IKK.

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-IKKα 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 IKK.

Whole-cell Cholesterol Esterification Assay—Macrophages were incubated in Dulbecco’s modified Eagle’s medium and 0.2% BSA containing 0.1 mM [14C]oleate complexed with albumin and 100 μg/ml acetyl-LDL for 5 h. The cells were washed twice with phosphate-buffered saline, and IL-6 mRNA levels were measured.
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 nucleophosmin, and the cytosolic marker α-tubulin.

B, FC-induced IKK activity.

C, FC-induced p85 NF-κB phosphorylation.

D, Effects of PS1145 on FC-induced p65 NF-κB phosphorylation.

E, Relative mRNA levels of TNF-α and IL-6 with different incubation times.
showed to be relevant for advanced atherosclerosis acyltransferase-1-deficient mice (not shown), which was also acetyl-LDL-incubated macrophages from acyl-CoA:cholesterol that macrophages incubated with acetyl-LDL plus the acyl-C- was measured using GST-I

eysignify S.E. values smaller than the graphic symbols.

ments unless stated otherwise. Absent error bars in the bar graphs

 USING the acetyl-LDL/58035 model, we assayed the mRNA

and rules out endotoxin contamination of the reagents as an

of FC-loaded cells but not in the medium of macrophages incubated with either acetyl-LDL or 58035 alone (Fig. 1B). 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.

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. 2A, IL-6 mRNA was elevated to the same degree in FC-loaded αNF-α/+ and αNF-α−/− macrophages. We also showed that IL-6 induction was not diminished by a TNF-α-blocking antibody (Fig. 2B). 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 macrophase 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. 4A, 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. 4B). 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. 4D), and it markedly suppressed FC-induced elevation of TNF-α and IL-6 (Fig. 4E). 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. 5A, 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. 5B 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. 5C), 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. 6A, the FC-induced TNF-α mRNA level was abolished in Mkk3−/− macrophages.

2 M. Gerhod-Giannone, Y. Li, A. Holleboom, D. Mangelsdorf, I. Tabas, and A. R. Tall, submitted manuscript.
TNF-\(\alpha\) and IL-6 Induction in FC-loaded Macrophages

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. 6B, when the cells were loaded with FC in the presence of 10 \(\mu\)M PD98058 or 2.5 \(\mu\)M U0126, IL-6 induction was abolished, whereas TNF-\(\alpha\) was not affected. The role of JNK1/2 MAP kinase signaling in the induction of TNF-\(\alpha\) and IL-6 was assessed using a specific inhibitor against JNK1/2, SP600125. Fig. 6C shows that when the macrophages were loaded with FC in the presence of 10 \(\mu\)M SP600125, TNF-\(\alpha\) 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-\(\kappa\)B and JNK1/2 is needed for the full induction of both TNF-\(\alpha\) and IL-6, whereas MKK3/p38 signaling is specifically involved in TNF-\(\alpha\) 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-\(\alpha\) and IL-6, IKK/NF-\(\kappa\)B 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-\(\alpha\) induction, IKK/NF-\(\kappa\)B 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-\(\kappa\)B, p38, and JNK1/2, which are upstream of TNF-\(\alpha\) 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-\(\alpha\) and/or IL-6. Among the three MAP kinase signaling pathways, JNK1/2 cooperates with MKK3/p38 to induce TNF-\(\alpha\) (Fig. 8, right panel) and with UPR/Erk1/2 to induce IL-6 (Fig. 8, right panel). The IKK/NF-\(\kappa\)B pathway is required for both TNF-\(\alpha\) 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-\(\kappa\)B, 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-\(XBP-1\)) 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-\(\kappa\)B 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
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. 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.
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-

![The CHOP branch of the UPR pathway is required for FC-induced elevation of IL-6 and for full activation of Erk1/2 MAPK.](http://www.jbc.org/)

**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, nucleophosmin, 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.
TNF-α and IL-6 Induction in FC-loaded Macrophages

**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 JNK, are involved in the increase in mRNA levels of both cytokines (both panels). One of the pathways, MKK3 \( \rightarrow p38 \), is specifically involved in the increase in TNF-α mRNA (left panel), and another of the pathways, UPR \( \rightarrow CHOP \rightarrow Erk1/2 \), 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”).

| REFERENCES |
|----------------|
| 1. Linton, M. F., and Fazio, S. (2003) Int. J. Obes. Relat. Metab. Disord. 27, Suppl. 3, 35–40 |
| 2. Ross, R. (1999) *N. Engl. J. Med.* 340, 115–126 |
| 3. Steinberg, D. (2002) *Nat. Med.* 8, 1211–1217 |
| 4. Ross, R. (1995) *Annu. Rev. Physiol.* 57, 791–804 |
| 5. Rapp, J. H., Connor, W. E., Lin, D. S., Inahara, T., and Porter, J. M. (1983) *J. Biol. Chem.* 258, 1329–1335 |
| 6. Small, D. M., Bond, M. G., Waugh, D., Prack, M., and Sawyer, J. K. (1984) *J. Clin. Invest.* 73, 1590–1605 |
| 7. Kruth, H. S. (1984) *Am. J. Pathol.* 114, 201–208 |
| 8. Tabas, I. (2002) *J. Clin. Invest.* 110, 905–911 |
| 9. Zhou, J., Chew, M., Ravn, H. B., and Falk, E. (1999) *Scand. J. Clin. Lab. Invest.* Suppl. 229, 3–11 |
| 10. L. Y., Gm, C., Guan, L., Kuriakose, G., Restor, E. J., Dura, M., Covey, D. F., Freed, J. H., Maxfield, F. R., Lytton, J., and Tabas, I. (2004) *J. Biol. Chem.* 279, 37030–37039 |
| 11. Harding, H. P., Calvin, M., Urasa, F., Novosa, I., and Ron, D. (2002) *Annu. Rev. Cell Dev. Biol.* 18, 575–599 |
| 12. Schroder, M., and Kaufman, R. J. (2005) *Mutat. Res.* 569, 29–63 |
| 13. Feng, B., Yao, F. M., Li, Y., Devlin, C. M., Zhang, D., Harding, H. P., Sweeney, M., Kong, J. X., Kuriakose, G., Fisher, E. A., Marks, A. R., Ron, D., and Tabas, I. (2003) *Cell Biol.* 5, 381–392 |
| 14. Aikawa, M., and Libby, P. (2004) *Circ. Res.* 94, 361–364 |
| 15. Fan, J., and Watanabe, T. (2005) *J. Atheroscler. Thromb.* 10, 63–71 |
| 16. Raine, E. S., and Ross, R. (1996) *BioEssays* 18, 271–282 |
| 17. Steffens, S., and Mach, F. (2004) *Herz* 29, 741–748 |
| 18. Hoss, K. J., Edel, H., and Bragdon, J. (1955) *J. Clin. Invest.* 34, 1345–1353 |
| 19. Bost, S. K., Goldstein, J. L., Anderson, R. G. W., and Brown, M. S. (1976) *Proc. Natl. Acad. Sci. U. S. A.* 73, 3178–3182 |
| 20. Ross, A. C., Go, K. J., Heider, J. G., and Rothblat, G. H. (1984) *J. Biol. Chem.* 259, 815–819 |
| 21. Hideshima, T., Chauhan, D., Richardson, P., Mitsiades, C., Mitsiades, N., Hayashi, T., Munashi, N., Dang, L., Castro, A., Palombella, V., Adams, J., and Anderson, K. C. (2002) *J. Biol. Chem.* 277, 10639–10647 |
| 22. Lu, H. T., Yang, D. D., Wys, M., Gatti, K., Mellem, I., Davis, R. J., and Flavell, R. A. (1999) *EMBO J.* 18, 1845–1857 |
| 23. Feng, B., Zhang, D., Kuriakose, G., Devlin, C. M., Kocks, M., and Tabas, I. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 12834–12839 |
| 24. Cook, A. D., Braine, E. L., and Hamilton, J. A. (2003) *J. Immunol.* 171, 4816–4823 |
| 25. Schwarz, P. E., Bennett, B. L., Manning, A. M., and Brenner, D. A. (2001) *Hepatology* 33, 81–90 |
| 26. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, J. R. (1951) *J. Biol. Chem.* 193, 265–275 |
| 27. Fazio, S., Major, A. S., Swift, L. L., Gleave, I. A., Accad, M., Linton, M. F., and Fearce, R. V., Jr. (2001) *J. Clin. Invest.* 107, 163–171 |
| 28. Azzolina, A., Bongiovanni, A., and Lampiasi, N. (2003) *Biochim. Biophys. Acta* 1643, 75–83 |
29. Jeong, H. J., Koo, H. N., Na, H. J., Kim, M. S., Hong, S. H., Eom, J. W., Kim, K. S., Shin, T. Y., and Kim, H. M. (2002) Cytokine 18, 252–259
30. Jiang, J. X., Zhang, Y., Ji, S. H., Zhu, P., and Wang, Z. G. (2002) Shock 18, 336–341
31. Kim, S. H., Kim, J., and Sharma, R. P. (2004) Pharmaco. Res. 49, 433–439
32. Yao, P. M., and Tabas, I. (2000) J. Biol. Chem. 275, 23807–23813
33. Yao, P. M., and Tabas, I. (2001) J. Biol. Chem. 276, 42468–42476
34. Hu, P., Han, Z., Couvillon, A. D., and Exton, J. H. (2004) J. Biol. Chem. 279, 49420–49429
35. Nguyen, D. T., Kebache, S., Fazel, A., Wong, H. N., Jenna, S., Emadali, A., Lee, E. H., Bergeron, J. J. M., Kaufman, R. J., Larose, L., and Chevet, E. (2004) Mol. Biol. Cell 15, 4248–4260
36. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001) Trends Biochem. Sci. 26, 63–67
37. Ron, D. (2002) J. Clin. Investig. 110, 1383–1388
38. Chen, C., Edelstein, L. C., and Gelin, C. (2000) J. Biol. Chem. 275, 2687–2695
39. Kucharczak, J., Simmons, M. J., Fan, Y., and Gelinas, C. (2004) Oncogene 23, 8858
40. Papanicolaou, D. A., Wilder, R. L., Manolagas, S. C., and Chrousos, G. P. (1998) Ann. Intern. Med. 128, 127–137
41. Pahl, H. L., Sester, M., Burgert, H. G., and Baeuerle, P. A. (1997) Trends Biochem. Sci. 22, 63–67
42. Kishimoto, T. (1989) J. Biol. Chem. 264, 1498–1505
43. Maier, W., Altwegg, L. A., Corti, R., Gay, S., Tanner, F. C., Eberli, F. R., Von Eckardstein, A., and Lascher, T. F. (2004) Eur. J. Heart J. 25, 450
44. Fishbein, M. C., Kaul, S., Cereb, B., Sharifi, B., and Shah, P. K. (1999) Circulation 99, 3103–3109
45. Schleef, R. R., Bevilacqua, M. P., Sawdey, M., Gimbrone, M. A., Jr., and Sokoloff, D. J. (1988) J. Biol. Chem. 263, 5797–5803
46. Fan, H., Turck, C. W., and Derynck, R. (2003) J. Biol. Chem. 278, 3493–3500
47. Fan, H., and Derynck, R. (1999) Eur. J. Immunol. 29, 2417–2424
48. Diaz-Rodriguez, E., Montero, J. C., Esparis-Ogando, A., Yuste, L., and Paniagua, A. (2002) Mol. Biol. Cell 13, 2031–2044
49. Scherer, S. F., Lauter, C. A., and Schindler, C. (2001) Curr. Opin. Lipidol. 12, 535–541
50. Cesari, M., Penninx, B. W., Newnman, A. B., Kritchevsky, S. B., Nicklas, B. J., Sutton-Tyrrell, K., Rubin, S. M., Ding, J., Simonsick, E. M., Harris, T. B., and Pahor, M. (2003) Circulation 108, 2517–2322
51. Cesari, M., Penninx, B. W. J. H., Newman, A. B., Kritchevsky, S. B., Nicklas, B. J., Sutton-Tyrrell, K., Tracy, R. P., Rubin, S. M., Harris, T. B., and Pahor, M. (2003) Am. J. Cardiol. 92, 522–528
52. Lind, L. (2003) Atherosclerosis 169, 203–214
53. Willerson, J. T., and Ridker, P. M. (2004) Circulation 109, II-2
54. Ron, D. (2002) J. Clin. Investig. 110, 1383–1388
55. Willerson, J. T., and Ridker, P. M. (2004) Atherosclerosis 169, 203–214
56. Schieffer, B., Selle, T., Hilfiker, A., Hilfiker-Kleiner, D., Grote, K., Tietge, U. J. F., Trautwein, C., Luchtefeld, M., Schmittkamp, C., Heeseman, J. W., and Brenner, D. A. (1998) J. Clin. Investig. 101, 802–811
57. Boesten, L. S., Zadelaar, A., van Nieuwkoop, A., Gijbels, M. J., de Winther, M. P., Havelkes, L. M., and van Vlijmen, B. J. (2005) Cardiovasc. Res. 66, 179–185
58. Huber, S. A., Nakatani, H., Hardin, N., and Tracy, R. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 2364–2367
59. Ron, D. (2002) J. Clin. Investig. 110, 1383–1388
60. Schieffer, B., Selle, T., Hilfiker, A., Hilfiker-Kleiner, D., Grote, K., Tietge, U. J. F., Trautwein, C., Luchtefeld, M., Schmittkamp, C., Heeseman, J. W., and Brenner, D. A. (1998) J. Clin. Investig. 101, 802–811
61. Iimuro, Y., Nishiura, T., Hellerbrand, C., Behrns, K. E., Schoonhoven, R., Grisham, J. W., and Brenner, D. A. (1998) J. Clin. Investig. 101, 802–811
62. Pahl, H. L., and Baeuerle, P. A. (1997) Trends Biochem. Sci. 22, 63–67
63. Fishbein, M. C., Kaul, S., Cereb, B., Sharifi, B., and Shah, P. K. (1999) Circulation 99, 3103–3109
64. Brown, M., Sully, G., Clark, A. R., and Saklatvala, J. (2000) FEBS Lett. 483, 57–61
65. Kotsyurbyov, A., and Gaestel, M. (2002) Biochem. Soc. Trans. 30, 959–963
66. Neinery, A., Kontoyiannis, D., Kotsyurbyov, A., Winzen, R., Eckert, R., Volk, H. D., Hollmann, H., Kollia, G., and Gaestel, M. (2002) J. Biol. Chem. 277, 3065–3068
67. Espel, E., Garcia-Sanz, J. A., Aubert, V., Menoud, Y., Sperisen, P., Fernandez-Diella, A. (2002) Eur. J. Immunol. 32, 2417–2424
68. Diaz-Rodriguez, E., Montoro, J. C., Estavess-Ogando, A., Yuste, L., and Paniagua, A. (2002) Mol. Biol. Cell 13, 2031–2044
69. Fan, H., Turck, C. W., and Derynck, R. (2003) J. Biol. Chem. 278, 18617–18627
70. Fan, H., and Derynck, R. (1999) EMBO J. 18, 6962–6972
71. Martinet, W., and Kockx, M. (2001) Curr. Opin. Lipidol. 12, 535–541

21772

TNF-α and IL-6 induction in FC-loaded macrophages
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

Yankun Li, Robert F. Schwabe, Tracie DeVries-Seimon, Pin Mei Yao, Marie-Christine Gerbod-Giannone, Alan R. Tall, Roger J. Davis, Richard Flavell, David A. Brenner and Ira Tabas

J. Biol. Chem. 2005, 280:21763-21772.
doi: 10.1074/jbc.M501759200 originally published online April 11, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M501759200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 71 references, 29 of which can be accessed free at http://www.jbc.org/content/280/23/21763.full.html#ref-list-1