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Identification and Characterization of *Chlamydia pneumoniae*-Specific Proteins That Activate Tumor Necrosis Factor Alpha Production in RAW 264.7 Murine Macrophages[▽]

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***Chlamydia pneumoniae* is a common respiratory pathogen, which activates macrophages to induce inflammatory cytokines that may promote atherosclerosis. However, the antigens that induce macrophage activation have not been well defined. In the current study, three chlamydial proteins which are recognized during human infection, outer membrane protein 2 (OMP2) and two 53-kDa proteins (Cpn 0980 and Cpn 0809), were investigated to determine whether they activate macrophages and, if they do, what mechanism they use for this activation. It was shown that these three proteins could (i) induce expression of tumor necrosis factor alpha (TNF-α) and tissue factor and (ii) induce phosphorylation of p44/42 mitogen-activated protein kinases (MAPK) and activation of early growth response factor 1 (Egr-1). Control proteins, the N-terminal fragment of polymorphic membrane protein 8 and the thioredoxin portion of the fusion protein, had no effect on macrophages. Treatment of cells with a MEK1/2 inhibitor, U0126, dramatically reduced the phosphorylation of ERK, activation of Egr-1, and expression of TNF-α in macrophages treated with recombinant proteins. Toll-like receptors (TLRs) act as sensors for microbial antigens and can signal via the MAPK pathway. Chlamydial protein-induced expression of TNF-α was significantly reduced in macrophages lacking TLR2 or TLR4. These findings suggest that *C. pneumoniae* may activate macrophages through OMP2, Cpn 0980, and Cpn 0809 in addition to cHSP60 and that activation occurs via TLR2 or TLR4, Egr-1, and MAPK pathways.**

Atherosclerosis is thought to result from a protective, inflammatory fibroproliferative response against chronic injury over a long period of time (43). The earliest stages of atherosclerosis are characterized by invasion of the intima by mononuclear phagocytes (44). Several lines of evidence indicate that *Chlamydia pneumoniae*, an obligate intracellular bacterium associated with respiratory tract infection, may play a role in progression of atherosclerosis (9, 47). An association between anti-*C. pneumoniae* antibodies and cardiovascular disease was found in several seroepidemiological studies (13, 46, 50). Recent clinical studies have suggested that infection with *C. pneumoniae* is associated with increased plaque instability and thrombogenesis during acute coronary events (14, 52). Although *C. pneumoniae* has been detected in smooth muscle cells and endothelial cells in atheroma (28a, 28b), it is localized mainly in macrophage-derived foam cells (28b). In vitro studies have shown that *C. pneumoniae* can induce macrophage foam cell formation (22, 23), and in vivo studies have shown that *C. pneumoniae* infection accelerates lesion progression in animal models (5, 8). It has been hypothesized that *C. pneumoniae* infection of macrophages is a key factor for dissemination to the vasculature, establishment of persistent infection in the atheroma, and exacerbation of lesion progression.

It has been reported that *C. pneumoniae* infection of monocytes/macrophages triggers the secretion of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) (19, 24), and production of tissue factor (TF) (4). TNF-α is found in atherosclerotic plaques and can induce a number of vascular cell functions relevant to atherogenesis (36). TNF-α is thought to play an important role in the inflammatory processes involved in atherogenesis (42). TF, a potent prothrombotic molecule, is thought to play an important role in stimulating thrombus formation after plaque rupture (4). The mechanisms by which *C. pneumoniae* activates monocytes to produce cytokines and TF have been investigated. *C. pneumoniae* has been shown to activate dendritic cells via NF-κB and Toll-like receptor 2 (TLR2)/TLR4-dependent pathways to express cytokines (38). However, Netea et al. reported that only the TLR2-dependent pathway stimulates cytokine production by sonicated *C. pneumoniae* (32). In vivo studies also showed that TLR2 and TLR4 are involved in *C. pneumoniae*-induced cytokine release (12, 40). *C. pneumoniae* induction of TF expression in mouse macrophages is mediated in part by early growth response factor 1 (Egr-1), signaling through TLR4, and activation of the MEK-ERK1/2 pathway. Egr-1 is a nuclear transcription factor and plays a role in the regulation of the expression of growth factors such as platelet-derived growth factor, cytokines such as TNF-α, and adhesion molecules such as ICAM. Thus, *C. pneumoniae* signaling through a TLR activates expression of chemokines and cytokines by macrophages that are key components of atherosclerosis.

However, the specific antigens of *C. pneumoniae* that mediate macrophage activation have not been well defined. Recent studies have shown only that cHSP60 can activate macrophages through the TLR4 and NF-κB pathway (7). To determine whether other relevant antigens also activate macrophages, three chlamydial proteins, outer membrane protein 2 (OMP2) and two 53-kDa proteins (Cpn 0980 and Cpn 0809),

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TABLE 1. Primers used for amplification of recombinant proteins

Protein	Forward primer (5′→3′)	Reverse primer (5′→3′)
OMP2	CGGATCCACATGTCCA AACTC	CAAGCTTGGATACACG TGGGT
Cpn 0980	ATGCTTAATCATGCTA AAAAG	CTCTTTTATTTTAGGA AGCTT
Cpn 0809	GGGAATTCGTCTATTT CATCTTC	GGGAATTCTGATTGCG GCATACG
PMP 8N	AGCATTGCAACTTACG GAGC	AGCTGTGTTCCCGCAT CTAT

that are recognized frequently during human infection (10, 35, 55) were investigated. The 53-kDa proteins appear to be putative outer membrane proteins (16). OMP2 is a cysteine-rich protein and a major component of the chlamydial cell wall. Although OMP2 is poorly surface accessible to antibody binding in intact cells (58), pronounced antibody responses to OMP2 occur following *C. pneumoniae* infection (34, 57). Therefore, in the current study, whether these antigens stimulate macrophage functions relevant to atherosclerosis and, if they do, what mechanism they use for stimulation were investigated.

MATERIALS AND METHODS

Cell culture. RAW 264.7 cells, a murine macrophage cell line (ATCC, Manassas, VA), were grown in Dulbecco modified Eagle medium (Gibco BRL, Langley, OK) containing 10% fetal bovine serum, 100 U/ml vancomycin, and 100 μg/ml streptomycin.

Isolation of murine peritoneal macrophages. To harvest peritoneal macrophages, mice were euthanized by cervical dislocation, and the peritoneal cavities were washed three times with 10 ml of macrophage culture medium. Peritoneal macrophages were plated into six-well culture plates containing coverslips at a concentration of 1×10^6 macrophages/well, which yielded 90 to 100% confluence. After overnight incubation, nonadherent cells were removed by washing with Hanks balanced salt solution. Adherent cells were cultured for 3 days with fresh macrophage culture medium.

Cloning and expression of the chlamydial protein genes and gene fragments. *C. pneumoniae* DNA was purified by using a standard protocol (49). The complete OMP2 and Cpn 0980 genes were amplified, while N-terminal fragments of the Cpn 0809 and polymorphic membrane protein 8 (PMP 8N) genes were amplified. The forward and reverse primers used are shown in Table 1. Cpn 0980 and Cpn 0809 recombinant protein genes were cloned and expressed as described previously (10). The remaining amplified products (OMP2 and PMP 8N genes) were ligated directly into the pBAD/Topo/Thio vector (Invitrogen, Carlsbad, CA) by TA cloning and expressed by using the Topo/Thio fusion expression system according to the directions of the manufacturer.

Production and purification of the recombinant proteins. Transformed *Escherichia coli* TOP10 cells (200-ml cultures) were induced with L-arabinose, grown to an optical density at 600 nm of 1.5, and centrifuged at $3,000 \times g$ for 15 min. Pellets were stored at −80°C until they were used. The cells were resuspended in 100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea (pH 8), lysed on ice for 30 min, sonicated, and centrifuged at $3,000 \times g$ for 15 min. The supernatant was mixed with 50% nickel-chelate affinity resin (Qiagen, Valencia, CA), incubated at room temperature for 1 h to bind the His-tagged recombinant proteins, and loaded onto a column. The column was washed four times with 100 mM NaH_2PO_4, 10 mM Tris-Cl, 2 M urea (pH 6.3) and four times with 100 mM NaH_2PO_4, 10 mM Tris-Cl, 2 M urea (pH 5.9). The His-tagged protein was then eluted with 100 mM NaH_2PO_4, 10 mM Tris-Cl, 2 M urea (pH 4.5). Elution fractions were collected and mixed with polymyxin B-agarose (Sigma, St. Louis, MO) at 4°C for 1 h to remove any lipopolysaccharide (LPS) contamination. After elution, the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard. The protein preparation was stored at −80°C until it was used.

Preparation of nuclear extracts. RAW 264.7 cell nuclear extracts were obtained by a micropreparation technique described previously (1). In brief, cells were pelleted for 10 s and resuspended in 400 μl of cold buffer A (10 mM HEPES-KOH [pH 7.9 at 4°C], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin). The cells were allowed to swell on ice for 10 min, vortexed for 10 s, and centrifuged for 10 s. The pellet was resuspended in 20 to 100 μl of cold buffer C (20 mM HEPES-KOH [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin) and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4°C, and the supernatant fraction was stored at −80°C.

Immunoblot analysis. Cytosolic and nuclear extracts were prepared from RAW 264.7 cells (5×10^5 cells/well) as described above. For analysis of phosphor-ERK protein expression, cells were harvested in lysis buffer (150 mM NaCl, 20 mM Tris-HCl, 1.0% Triton X-100, 0.5 mM EDTA, 50 mM NaF, 10% glycerol, 20 μg/ml of phenylmethylsulfonyl fluoride, 1 mM sodium vanadate) and incubated on ice for 10 min with occasional vortexing. Cells were frozen and stored at −80°C. Protein concentrations were determined using a Micro BCA protein assay reagent kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocol. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Egr-1 was visualized using a 1:1,000 dilution of an anti-Egr-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Phosphorylated ERK1/2 and total ERK were visualized using 1:1,000 dilutions of antibodies against phosphorylated ERK1/2 and against nonphosphorylated ERK1/2, respectively (Cell Signaling Technology, Beverly, MA). Bound antibody was detected by treatment with horseradish peroxidase-conjugated anti-immunoglobulin G, followed by treatment with ECL Western blotting substrate (Pierce, Rockford, IL).

TF activity assay. Macrophages were plated to obtain a concentration of 1×10^6 cells/well and infected with *C. pneumoniae* (multiplicity of infection, 10) or incubated with 5 μg/ml of each recombinant antigen for 12 and 24 h in 12-well tissue culture plates (Falcon, Franklin Lakes, NJ). The TF activity in the macrophages was measured using an enzyme-linked immunosorbent assay kit for TF (American Diagnostica Inc., Stamford, CT) according to the manufacturer's protocol.

TNF-α assay. Macrophages were plated at a concentration of 5×10^4 cells/well and incubated with 0.5 to 5 μg/ml of each of the recombinant antigens for 24 h in 96-well tissue culture plates (Falcon, Franklin Lakes, NJ). TNF-α levels in the macrophage culture supernatants were measured using an enzyme-linked immunosorbent assay kit for TNF-α (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's protocol. To determine whether the ERK1/2 pathway was involved in activation of TNF-α expression, cells were pretreated with the MEK inhibitor U0126 (Cell Signaling, Beverly, MA) at a concentration of 10 μM for 30 min and then maintained in the medium during the entire experiment.

Statistical analysis. Statistical analysis was performed by using the unpaired Student *t* test. A P value of <0.05 was considered statistically significant.

RESULTS

Chlamydial recombinant proteins induce expression of TNF-α and TF. RAW 264.7 cells are continuously growing cells that are activated by LPS to induce secretion of TNF-α (18, 26). It has been reported that *C. pneumoniae* can stimulate macrophages to secrete TNF-α (19, 24) and to express TF (4). To investigate whether these proteins elicit RAW 264.7 cells to secrete TNF-α, the individual recombinant proteins were incubated with RAW 264.7 cells for 24 h, and the supernatants were assayed for TNF-α. Stimulation of RAW 264.7 cells with LPS, the positive control, induced the secretion of TNF-α (Fig. 1A). All three recombinant proteins induced the expression of TNF-α in a dose-dependent manner, like LPS (Fig. 1A). In contrast, the negative controls, the thioredoxin portion of the fusion protein and PMP 8N, did not activate the expression of TNF-α. Similar to the induction of TNF-α expression, the expression of TF was also induced by Cpn 0809, Cpn 0980, OMP2, and LPS but not by PMP 8N, a recombinant protein which is known not to activate endothelial cells (33) (Fig. 1B).

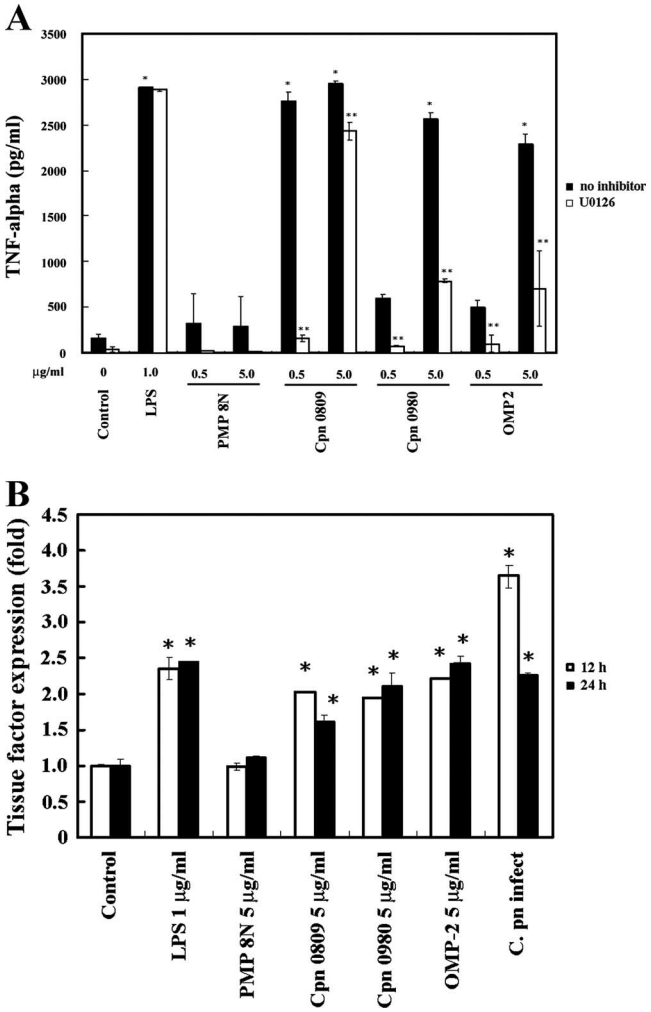

FIG. 1. Effects of recombinant proteins on TNF-α and TF expression in RAW 264.7 cells. (A) Effects of U0126 on the induction of TNF-α production by recombinant antigens. RAW 264.7 cells were preincubated with 10 μM U0126 for 30 min. After this, macrophages were washed and treated with recombinant proteins for 24 h in fresh medium. The culture supernatants were collected and assayed for TNF-α production. The data are the means ± standard deviations from two wells. One asterisk, $P < 0.05$ for a comparison of the control and treated cultures; two asterisks, $P < 0.05$ for a comparison of recombinant protein- and U0126-treated cultures. (B) Effects of recombinant proteins on TF expression. The data are the means ± standard deviations from two wells. One asterisk, $P < 0.05$ for a comparison of control and treated cultures. C. pn infect, *C. pneumoniae* infection.

Blockage of ERK1/2 phosphorylation inhibits the production of TNF-α. The induction of TNF-α expression by LPS is mediated by activation of the MEK-ERK1/2 pathway in macrophages (17). Our previous study showed that stimulation of TF production by *C. pneumoniae* occurred through the MEK-ERK1/2 and Egr-1 signaling pathway in RAW 264.7 cells (4). To determine if the MEK-ERK1/2 pathway is required for the induction of TNF-α expression by recombinant OMP2, Cpn 0809, and Cpn 0980 in macrophages, the induction of TNF-α was analyzed in the presence of U0126, an inhibitor of the upstream protein kinase, MEK1/2. Pretreatment with U0126 inhibited the recombinant antigen-induced production of TNF-α significantly (Fig. 1A). These results indicate that induction of TNF-α occurs through the MEK-ERK1/2 pathway. However, when cells were treated with a higher concentration of recombinant antigens (5.0 μg/ml), although TNF-α production was significantly decreased by U1026, it was not ablated. This suggests that TNF-α induction by OMP2, Cpn 0980, and especially Cpn 0809 can also occur through MEK-ERK1/2-independent pathways.

Recombinant antigens stimulate phosphorylation of p42/44 mitogen-activated protein kinases (MAPK) in mouse macrophages. The finding that TNF-α induction by recombinant proteins was inhibited by U0126 suggested that the recombinant antigens activate phosphorylation of ERK1/2. Immunoblot analysis showed that the expression of phosphor-ERK1/2 was activated by recombinant OMP2, Cpn 0809, and Cpn 0980 at concentrations ranging from 0.5 to 5 μg/ml (Fig. 2A). To standardize sample loading, total ERK was used as the internal control. A time-dependent increase in phosphor-ERK1/2 activity was observed from 15 min to several hours (Fig. 2B). As expected, PMP 8N did not have a stimulatory effect on ERK1/2 phosphorylation (Fig. 2B). To confirm the specificity of the recombinant antigen-induced phosphorylation of ERK1/2, macrophage cultures were preincubated with the MEK inhibitor U0126 for 30 min before treatment with recombinant proteins. U0126 inhibited induction of ERK1/2 phosphorylation (Fig. 2A). In contrast to the phosphor-ERK1/2 results, no phosphorylation of p38 and JNK was observed (Fig. 2C).

Induction of Egr-1 expression by recombinant antigens is mediated through the MEK-ERK1/2 pathway. Since it was previously shown that expression of TF by *C. pneumoniae* occurs through activation of Egr-1 and the MEK-ERK1/2 pathway (4), whether OMP2, Cpn 0809, and Cpn 0980 activated Egr-1 induction and TF expression was investigated. As shown in Fig. 3A, all three recombinant proteins induced Egr-1 protein expression in the nuclear fraction in a dose-dependent manner. The maximum induction was observed at 1 h (Fig. 3B). Proliferating cell nuclear antigen was used as an internal control for standardization (Fig. 3B). As expected, the total cellular Egr-1 was not affected (Fig. 3C). In contrast, bovine serum albumin, thioredoxin, and PMP 8N had no effect on Egr-1 induction (Fig. 3A and B). To determine if the MEK-ERK1/2 pathway was required for induction of Egr-1, the effect of U0126 on Egr-1 expression in RAW 264.7 cells was examined. As shown in Fig. 3B, U0126 inhibited induction of Egr-1. These data confirmed that the induction of the MEK-ERK1/2 pathway by *C. pneumoniae* antigens occurred through Egr-1.

TNF-α induction by chlamydial antigens is diminished in TLR2- and TLR4-deficient murine macrophages. Macrophages play a key role in early innate immune responses and production of proinflammatory cytokines upon activation. Therefore, to investigate the potential involvement of TLR2 and TLR4 signaling in the induction of TNF-α by OMP2, Cpn 0809, and Cpn 0980, macrophages from TLR2 or TLR4 knockout mice were tested and compared with wild-type macrophages. Macrophages deficient in TLR2 produced significantly reduced amounts of TNF-α following incubation with OMP2, Cpn 0809, and Cpn 0980 (Fig. 4). However, PMP 8N, the negative control, had no effect on TNF-α induction. In con-

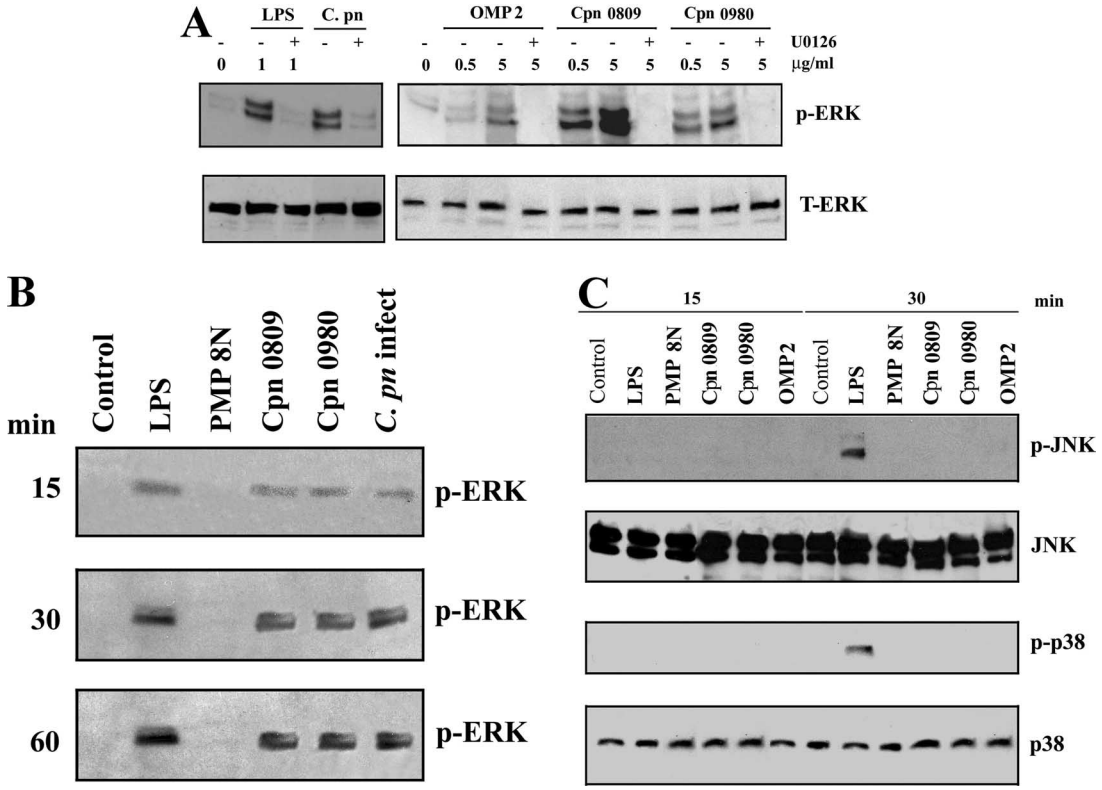

FIG. 2. Induction of ERK1/2 phosphorylation in RAW 264.7 cells. (A) Dose responses to recombinant proteins with a 1-h time course. (B) Kinetic responses to recombinant proteins (5 μg/ml) and LPS (1 μg/ml). Protein extracts were prepared at the indicated time points after treatment with recombinant proteins, separated by SDS-PAGE, and immunoblotted with antibody that specifically recognized phosphorylated ERK1/2. (C) Effects of recombinant antigens (5 μg/ml) on phosphorylation of JNK and p38 in RAW 264.7 cells following a 1-h exposure. C. pn, *C. pneumoniae*; *C. pn* infect, *C. pneumoniae* infection.

trast, LPS, which is known to signal through TLR4, stimulated TNF-α expression equally in TLR2 knockout and wild-type macrophages (41). Interestingly, TNF-α expression was also decreased in TLR4 knockout macrophages. These findings suggest that OMP2, Cpn 0809, and Cpn 0908 could stimulate TNF-α expression through both TLR2- and TLR4-mediated signaling pathways.

DISCUSSION

Strong evidence indicating that there is an association between *C. pneumoniae* and atherosclerosis has been presented previously. Since inflammatory processes are essential components of atherogenesis (36), induction of chronic inflammatory reactions by chlamydial infection may promote the progression of atherosclerosis. *C. pneumoniae* infection can trigger monocytes/macrophages to secrete proinflammatory cytokines such as TNF-α (19, 24) and TF (4). All three recombinant antigens stimulated the secretion of TNF-α (Fig. 1A) and TF (Fig. 1B) in macrophages. It has been reported that induction of TNF-α in macrophages with LPS is mediated by activation of the MEK-ERK1/2 pathway and NF-κB translocation (17). Activation of the MEK-ERK1/2 kinase by *C. pneumoniae* infection has been demonstrated in endothelial cells and smooth muscle cells (27, 51). Our previous study also showed that stimulation of TF production by *C. pneumoniae* was mediated through the MEK-ERK1/2 and Egr-1 signaling pathway in RAW 264.7 cells (4). However, the specific *C. pneumoniae* antigens that activate MEK-ERK1/2 were not determined.

Three such antigens, Cpn 0980, Cpn 0809, and OMP2, were identified in the current study (Fig. 2A). The specificity of the ERK1/2 activation was confirmed by treatment with the MEK inhibitor U0126 (Fig. 2A). Pretreatment of macrophages with U0126 also significantly decreased the expression of TNF-α production by chlamydial antigens (Fig. 1A). These findings indicate that chlamydial antigens could activate TNF-α secretion by macrophages via phosphorylation of the MEK-ERK1/2 pathway. This suggests that activation of MAPK pathways may be a common mechanism for *C. pneumoniae* infection to trigger cellular responses. In contrast, the effect of LPS on stimulation of TNF-α expression in RAW 264.7 cells was not affected by U0126. These findings are consistent with reports demonstrating that in addition to ERK 1/2, LPS-induced TNF-α expression occurs via p38 (2, 45) and JNK (3, 29) signaling. In contrast, *C. pneumoniae* antigens did not activate JNK and p38 kinases in this study (Fig. 2C). Our data differed from those reported by Krüll et al. (28), who showed that *C. pneumoniae* activated p38 in epithelial cells, suggesting that other *C. pneumoniae* antigens may stimulate different pathways. It also has been reported that *C. pneumoniae* (2, 38, 51), cHSP60 (2, 7), or chlamydial PMP 20 and 21 (33) activate

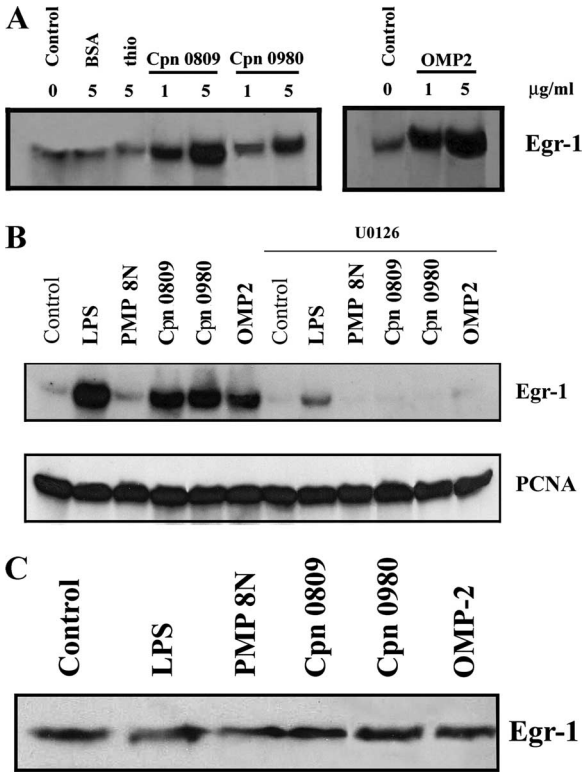

FIG. 3. Induction of Egr-1 expression by recombinant proteins is mediated in part through MEK-ERK1/2 pathways. (A) RAW 264.7 cells were treated with 1 μg/ml LPS or recombinant proteins (5 μg/ml). Nuclear extracts were harvested 1 h after treatment. Egr-1 expression was determined by Western blotting. (B) RAW 264.7 cells were pretreated for 30 min at 37°C with 10 μM U0126 to inhibit MEK phosphorylation. RAW 264.7 cells were then treated with recombinant proteins. Nuclear extracts were collected 1 h later. (C) RAW 264.7 cells were treated with 1 μg/ml LPS or recombinant proteins (5 μg/ml). Cell lysates were harvested 1 h after treatment. Egr-1 expression was determined by Western blotting. BSA, bovine serum albumin.

NF-κB translocation in several cell types, which was not addressed in this study.

Activation of ERK1/2 induces phosphorylation and activation of the transcription factors c-jun, c-*fos*, and Egr-1 (17, 54, 59). The activation of ERK1/2 by OMP2, Cpn 0809, and Cpn 0980 was correlated with an increase in the expression and phosphorylation of Egr-1 (Fig. 3A) in the nucleus. Furthermore, inhibition of MEK-ERK1/2 reduced expression of TNF-α and Egr-1 (Fig. 3B), further supporting the hypothesis that the ERK1/2 signal transduction cascade has a role in the activation of macrophages by these chlamydial antigens (Fig. 3B). This finding is consistent with the findings of a previous study performed by Guha et al. (17) showing that in human monocytes treated with *E. coli*-derived LPS there is activation of the MEK-ERK1/2 kinase pathway and increased expression of Egr-1 and TNF-α. Egr-1 is abundantly expressed in human atherosclerotic lesions, and its expression is induced in the aortas of low-density lipoprotein receptor-deficient mice fed a high-cholesterol diet (30). Egr-1 is known to regulate the expression of a plethora of proatherogenic factors (4). Thus, activation of Egr-1 in macrophages by *C. pneumoniae* is likely to have a broad effect on the atherogenic process.

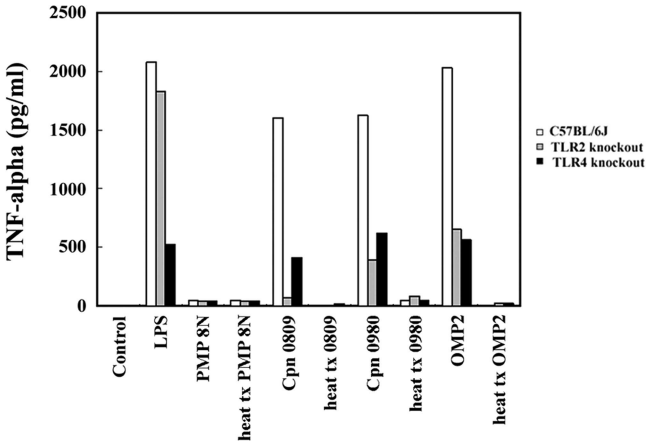

FIG. 4. TLR2 and TLR4 mediate in vitro macrophage response to treatment with chlamydial proteins. TNF-α was detected in culture supernatants of peritoneal macrophages from TLR2 and TLR4 knockout mice after 24 h of in vitro stimulation with PMP 8N (5 μg/ml), Cpn 0809 (5 μg/ml), Cpn 0980 (5 μg/ml), OMP2 (5 μg/ml), or LPS (1 μg/ml). heat tx, heat treated.

Binding of bacterial products to TLRs can activate the MAPK pathway (20). It has previously been shown that TLRs mediate translation of microbial recognition into expression of specific subsets of chemokines and cytokines by macrophages and dendritic cells (6, 21, 53, 54). Furthermore, TLR2 and TLR4 have been shown to induce different sets of chemokines and cytokines, implying that the innate immune response can be customized for different pathogens (11, 15, 39). Recent evidence has shown that *C. pneumoniae* activates the innate immune system via TLRs. However, different studies have reported differences in TLR utilization depending on whether live organisms, sonicated organisms, or individual antigens were tested. For example, recognition of live and sonicated organisms occurred mainly through TLR2, and TLR4 played a minor role in recognition of live organisms (32, 38). Bulut et al. (7) reported that cHSP60 activates macrophages and endothelial cells through TLR4, while Vabulas et al. (56) reported that chlamydial cHSP60 was recognized by both TLR2 and TLR4. In this study, OMP2 and two 53-kDa chlamydial proteins were shown to induce TNF-α signaling through both TLR2 and TLR4 (Fig. 4). Activation of macrophages by the recombinant antigens was not due to endotoxin contamination, since heat treatment abolished the increase in chlamydial antigen-induced TNF-α expression (Fig. 4). These findings suggest that chlamydial proteins activate macrophages through both TLR2 and TLR4. Because TLRs play a critical role in inflammatory signaling, the finding that several chlamydial antigens signal through TLR2 and TLR4 suggests one mechanism by which chronic infection promotes the inflammatory process of atherosclerosis.

In conclusion, the present study demonstrated that in addition to cHSP60, other chlamydial proteins recognized during human infection induce the expression of TNF-α in macrophages and that this induction involves Egr-1 and the MEK-ERK1/2 kinase pathway. TNF-α expression is predominantly dependent on binding of chlamydial proteins to the TLR2 but can occur in part through TLR4. These observations should

facilitate elucidation of the cellular and molecular mechanisms by which *C. pneumoniae* infection promotes atherosclerosis.

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REFERENCES

1. **Andrews, N. C., and D. V. Faller.** 1991. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res. **19:**2499–2500.
2. **Arrighi, J. F., M. Rebsamen, F. Rousset, V. Kindler, and C. Hauser.** 2001. A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers. J. Immunol. **166:**3837–3845.
3. **Ban, H. S., K. Suzuki, S. S. Lim, S. H. Jung, S. Lee, J. Ji, H. S. Lee, Y. S. Lee, K. H. Shin, and K. Ohuchi.** 2004. Inhibition of lipopolysaccharide-induced expression of inducible nitric oxide synthase and tumor necrosis factor-alpha by 2′-hydroxychalcone derivatives in RAW 264.7 cells. Biochem. Pharmacol. **67:**1549–1557.
4. **Bea, F., M. H. Puolakkainen, T. McMillen, F. N. Hudson, N. Mackman, C. C. Kuo, L. A. Campbell, and M. E. Rosenfeld.** 2003. *Chlamydia pneumoniae* induces tissue factor expression in mouse macrophages via activation of Egr-1 and the MEK-ERK1/2 pathway. Circ. Res. **92:**394–401.
5. **Blessing, E., L. A. Campbell, M. E. Rosenfeld, and C. C. Kuo.** 2002. *Chlamydia pneumoniae* and hyperlipidemia are co-risk factors for atherosclerosis: infection prior to induction of hyperlipidemia does not accelerate development of atherosclerotic lesions in C57BL/6J mice. Infect. Immun. **70:**5332–5334.
6. **Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, P. J. Brennan, B. R. Bloom, P. J. Godowski, and R. L. Modlin.** 1999. Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. Science **285:**732–736.
7. **Bulut, Y., E. Faure, L. Thomas, H. Karahashi, K. S. Michelsen, O. Equils, S. G. Morrison, R. P. Morrison, and M. Arditi.** 2002. Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway. J. Immunol. **168:**1435–1440.
8. **Campbell, L. A., and C. C. Kuo.** 1999. Mouse models of *Chlamydia pneumoniae* infection and atherosclerosis. Am. Heart J. **138:**S516–S518.
9. **Campbell, L. A., M. Rosenfeld, and C. C. Kuo.** 2000. The role of *Chlamydia pneumoniae* in atherosclerosis: recent evidence from animal models. Trends Microbiol. **8:**255–257.
10. **Campbell, L. A., S. Roberts, S. Inoue, L. Kong, and C. C. Kuo.** 2001. Evaluation of *Chlamydia pneumoniae* 43- and 53-kilodalton recombinant proteins for serodiagnosis by Western blot. Clin. Diagn. Lab. Immunol. **8:**1231–1233.
11. **Dabbagh, K., and D. B. Lewis.** 2003. Toll-like receptors and T-helper-1/T-helper-2 responses. Curr. Opin. Infect. Dis. **16:**199–204.
12. **Da Costa, C. U., N. Wantia, C. J. Kirschning, D. H. Busch, N. Rodriguez, H. Wagner, and T. Miethke.** 2004. Heat shock protein 60 from *Chlamydia pneumoniae* elicits an unusual set of inflammatory responses via Toll-like receptor 2 and 4 *in vivo*. Eur. J. Immunol. **34:**2874–2884.
13. **Davidson, M., C. C. Kuo, J. P. Middaugh, L. A. Campbell, S. P. Wang, W. P. Newman III, J. C. Finley, and J. T. Grayston.** 1998. Confirmed previous infection with *Chlamydia pneumoniae* (TWAR) and its presence in early coronary atherosclerosis. Circulation **98:**628–633.
14. **de Boer, O. J., A. C. van der Wal, M. A. Houtkamp, J. M. Ossewaarde, P. Teeling, and A. E. Becker.** 2000. Unstable atherosclerotic plaques contain T-cells that respond to *Chlamydia pneumoniae*. Cardiovasc. Res. **48:**402–408.
15. **Edwards, A. D., S. P. Manickasingham, R. Spörri, S. S. Diebold, O. Schulz, A. Sher, T. Kaisho, S. Akira, and C. Reis e Sousa.** 2002. Microbial recognition via Toll-like receptor-dependent and -independent pathways determines the cytokine response of murine dendritic cell subsets to CD40 triggering. J. Immunol. **169:**3652–3660.
16. **Freidank, H. M., A. S. Herr, and E. Jacobs.** 1993. Identification of *Chlamydia pneumoniae*-specific protein antigens in immunoblots. Eur. J. Clin. Microbiol. Infect. Dis. **12:**947–951.
17. **Guha, M., M. A. O'Connell, R. Pawlinski, A. Hollis, P. McGovern, S. F. Yan, D. Stern, and N. Mackman.** 2001. Lipopolysaccharide activation of the MEK-ERK1/2 pathway in human monocytic cells mediates tissue factor and tumor necrosis factor α expression by inducing Elk-1 phosphorylation and Egr-1 expression. Blood **98:**1429–1439.
18. **Haslberger, A., C. Romanin, and R. Koerber.** 1992. Membrane potential modulates release of tumor necrosis factor in lipopolysaccharide-stimulated mouse macrophages. Mol. Biol. Cell **3:**451–460.
19. **Heinemann, M., M. Susa, U. Simnacher, R. Marre, and A. Essig.** 1996. Growth of *Chlamydia pneumoniae* induces cytokine production and expression of CD14 in a human monocytic cell line. Infect. Immun. **64:**4872–4875.
20. **Kaisho, T., K. Hoshino, T. Iwabe, O. Takeuchi, T. Yasui, and S. Akira.** 2002. Endotoxin can induce MyD88-deficient dendritic cells to support Th2 cell differentiation. Int. Immunol. **14:**695–700.
21. **Kaisho, T., and S. Akira.** 2003. Regulation of dendritic cell function through Toll-like receptors. Curr. Mol. Med. **3:**373–385.
22. **Kalayoglu, M. V., and G. I. Byrne.** 1998. Induction of macrophage foam cell formation by *Chlamydia pneumoniae*. J. Infect. Dis. **177:**725–729.
23. **Kalayoglu, M. V., and G. I. Byrne.** 1998. A *Chlamydia pneumoniae* component that induces macrophage foam cell formation is chlamydial lipopolysaccharide. Infect. Immun. **66:**5067–5072.
24. **Kaukoranta-Tolvanen, S. S. E., A. M. Teppo, K. Laitinen, P. Saikku, K. Linnavuori, and M. Leinonen.** 1996. Growth of *Chlamydia pneumoniae* in cultured human peripheral blood mononuclear cells and induction of a cytokine response. Microb. Pathog. **21:**215–221.
25. Reference deleted.
26. **Kelly, N. M., L. Young, and A. S. Cross.** 1991. Differential induction of tumor necrosis factor by bacteria expressing rough and smooth lipopolysaccharide phenotypes. Infect. Immun. **59:**4491–4496.
27. **Krüll, M., A. C. Klucken, F. N. Wuppermann, O. Fuhrmann, C. Magerl, J. Seybold, S. Hippenstiel, J. H. Hegemann, C. A. Jantos, and N. Suttorp.** 1999. Signal transduction pathways activated in endothelial cells following infection with *Chlamydia pneumoniae*. J. Immunol. **162:**4834–4841.
28. **Krüll, M., P. Bockstaller, F. N. Wuppermann, A. C. Klucken, J. Mühling, B. Schmeck, J. Seybold, C. Walter, M. Maass, S. Rosseau, J. H. Hegemann, N. Suttorp, and S. Hippenstiel.** 2006. Mechanisms of *Chlamydophila pneumoniae*-mediated GM-CSF release in human bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. **34:**375–382.

28a. **Kuo, C.-C., A. M. Gown, E. P. Benditt, and J. T. Grayston.** 1993. Detection of Chlamydia pneumoniae in aortic lesions of atherosclerosis by immunocytochemical stain. Arterioscler. Thromb. **13:**1501–1504.

28b. **Kuo, C.-C., and L. A. Campbell.** 2000. Detection of Chlamydia pneumoniae in arterial tissue. J. Infect. Dis. **181:**S516–S518.

29. **Matsuguchi, T., T. Musikacharoen, T. R. Johnson, A. S. Kraft, and Y. Yoshikai.** 2001. A novel mitogen-activated protein kinase phosphatase is an important negative regulator of lipopolysaccharide-mediated c-Jun N-terminal kinase activation in mouse macrophage cell lines. Mol. Cell. Biol. **21:**6999–7009.
30. **McCaffrey, T. A., C. Fu, B. Du, S. Eksinar, K. C. Kent, Jr., H. Bush, K. Kreiger, T. Rosengart, M. I. Cybulsky, E. S. Silverman, and T. Collins.** 2000. High-level expression of Egr-1 and Egr-1-inducible genes in mouse and human atherosclerosis. J. Clin. Investig. **105:**653–662.
31. Reference deleted.
32. **Netea, M. G., B. J. Kullberg, J. M. Galama, A. F. Stalenhoef, C. A. Dinarello, and J. W. Van der Meer.** 2002. Non-LPS components of *Chlamydia pneumoniae* stimulate cytokine production through Toll-like receptor 2-dependent pathways. Eur. J. Immunol. **32:**1188–1195.
33. **Niessner, A., C. Kaun, G. Zorn, W. Speidl, Z. Türel, G. Christiansen, A. S. Pedersen, S. Birkelund, S. Simon, A. Georgopoulos, W. Graninger, R. de Martin, J. Lipp, B. R. Binder, G. Maurer, K. Huber, and J. Wojta.** 2003. Polymorphic membrane protein (PMP) 20 and PMP 21 of *Chlamydia pneumoniae* induce proinflammatory mediators in human endothelial cells in vitro by activation of the nuclear factor-kappa B pathway. J. Infect. Dis. **188:**108–113.
34. **Penttilä, T., J. M. Vuola, V. Puurula, M. Anttila, M. Sarvas, N. Rautonen, P. H. Mäkelä, and M. Puolakkainen.** 2000. Immunity to *Chlamydia pneumoniae* induced by vaccination with DNA vectors expressing a cytoplasmic protein (Hsp60) or outer membrane proteins (MOMP and Omp2). Vaccine **8:**1256–1265.
35. **Perez Melgosa, M. P., C. C. Kuo, and L. A. Campbell.** 1994. Isolation and characterization of a gene encoding a *Chlamydia pneumoniae* 76-kilodalton protein containing a species-specific epitope. Infect. Immun. **62:**880–886.
36. **Pfeffer, K.** 2003. Biological functions of tumor necrosis factor cytokines and their receptors. Cytokine Growth Factor Rev. **14:**185–191.
37. Reference deleted.
38. **Prebeck, S., C. Kirschning, S. Dürr, C. da Costa, B. Donath, K. Brand, V. Redecke, H. Wagner, and T. Miethke.** 2001. Predominant role of Toll-like receptor 2 versus 4 in *Chlamydia pneumoniae*-induced activation of dendritic cells. J. Immunol. **167:**3316–3323.
39. **Qi, H., T. L. Denning, and L. Soong.** 2003. Differential induction of interleukin-10 and interleukin-12 in dendritic cells by microbial Toll-like receptor activators and skewing of T-cell cytokine profiles. Infect. Immun. **71:**3337–3342.
40. **Rodriguez, N., N. Wantia, F. Fend, S. Dürr, H. Wagner, and T. Miethke.** 2006. Differential involvement of TLR2 and TLR4 in host survival during pulmonary infection with *Chlamydia pneumoniae*. Eur. J. Immunol. **36:**1145–1155.
41. **Romics, L., Jr., A. Dolganiuc, A. Velayudham, K. Kodys, P. Mandrekar, D. Golenbock, E. Kurt-Jones, and G. Szabo.** 2005. Toll-like receptor 2 mediates inflammatory cytokine induction but not sensitization for liver injury by Propionibacterium acnes. J. Leukoc. Biol. **78:**1255–1264.
42. **Ross, R.** 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature **362:**801–809.

43. **Ross, R.** 1993. Atherosclerosis: current understanding of mechanisms and future strategies in therapy. Transplant Proc. **25:**2041–2043.
44. **Ross, R.** 1999. Atherosclerosis is an inflammatory disease. Am. Heart J. **138:**S419–S420.
45. **Rutault, K., C. A. Hazzalin, and L. C. Mahadevan.** 2001. Combinations of ERK and p38 MAPK inhibitors ablate tumor necrosis factor-alpha (TNF-alpha) mRNA induction. Evidence for selective destabilization of TNF-alpha transcripts. J. Biol. Chem. **276:**6666–6674.
46. **Saikku, P.** 1999. Epidemiology of *Chlamydia pneumoniae* in atherosclerosis. Am. Heart J. **138:**S500–S503.
47. **Saikku, P.** 2000. *Chlamydia pneumoniae* in atherosclerosis. J. Intern. Med. **247:**391–396.
48. **Saikku, P. M. L., L. Tenkanen, and E. Linnanmaki.** 1992. Chronic *Chlamydia pneumoniae* infection as a risk factor for coronary heart disease in the Helsinki heart study. Ann. Intern. Med. **116:**273–277.
49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
50. **Sander, D., K. Winbeck, J. Klingelhöfer, T. Etgen, and B. Conrad.** 2001. Enhanced progression of early carotid atherosclerosis is related to *Chlamydia pneumoniae* (Taiwan acute respiratory) seropositivity. Circulation **103:** 1390–1395.
51. **Sasu, S., D. LaVerda, N. Qureshi, D. T. Golenbock, and D. Beasley.** 2001. *Chlamydia pneumoniae* and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via Toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. Circ. Res. **89:**244–250.
52. **Shimada, K., H. Mokuno, Y. Watanabe, M. Sawano, H. Daida, and H. Yamaguchi.** 2000. High prevalence of seropositivity for antibodies to *Chlamydia*-specific lipopolysaccharide in patients with acute coronary syndrome. J. Cardiovasc. Risk **7:**209–213.
53. **Sieling, P. A., and R. L. Modlin.** 2002. Toll-like receptors: mammalian "taste receptors" for a smorgasbord of microbial invaders. Curr. Opin. Microbiol. **5:**70–75.
54. **Sodhi, A., and G. Sethi.** 2003. Involvement of MAP kinase signal transduction pathway in UVB-induced activation of macrophages in vitro. Immunol. Lett. **90:**123–130.
55. **Sueur, J. M., K. Beaumont, T. Cabioch, J. Orfila, and F. Betsou.** 2006. Diagnostic value of an ELISA using a recombinant 54-kDa species-specific protein from *Chlamydia pneumoniae*. Clin. Microbiol. Infect. **12:**470–477.
56. **Vabulas, R. M., P. Ahmad-Nejad, C. da Costa, T. Miethke, C. J. Kirschning, H. Häcker, and H. Wagner.** 2001. Endocytosed heat shock protein 60s use TLR2 and TLR4 to activate the Toll/interleukin-1 receptor signaling pathway in innate immune cells. J. Biol. Chem. **276:**31332–31339.
57. **Wagar, E. A., J. Schachter, P. Bavoil, and R. S. Stephens.** 1990. Differential human serologic response to two 60,000 molecular weight *Chlamydia trachomatis* antigens. J. Infect. Dis. **162:**922–927.
58. **Watson, M. W., P. R. Lambden, J. S. Everson, and I. N. Clarke.** 1994. Immunoreactivity of the 60 kDa cysteine-rich proteins of *Chlamydia trachomatis*, *Chlamydia psittaci* and *Chlamydia pneumoniae* expressed in *Escherichia coli*. Microbiology **140:**2003–2011.
59. **Zhang, M., C. Miller, Y. He, J. Martel-Pelletier, J. P. Pelletier, and J. A. Di-Battista.** 1999. Calphostin C induces AP1 synthesis and AP1-dependent c-jun transactivation in normal human chondrocytes independent of protein kinase C-alpha inhibition: possible role for c-jun N-terminal kinase. J. Cell. Biochem. **76:**290–302.

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