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Chlamydial Heat Shock Protein 60 Activates Macrophages and Endothelial Cells Through Toll-Like Receptor 4 and MD2 in a MyD88-Dependent Pathway[1]

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Active inflammation and NF-κB activation contribute fundamentally to atherogenesis and plaque disruption. Accumulating evidence has implicated specific infectious agents including *Chlamydia pneumoniae* in the progression of atherogenesis. Chlamydial heat shock protein 60 (cHSP60) has been implicated in the induction of deleterious immune responses in human chlamydial infections and has been found to colocalize with infiltrating macrophages in atheroma lesions. cHSP60 might stimulate, enhance, and maintain innate immune and inflammatory responses and contribute to atherogenesis. In this study, we investigated the signaling mechanism of cHSP60. Recombinant cHSP60 rapidly activated NF-κB in human microvascular endothelial cells (EC) and in mouse macrophages, and induced human IL-8 promoter activity in EC. The inflammatory effect of cHSP60 was heat labile, thus excluding a role of contaminating LPS, and was blocked by specific anti-chlamydial HSP60 mAb. In human vascular EC which express Toll-like receptor 4 (TLR4) mRNA and protein, nonsignaling TLR4 constructs that act as dominant negative blocked cHSP60-mediated NF-κB activation. Furthermore, an anti-TLR4 Ab abolished cHSP60-induced cellular activation, whereas a control Ab had no effect. In 293 cells, cHSP60-mediated NF-κB activation required both TLR4 and MD2. A dominant-negative MyD88 construct also inhibited cHSP60-induced NF-κB activation. Collectively, our results indicate that cHSP60 is a potent inducer of vascular EC and macrophage inflammatory responses, which are very relevant to atherogenesis. The inflammatory effects are mediated through the innate immune receptor complex TLR4-MD2 and proceeds via the MyD88-dependent signaling pathway. These findings may help elucidate the mechanisms by which chronic asymptomatic chlamydial infection contribute to atherogenesis. *The Journal of Immunology,* 2002, 168: 1435–1440.

I t has been clearly established that an active inflammatory process contributes fundamentally to the pathogenesis and complications of atherosclerosis (1–4). Although precise triggers for inflammation in atherosclerosis are not fully understood, hypercholesterolemia, modified lipoproteins. and infection with organisms, such as *Chlamydia pneumoniae*, have all been implicated. Several studies have demonstrated a seroepidemiologic association of *C. pneumoniae* with atherosclerosis, and the organism has been detected in atherosclerotic lesions but not in normal tissue by immunohistochemistry, electron microscopy, PCR, and culture (5–11). Clinical studies suggest a correlation between serologic evidence of chronic *C. pneumoniae* infection and a proatherogenic lipid profile, further supporting the notion that *C. pneumoniae* infection can interact with traditional risk factors in the pathogenesis of atherosclerosis (12). Saikku et al. (13) demonstrated higher titers of anti-*C. pneumonia* Abs in patients with

atherosclerosis than in healthy controls. Animal models show that *C. pneumoniae* infection initiates vascular atheromatous changes in rabbits (14, 15) and promotes atheroma development in apo-E knockout mice (16, 17). Antibiotic treatment of infected rabbits inhibited these atheromatous changes (18).

Heat shock protein 60 (HSP60),[3] bacterial LPSs, and molecular mimicry have all been suggested as potential mediators linking infection to atherosclerosis (19, 20). Oxidation of low-density lipoprotein (LDL) into an atherogenic lipoprotein and production of foam cells from macrophages and smooth muscle cells are two key events in the initiation and development of atherosclerotic lesions. Recent studies have demonstrated that specific chlamydial molecules, such as chlamydial LPS and chlamydial HSP60 (cHSP60) can induce these activities. The component of *C. pneumoniae* that induces macrophage foam cell formation is chlamydial LPS and the component that induces oxidative modification of LDL is cHSP60 (21, 22). cHSP60 has been implicated in the induction of deleterious immune responses in human chlamydial infections and has been found to colocalize with infiltrating macrophages in the atheroma lesions (23). Collectively, these data support a potential role of *C. pneumoniae* as a cofactor in the development and progression of atherosclerosis. However, available data also underscore the current lack of a complete understanding of the molecular mechanisms that link *C. pneumoniae* infection to the activation of the innate immune system, which triggers the signals for enhanced inflammation and atherogenesis.

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[3] Abbreviations used in this paper: HSP, heat shock protein; LDL, low-density lipoprotein; TLR, Toll-like receptor; cLPS, chlamydial HSP; cHSP60, chalmydial HSP60; HMEC, human dermal microvessel endothelial cell; RsDPLA, Rhodobacter sphaeroides lipid A; EC, endothelial cells.

HSPs, a ubiquitous family of highly conserved proteins, function to stabilize cellular proteins during a variety of conditions such as heat shock, infection, and inflammation (24, 25). Atheromatous vessels contain both endogenous (human) HSP60 and cHSP60 (23). cHSP60 is abundantly produced during chronic chlamydial infection of the blood vessel, and might stimulate and enhance innate immune and inflammatory responses and contribute to atherogenesis (23). cHSP60 induces cytokine production by macrophages (23), and a recent study demonstrated that cHSP60 is the chlamydial Ag responsible for inducing the inflammatory responses by this microorganism (26). How cHSP60 interacts with host cells to transduce activating signals has only recently been investigated and is not completely delineated. Kol et al. (27) demonstrated that human HSP60 activates human PBMCs and monocyte-derived macrophages through CD14 signaling and p38 mitogen-activated protein kinase, sharing this pathway with LPS (27). Human HSP60 has been identified as an endogenous ligand for Toll-like receptor (TLR) 4 (27, 28). However Vabulas et al. (29) suggested that endogenous and cHSP60 are recognized by both TLR2 and TLR4. To better understand how cHSP60 activates cells of the immune system, including vascular endothelial cells and macrophages, we investigated the interaction of recombinant cHSP60 with human dermal microvessel endothelial cells (HMEC) and mouse macrophages. The results suggest that TLR4 but not TLR2 is the signaling receptor for recombinant chlamydial HSP60.

Materials and Methods

Cell cultures

Immortalized HMEC (generous gift from Dr. F. J. Candal of the Center for Disease Control and Prevention, Atlanta, GA) were cultured in MCDB-131 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 μg/ml penicillin and streptomycin in 24-well plates. Tissue culture reagents were purchased from Life Technologies (Rockville, MD). Mouse macrophage cell line, RAW 264.7 cells, and HEK 293 cell line cells were obtained from American Type Culture Collection (Manassas, VA) and were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 μg/ml penicillin and streptomycin in 24-well plates as previously described (30–33).

Chlamydial HSP60 and other reagents

Recombinant chlamydial HSP60 protein was isolated and purified as described earlier (34). *Chlamydia trachomatis* serovar A HSP60, fused with eight additional amino acids (arginine, serine, and six histidine residues) at the carboxyl terminus, was expressed in *Escherichia coli,* and recombinant protein was purified by affinity chromatography with Ni-NTA resin, as previously described (34). The endotoxin concentration of this preparation was <0.04 EU/μg, as determined by *Limulus* amebocyte lysate assay (Associates of Cape Cod, Falmouth, MA). Anti-chlamydial HSP60 mAbs (mAb A57-B9) (IgG1) were obtained from mice immunized with purified recombinant *C. trachomatis* serovar A HSP60, as described and characterized previously (35). Neutralizing anti-human TLR2 mAb was provided by T. Espevik (Trondheim, Norway) and neutralizing anti-human TLR4 mAb was provided by Kensuke Miyake (Saga Medical School, Nabeshima, Saga, Japan) as described earlier (31–33). Highly purified, phenol-water extracted, and protein-free (<0.0008% protein) *E. coli* K235 LPS was obtained from S. Vogel (Uniformed Services University, Bethesda, MD). The purity of this LPS preparation has been previously demonstrated (36, 37), and this preparation of LPS is active on TLR4-transfected HEK 293 cells and not on TLR2 transfectants (S. N. Vogel, unpublished observation). Purified Rhodobacter sphaeroides lipid A (RsDPLA) was obtained from N. Qureshi (University of Missouri, Kansas City, MO).

cDNA constructs and transient transfection

ELAM-NF-κB luciferase, human IL-8 promoter luciferase, pCMV-β-galactosidase, and dominant negative MyD88 vectors were used as described previously (30–33). Dominant-negative TLR2 constructs were obtained from C. J. Kirschning (Technical University of Munich, Munich, Germany), the C3H/HeJ TLR4 cDNA was obtained from B. Beutler (The Scripps Institute, La Jolla, CA), and were described earlier (30–33). C3H/HeJ mice

express dominant-negative TLR4 encoding a single missense mutation, which converts a cytoplasmic proline residue to histidine (P712H) (38). A FLAG-tagged mutant of the human TLR4 construct with a deletion in the intracellular domain (TLR4 Dicd) with COOH-terminal truncation was obtained from Tularik (San Francisco, CA) as described earlier (30, 31). A FLAG-tagged human MD2 cDNA construct was obtained from K. Miyake (Saga Medical Schhol, Saga, Japan). All constructs were verified by sequencing. Transient transfection of cultured cells was conducted by Fu-Gene 6 Transfection reagent (Roche Molecular Laboratories, Burlington, NC) as described earlier (31). All reagents were verified to be LPS free by the *Limulus* amebocyte lysate assay (Pyrotell, < 0.03 endotoxin units/ml; Associates of Cape Cod).

NF-κB and IL-8 promoter luciferase activation in HMEC and RAW 264.7

HMEC and/or RAW 264.7 were plated in 24-well plates and cotransfected the following day with FuGene 6 Transfection reagent (Roche Molecular Laboratories) following the manufacturer's instructions. Reporter genes pCMV-βgal (0.1 μg) and ELAM-NF-κB (0.5 μg), and pCMV empty vector (0.5 μg) or C3H/HeJ TLR4 (0.5 μg), an intracellular deletion mutant of TLR4 TLR4 Dicd (0.5 μg), dominant-negative TLR2 (0.5 μg), or dominant-negative MyD88 ΔMyD88 (0.1 μg) were cotransfected as described earlier (30–33). Total amount of DNA transfected to each cell was kept constant with pCMV empty vector. After overnight transfection, cells were stimulated for 5 h with LPS (20 ng/ml) or cHSP60 (10 μg/ml). Following a 5-h incubation, cells were washed once in PBS and lysed, and luciferase activity was measured with a Promega kit (Promega, Madison, WI) and a luminometer as described earlier (30). β-Galactosidase activity was determined using the colorimetric method to normalize transfection efficiency as described previously (30). Data shown are the mean and the SD of three independent experiments and are expressed as percent luciferase activity induced by LPS or cHSP60 (indicated as 100%).

Results

Recombinant cHSP60 induces NF-κB and IL-8 promoter luciferase activities in HMEC and macrophages in a dose-dependent manner

Activation of NF-κB is essential for the regulation of a variety of genes involved in the inflammatory and proliferative responses of cells critical to atherogenesis (39, 40). Both NF-κB and genes regulated by NF-κB are expressed in atherosclerotic lesions (40). Accumulating evidence indicates that chronic infection with *C. pneumoniae* may be an additional risk factor for atherosclerosis. Therefore, we investigated whether cHSP60 stimulated NF-κB activation in endothelial cells and macrophages and whether TLR4 was involved in this process. HMEC and RAW 264.7 cells were transiently transfected with a NF-κB-dependent ELAM promoter reporter or human IL-8 promoter luciferase construct. Transfected cells were stimulated with increasing concentrations of cHSP60 or LPS for 5 h. Cells were then lysed and assayed for luciferase and β-galactosidase activities as described in *Materials and Methods*. Recombinant cHSP60 induced a dose-dependent transactivation of NF-κB in HMEC (Fig. 1*A*) and RAW 264.7 (Fig. 1*B*). Chlamydial HSP60 also induced the activation of human IL-8 promoter luciferase activity in HMEC in a dose-dependent manner (Fig. 2). These experiments indicated that recombinant cHSP60 was able to induce NF-κB and IL-8 promoter transactivation in vascular endothelium and in macrophages, which is indicative of a robust inflammatory response.

Activation of NF-κB by cHSP60 in HMEC is TLR4 and MyD88 dependent

Recent studies have documented the role of the innate immune system and transmembrane TLRs in cellular activation by microbial pathogens (41–45). We (30, 31) and others (46, 47) have shown that LPS signals via TLR4 in HMEC and macrophages and that activation of NF-κB is MyD88 dependent. However, a MyD88-independent pathway of TLR4 activation has been described as well (48, 49). Although endogenous (human) HSP60
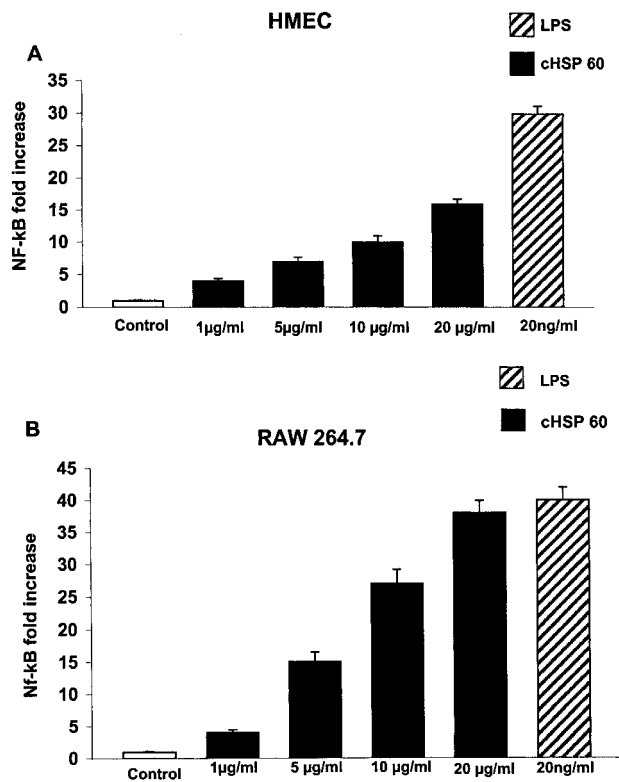
FIGURE 1. cHSP60 induces NF-κB activation in HMEC and RAW cells in a dose-dependent manner. HMEC (*A*) or mouse macrophages RAW 264.7 (*B*) were transiently transfected with ELAM-NF-κB and pCMV-β-galactosidase. The cells were then stimulated with LPS (20 ng/ml) or with increasing amounts of cHSP60 for 5 h. Luciferase and β-galactosidase assays were performed as described in *Materials and Methods*. Data shown are the mean and the SD of three independent experiments and are expressed as fold increase over unstimulated, empty vector-transfected control cells (indicated as vector).

FIGURE 2. cHSP60 induces human IL-8 promoter luciferase activation in HMEC in a dose-dependent manner. HMEC were transiently transfected with a human IL-8 promoter luciferase construct and pCMV-β-galactosidase. The cells were then stimulated with LPS (20 ng/ml) or with increasing amounts of cHSP60 for 5 h. Luciferase and β-galactosidase assays were performed as described in *Materials and Methods*. Data shown are the mean and the SD of three independent experiments and are expressed as fold increase over unstimulated, empty vector control cells.

has been reported to signal through TLR4 (27–29) as well as TLR2 (29), the signaling pathway stimulated by cHSP60 has not been fully delineated. A recent study suggested that cHSP60 signals via both TLR4 and TLR2, while another study showed that cHSP60 induces human vascular smooth muscle cell proliferation via TLR4 (50). To investigate the potential involvement of TLR4 and MyD88 signaling in cHSP60-induced inflammatory responses, we transiently transfected HMEC with nonsignaling, dominant-negative TLR4 constructs, such as C3H/HeJ TLR4 or intracellular deletion mutant of human TLR4 (TLR4 Dicd), and tested their response to cHSP60 and to LPS by monitoring expression of the luciferase activity driven by the NF-κB-responsive enhancer of the ELAM gene as described earlier (30). As shown in Fig. 3*A*, coexpression of C3H/HeJ TLR4 construct blocked cHSP60- as well as LPS-induced NF-κB activation in HMEC. Similarly, overexpression of an intracellular deletion mutant of TLR4 (TLR4 Dicd) with COOH-terminal truncation led to a significant inhibition of cHSP60-mediated NF-κB activation in HMEC (Fig. 3*A*). Coexpression of C3H/HeN TLR4 or a dominant-negative mutant of TLR2 had no effect on LPS- and cHSP60-mediated NF-κB activation (data not shown). A dominant-negative mutant of MyD88 (ΔMyD88), which abrogates IL-1- and LPS-induced NF-κB activation, significantly inhibited cHSP60-induced NF-κB activation as well (Fig. 3*A*). To further confirm the role of TLR4 in cHSP60-induced NF-κB activation in endothelial cells, we preincubated HMEC with function blocking mAbs against TLR2 or TLR4 and measured cHSP60-mediated cellular activation. The anti-TLR4
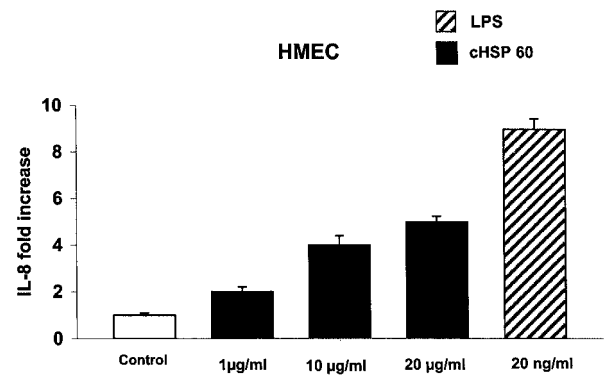
mAb significantly blocked LPS- and cHSP60-induced NF-κB activation (Fig. 3*B*), whereas an isotype-matched control Ab (data not shown) and the anti-TLR2 Ab were ineffective (Fig. 3*B*). Taken together, these results support the hypothesis that TLR4 is essential for cHSP60 signaling and that the cHSP60-TLR4 signaling to NF-κB proceeds through MyD88.

Based on LPS-binding studies, RsDPLA is believed to competitively antagonize LPS activity at the cell surface receptor level. We have previously shown that RsDPLA blocks LPS-induced NF-κB activation at the TLR4 level, but is unable to block TLR2 signaling by TLR2 ligands (31). Therefore, we next investigated whether RsDPLA is able to block cHSP60-induced signaling in HMEC. Pretreatment of HMEC with RsDPLA (1 μg/ml) for 60 min significantly inhibited cHSP60-induced NF-κB activation (Fig. 3*B*). These observations further support the observation that TLR4 is the signaling receptor for cHSP60 as well as LPS.

Activation of NF-κB by cHSP60/TLR4 is MD2 dependent

To respond efficiently to LPS, TLR4 requires an accessory protein, MD2 (51). MD2 is a 20- to 30-kDa glycoprotein, which binds to the extracellular domain of TLR4 (51). Photoaffinity labeling studies have recently shown that LPS binds directly to the TLR4-MD2 complex and that both molecules are in close proximity to the bound LPS (52). Although both LPS and Taxol have been shown to require MD2 to signal through TLR4, is it unknown whether cHSP60-TLR4 interaction requires the presence of MD2 as well for signaling. To investigate the requirement of MD2 in cHSP60-TLR4 signaling, NF-κB reporter gene- and TLR4-transfected 293 cells were stimulated either with increasing concentrations of cHSP60 or with LPS in the presence and absence of MD2 cotransfection. Fig. 4 shows that neither LPS nor cHSP60 is able to signal in the absence of MD2 and that both molecules require the presence of TLR4 as well as MD2 to activate NF-κB. cHSP60 induced a dose-dependent increase in NF-κB activation in 293 cells transfected with TLR4 and MD2 (Fig. 4). We conclude that MD2 is not specific for LPS-TLR4 signaling and is also required for another TLR4 ligand, i.e., cHSP60 signaling as well.

Chlamydial HSP60-induced NF-κB activation is not due to endotoxin contamination

In this study, we used recombinant cHSP60. The concentration of LPS measured in the undiluted recombinant protein was <0.06
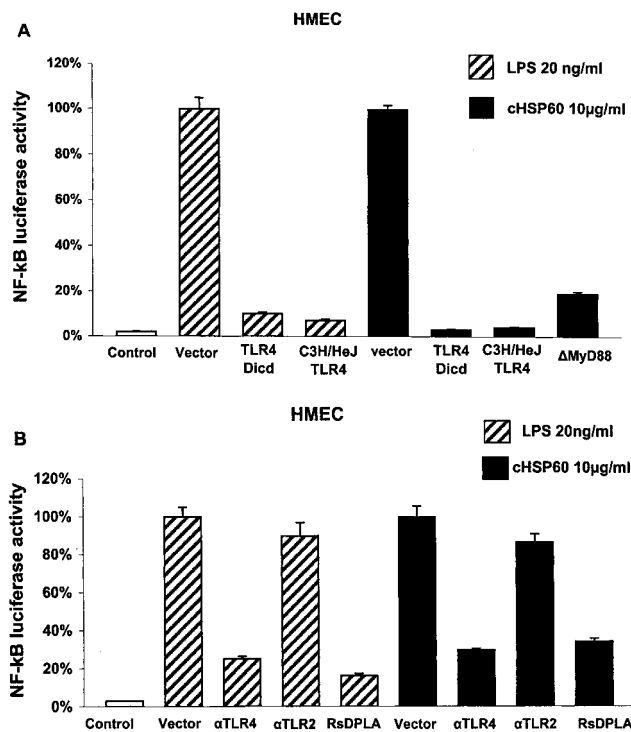
FIGURE 3. cHSP60 activates NFκB through TLR4 and MyD88. *A,* HMEC were transiently transfected with NF-κB luciferase and β-galactosidase reporter vectors and with either C3H/HeJ TLR4 or TLR4 Dicd or dominant-negative MyD88 cDNA constructs. The total amount of cDNA was kept constant with pCMV empty vector. The cells were then stimulated with LPS (20 ng/ml) or with c-HSP60 (10 μg/ml) for 5 h and lysed. NF-κB luciferase activity was measured with luciferase assay and normalized with β-galactosidase activity. *B,* HMEC were transiently transfected with NF-κB luciferase and β-galactosidase reporter vectors overnight and were either grown in media alone or pretreated with anti-TLR4 mAb (10 μg/ml), anti-TLR2 mAb(10 μg/ml), or with RsDPLA (1 μg/ml) for 1 h, and then stimulated with LPS (20 ng/ml) or with cHSP60 (10 μg/ml) for 5 h. Cells were lysed and NF-κB luciferase activity was measured with luciferase assay and normalized with β-galactosidase activity. Data shown are mean and SD obtained from three experiments and are expressed as percentage luciferase activity induced by LPS or cHSP60 in vector control cells (indicated as 100%).

ng/ml, which corresponds to <0.0012 ng/ml LPS in 10 μg of protein used in each well, a concentration well below what is needed for cellular activation. To further exclude the possibility of endotoxin contamination in the cHSP60-mediated responses that we observed, cHSP60 and *E. coli* LPS preparations were heat treated (100°C, 20 min) before incubation with cells. Heat treatment abolished the ability of cHSP60 to induce NF-κB activation, but did not affect the activation of NF-κB by LPS (Fig. 5), further suggesting that the observed activity of the recombinant cHSP60 was not due to endotoxin contamination. Furthermore, preincubation of cells with a specific mAb raised against cHSP60 (A57-B9, 50 μg/ml) significantly blocked cHSP60-induced NF-κB activation, whereas mouse IgG1 control (Fig. 4) and an isotype-matched (mouse IgG1) irrelevant control Ab did not block LPS signaling (data not shown). Also, anti-cHSP60 mAb did not inhibit LPS-induced cellular activation (data not shown). Together, these observations suggest that cHSP60-mediated NF-κB activation through TLR4 was not due to endotoxin contamination of this recombinant protein and that the responses observed were specific to cHSP60.
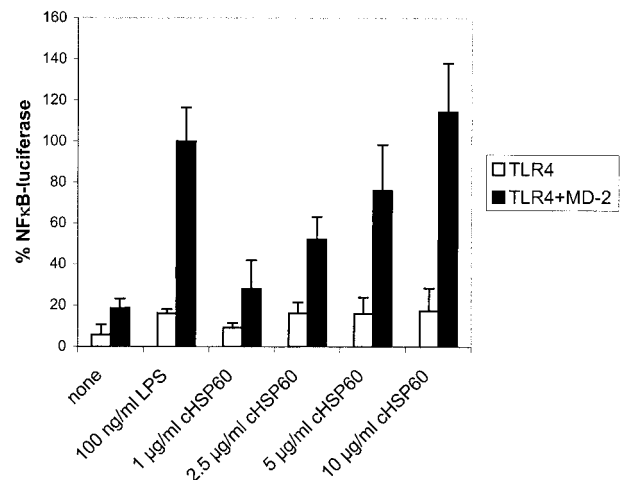

FIGURE 4. TLR4 and MD2 are required for cHSP60-induced NF-κB activation. 293 cells in 24-well plates (5×10^4 cells/well) were cotransfected with the NF-κB-luciferase (0.05 μg/well), β-galactosidase (0.075 μg/well), and either with TLR4 cDNA (0.03 μg/well) or with both TLR4 and MD2 (0.03 μg/well) constructs overnight and stimulated for 5 h at 37°C with either LPS (100 ng/ml) or various concentrations of cHSP60 (1–10 μg/ml). Total amount of DNA transfected was kept constant with empty vector. Cells were lysed and NF-κB luciferase activity was measured with luciferase assay and normalized with β-galactosidase activity. Data shown are mean and SD obtained from three experiments and are expressed as percentage of luciferase activity induced by 100 ng/ml LPS in 293 cells transfected with both TLR4 and MD2.

Discussion

Epidemiological, clinical and experimental animal studies show that *C. pneumoniae* infection is associated with cardiovascular disease; however it is unclear how the organism may initiate or promote atherosclerosis. It is postulated that *C. pneumoniae* triggers key atherogenic events through specific virulence determinants. For example, chlamydial LPS and cHSP60 induce mononuclear phagocyte foam cell formation and LDL oxidation, respectively, and therefore those chlamydial Ags may be involved in the pathogenesis of atherosclerosis (21, 22). cHSP60 also colocalizes with
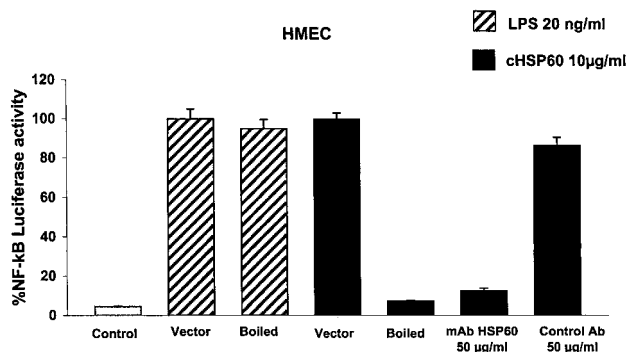
FIGURE 5. cHSP60-induced NF-κB activation is specific and not due to endotoxin contamination. HMEC were transiently transfected with NF-κB luciferase and β-galactosidase reporter vectors. Cells were pretreated with anti-cHSP60 mAb (A57-B9, 50 μg/ml) or mouse IgG1 control Ab (50 μg/ml) for 1 h and then stimulated with LPS (20 ng/ml) or c-HSP60 (10 μg/ml). In separate experiments, HMEC were stimulated with heat-treated c-HSP60 or LPS (100°C, 20 min) for 5 h. Luciferase and β-galactosidase assays were performed as described in *Materials and Methods.* Data shown are the mean and SD of three independent experiments and are expressed as percentage of luciferase activity induced by LPS or cHSP60 in empty vector control cells (indicated as 100%).

infiltrating macrophages within human atheromatous tissue (23). The 60-kDa cHSP60 is an inflammatory chlamydial protein implicated in the pathophysiology of chronic chlamydial infections (53) and induces secretion of inflammatory cytokines or expression of adhesion proteins by endothelial cells, smooth muscle cells, and macrophages (26). Anti-cHSP60 Ab isolated from patients with coronary artery disease cross-reacts with human HSP60 to mediate endothelial cytotoxicity in the presence of complement (54). Thus, several studies suggest indirectly that cHSP60 is involved in the pathogenesis of atherosclerosis. cHSP60 may function in two ways to promote atherosclerosis: first, by direct antigenic stimulation and, second, as a signal transducer that triggers activation of cells within atheromatous lesions.

HSPs or chaperonins are generally considered to act intracellularly to preserve cellular protein stability in response to conditions such as heat shock, nutrient deprivation, infections, and inflammatory reactions (24, 25). The ability of cHSP60 or human HSP60 to activate human vascular cells and to trigger NF-κB activation suggests a novel amplification loop in vascular inflammation. Chronic, persistent *C. pneumoniae* infection could provoke the expression of cHSP60 in the vessel wall. This protein could then amplify the ongoing inflammatory process through its actions on human vascular cells.

How cHSP60 interacts with host cells, including vascular endothelial cells, to transduce activating signals is not completely understood. Recent evidence supports the hypothesis that cHSP60 activates the innate immune system via TLRs, the sensors of innate immunity. TLRs are transmembrane proteins with an extracellular domain consisting of leucine-rich repeats involved in recognition of microbial components. To date, at least 10 TLRs have been identified in humans, but only a few of their ligands have been identified (41–45). Because of its potential role in the pathogenesis of atherosclerosis, several laboratories have begun to investigate the interaction of cHSP60 with cell surface receptors. Ohashai et al. (28) reported that human HSP60 stimulates TNF-α and NO production in mouse macrophages, whereas macrophages derived from C3H/HeJ mice that express a nonfunctional form of TLR4 were unresponsive to HSP60, supporting a role of TLR4 in human HSP60-induced cell activation (28). Also, CD14-dependent signaling by human HSP60 has been reported in U373 cells, an astrocytoma cell line that expresses TLR4 but not TLR2 (27). However, Vabulas et al. (29) reported that endogenous and cHSP60 are recognized by both TLR2 and TLR4. More recently, Sasu et al. (50) reported that *C. pneumoniae* elementary bodies and cHSP60 stimulate vascular smooth muscle cell proliferation in vitro. Proliferation in that system is attenuated by a TLR4 antagonist, suggesting TLR4-dependent signaling (50). In another recent study, *C. pneumoniae* was shown to be taken up by bone marrow-derived murine dendritic cells and the recognition of the organism by these cells depends largely on TLR2 and only to a minor extent on TLR4 (55).

To better understand how cHSP60 activates cells of the immune system, including endothelial cells (EC) and macrophages, we used EC and macrophage cell lines and various nonsignaling TLR constructs to identify potential receptors and signaling pathways stimulated by cHSP60. We demonstrated that TLR4 but not the TLR2 is the signaling receptor for recombinant cHSP60. cHSP60 induced NF-κB and human IL-8 promoter activity in both HMEC and macrophages in a dose-dependent manner. We have previously shown that HMEC respond to LPS through TLR4, but do not express TLR2 and are unresponsive to known TLR2 ligands (31, 33). cHSP60 induced a strong response in HMEC, thus excluding the role of TLR2 in this response. Overexpression of nonsignaling TLR4 constructs and anti-TLR4 mAb blocked cHSP60- as well as LPS-induced NF-κB activation, whereas the dominant-negative

TLR2 construct and anti-TLR2 mAb had no effect. These findings suggest that cHSP60 activates HMEC and macrophages through TLR4 and not TLR2. The biological effect of the recombinant cHSP60 was not due to endotoxin contamination, as the concentration of LPS in the recombinant cHSP60 preparation used was below the level needed for cellular activation, and heat treatment abolished the ability of cHSP60 but not of LPS to induce NF-κB activation. Furthermore, a mAb against cHSP60 significantly blocked cHSP60-mediated NF-κB activation, while an isotype-matched control Ab had no effect. Taken together, these observations suggest that the biological effects that we observed were specific to cHSP60.

Our results are consistent with the recent studies, which suggest that recombinant human HSP60 signals through TLR4 (27, 28), and with a more recent study by Sasu et al. (50) showing that cHSP60-mediated signaling proceeds via TLR4 as well. Our data differ from those reported by Vabulas et al. (29), who described that TLR2-transfected 293T cells are responsive to cHSP60. The purity of the cHSP60 protein used by these investigators was not noted, and therefore the response of TLR2-transfected 293T cells to cHSP60 in their study may have been due to contamination of HSP60 with lipoproteins which utilize the TLR2 pathway. Indeed, earlier studies with the TLR2 overexpression system in 293 cells found that TLR2 could transduce enteric LPS signaling (56). However, follow-up studies determined that the TLR2 signaling was triggered by contaminating lipoproteins in the commercial LPS preparations, while protein-free enteric LPS signals through TLR4 (57). We have used recombinant cHSP60 as opposed to purified protein from *Chlamydia* and observed that this protein signals via TLR4 and not through TLR2.

Recent studies have established MyD88-dependent and MyD88-independent pathways for TLR4 signaling (48, 49). Although both pathways induce NF-κB activation, only the MyD88-dependent activation pathway of NF-κB leads to cytokine production. The MyD88-independent activation pathway is linked to dendritic cell maturation (48, 58). Our data indicate that overexpression of a dominant-negative mutant of MyD88 (ΔMyD88), which inhibits both LPS- and IL-1-induced NF-κB activation, inhibits cHSP60-induced NF-κB activation. Our data further extend our current understanding of cHSP60-mediated signaling by demonstrating that this pathway is MyD88 dependent.

We recently demonstrated that TLR4 is preferentially expressed by infiltrating macrophages in murine and human lipid-rich atherosclerotic lesions where it may play a role to enhance and sustain the innate immune and inflammatory responses (59). Given that TLR4 plays a critical role in inflammatory signaling, and that chlamydial Ags such as cHSP60 signals through TLR4, TLR4 may represent a biochemical link between chronic infection and atherosclerosis. In this context, ultimately, understanding how cHSP60 interacts with host cells to transduce signals and promote atherogenic events such as LDL oxidation may elucidate how *C. pneumoniae* contributes to the pathogenesis of atherosclerosis.

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