Mycobacterium Tuberculosis Heat Shock Proteins Use Diverse Toll-like Receptor Pathways to Activate Pro-inflammatory Signals*

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Yonca Bulut‡§, Kathrin S. Michelsen§§, Linda Hayrapetian¶, Yoshikazu Naikii, Ralf Spallke¶, Mahvir Singh§, and Moshe Arditi**

From the Divisions of ‡Pediatric Critical Care, and §Pediatric Infectious Diseases, Cedars-Sinai Medical Center, David Geffen School of Medicine at UCLA, Los Angeles, California 90048 and ¶Lionex Diagnostics and Therapeutics GmbH, Mascheroder Weg 16, 38124 Braunschweig, Germany

Although the Toll-like receptors used by Mycobacterium tuberculosis membrane and secreted factors are known, the pathways activated by M. tuberculosis heat shock proteins are not. An efficient immune response against the intracellular pathogen M. tuberculosis is critically dependent on rapid detection of the invading pathogen by the innate immune system and coordinated activation of the adaptive immune response. Macrophage phagocytosis of M. tuberculosis is accompanied by activation of the transcription factor NF-κB and secretion of inflammatory mediators that play an important role in granuloma formation and immune protection during M. tuberculosis infection. The interaction between M. tuberculosis and the various Toll-like receptors is complex, and it appears that distinct mycobacterial components may interact with different members of the Toll-like receptor family. Here we show that recombinant, purified, mycobacterial heat shock proteins 65 and 70 induce NF-κB activity in a dose-dependent manner in human endothelial cells. Furthermore, we show that whereas mycobacterial heat shock protein 65 signals exclusively through Toll-like receptor 4, heat shock protein 70 also signals through Toll-like receptor 2. Mycobacterial heat shock protein 65-induced NF-κB activation was MyD88-, TIRAP-, TRIF-, and TRAM-dependent and required the presence of MD-2. A better understanding of the recognition of mycobacterial heat shock proteins and their role in the host immune response to the pathogen may open the way to a better understanding of the immunological processes induced by this important human pathogen and the host-pathogen interactions and may help in the rational design of more effective vaccines or vaccine adjuvants.

Mycobacterium tuberculosis continues to be a major global health problem, infecting nearly one-third of the world’s population and killing at least 3 million people each year (1, 2). The immune response mounted to the infection is generally successful in containing, although not eliminating, the pathogen (1). Traditionally, protective immunity to M. tuberculosis has been ascribed to T cell-mediated immunity, with CD4+ T cells playing a crucial role. This is primarily because the organism lives within cells, usually macrophages; thus T cell effector mechanisms, rather than antibody responses, are required to control or eliminate the bacteria (1). The breakdown of immune responses designed to contain the infection can result in reactivation and replication of the bacilli, with necrosis and damage to lung tissue (1). Recent immunological and genetic studies support the model that innate immunity plays an important role in host defenses against M. tuberculosis (3). An efficient immune response against the intracellular pathogen M. tuberculosis is critically dependent on the rapid detection of the invading pathogen by the innate immune system and the coordinated activation of the adaptive immune response, but a comprehensive understanding of the molecular mechanisms mediating these responses has only recently been addressed (4–11).

Emerging evidence suggests that Toll-like receptors (TLRs), which are critical pattern recognition molecules that alert the host to the presence of microbial pathogens, contribute to innate immunity by detecting M. tuberculosis-associated molecular patterns and mediating the secretion of various cytokines and antibacterial effector molecules (12). In addition, TLRs influence the adaptive immune response by up-regulating co-stimulatory molecules to support the development of a Th1 response (12). TLRs comprise a family of at least eleven cell-surface pattern recognition molecules that alert the host to the presence of microbial pathogens (12). TLR4 initiates signaling cascades in response to lipopolysaccharide (LPS) (13), a major component of the outer membrane of Gram-negative bacteria, as well as to Taxol (14) and endogenous (15) or chlamydial heat shock protein 60 (HSP60) (16–18), whereas TLR2 recognizes various fungal, Gram-positive (4, 13), and mycobacterial cell wall components (6, 19) such as peptidoglycan, lipoteichoic acid, soluble tuberculosis factor (STF), and lipoarabinomannan.

It appears that distinct mycobacterial components may interact with different members of the TLR family (19), thus increasing the likelihood that a pathogen will be recognized by several mechanisms. Indeed, recent studies have demonstrated that the M. tuberculosis 19-kDa lipoprotein, a potent inducer of T cell responses, activates murine and human macrophages to secrete TNF and nitric oxide (7) and inhibits major histocompatibility complex II antigen processing via interaction with TLR2 (8). Lipoarabinomannan, a glycolipid that is abundant in

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§ These authors contributed equally to this work.

¶ To whom correspondence should be addressed: Cedars-Sinai Medical Center, Division of Pediatric Infectious Diseases, 8700 Beverly Blvd., Rm. 4221, Los Angeles, CA. 90048. Tel.: 310-423-4471; Fax: 310-423-8284; E-mail: moshe.arditi@csbs.org.

1 The abbreviations used are: TLR, Toll-like receptor; Δ, dominant negative; HMEC, human dermal endothelial cell; HSP, heat shock protein; IL, interleukin; LPS, lipopolysaccharide; Mtb, mycobacterial; MyD88, myeloid differentiation factor 88; sCD14, soluble CD14; STF, soluble tuberculosis factor; TIR, Toll/IL-1 receptor; TRIF, TRAM domain-containing adapter protein; TRIF, TRAM domain-containing adapter inducing interferon-β; TNF, tumor necrosis factor; TRAM, TRIF-related adapter molecule.
the mycobacterial cell wall, is a key molecule in eliciting cytokine secretion by macrophages (9). Uncapped lipoarabinomannan (AraLAM) induces cell activation via TLR2 (5, 6). STF contains mannosylated phosphatidylinositol that possesses TLR2 agonist activity (20). Furthermore, we demonstrated previously a functional interaction between TLR2 and TLR6 in the cellular response to STF (21). TLR2 activation leads to the killing of intracellular M. tuberculosis in both mouse and human macrophages. Whereas in mouse macrophages this pathway is nitric oxide-dependent, in human monocytes and alveolar macrophages it is nitric oxide-independent (11).

In contrast to the findings in some earlier publications, more recent reports found that mycobacterial mannosylated phosphatidylinositol induces both TLR2- and TLR4-dependent signaling (10). Means et al. reported that both soluble and cell-associated mycobacterial factors can activate TLR-dependent signaling in a CD14-independent manner (5). They observed that a soluble, heat-stable, and protease-resistant mycobacterial factor mediated TLR2-dependent activation, whereas a heat-labile, cell-associated mycobacterial factor activated cells in a TLR4-dependent manner (5, 19). Therefore, whereas purified mycobacterial antigens such as 19-kDa lipoprotein, lipoarabinomannan, and STF preferentially interact with TLR2, infection with whole bacilli evokes a more complex activation pattern involving at least TLR2 and TLR4 and leads to differential activation of antibacterial effector pathways (5–8, 19, 22).

Several experimental animal models suggest that perhaps both TLR2 and TLR4 play a role in innate responses to M. tuberculosis infection in vivo (23–25). A recent study suggested that TLR2 and TLR4 are redundant to control M. tuberculosis infection and that only at extremely high infectious doses was survival reduced in TLR2-deficient mice (26). Another study demonstrated that both standard and high doses of aerosol infection with live M. tuberculosis resulted in significantly increased bacterial loads, defective granulomatous response, and chronic pneumonia in TLR2-deficient mice (27). Whereas studies with C3H/HeJ mice, a strain harboring a mutation in the signaling domain of TLR4 that renders mice unresponsive to LPS, showed that TLR4 may not play a significant role in immunity to M. tuberculosis (28, 29), other studies have shown that C3H/HeJ mice have a reduced capacity to eliminate mycobacteria from the lungs, with spreading of the infection to the spleen and liver, whereas the wild-type mice controlled the infection (10). These authors suggested that TLR4 signaling appears to be required to control the local growth and dissemination of M. tuberculosis infection from lungs (10, 23, 24). In summary, previous studies suggest that the interaction between M. tuberculosis and the various TLRs is complex, and it appears that distinct mycobacterial components may interact with different members of the TLR family.

Mycobacterial (Mt) HSPs may also participate in cytokine expression resulting from infection by M. tuberculosis (30). HSPs stabilize cellular proteins in response to diverse sources of stress or injury (31, 32). HSPs also have a number of immunological effects, including the induction of pro-inflammatory cytokines (15, 16, 31–34). We have shown that chlamydial HSP60 signals through TLR4 and MD-2 (16), and other studies suggest that HSP60 and HSP70 may signal through pathways dependent upon both TLR2 and TLR4 (33, 34). It is not known whether Mt HSPs are recognized by and signal through TLRs. Given the importance of Mt HSPs 65 and 70 in the pathogenesis of the M. tuberculosis infection, we sought to clarify the role of TLRs in the innate immune response to these ligands. Here we report that recombinant purified M. tuberculosis HSP65 signals exclusively through TLR4, whereas M. tuberculosis HSP70 signals through both TLR4 and TLR2 to activate the innate immune system in a myeloid differentiation factor 88 (MyD88)-, Toll/interleukin-1 receptor (TIR) domain-containing adapter protein (TIRAP)-, TIR domain-containing adapter-inducing interferon-β (TRIF)-, and TRIF-related adapter molecule (TRAM)-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Immortalized human dermal endothelial cells (HMECs) (a generous gift from Dr. Francisco J. Candal, Center for Disease Control and Prevention, Atlanta, GA) were cultured as described earlier (35). Tissue culture reagents were from Invitrogen. HEK 293 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured as recommended.

**Recombinant Purified M. tuberculosis HSP65 and HSP70**—Purified recombinant M. tuberculosis HSP65 and HSP70 were produced using a bacterial expression system. For Mt HSP70, protein was obtained from heat-induced (42 °C) cells of Escherichia coli strain M1592 with plasmid pKAM2101. Cells were lysed by sonication. After centrifugation, the recombinant 70-kDa protein was purified by anion exchange chromatography (Q-Sepharose), followed by ATP affinity chromatography and again by anion exchange chromatography. The purified protein was treated with proteinase K at an 10 nm ammonium bicarbonate (32, 36). For Mt HSP65, the protein was obtained from the heat-induced (42 °C) E. coli K12 strain M1546, which carries the plasmid pRB1300. Cells were lysed by sonication, and the supernatant was subjected to Q-Sepharose anion exchange chromatography. The fractions containing HSP65 were further purified on a Mono Q column. The buffer was exchanged to 10 mM ammonium bicarbonate by dialysis (32). All reagents were purchased from Sigma-Aldrich.

**Human Clinical Studies**—Human whole-blood cultures were used to assess the effects of mycobacterial factors on innate immune responses. Whole-blood cultures (200 μl) were stimulated with Mtb HSP65 (0.3–10 μg/ml), LPS (0.5 μg/ml; Escherichia coli K12 strain M1546, which carries the plasmid pRB1300), or recombinant 70-kDa protein (0.5 μg/ml; Associates of Cape Cod, East Falmouth, MA). Purified, protein-free E. coli K23S LPS was obtained from Dr. Kensuke Miyake (University of Tokyo). Transient transfection of cultured cells was conducted using FuGENE 6 transfection reagent (Roche Applied Science) as described earlier (21, 35, 37). A FLAG-tagged human MD-2 cdna construct was obtained from Dr. Renske Miyake (University of Tokyo). Transient transfection of cultured cells was conducted using FuGENE 6 transfection reagent (Roche Applied Science) as described earlier (21, 35, 37). HMECs were also stimulated in the presence or absence of serum with or without recombinant human soluble CD14 (sCD14) (0.15 μg/ml; R&D Systems, Minneapolis, MN). The plasmid DNA for pCMV-β-galactosidase (0.1 μg), ELAM-αN-f-B luciferase (0.5 μg), pcMV empty vector (0.5 μg), MyD88 (0.5 μg), TIRAP (0.5 μg), and ΔMyD88 (0.5 μg) were co-transfected as described earlier (21, 35, 38). The total amount of DNA transfected was kept constant with a pcMV empty vector. After overnight transfection, cells were stimulated for 5 h with LPS (20 ng/ml), Mt HSP65 (0.3–10 μg/ml), or Mt HSP70 (0.3–10 μg/ml). Luciferase and β-galactosidase activity were measured as reported previously (35, 37, 38).

**Generation of Bone Marrow-derived Macrophages from TLR2-, TLR4-, TRAM-, and TRIF-deficient Mice—TLR2-deficient mice, TRAM-deficient mice, and TRIF-deficient mice were kindly provided by Dr. Bruce Beutler (The Scripps Research Institute, La Jolla, CA). Bone marrow cells were flushed from femurs and tibias of mice by complete medium (Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and streptomycin) and washed three times with complete medium. Cells were cultured for 3 days in complete medium supplemented with murine macrophage colony-stimulating factor (M-CSF) (BIOSOURCE). On day 6 adherent cells were fed with fresh M-CSF-containing medium. On day 6 adherent cells were fed with complete Dulbecco’s modified Eagle’s medium and used in experiments on day 8. Briefly, macrophages were stimulated with Mt HSP65, Mt HSP70, LPS, or lipopeptide (2 μg/ml) (palmitoyl-Cys(RS)-2,3-di(palmitooylxyloxy)-propyl-Ala-Gly-OH; Bachem, Torrance, CA.) for 24 h. Supernatants were harvested and assessed for cytokine release using murine TNF-α or IL-6 enzyme-linked immunosorbent assay kits according to the manufacturer’s instructions (BD Biosciences).
**RESULTS**

*M. tuberculosis* HSP65 and HSP70 Induce NF-κB Activation in HMECs in a Dose-dependent Manner—HMECs express TLR4 but not functional TLR2 (35). We used this property to first examine the ability of *M. tuberculosis* HSP65 and HSP70 to stimulate NF-κB activation using a reporter gene assay. Cells were stimulated with increasing concentrations of Mt HSP65 (0.3–10 μg/ml), Mt HSP70 (0.3–10 μg/ml), or LPS (20 ng/ml) for 5 h. Data shown are means ± S.D. obtained from one representative experiment of three independent experiments.

Cells were lysed and assayed for luciferase activity induced by LPS or Mtb HSP65 and HSP70 in HMECs. These observations are consistent with the direct involvement of the TLR4 signaling pathway in *M. tuberculosis* HSP65- and HSP70-induced inflammatory responses, we co-transfected ΔTLR4, a dominant negative mutant of TLR4, and measured the response of HMECs to Mt HSP65, HSP70, and LPS by monitoring NF-κB activation. As shown in Fig. 2, the transfection of dominant negative TLR4 completely blocked the response to LPS and to Mt HSP65 and HSP70 in HMECs. These observations are consistent with the interpretation that Mt HSP65 and HSP70 activation of NF-κB is mediated through TLR4-dependent signaling.

Bone Marrow-derived Macrophages from TLR4-deficient or TLR2-deficient Mice Reveal Differences in Signaling Pathways in Response to *M. tuberculosis* HSP65 and HSP70—To examine the relative contributions of TLR2 and TLR4 in *M. tuberculosis* HSP65- and HSP70-induced NF-κB activation, we used complementary approaches. We asked whether cells deficient in TLR2 or TLR4 from the appropriate genetically deficient mouse recognized *M. tuberculosis* HSP65 and HSP70. TNF-α or IL-6 release was measured in response to Mt HSP65 and HSP70 in primary bone marrow macrophages derived from TLR4- or TLR2-deficient mice. Macrophages from TLR4 knockout mice did not respond to LPS and Mt HSP65, whereas the responses to Mt HSP65 and LPS were intact in the wild-type macrophages (Fig. 3A). For Mt HSP70 we observed significantly reduced but not absent responses in TLR2-deficient macrophages when compared with wild-type (TLR4+/+) macrophages (Fig. 3A). As expected, compared with wild-type macrophages the TLR2-deficient macrophages responded normally to LPS but not to synthetic lipopeptide, a TLR2 agonist (Fig. 3B). We observed no significant difference in cytokine release in TLR2-deficient versus wild-type macrophages in response to Mt HSP65 (Fig. 3B). However, we observed an ~50% inhibition in IL-6 secretion in response to Mt HSP70 in TLR2-deficient macrophages compared with wild-type macrophages (Fig. 3B). These data suggest that whereas Mt HSP65 signals exclusively through TLR4, Mt HSP70 signals through both TLR4 and TLR2. Furthermore, data from TLR4−/−

**FIG. 1.** *M. tuberculosis* HSP65 and HSP70 induce NF-κB activity in HMECs in a dose-dependent manner. HMECs were transiently transfected with NF-κB-luciferase. Transfected cells were stimulated with LPS (20 ng/ml) or increasing concentrations of Mt HSP65 (0.3–10 μg/ml) (panel A) or Mt HSP70 (0.3–10 μg/ml) (panel B) for 5 h. Cells were lysed and assayed for luciferase and β-galactosidase activities as described under “Experimental Procedures.” *M. tuberculosis* HSP65 and HSP70 induced a dose-dependent activation of NF-κB in HMECs. Data shown are means ± S.D. of one representative experiment of three independent experiments.

**FIG. 2.** Activation of *M. tuberculosis* HSP65- and HSP70-induced NF-κB activation in HMECs is blocked by ΔTLR4. HMEC were transiently transfected with NF-κB-luciferase and co-transfected with ΔTLR4 (0.5 μg). Cells were stimulated with Mt HSP65 (10 μg/ml), Mt HSP70 (10 μg/ml), or LPS (20 ng/ml) for 5 h. Data shown are means ± S.D. from one representative experiment of three independent experiments and are expressed as the percentage of luciferase activity induced by LPS (indicated as 100%).

TLR2 ligand (35, 38). These data demonstrate that Mt HSP65 and Mt HSP70 activation of NF-κB in HMECs is TLR2-independent and that overexpression of TLR2 does not augment NF-κB activation.

Activation of NF-κB by *M. tuberculosis* HSP65 and HSP70 in HMECs is TLR4-dependent—We next determined if NF-κB activation in response to Mt HSP65 and Mt HSP70 in HMECs was mediated by TLR4. To further characterize the direct involvement of the TLR4 signaling pathway in *M. tuberculosis* HSP65- and HSP70-induced inflammatory responses, we co-transfected ΔTLR4, a dominant negative mutant of TLR4, and measured the response of HMECs to Mt HSP65, HSP70, and LPS by monitoring NF-κB activation. As shown in Fig. 2, transfection of dominant negative TLR4 completely blocked the response to LPS and to Mt HSP65 and HSP70 in HMECs. These observations are consistent with the interpretation that Mt HSP65 and HSP70 activation of NF-κB is mediated through TLR4-dependent signaling.
macrophages also demonstrate some preservation of HSP70 signaling, lending further support to the interpretation that HSP70 initiates signals that are mediated by both TLR4 and TLR2.

*M. tuberculosis* HSP65 and HSP70 Utilize Both MyD88-dependent and MyD88-independent Signaling Pathways—TIRAP, the TIR domain-containing adapter protein, has been recently identified as an additional TIR domain-containing adapter molecule involved in LPS-induced activation of the MyD88-dependent pathway (40, 41). To determine whether NF-κB activation in response to stimulation with *Mtb* HSP65 and HSP70 proceeds through MyD88 or TIRAP-dependent pathways, HMECs were co-transfected with dominant negative constructs of MyD88 or TIRAP, respectively. MyD88, which abrogates IL-1 and LPS-induced NF-κB activation, significantly inhibited *Mtb* HSP65- and HSP70-induced NF-κB activation (Fig. 4). Similarly, ΔTIRAP inhibited LPS-, *Mtb* HSP65-, and *Mtb* HSP70-induced NF-κB activity (Fig. 4). These results suggest that, similar to LPS, NF-κB activation after stimulation with *M. tuberculosis* HSP65 and HSP70 is mediated through both MyD88- and TIRAP-dependent pathways. To investigate the involvement of MyD88-independent signaling pathways, we utilized bone marrow-derived macrophages from *Trif*^−/−^ mice (Fig. 5A) and TRAM-deficient mice (Fig. 5B). *Trif*^−/−^ macrophages were severely impaired in their ability to respond to HSP65 and HSP70 as measured by IL-6 release, whereas wild-type macrophages responded normally (Fig. 5B). Similarly, macrophages from TRAM^−/−^ mice did not respond to HSP65 or HSP70 whereas wild-type macrophages responded normally (Fig. 5B).

Activation of NF-κB in Response to Mtb HSP65 and HSP70 Is Not Due to Endotoxin Contamination—To exclude the possibility of endotoxin contamination in recombinant *M. tuberculosis* HSP65- and HSP70-mediated NF-κB activation, we used several different approaches. First, LPS, *Mtb* HSP65, and HSP70 proteins were boiled at 100 °C for 30 min before the stimulation of HMECs. As expected, boiling did not affect LPS-mediated cellular responses. In contrast, boiling abolished both HSP65- and HSP70-induced NF-κB activation in HMECs (Fig. 6). Second, when these ligands were treated with proteinase K prior to stimulation of HMECs there was a complete loss of both HSP65- and HSP70-mediated responses; but again,
LPS-mediated cellular activation remained intact as anticipated (Fig. 6). We also directly measured LPS concentrations in the recombinant Mtb HSP65 and HSP70 and found that the recombinant Mtb HSP65 protein contained 0.056 endotoxin units per 10 μg of protein, and Mtb HSP70 contained 0.03 endotoxin units per 10 μg of protein (1 endotoxin unit = 0.1 ng/ml E. coli LPS). These endotoxin concentrations are insufficient to activate HMECs.

Activation of NF-κB by Mtb HSP65 and HSP70 Is MD-2-dependent—To respond efficiently to LPS, TLR4 requires an accessory protein called MD-2. To investigate the role of MD-2 in the activation of NF-κB by Mtb HSP65 and HSP70, HEK 293 cells were transfected with NF-κB-luciferase and TLR4 cDNA with and without co-transfected MD-2. Cells were stimulated with either recombinant Mtb HSP65 (10 μg/ml) or LPS (20 ng/ml) for 5 h. Data shown are means ± S.D. of one representative experiment of three independent experiments and are expressed as fold increase over untreated cells (indicated as control).

**FIG. 4.** *M. tuberculosis* HSP65- and HSP70-induced NF-κB can be blocked by dominant negative MyD88 and dominant negative TIRAP. HMECs were co-transfected with ΔMyD88 (0.5 μg) or ΔTIRAP (0.1 μg) as well as with reporter genes. The total amount of DNA transfected was kept constant with empty vector. The cells were stimulated with LPS (20 ng/ml), Mtb HSP65 (10 μg/ml), or Mtb HSP70 (10 μg/ml) for 5 h. Data shown are means ± S.D. of one representative experiment of three independent experiments and are expressed as the percentage of luciferase activity (empty vector control transfections, stimulated with the corresponding ligands, have been set to 100%).

**FIG. 5.** *M. tuberculosis* HSP65 and HSP70 signal through the adaptor molecules TRIF and TRAM. Bone marrow-derived macrophages from *Trif*−/− mice (panel A) or TRAM-deficient mice (panel B) were stimulated with Mtb HSP65 (10 μg/ml), HSP70 (10 μg/ml), or LPS (20 ng/ml) for 24 h. Supernatants were harvested and assessed for IL-6 release using an enzyme-linked immunosorbent assay kit. Data shown are means ± S.D. of one representative experiment of three independent experiments.

**FIG. 6.** *M. tuberculosis*-derived HSP65- and HSP70-induced NF-κB activation is not due to endotoxin contamination. HMECs were transiently transfected with NF-κB-luciferase. LPS, Mtb HSP65, and HSP70 were either boiled at 100 °C for 30 min or treated with proteinase K (Prot K) prior to stimulation of HMECs. Cells were stimulated with heat-treated or proteinase K-digested Mtb HSP65 (10 μg/ml), Mtb HSP70 (10 μg/ml), or LPS (20 ng/ml) for 5 h. **no treat.**, no treatment. Data shown are means ± S.D. of one representative experiment of three independent experiments and are expressed as the percentage of luciferase activity induced by untreated LPS (indicated as 100%).

**FIG. 7.** Activation of NF-κB by Mtb HSP65 is MD-2-dependent. HEK 293 cells were transfected with NF-κB luciferase and TLR4 cDNA with and without co-transfected MD-2. Cells were stimulated with either recombinant Mtb HSP65 (10 μg/ml) or LPS (20 ng/ml). Data shown are means ± S.D. of one representative experiment of three independent experiments and are expressed as fold increase over unstimulated cells (indicated as control).

Activation of NF-κB by Mtb HSP65 and HSP70 Is MD-2-dependent—To respond efficiently to LPS, TLR4 requires an accessory protein called MD-2.
FIG. 8. Mtb HSP65 and HSP70 mediated endothelial cell activation is only partially serum- or sCD14-dependent. HMEC were transiently transfected with NF-κB luciferase. HMECs were stimulated with Mtb HSP65, Mtb HSP70, or LPS in the presence or absence of serum with or without the addition of recombinant human sCD14 (0.15 μg/ml). Data are shown as means ± S.D. of one representative experiment of three experiments and are expressed as the percentage of luciferase activity induced by LPS stimulation (indicated as 100%).

DISCUSSION

The current global epidemic of tuberculosis is responsible for 3–4 million deaths each year (44). Chronic inflammation is the hallmark of tuberculosis, and substantial efforts have been made to identify the bacterial components responsible. The immune response to M. tuberculosis is complex and incompletely understood. It appears that distinct mycobacterial components may interact with different members of the TLR family (5). Some attention has focused on M. tuberculosis HSPs 60, 65, and 70 in the pathology of tuberculosis because of their immunogenicity and ability to directly activate monocytes (45, 46).

HSPs are highly conserved intracellular proteins expressed in all prokaryotic and eukaryotic cells, both constitutively and under stress conditions. Several studies have identified HSPs as targets of immune responses during microbial infections (47, 48). Almost two decades ago, Srivastava and colleagues (reviewed in Ref. 49) discovered that some inducible stress proteins, such as HSP70, potently stimulated antigen-specific T cell responses. As HSPs are released from cells as a result of necrotic but not apoptotic death (50), they may be taken up by antigen-presenting cells (51), thereby providing a general pathway through which antigens from cancer cells or infectious agents may be presented to naïve T cells in the lymph nodes (52). Stress proteins such as endogenous HSP70 or M. tuberculosis-derived HSP70 are potent adjuvants and link innate and adaptive immune responses (53).

Dendritic cells and macrophages express CD91 and TLR4, both of which bind mammalian HSP70 (50, 54). Another receptor, CD40, was recently shown to bind HSP70 derived from M. tuberculosis but not mammalian HSP70 (55). Binding of Mtb HSP70 by CD40 leads human dendritic cells to release CC chemokines such as RANTES (regulated on activation normal T cell expressed and secreted) (55). Little is known about how these stress protein receptors induce APC differentiation, except that two or more pathways exist and that MyD88/IRAK/NF-κB is required for at least one of them (33, 50, 54). An increased understanding of recognition of mycobacterial HSPs may open the way to a better understanding of immunological processes induced by this important human pathogen and the host-pathogen interactions.

Our laboratory has been interested in the TLR response to microbial and endogenous HSPs. We demonstrated previously a functional interaction between TLR2 and TLR6 in the cellular response to STF (21). To extend these studies, we investigated the nature of the involvement of TLRs and their downstream adaptor molecules in Mtb HSP-induced signaling. Here we report that both Mtb HSP65 and HSP70 induced a strong response in endothelial cells. Our results demonstrate that Mtb HSP65 signals exclusively through TLR4, whereas Mtb HSP70 signals through both TLR4 and TLR2 to activate the innate immune system. Our data demonstrate that HSP65- and HSP70-induced NF-κB activation depended on MyD88-dependent and -independent signaling pathways. Whereas certain TLR4 ligands do not require CD14, it appears that TLR4-dependent recognition of HSP65 and HSP70 is enhanced by the addition of sCD14. Our findings are consistent with a recent study by Scanga et al., who demonstrated that MyD88-deficient mice fail to control mycobacterial replication and rapidly succumb to M. tuberculosis infection (36). Our results are also in accordance with previous reports showing that TLR4 plays a protective role in host defense against pulmonary tuberculosis (10, 25).

Gao and Tax suggested that the inflammatory cytokine responses to human HSP60 might be confounded by the pres-
ence of small amounts of LPS, because polymyxin B treatment abolished TNFα expression in response to stimulation with HSP60 (43). Although certain recombinant human HSP products may be contaminated with LPS, we have shown previously that chlamydial HSP60 activation of NF-κB and IL-8 activity in macrophages and endothelial cells was not due to LPS contamination (16). In our present study, both heat treatment and proteinase K digestion abolished the ability of Mtb HSP 65 and HSP70 to induce NF-κB activation. It therefore appears unlikely that our results might have been influenced by LPS contamination in our experiments.

A better understanding of the complex network of immune responses to pathogenic components of *M. tuberculosis*, including the role of Mtb HSPs, is critical to the development of effective vaccines and more efficacious treatments for tuberculosis. We conclude that Mtb HSP65 signals through TLR4, whereas Mtb HSP70 utilizes both TLR4 and TLR2. Both effective vaccines and more efficacious treatments for tuberculosis, the role of Mtb HSPs, is critical to the development of responses to pathogenic components of contamination in our experiments.

HSP70 to induce NF-

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