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Toll-like Receptor 4 (TLR4) Is Essential for Hsp70-like Protein 1 (HSP70L1) to Activate Dendritic Cells and Induce Th1 Response*

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Toll-like receptors (TLRs) play important roles in initiation of innate and adaptive immune responses. Emerging evidence suggests that TLR agonists can serve as potential adjuvant for vaccination. Heat shock proteins (HSPs), functionally serving as TLR4 agonists, have been proposed to act as Th1 adjuvant. We have identified a novel Hsp70 family member, termed Hsp70-like protein 1 (Hsp70L1), shown that Hsp70L1 is a potent T helper cell (Th1) polarizing adjuvant that contributes to antitumor immune responses. However, the underlying mechanism for how Hsp70L1 exerts its Th1 adjuvant activity remains to be elucidated. In this study, we found that Hsp70L1 binds directly to TLR4 on the surface of DCs, activates MAPK and NF-κB pathways, up-regulates I-a^b, CD40, CD80, and CD86 expression and promotes production of TNF-α, IL-1β, and IL-12p70. Hsp70L1 failed to induce such phenotypic maturation and cytokine production in TLR4-deficient DCs, indicating a role for TLR4 in mediating Hsp70L1-induced DC activation. Furthermore, more efficient induction of carcinoembryonic antigen (CEA)-specific Th1 immune response was observed in mice immunized by wild-type DCs pulsed with Hsp70L1-CEA$_{576-669}$ fusion protein as compared with TLR4-deficient DCs pulsed with same fusion protein. In addition, TLR4 antagonist impaired induction of CEA-specific human Th1 immune response in a co-culture system of peripheral blood lymphocytes (PBLs) from HLA-A2.1$^+$ healthy donors and autologous DCs pulsed with Hsp70L1-CEA$_{576-669}$ *in vitro*. Taken together, these results demonstrate that TLR4 is a key receptor mediating the interaction of Hsp70L1 with DCs and subsequently enhancing the induction of Th1 immune response by Hsp70L1/antigen fusion protein.

Toll-like receptors (TLRs)[4] are a crucial family of conserved pattern recognition receptors well known for their roles in recognizing specific microbial patterns and allowing the host cells to distinguish between self and non-self molecules. The activation of TLRs by microbial ligands triggers innate immune responses and primes antigen-specific adaptive immunity toward exogenous pathogens (1, 2). Relatively recent appreciation of the ability of TLRs to link innate and adaptive immunity offers a new prospect to consider agonists that engage TLR signaling in vaccine development. There is a growing interest in TLR ligands as agonist for the prevention and treatment of infectious diseases, cancer, and autoimmune diseases. The developments utilizing TLR agonists as vaccine adjuvant are derived mainly from pathogen-associated molecular patterns (PAMPs) like BCG peptidoglycan (3) (recognized by TLR2), poly I:C (4) (by TLR3), CpG motif containing DNA (5) (by TLR9), and monophosphoryl lipid A (MPLA) (6), and Adjuvant System 04 (AS04) (7) (recognized by TLR4). These natural or synthetic agonists elicit a synergistic effect that is attributed to use as vaccine adjuvant. The therapeutic potential of synthetic BCG peptidoglycan, poly I:C, CpG DNA and MPLA have been well documented in different pathophysiological conditions like cancer and infectious diseases (8–10).

In addition to PAMPs, an increasing number of endogenous damage-associated molecular pattern molecules (DAMPs) are being reported as candidate agonists of TLRs. DAMPs that serve as agonists of TLR4 include heat shock proteins (Hsp60, Hsp70, Hsp90, HspB8, and endoplasmin) (11–14), high mobility group box 1 (HMGB1) (15, 16), and uric acid crystals (17). Among those molecules, HSPs, which chaperones a wide array of peptides generated in cells, has attracted increasing attention. HSP-peptide complexes isolated from tumor cells, or reconstituted by covalent cross-link or fusion-protein strategies are critical for loading of MHC-I with epitopes, triggering tumor-specific T-cell responses and antitumor effects via cross-presentation by DCs. Therefore, HSPs-based tumor vaccines have been extensively investigated in both established and experimental vaccines for cancer treatment. HSP-peptide complex 96 (HSPPC-96)-based vaccine, the first HSP-associated autologous cancer vaccine isolated from individual patients' tumors, has shown to be safe and exhibits encouraging results in clinical trials treating melanoma and kidney cancer (Phase III) (18, 19). Thus, HSPs-based vaccines are being considered as a novel therapeutic approach with an important role in cancer treatment.

Hsp70-like protein 1 (Hsp70L1) is a member of Hsp70 family cloned from a cDNA library of human DCs by our laboratory (20). Our previous studies have shown that the Hsp70L1 interacts with DCs and promotes DC maturation and activation

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[4] The abbreviations used are: TLR, Toll-like receptors; HSPs, heat shock proteins; Hsp70L1, Hsp70-like protein 1; DCs, dendritic cells; CEA, carcinoembryonic antigen.

including the stimulation of DCs to secrete several cytokines and chemokines (20). Recombinant fusion protein of carcinoembryonic antigen ($CEA_{576-669}$)-Hsp70L1 enhances the induction of CEA-specific anti-tumor immunity efficiently both *in vitro* and *in vivo* by DCs once pulsed with this fusion protein (21). These previous findings directly highlight the potential application of Hsp70L1 as a novel adjuvant to induce cytotoxic T lymphocytes (CTLs) and Th1 response against cancer cells. However, the molecular mechanisms by which Hsp70L1 engages DCs remain unclear.

Here we investigate the key receptor mediating the interaction of Hsp70L1 with DCs and the mechanism of induction of anti-tumor immunity stimulated by Hsp70L1-$CEA_{576-669}$-pulsed DCs. Our study shows that Hsp70L1 directly binds to TLR4 on the surface of DCs, induces DCs to secrete proinflammatory cytokine and up-regulates costimulatory molecules on DCs via the TLR4 signaling pathway. The CEA-specific Th1 response is also enhanced both *in vitro* and *in vivo* by DCs pulsed with Hsp70L1-$CEA_{576-669}$ fusion protein through the interaction of Hsp70L1 with TLR4 on DCs. Our data demonstrate a key role for TLR4 in mediating Hsp70L1 interaction with DCs and provides further evidence for the rationale of devising strategies to incorporate Hsp70L1 in the development of cancer vaccines.

EXPERIMENTAL PROCEDURES

Animals and Cell Lines—C57BL/6 (H-$2K^b$) wild-type (WT) mice, 6–8 weeks of age, were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. TLR4-deficient mice ($TLR4^{-/-}$, C57BL/6 strain) were kindly provided by Dr. S. Akira (Osaka University, Japan) (22). All mice were housed in a specific pathogen-free facility for all experiments. HEK293 cells and SW480 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured according to American Type Culture Collection instructions. HEK293 cells stably transfected with TLR4 (293-TLR4) were purchased from Invivogen Company.

Proteins—GST-Hsp70L1 expression vector was constructed by inserting the full-length encoding region of Hsp70L1 cDNA into the eukaryotic expression vector pGEX-4T-2 resulting in production of GST-Hsp70L1 fusion protein with a GST tag at the N terminus. The proteins were purified by Source15Q chromatography and GSTrap^{TM}FF chromatography (Amersham Biosciences), achieving >90% purity as confirmed by silver stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified recombinant Hsp70L1′$CEA_{576-669}$ and Hsp70L1-$CEA_{576-669}$ were obtained as described previously (20, 21). Lipopolysaccharide (LPS) contamination was determined <0.1 EU/μg protein by the Limulus amebocyte lysate assay (BioWhittaker).

Generation of DCs—Human peripheral blood monocyte-derived DCs were prepared from healthy volunteers (HLA-$A2.1^+$) as described previously by us (23). Mouse bone marrow-derived DCs (BMDCs) were prepared as described previously (24).

Extraction of Immature DC Membrane Proteins—Human DCs were cultured for 5 days, collected, washed with PBS, and prepared for membrane protein extraction following the manufacturer's procedures with Mem-PER Eukaryotic Membrane Protein extraction reagent kit (Pierce) (25).

FITC-labeled Hsp70L1 Binding with 293-TLR4 Cells—Hsp70L1 proteins were conjugated to fluorescein isothiocyanate (FITC) using FluoroTag FITC conjugation kits (Merck), and the Molar F/P (the ratio of FITC molecules and Hsp70L1 protein) was 6.5. Cell surface binding was performed as described (24). Briefly, HEK293 cells and 293-TLR4 cells were fixed in 2% paraformaldehyde then incubated with 5 μg/ml FITC-Hsp70L1 in phosphate-buffered saline (PBS) for 30 min at 4 °C. Cells were washed twice and analyzed using a FACS Calibur flow cytometer (Becton Dickinson). HEK293 cells and 293-TLR4 cells were cultured on coverslips until the cell density reached 50%, and then transiently transfected with Mem-DsRed vector (Clontech) for 24 h. This vector (pDSRed-Monomer-Mem) expresses a red fluorescent fusion protein that contains a signal for post-translational palitoylation of cysteines, which facilitates targeting of DsRed-Monomer to the cellular membrane. After fixing with 4% paraformaldehyde treatment for 30 min at 4 °C, the cells were stained with 5 μg/ml FITC-labeled Hsp70L1 and analyzed by confocal microscopy (Zeiss LSM, Leica).

Western Blotting—Proteins extracted from HEK293 and 293-TLR4 whole cell lysates were resolved on 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane. After the membrane was blocked at room temperature for 2 h, the membrane was incubated for 6 h with various primary Abs specific for phosphorylated and non-phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK1/2), p38, I-κB kinase (IKK), and I-$\kappa B\alpha$ respectively (Cell Signal Technology). After incubation with peroxidase-conjugated secondary Abs for 1 h at room temperature, the signals were visualized by diaminobenzidine detection (Boster Biotech) according to the manufacturer's instruction, and the bands of protein were scanned and quantified with the Gel-pro Analyzer software (Media Cybernetics) (26).

GST Pull-down—GST- Hsp70L1 bound to glutathione beads (Qiagen) was incubated with total immature DC membrane proteins at 4 °C for 2 h, and then washed with PBS/0.5% Triton X-100 three times. The complexes were released by boiling coated beads in 1× SDS loading buffer and analyzed by SDS-PAGE followed by immunoblotting with an anti-TLR4 antibody (Abcam).

Functional Assessment of Mouse DCs—5-Day DCs from C57BL/6 WT and $TLR4^{-/-}$ mice were cultured with or without 10 μg/ml Hsp70L1 at the concentration 5×10^5 cells/ml for 6, 24, or 48 h, respectively. Secretion of interleukin (IL)-1β, tumor necrosis factor-a (TNF-α), and IL-12p70 into supernatants of DCs was quantified using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems). After 48 h of stimulation with or without 10 μg/ml Hsp70L1, DCs were stained with phycoerythrin (PE)-conjugated anti-CD80, (PE)-conjugated anti-CD40, FITC conjugated anti-Ia^b, or FITC conjugated anti-CD86 monoclonal antibody (mAb, eBioscience) and then analyzed by FACS (Becton Dickinson, Mountain View, CA) and CellQuest software (Becton Dickinson).

Vaccination of Mice and Generation of Antigen-specific Lymphocytes—BMDCs from C57BL/6 WT and TLR4$^{-/-}$ mice were generated as described previously (24). On day 5, BMDCs were harvested and pulsed with 10 μg/ml Hsp70L1-CEA$_{576-669}$, Hsp70L1, or CEA$_{576-669}$ at the concentration of 2×10^6/ml for 6 h. WT and TLR4$^{-/-}$ mice were injected subcutaneously with 1×10^6 protein-pulsed BMDCs per mouse. Three immunizations were given at 1-week intervals. Seven days after the last immunization, splenocytes from mice were harvested and stimulated *in vitro* with syngeneic BMDCs prepulsed with 50 μg/ml SW480 (CEA$^+$) tumor cell lysate antigen (prepared with freezing and thawing SW480 repeatedly) in medium containing 50 IU/ml recombinant human IL-2 (rhIL-2; Sigma).

Generation of Human Antigen-specific Lymphocytes—DCs from healthy donors cultured for 5 days were treated with and without 30 μg/ml of the TLR4 antagonist mAb HTA125(27) (HBT company) and pulsed with 10 μg/ml Hsp70L1-CEA$_{576-669}$, Hsp70L1, or CEA$_{576-669}$ for 6 h. After twice washing, peripheral blood lymphocytes (PBL; 2×10^6) and protein-pulsed autologous DCs (2×10^5) were co-cultured in 1 ml of RPMI 1640 supplemented 10% FBS in 24-well plates. The cells were re-stimulated with fresh protein-pulsed autologous DCs every 7 days for three times. On day 3 after second stimulation, recombinant human IL-2 (Sigma) was added to the final concentration at 20 IU/ml. On day 7 after the last stimulation, cells were harvested and prepared for analysis.

ELISPOT Assay—Splenocytes from C57BL/6 WT and TLR4$^{-/-}$ mice were re-stimulated with 50 μg/ml SW480 (CEA$^+$) tumor cell lysate antigen for 72 h and used as effector cells. 1×10^5 DCs pulsed with SW480 tumor lysate antigen, as stimulator cells, were co-cultured with the 2×10^5 effector cells and seeded into 96-well polyvinylidene difluoride-backed microplates coated with anti-mouse IFN-γ mAb or anti-mouse Granzyme B mAb. After incubation at 37 °C for 24 h, cells were removed, and the plates processed following the manufacturer's protocol of ELISPOT kit (R&D systems). Human antigen-specific lymphocytes induced as described above were used as effector cells. 1×10^5 SW480 tumor cells, as stimulator cells, were co-cultured with the 2×10^5 effector cells and seeded into 96-well PVDF-backed microplates coated with anti-human IFN-γ mAb or anti-human perforin mAb. After incubation at 37 °C for 24 h, cells were removed and the plates processed following the manufacturer's protocol. Resulting spots were counted with ImmunoSpot Analyzer (Cellular Technology Ltd.).

Statistical Analysis—Statistical significance was determined by Student's *t* test, with a value of $p < 0.05$ considered to be statistically significant.

RESULTS

Hsp70L1 Binds TLR4 on the Cell Surface and Activates TLR4 Signaling Pathways—To explore whether Hsp70L1 interacts with TLR4 expressed on cell surface, we employed HEK-293 cells that lack TLR4 expression and HEK-293 cells engineered to express TLR4 (293-TLR4). As shown in Fig. 1*A*, the FITC-labeled Hsp70L1 binds to the surface of 293-TLR4 cells, but is unable to bind to the surface of HEK-293 cells. The association of HSP70L1 with TLR4-expressing cells was further investigated using confocal microscopy. Mem-DsRed serves as a marker of the plasma membrane. FITC-labeled Hsp70L1 clearly associates with TLR4-expressing 293-TLR4 cells and areas of co-localization with the plasma membrane marker are observed (Fig. 1*B*). FITC-labeled Hsp70L1 fails to associate with TLR4 negative HEK-293 cells (Fig. 1*B*). Taken together, these results suggest that Hsp70L1 interacts with membrane TLR4 on the cell surface.

Next, we investigated whether Hsp70L1 was capable of activating downstream signaling pathways through engagement of TLR4. It is well known that both mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) pathways are necessary for transmitting a TLR-triggered immune response (28, 29). We examined ERK1/2, JNK1/2, p38, IKK, and I-κBα activation within both 293-TLR4 and HEK293 cells in the presence and absence of Hsp70L1. Hsp70L1 induces activation of the ERK1/2, JNK1/2, p38, in 293-TLR4 cells but not in HEK 293 cells (Fig. 2*A*). Many signals are capable of triggering phosphorylation of I-κB directly, which leads to I-κB ubiquitination and degradation. This event is followed by NF-κB activation and translocation to the nucleus, where NF-κB can initiate the production of a select set of genes (26). Our results reveal that Hsp70L1 triggers IKK and I-κBα phosphorylation in 293-TLR4 cells, but not in HEK293 cells (Fig. 2*B*). This data suggests that the interaction between Hsp70L1 and TLR4 is critical for the ability of Hsp70L1 to activate MAPK and NF-κB signaling pathways.

Membrane TLR4 Mediates Binding of Hsp70L1 with DCs— Our previous studies demonstrated that Hsp70L1 associates with and subsequently activates DCs. However, the receptor mediating Hsp70L1 interaction with DCs has not yet been identified. To determine whether TLR4 expressed by DCs associates with Hsp70L1, we constructed a GST-Hsp70L1 fusion protein and purified the GST-Hsp70L1 fusion protein by Source15Q and GSTrap^{TM}FF chromatography and visualized by SDS-PAGE (Fig. 3*A*). Equal GST-Hsp70L1, GST and bare-glutathione beads were incubated with immature DC membrane protein in GST pull-down experiments (Fig. 3*B*). TLR4 is detected in total DC membrane protein (Fig. 3*B*, *lane 4*) and found to specifically associate with GST-Hsp70L1 (Fig. 3*B*, *lane 1*), but fails to associate with GST alone or the bare glutathione beads (Fig. 3*B*, *lanes 2* and *3*). Therefore, TLR4 expressed on the membranes of DCs is capable of binding Hsp70L1.

Hsp70L1 Induces Maturation of DCs in a TLR4-dependent Manner—Induction of DC maturation by Hsp70L1 was previously reported by us (20). In order to investigate the role of TLR4 in the mediation of DC maturation by Hsp70L1, we prepared DCs from WT or TLR4$^{-/-}$ mice, and examined phenotypic changes of each set of DCs following stimulation with Hsp70L1. We found that Hsp70L1 treatment promotes the up-regulation of I-a^b, CD40, CD80, and CD86 expression on WT DCs. In contrast, Hsp70L1 treatment of TLR4$^{-/-}$ DCs fails to promote any significant up-regulation of I-a^b, CD40, CD80, and CD86 expression (Fig. 4*A*). This result suggests that TLR4 serves as a functional receptor linking maturation of DCs with exposure to Hsp70L1.

Next, we explored whether the ability of Hsp70L1 to stimulate cytokine production in DCs was dependent on the presence of

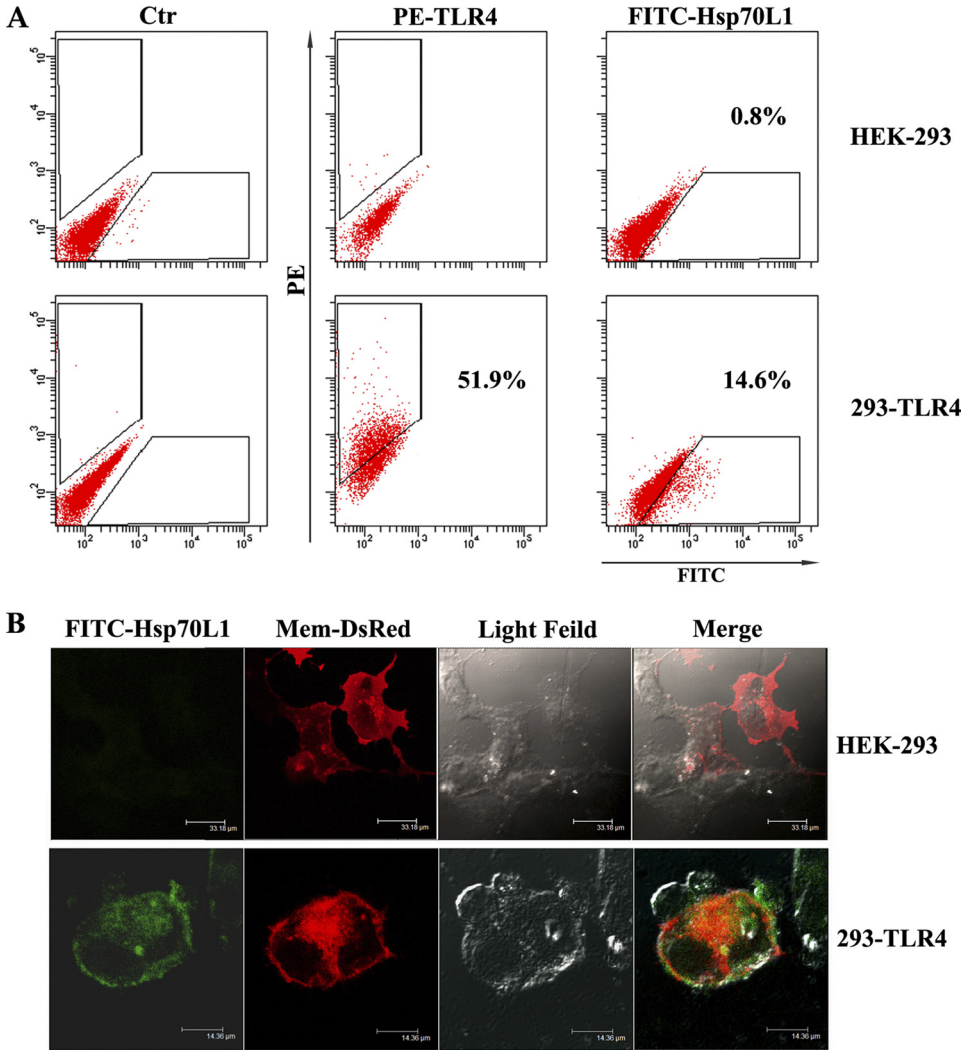

FIGURE 1. **Hsp70L1 binds 293-TLR4 on the cell surface.** *A*, HEK 293 cells and 293-TLR4 cells were incubated with 2 μg/ml PE-TLR4 or 5 μg/ml FITC-labeled Hsp70L1 for 30 min at 4 °C. After washing, the cells were analyzed by flow cytometry. *Column 1*, isotype controls (HEK 293 cells, *upper*, and 293-TLR4 cells, *lower*); *column 2*, HEK 293 cells (*upper*) and 293-TLR4 cells (*lower*) stained with PE-conjugated anti-TLR4 mAb; *column 3*, HEK 293 cells(*upper*) and 293-TLR4 cells (*lower*) incubated with FITC-labeled Hsp70L1. *B*, HEK 293 cells and 293-TLR4 cells were harvested and cultured on coverslips at the density of 50%, and then transiently transfected with Mem-DsRed vector. After 24 h, the cells were fixed by 4% paraformaldehyde for 30 min at 4 °C. After washing, the cells were stained with 2 μg/ml PE-TLR4 or 5 μg/ml FITC-labeled Hsp70L1 and analyzed by confocal microscope. Green fluorescence represents FITC-labeled Hsp70L1, and red fluorescence represents the cell membrane.

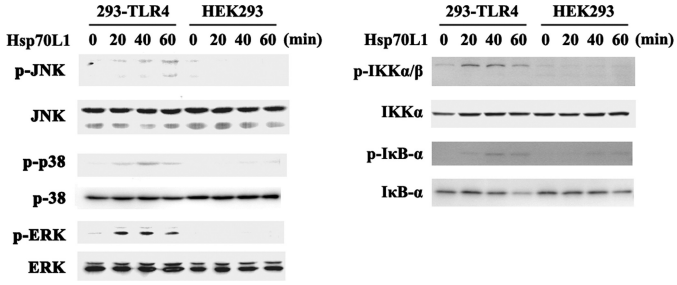

FIGURE 2. **Activation of signaling pathways in 293-TLR4 cells by Hsp70L1.** HEK293 cells and 293-TLR4 cells were stimulated with 10 μg/ml Hsp70L1 for the indicated time. Phospho-ERK, phospho-38, phospho-JNK, phospho-IKKα/β, and phosphor-IκB-α as well as total ERK, p38, JNK, IKKα, and IκB-α were detected by immunoblot.

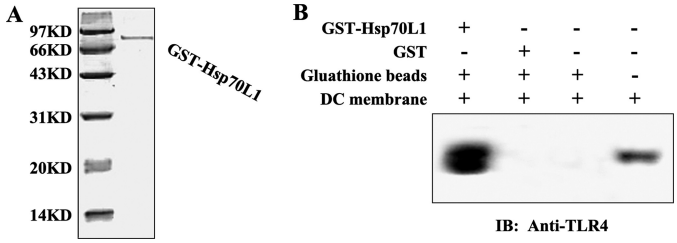

FIGURE 3. **Binding of Hsp70L1 to membrane TLR4 of DCs.** *A*, GST-Hsp70L1 fusion protein was purified and assessed by SDS-PAGE. *B*, for *lane 1* to *lane 3*, GST-Hsp70L1 fusion proteins and GST protein were combined to glutathione-beads, and incubated with membrane protein (1.1 mg/ml, 200 μl) prepared from immature DC. After washing with 20 μl of buffer, complexes were released by boiling, electrophoresed on 10% SDS-PAGE, and subjected to immunoblot with anti-TLR4 antibody. For *lane 4*, membrane protein complexes (1.1 mg/ml, 20 μl) prepared from immature DC were released by boiling, electrophoresed on 10% SDS-PAGE, and subjected to immunoblot with anti-TLR4 antibody to identify TLR4 existence.

TLR4. We observed that TNF-α, IL-1β, and IL-12p70 secretion increases significantly in WT DCs stimulated with Hsp70L1. However, there is no significant production of TNF-α, IL-1β, and IL-12p70 in TLR4$^{-/-}$ DCs stimulated by Hsp70L1 (Fig. 4, *B–D*). The observation that Hsp70LI stimulates cytokine production as well as induces phenotypic maturation in a TLR4-dependent manner further establishes the notion that TLR4 serves as a functional receptor for transducing the affects of Hsp70L1 on DCs.

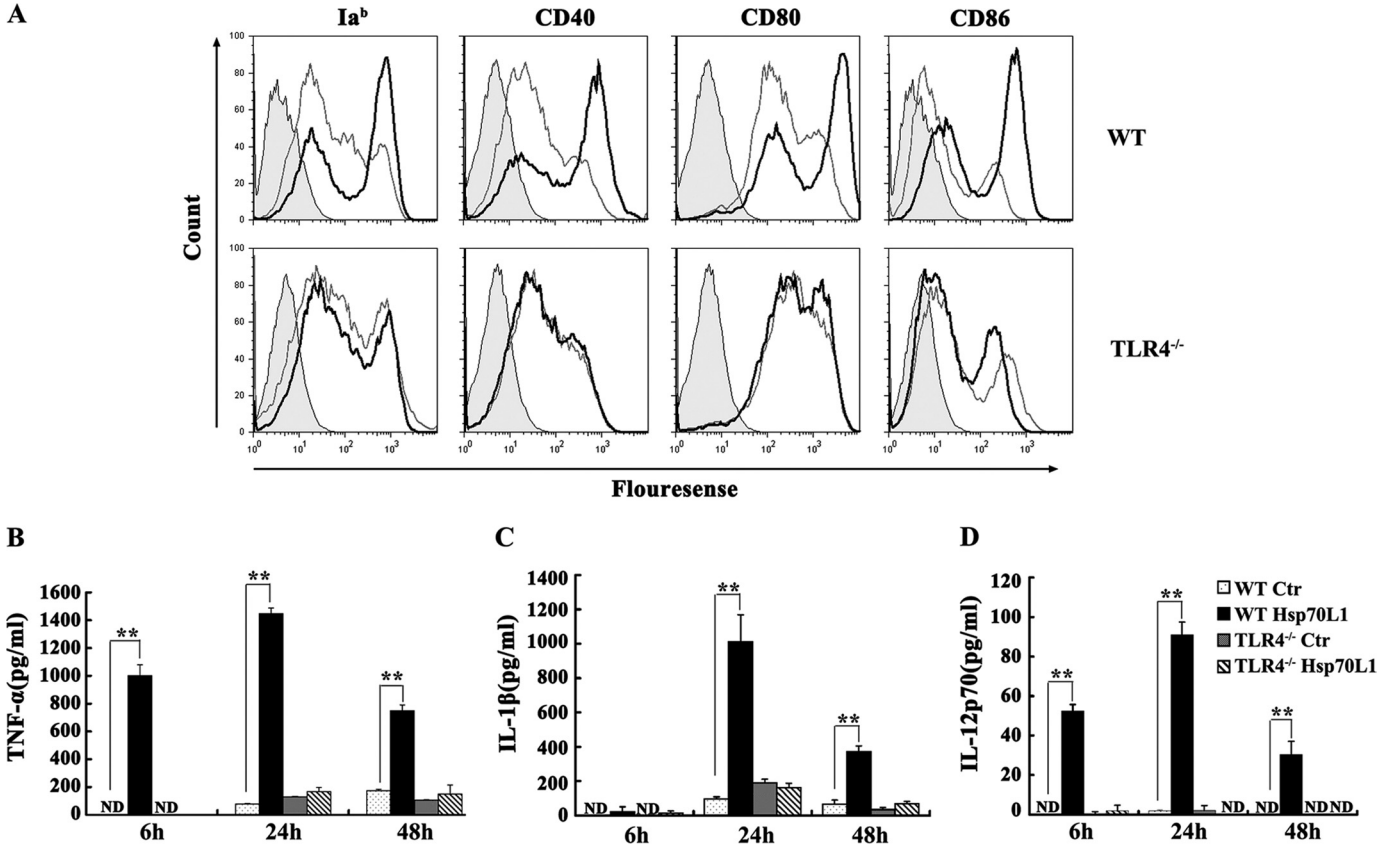

FIGURE 4. **TLR4 is required for phenotypic and functional maturation of mouse DCs induced by Hsp70L1.** *A*, 5 day bone marrow-derived DCs (5×10^5/ml) were stimulated with 10 μg/ml Hsp70L1 for 48 h, then collected for FACS analysis of CD40, CD80, CD86, and I-a^b expression. *Gray* histograms indicate negative control; *open thin line* histograms, unstimulated DCs; and *open thick line* histograms, Hsp70L1-stimulated DCs. Experiments were performed independently at least three times. *B–D*, cytokine production of DCs stimulated with Hsp70L1 protein. Wild type DCs (*WT*) and TLR4-deficient DCs ($TLR4^{-/-}$) were cultured for 5 days and stimulated with or without 10 μg/ml Hsp70L1 respectively for 6, 24, and 48 h. The levels of TNF-α, IL-1β, and IL-12p70 in the supernatants were measured by ELISA. Results are presented as mean S.D. of triplicate samples. (*ND*, not detected) (**, $p < 0.01$).

DCs Pulsed with Hsp70L1-antigen Fusion Protein Induces a Specific T Cell Response in the Mice through a TLR4-dependent Process—Hsp70 has been shown to be a Th1 polarizing adjuvant and is capable of promoting cross-priming of DCs (20, 21). In order to investigate whether Hsp70L1 exhibits such Th1 adjuvant activity, we generated a recombinant Hsp70L1-antigen fusion protein by fusing Hsp70L1 with $CEA_{576-669}$ (Hsp70L1-$CEA_{576-669}$). $CEA_{576-669}$ is a fragment of CEA which contains HLA-A2.1 restricted CTL epitopes. We hypothesize that TLR4 is required to induce a CEA-specific T cell response *in vivo* by DCs pulsed with Hsp70L1-$CEA_{576-669}$ fusion protein. To explore this hypothesis, we immunized WT and $TLR4^{-/-}$ mice, respectively, with syngeneic DCs pulsed with Hsp70L1-$CEA_{576-669}$, Hsp70L1, or $CEA_{576-669}$ proteins. After three rounds of immunization, splenocytes were isolated, stimulated, and assayed for CEA-specific IFN-γ-producing cells and Granzyme B-producing cells by ELOSPOT. As shown in Fig. 5, IFN-γ-producing cells and Granzyme B-producing cells are significantly induced in WT mice immunized with syngeneic DCs pulsed with Hsp70L1-$CEA_{576-669}$ compared with WT mice immunized with syngeneic DCs pulsed with either Hsp70L1 or $CEA_{576-669}$ alone($p < 0.01$). In contrast, IFN-γ-producing cells and Granzyme B-producing cells are not induced in $TLR4^{-/-}$ mice immunized by syngeneic DCs pulsed with Hsp70L1-$CEA_{576-669}$. Thus, TLR4 is required for the induction of T cell responses facilitated by Hsp70L1-$CEA_{576-669}$.

Human DCs Pulsed with Hsp70L1-antigen Fusion Protein Induces a Specific T Cell Response in Vitro in a TLR4-dependent Manner—Finally, we went further to determine whether human DCs presenting Hsp70L1-$CEA_{576-669}$ promote a response characteristic of the Th1 phenotype in a TLR4-dependent manner. PBL from HLA-$A2.1^+$ healthy donors were incubated with autologous DCs pulsed with Hsp70L1-$CEA_{576-669}$. We employed the mAb HTA125, a TLR4 antagonist to interfere with the interaction of TLR4 and Hsp70L1. As shown in Fig. 6, the number of IFN-γ-producing cells and perforin-producing cells induced by DCs pulsed with Hsp70L1-$CEA_{576-669}$ increases significantly when compared with DCs alone or DCs pulsed with either Hsp70L1 or $CEA_{576-669}$ alone($p < 0.01$). The enhancement in number of IFN-γ-producing cells and perforin-producing cells following incubation with DCs pulsed with Hsp70L1-$CEA_{576-669}$ is completely ablated in the presence of the TLR4 antagonist HTA125. These results further demonstrate that TLR4 is critical for the adjuvant attributes of Hsp70L1, likely as a result of their direct interaction facilitating DC activation and efficiently triggering a potent CEA-specific T cell response.

DISCUSSION

A prominent property of the TLRs is to link innate and adaptive immunity, which underlies a novel prospect for developing vaccines aimed at engaging TLR signaling. The presence of TLR ligands as adjuvant in the vaccine strategy has the potential to

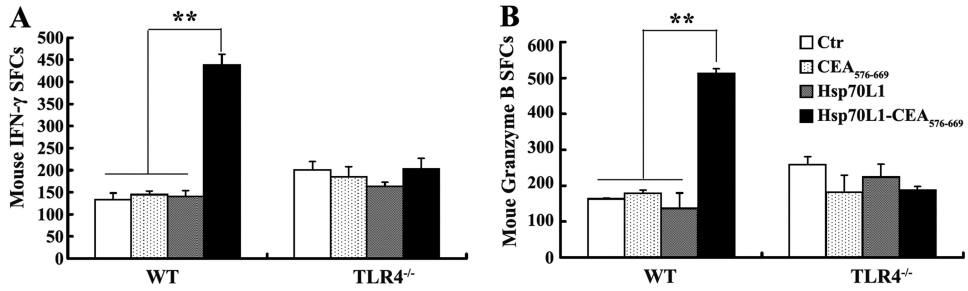

FIGURE 5. **TLR4 mediates *in vivo* induction of specific Th1 immune responses by immunization with Hsp70L1-CEA$_{576-669}$-pulsed DCs.** Wild type (*WT*) mice and TLR4-deficient (TLR4$^{-/-}$) mice were immunized with syngeneic DCs pulsed with Hsp70L1-CEA$_{576-669}$, Hsp70L1, or CEA$_{576-669}$. Then splenocytes were isolated and used to assay specific Th1 immune responses by IFN-γ ELISPOT (*A*) and Granzyme B ELISPOT (*B*). The results were indicated by the number positive SFCs/2 × 10^5 splenocytes. Columns, mean of three independent experiments; bars, S.E. **, $p < 0.01$.

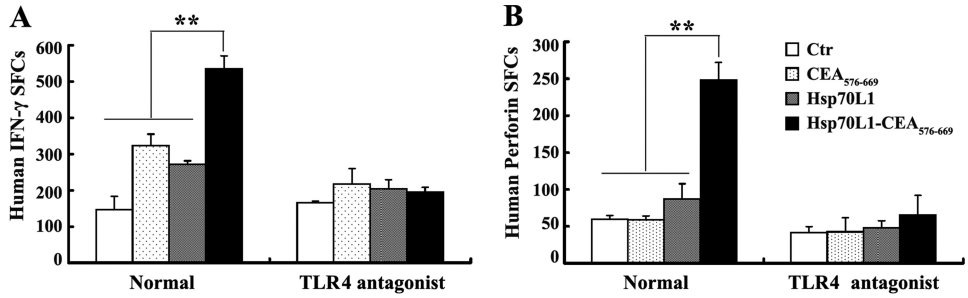

FIGURE 6. **Human DCs pulsed with Hsp70L1-CEA$_{576-669}$ induces specific T cell response through TLR4-dependent manner.** Human PBLs from HLA-A2.1$^+$ healthy donors were stimulated with autologous DCs pulsed with Hsp70L1-CEA$_{576-669}$, Hsp70L1, or CEA$_{576-669}$. For TLR4 blockade experiment, the autologous DCs were blocked with 30 μg/ml TLR4 antagonist HAT125 1 h before DC pulsing. The IFN-γ (*A*) and Perforin ELISPOT (*B*) were used to analyze Th1 immune response. The results were indicated by the number positive SFCs/2 × 10^5 lymphocytes. Columns, mean of three independent experiments; *bars*, S.E. **, $p < 0.01$.

increase the efficacy of immunization toward a given antigen. TLR activation strategies have been used in both established and experimental vaccines for infectious or non-infectious diseases, as well as cancer treatment. The choice of the TLR agonist and the subsequent effects of the vaccine thus become a crucial point in vaccine approach. In addition to well-known microbial ligands, an increasing number of endogenous proteins are being reported as candidate stimulators of TLRs (in particular of TLR2 and TLR4), such as HMGB1 (15, 16), HSPs including Hsp60, Hsp70, endoplasmin, and HspB8 (11–14). During chemotherapy or radiotherapy, HMGB1, which is released from dying tumor cells, can activate TLR4-MyD88 signaling pathway and induce tumor antigen-specific T cell immunity against tumor cells, delineating a clinically relevant immuno-adjuvant pathway triggered by DAMPs (30).

We have identified the Th1 adjuvant effects of Hsp70L1 previously. The effects depended on the interaction with DCs. It becomes critical to find and confirm the binding proteins or the receptors of Hsp70L1 on DCs to deeply reveal the mechanism of Hsp70L1 biofunctions. GST-pull down assay is a useful tool for identification of protein-protein interactions, and also for identification of receptors and even novel receptors. Here we constructed GST-Hsp70L1 fusion protein to affinity capture one or more proteins (either defined or unknown) in DC membrane protein preparations and subsequently to isolate the interacting proteins through the binding of GST to glutathione-coupled beads. Actually we found a string of proteins which could bind to Hsp70L1, including TLR4, TLR2, CD40, Lox-1, etc. These receptors are also speculated to be important in exertion of Hsp70L1 Th1 adjuvant effects. CD40, a member of TNF-α superfamily, plays an important part in T-cell mediated immune response and Hsp70 stimulation of CC-chemokines by DCs and monocytes. Lox-1 and CD91, as scavenger receptors, are also two of the main HSPs binding structures on human dendritic cells, being involved in *in vivo* specifically endocytosis and antigen cross-priming. Distinguished from Lox-1 and CD91, TLR2/4 has been suggested to be involved in Hsp-mediated signaling and activation of APCs but not in representation of HSP-chaperoned peptides. Dybdahl *et al.* (31) found that DCs from TLR2 knock-out mice produced normal amounts of IL-12p40 and TNF-α, whereas DCs from TLR4 knock-out mice did not produce either cytokine. In our study, Hsp70L1 failed to stimulate TLR4$^{-/-}$ DCs, suggesting that the Hsp70L1 protein's effect on DC is primarily TLR4-mediated. So, in current study we focus on TLR4, one of the most important members in TLR family, and identified that TLR4 plays an essential role in mediating the interaction of Hsp70L1 with DCs and subsequent effects.

Here, we revealed that Hsp70L1could bind to TLR4 on the surface of 293-TLR4 cells, but failed to bind to TLR4 negative HEK-293 cells, as demonstrated by both FACS analysis and confocal microscopy. The results are suggestive of Hsp70L1 serving as a novel TLR4 agonist. It is well known that both MAPKs and NF-κB pathways are necessary for TLR-triggered immune response (28, 29). The activation of TLR4 downstream signaling pathways by Hsp70L1 stimulation was observed in 293-TLR4 cells but not in HEK 293 cells. Enhanced phosphorylation of ERK1/2, JNK1/2, p38, and I-κB were all observed in 293-TLR4 cells treated with Hsp70L1. Activation of these kinases are essential to bridge TLR activation with the orchestration of host innate and adaptive immune responses. Our studies herein have thus revealed mechanistic insight into the

biological activity of Hsp70L1, which requires interaction with TLR4.

We previously reported that Hsp70L1 can promote DCs maturation, activate DCs to produce cytokines such as IL-12, IL-1β, and TNF-α, and chemokines such as MIP-1α, MIP-1β, and RANTES, however, the underlying mechanism remained to be elucidated (20, 21). In this study, we constructed a GST-Hsp70L1 fusion protein to determine whether Hsp70L1 interacts with TLR4 expressed by DCs. Indeed, we found that TLR4 present among total DC membrane proteins bound specifically to GST-Hsp70L1, but not to GST or the bare glutathione beads. Furthermore, we found phenotypic changes of up-regulation of I-a^b, CD40, CD80, and CD86 expression and significantly increased secretion of TNF-α, IL-1β, and IL-12p70 by WT DCs, but not $TLR4^{-/-}$ DCs after Hsp70L1 stimulation. Our observations in aggregate provide evidence that Hsp70L1 induces phenotypic and functional maturation of DCs in a TLR4-dependent manner, indicting TLR4 is one of the most important functional receptors mediating biological activities of Hsp70L1.

The activation of TLRs by their cognate ligands leads to inflammatory cytokine production and up-regulation of co-stimulatory signals and MHC molecules, thereby linking innate recognition with adaptive T and B cell immune responses, as well as memory responses after immune system encounters any pathogen (32, 33). Thus, the emerging concept of utilizing TLR agonists as vaccine adjuvant and strategizing to manipulate TLR signaling in this regard are considered herein. Discovery and/or design of novel potent TLR-associated adjuvant will likely contribute to the repertoire of options when devising a vaccine profile. Our previous studies have shown that Hsp70L1 fused with $CEA_{576-669}$, a fragment of CEA containing HLA-A2.1 restricted CTL epitopes, enhances the CEA-specific anti-tumor immunity efficiently both *in vitro* and *in vivo* by DCs pulsed with this fusion protein(20,21). Here, we focused on whether Hsp70L1 exerts an adjuvant effect in a TLR4-dependent manner by exploring whether TLR4 plays a key role in the induction of CEA-specific T cell response by DCs pulsed with Hsp70L1-$CEA_{576-669}$ fusion protein. Results showed that IFN-γ- or Granzyme B-producing cells were induced more significantly in the WT mice immunized with syngeneic DCs pulsed with Hsp70L1-$CEA_{576-669}$, but not in the $TLR4^{-/-}$ mice immunized by syngeneic DCs pulsed with Hsp70L1-$CEA_{576-669}$. HLA-$A2.1^+$ DCs derived from healthy donors and pulsed with Hsp70L1-$CEA_{576-669}$ induced both IFN-γ- and perforin-producing cells in PBLs. However, no induction was observed in the presence of mAb HTA125 utilized to interfere with TLR4 function. We speculate that Hsp70L1-$CEA_{576-669}$ engages DCs via TLR4 recognition of Hsp70L1 facilitating uptake of the fusion protein and DC activation. $CEA_{576-669}$ antigen epitope likely becomes more efficiently presented by DCs to T cells, which triggers a more potent CEA-specific T cell response. Our studies therefore suggest that TLR4 is critical for the adjuvant effect of Hsp70L1 and is required for potent induction of specific T cell responses by Hsp70L1-$CEA_{576-669}$.

Although we used $CEA_{576-669}$ as a model antigen to confirm the adjuvant effects of Hsp70L1 in this study, it is reasonable to predict that Hsp70L1 can be fused with a variety of antigenic peptides or proteins, conferring special adjuvant properties to such antigens in a TLR4-dependent manner. The application of Hsp70L1 in immunotherapeutic strategies, as a new and potent TLR4 agonist adjuvant of mammalian origin, would likely serve to evoke a robust and durable immune response toward a wide range of cancers, as well as infectious diseases.

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