Class I Presentation Pathway

Protein 60-Fused Antigen into the MHC

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Lipoprotein Receptor-1 Delivers Heat Shock
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Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 Delivers Heat Shock Protein 60-Fused Antigen into the MHC Class I Presentation Pathway

Jianhui Xie,* Haiyan Zhu,* Liang Guo,* Yuanyuan Ruan,* Lan Wang,* Lingling Sun,* Lei Zhou,* Weibin Wu,* Xiaojing Yun,* Aiguo Shen,† and Jianxin Gu*  

Heat shock protein (Hsp) 60 elicits a potent proinflammatory response in the innate immune system and has been proposed as a danger signal of stressed or damaged cells to the immune system. Previous studies reported CD14, TLR2, and TLR4 as mediators of signaling but probably not of binding. Although the receptor for Hsp60 was proposed to be saturable and specific on macrophages, it is not well defined. In the current study, we found that lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), as a receptor for Hsp60, could bind and internalize Hsp60 via the C terminus of Hsp60. Yeast two-hybrid assay revealed that the second β-sheet containing the long-loop region of LOX-1 played an important role in this interaction. Furthermore, LOX-1 might be engaged as a common receptor for different Hsp60 species. Bone marrow-derived dendritic cells could cross-present Hsp60-fused OVA Ag on MHC class I molecules via LOX-1. Inhibition of the recognition of Hsp60 by LOX-1 decreases Hsp60-mediated cross-presentation of OVA and specific CTL response and protective tumor immunity in vivo. Taken together, these results demonstrate that LOX-1 functions as a receptor for Hsp60 and is involved in the delivery of Hsp60-fused Ag into the MHC class I presentation pathway. The Journal of Immunology, 2010, 185: 2306–2313.

C-type lectin-like receptors, or nonclassical C-type lectins, share structural homology with C-type lectins, but they lack the residues involved in calcium binding in the C-type lectin-like domain (CTLD) (1, 2). Many members of this family have evolved to recognize nonsugar ligands, but some may be able to recognize carbohydrates via alternative mechanisms (3). The myeloid-expressed receptors seem to have a far more diverse range of ligands and cellular functions, compared with the C-type lectin-like receptors, on NK cells. Accumulating evidence suggests that many C-type lectin-like receptors on myeloid cells recognize endogenous ligands and exogenous structures, which results in numerous cellular responses (4). Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is a member of the scavenger receptor family and is predominantly expressed on the surface of endothelial cells, smooth muscle cells, and dendritic cells (DCs) (5). LOX-1 activation can lead to intracellular signaling, causing endothelial activation or dysfunction, cell proliferation, apoptosis, and atherosclerosis. Recently, LOX-1 was also reported to be associated with the binding of heat shock protein (Hsp) 70 to DCs and involved in Ag cross-presentation (6, 7). Additionally, LOX-1 can bind various ligands, such as apoptotic cells, activated platelets, and bacteria (8–10). However, these surface molecules remain to be identified to further understand their physiological functions. Analogous to the missing self or altered self-ligands that C-type lectin-like receptors on NK cells recognize, the ligands that LOX-1 recognizes are probably expressed at low levels or at specific sites or are expressed only during cellular stress, transformation, or even cell death.

Human Hsp60 is a member of a highly conserved Hsp60 family that includes molecular chaperones from several species, such as human Hsp60, Escherichia coli GroEL, and Mycobacterium bovis Hsp65 (11). Monomer of Hsp60 has three topological domains: an apical domain, to which the substrate and Hsp10 bind; an equatorial domain, which contains a binding site for ATP and the contacts for ring binding; and the intermediate domain, which connects these two domains (12). The N and C terminus of each monomer are involved in the well-ordered and highly helical architecture of the equatorial domain. Human Hsp60 is localized in the mitochondrial matrix, and synthesis of Hsp60 is markedly upregulated during stress (13). Moreover, exposures on the cell surface and release into the extracellular space of Hsp60 have been observed in response to a variety of cellular stress conditions (14). Numerous studies demonstrated that extracellular Hsp60 act as potent intercellular signaling molecules that serve as danger signals to the innate immune system (15). A functional interaction...
of Hsp60 with TLR2 and TLR4 was demonstrated and leads to a proinflammatory response (16–18). However, binding and internalization of Hsp60 to innate immune cells seem to be independent of TLR2, TLR4, or CD14. Although the receptor(s) for Hsp60 were proposed to be saturable and specific on macrophages, the exact surface molecule remains to be elucidated (19).

In this study, we identified Hsp60 as an interaction partner for LOX-1 by a yeast two-hybrid assay. Further investigation reveals that LOX-1 acts as a common receptor for different Hsp60 species and recognizes the C terminus of Hsp60. Furthermore, Hsp60-fused OVA could be delivered into the MHC class I presentation pathway by LOX-1.

Materials and Methods

**Mice and reagents**

C57BL/6 mice were provided by the Shanghai Laboratory Animal Center. OT-I mice were from the Institute of Immunology, Tsinghua University. Anti–LOX-1 mAb 23C11 was from Hycult Biotechnology (Uden, The Netherlands), and 25D1,16 mAb was from eBioscience (San Diego, CA). Anti-MyC Ab was purchased from Invitrogen, and Hsp70 was from StressGen Biotechnologies (Vancouver, British Columbia, Canada). Other Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). All transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions.

**Cell culture**

Wild CHO-K1 cells and stable CHO transfectant cells were grown in Ham’s F-12 medium supplemented with 10% FBS. Stable CHO-LOX-1 and CHO-Dectin-1 cells were produced through transfection of pcDNA3.1-LOX-1 and pcDNA3.1–Dectin-1, respectively, and populations of GA418 (0.8 mg/ml)-resistant cells were generated after 2 wk of cell culture, followed by identification by FACS analysis.

**Yeast two-hybrid assay**

Matchmaker GAL4 two-hybrid system 3 (BD Clontech, Palo Alto, CA) was used to perform yeast two-hybrid screening, according to the manufacturer’s instructions. Briefly, the complete CTLD of LOX-1 was cloned into the pGBK7T vector as the bait. A total of 2.8 × 10⁸ transformants from a human thymus cDNA library (Clontech) were screened in the yeast strain AH109. The plasmids from positive clones were isolated and introduced into E. coli strain DH5α, according to the manufacturer’s instructions. Finally, the clones, harboring target cDNA, were isolated, and cDNA sequences were determined.

**Preparation of recombinant proteins**

The cDNA-coding regions for human Hsp60 (aa 27–573), the N terminus of Hsp60 (Hsp60-N), the apical domain plus intermediate domain of Hsp60 (Hsp60-AM), the C terminus of Hsp60 (Hsp60-C), GroEL, Hsp65, OVA30–359 (aa 230–359), and the extracellular regions of LOX-1 (L-ECDF) and Dectin-1 (D-ECDF) were in-frame cloned into PET22b vector. Hsp60 with C-terminal fusion of OVA30–359 (Hsp60.OVA) and Hsp65 with C-terminal fusion of OVA30–359 (Hsp65.OVA) fusion constructs were generated by cloning the coding sequence for OVA30–359 into the PET22b vector between Hsp60 and C-terminal His tag. Recombinant constructs and pGEX-4T-1 vector were introduced into E. coli BL21. Cells harboring expression vectors were induced with addition of isopropyl β-D-thiogalactoside to a final concentration of 0.1 mM, followed by growth of the culture for 5 h at 30°C. GST was purified using glutathione-Sepharose 4B (General Electric, Fairfield, CT). The inclusion bodies of L-ECDF and D-ECDF were bound to a Ni-NTA column (General Electric) and eluted, followed by dialysis against sequential refolding buffer. All proteins were isolated, and cDNA sequences were determined. cDNA sequences were determined.

**Western blot analysis**

Proteins were immobilized to Ni-NTA Sepharose and incubated with conditioned culture medium from CHO-K1 cells transfected with the soluble LOX-1–Fc chimera generated by cloning the ectodomain of human LOX-1 into the pMuse-mFc2 vector (Invivogen, San Diego, CA) in frame. Beads were washed three times with PBS and then resuspended in SDS sample buffer, boiled for 5 min, and subjected to Western blotting analysis.

**Generation of bone marrow-derived DCs**

To generate bone marrow-derived DCs (BMDCs) from C57BL/6 mice, bone marrow was flushed from the femur and tibia. RBCs were lysed, and the remaining cells were cultured at 1 × 10⁶ cells/ml in complete RPMI 1640 with 10% heat-inactivated PBS and 20 ng/ml GM-CSF. On day 3, fresh medium with GM-CSF was added to the plates, and the cells (immature DCs) were harvested for use on day 5.

**Ag-presentation assays**

A total of 1 × 10⁶/ml BMDCs in FBS-free medium were pulsed with the indicated Ags at various concentrations for 8 h. After being washed and fixed, BMDCs were cultured at 5 × 10⁵/ml with B3Z T cell hybridoma or OT-1 T cells purified from spleens and lymph nodes (5 × 10⁵/ml) in complete RPMI 1640 for 16 h. The stimulation of B3Z T cell hybridomas by BMDCs was monitored by IL-2 release in the supernatants, which was determined by ELISA. For the inhibition assays, BMDCs were incubated with the indicated inhibitors for 30 min prior to the addition of Ags. The effect of SHIFK-1 peptide on IL-2 release by B3Z cells and expression of MHC class I–SHIFK-1 peptide complexes on DCs were evaluated as a control (Supplemental Fig. 4).

**Measurement of induction of CTL and production of IFN-γ**

Groups of four mice were immunized s.c. with PBS, Hsp65, OVA30–359, or Hsp65.OVA at the indicated dosages and were boosted 1 wk later without adjuvant. In some cases, Hsp65.OVA was preincubated at 4°C for 1 h with L-ECDF, D-ECDF, or GST before immunization. One week after the last immunization, single-cell suspensions of pooled spleens were prepared in complete RPMI 1640 medium. Spleen cells were restimulated in complete medium by incubation with 2 × 10⁶ viable lymphoid cells together with 1 μg/ml SHIFK-1 peptide for 7 d in an upright T-25 tissue-culture flask (10 ml). After restimulation, effecter cells were harvested and cultured with 51Cr-labeled E.G7 target cells at the indicated E:T cell ratios. After

**Protein labeling**

Proteins with fluorescent dye were performed using the Alexa Fluor 488 Protein Labeling Kit (Invitrogen), according to the manufacturer’s instructions. Briefly, 1 mg protein was incubated with Alexa Fluor 488 in 0.1 M sodium bicarbonate for 1 h at room temperature, followed by incubation for 3 h at 4°C. Labeled proteins were purified by purification resin. The number of dye molecules bound per protein molecule was determined by measuring the OD at 280 and 494 nm. It was calculated that four to nine Alexa Fluor molecules bound to each protein molecule.

**Flow-cytometric analysis**

Cells were harvested with EDTA and resuspended in PBS for the binding assay (4°C). Cells were incubated with various concentrations of Alexa Fluor 488-labeled proteins or Abs for 45 min. For the competition assays, cells were preincubated with indicated inhibitors for 30 min (4°C) and then the labeled protein was added, and the incubation was continued for 45 min. Subsequently, cells were washed with PBS and resuspended in PBS containing 1% paraformaldehyde. Samples were evaluated using a FACSCalibur flow cytometer. The affinity of Hsp60 and LOX-1 was calculated by subtracting the nonspecific binding from Hsp60 binding.

**Confocal microscopy**

Cells seeded on coverslips were washed twice in PBS and treated with anti-MyC Ab and Alexa Fluor 488-labeled protein at 4°C for 45 min. Cells were washed in PBS and stained with rhodamine-tandem-conjugated anti-mouse Ab at 4°C for 45 min to mark LOX-1 expression. Cells were washed in PBS, followed by fixation with 1% paraformaldehyde for 10 min. For the uptake studies, cells were washed and subsequently incubated for 15 min at 37°C before fixation. Finally, cells were washed in PBS and mounted on glass slides using Fluoromount mounting medium (Sigma-Aldrich, St. Louis, MO). Labeled cells were visualized using a Zeiss LSM 510 confocal microscope.

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Hsp60 binds to LOX-1 on CHO cells. A, CHO-K1 cells were stably transfected with pcDNA3.1/LOX-1 and pcDNA3.1/Dectin-1, and expression was confirmed by FACS analysis with anti-Myc Ab. CHO-K1 cells stably transfected with pcDNA3.1 vector (Mock) was used as a control.

OVA was preincubated at 4°C for 1 h with molar-equivalent of L-ECD or GST before injection. One week after the last immunization, mice were challenged s.c. with $1 \times 10^5$ E.G7 cells on the right flank. Tumor growth was assessed at different time points, and tumor volume was determined by the formula: $0.5 \times \text{length} \times \text{width}^2$.

**Statistical analysis**
Data were expressed as the mean ± SD. Statistical analysis was performed using the Student two-tailed t test. Differences were considered statistically significant at $p < 0.05$.

**Results**

Interaction of the C terminus of Hsp60 with CTLD of Dectin-1 and LOX-1 in yeast

Yeast two-hybrid analysis was carried out to screen human thymus cDNA library with the complete CTLD of LOX-1 (residues 2308–2343) and Dectin-1 (residues 156–234). Results are expressed as the percentage of corrected lysis, calculated using the formula: 100% × (experimental release − spontaneous release)/(maximal release − spontaneous release). Maximum release was obtained by adding 1% Triton X-100 to target cells, and spontaneous release was determined with target cells incubated in culture medium.

To measure the production of IFN-γ in response to SIINFEKL, splenocytes were plated on 96-well plates at $5 \times 10^5$ cells/well with 1 µg/ml peptide. Supernatants were removed 72 h later, and IFN-γ was measured by ELISA.
binding of proteins to cells was detected by FACS. To evaluate the binding of Hsp60 to LOX-1, CHO-K1 cells stably transfected with the LOX-1 construct (CHO–LOX-1 cells) were established, and the level of expression was verified by FACS analysis (Fig. 1A). Next, the binding of Hsp60 to LOX-1 was investigated. At 4˚C, CHO–LOX-1 cells were incubated with Alexa Fluor 488-Hsp60, and FACS analysis was performed. As shown in Fig. 1B, Hsp60 significantly bound to CHO–LOX-1 cells. As controls, Alexa Fluor 488-BSA did not bind to these cells (Supplemental Fig. 3A). In contrast, there was a relatively weak binding of Hsp60 to CHO-K1 cells stably transfected with Dectin-1 (Fig. 1B). The weak binding of Hsp60 to Dectin-1 was not due to the low expression of Dectin-1 because FACS analysis revealed a much greater expression of Dectin-1 than LOX-1 on CHO cells (Fig. 1A). Saturable binding of Hsp60 to LOX-1 was determined by FACS analysis. There was a dose-dependent increase in Alexa Fluor 488-Hsp60 binding to CHO–LOX-1 cells (Fig. 1C). Saturable binding of Hsp60 to CHO–LOX-1 cells was reached at ∼800 nM with a K_d of 199 nM. To evaluate the specificity of Hsp60 binding to LOX-1, CHO–LOX-1 cells were pretreated with 50 μg/ml the anti–LOX-1 mAb 23C11 before incubation with Alexa Fluor 488-Hsp60. As shown in Fig. 1D, 23C11, but not isotype control IgG1 mAb, inhibited the binding of Hsp60 to CHO–LOX-1 cells, indicating that this mAb bound at or near the Hsp60-binding site.

**Binding of Hsp60 to Dectin-1 and LOX-1**

To determine the region of LOX-1 recognized, three recombinant proteins were generated as described in Materials and Methods. We first preformed a pull-down assay to test the interaction between LOX-1 and three recombinant variants. Hsp60 and three recombinant variants immobilized to Ni-NTA Sepharose were incubated with conditioned culture medium containing LOX-Fc, a soluble chimeric protein containing the ectodomain of human LOX-1 fused to the Fc portion of mouse IgG1, followed by Western blot analysis. As
shown in Fig. 2A, Hsp60 and Hsp60-C, but not Hsp60-N and Hsp60-AM, were associated with LOX-1-Fc. Furthermore, results from FACS analysis showed that Alexa Fluor 488–Hsp60-C, but not Alexa Fluor 488–Hsp60-N or Hsp60-AM, bound to CHO–LOX-1 cells, which is in agreement with the observation revealed in the pull-down assay (Fig. 2B). To further quantify the contribution of individual domains to the binding properties of full-length Hsp60, competition with unlabeled proteins, each at a 10-fold excess, was performed by FACS analysis. Quantification of these binding studies showed that preincubation with unlabeled Hsp60 or Hsp60-C resulted in 85% and 76%, respectively, inhibition of the binding of Hsp60-Alexa Fluor 488 (Fig. 2C). In contrast, preincubation with Hsp60-N or Hsp60-AM led to a negligible inhibition of Hsp60 binding. Taken together, these data suggest that LOX-1 recognizes Hsp60 via the C terminus of Hsp60.

LOX-1–mediated Hsp60 endocytosis

The specific binding of Hsp60 to LOX-1 was further confirmed by the confocal microscopy assay. CHO–LOX-1 cells were incubated with Alexa Fluor 488-Hsp60 and anti-Myc Ab at 4˚C for 45 min. Cells were washed and stained with rhodamine-conjugated anti-mouse Ab at 4˚C to mark LOX-1 expression, followed by fixation and confocal microscopy assay. Representative confocal images (Fig. 3) revealed that Hsp60 could bind to the cell surface. LOX-1 was also detected on the cell surface and extensively colocalized with Hsp60, as revealed by merged images. However, the binding of Alexa Fluor 488-Hsp60 to CHO-K1 mock cells was not detected (data not shown). Hsp60 internalization was initiated by incubating for an additional 15 min at 37˚C to trigger energy-dependent Hsp60 uptake. As seen in Fig. 3, Hsp60 colocalized with LOX-1 after 15 min of internalization, suggesting that LOX-1 was functioning to endocytose Hsp60, rather than exclusively binding it on the cell surface. A confocal microscopy assay was subsequently conducted to investigate the binding and internalization of Hsp60-C by LOX-1. As shown in Fig. 3, Hsp60-C could bind to the cell surface and was colocalized with LOX-1 at 4˚C. At 37˚C, Hsp60-C was internalized and colocalized with LOX-1 inside the cells.

Engagement of LOX-1 as a common receptor for Hsp60 family members

Hsp60 are highly conserved intracellular proteins expressed in all prokaryotic and eukaryotic cells. We next investigated whether LOX-1 is engaged as a common receptor for Hsp60 family members. Given that other mammalian Hsp60s share higher sequence homology with human Hsp60 than with microbial Hsp60, we used E. coli GroEL and M. bovis Hsp60.2 (Hsp65) to address this issue. GroEL and Hsp65 were labeled with Alexa Fluor 488, and FACS analysis was performed. As shown in Fig. 4A, Alexa Fluor 488-GroEL and Alexa Fluor 488-Hsp65 could significantly bind to CHO–LOX-1 cells. This binding was further confirmed by the confocal microscopy assay (Fig. 4B). Alexa Fluor 488-GroEL and Alexa Fluor 488-Hsp65 could bind to CHO cells and colocalized with LOX-1 on the cell surface. There was no binding of GroEL and Hsp65 to the cell surface of CHO cells without expression of LOX-1 (Fig. 4B). In contrast, Alexa Fluor 488-BSA did not bind to cells with or without expression of LOX-1 (Supplemental Fig. 3B). Furthermore, FACS-based analysis for competition experiments revealed that different Hsp60 species could bind competitively to CHO–LOX-1 cells, which further verifies that LOX-1 is engaged as a common receptor for different Hsp60 species (Fig. 4C). Additionally, preincubation with Hsp70 inhibited binding of Hsp65 to CHO–LOX-1 cells, which is consistent with previous reports that Hsp70 and Hsp60 share common receptors expressed on human monocyte-derived DCs (24).

Involvement of LOX-1 in cross-presentation of Hsp60-fused Ag

To investigate the functional significance of the interaction between Hsp60 and LOX-1, the binding of Hsp60 to immature BMDCs was assessed. Results from FACS analysis revealed the binding of Alexa Fluor 488-Hsp60 to BMDCs (Fig. 5A). Furthermore, pre-treatment with anti–LOX-1 mAb 23C11 partially prevented the binding of Alexa Fluor 488-Hsp60 to BMDCs, which implies that other unidentified receptors are likely involved in the binding of Hsp60 (Fig. 5A).

We next evaluated whether LOX-1 could deliver an Hsp60-fused Ag into the MHC class I presentation pathway. BMDCs were incubated with Hsp60-OVA fusion protein, composed of Hsp60 with C-terminal fusion of OVA230–359, as previously described (25), and were then tested for the ability to activate the T cell hybridoma B3Z, which secretes IL-2 in response to OVA257–264.
and H-2Kb complexes, without costimulatory signaling. As shown in Fig. 5B, Hsp60.OVA induced the release of IL-2 significantly more potently than did rOVA230–359. In contrast, no IL-2 was produced in response to Hsp60 stimulation at the same concentrations as Hsp60.OVA, indicating that Hsp60 could enhance cross-processing of the linked antigenic sequence. Also, the effect of Hsp60.OVA on IL-2 release is dependent on the fusion with Hsp60, because an equal amount of Hsp60 and OVA230–359 did not significantly induce IL-2 release (Fig. 5B). To explore the role of LOX-1 in this process, BMDCs were pretreated with anti-LOX-1 mAb 23C11 prior to the addition of Hsp60.OVA. As shown in Fig. 5C, the release of IL-2 from B3Z cells was inhibited by 23C11. In contrast, control IgG1 mAb did not result in a reduction of IL-2 release. Furthermore, OT-1 cells were used to confirm that LOX-1 could cross-present Hsp60.OVA into the MHC class I presentation pathway. As shown in Fig. 5D, Hsp60.OVA, but not OVA230–359, could induce IL-2 release by OT-I cells, and 23C11, but not IgG1, could inhibit IL-2 release, which is consistent with the above observation. FACS analysis with 25D1.16 mAb, a specific Ab for SIINFEKL-Kb complex (26), was performed to verify that LOX-1 could cross-present Hsp60.OVA on MHC class I molecules. These results showed visible staining of cells incubated with Hsp60.OVA but not with control OVA230–359 (Fig. 5E). Furthermore, preincubation with 23C11 reduced staining of 25D1.16.

Role of LOX-1 in specific CTL response induced by Hsp65-fused Ag

Among different Hsp60 species, Hsp65 fusion proteins are extensively used, and it was reported that Hsp65 fusion proteins could generate Ag-specific CTL activity and elicit protective immunity against tumors in vivo (27, 28). We next evaluated the role of LOX-1 in these effects using Hsp65.OVA fusion protein with C-terminal fusion of OVA230–359. C57BL/6 mice were immunized s.c. with Hsp65, OVA, or Hsp65.OVA, as described in Materials and Methods. Seven days after the last immunization, spleen cells were restimulated in vitro for 1 wk; CTL activity was assessed using a 51Cr-release assay with E.G7 cells as targets. As shown in Fig. 6A, cells from mice primed with Hsp65.OVA were able to lyse E.G7 cells. In contrast, cells from mice injected with Hsp65 or OVA inefficiently lysed target cells. A dose-dependent effect of spleen cell activation was also observed when different dosages of Hsp65.OVA were used (Fig. 6A), which is in agreement with previous reports (27). To investigate the role of LOX-1, Hsp65.OVA was preincubated with molar-equivalent of L-ECD for 1 h at 4°C to block the recognition site of LOX-1 prior to injection. Preincubation with L-ECD, but not with GST or D-ECD, reduced specific CTL activity and IFN-γ release primed by Hsp65.OVA (Fig. 6B, 6C, Supplemental Fig. 5B, 5C). Furthermore, a treble L-ECD more potently inhibited the effect of Hsp65.OVA (Fig. 6B, 6C).

Subsequently, the role of LOX-1 in protective tumor immunity of Hsp65.OVA was examined. Mice were immunized with PBS or Hsp65.OVA and challenged with 1 × 107 E.G7 cells intradermally. As shown in Fig. 6D, mice immunized with Hsp65.OVA had significantly decreased tumor burden relative to the PBS-treated group. However, preincubation of Hsp65.OVA with L-ECD, but not with GST, reduced protective tumor immunity of Hsp65.OVA. These results suggest that LOX-1 plays an important role in Ag-specific CTL activity and protective immunity of Hsp65 fusion protein in vivo.

Discussion

In the current study, our results provide strong evidence for LOX-1, which was previously known as an oxidized LDL receptor,
as a receptor structure for Hsp60. Specificity of binding, saturability of this interaction, and the ability of the ligand to compete with itself are essential features of a ligand–receptor interaction and differentiate it from nonspecific adherence or endocytosis (19). These characteristics were well demonstrated for the binding of human Hsp60 to LOX-1 in this study.

The sequence of CTLD has diversified to accommodate ligands other than calcium or carbohydrates, but some may be able to recognize carbohydrates via alternative mechanisms. The long-loop region residing in the second β-sheet plays a key role in specific ligand binding for CTLTD (29). Although the regions β0-β1-α1 and α2-β2'-β3-β4 of LOX-1 and Dectin-1 interact with Hsp60-C in yeast, the region β0-β1 of Dectin-1 and LOX-1 loses this ability, and the region β2'-β3-β4 of Dectin-1 weakly interacts with Hsp60-C, which suggest that the α1 and α2 helix interact with Hsp60-C in yeast. However, Dectin-1 on the cell surface binds very weakly to Hsp60, suggesting that the α1 and α2 helix do not play a role in this interaction or do not form a proper fold in yeast compared with on CHO cells. The interaction between Hsp60-C and LOX-1 was confirmed by FACS analysis, implying that the second β-sheet (β2'-β2-β3-β4) forms a proper fold to interact with its ligand in yeast.

Furthermore, the significant binding of Hsp60 to LOX-1 shows that the second β-sheet plays a crucial role in this interaction, because the second β-sheet of LOX-1, but not of Dectin-1, strongly interacts with Hsp60-C revealed in yeast. There may be some specific amino acids in the second β-sheet of LOX-1 that are involved in this binding, because the second β-sheet of CLEC-1, another C-type lectin-like receptor with a low homolog, completely loses the ability to interact with Hsp60-C (Supplemental Fig. 2B).

The observation that LOX-1 mediated the binding and internalization of Hsp60 via its C terminus further defines the interaction between Hsp60 and LOX-1. The addition of anti–LOX-1 Ab incompletely inhibited the binding of Hsp60 to BMDCs, implying that other unidentified receptors on DCs are likely to be involved in the recognition of Hsp60. Previous studies demonstrated that the receptors for Hsp60 are cell-type specific, and different regions of the Hsp60 molecule are engaged in interactions with adequate receptor structures on innate immune cells (30, 31). Four epitopes, aa 241–260 in the apical domain, aa 391–410 in the intermediate domain, and aa 461–480 and aa 481–500 in the C-terminal region of Hsp60, were identified to be involved in the binding of Hsp60 to innate immune cells, which supports our observation. However, it remains elusive whether aa 461–480 and/or aa 481–500 in the C-terminal region of the Hsp60 is responsible for the interaction of Hsp60 with LOX-1.

Hsps are highly conserved intracellular proteins expressed in all prokaryotic and eukaryotic cells and share high sequence homology. Extracellular release of Hsps and their modulated access on the cell surface can be considered a putative danger signal. Receptors on the innate immune cells play a key role in the recognition and proinflammatory activity of Hsps (17, 32). In this article, we demonstrated that LOX-1 is able to recognize different Hsp60 species, although we did not test all of them. Given that there is a high degree of amino acid sequence homology between different species, it is possible that LOX-1 is involved in the recognition of other members of Hsp60 family and is engaged as a common receptor. However, Habich et al. (33) reported that different Hsp60 species share proinflammatory activity and do not compete for the same binding site on macrophages. This might be due to a low expression of LOX-1, which does not play a major role in the binding of Hsp60 on RAW264.7 cells. Cross-presentation of exogenous Ags in the context of MHC class I molecules has recently attracted a lot of research interest because it may prove crucial for vaccine development (34, 35). Hsps are molecular chaperones and can bind a wide spectrum of polypeptides, which allows antigenic polypeptides to be cross-presented on MHC class I molecules (35). In comparison with Hsp70 and Hsp90, Hsp60 does not bind peptides directly and, thus, it does not cross-prime CD8+ T cells (36). Recently, several members of the Hsp60 family were shown to cross-prime Ag-specific CD8+ cytotoxic T cell activity when used as a fusion protein partner (27, 28). However, little is known about the mechanisms of Hsp60 fusion proteins to promote cytotoxic T cell activity. We demonstrated that Hsp60 could deliver a fused Ag into the MHC class I presentation pathway via LOX-1. Inhibition of the binding to LOX-1 with 23C11 reduces the cross-presentation of Hsp60-fused OVA on MHC class I molecules. Blocking the binding site for LOX-1 with L-EC decreased OVA-specific CTL activity and protective immunity against tumors to a certain extent in vivo. However, there are several binding sites on Hsp60 and we cannot rule out that other unidentified receptors for Hsp60 also play a role in the cross-presentation of Hsp60-fused Ag on MHC class I molecules. Further studies are required to identify and clarify the receptors for Hsp60 in the cross-presentation of Hsp60-fused Ag.

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Disclosures

The authors have no financial conflicts of interest.

References

**Figure S1. SDS-PAGE analysis of recombinant proteins and detection of the functional activity of refolding Hsp60-N and Hsp60-AM.** Indicated recombinant proteins were resuspended in SDS sample buffer, boiled for 5 min and subjected to SDS-PAGE analysis (A, B, and C). Hsp60-N, N-terminus of Hsp60; Hsp60-AM, the apical domain plus intermediate domain of Hsp60; Hsp60-C, C-terminus of Hsp60; L-ECD, the extracellular domain of LOX-1; D-LED, the extracellular domain of Dectin-1; Hsp60.OVA, Hsp60 with C-terminal fusion of OVA230-359; Hsp65.OVA, Hsp65 with C-terminal fusion of OVA230-359. (D) Interaction between refolding Hsp60-N and Hsp60-C. Biotinylated unfolding or refolding Hsp60-N was incubated with refolding Hsp60-C for 2 h and mixed with streptavidin agarose beads for 4h at 4 °C. Beads were washed in PBS and resuspended in SDS sample buffer, boiled for 5 min and subjected to western blot analysis with an anti-Hsp60 antibody (K-19) against a peptide mapping near the C-terminus of Hsp60 (upper panel). The unfolding and refolding Hsp60-N were revealed by SDS-PAGE analysis (bottom panel) and the refolding Hsp60-C was revealed in Fig. S1A. (E) Refolding Hsp60-AM binds the nonnative substrate rhodanese. Unfolding or refolding Hsp60-AM was incubated with denatured bovine mitochondrial rhodanese (Sigma) for 2 h and mixed with Ni-NTA agarose beads for 4h at 4 °C. Beads were washed in PBS and resuspended in SDS sample buffer, boiled for 5 min and subjected to western blot analysis with an anti-rhodanese antibody (upper panel). Western blot analysis for rhodanese was shown (middle panel) and the unfolding and refolding Hsp60-AM were revealed by SDS-PAGE analysis (bottom panel).

**Figure S2. Structure of CTLD of LOX-1 and Interaction between Hsp60-C and the second β sheets of LOX-1, Dectin-1, and CLEC-1 in yeast.** (A) A schematic structure
of CTLD of LOX-1 is shown. The regions of the first β sheet are indicated in gray and the pinked region is the second β sheet. The secondary structure in the first β sheet is shown. (B) The interaction between Hsp60-C and the second β sheet s of LOX-1, Dectin-1, and CLEC-1 in yeast at day four is shown. Clone 1 and 2: positive control; Clone 3 and 4, negative control; Clone 5 and 6: Interaction between the second β sheet of LOX-1 and Hsp60-C; Clone 7 and 8: Interaction between the second β sheet of Dectin-1 and Hsp60-C; Clone 9 and 10: Interaction between the second β sheet of CLEC-1 (aa195-251) and Hsp60-C.

**Figure S3. The binding of Alexa488-BSA with LOX-1.** (A) CHO-LOX-1 cells were incubated with 100nM Alexa488-BSA at 4 °C for 45 min and the binding of Alexa488-BSA to cells was detected by FACS analysis. Gray histogram corresponds to CHO-LOX-1 cells without addition of Alexa488-BSA and the binding of Alexa488-BSA to CHO-LOX-1 cells is shown by the black line. (B) CHO-K1 cells were transiently transfected with LOX-1 and incubated with Alexa488-BSA. Cells were washed to remove unbound ligand and fixed, followed by staining with anti-Myc antibody.

**Figure S4. The binding of SIINFEKL peptide to MHC class I and the effect of on IL-2 release.** (A) BMDCs were incubated with different concentrations of SIINFEKL peptide for 2h. Expression of MHC class I-peptide complexes was evaluated using a mAb 25D1.16 by FACS analysis and the mean fluorescence intensity was shown. (B) BMDCs were incubated for 2h in serum-free medium with different concentrations of SIINFEKL peptide. After washing, cells were cultured with the B3Z T cell hybridoma for 16 h. The release of IL-2 was detected by ELISA.

**Figure S5. The binding of Hsp60 with recombinant L-ECD and D-ECD and the**
inhibition of D-ECD on the effect of Hsp65.OVA. (A) L-ECD, D-ECD and GST (1μg/ml) were coated onto 96-well plates, respectively. The binding of Hsp60 (6μg/ml) to L-ECD, D-ECD or GST was detected with anti-Hsp60 antibody. The ELISA was developed with the goat anti-mouse IgG-HRP and the absorbance at 450 nm was measured. Graph represents mean ±SD from triplicate samples. (B) Mice were immunized with PBS, Hsp65.OVA in the absence or presence of D-ECD or GST. Single spleen cell suspensions were prepared. For cytotoxic activity analysis, splenocytes were restimulated and were incubated with 51Cr-labelled E.G7 target cells at the indicated effector: target cell ratios. (C) For quantification of IFN-γ production, splenocytes were incubated for 72h in the presence of SIINFEKL and IFN-γ released into the culture medium was measured by ELISA. Graph represents mean ±SD from triplicate samples.
Figure S4

A

Mean Fluorescence Intensity

SIINFEEKL peptide

0 10pM 50pM 100pM 200pM 500pM

B

IL-2 (pg/mL)

SIINFEEKL peptide

0 10pM 50pM 100pM 200pM
Figure S5

A

B

C

PBS

1.6nmol Hsp65.OVA

1.6nmol Hsp65.OVA
+1.6nmol GST

1.6nmol Hsp65.OVA
+1.6nmol D-ECD