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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.

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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 Hybcult Biotechnology (Uden, The Netherlands), and 2D1,16 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 G418 (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 (aa 140–273) was cloned into the pGBK7T vector as the bait. A total of 2.8 × 10^5 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, OVA_{230–359} (aa 230–359), and the extracellular regions of LOX-1 (L-ECOD) and Dectin-1 (D-ECOD) were in-frame cloned into PET22b vector. Hsp60 with C-terminal fusion of OVA_{230–359} (Hsp60.OVA) and Hsp65 with C-terminal fusion of OVA_{230–359} (Hsp65.OVA) fusion constructs were generated by cloning the coding sequence for OVA_{230–359} 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 by addition of isopropyl β-d-thiogalactoside to a final concentration of 0.1 mM, followed by growth for 5 h at 30˚C. GST was purified using glutathione-Sepharose 4B (General Electric, Fairfield, CT). The inclusion bodies of L-ECOD and D-ECOD were bound to a Ni-NTA column (General Electric) and eluted, followed by dialysis against sequential refolding buffers, according to a previous description (20). Other recombinant proteins were purified using Ni-NTA Sepharose, as described previously (21). Briefly, the inclusion body fraction was denatured and bound to the Ni-NTA affinity column, followed by elution and dialysis against sequential refolding buffer. All proteins bound to beads were washed to remove contaminating LPS and other hydrophobic materials with polyoxymethylene B (Pierce, Rockford, IL). The preparations of all recombinant proteins were confirmed on SDS-PAGE (Supplemental Fig. 1). All proteins contained <10 endotoxin units LPS/mg of protein, as determined by Limulus amebocyte lysate assay. The functional activity of refolding Hsp60-N, Hsp60-AM, and Hsp60-C was detected by pull-down assay (Supplemental Fig. 1D, 1E). The binding activity of L-ECOD, D-ECOD, and GST with Hsp60 was detected by ELISA (Supplemental Fig. 4A).

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-tied 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.

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 pFUSE-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. BMDCs were lysed, and the remaining cells were cultured at 1 × 10^6 cells/ml in complete RPMI 1640 with 10% heat-inactivated FBS 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 × 10^6/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 with 5 × 10^5/ml B3Z T cell hybridoma or OT-1 T cells purified from spleens and lymph nodes (5 × 10^6/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 quantified 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 SINFEKL peptide on IL-2 release by B3Z cells and expression of MHC class I-SINFEKL 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, OVA_{230–359}, 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-ECOD, D-ECOD, or GST before immunization. One week after the immunization, groups of four mice were immunized s.c. with PBS, Hsp65, OVA_{230–359}, 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-ECOD, D-ECOD, 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 incubating 2 × 10^6 viable lymphoid cells together with 1 μg/ml SINFEKL peptide for 7 d in an upright T-25 tissue-culture flask (10 ml). After restimulation, effector cells were harvested and cultured with 51Cr-labeled E.G7 target cells at the indicated E:T cell ratios. After
5 h, 100 μl the culture supernatants was collected, and the released radioactivity was determined by scintillation counting. 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 × 10^5 cells/well with 1 μg/ml peptide. Supernatants were removed 72 h later, and IFN-γ was measured by ELISA.

**Tumor-protection assay**

C57BL/6 mice (six per group) were immunized s.c. with PBS or 1.6 nmol Hsp65.OVA without adjuvant and boosted 1 wk later. In some cases, Hsp65 was set to 100%. Data represent mean ± SD from triplicate samples.

**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 140–273) and Dectin-1 (residues 112–247) (Table I).

**Table I. Interaction of the C terminus of Hsp60 with CTLDs of Lox-1 and Dectin-1**

<table>
<thead>
<tr>
<th>Bait</th>
<th>CTLD</th>
<th>−β5</th>
<th>−β2′−β2−β3−β4−β5’</th>
<th>β0−β1−α1</th>
<th>β0−β1</th>
<th>α2−β2′−β2−β3−β4</th>
<th>β2′−β2−β3−β4</th>
</tr>
</thead>
</table>

**Interaction stringency**

<table>
<thead>
<tr>
<th>Bait</th>
<th>Stringency</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOX-1</td>
<td>+++</td>
</tr>
<tr>
<td>Dectin-1</td>
<td>+++</td>
</tr>
</tbody>
</table>

**A** — indicates a lack of this region.

**The stringency of interaction between bait and prey was evaluated by the size of colonies and the degree of blue color. The stringency of interaction between P53 and Tam as a negative control is evaluated as −.

**The colonies appeared after 4 d and turned very light blue.**

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. B, CHO-LOX-1 and CHO-Dectin-1 cells were incubated with 6 μg/ml (100 nM) Alexa Fluor 488-Hsp60 to cells was detected by FACS. The binding of Alexa Fluor 488-Hsp60 to CHO-Mock cells was used as a control. C, CHO-LOX-1 cells were incubated at 4°C with the indicated concentrations of Alexa Fluor 488-Hsp60 or Alexa Fluor 488-BSA for 45 min and washed, followed by FACS analysis. Mean fluorescence intensity represents mean ± SD from triplicate samples. D, The specificity of Hsp60 binding to LOX-1. CHO-LOX-1 cells were pretreated with 50 μg/ml anti-LOX-1 mAb 23C11 or isotype control IgG1 mAb before incubation with Alexa Fluor 488-Hsp60, and Hsp60 binding was detected by FACS analysis.

LOX-1 recognizes Hsp60 via the C terminus of Hsp60. A, N1NTA Sepharose was left untreated (Control) or preincubated with recombinant proteins and then incubated with soluble LOX-1–Fc chimera, followed by Western blot analysis. B, CHO-LOX-1 cells were left untreated (gray) or incubated at 4°C with 1 × 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 × length × width^2.

**FIGURE 1.** 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. B, CHO-LOX-1 and CHO-Dectin-1 cells were incubated with 6 μg/ml (100 nM) Alexa Fluor 488-Hsp60 to cells was detected by FACS. The binding of Alexa Fluor 488-Hsp60 to CHO-Mock cells was used as a control. C, CHO-LOX-1 cells were incubated at 4°C with the indicated concentrations of Alexa Fluor 488-Hsp60 or Alexa Fluor 488-BSA for 45 min and washed, followed by FACS analysis. Mean fluorescence intensity represents mean ± SD from triplicate samples. D, The specificity of Hsp60 binding to LOX-1. CHO-LOX-1 cells were pretreated with 50 μg/ml anti-LOX-1 mAb 23C11 or isotype control IgG1 mAb before incubation with Alexa Fluor 488-Hsp60, and Hsp60 binding was detected by FACS analysis.

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 × 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 × length × width^2.

**FIGURE 2.** LOX-1 recognizes Hsp60 via the C terminus of Hsp60. A, N1NTA Sepharose was left untreated (Control) or preincubated with recombinant proteins and then incubated with soluble LOX-1–Fc chimera, followed by Western blot analysis. B, CHO-LOX-1 cells were left untreated (gray) or incubated at 4°C with 1 × 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 × length × 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.
interaction in yeast (Table I). The region Hsp60-C. A lack of were generated to define the region of CTLD interacting with (22). Based on this structure, mutant structures mental Fig. 2 representative of three independent experiments. Data (10-fold molar excess). FACS analysis was performed, absence or presence of different unlabeled inhibitors incubated with 100 nM Alexa Fluor 488-Hsp65 in the absence of any competitor was set to 100%. Data (3x magnification) are shown (original magnification ×64).

FIGURE 3. LOX-1 mediates the binding and endocytosis of Hsp60. CHO–LOX-1 cells were incubated with Alexa Fluor 488-Hsp60 and Hsp60-C in the presence of anti-Myc Ab at 4°C, followed by staining with rhodamine-labeled Ab to mark LOX-1 expression. Cells were washed to remove unbound ligand and Ab, followed by fixation. For uptake studies, cells were warmed to 37°C for 15 min to allow ligand internalization before fixation. Confocal microscopy assay was performed and the localization of LOX-1 (red) and Hsp60/Hsp60-C (green) and their colocalization (yellow) are shown (original magnification ×64).

140–273) as bait. Subsequent sequence analysis revealed that two sequences from isolated positive clones encode aa 405–573 and aa 394–573 of human Hsp60. Further investigation revealed that Hsp60-C (aa 436–573), but not Hsp60-N (aa 27–152) or Hsp60-AM (aa 153–435), could interact with CTLD of LOX-1 (data not shown).

The CTLD fold of LOX-1 is similar to that of other long-form CTLDs, consisting of two antiparallel β-sheets, β0-β1-β5-β1’ and β2’-β2-β3-β4, flanked by two α helices, α1 and α2 (Supplemental Fig. 2A) (22). Based on this structure, mutant structures were generated to define the region of CTLD interacting with Hsp60-C. A lack of β5 or β2’-β2-β3-β4-β5 did not impair this interaction in yeast (Table I). The region β0-β1-α1 or α2-β2’-β2-β3-β4 could interact with Hsp60-C. Furthermore, only the second β-sheet (β2’-β2-β3-β4) of LOX-1 could also interact with Hsp60-C, whereas the region β0-β1 lost the ability to interact with Hsp60-C. Although the CTLD and the region β0-β1-α1 or α2-β2’-β2-β3-β4 of Dectin-1, which is a receptor for β-1, 3/β-1, 6-linked glucans and shares the greatest structural homology with LOX-1 among C-type lectin-like receptors (23), interact with Hsp60-C, a difference from LOX-1 was observed, in that the region β2’-β2-β3-β4 of Dectin-1 weakly interacts with Hsp60-C, which implies the binding profile of Hsp60 to LOX-1 is different from it to Dectin-1 (Supplemental Fig. 2B).

Binding of Hsp60 to Dectin-1 and LOX-1

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 Keq 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 LOX-1 via the C terminus of Hsp60

To determine that the region 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).

**FIGURE 5.** LOX-1 mediates in vitro Hsp60-fused Ag cross-presentation. A, Immature BMDCs were incubated with 50 μg/ml anti–LOX-1 or isotype control mAb prior to the addition of 10 μg/ml Alexa Fluor 488-Hsp60, followed by FACS analysis. B, Immature BMDCs were incubated for 8 h in serum-free medium with different concentrations of indicated Ags. After washing, cells were cultured with the B3Z T cell hybridoma for 16 h. The release of IL-2 was detected by ELISA. C, Immature BMDCs were pulsed for 8 h with 100 nM Hsp60.OVA in the presence of anti–LOX-1 mAb or control mAb at the indicated concentrations. After washing, cells were cultured with the B3Z T cell hybridoma for 16 h. The release of IL-2 was detected by ELISA. Bar graph represents mean ± SD from triplicate samples. All results are representative of three independent experiments. D, Immature BMDCs were left untreated or pulsed for 8 h with 100 nM OVA230–359 or Hsp60.OVA in the absence or presence of 50 μg/ml anti–LOX-1 mAb or control IgG1. After washing, cells were cultured with OT-I cells for 16 h. The release of IL-2 was detected by ELISA. E, BMDCs were incubated with 100 nM OVA230–359 or 100 nM Hsp60.OVA in the absence or presence of 50 μg/ml anti–LOX-1 mAb or control mAb for 16 h. Expression of MHC class I-peptide complexes was evaluated using mAb 25D1.16. Gray graph represents un pulsed cells.
and H-2K\textsuperscript{b} complexes, without costimulatory signaling. As shown in Fig. 5\textit{B}, Hsp60.OVA induced the release of IL-2 significantly more potently than did rOVA\textsubscript{230-359}. 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 OVA\textsubscript{230-359} did not significantly induce IL-2 release (Fig. 5\textit{B}). 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. 5\textit{C}, 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-I cells were used to confirm that LOX-1 could cross-present Hsp60.OVA into the MHC class I presentation pathway. As shown in Fig. 5\textit{D}, Hsp60.OVA, but not OVA\textsubscript{230-359}, 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-K\textsuperscript{b} 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 OVA\textsubscript{230-359} (Fig. 5\textit{E}). Furthermore, preincubation with 23C11 reduced staining of 25D1.16.

\textit{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 OVA\textsubscript{230-359}. 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 \textsuperscript{51}Cr-release assay with E.G7 cells as targets. As shown in Fig. 6\textit{A}, 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. 6\textit{A}), 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-\(\gamma\) release primed by Hsp65.OVA (Fig. 6\textit{B}, 6\textit{C}). Furthermore, a treble L-ECD more potently inhibited the effect of Hsp65.OVA (Fig. 6\textit{B}, 6\textit{C}).

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 \(1 \times 10^7\) E.G7 cells intradermally. As shown in Fig. 6\textit{D}, 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.

\textbf{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, satura-
ility 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 CTLD (29). Although the regions
β0-β1-a1 and α2-β2-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, suggest-
ing 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 homol, completly
loses the ability to interact with Hsp60-C (Supplemental Fig. 2B).

The observation that LOX-1 mediated the binding and internal-
ization of Hsp60 via its C terminus further defines the interaction
between Hsp60 and LOX-1. The addition of anti–LOX-1 Ab in-
completely 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 interac-
tion of Hsp60 with LOX-1.

Hsp60 are highly conserved intracellular proteins expressed in all
prokaryotic and eukaryotic cells and share high sequence ho-
ology. 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 rec-
ognition 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 recog-
nition 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-spe-
cific 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 bind-
ing 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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