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Extracellular HSP70 binding to surface receptors present on antigen presenting cells and endothelial/epithelial cells

Jimmy R. Thériault[a], Salamatu S. Mambula[a], Tatsuya Sawamura[b], Mary Ann Stevenson[a], Stuart K. Calderwood[a,*]

[a] *Department of Radiation Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, 21-27 Burlington Avenue, Boston, MA 02215, USA*
[b] *Department of Bioscience, National Cardiovascular Center Research Institute, Fujishirodai, Suita, Osaka, Japan*

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Abstract Extracellular HSP70 has been found to participate in both innate and adaptive immune responses. However, little is known about the molecular mechanisms that mediate this process. Previous reports suggest that HSP70 interacts with antigen presenting cells (APC) through a plethora of surface receptors. In this study, we have examined the relative binding of potential HSP70 receptors and found high affinity binding to LOX-1 but not other structures with a role in HSP70–APC interactions such as LRP/CD91, CD40, TLR2, TLR4 or another c-type lectin family member (DC-SIGN) closely related to LOX-1. In addition to APC, HSP70 can avidly bind to non-APC cell lines, especially those from epithelial or endothelial background.

Keywords: Heat shock protein 70; LOX-1; Toll-like receptor; CD40; Endothelial

1. Introduction

Heat shock protein 70 (HSP70) has been well characterized as an intracellular molecular chaperone involved in nascent and damaged intracellular protein refolding [1,2]. However, a novel extracellular role has been ascribed to this protein since HSP70-derived tumor preparations can elicit in vivo tumor rejection [3,4]. HSP70 has been shown to capture antigenic peptides and trigger their cross-presentation through immune effector cells [5]. HSP70 also mediates both cytokine expression and maturation of antigen presenting cell (APC) [5–9]. This HSP70 extracellular function is also conserved among several other HSP family members including gp96/GRP94, calreticulin and HSP60 [4]. Although, HSP70 shows exciting potential as an adjuvant molecule, the mechanism(s) through which it exerts this function remains elusive. Previous studies have demonstrated that the anti-tumoral immune function of HSPs is activated through receptor-mediated endocytosis because of the low level (nanomolar) of HSPs needed to see an immune response and the saturability of the process [10,11]. A growing number of surface receptors for HSPs such as scavenger receptors LOX-1, CD94 and scavenger receptor A (SR-A), the LDL-receptor-related protein/α2-macroglobulin CD91 receptor, the Toll-like receptor (TLR) 2 and 4 and CD40 have been associated with endocytosis/phagocytosis and/or are involved in the induction of the pro-inflammatory response [12–16].

SRs are membrane endocytic receptors recognizing modified or altered molecules such as lipoproteins and polyanionic ligands [17–19]. Delneste et al. [12] have shown convincingly that LOX-1, a class E SR, is an important HSP70 binding structure present on the surface of dendritic cells (DC). This type II calcium-dependent SR also belongs to c-type lectin domain receptor family comprising other members, such as DC-SIGN, CD94 and DECTIN-1 [20,21]. LOX-1 has been associated with functions related to immunity including leukocyte homing as the tethering receptor responsible for leukocyte adhesion rolling on endothelial cells [22]. LOX-1 also binds and internalizes oxidized LDL (ox-LDL), an important process involved in atherosclerosis [23]. Interestingly, another c-type lectin receptor found on Natural Killer (NK) cells CD94 can bind HSP70 and the endoplasmic reticulum (ER) resident Gp96 interacts to SR-A illustrating the importance of SRs in HSP-mediated immune function [14].

LRP/CD91 has also been proposed as a HSP70 receptor [15]. LRP/CD91 is a multifunctional plasma membrane receptor recognizing as well as SR family members various ligands, such as lipoproteins, bacterial toxins and more specifically α2-macroglobulin [24]. LRP/CD91 is synthesized as a 600-kDa precursor processed into 515-kDa (alpha) and 85-kDa (beta) subunits [24]. The 85-kDa subunit (beta) possesses a transmembrane domain and binds the extracellular 515-kDa subunit (alpha) through non-covalent interactions [25]. The HSP70 binding site on CD91 has been mapped to the first part of the alpha subunit and HSP70/CD91 interaction can be competed by α2-macroglobulin and receptor-associated protein (RAP) [15,16,26]. LRP/CD91 binding is evidently not restricted to HSP70 but encompasses other HSPs (gp96/GRP94, calreticulin and HSP90) [15]. Interestingly, CD91/calreticulin complexed with surfactant proteins (SP-A and SP-D) associated with foreign debris can initiate phagocytosis and pro-inflammatory response in lungs [27].

HSP70–peptide complex binding is followed by antigen cross-presentation in APC. HSP70–peptide complexes

*Corresponding author. Fax: +1 617 632 0635.
E-mail address: scalderw@bidmc.harvard.edu (S.K. Calderwood).

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proceed through specific routes for peptide representation on the cell surface by major histocompatibility complex (MHC) receptor (cross-presentation) [10,11]. Such peptides are taken up into the ER through ABC family transport system that involves the transporters associated with antigen processing (TAP)-TAP1 and TAP2 proteins. TAP1 and TAP2 form a complex that transports peptides across the ER membrane and delivers them to MHC class I (MHCI) protein complexes [28,29]. These MHC I complexes are transported to the cytoplasm via a vesicular system and displayed on the cell surface where they are subject to surveillance by cytotoxic, CD8+ lymphocytes [30].

In parallel with cross-presentation, HSP70 can initiate a potent innate immune response [6,8,31]. After HSP70 binding to APC, a pro-inflammatory response is generated through the activation of various immune processes, such as cytokine production (IL-1β, TNF-α and IL-6, etc.), co-stimulatory molecule expression (MHC class II and CD86) and nitric oxide (NO) release. Our group and others have demonstrated that two members of the Toll-like receptor family TLR2 and 4 activate HSP-mediated pro-inflammatory cytokine production via the MyD88/IRAK/NF-κB signal transduction pathway in a CD14-dependent fashion [6–8]. Extracellular HSP70 also stimulates intracellular calcium mobilization. Artificial depletion of the calcium content using intracellular Ca^{2+} chelator BAPTA-AM interferes with the IκBα/NF-κB pathway leading to cytokine expression inhibition. In addition to TLRs, other HSP70 signaling receptors, such as CD40 have shown some pro-inflammatory activity [32]. CD40 plays an essential role in the development of HSP70-induced autoimmune form of diabetes [33]. In this particular context, HSP70 promotes cytokine production through a CD40-dependent mechanism but induces functional maturation of bone marrow-derived DC in the absence of a "standard" phenotypic maturation [33].

In this study, the relative binding affinity of known and unknown HSP70 receptors, such as LOX-1, DC-SIGN, LRP/CD91, TLR2, TLR4 and CD40 in HSP70–cell surface interaction has been evaluated using stable transfectants overexpressing these receptors in non-APC cells. Among the receptors proposed to specifically bind HSP70, only c-type lectin receptor LOX-1 showed significant HSP70 binding affinity. Intriguingly, endothelial/epithelial cells possessed membrane HSP70 receptor(s) suggesting a role of HSP70 in other cellular function(s) than anti-tumor immune response.

2. Materials and methods

2.1. Cell culture

THP-1, RAW 264.7, wild-type K562 and K562 overexpressing human DC-SIGN were grown in RPMI 1640 medium 10% FBS supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin [34]. IMR90 were cultured in minimal essential medium (MEM) 15% FBS with 2 mM glutamine. Human umbilical vein endothelial cells (HUVEC) were maintained in endothelial basal medium-2 (EBM-2) supplemented with Clonetics™ SingleQuot® (Cambrex/Biowittaker). The melanoma A375, wild-type HEK293 (293), HEK293 overexpressing human TLR2 or 4 (293-TLR2, 293-TLR4) (G418), HEK293 overexpressing the PMX empty vector (293 EV Ley) (G418), HEK293 overexpressing murine CD40 (293 CD40 Ley) (G418), HeLa, HeLa S3 and MCF-7 were grown in DMEM 10% FBS [8,35]. Prostate cancer cells PC-3, Chinese Hamster Ovary (CHO), CHO overexpressing human LOX-1 (CHO-LOX-1), CHO LRP null, CHO LRP null overexpressing the second extracellular binding region of the alpha subunit of human LRP linked to the beta subunit (LRP2) (aa. 787–1164 + 3765–4525) (G418) or the fourth region of the alpha subunit fused with the beta subunit (LRP4) (aa. 3274–4525) (G418) were maintained in F-12 (Ham) nutrient mixture with 10% FBS [36]. In the case of CHO-LOX-1, clonal selection was kept with 10 μg/ml blasticidin S. For all stable transfectants using G418 as a selecting agent, cells were incubated with G418 at a final concentration of 0.4 μg/ml. Each experiment was carried out in cell lines grown in exponential conditions.

2.2. Thioglycollate-elicited peritoneal macrophages

Isolation of peritoneal macrophages was done as previously described [37]. Briefly, peritoneal macrophages were isolated from 6–10-week-old C57BL/6 background mice. The mice were injected intraperitoneally with 3 ml of thioglycollate, and after 4 days peritoneal exudate cells were harvested by lavage with 10 ml of RPMI 1640 medium supplemented with 10% heat-inactivated FBS and penicillin/streptomycin.

2.3. Alexa 488-labeled purified HSP70 preparation

Human melanoma cells A375-MEL or mouse MISA cells have been used as starting material for HSP70–peptide complexes (HSP70.PC) preparation because high endogenous HSC70 and/or HSP70 levels were detected in these cell types. HSP70.PC purification was executed as previously reported [38]. Briefly, a 10-ml cell pellet of tumor cells was homogenized in 40 ml hypotonic buffer (10 mM $NaHCO_3$, 0.5 mM PMSF, pH 7.1) by Dounce homogenization. The homogenate was first centrifuged at $10000 \times g$ for 30 min and the supernatant was recentrifuged for 60 min at $100000 \times g$. The sample buffer was changed to buffer D (20 mM Tris–acetate, 20 mM NaCl, 15 mM β-mercaptoethanol, 3 mM $MgCl_2$, and 0.5 mM PMSF, pH 7.5) using PD-10 column (Amersham-Biosciences). The sample was applied directly to a 5-ml ADP–agarose column (Sigma–Aldrich) which was equilibrated with buffer D. HSP70.PC was eluted from ADP–agarose column with 3 mM ADP in buffer D. The sample buffer was changed to FPLC buffer (20 mM sodium mono- and diphosphate, 20 mM NaCl, pH 7.0) with PD-10 column. The supernatant was applied to a DEAE anion exchange column equilibrated with FPLC buffer (Amersham-Biosciences). HSP70.PC was eluted with the FPLC buffer containing 150 mM NaCl. All proteins were quantitated with Bradford assay. Alexa 488 labeling on HSP70.PC was carried out according to the manufacturer's instructions (Molecular Probes, USA). BSA was used as negative control. No degradation of the HSP70.PC purified preparation was observed by Coomassie staining and the presence of HSP70 in the preparation was confirmed by Western blotting using a mouse monoclonal antibody specific against HSP70 (SPA-810, Stressgen).

2.4. HSP70.PC binding assay

Non-trypsinized cells (2×10^5) were washed twice in PBS containing 0.5% FBS, 0.05% NaN_3 and 1 mM $CaCl_2$ (PFNC) and incubated with 150 nM Alexa 488-labeled BSA or HSP70.PC for 30 min on ice with gentle shaking. The cells were washed in PFNC twice and Alexa 488-labeled HSP70.PC binding was monitored by flow cytometry (Becton Dickinson). For blocking experiments, cells were pre-incubated with 120 μg/ml of anti-human LOX-1 (JTX92) (Dr. Sawamura) for 30 min at 37 °C and then washed twice with PFNC before HSP70.PC binding assessment.

3. Results

3.1. Exogenous HSP70.PC can specifically bind to APC

As mentioned earlier, HSP70 anti-tumoral immune function is probably activated through its interaction with a surface membrane receptor [10]. Our group and others have demonstrated that exogenous HSP70 can bind to the surface of human monocytes, splenocytes and DC [6,15,39]. We have analyzed by flow cytometry HSP70 binding to a human pre-monocytic cell line (THP-1), to mature murine macro-

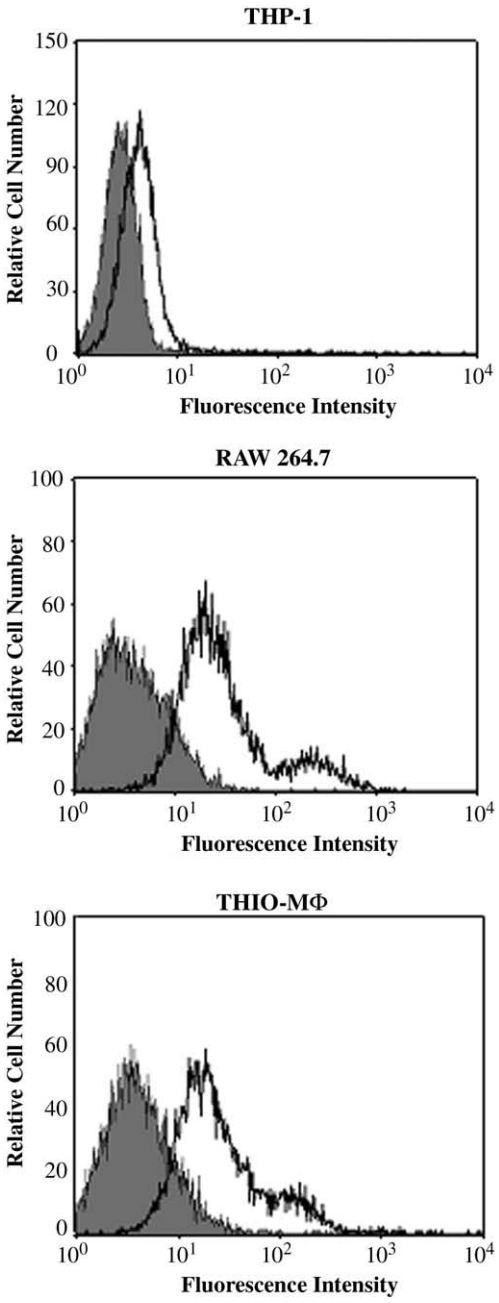

Fig. 1. Exogenous HSP70.PC specifically binds to monocytes and macrophages. Pre-monocytic THP-1, mature macrophages RAW 254.7 or thioglycollate-elicited peritoneal macrophages were incubated with Alexa 488-labeled BSA or HSP70.PC at a concentration of 10 μg/ml (150 nM) on ice for 30 min with gentle shaking. HSP70.PC binding was monitored by flow cytometry (FL1). Shaded gray and thick black line histograms corresponded, respectively, to BSA and HSP70.PC binding. Experiments were carried out three times with similar results.

phages (RAW 264.7) and to thioglycollate-elicited peritoneal macrophages using human HSP70.PC (Fig. 1). HSP70 binding intensity differed between each cell type reflecting probably differences in HSP70 receptor(s) expression. Interestingly, at least two peaks were generated when HSP70.PC was associated with RAW 264.7 cells and thioglycollate-elicited macrophages suggesting the possibility of multiple HSP70 receptors on macrophages (Fig. 1).

3.2. Absence of association between HSP70.PC and LRP/CD91 or TLR 2, 4 or CD40

It has been suggested that HSP70 can interact directly with LRP/CD91 [15]. Nonetheless, some skepticism has been directed to the involvement of CD91 in HSP binding since independent studies conducted by Berwin et al. [40] showed that CHO cells, which express CD91, do not bind gp96. LRP/CD91 is composed of more than 4500 amino acids making difficult the expression of the full-length protein in an artificial system (stable transfection). To overcome this problem, LRP/CD91 mini-receptors have been prepared in CHO LRP null line to avoid the possibility of background binding to endogenous LRP/CD91 [41]. LRP/CD91 ligands mainly interact with the second and/or the fourth binding domain present on its alpha subunit (CHO LRP 2 and LRP 4) [41]. A residual HSP70.PC binding was seen on wild-type CHO (CHO-K1) suggesting the presence of endogenous HSP70 receptors. Surprisingly, no sign of high affinity HSP70.PC binding was observed in CHO-LRP 2 and 4 (Fig. 2). Also, there was no significant difference in HSP70.PC binding between wild-type CHO cells (CHO-K1) and CHO cells devoid of a functional LRP/CD91 gene suggesting that LRP/CD91 was not the high affinity HSP70 receptor present on CHO (Fig. 2).

The HSP70-induced pro-inflammatory response has been functionally related to TLR2 and/or TLR4 in collaboration with CD14 suggesting these molecules as potential HSP70 receptors [6–8]. Previous experiments have ruled out the possibility that CD14 is a HSP70 receptor since no interaction is observed between HSP70 and CD14-overexpressing CHO cells [12]. Moreover, a specific neutralizing anti-CD14 antibody (clone MY4) does not inhibit HSP70 binding to APC [42]. In order to determine whether TLR2 and TLR4 are HSP70 important receptors, we have stably transfected expression vectors encoding those receptors in HEK293 cells. Such cells showed enhanced activation of NF-κB activity when challenged with *E. coli* LPS or mammalian HSP70 indicating efficient expression of the proteins in HEK293 [8]. Intriguingly, wild-type HEK293 cells can significantly bind exogenous HSP70 showing that HSP70 binding was not restricted to APC (Fig. 3A). Nonetheless, no greater association was obtained when human TLR2 or TLR4 was overexpressed in these cells (Fig. 3A).

Concerning CD40, HSP70 binding to this molecule was reported to be ADP-dependent requiring only the HSP70 ATPase domain [32]. This interaction was evidently further stabilized with the presence of HSP70 peptide substrate. Nevertheless, in our studies, HSP70.PC binding assessment to HEK293 embryonic kidney cells overexpressing the murine form of CD40 revealed no significant interaction between HSP70.PC and CD40 even when HSP70 binding assays were carried out at high concentrations (up to 50 μg/ml) (Fig. 3B) (data not shown). Surface expression of CD40 was confirmed in these cells using an anti-CD40 antibody in Western Blotting and by flow cytometry signifying that CD40 does not play a major role as a HSP70 receptor (data not shown) [35]. The HSP70 binding affinity of HEK293 overexpressing an empty vector was significantly reduced in comparison from the one obtained with wild-type HEK293 (Figs. 3A and B). It is possible that the provenance of these cell lines, different culture conditions or selection of stably overexpressing clone has favored the establishment of different HEK293 cell populations

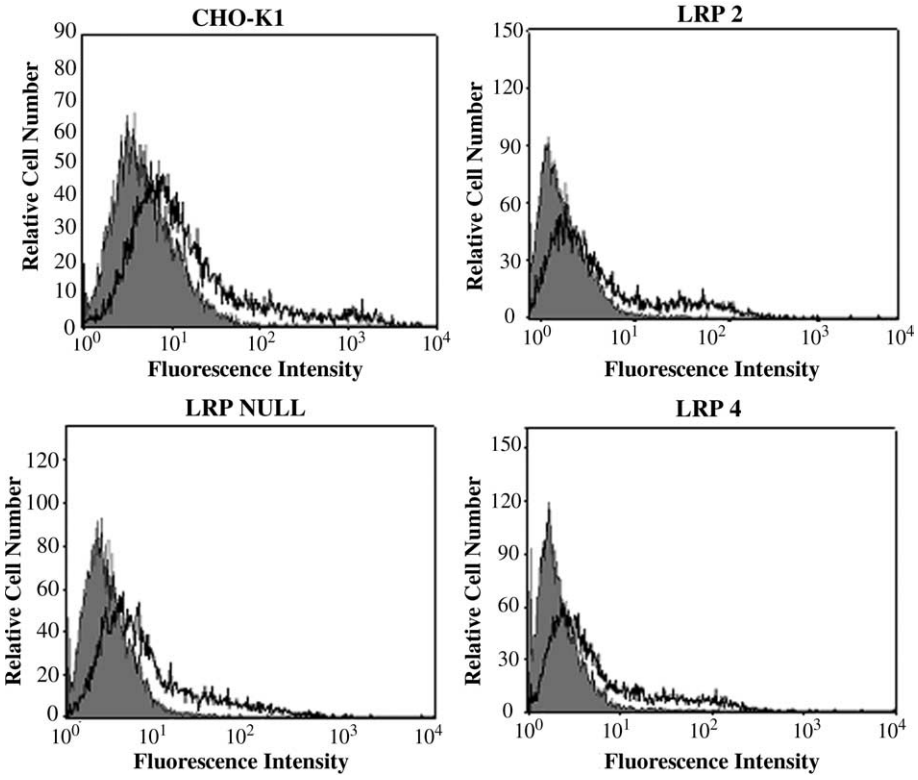

Fig. 2. Extracellular HSP70.PC does not interact with LRP/CD91. Wild-type CHO, CHO devoid of a functional LRP/CD91 gene (LRP NULL) or overexpressing the second or the fourth LRP/CD91 binding domain present on the alpha subunit (LRP 2 or LRP 4) were subjected to a HSP70 binding assay as mentioned in Section 2. Shaded gray and thick black line histograms corresponded to BSA and HSP70.PC binding, respectively. Experiments were carried out three times with similar results.

expressing variable amounts of endogenous HSP70 receptor(s). However, in each cell population, independent controls were carried out, indicating minimal high affinity binding of HSP70 to TLR2, TLR4 and CD40 (data not shown).

3.3. C-type lectin receptor LOX-1 interacted with HSP70.PC

We next examined the role of SR LOX-1 which has been previously associated with HSP70.PC binding capacity [12]. We observed abundant HSP70.PC-LOX-1 interaction in stable transfectant overexpressing the human LOX-1 receptor on CHO (Fig. 4) [36]. CD94, another member of c-type lectin receptor family member expressed on NK cells, possesses some HSP70 binding affinity [13]. Since the extracellular domains of both proteins comprise almost exclusively the c-type lectin domain, we wanted to determine if other c-type lectin family member such as DC-SIGN could interact with HSP70. As illustrated in Fig. 4, no clear HSP70 binding was seen on the lymphoblastic cell line K562 when human DC-SIGN was overexpressed at the HSP70 concentration tested suggesting that DC-SIGN was not a high affinity HSP70.PC binding molecule [34]. These experiments indicate some specificity in the interaction of the c-type lectin family with HSP70.PC.

3.4. HSP70.PC binding to endothelial/epithelial cells

Since significant HSP70 binding to HEK293 embryonic kidney cells was observed, we examined whether this observation could be extended to other non-APC cell types. In fact, previous studies had suggested that extracellular HSP70 might have properties independent of the immune response, in for instance, cytoprotection in neuronal cells [43]. To explore the extent of this possibility, HSP70 binding assays using HSP70.PC at a concentration of 10 μg/ml were performed on various cell lines including endothelial/epithelial, melanoma cells and fibroblasts. Extracellular HSP70.PC bound avidly to human endothelial cells HUVEC, prostate cancer PC-3 cells and HeLa cells while binding to human fibroblasts IMR90, breast cancer cells MCF-7 or melanoma cells A375 was minimal (Figs. 5A and B). As observed for HEK293, HeLa coming from two different backgrounds (HeLa S3 or HeLa) appeared to bind with different avidity to HSP70.PC (Fig. 5A).

HSP70 binding to HUVEC correlates with previous reports showing surface expression of the c-type lectin receptor LOX-1 on endothelial cells [44]. As our studies indicate that LOX-1 is an effective HSP70 receptor, we tested whether LOX-1 could account for HSP70.PC binding to HUVEC. We performed an inhibition experiment using an anti-human LOX-1 blocking antibody (JTX92) on CHO LOX-1 and HUVEC cells. As seen in the Fig. 5C, HSP70.PC binding to CHO LOX-1 was inhibited by approximately 70% by the JTX92 antibody but not a specific control antibody against DC-SIGN (data not shown). Nevertheless, JTX92 antibody had no effect on HSP70.PC binding to HUVEC indicating that LOX-1 does not play a major role in HSP70-HUVEC binding. Interestingly, HSP70.PC binding to PC-3 and HeLa was also not affected by pre-incu-

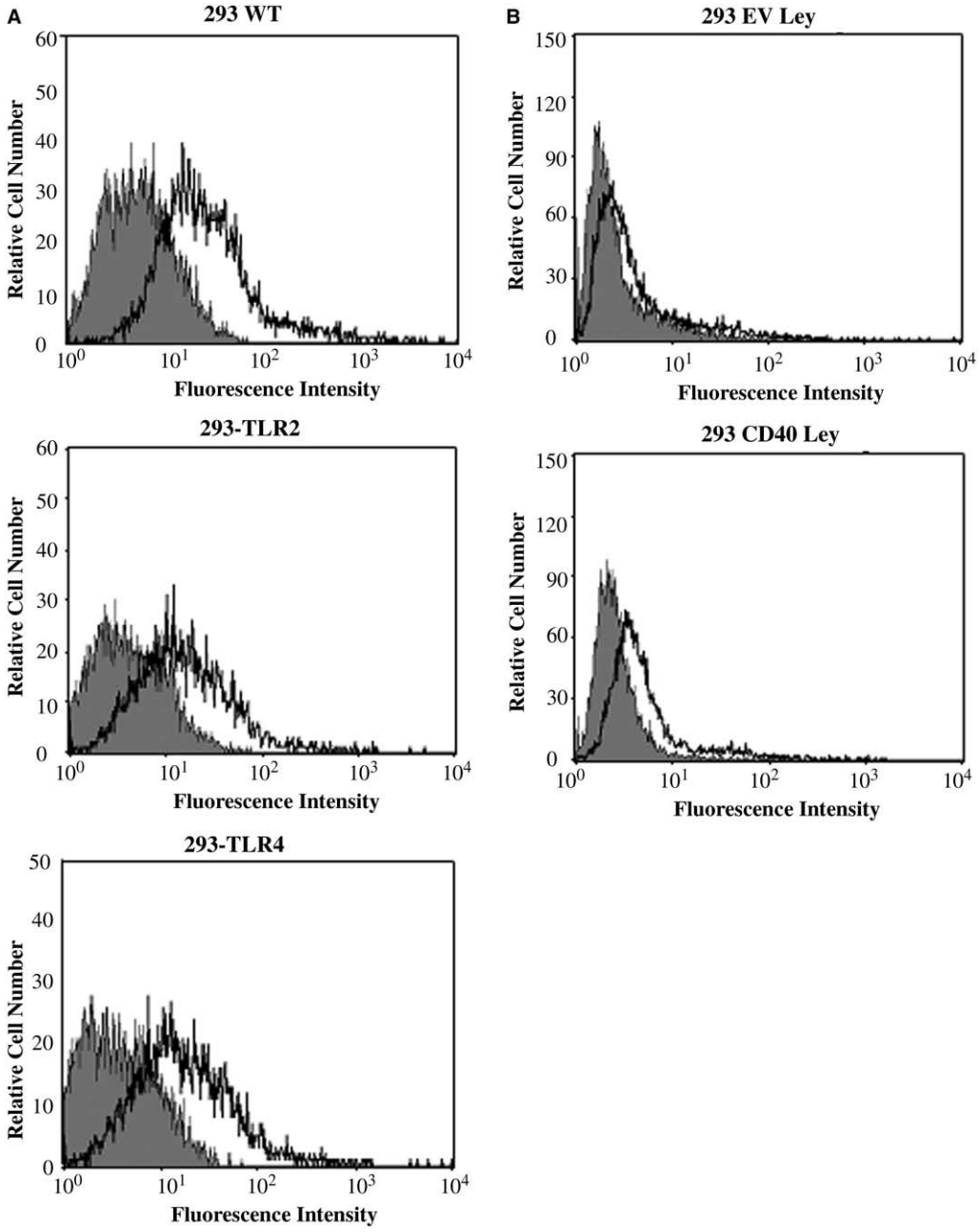

Fig. 3. Absence of association between HSP70.PC and signaling receptors such as TLR 2, 4 and CD40. (A) Wild-type HEK293, HEK293 overexpressing the TLR 2 or 4 (293 TLR2 or TLR4), (B) HEK293 overexpressing an empty vector (293 EV Ley) and HEK293 overexpressing the murine CD40 (293 CD40 Ley) (kindly provided by Dr. Steve C. Ley) were subjected to a HSP70 binding assay as mentioned in Section 2. Shaded gray and thick black line histograms corresponded to BSA and HSP70.PC binding, respectively. Experiments were carried out three times with similar results.

bating them with JTX92 antibody suggesting that these cells may possess a number of potential receptors for HSP70 excluding LOX-1 and these are widely distributed in different cell types (Fig. 5C).

4. Discussion

There has been considerable recent interest in the biological role of extracellular HSP molecular chaperones particularly in the immune response. Such HSP are evidently able to interact with target cells and evince molecular and biological responses. Extracellular HSPs may be released from cells under a range of conditions and travel through the circulation to cellular targets. HSPs are particularly interesting as potential danger signals due to their massive induction by sub-lethal and lethal stresses and their ability to bind to immune effector cells [1,6,15,45]. Previous studies suggest that HSP70 binds to a number of cell surface proteins [12,13,15,32]. Even though specific receptors have shown to

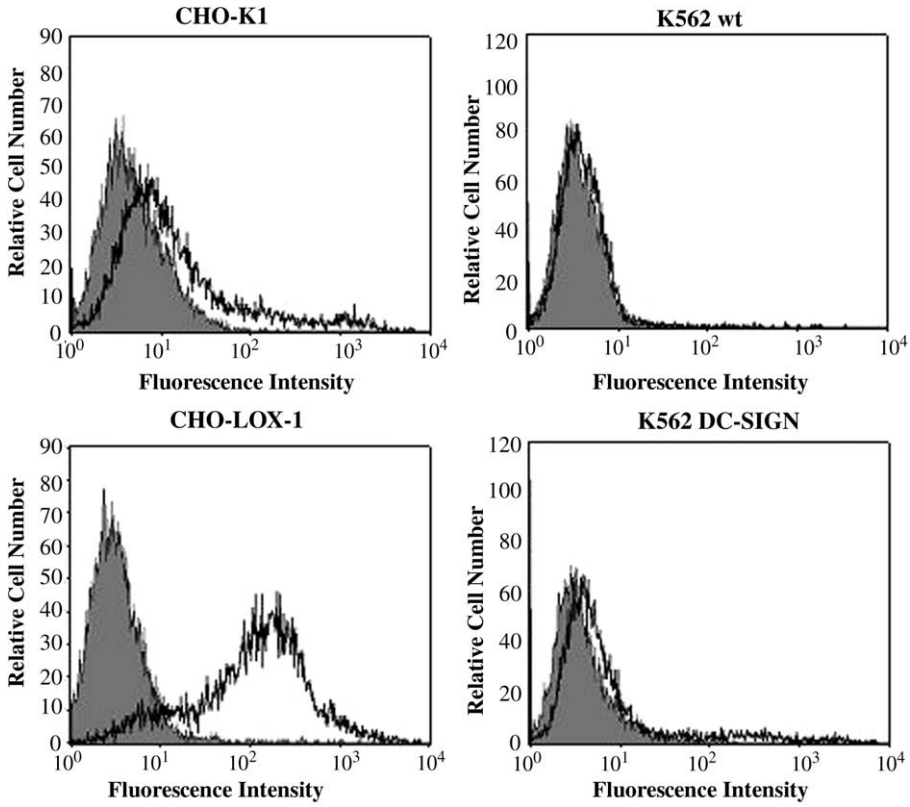

Fig. 4. C-type lectin LOX-1 but not DC-SIGN interacts with HSP70.PC. Wild-type CHO-K1 and CHO overexpressing human receptor LOX-1 (also known as the ox-LDL receptor 1) or wild-type lymphoblastic K562 and K562 overexpressing human DC-SIGN were incubated with Alexa 488-labeled BSA or HSP70.PC as reported in Section 2. Shaded gray and thick black line histograms corresponded to BSA and HSP70.PC binding, respectively. Experiments were carried out three times with similar results.

possess significant HSP70 binding affinity, their relative contribution in cell surface association has not been determined. In this study, we show strong HSP70 binding affinity to SR LOX-1 as first demonstrated by [12]. The specificity of this association seems to be restricted to LOX-1 since no significant binding occurs between HSP70 and a closely related c-type lectin family member known as DC-SIGN or SRs coming from other classes (ex. CLA-1, SR-A1, MARCO and CD36) [12]. As mentioned earlier, the c-type lectin receptor CD94 also shows some binding affinity for HSP70. However, the HSP70 relative affinity of CD94 for HSP70 seems to be weaker than for LOX-1 since more HSP70 (five times more) is needed to see a clear binding to CD94 indicating that conserved residues defining the c-type lectin domain are not the sole determinant necessary for HSP70 interaction [12,13].

LRP/CD91 binds a wide spectrum of ligands and, in every case, ligands interact, in part or entirely, with the second and/or the fourth LRP/CD91 ligand binding site located in the alpha subunit [24,41]. RAP ligand binds exclusively these two domains and can block HSP binding to LRP/CD91 while our studies show that HSP70.PC does not interact with these domains [26,41]. Although, LRP/CD91 does not seem to participate directly in HSP70.PC interaction with cells, it may however be involved at a subsequent stage in HSP70.PC-mediated cross-presentation [26].

From initial experiments showing HSP70-mediated pro-inflammatory cytokine production, HSP70 participation in the innate immune response has been linked to both TLR2 and TLR4 in a CD14-dependent fashion [6–8]. HSP70, HSP90, CXCR4, GDF5 and TLR4 are also mediators of cytokine release in a CD14-independent LPS-activated complex in monocyte [46]. However, no specific interaction is seen between HSP70 and TLR2/4 or with CD14 (Fig. 3A) [42]. It is possible that HSP70-mediated pro-inflammatory cytokine production does not require a direct association between TLRs and HSP70 but involve a putative cross-talk between TLRs and other HSP70 receptors. Supporting this idea, TLR2-mediated inflammatory response can be enhanced through synergic collaboration with endocytic receptors such the c-type lectin receptor DECTIN-1 (also known as the β-glucan receptor) in response against, for example, a yeast cell wall preparation called Zymosan [47,48].

The failure to see HSP70 binding to CD40 was quite unexpected since our HSP70 is associated with ADP and likely contains an array of endogenous mammalian peptide antigens, properties evidently required for HSP70–CD40 interaction [32]. No accurate prediction can be made about the proportion of peptide bound to HSP70. Nonetheless, ADP seems to be the major element required for the direct binding to CD40. However, only one report has proposed mamma-

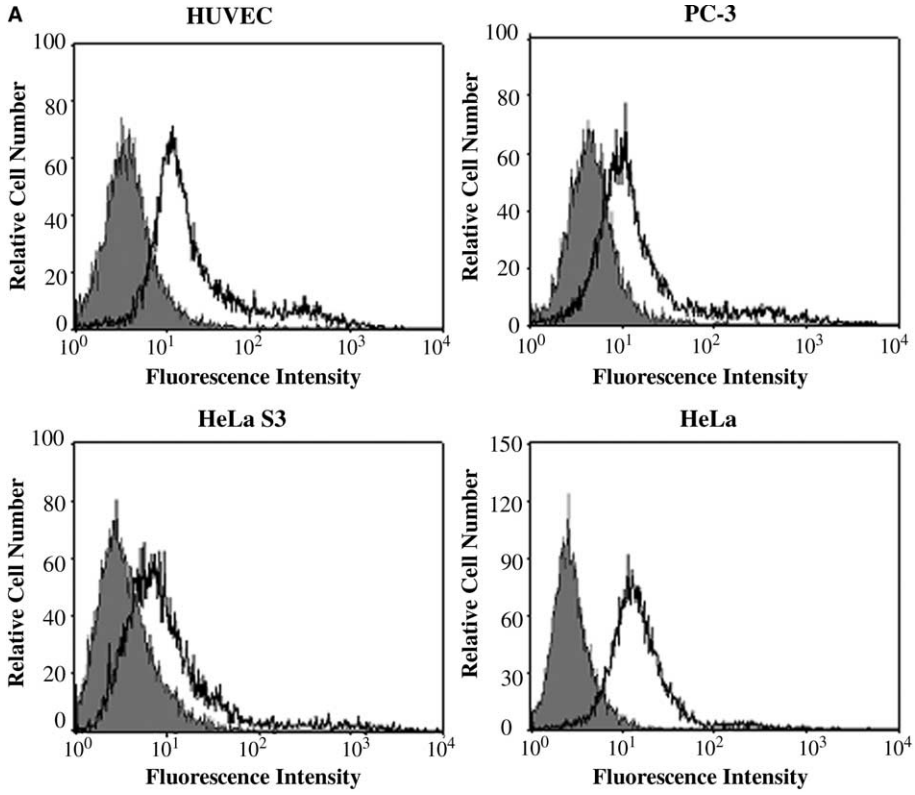

Fig. 5. HSP70.PC interacts with endothelial/epithelial cells but not other non-APC cell types. (A) HSP70 binding assay on human endothelial HUVEC, prostate cancer cell line PC-3 and HeLa from two different backgrounds. (B) No or few interaction between HSP70 and other non endothelial/epithelial cell lines IMR90 (human fibroblasts), MCF-7 (breast cancer cells) and A375 (melanoma cells). Shaded gray and thick black line histograms corresponded to BSA and HSP70.PC binding, respectively. Experiments were carried out three times with similar results. (C) HSP70.PC binding blocking experiments (Section 2) using anti-human LOX-1 (JTX92) (αLOX-1) on CHO-LOX-1, HUVEC, PC-3 and HeLa ($n = 3$). MFI: mean fluoresence intensity.

lian HSP70 as ligand for CD40 whereas other studies suggest instead that only mycobacterial hsp70 is able to transduce signal directly through CD40 while mammalian HSP70 is inactive [49]. The latter study utilized similar experimental conditions used here, involving overexpression of the human CD40 in HEK293 cells.

Previous reports have exclusively attributed surface HSPs binding affinity to immune effector such monocytes, macrophages and DC [6,15,39]. Indeed, we show here HSP70.PC interaction with pre-monocytic THP-1, mature macrophages RAW 267.4 and thioglycollate-elicited macrophages (Fig. 1). Surprisingly, we also found that HSP70.PC interacts with non-APC cells especially those from endothelial/epithelial background (Fig. 5A). LOX-1 does not however play a major role in HSP70.PC binding to HUVEC, PC-3 and HeLa suggesting the involvement of, at least, another HSP70 receptor. Concerning the HUVEC cells, one possible explanation is that LOX-1 levels were insufficient to mediate HSP70 binding. The significance of the HSP70 interaction with non-APC cells remains undefined but HSP70 binding to these cells may mediate other non-immune purposes or applications. In fact, HSP70 uptake may participate in the response to external stresses in HSP70-deficient cell types unable to mount a heat shock response such as neurons [43]. Indeed, it has been found that glial cells can release HSP70 in normal conditions or during stress such as heat shock [43]. HSP70 released from glial cells is taken up by adjacent neuronal cells, which in turn develop a higher resistance to stress-induced apoptosis. Interestingly, HSP60 has been shown to induce endothelial cytokine production and adhesion molecule expression [50]. Moreover, HSP70 is aberrantly expressed at the surface on DC and/or secreted in a rheumatoid arthritis model suggesting a role of HSPs in autoimmune diseases [51]. Thus, identification and characterization of endocytic and signaling HSP70 receptors involved in HSP70-mediated tumor antigen cross-presentation will not only help to decipher the role of HSP70 as a self-adjuvant but may pave the way to elucidate the involvement of extracellular HSP70 in other non anti-tumor immune functions such as atherosclerosis and arthritis.

Our experiments therefore indicate that LOX-1 binds with high affinity to HSP70, but other cell surface structures that appear to mediate immune effects of HSP70, such as LRP/CD91, CD40, CD14, TLR2 and TLR4 do not show significant binding to HSP70. It is, however, evident that other perhaps unidentified HSP70 receptors exist as binding to HUVEC, PC-3 and HeLa cells does not involve a major contribution from LOX-1 and our future studies will address this question.

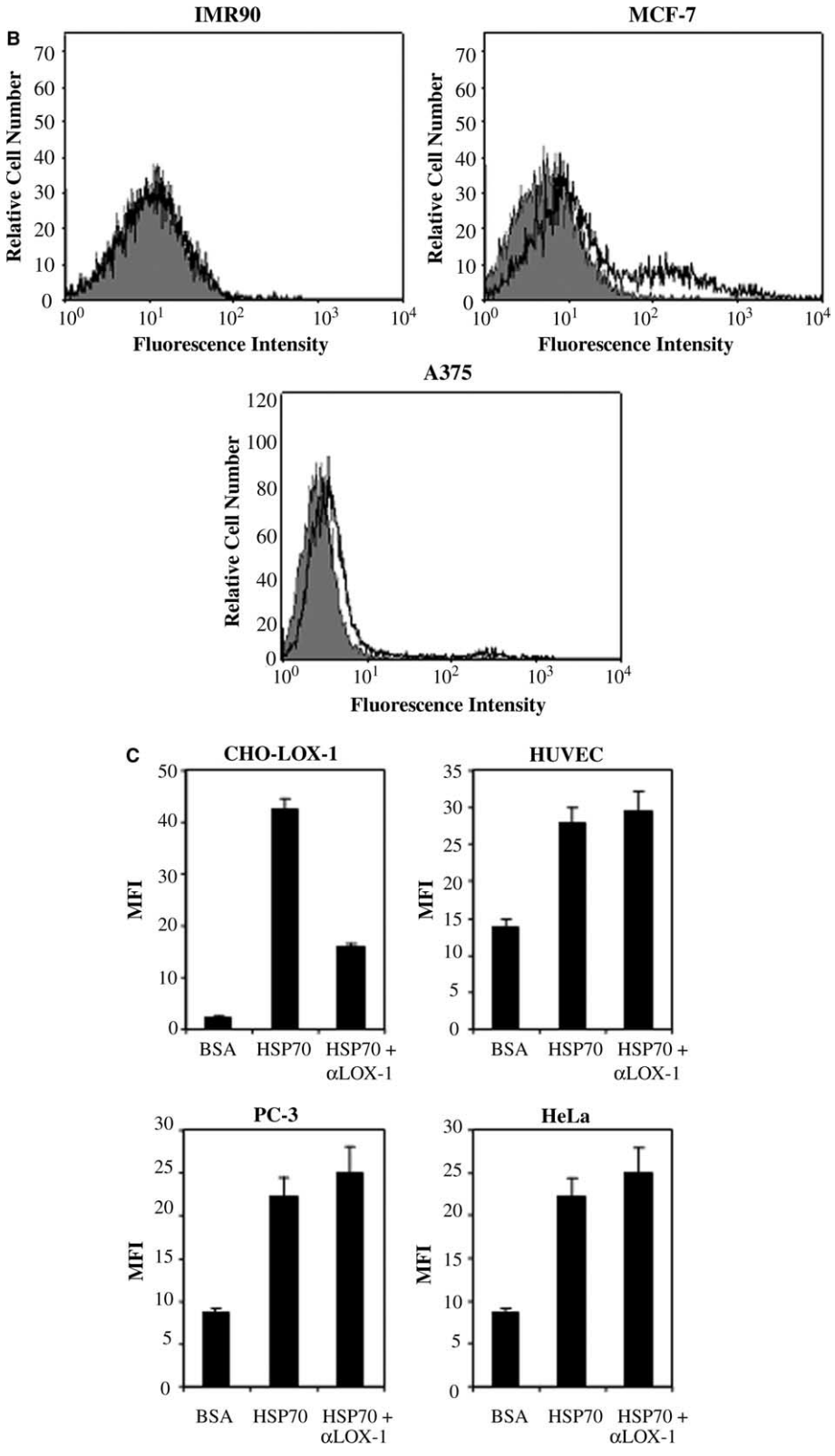

Fig. 5 (*continued*)

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References

[1] Lindquist, S. and Craig, E.A. (1988) The heat-shock proteins. Annu. Rev. Genet. 22, 631–677.
[2] Georgopoulos, C. and Welch, W.J. (1993) Role of the major heat shock proteins as molecular chaperones. Annu. Rev. Cell Biol. 9, 601–634.
[3] Udono, H. and Srivastava, P.K. (1993) Heat shock protein 70-associated peptides elicit specific cancer immunity. J. Exp. Med. 178, 1391–1396.
[4] Srivastava, P. (2002) Roles of heat-shock proteins in innate and adaptive immunity. Nat. Rev. Immunol. 2, 185–194.
[5] Baker-LePain, J.C., Reed, R.C. and Nicchitta, C.V. (2003) ISO: a critical evaluation of the role of peptides in heat shock/chaperone protein-mediated tumor rejection. Curr. Opin. Immunol. 15, 89–94.
[6] Asea, A., Kraeft, S.K., Kurt-Jones, E.A., Stevenson, M.A., Chen, L.B., Finberg, R.W., Koo, G.C. and Calderwood, S.K. (2000) HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat. Med. 6, 435–442.
[7] Vabulas, R.M., Ahmad-Nejad, P., Ghose, S., Kirschning, C.J., Issels, R.D. and Wagner, H. (2002) HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. J. Biol. Chem. 277, 15107–15112.
[8] Asea, A., Rehli, M., Kabingu, E., Boch, J.A., Bare, O., Auron, P.E., Stevenson, M.A. and Calderwood, S.K. (2002) Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J. Biol. Chem. 277, 15028–15034.
[9] Kuppner, M.C., Gastpar, R., Gelwer, S., Nossner, E., Ochmann, O., Scharner, A. and Issels, R.D. (2001) The role of heat shock protein (hsp70) in dendritic cell maturation: hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors. Eur. J. Immunol. 31, 1602–1609.
[10] Singh-Jasuja, H., et al. (2000) Cross-presentation of glycoprotein 96-associated antigens on major histocompatibility complex class I molecules requires receptor-mediated endocytosis. J. Exp. Med. 191, 1965–1974.
[11] Castellino, F., Boucher, P.E., Eichelberg, K., Mayhew, M., Rothman, J.E., Houghton, A.N. and Germain, R.N. (2000) Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. J. Exp. Med. 191, 1957–1964.
[12] Delneste, Y., et al. (2002) Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. Immunity 17, 353–362.
[13] Gross, C., Hansch, D., Gastpar, R. and Multhoff, G. (2003) Interaction of heat shock protein 70 peptide with NK cells involves the NK receptor CD94. Biol. Chem. 384, 267–279.
[14] Berwin, B., Hart, J.P., Rice, S., Gass, C., Pizzo, S.V., Post, S.R. and Nicchitta, C.V. (2003) Scavenger receptor-A mediates gp96/GRP94 and calreticulin internalization by antigen-presenting cells. EMBO J. 22, 6127–6136.
[15] Basu, S., Binder, R.J., Ramalingam, T. and Srivastava, P.K. (2001) CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity 14, 303–313.
[16] Binder, R.J., Han, D.K. and Srivastava, P.K. (2000) CD91: a receptor for heat shock protein gp96. Nat. Immunol. 1, 151–155.
[17] Krieger, M. (2001) Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. J. Clin. Invest. 108, 793–797.
[18] Gough, P.J. and Gordon, S. (2000) The role of scavenger receptors in the innate immune system. Microbes Infect. 2, 305–311.
[19] Peiser, L., Mukhopadhyay, S. and Gordon, S. (2002) Scavenger receptors in innate immunity. Curr. Opin. Immunol. 14, 123–128.
[20] Cambi, A. and Figdor, C.G. (2003) Dual function of C-type lectin-like receptors in the immune system. Curr. Opin. Cell Biol. 15, 539–546.
[21] Figdor, C.G., van Kooyk, Y. and Adema, G.J. (2002) C-type lectin receptors on dendritic cells and Langerhans cells. Nat. Rev. Immunol. 2, 77–84.
[22] Hayashida, K., Kume, N., Minami, M. and Kita, T. (2002) Lectin-like oxidized LDL receptor-1 (LOX-1) supports adhesion of mononuclear leukocytes and a monocyte-like cell line THP-1 cells under static and flow conditions. FEBS Lett. 511, 133–138.
[23] Chen, M., Masaki, T. and Sawamura, T. (2002) LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: implications in endothelial dysfunction and atherosclerosis. Pharmacol. Ther. 95, 89–100.
[24] Herz, J. and Strickland, D.K. (2001) LRP: a multifunctional scavenger and signaling receptor. J. Clin. Invest. 108, 779–784.
[25] Li, Y., Wood, N., Parsons, P.G., Yellowlees, D. and Donnelly, P.K. (1997) Expression of alpha2-macroglobulin receptor/low density lipoprotein receptor-related protein on surfaces of tumour cells: a study using flow cytometry. Cancer Lett. 111, 199–205.
[26] Binder, R.J. and Srivastava, P.K. (2004) Essential role of CD91 in re-presentation of gp96-chaperoned peptides. Proc. Natl. Acad. Sci. USA 101, 6128–6133.
[27] Gardai, S.J., Xiao, Y.Q., Dickinson, M., Nick, J.A., Voelker, D.R., Greene, K.E. and Henson, P.M. (2003) By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. Cell 115, 13–23.
[28] Shepherd, J.C., Schumacher, T.N., Ashton-Rickardt, P.G., Imaeda, S., Ploegh, H.L., Janeway Jr., C.A. and Tonegawa, S. (1993) TAP1-dependent peptide translocation in vitro is ATP dependent and peptide selective. Cell 74, 577–584.
[29] Schumacher, T.N., et al. (1994) Peptide length and sequence specificity of the mouse TAP1/TAP2 translocator. J. Exp. Med. 179, 533–540.
[30] Neefjes, J.J., Momburg, F. and Hammerling, G.J. (1993) Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. Science 261, 769–771.
[31] Asea, A., Kabingu, E., Stevenson, M.A. and Calderwood, S.K. (2000) HSP70 peptide bearing and peptide-negative preparations act as chaperokines. Cell Stress Chaperones 5, 425–431.
[32] Becker, T., Hartl, F.U. and Wieland, F. (2002) CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. J. Cell Biol. 158, 1277–1285.
[33] Millar, D.G., Garza, K.M., Odermatt, B., Elford, A.R., Ono, N., Li, Z. and Ohashi, P.S. (2003) Hsp70 promotes antigen-presenting cell function and converts T-cell tolerance to autoimmunity in vivo. Nat. Med. 9, 1469–1476.
[34] Relloso, M., et al. (2002) DC-SIGN (CD209) expression is IL-4 dependent and is negatively regulated by IFN, TGF-beta, and anti-inflammatory agents. J. Immunol. 168, 2634–2643.
[35] Coope, H.J., et al. (2002) CD40 regulates the processing of NF-kappaB2 p100 to p52. EMBO J. 21, 5375–5385.
[36] Moriwaki, H., et al. (1998) Ligand specificity of LOX-1, a novel endothelial receptor for oxidized low density lipoprotein. Arterioscler. Thromb. Vasc. Biol. 18, 1541–1547.
[37] Mambula, S.S., Sau, K., Henneke, P., Golenbock, D.T. and Levitz, S.M. (2002) Toll-like receptor (TLR) signaling in response to *Aspergillus fumigatus*. J. Biol. Chem. 277, 39320–39326.
[38] Peng, P., Menoret, A. and Srivastava, P.K. (1997) Purification of immunogenic heat shock protein 70-peptide complexes by ADP-affinity chromatography. J. Immunol. Methods 204, 13–21.
[39] Noessner, E., et al. (2002) Tumor-derived heat shock protein 70 peptide complexes are cross-presented by human dendritic cells. J. Immunol. 169, 5424–5432.
[40] Berwin, B., Hart, J.P., Pizzo, S.V. and Nicchitta, C.V. (2002) Cutting edge: CD91-independent cross-presentation of GRP94 (gp96)-associated peptides. J. Immunol. 168, 4282–4286.
[41] Obermoeller-McCormick, L.M., Li, Y., Osaka, H., FitzGerald, D.J., Schwartz, A.L. and Bu, G. (2001) Dissection of receptor folding and ligand-binding property with functional

minireceptors of LDL receptor-related protein. J. Cell Sci. 114, 899–908.
[42] Lipsker, D., et al. (2002) Heat shock proteins 70 and 60 share common receptors which are expressed on human monocyte-derived but not epidermal dendritic cells. Eur. J. Immunol. 32, 322–332.
[43] Guzhova, I., Kislyakova, K., Moskaliova, O., Fridlanskaya, I., Tytell, M., Cheetham, M. and Margulis, B. (2001) In vitro studies show that Hsp70 can be released by glia and that exogenous Hsp70 can enhance neuronal stress tolerance. Brain Res. 914, 66–73.
[44] Sawamura, T., et al. (1997) An endothelial receptor for oxidized low-density lipoprotein. Nature 386, 73–77.
[45] Broquet, A.H., Thomas, G., Masliah, J., Trugnan, G. and Bachelet, M. (2003) Expression of the molecular chaperone Hsp70 in detergent-resistant microdomains correlates with its membrane delivery and release. J. Biol. Chem. 278, 21601–21606.
[46] Triantafilou, K., Triantafilou, M. and Dedrick, R.L. (2001) A CD14-independent LPS receptor cluster. Nat. Immunol. 2, 338–345.
[47] Brown, G.D., Taylor, P.R., Reid, D.M., Willment, J.A., Williams, D.L., Martinez-Pomares, L., Wong, S.Y. and Gordon, S. (2002) Dectin-1 is a major beta-glucan receptor on macrophages. J. Exp. Med. 196, 407–412.
[48] Gantner, B.N., Simmons, R.M., Canavera, S.J., Akira, S. and Underhill, D.M. (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. J. Exp. Med. 197, 1107–1117.
[49] Wang, Y., et al. (2001) CD40 is a cellular receptor mediating mycobacterial heat shock protein 70 stimulation of CC-chemokines. Immunity 15, 971–983.
[50] Kol, A., Bourcier, T., Lichtman, A.H. and Libby, P. (1999) Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. J. Clin. Invest. 103, 571–577.
[51] Martin, C.A., Carsons, S.E., Kowalewski, R., Bernstein, D., Valentino, M. and Santiago-Schwarz, F. (2003) Aberrant extracellular and dendritic cell (DC) surface expression of heat shock protein (hsp)70 in the rheumatoid joint: possible mechanisms of hsp/DC-mediated cross-priming. J. Immunol. 171, 5736–5742.