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Receptors for extracellular heat shock proteins

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Abstract

Extracellular heat shock proteins (HSP) play important roles in cell signaling and immunity. Many of these effects are mediated by cell surface receptors expressed on a wide range of cell types. We have investigated the nature of such proteins by cloning candidate receptors into cells (CHO-K1) with the rare property of being null for HSP binding. Using this approach we have discovered that Hsp70 binds to a least two classes of receptor: c-type lectin receptors (CLR) and scavenger receptors (SR). However the nature of the receptor-ligand interactions is not yet clear. Hsp70 can bind to LOX-1 (a member of both the CLR and SR), with the c-type lectin binding domain (CTLD) as well as the SR family members SREC-I and FEEL-1/CLEVER-1/STABILIN-1, which by contrast have arrays of EGF-like repeats in their extracellular domains. In this chapter we discuss (1) methods for determining HSP receptors, (2) approaches to study of individual receptors in cells that contain multiple such receptors and (3) methods for investigating HSP receptor function *in vivo*.

Keywords

extracellular; heat; shock; protein; scavenger; receptor; immunity

Introduction

It is apparent that heat shock proteins (HSP) play significant signaling roles in the extracellular microenvironment¹. HSP have been found in human serum particularly after disease or stress^{2, 3}. The 70 kilodalton heat shock protein (Hsp70) has been shown to be released from cells after acute stress as well as being secreted after exposure to a number of stimuli^{4, 5}. Extracellular HSPs may thus be able to play the role of danger signal (danger activated molecular pattern or DAMP)⁶. In this context, they may interact with pattern

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(1)*Purified HSP.* Most heat shock proteins are encoded by multigene families. This is particularly true for Hsp70 in which there are, depending on species at least 12 closely related gene family members each of which may bind to cells with differing affinities³⁹. This complication could be remedied by the use of recombinant proteins. However, if recombinant proteins are produced in *E. coli*, great care must be taken to remove contaminating PAMPs such as lipopolysaccharides or peptidoglycans¹¹. We have prepared recombinant HSPs using the baculovirus/sf9 system to avoid these problems. However, LPS in particular can be introduced into protein preparations from contaminated glassware and we have been very careful to assay all batches of HSP by the *limulus* assay.

(2)HSP bind with only moderate affinity to the receptors uncovered so far and these are promiscuous in terms of binding partners. In most cases the structural basis for HSP-receptor binding is not known. Thus much needs to be learned regarding HSP-receptor interaction. Future *in vitro* studies should address questions of three dimensional structure of HSP-receptor complexes and the exact affinities of the interactions.

(3)Many cells express multiple HSP receptors and great care must be taken to isolate the properties of individual receptors. It seems likely that other as yet unknown receptors exist.

recognition receptors (PRR) such as Toll-like receptors (TLR) and activate pro-inflammatory signaling and transcription^{7, 8}. Proteins including Hsp60, Hsp70, Grp 96 have been implicated as DAMPs^{9, 10}. However, interpretation of such experiments however requires caution and careful control as some HSPs have the ability to bind to prokaryotic molecules that activate TLR signaling, such as lipopolysaccharides¹¹. In addition, many members of the HSP family can participate in adaptive immunity by binding to antigenic peptides and transporting them into antigen presenting cells (APC)^{12, 13}. HSPs mediate the process of *antigen cross presentation*¹⁴ by facilitating internalization of antigens and permitting their delivery to major histocompatibility class I (MHC class I) molecules. MHC I-peptide complexes can then stimulate cognate T cell receptors on T lymphocytes and initiate the activation of clones of such powerful immune effectors. HSP may thus play a versatile role in anti-tumor immunity by activating the innate and adaptive arms. HSP can additionally activate natural killer cells and lead to tumor cell killing and CD25+ immune regulatory T cells^{15–17}. HSP can thus upregulate or down regulate immunity depending on context.

Many studies have suggested that HSP activate immunity by binding to receptors on the cell surface^{18–26}. HSP binding is saturable and competed for by unlabeled ligand, properties of receptor-mediated signaling. However, this is where consensus seems to end and some controversy exists as to the most significant HSP receptors. We have attempted to address this issue by screening the various contenders for binding to Hsp70 and Hsp90.

Materials

Plasmids

pET23 *hsp90a* plasmid
 pDESTTM10
 AC-to-BAC Baculovirus transfection kit
 pCDNA3.1 eukaryotic expression vector

Cell lines

Chinese Hamster Ovary-K1 cells (CHO-K1).
 A375 human melanoma
 MISA human breast carcinoma cells
 Sf9 insect cells
 MC38 cells stably expressing the MUC1 tumor antigen
 B16 melanoma cells
 B16 melanoma cells stably expressing the MUC1 tumor antigen

Mouse Systems and primary murine cells

Wild type mice C57BL/6 and *tlr2*^{−/−} / *tlr4*^{−/−} double knockouts
 Primary mouse bone marrow dendritic cells were prepared from C57BL/6 as in text.

Splenocytes and/or lymph node cells (LNC) were isolated from mice immunized with Hsp70.PC fusion vaccine as described²⁷

Chromatography

Ni-NTA purification system (Quiagen)
 10 ml Sephadex G-25 in PD10 column (Sigma-Aldrich)
 5.0 ml ADP-agarose column (Sigma-Aldrich)
 20 ml DEAE-cellulose anion exchange column (Pierce Chemicals)

Buffers & reagents

Hypotonic Buffer: 10 mM NaHCO₃, 0.5 mM PMSF, pH 7.1
Buffer D: 20 mM Tris-acetate, 20 mM NaCl, 15 mM b-mercaptoethanol, 3 mM MgCl₂, 0.5 mM PMSF, pH 7.5
ADP-agarose Elution buffer: 3.0 mM ADP in buffer D
FPLC buffer: 20 mM sodium mono and diphosphate, 20 mM NaCl, pH 7.0
DEAE-cellulose elution buffer: 150 mM NaCl in FPLC buffer.
Hsp70 binding buffer (PFNC): 0.5% FBS, 0.05% NaN₃ and 1mM CaCl₂.
 Hanks' Buffered Saline Solution.

Antibodies

Anti-Hsp70 antibody (SPA-810, Assay Designs Inc).

Chromophores

Alexa488 (Molecular Probes)

shRNA to SREC-I

MISSION™ shRNA plasmids (shRNA) were purchased (Sigma-Aldrich, St. Louis, MI) and the Lentivirus generation and transduction were performed according to the manual of ViralPower™ Lentiviral Expression Systems (Invitrogen).

(1) Screening for HSP receptors—We have screened receptors for HSP binding in the context of cell surface expression, by expressing candidate receptors in cells null for Hsp70 binding. A number of primary and tissue culture cells were therefore screened for lack of capacity to bind to Hsp70. We screened both primary cells and established cell lines (Table 1). Maintenance of established cell lines is described previously²⁸. Human Umbilical Vein Endothelial Cells (HUVEC) were maintained in Endothelial Basal Medium-2 (EBM-2) supplemented with Clonetics™ SingleQuot® (Cambrex/Biowittaker). Isolation of peritoneal macrophages was carried out as previously described²⁹. 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 and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and penicillin streptomycin. Bone marrow-derived dendritic cells (BMDCs) were generated from the femur and tibiae of C57BL/6

mice. The bone marrow was flushed out and cultured in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS) and 40ng/ml GMCSF for 6 days. On day 3, a third of the media was replaced by fresh growth media.

1.2 Alexa 488-labeled purified HSP70 preparation—Human melanoma cells A375-MEL or mouse MISA cells were used as starting material for Hsp70 preparation because high endogenous HSC70 and/or HSP70 levels were detected in these cell types (J. Theriault & SK Calderwood, unpublished). In addition, for some experiments we used minced mouse liver as an abundant source for Hsp70. The Hsp70 purification protocol was based on previous studies³⁰. Briefly, a 10-ml cell pellet of tumor cells or minced liver was homogenized in 40 ml hypotonic buffer by Dounce homogenization. The homogenate was then spun at 10,000×g for 30 min and the supernatant was further treated for 60 min. at 100,000×g. The sample buffer was changed to buffer D using a PD-10 desalting column (Amersham-Biosciences). The material was then applied directly to a 5-ml ADP-agarose column pre-equilibrated with buffer D. Hsp70 was eluted from the ADP-agarose column with 3 mM ADP in buffer D. The sample buffer was then changed to FPLC buffer with PD-10 column. The supernatant was applied to a DEAE anion exchange column equilibrated with FPLC buffer (Amersham-Biosciences). Hsp70 was eluted with the FPLC buffer containing 150 mM NaCl. Protein concentrations were determined by *Bradford* assay. Purified Hsp70 was then labeled with fluorophore Alexa 488 according to the manufacturer's instructions (Molecular Probes, USA). Intactness and purity of the labeled Hsp70 was checked by SDS-PAGE, Coomassie staining and the presence of Hsp70 in the preparation confirmed by Western blotting using a mouse monoclonal antibody specific for HSP70.

1.3 Alexa 488-labeled purified Hsp90 preparation—Hsp90 alpha DNA was prepared by PCR amplification from the pET23 plasmid and cloned into pDESTTM10. Overexpression of Hsp90 alpha in Sf9 cells was achieved according to the BAC-to-BAC Transfection kit protocol of *Invitrogen*. Transfer vector was transformed into DH10BAC competent cells containing bacmid DNA. Later, colonies containing recombinant bacmid were identified and prepared. The bacmid DNA was then transfected into Sf9 cells using CellFECTIN (Invitrogen) to make recombinant baculovirus according to manufacturer's protocol.

Sf9 cells were grown in Sf900II serum-free medium (Invitrogen) supplemented with 100 U/ml penicillin-streptomycin and 2mM of L-glutamine in suspension cultures with continuous shaking at 150 rpm at 27°C in a non-humidified environment. The insect cultures were infected in the log phase of growth with recombinant baculovirus. Cells were harvested 48 hr post infection, washed with Hank's buffered saline solution and protein was purified using the Ni-NTA purification system according to the manufacturer's protocol (Invitrogen). AcTEV protease was used to cleave the 6× His tag from the fusion protein generated using pDESTTM10 after purifying the recombinant protein on a nickel chelating resin. Purified Hsp90 was then labeled with Alexa 488 as above. Intactness and purity of the labeled Hsp90 was checked by SDS-PAGE, Coomassie Blue staining and the presence of Hsp90 in the preparation confirmed by immunoblot.

1. 4 HSP binding assay—Cells were first screened by binding to Alexa 488-labeled HSP *in vitro* and analysis by flow cytometry. 2×10^5 non-trypsinized cells were washed twice in PFNC buffer and incubated with 150 nM Alexa 488-labeled BSA (negative control), Hsp70 or Hsp90 for 30 min on ice with gentle shaking. The cells were washed twice in PFNC buffer and Alexa 488-labeled protein binding was monitored by flow cytometry (Becton Dickinson).

Experiments utilizing flow cytometry were next confirmed by confocal fluorescence microscopy. Alexa Fluor conjugated BSA, Hsp70 or Hsp90 were prepared as above. Cells were labeled with ligand for 20–30 min on ice. Cells were later washed with ice-cold stripping buffer to remove unbound Hsp90. Cells were then fixed with 4% para-formaldehyde and permeabilized with 0.1% TritonX-100. Fluorescence was then visualized using a Zeiss 510 confocal microscope (Carl Zeiss GmbH, Jena, Germany). Fluorophores were visualized using 488nm excitation and a band pass 505–530 emission filter for Alexa 488. Images were taken using a 63 × numerical aperture (NA) 1.4 oil immersion objective lens.

To assay individual receptors for HSP binding, we selected CHO-K1 cells as null for HSP binding in the wild-type state: Cells were then transfected with expression plasmids for individual receptors following the protocol used for study of LOX-1.

2.5×10^5 CHO-K1 cells were transiently transfected with 5 ug of empty vector (pCDNA3) or pCDNA3 plasmids encoding Myc-tagged LOX-1, for 48 hours using the Superfect transfection reagent according to the manufacturer's instructions (QIAGEN). Expression of recombinant proteins were analyzed after transfection by SDS-PAGE and immunoblot with the mouse monoclonal anti-Myc antibody (clone 9E10, Stratagene, USA). Cell lines were maintained by selection with neomycin and checked routinely for expression of Myc-tagged product. In addition, we examined the cell surface location of the candidate receptors using antibodies to the extracellular domains of such proteins.

Using this approach, we have examined a number of candidate receptors. As previous studies had suggested a role for LOX-1 in immune responses to Hsp70, we began with study of this protein and have confirmed that Hsp70 binds avidly to CHO-LOX-1 cells (Table 2)^{21, 31}. LOX-1 has been assigned to at least two distinct protein families, the c-type lectins and the scavenger receptors (SR)^{31, 32}. C-type lectin receptors (CLR) are a large family of receptors characterized by the possession of a common binding domain – the Ca^{++} dependent carbohydrate binding motif (CTLD)^{33, 34}. Binding to protein ligands can be inhibited by use of hapten sugars that differ between different CLR family members. In the case of LOX-1, fucoidin is a hapten sugar that interacts with its CTLD domain and inhibits Hsp70 binding to LOX-1²¹. Scavenger receptors (SR) have been studied mostly in endothelial cells but are expressed in dendritic cells and macrophages also (J. Gong, A Murshid & SK Calderwood, in preparation). SR are a group of proteins that are clustered according to their function in cells- their ability to interact with chemically modified proteins in the extracellular fluid, as exemplified by binding oxidized low density lipoprotein and acetylated or maleylated bovine serum albumin (BSA)^{35–38}. Binding of HSP to SR can initially be screened by competition assay using known SR binding proteins such as maleylated BSA, oxidized LDL, acetylated LDL, apolipoprotein B or polyanions such as polyinosine²¹. In this approach, one ligand is labeled (fluorescently for flow cytometry) and the other one remains unlabeled. The competition assay is done with a constant concentration of labeled HSP and varying concentration of unlabeled ligands such as mBSA, AcLDL. The basis for this interaction as well as HSP binding is not well understood as the extracellular domains of individual SR are highly divergent³⁸.

We screened members of these receptor families and confirmed that Hsp70 binds to LOX-1 and as well as two other SR family members, SREC-I and FEEL-1 / Stabilin1 / CLEVER-1 when expressed in CHO-K1 (Table 2)^{39–41}. Others have subsequently shown that another SR family member – scavenger receptor A can interact with HSP family members which however fail to bind CD36, MARCO and CLA-1^{21, 42}. Hsp70 also binds to other members of the CLR family such as NKG2D that are expressed in natural killer cells (Table 2)³⁹. However, in our studies, Hsp70 failed to bind to a number of major CLR family members

including Dectin-1, DC-SIGN, CLEC-1 and CLEC-2 (Table 2)³⁹. Some studies also indicate a role for the LDL receptor-related protein (LRP) or CD91 in HSP binding. LRP/CD91 contains four clusters of binding repeats that mediate association with at least 30 different ligands including apolipoprotein E, α_2 macroglobulin, pro-urokinase and others^{43,44}. Most ligands bind specifically to two of these clusters of binding repeats within domains II and IV⁴³. However, we could not detect Hsp70 binding to either domain II or IV when expressed in CHO-K1 cells, and in addition, LRP null cells appeared to bind Hsp70 as well as wild-type cells (Table 2)^{20,39,45}. Endocytosis of the molecular chaperone calreticulin was also not decreased in CD91^{-/-} cells casting some doubt on CD91 as a universal endocytic receptor for HSPs⁴⁶. By contrast scavenger receptor SRA has been shown to be required for a large proportion of gp96 and calreticulin uptake¹⁹. In the case of Hsp90, neither fucoidin (LOX-1 agonist) nor α_2 -macroglobulin (LRP/CD91 agonist) were able to block representation of a peptide bound to Hsp90⁴⁷. Nonetheless others have shown that inactivation of CD91 can lead to loss of antigen re-presentation ability in cells exposed to gp96 / peptide complexes⁴⁸. The large heat shock proteins Hsp110 and Grp170, that have potent immune properties, can bind to SRA and SREC-I and there is also some evidence for binding to LRP/CD91²². There is additionally evidence indicating a possible role for LRP/CD91 in Hsp70 binding to macrophages²¹. Inhibition by α_2 macroglobulin competition has often been used as a criterion for HSP binding to LRP/CD91²². In addition, our unpublished experiments indicate that levels of SREC-I and LOX-1 are very low in unstimulated murine macrophages in which CD91 may play a significant role. The usage of HSP receptors could thus vary with the nature of the chaperone ligand, immune cell type and the activation state of the cell. Our studies *in vivo* indicate that TLR signaling is essential for SREC-I-expressing DC to traffic to afferent lymph nodes after vaccination with Hsp70 vaccine²⁷. Hsp70 can itself induce SREC-I expression in TLR pathway proficient murine DC suggesting a feed-forward mechanism in which Hsp70 induces its own receptors (SR) and amplifies immune effects of Hsp70-peptide complexes²⁷. There are also indications that Hsp70 activates signaling receptors such as TLR2, TLR4 and CD40 and may be involved in inducing inflammation and innate immunity^{9,10,49}. However, using the CHO transfection system described above, we were unable to confirm direct binding of Hsp70 to these molecules (Table 2). At least in the case of TLR2, indirect activation of this receptor downstream of both LOX-1 and SREC-I is observed after receptor binding to bacterial protein OMP1⁵⁰.

Figure 1 shows a cartoon indicating that at least 4 HSP receptors exist and could potentially be co-expressed in a single cell. In addition, a range of other ligands can interact with such receptors. The existence of multiple receptors therefore complicates interpretation of experiments probing the function of extracellular HSP. This difficulty is exacerbated by the findings that, while SREC-I and LOX-1 have pro-immune functions, SRA-1 appears to be inhibitory to the immune response by inhibiting the activity of TLR4^{21,27,42,51}. The receptors may thus have both additive and confounding effects.

With this plethora of receptors, the nature of the HSP-receptor interaction is still in some doubt. However, the crystal structure of ligand-bound LOX-1 has recently been determined. These studies indicate that the ligand (oxidized LDL) binding surface is hydrophobic except for a basic spine composed of arginine residues essential for ligand binding^{52,53}. These positively charged arginine residues together with the hydrophobic residues appears to confer the specificity of LOX-1 for negatively charged lipids and lipoproteins⁵². LOX-1 binds to its ligands as a homodimer with an intramolecular disulfide bond⁵³. It is not clear to what degree the ligand binding properties of SREC-I and FEEL-1 / CLEVER1 resemble those of LOX-1. The extracellular domains of these two SR do not contain CTLD, but consist mostly of multiple EGF-like repeats⁵⁴. These repeat domains have a length of approximately 40 amino acids and are characterized by conserved arrangement of six

cysteine residues found in EGF itself^{55, 56}. It has been shown that at least four tandem repeats of EGF-like regions are required for the FEEL-1 homolog FEEL-2 (STABILIN-2) to bind the acidic lipid phosphatidylserine⁵⁷. It is apparent therefore that much is left to be learnt regarding the specificity and sequence requirements for HSP binding to these candidate receptors.

2. Studying HSP-SREC-I interaction *in vivo*—As we currently have not developed SREC-I knockout mice, we took the approach of knocking down SREC-I in dendritic cells by shRNA. Our studies had indicated that in mice with TLR2 / TLR4 knockdown, responses to Hsp70 vaccine (Hsp70-FC) were strongly inhibited^{27, 58}. We utilized this finding to study the significance of SREC-I in responses to Hsp70 *in vivo*. To address this issue, we first determined whether the decreased immunity in TLR knockout cells could be reversed by adoptive transfer of wild type DC (WT-DC) into *tlr2*^{-/-} / *tlr4*^{-/-} mice²⁷. Indeed transfer of Hsp70-FC pulsed DC was able to induce immunity to tumors in naïve WT mice and support tumor specific cytotoxicity²⁷. We next showed that immunization of *tlr2*^{-/-} / *tlr4*^{-/-} mice with WT DC that had been pulsed with Hsp70-FC also increased the cytotoxic lymphocyte (CTL) activity and CTL frequency of such mice²⁷. This implies that WT donor DC were able to compensate for the endogenous, inactive DC in *tlr2*^{-/-} / *tlr4*^{-/-} mice. Groups of such knockout mice were next immunized with DC generated from WT mice that had been infected with SREC-I shRNA or control shRNA constructs and then pulsed with Hsp70-FC. SREC-I knockdown inhibited the ability of WT DC to compensate for TLR knockout indicating that this is a viable approach for studying SREC-I function *in vivo*. These experiments therefore suggested that SREC-I is essential for antigen presentation by DC exposed to Hsp70-FC. These findings were confirmed by assessing the frequency of antigen-specific T cells induced using an MHC-class-I/peptide tetramer (iTag). The 8-mer peptide (SAPDTRPA) is a dominant epitope from MUC1 that binds to C57BL/6 MHC class I, H-2K^b⁵⁹. MUC1 is one of the most prominent tumor antigens present in the Hsp70-FC vaccine²⁷. The MUC1-8 iTag was used to identify and assess the tetramer-positive T cells. The numbers of MUC1-8 iTag-positive T cells from *tlr2*^{-/-} / *tlr4*^{-/-} immunized with SREC-I knockdown DC were significantly decreased compared with those from mice transferred with DC infected with control virus. Immunization of *tlr2*^{-/-} / *tlr4*^{-/-} mice either with Hsp70-FC pulsed DC after SREC-I knockdown or after infection with control virus resulted 1.45% and 3.51% CD8 T cells positive for MUC1-8 indicating that that SREC-I plays an important role in the induction of antigen-specific T cells by Hsp70-based vaccines.

2.1 shRNA directed against SREC-I: MISSION™ shRNA plasmids (shRNA) were purchased (Sigma-Aldrich, St. Louis, MI) and the Lentivirus generation and transduction were performed according the manual of ViralPower™ Lentiviral Expression Systems (Invitrogen). The effectiveness of shRNA for knockdown of murine SREC-I (five clones: TRCN0000067873; TRCN0000067874; TRCN0000067875; TRCN0000067876 and TRCN0000067877) was examined Real-Time PCR and immunoblot. The most effective construct for mSREC-I mRNA knockdown (TRCN0000067875) was used. The Lentivirus (insert sequence was 5'-CCGCAGGTATGCACGCGT-3' which does not target any mouse genes, but will activate RISC and the RNAi pathway in the cells) was used as negative control.

Three-day-old DC were collected, purified, placed in 96 well round bottom plates with 1×10^6 cell per well for O/N culture in medium containing GM-CSF. On the second day, half of the medium (100 μ l) was removed and 100 μ l Lenti-virus supernatant (1 virus :1 DC) was added. After 20 hours, 150 μ l of medium from each well was replaced by fresh GM-CSF medium for additional culture. DC infected with SREC-I shRNA or control constructs or un-

infected DC were collected for SREC1 expression and T cell stimulation assay. Significant SRECI knockdown at the protein level was achieved in 90–100 hours.

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Figure 1.

A cell expressing four HSP receptors that could bind simultaneously to extracellular HSP. We also depict three of the potential ligands in the extracellular fluid, including HSP, oxidized LDL particles and chemically modified proteins. We depict relative lengths of the intracellular and extracellular domains of the proteins (■).

TABLE 1**Binding to Hsp70**

Cell type	Species	Hsp70 binding
THP1, monocyte	Human	+
RAW264.7, macrophage	mouse	++
Primary macrophage	mouse	++
Primary dendritic cell	mouse	++
HEK293 embryonic kidney	Human	+
Vacular endothelial	Human	++
PC-3, prostate carcinoma	Human	+
HeLa, cervical carcinoma	Human	++
Hela S3 cervical carcinoma	Human	+
MCF7, mammary Cancer	Human	+
IMR90, fibroblast	Human	–
K562, pluripotent leukemia	Human	–
A375, melanoma	Human	–
CHO K1, ovarian cells	Chinese hamster	–

TABLE 2

Candidate HSP receptors

Receptor	Type	Expressed in	Hsp70	Hsp90
TLR2	Signaling	APC etc	–	ND
TLR4	Signaling	APC etc	–	ND
CD14	Signaling	APC etc	–	ND
CD40	Signaling	APC	–	ND
CD91	Internalizing	many	–	ND
LOX-1	scavenger/CTL	Endo, APC	+	++
DC-SIGN	scavenger/CTL	APC	–	ND
Dectin 1	scavenger	APC	–	ND
CLEC-1	scavenger	APC	–	ND
CLEC2	scavenger	APC	–	ND
SREC-1	scavenger	APC	++	++
FEEL-1	scavenger	APC	++	++
NKG2A	CTL	NK	++	ND
NKG2C	CTL	NK	++	ND
NKG2D	CTL	NK, T cell	++	ND

Binding to receptors was assayed in CHO transfectants with two exceptions, which are DC-SIGN, which was in K-562 (also HSP binding null) and the TLR, which were in HEK293. Ability to compete with 25-fold XS cold HSP70 is indicated in last column.