Identification of the heat shock protein 60 epitope involved in receptor binding on macrophages

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Abstract In the present study, we identified the human heat shock protein 60 (HSP60) epitope responsible for binding to macrophages. Studies using overlapping 15- and 20-mer peptides of the human HSP60 sequence to compete with binding of HSP60 to macrophages indicated that surface binding was accounted for by the region aa481–500. Deletion mutants of HSP60, lacking the N-terminal 137, 243 or 359 amino acids, strongly inhibited HSP60 binding to macrophages. Monoclonal antibodies addressing regions aa1–200, aa335–366 or aa383–447 did not block HSP60 binding. We conclude that a single C-terminal region, aa481–500, accounts for the binding of HSP60 to macrophages.

Keywords: Heat shock protein 60; Binding epitope; Receptor; Macrophage

1. Introduction

Heat shock protein (HSPs) are highly conserved proteins expressed in all pro- and eukaryotic cells. They play important roles in the correct processing of newly synthesized proteins [1] and exert cytoprotective functions such as preventing the aggregation of denatured proteins, initiating their refolding or proteolytic degradation [2]. Under unphysiological conditions like high temperature, viral or bacterial infections, cellular HSP synthesis is upregulated [2].

Recently, the HSP60, HSP70, and HSP90 subfamilies have attracted increasing attention because of their potential roles in immunologically relevant processes. Several studies have identified HSPs as targets of immune responses during microbial infections [3,4]. Because of the high sequence homology between microbial HSPs and endogenous HSPs derived from damaged or stressed tissue, immunological cross-reactivity was suggested to contribute to the development of autoimmune disorders including rheumatoid arthritis and diabetes [5,6].

Furthermore, human HSP60 has been reported to induce pro-inflammatory reactivity in human and murine innate immune cells [7–9]. This response includes the release of the inflammatory mediators tumor necrosis factor α, nitric oxide, and interleukin-6 (IL-6). In addition, HSP60 was found to induce gene expression of the cytokines IL-12(p70) and IL-15, promoting a T helper 1 phenotype [7]. These findings suggest a role of HSP60 as danger signal for the innate immune system.

Recently, we have characterized the binding receptor for human HSP60 on macrophages. Binding of fluorescence-labeled HSP60 was found to be saturable, occurred with submicromolar affinity and could be competed by unlabeled HSP60 [10]. Furthermore, we could demonstrate that pro- and eukaryotic HSP60 species bind to different sites on macrophages. Interestingly, this study indicated that HSP60 molecules of most mammalian species, including human, mouse and rat, share the same binding site on macrophages, with identical competition characteristics [11]. Moreover, our data indicate that the binding receptor for HSP60 is different from the common receptor for HSP70, HSP90 and glycoprotein 96 (gp96), recently identified as the α_2-macroglobulin receptor [10,12–14].

So far, the potential epitope(s) on the HSP60 molecule for receptor binding on macrophages are unknown. Therefore, the present study was designed to identify the epitope of HSP60, which is responsible for binding to the receptor on macrophages. Our results identified the C-terminal region of aa481–500 as the single HSP60 epitope involved in receptor binding.

2. Materials and methods

2.1. Cell lines

The mouse macrophage cell line J774A.1 was purchased from the German Collection of Microorganisms and Cell Culture (Braunschweig, Germany) and was cultured as described previously [10].

2.2. Reagents

Recombinant human HSP60 was obtained from Peptor Inc. (Rehovot, Israel). A complete set of overlapping peptides of 20 amino acids, spanning the sequence of the unprocessed precursor of human

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Abbreviations: HSP, heat shock protein; IL, interleukin; gp96, glycoprotein 96; BSA, bovine serum albumin; mAbs, monoclonal antibodies; BLU, Boehringer luminescence units; TLR, toll-like receptor

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HSP60 from aa1–560 (Swiss-Prot. P10809), was from the Department of Immunohematology and Blood Transfusion (Leiden University Medical Center, Leiden, The Netherlands). Selected overlapping 15-mer peptides, covering the region aa471–510 of the human HSP60 sequence (GenBank M22382), were synthesized by automated simultaneous multiple peptide synthesis as described previously [15]. A modified peptide, pep481–500 (Ile490 → Ala490, Ser497 → Gly497, Ser499 → Gly499), was synthesized by Biotrend (Cologne, Germany). Mutants of human HSP60, N-terminally deleted and covering the human HSP60 sequence from aa138–573, aa244–573 or aa360–573, were prepared by the following method. The nucleotide sequences of the human HSP60 deletions were amplified from plasmid pGA8, encoding human HSP60, using human HSP60 oligonucleotide primers. The PCR product was ligated into a pQE60 vector (Qiagen, Dorking, Surrey, UK), which was subsequently transfected into *Escherichia coli* ultracompetent XL-2 blue cells (Stratagene, Cambridge, UK) using a heat shock method [16]. Bovine serum albumin (BSA) was from Sigma (Deisenhofen, Germany). Murine anti-human HSP60 monoclonal antibodies (mAbs) were from StressGen Biotechnologies (Victoria, Canada; clone LK1), Dianova (Hamburg, Germany; clone 4B9/89) and BD Transduction Laboratories (San Diego, CA; clone 24). Goat anti-mouse IgG antibody was used as isotype control for all murine mAbs (Sigma).

2.3. Protein labeling

Labeling of human HSP60 with fluorescence dye was performed as described previously [10] using the Alexa Fluor488 Protein Labeling kit (Molecular Probes, Leiden, The Netherlands).

2.4. HSP60 binding and inhibition studies

Binding and inhibition studies were performed as described previously [10]. Briefly, J774A.1 cells (1×10^6 cells/ml) were incubated in a total volume of 100 μl in the presence of HSP60–Alexa488 for 45 min on ice for the binding studies. Subsequently, cells were washed with PBS/1% BSA and resuspended in PBS containing 1% paraformaldehyde. For the inhibition studies the macrophages were preincubated with unlabeled HSP60, the different peptides or HSP60 mutants in the indicated concentrations for 30 min at 4 °C. Then, HSP60–Alexa488 was added and the incubation was continued for another 45 min on ice. In experiments using different anti-HSP60 antibodies, HSP60–Alexa488 was preincubated together with the mAbs for 30 min at room temperature, the mixture was added to the cells and incubation was continued for another 45 min on ice. After washing and fixation steps, the samples were evaluated using a FACSCalibur flow cytometer (BD Bioscience, Rockville, CA). Cell surface binding of HSP60–Alexa488 was calculated using the geometric mean fluorescence value after subtracting the autofluorescence of the cells.

2.5. Dot blot analysis

For dot blot analysis 140 ng human HSP60 or 7.5 μg of single 20-mer peptides with 10aa overlap corresponding to the regions aa321–380 or aa471–560 of human HSP60 were spotted onto Hybond ECL membranes (Roche Diagnostics, Mannheim, Germany). After blocking in 5% skimmed milk solution, membranes were incubated with murine anti-human HSP60 antibody clone 4B9/89 (0.2 μg/ml, overnight). In another approach antibody clone 4B9/89 (0.5 μg/ml) was preincubated with a mixture of 20-mer peptides (1 μM) covering the regions aa141–230, aa321–380 or aa471–560, with the individual 20-mer peptides (10 μM) or with PBS (control) for 1 h. Subsequently, these mixtures were incubated with human HSP60 dotted onto membranes for 30 min. The detection was performed with rabbit peroxidase-labeled anti-mouse IgG antibody (1 μg/ml, 45 min, DakoCytomation, Hamburg, Germany) using the ECL detection system (Amersham Pharmacia Biotech, Freiburg, Germany). Quantitative analysis was performed by Lumi-Imager (Boehringer, Mannheim) and shown as Boehringer luminescence units (BLU).

2.6. Statistical analysis

Data were expressed as mean values ± S.D. Statistical analysis was performed using the Student's t test, two-tailed. Differences were considered statistically significant with $P < 0.05$.

3. Results

3.1. Identification of the HSP60 binding epitope by inhibition studies with HSP60 peptides

Inhibition experiments with a complete set of overlapping 20-mer peptides, covering the human HSP60 sequence from aa1–560, were performed to identify regions of the molecule involved in surface binding to macrophages. Peptides were screened at 125 μM concentration for inhibiting binding of fluorescence-labeled human HSP60 (HSP60–Alexa488) to cells of the mouse macrophage line J774A.1. At this high concentration, only one peptide corresponding to aa481–500, pep481–500, was found to induce strong inhibition of HSP60–Alexa488 binding, i.e., more than 50% (Fig. 1). A more detailed analysis using pep471–490, pep481–500 and pep491–510 showed that only pep481–500 resulted in a significant ($P < 0.05$) dose-dependent reduction of HSP60–Alexa488 binding to J774A.1 cells to 28% at a concentration of 125 μM (Fig. 2). The adjacent peptides, pep471–490 and pep491–510, did not compete with HSP60–Alexa488 binding.

To exclude that the observed inhibitory effect of pep481–500 is mediated by specific conformational properties of the 20-mer peptide, we further analyzed the effect of selected 15-mer peptides, covering the region around the potential binding epitope of HSP60, aa471–510 (Fig. 3). Only one peptide, pep481–495, interfered with binding of HSP60–Alexa488 to J774A.1 macrophages, but to a lesser extent as pep481–500 (51% versus 28% HSP60–Alexa488 binding). None of the adjacent peptides, i.e., pep471–485, pep476–490, pep486–500, pep491–505 and pep496–510, blocked HSP60 binding. Furthermore, when testing pep481–500, modified at amino acid positions 490, 497 and 499, the inhibitory effect of the peptide was almost lost (72% versus 21% inhibition of HSP60–Alexa488 binding). Taken together, these results indicate that the region aa481–500 of the HSP60 molecule is responsible for the binding of HSP60 to macrophages.

3.2. Mapping of the HSP60 binding epitope by HSP60 deletion mutants

The findings obtained with peptides were further supported by the use of three different deletion mutants of human HSP60. All mutants had a preserved region aa481–500, but were deleted for the N-terminal 137, 243 or 359 amino acids. When testing these HSP60 mutants at a concentration of 3.5 μM for inhibiting binding of HSP60–Alexa488 to J774A.1 macrophages, all three mutants led to a considerable reduction of HSP60 binding, i.e., mean fluorescence intensity of the cells decreased from 9.1 to 5.4 (Fig. 4). This corresponds to an inhibitory effect of these three HSP60 mutants on HSP60–Alexa488 binding in the range of 57–62%, thereby confirming the location of the binding epitope in the C-terminal region of HSP60.

3.3. Mapping of the HSP60 binding epitope by antibodies

Finally, we investigated the effect of commercially available mAbs, directed against different epitopes of the native human HSP60 protein, i.e., clone 24 (aa1–200), clone 4B9/89 (aa335–366 or aa484–547) or clone LK1 (aa383–447), on the binding of HSP60–Alexa488 to J774A.1 macrophages (Table 1). When analyzing these antibodies at concentrations of 8 and 20 μg/ml in the binding assay, none of them competed with the binding of HSP60–Alexa488 to the cells.

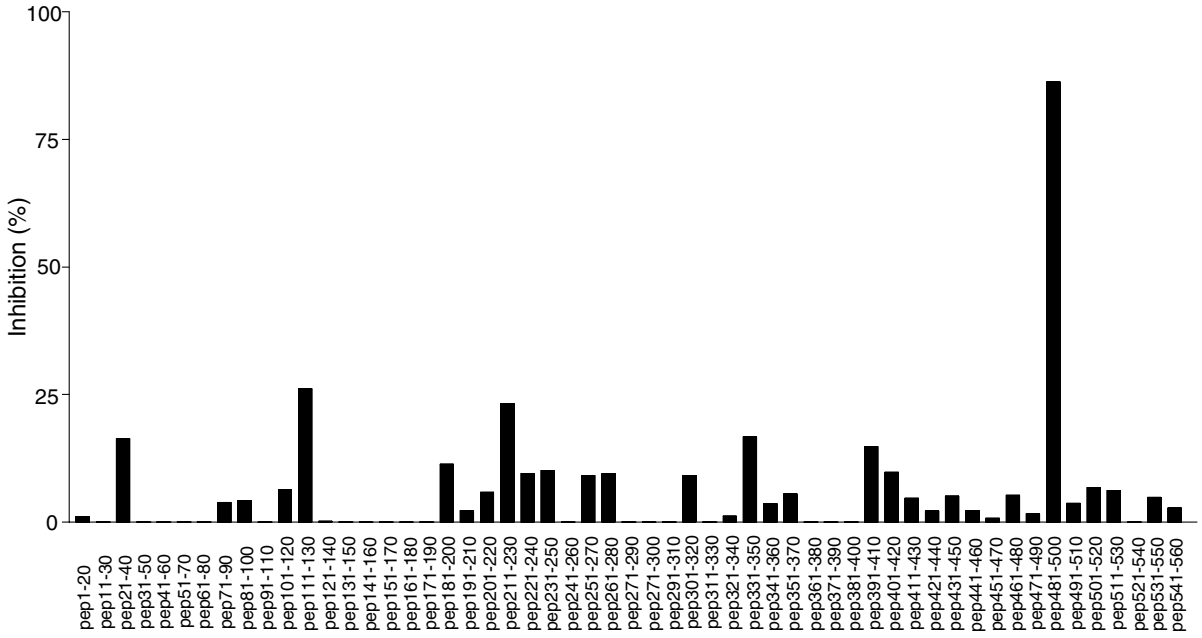

Fig. 1. Effect of 20-mer peptides on HSP60 binding. J774A.1 cells were preincubated with different 20-mer peptides (125 μM, 30 min). Subsequently, 350 nM HSP60–Alexa488 was added and incubation was continued for another 45 min on ice. The analysis was performed by flow cytometry. Inhibition of HSP60–Alexa488 binding is indicated as %.

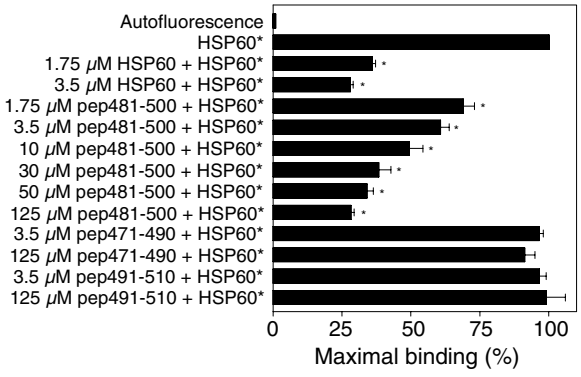

Fig. 2. Effect of selected 20-mer peptides on HSP60 binding. J774A.1 cells were incubated in the absence (autofluorescence) or presence of 350 nM HSP60–Alexa488 (HSP60*) for 45 min on ice. For inhibition studies cells were preincubated with unlabeled HSP60, pep481–500, pep471–490 or pep491–510 at the indicated concentrations (30 min), followed by incubation with HSP60*. The analysis was performed by flow cytometry and binding of HSP60* in the absence of inhibitors was set 100%. Error bars represent means + S.D. of three independent experiments. Significant inhibition of binding is indicated as *, $P < 0.05$.

Peptides	Binding of HSP60-Alexa488 (%)
$_{481}$KNAGVEGSLIVEKIMQSSSE$_{500}$	28 ± 2
$_{471}$TLKIPAMTIAKNAGV$_{485}$	104 ± 12
$_{476}$AMTIAKNAGVEGSLI$_{490}$	110 ± 4
$_{481}$KNAGVEGSLIVEKIM$_{495}$	51 ± 11
$_{486}$EGSLIVEKIMQSSSE$_{500}$	100 ± 3
$_{491}$VEKIMQSSSEVGYDA$_{505}$	110 ± 3
$_{496}$QSSSEVGYDAMAGDF$_{510}$	110 ± 6
$_{481}$KNAGVEGSL**A**VEKIMQ**G**SG**E**$_{500}$	79 ± 2

Fig. 3. Effect of selected 15-mer and 20-mer peptides on HSP60 binding. J774A.1 cells were preincubated 30 min with 125 μM of the different unlabeled 15-mer or 20-mer peptides, followed by incubation with 350 nM HSP60–Alexa488 (45 min on ice). The analysis was performed by flow cytometry. Binding of HSP60–Alexa488 is indicated as %. Error bars represent means ± S.D. of two to three independent experiments. Amino acids exchanged in comparison to the original sequence of pep481–500 are underlined.

Since the HSP60 antibody clone 4B9/89 is suggested to recognize either the HSP60 region aa335–366 or the region aa484–547 [17], including the putative binding region, we further analyzed the epitope specificity of this antibody. Dot blot experiments analyzing the effect of mixtures of distinct 20-mer peptides on the binding of antibody clone 4B9/89 to HSP60 (Fig. 5A) revealed that preincubation of the peptide mixture covering the region aa321–380 of HSP60 with antibody clone 4B9/89 resulted in the inhibition of antibody binding to HSP60. Neither the mixture of peptides from a region not recognized by the antibody (aa141–230), nor the peptide mixture covering the region aa471–560 inhibited antibody binding to HSP60. Next, we investigated the effect of the individual 20-mer peptides on the binding of antibody clone 4B9/89 to HSP60 by dot blot analysis (Fig. 5B). Preincubation of pep361–380 with antibody clone 4B9/89 inhibited binding of the antibody to HSP60, whereas none of the other peptides interfered with antibody binding to HSP60. These results were confirmed by additional dot blot analysis with single 20-mer peptides, which showed that only the peptide corresponding to region aa361–380 was recognized by antibody clone 4B9/89 (2.06×10^6 BLU) in a similar intensity as human HSP60 (2.65×10^6 BLU). None of the other tested 20-

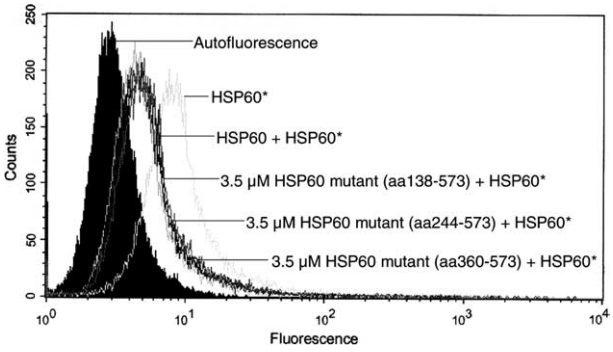

Fig. 4. Effect of selected HSP60 deletion mutants on HSP60 binding. J774A.1 cells were incubated in the absence (autofluorescence) or presence of 350 nM HSP60–Alexa488 (HSP60*) for 45 min on ice. For inhibition studies cells were preincubated (30 min) with 3.5 μM unlabeled HSP60 or 3.5 μM of the unlabeled HSP60 mutants (aa138–573, aa244–573 or aa360–573), followed by incubation with HSP60*. The analysis was performed by flow cytometry and fluorescence intensities of the cells (*x*-axis) were plotted against cell counts.

Table 1
Effect of anti-HSP60 antibodies on HSP60 binding to macrophages

Anti-human-HSP60 antibody		Concentration (μg/ml)	Binding of HSP60–Alexa488 (%)
Clone	Recognized epitope		
–	–	–	100
Clone 24	aa1–200	8	97 ± 9
		20	102 ± 8
4B9/89	aa335–366 or aa484–547	8	130 ± 41*
		20	125 ± 5*
LK1	aa383–447	8	100 ± 20
		20	99 ± 5
IgG isotype Control		8	111 ± 8
		20	106 ± 1

HSP60–Alexa488 (350 nM) was preincubated with the different mAbs (30 min) and then added to J774A.1 macrophages.
The analysis was performed by flow cytometry and HSP60–Alexa488 binding in the absence of antibodies was set 100%. The data represent means ± S.D. of three independent experiments. Significant differences to binding of HSP60–Alexa488 are indicated as *, $P < 0.05$.

mer peptides were detected by antibody clone 4B9/89 ($<0.1 \times 10^6$ BLU). By assigning the epitope specificity of clone 4B9/89 to region aa335–366, we could confirm the location of the binding epitope of HSP60 to the C-terminal region of the molecule.

4. Discussion

When using a peptide library to characterize epitope(s) of HSP60 involved in receptor binding on macrophages, we identified a peptide corresponding to aa481–500 of the human HSP60 sequence. This peptide mediated strong dose-dependent inhibition of HSP60 binding to macrophages. Significant inhibition of binding was already reached at low micromolar concentrations of the peptide pep481–500. No inhibitory effect, even at 125 μM, was observed for the overlapping peptides pep471–490 or pep491–510.

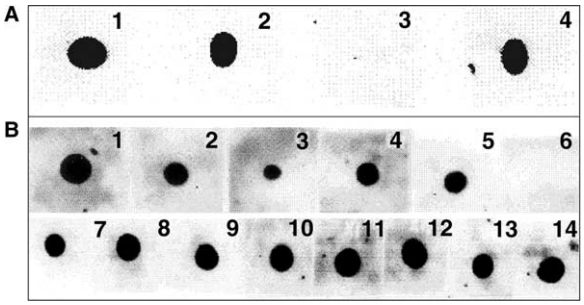

Fig. 5. Epitope specificity of HSP60 antibody clone 4B9/89. (A) PBS (1), 20-mer peptide mixtures (1 μM), covering the regions aa141–230 (2), aa321–380 (3) or aa471–560 (4) of the human HSP60 molecule or (B) PBS (1) or individual 20-mer peptides (10 μM), covering the regions aa321–380: pep321–340 (2), pep331–350 (3), pep341–360 (4), pep351–370 (5), pep361–380 (6) and region aa471–560: pep471–490 (7), pep481–500 (8), pep491–510 (9), pep501–520 (10), pep511–530 (11), pep521–540 (12), pep531–550 (13), pep541–560 (14) of the human HSP60 molecule, were preincubated with murine anti-human HSP60 antibody clone 4B9/89 (0.5 μg/ml) for 1 h. Subsequently these mixtures were incubated on membranes spotted with HSP60 (140 ng) for 30 min. After incubation with peroxidase-labeled rabbit anti-mouse IgG antibody (1 μg/ml, 45 min), blots were developed using ECL substrate.

Based on these findings, we performed further analysis using 15-mer peptides of human HSP60 with an overlap different from that of the 20-mer peptides, thereby excluding that conformational properties of pep481–500 mediated the inhibitory effect on HSP60 binding. When analyzing the inhibitory effect of selected 15-mer peptides, covering the region 471–510 of the human HSP60 sequence, only pep481–495 blocked HSP60 binding to J774A.1 macrophages. The inhibition of HSP60–Alexa488 binding to the cells by pep481–495 was lower (49%) compared to that caused by pep481–500 (72%). We obtained further support of our findings by the use of a modified peptide covering the region aa481–500 that lost its inhibitory effect on HSP60 binding to macrophages. At amino acid positions 497 and 499 conservative amino acid substitutions were performed (serine → glycine), which should not lead to major alterations of the peptide structure. By contrast, replacement of isoleucine by alanine at position 490 leading to the loss of ethyl- and methyl-groups, may result in an alteration of the structure or the physical properties of the peptide, thereby abolishing its inhibitory effect. These results indicate a major contribution of the HSP60 region around amino acid 490 to binding of HSP60 on macrophages.

As an independent proof of our findings described above, we analyzed the effect of three different HSP60 mutants, truncated from the N-terminal end, in our binding assay. All three HSP60 mutants, possessing a preserved region aa481–500, competed with HSP60–Alexa488 binding to J774A.1 macrophages, thereby confirming that the relevant binding region on the HSP60 molecule is located at the C-terminal region of HSP60. Furthermore, by this approach we could exclude that pep481–500 mediated its inhibitory effect by blocking a potential binding site on the N-terminal region of the HSP60 molecule up to amino acid position 359.

Further support of our findings was gained from inhibition studies with mAbs recognizing different regions of the native HSP60 molecule (aa1–200, aa335–366/aa484–547, and aa383–447). None of these antibodies interfered with HSP60 binding to J774A.1 cells. One of these antibodies, clone 4B9/89, has

been described to either bind to region aa335–366 or aa484–547 of the human HSP60 sequence [17], which means that this antibody possibly recognizes the region of interest, aa481–500. Further mapping done here revealed that the antibody binds to region aa335–366. Hence, these results demonstrate that the binding region on HSP60 is localized outside the regions recognized by the analyzed antibodies. These findings together with our results discussed above demonstrate that the binding epitope of HSP60 to macrophages is restricted to the aa481–500 in the C-terminal region of the HSP60 molecule.

The identification of a specific binding epitope on the HSP60 molecule will facilitate the identification of the macrophage surface receptor involved, and signaling events and functional consequences of binding. Previous studies have indicated that toll-like receptor 4 (TLR4) and CD14 are involved in the inflammatory signaling of HSP60 [18,19], but that TLR4 is not involved in HSP60 binding [10]. These observations suggest that the interaction of HSP60 with macrophages is a highly complex process. Similar observations were made for other HSPs. The binding receptor for the endoplasmic reticulum resident gp96 on innate immune cells has been reported as the α_2-macroglobulin receptor, also known as CD91, whereas gp96 signaling is TLR2/4-dependent [12–14,20]. CD91 and CD40 have been described as binding receptors for human HSP70 on innate immune cells [12–14,21], but CD14 [22], TLR2, and TLR4 [23] have been found to be involved in HSP70 signaling.

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