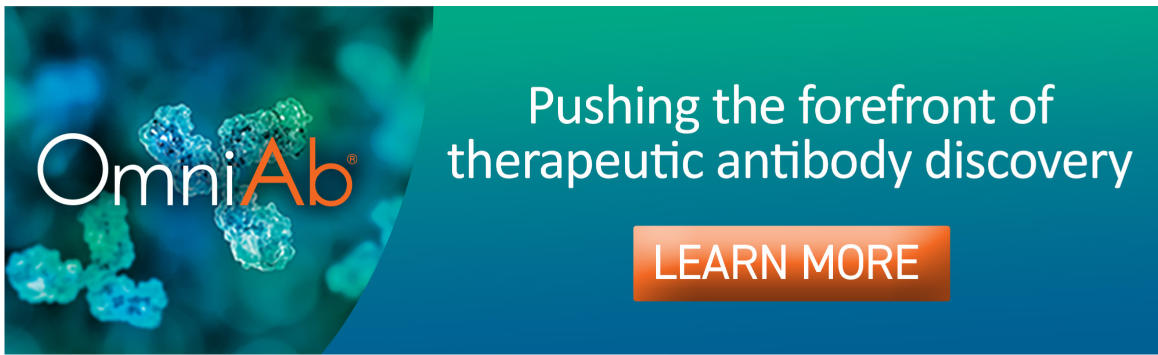

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Bacterial Heat Shock Proteins Promote CD91-Dependent Class I MHC Cross-Presentation of Chaperoned Peptide to CD8^{+} T Cells by Cytosolic Mechanisms in Dendritic Cells versus Vacuolar Mechanisms in Macrophages[1]

Aaron A. R. Tobian,* David H. Canaday,† W. Henry Boom,† and Clifford V. Harding[2]*

APCs process mammalian heat shock protein (HSP):peptide complexes to present HSP-chaperoned peptides on class I MHC (MHC-I) molecules to CD8^{+} T cells. HSPs are also expressed in prokaryotes and chaperone microbial peptides, but the ability of prokaryotic HSPs to contribute chaperoned peptides for Ag presentation is unknown. Our studies revealed that exogenous bacterial HSPs (*Escherichia coli* DnaK and *Mycobacterium tuberculosis* HSP70) delivered an extended OVA peptide for processing and MHC-I presentation by both murine macrophages and dendritic cells. HSP-enhanced MHC-I peptide presentation occurred only if peptide was complexed to the prokaryotic HSP and was dependent on CD91, establishing CD91 as a receptor for prokaryotic as well as mammalian HSPs. Inhibition of cytosolic processing mechanisms (e.g., by transporter for Ag presentation deficiency or brefeldin A) blocked HSP-enhanced peptide presentation in dendritic cells but not macrophages. Thus, prokaryotic HSPs deliver chaperoned peptide for alternate MHC-I Ag processing and cross-presentation via cytosolic mechanisms in dendritic cells and vacuolar mechanisms in macrophages. Prokaryotic HSPs are a potential source of microbial peptide Ags during phagocytic processing of bacteria during infection and could potentially be incorporated in vaccines to enhance presentation of peptides to CD8^{+} T cells. ***The Journal of Immunology,* 2004, 172: 5277–5286.**

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Heat shock proteins (HSPs)[3] are molecular chaperones expressed by prokaryotes and eukaryotes that bind polypeptide chains, prevent aggregation, and support protein folding (1). Expression of many HSPs is increased with stress (e.g., heat, anoxia, glucose starvation). Members of the HSP70 family are constitutively expressed in eukaryotic and prokaryotic cells. This family includes *Escherichia coli* DnaK and *Mycobacterium tuberculosis* (MTB) HSP70. These HSPs bind the hydrophobic regions of nascent polypeptides and unfold or disaggregate misfolded proteins to yield productive folding intermediates.

Recently, it was discovered that mammalian HSPs have immunological functions (2). Mammalian HSPs, e.g., HSP70 (3–6), HSP90 (4, 7), gp96 (4, 7–11), calreticulin (12, 13), and HSP110 (14), bind peptides to form highly immunogenic HSP:peptide complexes (15). Macrophages and dendritic cells process HSP-chaperoned peptides for presentation by class I MHC (MHC-I) molecules to activate CD8^{+} T cells (5, 16). HSPs assist in cross-priming (11) and immunization to induce antiviral immunity (6). Immunization with HSPs purified from tumor cells protects mice from subsequent challenge with the same tumor (2–4, 10, 12, 14). The enhanced immunogenicity conferred by mammalian HSPs requires binding of antigenic peptide to the HSP (3), and the immunogenicity of empty HSPs can be reconstituted by loading them with peptides (17). It appears that HSP-chaperoned peptides are processed by APCs for presentation of constituent epitopes, and the peptides presented by MHC-I may be smaller proteolytic fragments of HSP-chaperoned peptides that are produced by Ag-processing mechanisms.

Endogenous cytosolic Ags are processed via the conventional MHC-I Ag-processing pathway that involves cytosolic proteasome-mediated proteolysis and transport of peptides into the endoplasmic reticulum (ER) via the transporter for Ag presentation (TAP) to bind MHC-I. Exogenous Ags are internalized into vacuolar compartments and then may be processed for MHC-I cross-presentation by alternate MHC-I-processing mechanisms that involve either transit to the cytosol for cytosolic processing (similar to the conventional pathway) or exclusively vacuolar processing. Exogenous HSPs may use cytosolic processing mechanisms, perhaps by enhancing delivery of HSP-chaperoned peptides to the APC cytosol, thereby achieving access to proteasome-dependent processing and TAP-dependent entry into the ER to bind MHC-I. Studies that support cytosolic mechanisms report that HSP enhancement of peptide presentation is inhibited by brefeldin A (which inhibits anterograde Golgi transport and transport of peptide MHC-I complexes from the ER to the cell surface), proteasome inhibitors (e.g., lactacystin), and absence of TAP (16, 18). In contrast, other studies report that HSP-enhanced MHC-I processing of chaperoned peptides may occur by vacuolar mechanisms independent of TAP or cytosolic processing (19, 20), and that

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[3] Abbreviations used in this paper: HSP, heat shock protein; MTB, *Mycobacterium tuberculosis*; MHC-I, class I MHC; ER, endoplasmic reticulum; TAP, transporter for Ag presentation; MyD88, myeloid differentiation factor 88; TLR, Toll-like receptor; MFV, mean fluorescence value.

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mammalian HSPs traffic to endosomes to deliver peptides for MHC-I processing and presentation (8, 21). These observations suggest that mammalian HSPs may contribute to vacuolar as well as cytosolic alternate MHC-I Ag-processing mechanisms.

Because HSPs are expressed by prokaryotic as well as eukaryotic organisms, bacterial HSPs could potentially play an important role in antibacterial immunity. MTB HSP70 enhances production of IL-12 and RANTES (22), and MTB HSP70 fusion proteins have been shown to elicit $CD8^+$ T cell responses (23–26). However, Ag-processing functions have not been studied with prokaryotic HSPs, and it is not known whether microbial peptides chaperoned by prokaryotic HSPs can generate immune responses.[4]

Peptides naturally associated with mammalian HSPs include self peptides, tumor peptides, or viral peptides, because these are derived from proteins synthesized in mammalian cells. In contrast, peptides naturally associated with bacterial HSPs include bacterial Ags, suggesting that bacterial HSPs may contribute to antibacterial $CD8^+$ T cell responses or could be used therapeutically to generate such responses. These mechanisms could be especially important to augment host immunity to intracellular bacterial pathogens (e.g., MTB, *Mycobacterium leprae*, and *Salmonella*), because $CD8^+$ T cell responses play a role in infection with these pathogens.

This study reveals that two bacterial HSPs, *E. coli* DnaK and MTB HSP70, are capable of delivering an extended synthetic peptide for enhanced processing and MHC-I presentation of a constituent epitope. We report that *E. coli* DnaK and MTB HSP70 promote alternate MHC-I Ag processing through vacuolar mechanisms in macrophages and cytosolic mechanisms in dendritic cells. The dichotomy in processing mechanisms observed in these studies may explain disparities among other studies regarding the relative roles of cytosolic and vacuolar processing for MHC-I cross-presentation of exogenous Ags. We propose that bacterial HSPs may deliver HSP-bound bacterial peptides during phagocytic processing of bacteria, thereby promoting MHC-I presentation of bacterial Ags and $CD8^+$ T cell responses during infection with bacterial pathogens. Furthermore, this mechanism could provide a basis for the use of prokaryotic HSPs in vaccines to enhance efficacy in priming of $CD8^+$ T cell responses.

Materials and Methods

Cells and media

Unless otherwise specified, incubations were at 37°C and 5% CO_2 in standard medium containing DMEM (Life Technologies, Carlsbad, CA), 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 50 μM 2-ME, 1 mM sodium pyruvate, 10 mM HEPES buffer, and antibiotics. B6D2F1/J, C57BL/6, and $TAP1^{-/-}$ (C57BL/6 background) female mice were from The Jackson Laboratory (Bar Harbor, ME). Myeloid differentiation factor 88 $(MyD88)^{-/-}$ mice were generously provided by O. Takeuchi and S. Akira (Osaka University, Osaka, Japan) (27) and bred onto C57BL/6 background for five to seven generations. B6D2F1/J mice were used for all experiments except those involving knockout models, which used $TAP1^{-/-}$ or $MyD88^{-/-}$ mice with C57BL/6 mice for wild-type controls. Macrophages were derived from bone marrow cells cultured in bacterial grade dishes for 7–10 days in 20% LADMAC cell-conditioned medium (containing M-CSF (28)). To produce dendritic cells (29, 30), femur marrow cells were resuspended for 10 min in 0.83% NH_4Cl to lyse erythrocytes, incubated for 1 h at 4°C with combined supernatants of B hybridomas GK1.5 (anti-CD4), 53-6.72 (anti-CD8), RA3-3A1/61 (anti-B220), and 34-5-3S (anti-I-$A^{b/d}$) (American Type Culture Collection, Manassas, VA), and resuspended for 1 h at 37°C in complement (Accurate, Westbury, NY). Cells were resuspended in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 5% FCS, 50 mM 2-ME, 25 mM HEPES (Life Technologies), 20 μg/ml gentamicin (Life Technologies), and 4 ng/ml GM-CSF (R&D Systems, Minneapolis, MN). Cells were cultured in six-well plates (3×10^6 cells/well), and nonadherent cells were removed every 2 days by gentle swirling and replacement of half of the volume with fresh medium containing GM-CSF. Dendritic cells were harvested by pipetting on day 5, incubated with anti-murine CD11c microbeads (100 μl beads/4×10^7 cells; Miltenyi Biotec, Auburn, CA) for 20 min at 4°C, resuspended in PBS with 0.5% BSA and 2 mM EDTA (4×10^7 cells/0.5 ml), isolated with a MACS MS column (Miltenyi Biotec), washed, and resuspended in standard medium.

HSPs, Abs, and reagents

E. coli DnaK (StressGen, Victoria, BC, Canada) was >90% pure by SDS-PAGE analysis. MTB HSP70 for most experiments was obtained from Lionex (Braunschweig, Germany) and was >95% pure by SDS-PAGE analysis. We also prepared MTB HSP70 from *E. coli* BL-21 pLys transformed with MTB HSP70 in pET-23 (Novagen, Madison, WI), obtained through the Tuberculosis Research Materials and Vaccine Testing Contract (Colorado State University, Fort Collins, CO), which drives expression of His-tagged MTB HSP70 (His tag on C terminus of MTB HSP70). *E. coli* were induced with isopropyl β-D-thiogalactoside for 4 h and lysed with BugBuster (Novagen). His-tagged MTB HSP70 was purified under native conditions with nickel columns (Qiagen, Valencia, CA). Similar results were obtained with native MTB HSP70 (Lionex) and His-tagged MTB HSP70. Although LPS contamination was detected in HSP preparations with the E-TOXATE LAL assay (Sigma-Aldrich, St. Louis, MO) with maximum experimental LPS concentrations of 0.22–1.1 μg/ml for *E. coli* DnaK and <0.14 μg/ml for MTB HSP70, control experiments showed that addition of LPS (from *E. coli* O127:B8; Difco, Detroit, MI) at concentrations in this range or higher (1.5 μg/ml) did not alter the results of our experiments.

FITC-labeled 18-mer extended OVA peptide EQLESIINFEKLLV-LLKK (FITC conjugation at N terminus; 90% pure by HPLC) was from Bio-Synthesis (Lewisville, TX). The sequence included 12 residues at the N terminus reflecting OVA sequence (including the SIINFEKL K^b-binding motif) plus 6 residues including the LVLL DnaK binding motif (31) and 2 K residues that assist in binding DnaK (32). FITC-EQLESIINFEKLLV-LLKK (0.04 ml at 1 mM in H_2O) was incubated with 0.4 ml of *E. coli* DnaK (StressGen) or MTB HSP70 at 1 mg/ml in 40 mM Tris-HCl (pH 7.5) at 37°C with rotation for 1 h (final prokaryotic HSP concentration of 0.91 mg/ml). Unbound peptide was removed using a Centricon YM-10 or YM-30 centrifugal filter device (Millipore, Bedford, MA) three times for 20 min at 14,000 rpm with washes in 40 mM Tris-HCl. A negative control sample of 0.4 ml of 40 mM Tris-HCl (pH 7.5) and 0.04 ml of uncomplexed peptide (1 mM) was processed to insure that unbound peptide was removed. The HSP:FITC-peptide solution was analyzed with a Spectra Fluor Plus plate fluorometer (Tecan, Research Triangle Park, NC) to determine HSP-bound peptide concentration. DnaK and MTB HSP70 bound similar amounts of peptide with ~0.1 mol of peptide bound per mole of HSP (10% loading). In comparison, studies with eukaryotic HSPs reported 1–5% loading with peptide sequences from model Ag (17, 33) and 20% loading with Ag peptide containing sequences known to promote HSP binding (5). A previous study with prokaryotic HSP reported 30% loading with sequence optimized for HSP binding (34). In our studies, HSP loading may have been enhanced by design of the extended OVA peptide to include C-terminal residues containing an optimized DnaK binding motif (31, 32). To make latex bead-DnaK:peptide, protein G-Fluoresbrite YG carboxylate microspheres (Polysciences, Warrington, PA) were incubated overnight at 4°C with anti-DnaK Ab (Stressgen), washed, incubated with DnaK:peptide for 2 h at 4°C, and washed with PBS.

Ag-processing and -presentation assays

Macrophages were detached with trypsin-versene (BioWhittaker), plated in 96-well flat-bottom plates at 10^5 cells/well, and incubated with 2 ng/ml recombinant IFN-γ (Genzyme, Cambridge, MA) for 48 h. Cells were incubated with HSP:peptide (for 2 h unless otherwise stated), fixed with 0.5% paraformaldehyde, washed, and incubated for 24 h with CD8OVA1.3 T hybridoma cells (10^5 cells/well), which are specific for SIINFEKL:K^b (35). Supernatants (100 μl) were frozen, thawed, and assessed for IL-2 using a colorimetric CTLL-2 bioassay (29, 36). CTLL-2 cells (5×10^3/well) were incubated with supernatants for 24 h at 37°C, Alamar blue (Trek Diagnostic Systems, Cleveland, OH) was added (15 μl/well) for 24 h, and Alamar Blue reduction was determined by difference in OD at 550 and 595 nm using a Bio-Rad (Hercules, CA) model 550 microplate spectrophotometer. Dendritic cells were plated in 96-well flat-bottom plates at 10^5 cells/well and subjected to the same protocol except that plates were spun at 1800 rpm for 5 min between each wash step. Inhibitors used to probe processing mechanisms included brefeldin A (1 μg/ml; Sigma-Aldrich),

[4] A paper by MacAry et al. (49) published while this manuscript was in review reported that mycobacterial HSP70-associated peptides can generate CTL responses in vivo and provided structure-function studies with mutant HSP70 molecules.

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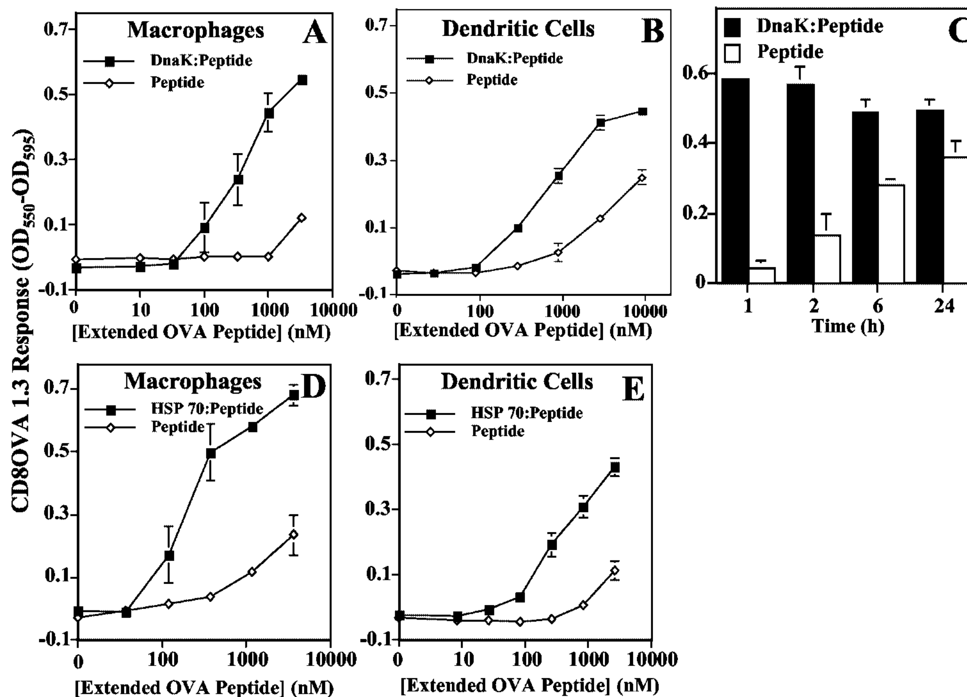

FIGURE 1. *E. coli* DnaK and MTB HSP70 enhance alternate MHC-I Ag processing in both macrophages and dendritic cells. Cells were incubated for 2 h with FITC-labeled extended OVA peptide complexed to *E. coli* DnaK or MTB HSP70, fixed, and incubated with MHC-I-restricted CD8OVA1.3 T hybridoma cells that recognize SIINFEKL:K^b complexes. Supernatants were assessed for IL-2 using a colorimetric CTLL-2 bioassay. *A* and *B*, *E. coli* DnaK enhances processing and presentation of extended OVA peptide by bone marrow-derived macrophages (*A*) and dendritic cells (*B*). *C*, *E. coli* DnaK produces rapid enhancement of extended OVA peptide processing by macrophages. Macrophages were incubated with 3 μM OVA peptide complexed to *E. coli* DnaK for the indicated periods. *D* and *E*, MTB HSP70 enhances processing and presentation of extended OVA peptide by macrophages (*D*) and dendritic cells (*E*). The results in each panel are representative of at least three independent experiments. Data points represent means of triplicate samples with SD.

lactacystin (20 μM; purchased from E. Corey (Harvard University, Cambridge, MA)), 2-deoxy-D-glucose (Sigma-Aldrich), and sodium azide. The mAb to CD91 (5A6) was from Progen Biotechnik (Heidelberg, Germany), and isotype control IgG1 was from Zymed (San Francisco, CA). The mAb to mouse CD40 and isotype control Armenian hamster IgM were from BD Biosciences (San Diego, CA). Latex-OVA beads were made by noncovalent conjugation of chicken egg OVA (Sigma-Aldrich A-5503) to 2-μm latex beads (Polysciences).

To assess conventional MHC-I processing, macrophages activated with 2 ng/ml recombinant IFN-γ for 48 h were suspended at 2×10^6/ml in 0.5 ml of DMEM with or without brefeldin A (1 μg/ml) or lactacystin (20 μM), incubated for 10 min at 37°C in polypropylene tubes with mixing, and cooled to 4°C. OVA protein (Sigma Aldrich A-5503) was added (0.6 mg/ml final concentration), and macrophages were electroporated at 4°C in 4-mm-gap electroporation cuvettes (Life Technologies) with a Cell-Porator (Life Technologies) at 200 V, 800 μF, and low resistance settings (37–39). Macrophages were washed, plated (10^5/well) for 2 h, and fixed for T cell assays.

Flow cytometry

Macrophages were incubated in 24-well plates (6.7×10^5 cells/well) for 48 h at 37°C with 2 ng/ml IFN-γ. HSP:FITC-labeled peptide complexes were added for 30 min. Cells were then washed in PBS, detached by scraping, fixed in 1% paraformaldehyde, and analyzed by flow cytometry.

Results

E. coli *DnaK and MTB HSP70 promote processing and MHC-I presentation of chaperoned extended OVA peptide containing the SIINFEKL epitope*

Mammalian HSPs have been shown to promote immune responses to chaperoned peptides that are derived from proteins synthesized in mammalian cells, including self peptides, tumor peptides, and viral peptides. Bacterial HSPs are associated with peptides derived from bacterial proteins, including bacterial Ags, suggesting that bacterial HSPs may contribute to antibacterial $CD8^+$ T cell responses or could be used therapeutically to generate such responses. These mechanisms could be especially important to augment host immunity to intracellular bacterial pathogens, because $CD8^+$ T cell responses play a role in infection with these pathogens. However, Ag-processing functions have not been studied with prokaryotic HSPs, and it is not known whether peptides chaperoned by prokaryotic HSPs can generate immune responses.[4] We propose that bacterial HSPs deliver exogenous peptides for alternate MHC-I processing and presentation of constituent epitopes.

To test this hypothesis, we studied the ability of bacterial HSPs to deliver 18-mer extended OVA peptide (EQLESIINFEKLLVLLKK) for processing and presentation of a constituent epitope (SIINFEKL) by K^b. This extended OVA peptide has been used in studies with mammalian HSPs (7, 16, 26, 33) and requires processing for efficient presentation of the SIINFEKL epitope. The extended OVA peptide itself is presented poorly by APCs, presumably because the extended sequence precludes interactions of N- and C-terminal groups with sites in the MHC-I peptide-binding groove. We determined that the minimal SIINFEKL peptide was presented much more efficiently than extended OVA peptide in the absence of HSP (minimum concentrations for CD8OVA1.3 T hybridoma responses were 1 pM for SIINFEKL and 100,000 pM for extended OVA peptide). This observation suggests that extended OVA peptide is poorly presented unless it is processed to generate SIINFEKL peptide for presentation by K^b (this conclusion is further supported by studies of processing requirements presented later in this paper). Therefore, the extended OVA peptide allows study of delivery and processing of an HSP-chaperoned peptide that requires active processing for efficient presentation.

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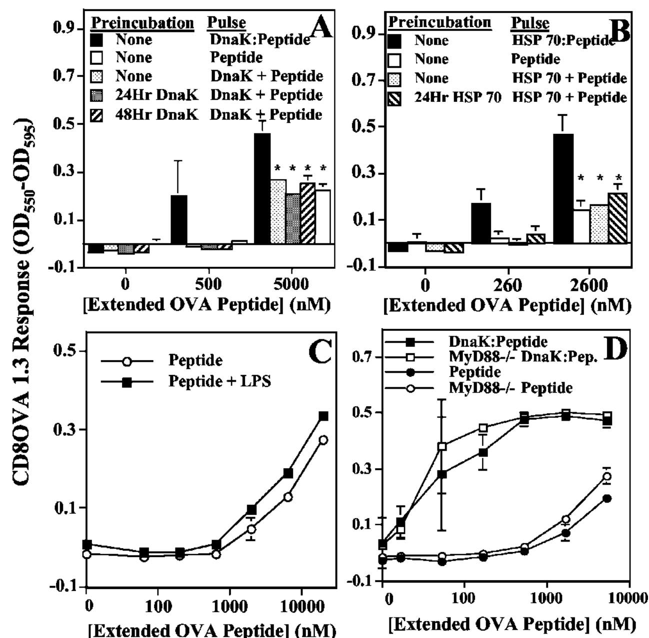

FIGURE 2. Bacterial HSPs specifically enhance processing and MHC-I presentation of chaperoned peptide. After incubation with HSPs and extended OVA peptide, macrophages were assessed for presentation of SIINFEKL:K^b complexes as in Fig. 1. *A*, Macrophages were incubated for 2 h with DnaK complexed to extended OVA peptide (DnaK:Peptide) or uncomplexed extended OVA peptide. Alternatively, macrophages were preincubated for 0, 24, or 48 h with DnaK (not loaded with OVA peptide) and then incubated for 2 h with uncomplexed extended OVA peptide and DnaK (DnaK + Peptide). *B*, Macrophages were incubated for 2 h with extended OVA peptide complexed with MTB HSP70 (HSP70:peptide) or uncomplexed extended OVA peptide. Alternatively, macrophages were preincubated for 0 or 24 h with MTB HSP70 and then incubated for 2 h with uncomplexed extended OVA peptide and MTB HSP70 (HSP70 + Peptide). *C*, Macrophages were incubated with uncomplexed extended OVA peptide with or without 1.5 μg/ml LPS for 2 h. *D*, Macrophages from MyD88$^{-/-}$ and wild-type C57BL/6 mice were incubated for 2 h with extended OVA peptide, either alone or complexed with *E. coli* DnaK. The results in each panel are representative of three independent experiments. Data points represent means of triplicate samples with SD. A two-tailed *t* test was used to calculate *p* values for the difference between HSP:peptide and uncomplexed peptide (*, $p < 0.05$).

To determine whether an *E. coli* HSP, DnaK, can deliver extended OVA peptide for alternate MHC-I processing and presentation, APCs were incubated for 2 h with uncomplexed extended OVA peptide or *E. coli* DnaK:extended OVA peptide complexes. The cells were then fixed and incubated with CD8OVA1.3 T hybridoma cells to detect SIINFEKL:K^b complexes. Association of extended OVA peptide with *E. coli* DnaK significantly enhanced presentation of the SIINFEKL epitope from the extended peptide by both macrophages (Fig. 1*A*) and dendritic cells (*B*). The greatest relative enhancement of peptide presentation by *E. coli* DnaK with our readout assay was observed within 1–2 h of processing (Fig. 1*C*). Plateau signal (maximum response of our T cell assay) was achieved in this time frame at typical concentrations of DnaK: peptide, whereas presentation of uncomplexed extended OVA remained poor at 1–2 h but gradually increased at longer times (beyond 1–2 h, it is likely that DnaK:peptide continued to produce peptide:MHC-I complexes that were not evident due to the signal plateau). We conclude that *E. coli* DnaK can efficiently deliver chaperoned peptide for processing and MHC-I presentation of constituent epitopes.

Additional studies were performed with an MTB HSP70 to determine whether the above findings are idiosyncratic to *E. coli* DnaK or reflect properties shared by other bacterial HSPs. In addition, MTB is an important intracellular bacterial pathogen for humans, so it is important to understand potential roles of MTB HSPs in generating immune responses. To determine whether MTB HSP70 enhances alternate MHC-I Ag processing and cross-presentation of chaperoned peptides, APCs were incubated with MTB HSP70:extended OVA peptide complexes for 2 h, fixed, and incubated with CD8OVA1.3 T hybridoma cells to detect SIINFEKL:K^b complexes. MTB HSP70 significantly enhanced MHC-I peptide presentation in both macrophages (Fig. 1*D*) and dendritic cells (*E*). Thus, bacterial HSPs from both *E. coli* and MTB have the ability to deliver extended peptide for enhanced alternate MHC-I Ag processing and MHC-I presentation of a constituent epitope.

HSP-enhanced processing and MHC-I presentation require extended peptide to be complexed to bacterial HSP and are not due to signaling initiated by bacterial HSP alone

Contributions to MHC-I Ag processing and presentation have been examined for mammalian HSPs (16, 40) but not prokaryotic HSPs. Nonetheless, some immune functions have been attributed to prokaryotic HSPs. Bacterial HSPs can serve as Ags to elicit immune responses; MTB HSP70 and chlamydial HSP60 stimulate cytokine secretion through CD40 and Toll-like receptor 4 (TLR4), respectively (22, 41). The latter observations suggested the possibility that bacterial HSP modulation of alternate MHC-I Ag processing could be explained by HSP signaling to produce general enhancement of Ag processing and presentation of peptides other than those chaperoned by the HSP.

To distinguish generalized effects of HSP signaling from enhanced processing specific to HSP-chaperoned peptide, we explored the requirement for complexing of extended peptide to HSP in our system. Macrophages were preincubated with *E. coli* DnaK or MTB HSP70 for 0, 24, or 48 h, and uncomplexed extended OVA peptide was then added in the presence of HSP for 2 h (for the 0-h point, uncomplexed HSP and extended OVA peptide were added at the same time). Alternatively, macrophages were incubated with HSP-complexed extended OVA peptide or uncomplexed peptide for 2 h (without HSP preincubation). APCs were fixed and assessed for presentation of SIINFEKL:K^b complexes. Preincubation with or the presence of *E. coli* DnaK (Fig. 2*A*) or MTB HSP70 (*B*) did not enhance processing and presentation of uncomplexed extended OVA peptide; SIINFEKL presentation was enhanced only if exogenous extended peptide was complexed to the HSP. These data suggest that bacterial HSPs specifically deliver their bound or chaperoned peptide for processing.

HSPs can signal through TLRs and MyD88 adaptor protein to stimulate proinflammatory cytokines (41–43). We performed experiments to determine whether TLR- and MyD88-dependent signaling by HSPs or potential microbial contaminants of bacterial HSP preparations (e.g., LPS) altered APC function to cause HSP enhancement of peptide presentation. LPS was excluded as a significant factor in our system, because LPS (up to 1.5 μg/ml, higher than maximal levels of LPS from HSP preparations) did not affect the processing of extended OVA peptide under conditions that allowed HSP enhancement of peptide processing and MHC-I presentation (Fig. 2*C*). Furthermore, enhancement of MHC-I peptide presentation by DnaK was identical with wild-type and MyD88$^{-/-}$ macrophages (Fig. 2*D*). Thus, enhancement of extended peptide processing and MHC-I presentation was not the

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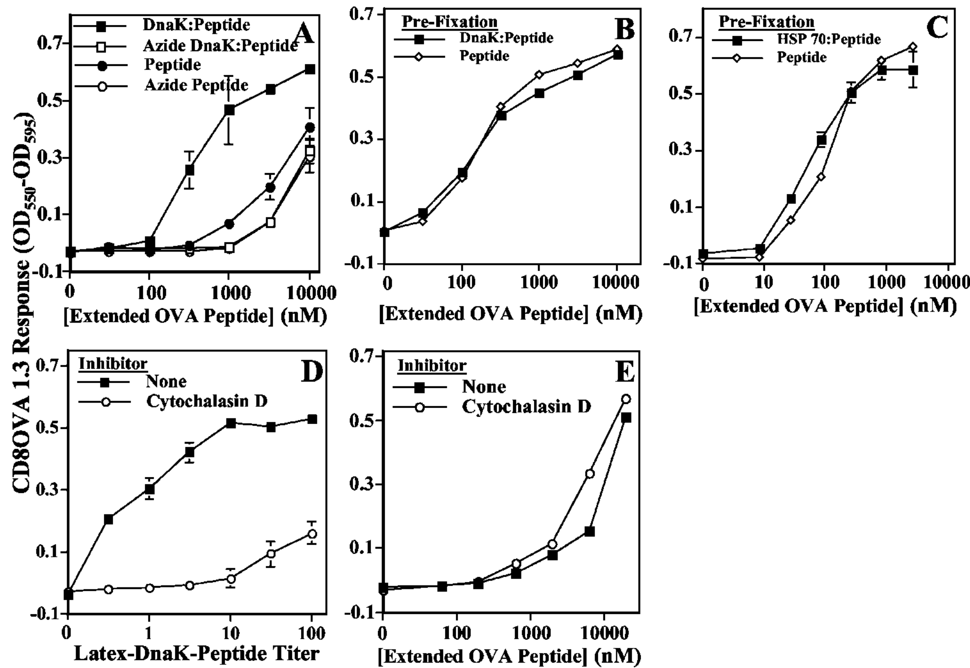

FIGURE 3. Active processing by APCs is required for bacterial HSPs to enhance MHC-I peptide presentation. Macrophages were exposed to inhibitors before and during incubation with HSP:extended OVA peptide complexes for 2 h. The cells were then fixed and assessed for presentation of SIINFEKL:K^b complexes as in Fig. 1. *A*, Macrophages were exposed to 30 mM sodium azide and 5 mM deoxy-D-glucose for 90 min before and during incubation with *E. coli* DnaK:peptide complexes. *B* and *C*, Macrophages were fixed with 0.5% paraformaldehyde before incubation with *E. coli* DnaK:peptide complexes (*B*) or MTB HSP70:peptide complexes (*C*). *D*, Macrophages were exposed to 10 μg/ml cytochalasin D for 15 min before and during a 30-min incubation with *E. coli* DnaK:peptide complexes linked to protein G-coated latex beads by anti-DnaK Ab. Macrophages were then washed and incubated for 90 min in standard medium with or without cytochalasin D before fixation. *E*, Macrophages were exposed to 10 μg/ml cytochalasin D for 15 min before and during incubation with soluble extended OVA peptide and processed as in *D*. The results in each panel are representative of three independent experiments. Data points represent means of triplicate samples with SD.

result of signaling by HSPs or bacterial contaminants to alter overall processing functions of APCs; rather, HSP-enhanced processing was specific to the HSP-chaperoned extended peptide that was delivered for processing and MHC-I presentation.

Active processing by APCs is necessary for enhanced presentation of HSP-chaperoned extended peptide

We tested whether bacterial HSP-chaperoned peptides required active processing, e.g., through endocytosis by viable APCs, or could be delivered directly to cell surface MHC-I molecules. To distinguish active processing from cell surface events, we inhibited uptake and processing by metabolic inhibition or fixation of APCs. Macrophages were exposed to sodium azide and 2-deoxy-D-glucose for 90 min to deplete ATP and subsequently incubated with DnaK:extended OVA peptide complexes for 2 h. This metabolic inhibition blocked enhancement of extended OVA peptide processing and presentation by *E. coli* DnaK (Fig. 3*A*). In other experiments, fixation of macrophages before the addition of extended OVA peptide prevented enhancement of processing and MHC-I presentation by *E. coli* DnaK (Fig. 3*B*) and MTB HSP70 (*C*) (the sensitivity of the Ag dose curve is shifted in this experiment, because prefixation with paraformaldehyde enhances MHC-I stability and peptide presentation (44)). Thus, active APC functions were required for HSP enhancement of peptide processing, which was prevented by prior fixation or metabolic inhibition.

To confirm that bacterial HSPs enhance Ag processing through an active process involving cellular uptake, we attached HSP:peptide complexes to latex beads and used cytochalasin D to inhibit actin-dependent phagocytosis of these beads (Fig. 3*D*). Macrophages were incubated with or without cytochalasin D for 15 min before and during incubation with bead-conjugated DnaK:extended OVA peptide. Cytochalasin D significantly inhibited the processing of DnaK:extended OVA peptide (Fig. 3*D*). Control samples showed that cytochalasin D completely blocked phagocytic alternate MHC-I processing of whole OVA protein conjugated to latex beads (data not shown). Presentation of uncomplexed extended OVA peptide was unaltered by cytochalasin D, indicating that cytochalasin D did not cause loss of macrophage ability to present peptide (Fig. 3*E*). Because bacterial HSP enhancement of extended OVA peptide processing and presentation is abolished in macrophages that are unable to internalize or actively process HSP:peptide complexes, we conclude that *E. coli* DnaK and MTB HSP70 peptide complexes must enter the cell to access alternate MHC-I Ag-processing mechanisms to which HSP-chaperoned Ag can contribute.

Bacterial HSPs enhance uptake of Ag

Enhancement of alternate MHC-I Ag processing by bacterial HSPs in our system could be mediated by enhanced uptake of peptide Ag as well as enhanced intracellular processing mechanisms. To assess whether *E. coli* DnaK or MTB HSP70 enhance uptake of extended OVA peptide, macrophages were incubated with FITC-labeled extended OVA, either uncomplexed or complexed with bacterial HSP. Flow cytometry was used to determine the level of FITC-peptide uptake. In negative control samples, APCs were incubated with metabolic inhibitors (azide and 2-deoxy-D-glucose) for 90 min before and during the peptide incubation to inhibit endocytosis. MTB HSP70:peptide complexes produced a strong signal for peptide uptake with mean fluorescence value (MFV) of 196, and metabolic inhibitors reduced uptake to ∼20% of this level

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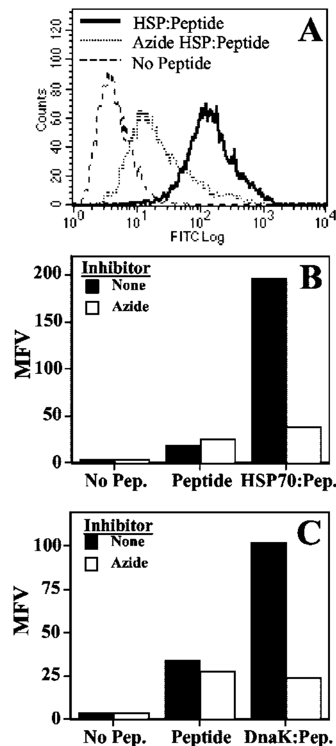

FIGURE 4. HSPs enhance endocytosis of chaperoned peptides. Macrophages were incubated with or without 30 mM sodium azide and 5 mM deoxy-D-glucose for 90 min before and during incubation for 30 min with FITC-labeled extended OVA peptide bound to HSP. Macrophages were incubated with 7.2 μM OVA peptide complexed to MTB HSP70 or 18.5 μM OVA peptide complexed to *E. coli* DnaK. The cells were then washed, fixed, and analyzed for uptake of FITC-labeled extended OVA peptide by flow cytometry. *A*, Representative histogram of MTB HSP70-enhanced uptake of extended OVA peptide. *B*, MFVs for MTB HSP70 enhancement of peptide uptake. *C*, MFVs for *E. coli* DnaK enhancement of peptide uptake. Each panel is representative of two independent experiments.

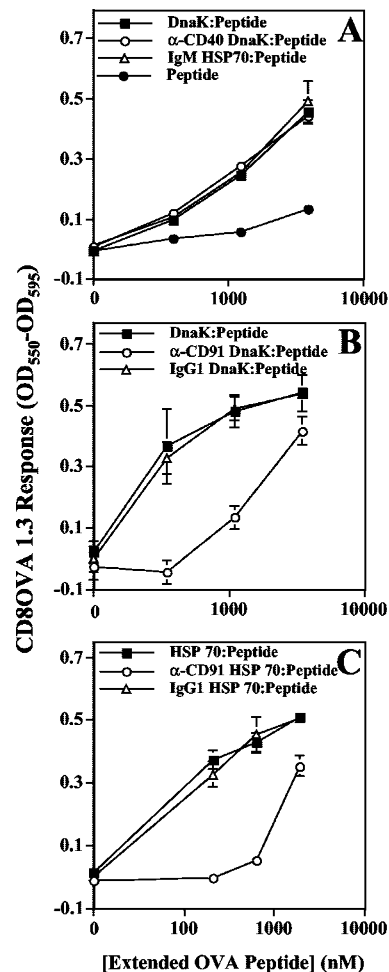

FIGURE 5. HSPs enhance alternate MHC-I Ag processing through CD91. Macrophages were preincubated with blocking mAbs to CD91 (α-CD91; 50 μg/ml) or CD40 (α-CD40; 50 μg/ml) or isotype controls mouse IgG1 (IgG1; 50 μg/ml) or Armenian hamster IgM (IgM; 50 μg/ml) for 30 min before and during a 90-min incubation with HSP:extended OVA peptide complexes. The cells were fixed and assessed for presentation of SIINFEKL:K^b complexes as in Fig. 1. *A*, Enhancement of processing by *E. coli* DnaK was unaffected by blocking CD40. *B* and *C*, Enhancement of processing by *E. coli* DnaK (*B*) or MTB HSP70 (*C*) was abolished by blocking CD91. The results in each panel are representative of three independent experiments. Data points represent means of triplicate samples with SD.

(MFV = 39) (Fig. 4, *A* and *B*). In contrast, uptake of extended OVA peptide without HSP was much lower (MFV = 19) and was not substantially affected by addition of metabolic inhibitors (MFV = 25). Similarly, *E. coli* DnaK:extended OVA peptide promoted peptide uptake (MFV = 114) that was reduced by metabolic inhibitors to 22% of normal uptake (MFV = 25) (Fig. 4*C*). In addition, MTB HSP70 enhanced uptake of chaperoned peptides by dendritic cells, similar to the results seen with macrophages (data not shown). In summary, *E. coli* DnaK and MTB HSP70 enhanced uptake of chaperoned extended OVA peptide through a mechanism that was blocked by metabolic inhibitors. Thus, bacterial HSPs mediate enhanced delivery of peptide Ags to APCs, and this mechanism may contribute to enhancement of either vacuolar or cytosolic alternate MHC-I Ag processing.

HSP enhancement of peptide presentation is dependent on CD91 (α_2-macroglobulin receptor)

The enhancement of peptide uptake by *E. coli* DnaK and MTB HSP70 suggests a receptor-mediated process. CD40 is involved in MTB HSP70 induction of cytokine secretion (22), and mammalian HSPs gp96, HSP90, HSP70, and calreticulin all use a common receptor, CD91, also known as α_2-macroglobulin receptor (7, 9, 18), which is present on APCs. Once these mammalian HSPs bind to the surface receptor, they are internalized by receptor-mediated endocytosis (21). Other unknown receptors may also be involved in internalization of mammalian HSPs. In our studies, processing of DnaK:peptide complexes was not altered by the addition of anti-CD40 blocking Ab (Fig. 5*A*), whereas anti-CD91 blocking Ab inhibited processing and presentation of extended peptide complexed to both *E. coli* DnaK (*B*) and MTB HSP70 (*C*). Thus, the ability of exogenous bacterial HSPs to contribute to MHC-I Ag processing and presentation is dependent on CD91. This finding reveals for the first time that CD91 can serve as a receptor for a wide range of HSPs, spanning prokaryotic as well as eukaryotic species.

Bacterial HSPs enhance alternate MHC-I Ag processing through vacuolar mechanisms in macrophages and cytosolic mechanisms in dendritic cells

It is still unclear whether HSPs enhance cytosolic or vacuolar mechanisms of alternate MHC-I Ag processing. Some researchers suggest that mammalian HSPs enhance processing through vacuolar mechanisms after endocytosis (8, 21), and, in some cases,

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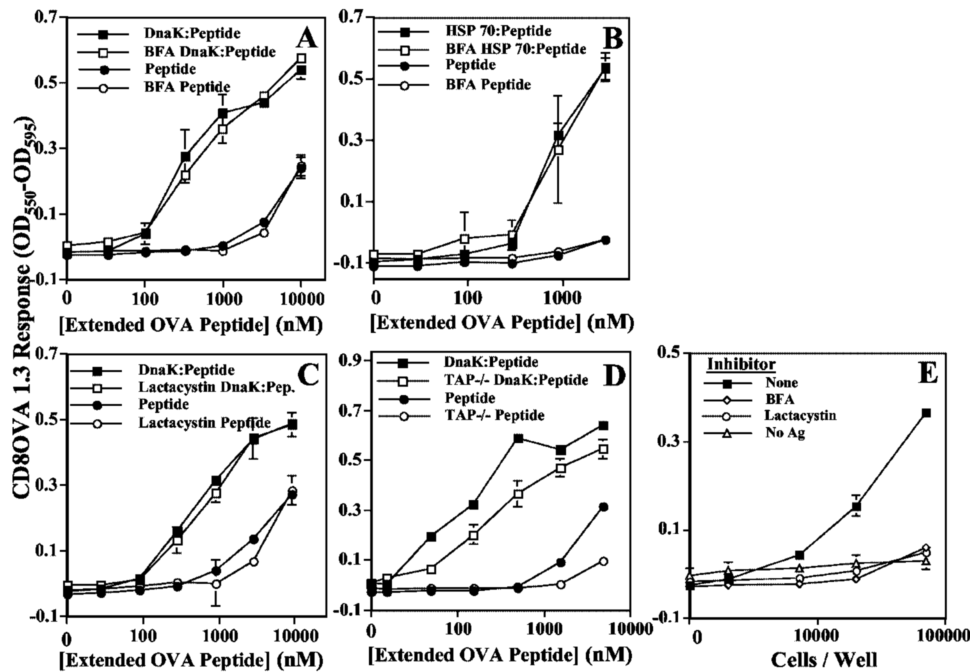

FIGURE 6. HSPs enhance macrophage alternate MHC-I Ag processing through noncytosolic mechanisms. Macrophages were exposed to inhibitors for 10 min before and during a 2-h incubation with HSP:extended OVA complexes. The cells were fixed and assessed for presentation of SIINFEKL:K^b complexes as in Fig. 1. *A* and *B*, Macrophages were incubated with *E. coli* DnaK:peptide (*A*) or MTB HSP70:peptide (*B*) with or without brefeldin A (1 μg/ml). *C*, Macrophages were incubated with *E. coli* DnaK:peptide with or without lactacystin (20 μM). *D*, Macrophages from TAP-deficient and wild-type C57BL/6 mice were incubated with *E. coli* DnaK:extended OVA peptide for 2 h. *E*, Macrophages were incubated with brefeldin A (1 μg/ml) or lactacysin (20 μM) for 10 min, electroporated to introduce OVA into the cytosol, washed, incubated for 2 h at 37°C with the appropriate inhibitor, and fixed. "None" refers to cells that were not exposed to inhibitor. "No Ag" refers to cells that were electroporated with no Ag present. The results in each panel are representative of at least three independent experiments. Data points represent means of triplicate samples with SD.

such processing is TAP independent (19, 20). However, others propose that mammalian HSPs enhance cytosolic processing, because their effect appears TAP dependent and inhibited by brefeldin A (an inhibitor of anterograde Golgi transport) and lactacystin (a proteasome inhibitor) (16, 18). One caveat is that these inhibitors and the TAP-deficient state decrease post-Golgi MHC-I levels and thereby inhibit vacuolar as well as cytosolic alternate MHC-I Ag-processing mechanisms (38, 45–47). Thus, it is still unclear whether mammalian HSPs deliver Ags for processing by cytosolic mechanisms, vacuolar mechanisms, or both, and this question is completely unaddressed for prokaryotic HSPs.

To determine the relative roles of vacuolar and cytosolic mechanisms in macrophage processing of HSP-associated Ag, cells were incubated with brefeldin A or lactacystin, incubated with bacterial HSP:extended OVA peptide complexes in the continued presence or absence of inhibitor, fixed, and incubated with CD8OVA1.3 T hybridoma cells. Brefeldin A did not substantially inhibit peptide processing and presentation by *E. coli* DnaK (Fig. 6*A*) or MTB HSP70 (*B*). In addition, lactacystin did not affect the *E. coli* DnaK enhancement of Ag processing (Fig. 6*C*). Finally, *E. coli* DnaK-enhanced processing was slightly diminished in TAP-deficient macrophages compared with wild-type C57BL/6 macrophages, but substantial TAP-independent processing activity was evident (Fig. 6*D*). As a positive control for activity of the inhibitors, we determined that conventional cytosolic MHC-I processing of OVA electroporated into the cytosol of macrophages was inhibited by similar concentrations of brefeldin A or lactacystin over a similar period (Fig. 6*E*). These data suggest that bacterial HSPs can contribute to vacuolar alternate MHC-I processing mechanisms in macrophages.

Dendritic cells are potent APCs that have been suggested to be unique in their capacity to deliver Ags from vacuolar compartments to the cytosol for cytosolic alternate MHC-I Ag processing (48). Dendritic cells were examined to test whether they differ from macrophages in the mechanisms of alternate MHC-I processing to which bacterial HSPs contribute. Dendritic cells were incubated with bacterial HSP:extended OVA peptide complexes with or without exposure to brefeldin A or lactacystin, fixed, and incubated with CD8OVA1.3 T hybridoma cells. Brefeldin A inhibited processing and presentation of extended OVA peptide chaperoned by *E. coli* DnaK (Fig. 7*A*) or MTB HSP70 (*B*), suggesting cytosolic processing. Lactacystin inhibited DnaK enhancement of extended OVA peptide processing, but to a smaller degree than brefeldin A (Fig. 7*C*); we speculate that some extended OVA peptide may be processed to present SIINFEKL without requiring a proteasome-mediated cleavage. Finally, DnaK-enhanced processing was reduced in TAP-deficient dendritic cells to a much greater degree than observed in macrophages (Fig. 7*D*), consistent with a cytosolic processing mechanism. Thus, alternate MHC-I Ag processing to which bacterial HSPs contribute in dendritic cells appears to involve cytosolic mechanisms that are highly dependent on TAP and very sensitive to brefeldin A. We conclude that the extent to which vacuolar and cytosolic processing mechanisms contribute to alternate MHC-I Ag processing is dependent on cell type with cytosolic mechanisms used more by dendritic cells and vacuolar mechanisms used more by macrophages.

Discussion

Mammalian HSPs enhance alternate MHC-I Ag processing of chaperoned self, tumor, or viral peptides (reflecting the range of proteins synthesized by mammalian cells) to promote MHC-I cross-presentation and $CD8^+$ T cell responses (2–6, 10–12, 16,

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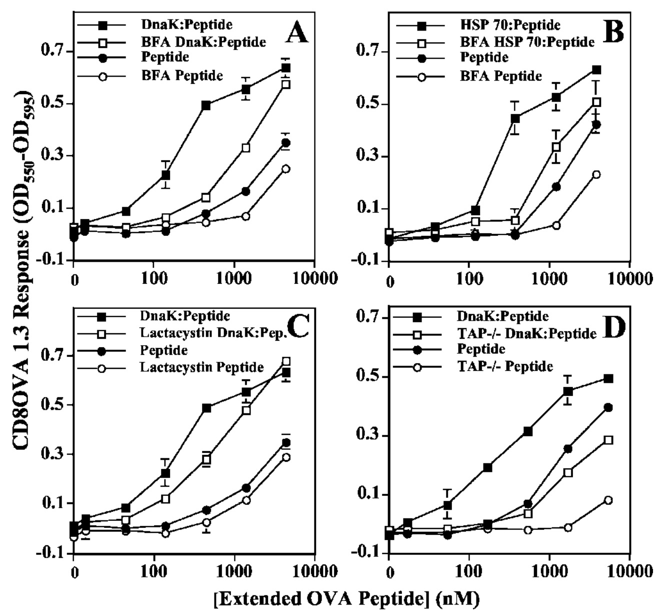

FIGURE 7. HSPs enhance dendritic cell alternate MHC-I Ag processing through cytosolic mechanisms. Dendritic cells were incubated with inhibitors and HSP:extended OVA peptide complexes as in Fig. 6. *A* and *B*, Dendritic cells were incubated with *E. coli* DnaK:peptide (*A*) or MTB HSP70:peptide (*B*) in the presence or absence of brefeldin A. *C*, Dendritic cells were incubated with *E. coli* DnaK:peptide with or without lactacystin. *D*, Dendritic cells from TAP-deficient and C57BL/6 wild-type mice were incubated with *E. coli* DnaK:peptide for 2 h. The results in each panel are representative of three independent experiments. Data points represent means of triplicate samples with SD.

17). In contrast, there have been no studies to test whether prokaryotic HSPs, which are naturally associated with microbial peptides, deliver chaperoned peptides for MHC-I Ag processing and cross-presentation. Some immunological effects of prokaryotic HSPs or HSP fusion proteins have been described, and MTB HSP70 fusion proteins are known to elicit HSP70-specific $CD8^+$ T cell responses (23, 24), but previous studies do not address the role of noncovalently associated peptides chaperoned by prokaryotic HSPs or the processing mechanisms for these peptides. The microbial peptides that are chaperoned by prokaryotic HSPs are an unexplored and potentially important source of Ags to promote antimicrobial immunity.

We propose that microbicidal mechanisms cause release of bacterial HSPs from phagocytosed bacteria, potentially delivering antigenic HSP-chaperoned bacterial peptides for processing and binding to MHC-I. In addition, bacterial HSPs released into the extracellular space may be internalized by APCs, allowing alternate MHC-I processing and cross-presentation of HSP-chaperoned bacterial Ags. In this study, we established that exogenous bacterial HSPs (*E. coli* DnaK and MTB HSP70) enhance alternate MHC-I Ag processing of a chaperoned model peptide, and we characterized the mechanisms (cytosolic vs vacuolar) by which processing occurs in both macrophages and dendritic cells.

Although some HSPs may stimulate signaling (e.g., chlamydial HSP60 signaling via TLR4 (41)) to increase expression of accessory factors (e.g., cytokines or costimulators), our data establish a different mechanism whereby prokaryotic HSPs contribute directly to generation of peptide:MHC-I complexes from HSP-chaperoned peptide Ag. This mechanism requires that peptides be complexed to the HSP and is independent of MyD88. Thus, bacterial HSPs are not signaling the cell to generally enhance MHC-I peptide presentation, but are specifically delivering chaperoned peptides for processing and presentation by MHC-I.

Enhancement of peptide presentation by prokaryotic HSPs requires internalization and active processing of HSP:peptide complexes via alternate MHC-I Ag-processing mechanisms. These mechanisms may include vacuolar or cytosolic processing. In vacuolar Ag processing, Ag is phagocytosed and degraded within vacuolar compartments to produce antigenic peptides that bind post-Golgi MHC-I molecules. In cytosolic Ag processing, exogenous Ag enters the APC cytosol, thereby achieving access to proteasome-dependent processing and TAP-dependent entry into the ER to bind MHC-I molecules. Studies of alternate MHC-I processing of other (non-HSP-associated) exogenous Ag point to vacuolar processing in some cases and cytosolic processing in others. Some researchers have suggested that mammalian HSPs enhance processing through vacuolar mechanisms because they interact with MHC-I molecules after receptor-mediated endocytosis (8, 21) and enhance processing independent of TAP in RMA-S cells (19, 20). However, others have proposed that mammalian HSPs enhance cytosolic processing, because their effect has been reported to be dependent on TAP and inhibited by brefeldin A (an inhibitor of anterograde Golgi transport) and lactacystin (a proteasome inhibitor) (16, 18). Castellino et al. (5) found evidence for both cytosolic and vacuolar processing mechanisms, depending on the sequence of HSP-associated antigenic material. Whereas the processing mechanisms for mammalian HSPs remain controversial, the mechanisms accessed by prokaryotic HSPs have not even been explored.

Our data indicate that bacterial HSPs enhance vacuolar processing in macrophages, because the enhancement was unaffected by exposure to brefeldin A or lactacystin, and there was little impact of TAP deficiency. In contrast, bacterial HSPs used cytosolic processing mechanisms in dendritic cells, as manifested by dependence on TAP and inhibition by brefeldin A. The presence of lactacystin produced mild inhibition of bacterial HSP:peptide processing in dendritic cells, suggesting that some extended OVA peptide may be presented without proteasomal processing (proteolysis by nonproteasomal mechanisms may contribute). We conclude that the extent to which vacuolar and cytosolic processing mechanisms contribute to alternate MHC-I Ag processing is dependent on cell type with cytosolic mechanisms used more by dendritic cells and vacuolar mechanisms used more by macrophages. This diversity of processing mechanisms may explain differences among other studies regarding the relative roles of cytosolic and vacuolar processing for MHC-I cross-presentation of exogenous Ags, including peptides chaperoned by mammalian HSPs.

HSPs, both mammalian and prokaryotic, may play important roles in generating immune responses in vivo. It is well established that immunization with mammalian HSPs elicits MHC-I-restricted $CD8^+$ T cell responses to HSP-chaperoned peptides. For example, Blachere et al. (17) demonstrated that mammalian HSP70 and gp96 bind OVA peptides in vitro and prime OVA-specific $CD8^+$ T cell responses in vivo. Studies with prokaryotic HSPs have not focused on responses to chaperoned peptides (excepting a report published after submission of this paper (49)),[4] but fusion proteins containing HSP sequence linked to Ag have been shown to produce substantially greater $CD8^+$ T cell responses than Ag alone. We have observed that fusion proteins containing sequence from OVA linked to MTB HSP70 prime $CD8^+$ T cells in vivo, as revealed by production of IFN-γ upon restimulation in vitro with OVA protein or SIINFEKL peptide (our unpublished observations). Similar results have been reported in prior studies (23, 24, 26). Our current in vitro results indicate that prokaryotic HSPs also promote MHC-I Ag processing and presentation of chaperoned

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peptides, similar to mammalian HSPs. Overall, observations from several studies support the significance of both mammalian and prokaryotic HSPs in generating $CD8^+$ T cell responses in vivo.

We propose that bacterial HSPs deliver antigenic HSP-chaperoned peptide Ags that contribute to the generation of $CD8^+$ T cell responses during infection of mammalian hosts with bacterial pathogens. This mechanism could be particularly important during infection with certain intracellular bacterial pathogens, for which $CD8^+$ T cell responses contribute to host immunity. APCs may encounter bacterial HSPs following bacterial phagocytosis and phagolysosomal degradation, which may release bacterial HSPs directly into the phagosomal Ag-processing environment. Alternatively, bacterial HSPs that have been released in the extracellular space may be internalized by receptor-mediated endocytosis for subsequent intracellular processing and MHC-I cross-presentation. CD91 may be one receptor involved in this process, and our data provide the first evidence that prokaryotic HSPs are internalized by CD91, driving enhanced delivery and presentation of HSP-chaperoned peptide. In addition to physiological roles in processing of bacterial Ags, bacterial HSPs have the potential to contribute to vaccine efficacy. HSPs, including prokaryotic HSPs, could be incorporated in vaccines to stimulate $CD8^+$ T cell responses that are crucial to immune responses against viruses, tumors, and certain intracellular bacteria.

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