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Stimulation of Th1-Polarizing Cytokines, C-C Chemokines, Maturation of Dendritic Cells, and Adjuvant Function by the Peptide Binding Fragment of Heat Shock Protein 70

Yufei Wang,* Charles G. Kelly,† Mahavir Singh,‡ Edward G. McGowan,§ Anne-Sophie Carrara,‡ Lesley A. Bergmeier,‡ and Thomas Lehner²*

The peptide binding C-terminal portion of heat shock protein (HSP)70 (aa 359–610) stimulates human monocytes to produce IL-12, TNF-α, NO, and C-C chemokines. The N-terminal, ATPase portion (HSP701–358) failed to stimulate any of these cytokines or chemokines. Both native and the truncated HSP70359–610 stimulation of chemokine production was mediated by the CD40 costimulatory molecule. Maturation of dendritic cells was induced by stimulation with native HSP70, was not seen with the N-terminal HSP701–358, but was enhanced with HSP70359–610, as demonstrated by up-regulation of CD83, CCR7, CD86, CD80, and HLA class II. In vivo studies in macaques showed that immunization with HSP70359–610 enhances the production of IL-12 and RANTES. Immunization with peptide-bound HSP70359–610 in mice induced higher serum IgG2a and IgG3 Abs than the native HSP70-bound peptide. This study suggests that the C-terminal, peptide-binding portion of HSP70 is responsible for stimulating Th1-polarizing cytokines, C-C chemokines, and an adjuvant function. The Journal of Immunology, 2002, 169: 2422–2429.

Materials and Methods

Reagents

The recombinant Mycobacterium tuberculosis HSP70, HSP70359–610, and HSP701–358 were prepared from the Escherichia coli pop strain. DNA encoding the N-terminal ATPase (aa 1–358) domain of HSP70 was cloned and expressed in E. coli using the pET 22b vector. DNA encoding the C-terminal peptide-binding domain (aa 359–610) was expressed using pJLA603 vector (22). Cloned inserts were verified by DNA sequence analysis. In both cases, recombinant polypeptides were prepared by affinity chromatography using Ni²⁺-chelating resin, and identity of the polypeptides was confirmed by N-terminal sequence analysis (10 cycles for each). The proteins were purified by Q-Sepharose followed by ATP affinity chromatography. The HSP preparations were further treated with polyvinyl B-coated beads (Sigma-Aldrich, Dorset, U.K.) to remove LPS. The LPS content of the HSP preparations was determined by the Limulus amebocyte lysate assay (Sigma-Aldrich), and showed <0.006 U/μg of HSP70 or 5 pg/μg of the HSP preparations. Soluble CD40LT was kindly donated by Dr. G. Geckeler, University of Freiburg, Germany.

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Molecular chaperons of the heat shock protein (HSP) family are conserved proteins that modulate intracellular protein folding. By binding to unfolded or folding intermediate polypeptides, chaperons prevent misfolding and aggregation, and promote folding and translocation (1). The peptide-binding function allows chaperon proteins such as HSP70, HSP90 (2), gp96 (3, 4), and calreticulin (5) to acquire antigenic proteins within cells when administered outside the cell to induce priming of CD8⁺ T lymphocytes in vivo. Immunization with HSP70 loaded in vitro with antigenic peptides (6) or with antigenic proteins fused with HSP65 (7) or HSP70 (8, 9) also induces MHC class I-restricted CD8⁺ T cell responses. The capacity of HSP to deliver exogenous protein Ags into the endoplasmic reticulum-associated MHC class I pathway has been recognized as an important mechanism of cross-priming (10–12). Presentation of HSP70-associated peptide by MHC class I molecule is mediated by α5 macroglobulin receptor CD91 (13, 14). HSP70 is also able to translocate across cellular membranes and gains cytoplasmic entry (15), which allows the protein Ags to be processed by cytoplasmic proteasomes, and subsequently, to enter the MHC class I pathway.

In addition to peptide binding and delivery, HSP70 can serve as a carrier of peptides or proteins that are effective immunogens for B cells, CD4⁺, and CD8⁺ T cells without requiring an adjuvant (8, 16, 17). Several studies have shown that mycobacterial HSP can induce inflammatory cytokines (18–20) and C-C chemokines (17). The CD40R mediates mycobacterial HSP70 stimulation of monocytes or dendritic cells (DCs), generating the C-C chemokines RANTES, macrophage inflammatory protein (MIP)-1α, and MIP-1β (21). Stimulation of CD40 on APCs may account for HSP70 functioning as an effective Ag carrier that elicits MHC class I-restricted CTL responses without an additional adjuvant.

In this study, we generated two major fragments of HSP70: an N-terminal 44-kDa ATPase portion (HSP701–358), and a C-terminal 28-kDa portion (HSP70359–610), which contains the 18-kDa peptide-binding region (aa 359–540). The data suggest that stimulation of human monocytes with HSP70 is mediated by the C-terminal HSP70359–610 which binds CD40 and elicits IL-12, TNF-α, NO, and C-C chemokines. Maturation of human DC was induced by stimulation with native HSP70, but was enhanced by HSP70359–610 and was comparable with that stimulated by CD40 ligand trimer (CD40LT). Immunization with HSP70359–610 elicits Th1-polarizing cytokines, C-C chemokines, and adjuvant function.

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1 United Kingdom; ‡ Lionex Diagnostics and Therapeutics, Braunschweig, Germany; † Department of Oral Medicine and Pathology, Guy’s, King’s, & St. Thomas’ Hospital Medical and Dental Schools, London, United Kingdom; §§ Peter Gorer Department of Immunobiology, and ¶ Department of Oral Medicine and Pathology, Guy’s, King’s, & St. Thomas’ Hospital Medical and Dental Schools, London, United Kingdom; ‡ Lionex Diagnostics and Therapeutics, Braunschweig, Germany

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The assays were conducted sequentially at days 2, 3, 4, and 5 after trans-CD40 was detected by protocol, and Transfection was performed according to the manufacturer transfected using LipofectAmine Plus (Life Technologies, Paisley, U.K.).

The supernatants collected from THP1 cells were diluted 10 times and the medium was replaced every 3–4 days. Human embryonic/H9262 CD40LT (10^4 cells were incubated with 10 μg/ml of penicillin and streptomycin, and 2 mM of glutamine; and the medium was replaced every 3–4 days. Human embryonic kidney (HEK) cell line 293 cells acquired from National Institutes of Health (Rockville, MD) were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 μg/ml of penicillin and streptomycin, and passaged every 3–4 days.

Transfection of HEK 293 cells with CD40

Full-length human CD40 cDNA in the pCDM8 plasmid vector (Invitrogen, San Diego, CA) was a kind gift from Dr. B. Seed (23). pCDM8 encoding the E. coli β-galactosidase (lacZ) was used as a control. The cells were cultured in 25-cm² flasks or 24-well plates until 30–50% confluence, and transfected using LipofectAmine Plus (Life Technologies, Paisley, U.K.). Transfection was performed according to the manufacturer’s protocol, and CD40 was detected by flow cytometry using PE-conjugated CD40 mAb. The assays were conducted sequentially at days 2, 3, 4, and 5 after transfection, and the percentage of CD40⁺ cells was consistently >65%.

Preparation of human DCs

PBMC were isolated from healthy donors by centrifugation on a Ficoll-Hypaque density gradient (Amersham Biosciences, Little Chalfont, U.K.). The CD14⁺ monocytes were enriched by depletion of CD14⁻ cells using Monocytes Isolation Kit (MACS; Miltenyi Biotec, Surrey, U.K.). Human DCs were generated by culturing monocytes with GM-CSF (400 U/ml) and IL-4 (100 U/ml) for 5 days in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, and 100 μg/ml of penicillin and streptomycin. The supernatant was assayed for the C-C chemokines RANTES and MIP-1α, and MIP-1β by a mechanism dependent on the cell surface expression of CD40 (20). In an attempt to identify the C-C chemokine-stimulating domains of HSP70, we have generated two subdomains of the N-terminal ATPase portion of HSP70, one of which retains the peptide binding activity (25). THP1 cells were incubated with these two fragments as well as native HSP70, and after 3 days of stimulation, the supernatant was used to assay C-C chemokines, or the cytokines IL-12 and TNF-α.

Assay of RANTES, MIP-1α, IL-12, and TNF-α

The supernatants collected from THP1 cells were diluted 10 times and HEK 293 cell culture supernatant two times. Specific ELISA–paired Ab kits were used for C-C chemokines (R&D Systems, Oxon, U.K.) or IL-12 and TNF-α (BD Pharmingen, Oxford, U.K.). The results were expressed in picograms per milliliter.

Flow cytometry analysis

Aliquots of 2 x 10⁶ cells were incubated with 10 μl of a panel of FITC-conjugated Abs to DC markers. For unconjugated primary Ab, the cells were further incubated with FITC-conjugated goat anti-mouse Fab. After washing twice, the cells were fixed in 1% formaldehyde PBS solution before flow cytometry analysis. The cells were analyzed on an Epics II flow cytometer (Coulter, High Wycombe, U.K.) and the data was analyzed on a Software WinMD.

Induction of RANTES in nonhuman primates

Simian PBMC were obtained from either HSP70, HSP70₃₅₉₋₆₁₀-immunized, or naïve rhesus macaques, and were stimulated in vitro with 0–0.5 μM of native HSP70, HSP70₃₅₉₋₆₁₀ or HSP70₃₅₈₋₃₅₉. After a 3-day culture, the supernatant was collected for assaying RANTES.

Immunization of mice

HSP70 or HSP70₃₅₉₋₆₁₀ was loaded with synthetic peptides by incubation with 20-fold molar excess of peptide, in PBS supplemented with 2 mM MgCl₂, for 1–2 h at 37°C. Unbound peptide was removed by washing in a centrifugal concentrator with PBS. Groups (n = 4) of C57BL/6J mice were immunized i.p. with 50 μg equivalent of HSP70 or HSP70₃₅₉₋₆₁₀ complexed with peptide in PBS. A second identical immunization was given after 4 wk, and mice were bled after a further 3 wk. Serum Abs were determined by ELISA.

ELISA for serum Abs

Proteins (HSP70 or HSP70₃₅₉₋₆₁₀ 2 μg/ml) or peptides (10 μg/ml) in PBS were adsorbed to wells of polystyrene microtiter plates (Dynatech Laboratories, Chantilly, VA), which were then blocked by treatment with 1.5% BSA (1 h at room temperature). Bound Abs were incubated with serially diluted sera in duplicate. Bound IgG Abs were determined by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma-Aldrich), and subsequent reaction with paranitrophenol phosphate. Plates were read at 405 nm with the microplate reader model 450 (Bio-Rad, Hercules, CA). Each serum was analyzed three times. A synthetic peptide derived from the N-terminal (residues 1–20) of CCR5 was made as a control of specificity.

Results

Stimulation of C-C chemokines by the HSP70 C-terminal fragment

We have previously shown that mycobacterial HSP70 stimulates the human monocytic (THP1) cells, dendritic-like (KG1) cell lines, and monocytes isolated from PBMC to produce RANTES, MIP-1α, and MIP-1β by a mechanism dependent on the cell surface expression of CD40 (20). In an attempt to identify the C-C chemokine-stimulating domains of HSP70, we have generated two subdomains of the N-terminal ATPase portion of HSP70, one of which retains the peptide binding activity (25). THP1 cells were incubated with these two fragments as well as native HSP70, and after 3 days of stimulation, the supernatant was used to assay C-C chemokines RANTES and MIP-1α. HSP70₃₅₉₋₆₁₀ or native HSP70 stimulates a dose-dependent increase in RANTES and MIP-1α at concentrations of 0–0.1 μM, and a plateau was reached at a dose of ~0.2 μM (Fig. 1). In contrast with native HSP70, stimulation of RANTES and MIP-1α was greatly enhanced by HSP70₃₅₉₋₆₁₀ with a 20-fold increase in RANTES (Fig. 1A) and 40-fold increase in MIP-1α (Fig. 1B). In contrast, treatment with HSP70₁₋₃₅₈ (up to 1 μM) elicited a negligible rise in the concentration of RANTES (Fig. 1A) or MIP-1α (Fig. 1B). It is noteworthy that <1 μM of the native or C-terminal HSP70 elicited most of the chemokine production.

Mycobacterial HSP70 stimulation of C-C chemokines is dependent on the cell surface expression of CD40 (20). To determine whether CD40 also mediates HSP70₃₅₉₋₆₁₀ stimulation, CD40 and control lacZ HEK 293-transfected cells were incubated with HSP70₃₅₉₋₆₁₀ or HSP70. HSP70₃₅₉₋₆₁₀ elicited a significant increase in the concentration of RANTES in CD40-transfected HEK 293 cells (Fig. 2A). This was 2- to 3-fold greater than that found with native HSP70, but was similar to that elicited with the control CD40LT (Fig. 2A). Native HSP70, HSP70₃₅₉₋₆₁₀, and CD40LT were not able to stimulate the control lacZ transfectected 293 cells to generate RANTES.

To rule out the role of LPS in HSP70₃₅₉₋₆₁₀ stimulation, we used polymixin B, an inhibitor of LPS, which previously showed no effect on HSP70 stimulation (21). In this study, we found that neither native HSP70 nor HSP70₃₅₉₋₆₁₀ stimulation of RANTES production was affected by addition of polymixin B, whereas in the same experiments, polymixin B completely abrogated LPS (500 ng/ml) stimulation of RANTES (Fig. 2B). The maximum amount of LPS in 10 μg of the HSP preparations was 50 pg, whereas the minimum amount of LPS necessary for stimulation of RANTES production was >10,000 pg/ml, as demonstrated previously and in this study (21).
Induction of IL-12, TNF-α, and NO by HSP70

Production of the proinflammatory cytokines IL-12 and TNF-α was analyzed in the culture supernatants of monocytic THP1 cells, following treatment with native HSP70, HSP70_1–358, or HSP70_359–610 fragment. Unstimulated THP1 cells produced little IL-12 (<50 pg/ml), and stimulation with increasing concentrations of either native HSP70 or HSP70_1–358 fragment failed to induce a significant level of IL-12 (Fig. 3A). However, a dose-dependent increase in the concentration of IL-12 was generated by stimulating THP1 cells with the HSP70_359–610 fragment. An increase in IL-12 was detected with 0.01 μM followed by a marked increase at concentrations between 0.05 and 0.1 μM of HSP70_359–610. IL-12 was

**FIGURE 2.** A, Stimulation of RANTES production by native HSP70, HSP70_359–610, and CD40LT in CD40 or lacZ-transfected HEK 293 cells. Subconfluent (80%) HEK 293 cells in 24-well plates were transfected with human CD40 or lacZ cDNA. Three days after transfection, HEK 293 cells were stimulated with 10 μg/ml of HSP70_359–610, HSP70, or CD40LT for 4 days before testing for RANTES in the supernatant. B, Effects of Polymixin B on native HSP70, HSP70_359–610 and LPS stimulation of RANTES. THP1 cells were stimulated with 10 μg/ml of HSP70_359–610, HSP70, or 500 ng/ml LPS in the presence of 50 μg/ml polymixin B. Three days after culture, the supernatants were collected to assay RANTES. RANTES (C) and IL-12 (D) production by monocyte-derived DC following stimulation with HSP70, HSP70_359–610, CD40LT, and LPS. Monocyte-derived DC (1 × 10⁵) were incubated with 0.5 μM of HSP70, HSP70_1–358, and HSP70_359–610 10 μg/ml of CD40LT, and 500 ng/ml of LPS for 2 days. The supernatants were used to assay RANTES and IL-12. The data represent the mean ± SEM of four independent experiments.
detected as early as 18 h after stimulation (data not shown), and maximal production of 3329 ± 469 pg/ml of IL-12 was reached after 3 days treatment with 0.2–1 μM of HSP70359–610 (Fig. 3A).

Unstimulated THP1 cells produced very low levels of TNF-α (10.5 ± 4.4 pg/ml), and stimulation with the HSP70359–610 fragment again failed to increase the concentration of TNF-α (Fig. 3B). Stimulation with increasing concentrations of native HSP70 led to a small increase in production of TNF-α from 6.6 ± 7.9 pg/ml to 129.5 ± 72.6 pg/ml with 0.5 μM HSP70 (Fig. 3B). However, treatment of THP1 cells with HSP70359–610 elicited a dose-dependent increase in TNF-α production. TNF-α increased >100-fold to 1045.1 ± 237.5 pg/ml after stimulation with 0.1 μM, as compared with the unstimulated control (Fig. 3B).

Production of NO was determined by an assay of nitrite production by monocyctic THP1 cells following activation with native HSP70, HSP70359–610, and HSP701–358 fragments. After a 3-day culture, the supernatant was taken for analysis of nitrite. Resting cultures of THP1 cells produced low levels of nitrite, and this was unchanged with increasing concentration of HSP701–358 fragment (Fig. 3C). However, native HSP70, and to a greater extent HSP70359–610 fragment, induced a dose-dependent increase in the concentration of nitrite. This was comparable with that of LPS, known to be a potent inducer of NO, although the dose response was different from that of HSP70 or HSP70359–610. The production of NO induced by native HSP70 or HSP70359–610 was specific and dependent on inducible NO synthase as 1-NAME, the inducible NO synthase-specific inhibitor suppressed NO production (data not shown).

Production of RANTES and IL-12 by mature DC was also determined following native HSP70, HSP70359–610, or HSP701–358 stimulation. Unstimulated immature DC or those stimulated by HSP701–358 produced negligible amounts of RANTES and some IL-12–610 (10.5 ± 4.4 pg/ml), and stimulation with the HSP701–358 fragment failed to increase the concentration of TNF-α (Fig. 3B). Stimulation with increasing concentrations of native HSP70 led to a small increase in production of TNF-α from 6.6 ± 7.9 pg/ml to 129.5 ± 72.6 pg/ml with 0.5 μM HSP70 (Fig. 3B). However, treatment of THP1 cells with HSP70359–610 elicited a dose-dependent increase in TNF-α production. TNF-α increased >100-fold to 1045.1 ± 237.5 pg/ml after stimulation with 0.1 μM, as compared with the unstimulated control (Fig. 3B).

FIGURE 3. The effect of stimulation of DCs with HSP70359–610 compared with HSP701–358 and native HSP70 on the phenotypic expression and maturation of DC.

The effect of stimulation of DCs with HSP70359–610 compared with HSP701–358 and native HSP70 on the phenotypic expression and maturation of DC

Culture of CD14+ monocytes with GM-CSF and IL-4 differentiated the cells into nonadherent, immature DC with typical DC morphology. These cells expressed 13.5% CD83+, 3.0% CCR7, 83.7% CD80, and 15.7% CD86 (Fig. 4). Stimulation with HSP701–358 showed no increase in any of the five maturation phenotypes; CD86 showed a slight increase to 25.9% (but not in MFI), compared with 95 and 93.7% stimulated by HSP70359–610 and native HSP70, respectively (Fig. 4). Treatment with HSP70 (5.0 μM) up-regulated the expression of CD83 to 85.3%, CCR7 to 20.7%, CD80 to 92.3%, and CD86 to 93.7% (Fig. 4). However, HSP70359–610 (0.3 μM) showed an enhanced effect on the expression of CD83, CCR7, CD80, and CD86, which was similar to that found with the C-C chemokines and cytokines. Up-regulation of these phenotypic markers was very similar to those found on treatment with CD40L (or LPS; data not shown), except that the CCR7 was up-regulated to 61.7% (LPS showed 26.9%). The HLA class II remained unchanged at 96–99%, but the median fluorescence intensity increased 2- to 3-fold after stimulation with native HSP70, HSP70359–610, or CD40L.

The effect of ATPase on native HSP70 and HSP70359–610 stimulation of chemokines and cytokines

A potential mechanism to account for the enhanced capacity of HSP70359–610 to stimulate monocytic THP1 cells after removal of the N-terminal ATPase is that the latter may have suppressed HSP70 stimulation of C-C chemokines and cytokines. We tested this possibility by adding the ATPase fragment of HSP701–358 to HSP70359–610, but this failed to suppress the production of RANTES, TNF-α, or IL-12 (Table I). The role of ATPase in regulating HSP70 stimulation was further studied by treatment of native or the C-terminal fragment of HSP70 with 0.5 mM of the nonhydrolysable ATP analog ATP-γ-S. ATP-γ-S failed to affect significantly the concentration of RANTES stimulated by either native HSP70 or HSP70359–610 (Table I).

The effect of immunization with native HSP70 as compared with the HSP70359–610 fragment on the production of RANTES in macaques

To compare the effect of immunization with HSP701–358 with that of the native HSP70, one group of rhesus monkeys was immunized (three times) with HSP70, a second group with HSP70359–610 and a third group was not immunized. The concentration of RANTES was...

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assayed after specific stimulation of PBMC in vitro for 3 days with the native HSP70, HSP70, or HSP70 immunized by the native HSP70, HSP70, or HSP70 immunized by the native HSP70. A small dose-dependent increase in the concentration of RANTES was elicited by the PBMC from naive macaques, which was higher when stimulated with the C-terminal than the native HSP70, but the N-terminal fragment failed to elicit any change (Fig. 5A). Macaques immunized with native HSP70 elicited an increased production of RANTES when stimulated with native HSP70 (Fig. 5B), and this response was further enhanced in HSP70-immunized macaques when stimulated by either native or HSP70 immunized macaques (Fig. 5C). Stimulation with 0.5 μM of HSP70 induced a 12-fold increase in RANTES in naive PBMC (222.1 ± 33.2 pg/ml) compared with a 7-fold increase when stimulated by native HSP70 (132.8 ± 25.3 pg/ml; Fig. 5A). However, PBMC from HSP70 primed macaques stimulated with HSP70 elicited a 30-fold increase of RANTES (525.6 ± 142.4 pg/ml) compared with a 7-fold increase stimulated by the native HSP70 (284 ± 35.3 pg/ml). Surprisingly, the HSP70-immunized macaques showed a 5-fold increase in RANTES in the unstimulated PBMC (81.9 pg/ml) as compared with a 7-fold increase when stimulated by native HSP70 (1457.2 and 1458 pg/ml), but these were statistically significant (p < 0.05). The effect of immunization with native HSP70 compared with HSP70 or HSP70 on native HSP70 stimulation of monocytes to produce IL-12, and TNF-α (given in picograms per milliliter).

**Table 1. Effects of the HSP70, ATPase domain (1–358) or ATPase inhibitor (ATP-γ-S) on native HSP70 or HSP70 stimulation of monocytes to produce RANTES, IL-12, and TNF-α.**

<table>
<thead>
<tr>
<th>Monocyte Stimulation</th>
<th>Nil</th>
<th>HSP70</th>
<th>ATP-γ-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANTES</td>
<td>1861.9 ± 196</td>
<td>2138.3 ± 231.5</td>
<td>1943.3 ± 45</td>
</tr>
<tr>
<td>IL-12</td>
<td>65.5 ± 8.9</td>
<td>76.4 ± 9.1</td>
<td>66.3 ± 11.3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>123 ± 4.3</td>
<td>132.1 ± 12.1</td>
<td>113.6 ± 2.8</td>
</tr>
</tbody>
</table>

**Induction of IL-12 by immunization with HSP70 or HSP70 in macaques**

IL-12 production in PBMC was also studied in naive and immunized macaques (Fig. 5D). No significant levels of IL-12 were detected in naive macaque PBMC, whether unstimulated or following in vitro stimulation with HSP70 and fragments. However, HSP70 and HSP70 did not elicit any change (Fig. 5A). However, PBMC from HSP70 primed macaques stimulated with HSP70 elicited a 30-fold increase of RANTES (525.6 ± 142.4 pg/ml) compared with a 7-fold increase stimulated by the native HSP70 (284 ± 35.3 pg/ml). Surprisingly, the HSP70-immunized macaques showed a 5-fold increase in RANTES in the unstimulated PBMC (81.9 pg/ml) as compared with a 7-fold increase when stimulated by native HSP70 (1457.2 and 1458 pg/ml), but these were statistically significant (p < 0.05). The effect of immunization with native HSP70 compared with HSP70 or HSP70 on native HSP70 stimulation of monocytes to produce IL-12, and TNF-α (given in picograms per milliliter).

**The effect of immunization with native HSP70 compared with the HSP70 fragment loaded with CCR5 peptide on stimulating Abs in mice**

To compare the adjuvanticity of HSP70 with that of intact HSP70 in stimulating serum Ab responses, mice were immunized with a synthetic peptide corresponding to the first extracellular
loop of the chemokine receptor CCR5 bound noncovalently to HSP70 (aa 359–610) or to native HSP70. Groups of four C57BL/6j mice were immunized i.p. with a boost after 4 wk, and the serum Ab response was determined by ELISA. Immunization with HSP70 noncovalently associated with the first loop peptide induced responses to the peptide (1 in 8000), native HSP70 (1 in 32,000), and HSP70 359–610 (1 in 8000). However, immunization with HSP70 359–638 linked to the same peptide resulted in an increased serum Ab response to the first loop peptide (1 in 32,000), but no detectable responses (<100) to either HSP70 or HSP70 359–610 (Table II). The IgG subclass of Ab induced to the first loop peptide (1 in 8000) was higher than those of IgG1, the IgG2a:IgG1 ratio was greater for HSP70 (4.1; Table IIB), consistent with a Th1 Ab response. The higher titers of total IgG in this assay reflect the inclusion of an extra (IgG subclass-specific) Ab in the assay.

**Discussion**

In addition to the well-documented role of eliciting adoptive immune responses to HSP-chaperoned peptides, evidence has emerged that HSP stimulates innate immunity. Indeed, HSP can activate macrophages to produce proinflammatory cytokines, chemokines, and up-regulation of costimulatory molecules (18–20, 26). We have shown that stimulation of monocytes by microbial HSP70 produces C-C chemokines (17, 21). In this study, we report that removing the ATPase portion (aa 1–358) and stimulating with the remaining C-terminal portion of HSP70 (aa 359–610) induces IL-12, TNF-α, and NO production, as well as enhancing the production of C-C chemokines. In contrast, the ATPase domain of HSP70 (aa 1–358) lacked the capacity to stimulate chemokines or cytokines and the native HSP70 either failed or stimulated production of very low concentrations of IL-12, TNF-α, and NO.

TNF-α is an important factor mediating innate immunity and is involved in inflammation (27). NO has a broad antimicrobial activity and also plays a role in regulation of adaptive immune responses (28, 29). IL-12 plays an essential role in controlling and maintaining a long-lasting protective immunity against viral and intracellular bacterial infections (30). Because IL-12 is one of the most potent cytokines inducing type 1 polarization (31), these findings may have important implications in using the HSP70 359–610 fragment as a Th1-polarizing adjuvant. Indeed, HSP70 359–610-linked peptide elicited higher serum IgG, and IgG3 subclasses of Abs than native HSP70-bound peptide, consistent with a Th1-polarizing response. Furthermore, the Th2 type of cytokine (IL-4) was not produced in immunized macaques. Thus, HSP70 359–610 might be used as a microbial adjuvant that attracts the entire immunological repertoire of cells by virtue of stimulating the production of C-C chemokines and elicits a Th1 response by generating IL-12. However, the peptide/protein binding properties of

**Table II. Serum Ab responses in C57BL/6j mice after immunization with a synthetic peptide derived from the sequences of the first loop of CCR5, noncovalently complexed with HSP70 or HSP70 (359–610)**

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Ag</th>
<th>IgG Subclass Responses to First Loop (aa 88–102)</th>
<th>Total IgG Response</th>
<th>IgG Subclass Titre</th>
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<tr>
<td></td>
<td></td>
<td>Peptide</td>
<td>HSP70</td>
<td>HSP70 359–610</td>
</tr>
<tr>
<td>HSP70 + first loop</td>
<td>160</td>
<td>8 × 10^3</td>
<td>32 × 10^3</td>
<td>8 × 10^3</td>
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<tr>
<td>HSP70 359–610 + first loop</td>
<td>200</td>
<td>32 × 10^3</td>
<td>&lt; 500</td>
<td>1 × 10^3</td>
</tr>
<tr>
<td>None</td>
<td>200</td>
<td>&lt; 100</td>
<td>&lt; 100</td>
<td>&lt; 100</td>
</tr>
</tbody>
</table>

*Ab titres are expressed as the highest dilution resulting in OD 405 > 0.2.

*Synthetic peptides corresponding to the sequence of CCR5.*

**FIGURE 5.** Induction of RANTES in simian PBMC by HSP70, HSP70 359–610, and HSP70 359–610. Simian PBMC were collected from either naive or immunized macaques, and 3 × 10^6/ml of cells were cultured either in medium or treated with 0.05, 0.1, and 0.5 μM of HSP70, HSP70 359–610, and HSP70 359–610. Three days after cultures, the supernatants were removed for the assay of RANTES or MIP-1α.
HSP70 have to be first determined, for if they do not have the hydrophobic motif necessary for noncovalent binding with the HSP70 binding groove, the peptide or protein has to be covalently linked to HSP70. The possibility that HSP70 may undergo intracellular enzyme digestion to the C-terminal fragment will need to be investigated.

The peptide binding function of the C-terminal portion of constitutive HSP70 has been documented previously, especially as removal of ATPase retained high-affinity binding of peptides (25). In this study, we demonstrate that removal of the ATPase fragment of mycobacterial HSP70 is required for effective HSP70 stimulation of monocytes to produce IL-12, TNF-α, and NO, and to enhance C-C chemokine production. Indeed, a suppressive role of mycobacterial HSP70 ATPase domain has been demonstrated in rats with the production of IL-10 and TGF-β (32, 33). A cytotoxic cell-induced function was also demonstrated in the ATPase portion (aa 161–370) of mycobacterial HSP70 when fused to a protein (9). Other studies suggest that the native HSP70 might conceal the stimulating epitope (7). We have failed to demonstrate an inhibitory role of the ATPase fragment of HSP70, by directly adding it to the native HSP70 or C-terminal HSP70, 610 fragment. Furthermore, addition of an ATPase inhibitor (ATP-γ-S) also failed to affect stimulation by native HSP70 or the C-terminal HSP70, 610 fragment.

In vivo administration of HSP70, 610 in nonhuman primates also elicited IL-12 produced by PBMC when stimulated in vitro by either native HSP70 or HSP70, 610. Immunization with native HSP70 also elicited IL-12, although to a lesser extent. This is in contrast with the in vitro stimulation of human monocytic THP1 cells or peripheral blood monocytes, that HSP70, 610, but not the native HSP70, was capable of eliciting IL-12. This difference suggests that presensitized T cells in vivo were involved in producing IL-12, following in vitro stimulation with either HSP70 or HSP70, 610 as was found previously with HSP70 (17). Enhanced adjuvant function of HSP70, 610 compared with native HSP70, was also demonstrated in mice immunized with a peptide (aa 88–102) derived from the first loop of CCR5, which was noncovalently linked to HSP70. Surface plasmon resonance was used to demonstrate that the peptide could bind to both HSP70 and HSP70, 610, however, HSP70, 610 induced a significantly lower Ab titer to intact HSP70 or HSP70, 610 than that induced by HSP70. Using a truncated form of HSP70, rather than the native HSP70 as an adjuvant, appears to reduce the likelihood of inducing responses to self HSP70, which is desirable for a vaccine carrier.

DCs are important cells of the innate immune system and play a primary role in regulation of the adaptive immune responses (34). Immature DCs reside in epithelia and can terminally differentiate into mature DCs by various stimuli, such as microbial pathogens, inflammatory cytokines, or other “danger” signals (35). Maturation of DC is critical in the initiation of the immune response. HSP70 derived from necrotic cells (36) or recombinant human HSP70 induced DC maturation (37). In this study, we demonstrate that native mycobacterial HSP70 induces DC maturation, as assayed for the cell surface expression of CD83, CCR7, and HLA-DR, and the expression of the costimulatory molecules (CD80 and CD86). However, maturation of DC was enhanced by stimulation with the HSP70, 610 fragment, which up-regulated the maturation markers CD83, CCR7 HLA-DR, and CD80, CD86. These phenotypic changes were comparable with those elicited by CD40LT or LPS, so HSP70, 610 is a potent inducer of DC maturation.

In summary, we demonstrate that the cytokine and chemokine stimulatory domain of HSP70 resides within the C-terminal fragment which elicits IL-12, TNF-α, NO, and C-C chemokines, and functions as a Th1 type adjuvant.

References

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