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

Yuefei Wang, Charles G. Kelly, Mahavir Singh, Edward G. McGowan, Anne-Sophie Carrara, Lesley A. Bergmeier and Thomas Lehner

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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 is 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.


Stimulation of Th1-Polarizing Cytokines, C-C Chemokines, Maturation of Dendritic Cells, and Adjuvant Function by the Peptide Binding Fragment of Heat Shock Protein 701

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 is 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.

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 Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) 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 affinity chromatography. The HSP preparations were further treated with polyvinylidene difluoride 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 affinity chromatography. The HSP preparations were further treated with polyvinylidene difluoride 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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Dr. F. Villinger (Emory University, Atlanta, GA). Synthetic peptides corresponding to CCR5 residues 1–20 (N-terminal: MDYQVSSPYIDNYTSEPC) and 88–102 (first loop: HYAAAQDFGNTMCGC) were purchased from NeoSystem (Strasbourg, France). Adenosine 5′-O-(3-thiotriphosphate (ATP-γS), LPS, and polyoxymin B were obtained from Sigma-Aldrich. FITC-conjugated mouse mAbs to CD14 (IgG2a) and CD83 (IgG2b) were obtained from Immunotech (Oxford, U.K.). FITC-conjugated Abs to CD80(IgG1), CD86(IgG1), CCR7(IgG1), and HLA-DR (IgG2a) and mouse control IgG isotypes were purchased from Serotech (Oxford, U.K.).

**Cell lines**

Monocytic THP1 cell lines were obtained from MRC (National Institute for Biological Standards and Control, Potters Bar, U.K.). The nonadherent THP1 cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 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 derived 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 (33). 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 of glutamine, and 100 μg/ml of penicillin and streptomycin, and passaged every 3–4 days.

**Induction of C-C chemokines and cytokines**

THP1 cells (2 × 10⁶/ml), DC, or transfected 293 cells (80–90% confluent) were cultured in 24-well plates and incubated with HSP70, HSP70₃₅⁹₋₆₁₀, and HSP70₃₅⁹ at concentrations of 0–0.5 μM, or CD40LT (0.1–10 μg/ml). After 3–5 days, 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 and human 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 PhaMingen, Oxford, U.K.). The results were expressed in picograms per milliliter.

**Flow cytometry analysis**

Aliquots of 2 × 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 WinMID.

**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 HSP70₃₅⁹. 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 μM 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 para-nitrophenol 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 monocyes isolated from PBMC to produce RANTES, MIP-1α, and MIP-1β by a mechanism dependent on the cell surface expression of CD40 (21). 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 HSP70₃₅⁹₋₆₁₀ and the C-terminal portion of HSP70₃₅⁹₋₆₁₀, 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 assayed for RANTES (Fig. 1). THP1 cells were incubated with these two fragments as well as native HSP70, and after 3 days of stimulation, the supernatant was assayed for RANTES, MIP-1α, HSP70₃₅⁹₋₆₁₀, and native HSP70 stimulates a dose-dependent increase in RANTES and MIP-1α. HSP70₃₅⁹₋₆₁₀ and native HSP70 stimulate 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 HSP70₁₃₅⁸ (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 (21). 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 transfected 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 359–610

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 1.** Induction of RANTES (A), and MIP-1α (B) by HSP70 and its fragments. THP1 cells (2 × 10⁶/ml) were treated with various concentrations (0–1 μM) of HSP70 (○), HSP70 359–610 (●), or HSP70 1–358 (▲). Three days after cultures, the supernatants were collected for assays of RANTES and MIP-1α. The results of three experiments were presented as mean ± SEM.

Induction of IL-12, TNF-α, and NO by HSP70 359–610

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, or HSP70 1–358 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 1-358, 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 HSP701358-610 (Fig. 3A).

Unstimulated THP1 cells produced very low levels of TNF-α (10.5 ± 4.4 pg/ml), and stimulation with the HSP701358 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 HSP701358-610 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 monocytic THP1 cells following activation with native HSP70, HSP701358-610, and HSP7011-238 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 HSP7011-238 fragment (Fig. 3C). However, native HSP70, and to a greater extent HSP7011-610 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 concentration of nitrite. This was comparable with that of LPS, known to be a potent inducer of NO, although the dose response concentration of nitrite was different from that of HSP70 or HSP701358-610. The production of NO induced by native HSP70 or HSP701358-610 was specific and dependent on inducible NO synthase (iNOS) synthesis. The inducible NO synthase-specific inhibitor suppressed NO production (data not shown).

Production of RANTES and IL-12 by IL-12 by mature DC was also determined following native HSP70, HSP701358-610, or HSP7011-238 stimulation. Unstimulated immature DC or those stimulated by HSP7011-238 produced negligible amounts of RANTES and some IL-12 (174.1 ± 110 and 48.5 ± 9.6 pg/ml; Fig. 2, C and D). Native HSP70 induced RANTES production (2432.5 ± 243 pg/ml) as well as low levels of IL-12 (1111.7 ± 94.6 pg/ml) by DC. This is consistent with HSP70-induced DC maturation as defined by cell surface expression of CD83, CD80, and CD86 (Fig. 4). However, HSP701358-610 showed greatly enhanced stimulation of both RANTES (8090.4 ± 845 pg/ml) and IL-12 (4974.1 ± 203.1 pg/ml) in comparison with native HSP70 or HSP7011-238. The control CD40LT stimulated DC to produce higher levels of RANTES or IL-12 than the native HSP70, but not HSP701358-610. LPS at 500 ng/ml gave similar results (Fig. 2, C and D).

The effect of stimulation of DCs with HSP701358-610 compared with HSP7011-238 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 HSP701358 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 HSP701358-610 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, HSP701358-610 (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 CD40LT (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, HSP701358-610, or CD40LT.

The effect of ATPase on native HSP70 and HSP701358-610 stimulation of chemokines and cytokines

A potential mechanism to account for the enhanced capacity of HSP701358-610 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 HSP7011-238 to HSP701358-610, 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 HSP701358-610 (Table I).

The effect of immunization with native HSP70 as compared with the HSP701358-610 fragment on the production of RANTES in macaques

To compare the effect of immunization with HSP7011-610 with that of the native HSP70, one group of rhesus monkeys was immunized (three times) with HSP70, a second group with HSP7011-610, and a third group was not immunized. The concentration of RANTES was
assayed after specific stimulation of PBMC in vitro for 3 days with the native HSP70, HSP70(359–610), or HSP70(1–358). 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(359–610)-immunized macaques when stimulated by either native or HSP70(359–610) (Fig. 5C). Stimulation with 0.5 μM of HSP70(359–610) 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(359–610) 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(359–610)-immunized macaques showed a 5-fold increase in RANTES in the unstimulated PBMC (81.9 pg/ml) as compared with the native HSP70 (17.3 ± 3.0 pg/ml) or unimmunized (18.3 ± 17.0 pg/ml) macaques (Fig. 5). In vitro stimulation with HSP70 or HSP70(359–610) induced comparable levels of RANTES (1457.2 pg/ml) compared with a 7-fold increase when stimulated by native HSP70(1–358) or HSP70(359–610) stimulation of monocytes to Th1-polarized immune responses.

Induction of IL-12 by immunization with HSP70 or HSP70(359–610) 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(359–610) but not HSP70(1–358) stimulated PBMC from HSP70-immunized macaques to produce a significant amount of IL-12 in comparison with naive macaques. Increased levels of IL-12 production were found in PBMC from HSP70(359–610)-immunized macaques when stimulated in vitro with HSP70, and to a greater extent when stimulated with HSP70(359–610) (Fig. 5D). We failed to detect IL-4 in either unstimulated or HSP70-stimulated PBMC in all three groups of macaques (data not shown), consistent with HSP70 or HSP70(359–610) eliciting Th1-polarized immune responses.

The effect of immunization with native HSP70 compared with the HSP70(359–610) fragment loaded with CCR5 peptide on stimulating Abs in mice

To compare the adjuvancticity of HSP70(359–610) with that of intact HSP70 in stimulating serum Ab responses, mice were immunized with a synthetic peptide corresponding to the first extracellular

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Table 1. Effects of the HSP70, ATPase domain (1–358) or ATPase inhibitor (ATP-γ-S) on native HSP70 or HSP70(359–610) stimulation of monocytes to produce RANTES, IL-12, and TNF-α (given in picograms per milliliter)

<table>
<thead>
<tr>
<th></th>
<th>Native HSP70</th>
<th>HSP70(359–610)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Nil</td>
<td>HSP70(1–358)</td>
</tr>
<tr>
<td>RANTES</td>
<td>1861.9 ± 196</td>
<td>2138.3 ± 231.5</td>
</tr>
<tr>
<td>IL-12</td>
<td>65.5 ± 8.9</td>
<td>76.4 ± 9.1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>123 ± 4.3</td>
<td>132.1 ± 12.1</td>
</tr>
</tbody>
</table>

|                  | Nil          | HSP70(1–358)   | ATP-γ-S          |
|                  | HSP70(359–610)| 6437 ± 853.8  | 5972.1 ± 341.1  |
| RANTES           | 4964 ± 54.9  | 4301.6 ± 116.3 | 4248.1 ± 758.6  |
| IL-12            | 823.9 ± 38   | 1121 ± 98.7    | 735.9 ± 131.5   |

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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 HSP70359–610 fragment as a Th1-polarizing adjuvant. Indeed, HSP70359–610-linked peptide elicited higher serum IgG2a 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, HSP70359–610 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 HSP70359–610.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Control peptide</th>
<th>First loop (aa 88–102)</th>
<th>HSP70</th>
<th>HSP70359–610</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP70 + first loop</td>
<td>160</td>
<td>8 × 10^3</td>
<td>32 × 10^3</td>
<td>8 × 10^3</td>
</tr>
<tr>
<td>HSP70359–610 + 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>

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IgG Subclass Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG1</td>
</tr>
<tr>
<td>HSP70</td>
<td>8 × 10^3</td>
</tr>
<tr>
<td>HSP70359–610</td>
<td>2 × 10^3</td>
</tr>
</tbody>
</table>

*Ab titers are expressed as the highest dilution resulting in OD_{405} > 0.2.

*Synthetic peptides corresponding to the sequence of CCR5.
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 cytotokic cell-inducing 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 HSP701-358 by directly adding it to the native HSP70 or C-terminal HSP70359–610 fragment. Furthermore, addition of an ATPase inhibitor (ATP–γ-S) also failed to affect stimulation by native HSP70 or the C-terminal HSP70359–610 fragment.

In vivo administration of HSP70359–610 in nonhuman primates also elicited IL-12 produced by PBMC when stimulated in vitro by either native HSP70 or HSP70359–610. 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 producing IL-12, following in vitro stimulation with either HSP70 or 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 HSP70359–610.

In vivo administration of HSP70359–610 in nonhuman primates also elicited IL-12 produced by PBMC when stimulated in vitro by either native HSP70 or HSP70359–610. 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 HSP70359–610, 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 HSP70359–610 as was found previously with HSP70 (17). Enhanced adjuvant function of HSP70359–610 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 HSP70359–610. However, HSP70359–610 induced significantly lower Ab titer to intact HSP70 or HSP70359–610 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 recombiant 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 HSP70359–610 fragment, which up-regulated the maturation markers CD83, CCR7 HLA-DR, and CD80, CD86. These phenotypic changes were comparable with those elicited by CD40L or LPS, so HSP70359–610 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