# Hsp70-Like Protein 1 Fusion Protein Enhances Induction of Carcinoembryonic Antigen–Specific CD8$^+$ CTL Response by Dendritic Cell Vaccine

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## Abstract

**Heat shock proteins (HSP) have been revealed to interact with antigen-presenting cells and have potent adjuvant capability to induce antigen-specific CD8$^+$ CTL and Th1 responses. Our previous work shows how Hsp70-like protein 1 (Hsp70L1), as a new member of the Hsp70 subfamily, acts as potent Th1 adjuvant. Here, we report the efficient induction of tumor antigen-specific immune response by dendritic cells pulsed with recombinant fusion protein of Hsp70L1 and CEA$_{576-669}$ fragment of the carcinoembryonic antigen (CEA) containing CAP-1 (a HLA-A2–restricted CTL epitope). Fusion protein CEA$_{576-669}$-Hsp70L1 can promote dendritic cell maturation and activate dendritic cells to produce cytokines, such as interleukin-12, interleukin-1β, and tumor necrosis factor-α, and chemokines, such as macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, and regulated on activation, normal T expressed and secreted, indicating the adjuvant ability of Hsp70L1 in the fusion protein. CEA-specific HLA-A2.1–restricted CD8$^+$ CTLs either from patients with CEA$^+$/HLA-A2.1$^+$ colon carcinoma or from splenocytes of immunized HLA-A2.1/K$^b$ transgenic mice can be generated more efficiently after stimulations or immunizations with dendritic cells pulsed by CEA$_{576-669}$-Hsp70L1 than with dendritic cells pulsed by CEA$_{576-669}$ alone, resulting in secreting more Th1 cytokine IFN-γ and killing target cells more potently in an antigen-specific and HLA-A2.1–restricted manner. Adoptive transfer of splenocytes from transgenic mice immunized with CEA$_{576-669}$-Hsp70L1–pulsed dendritic cells can inhibit tumor growth and prolong survival in nude mice bearing CEA$^+$/HLA-A2.1$^+$ human colon carcinoma more markedly. Therefore, Hsp70L1 has potent adjuvant effect in form of fusion protein, indicating that Hsp70L1 may be widely used as Th1 adjuvant to prepare antigenic fusion protein for the therapeutics of cancer or infectious diseases.** (Cancer Res 2005; 65(11): 4947-54)

## Introduction

Heat shock proteins (HSP) are reported to act as an effective adjuvant to enhance the induction of antigen peptide-specific cellular immunity (1, 2). HSP-peptide complexes can be taken quite efficiently in a receptor-mediated manner by antigen-presenting cells, such as dendritic cells (2–4). Meanwhile, the complexes or HSPs in the complexes interact with dendritic cells and stimulate dendritic cells to express MHC class II molecules, to secrete cytokines and chemokines, resulting in maturation of dendritic cells with migration to draining lymph nodes where they present antigen to T cells and initiate T-cell responses (1–7). Therefore, vaccination with HSP preparations based on their adjuvant effects has attracted more attention in the immunotherapy of cancer or infectious diseases. HSP-peptide complexes isolated from intact tumor cells or virus-infected cells or reconstituted by covalent cross-link or fusion-protein strategies are all capable of eliciting potent CD8$^+$ CTL responses to the antigenic peptides bound to the HSP (1, 2, 8–11). Vaccination approaches have even been evaluated in phase III clinical trials for the treatment of human papillomavirus–related carcinoma (12).

Hsp70-like protein 1 (Hsp70L1) is structurally and functionally similar to Hsp70 and has the ability to promote dendritic cell maturation and activation as described previously by us (13). Interestingly, it may be potential as a novel adjuvant in peptide immunizations for the treatment of cancer and infectious diseases.

Carcinoembryonic antigen (CEA) is a membrane oncofetal glycoprotein with an approximate molecular weight of $M_r$ 180,000 expressed on most adenocarcinoma of the gastrointestinal tract, on 70% of non–small cell lung cancers, and on 50% of breast cancers. The high expression makes it an attractive tumor-associated antigen for immunotherapeutic purposes (14). CEA as a target antigen of tumor therapy has been extensively evaluated in murine models and human by means of vaccinating recombinant CEA proteins (15), recombinant viruses carrying the CEA gene (16), CEA anti-idiotype antibodies (17), dendritic cells transfected with CEA RNA (18), or dendritic cells pulsed with agonist epitopes of CEA (14, 19–21). Although CEA is a self-antigen with a tendency to be immunologically tolerant, a HLA-A2.1-restricted CTL epitope peptide of CEA, CAP-1 (CEA$_{605-613}$, YLSGANLNL), is a target for T cell lines derived from HLA-A2.1-positive patients with CEA-expressing malignancies (14, 21). Among vaccine approaches, dendritic cell–based active immunotherapy for CEA-positive human carcinomas is quite promising (18–21). To further improve therapeutic efficacy of dendritic cell–based vaccines, some adjuvants have been used in combinations (22, 23). Among these adjuvants, more attention has particularly been focused on HSPs that can potently stimulate a CTL and Th1 polarized response (1, 2, 8).

Here, we fused Hsp70L1 with CEA$_{576-669}$, one fragment of CEA containing the CAP-1 (a HLA-A2-restricted CTL epitope), to prepare a recombinant fusion protein CEA$_{576-669}$-Hsp70L1. We investigated whether this fusion protein could activate dendritic cells and evaluated whether CEA-specific antitumor response could be

**Note:** Y. Wu and T. Wan contributed equally to this work.

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induced efficiently both *in vitro* and *in vivo* by dendritic cells pulsed with this fusion protein. We showed that the efficiency of presentation of antigenic epitopes in $CEA_{576-669}$ was enhanced by its fusion with Hsp70L1 through unique adjuvant effects on dendritic cells and consequently, leading to more significant induction of epitope-specific $CD8^+$ CTLs that specifically recognized and killed CEA-expressing tumor cells. These observations, therefore, ascertain that Hsp70L1 is a potent adjuvant in vaccination for immunotherapy of cancer.

## Materials and Methods

**Animals and cell lines.** HLA-A2.1/$K^b$ transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 nude mice were obtained from the Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). All of the mice were 6- to 8-week-old females bred and maintained under specific pathogen-free conditions. T2 cell line (TAP-deficient, HLA-A2.1$^+$) and human colorectal adenocarcinoma cell lines LS-174T (CEA$^+$, HLA-A2.1$^+$), SW480 (CEA$^+$, HLA-A2.1$^+$), and LoVo (CEA$^+$, HLA-A2.1$^-$) were obtained from American Type Culture Collection (Manassas, VA) and cultured according to American Type Culture Collection instructions.

**Proteins and peptides.** The coding sequence of $CEA_{576-669}$ was amplified by PCR from a plasmid containing the CEA cDNA sequence cloned from LS-174T cell line using the forward primer 5′-ATTGAGCTCGTGAGTG-CAAACCGCAGTGACC-3′ and the reverse primer 5′-TATGTCGACGAC-TATGGAATTATTGCGGCC-3′. Then, the $CEA_{576-669}$ fragment was ligated to the $NH_2$ terminus of Hsp70L1 coding sequence by *Sal*I restriction site. Recombinant expression vectors for $CEA_{576-669}$-Hsp70L1 fusion protein and $CEA_{576-669}$ protein with a 6xHis-tag at the $NH_2$ terminus were obtained by inserting $CEA_{576-669}$-Hsp70L1 or $CEA_{576-669}$ coding sequence into pQE30 expression vector (Qiagen, Valencia, CA). These two recombinant proteins were expressed in *Escherichia coli* strain M15 (pREP4) and then purified using His-Trap metal chelation chromatography and DEAE chromatography (Amersham Biosciences, Uppsala, Sweden). Purified recombinant human Hsp70L1 protein (Hsp70L1) was obtained as described previously (13). The purity of recombinant $CEA_{576-669}$-Hsp70L1 fusion protein ($CEA_{576-669}$-Hsp70L1) and recombinant $CEA_{576-669}$ protein ($CEA_{576-669}$) were >95% as confirmed by silver-stained SDS-PAGE analysis. Lipopolysaccharide (LPS) contamination was <0.1 EU/μg protein as determined by the *Limulus amebocyte* lysate assay (BioWhittaker, Walkersville, MD).

CAP-1 and $Tyr_{368-376}$ (YMDGTMSQV, a HLA-A2.1-restricted CTL epitope derived from tyrosinase) peptides were synthesized at GL Biochem Ltd. (Shanghai, China) and analyzed to be >95% purity by reverse-phase high-performance liquid chromatography as confirmed by mass spectrometry.

**Generation of human dendritic cells.** Peripheral blood mononuclear cells were isolated from patients with CEA-expressing colon carcinoma by Ficoll-Hypaque (Sigma, St. Louis, MO) density gradient centrifugation. Human peripheral blood monocyte-derived dendritic cells were generated as described previously by us (24).

**Assays for cytokine and chemokine production.** Human dendritic cells were cultured for 5 days and adjusted to $5 \times 10^5$ cells/mL in 24-well plates; then, 10 μg/mL $CEA_{576-669}$-Hsp70L1, $CEA_{576-669}$, Hsp70L1, 1 μg/mL LPS (Sigma), or PBS were added. Supernatants from designated wells were harvested after 48 hours for quantification of cytokines, such as interleukin (IL)-12p40, IL-1β, and tumor necrosis factor-α (TNF-α), and chemokines, such as macrophage inflammatory protein (MIP)-1α, MIP-1β, or regulated on activation, normal T expressed and secreted (RANTES), using ELISA kits (R&D Systems, Minneapolis, MN). Polymyxin B (50 μg/mL, a LPS inhibitor; Sigma) was used to exclude the possibility of LPS contamination in $CEA_{576-669}$-Hsp70L1 fusion protein.

**Flow cytometry analysis and mixed lymphocyte reaction.** Cultured for 5 days, human dendritic cells were stimulated with 10 μg/mL $CEA_{576-669}$-Hsp70L1, $CEA_{576-669}$, Hsp70L1, or 1 μg/mL LPS for 48 hours and then collected, washed with PBS, and stained with phycoerythrin (PE)–conjugated anti-CD80, anti-CD83, anti-CD86, anti-CD40, or anti-HLA-DR monoclonal antibody (mAb; PharMingen, San Diego, CA) for analysis by a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and CellQuest software (Becton Dickinson). As stimulator cells for mixed lymphocyte reaction (MLR) assessment, irradiated dendritic cells [30 Gy (3,000 rad)] were incubated with T lymphocytes, used as responder cells enriched from blood of another donor using anti-CD3 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), at a series of responder/stimulator ratios in 96-well round-bottomed tissue culture plates. On day 4, 1 μCi (0.037 MBq) [$^3$H]thymidine was added to each well; then, cells were harvested 18 hours later and the proliferation was evaluated based on incorporated [$^3$H]thymidine tested by liquid scintillation spectroscopy.

**Generation of human CTLs and purification of $CD8^+$ T cells.** Cultured for 5 days, dendritic cells were harvested, stimulated with 10 μg/mL $CEA_{576-669}$-Hsp70L1, $CEA_{576-669}$, Hsp70L1, or $CEA_{576-669}$ mixed with Hsp70L1 for 4 hours, and then washed twice in serum-free RPMI 1640. Peripheral blood lymphocytes (PBL; $2 \times 10^6$) and protein-pulsed autologous dendritic cells ($2 \times 10^5$) were cocultured in 1 mL RPMI 1640 supplemented 10% FCS in 24-well plates. The cells were restimulated with fresh protein-pulsed autologous dendritic cells every 7 days for two to three times. On day 3 after second stimulation, recombinant human IL-2 (20 IU/mL, Sigma) was supplemented. Media were changed every 3 days with half-fresh medium in the presence of recombinant human IL-2 (20 IU/mL) and expanded as necessary. On day 7 after the last stimulation, cells were harvested and tested by cytotoxicity assay, ELISPOT assay, and tetramer staining. For ELISPOT assay and cytotoxicity assays, $CD8^+$ T lymphocytes were enriched by positive selection using immunobeads (Miltenyi Biotec) following the procedure recommended by the manufacturer.

**Vaccination of HLA-A2.1/$K^b$ transgenic mice.** HLA-A2.1 transgenic mice are a well-established model for studying HLA-A2.1-restricted CTL epitopes and vaccine development (25). In this study, mouse bone marrow–derived dendritic cells were generated from HLA-A2.1/$K^b$ transgenic mice as described previously by us (26). On day 5, bone marrow–derived dendritic cells were harvested and pulsed with 10 μg/mL $CEA_{576-669}$-Hsp70L1, Hsp70L1, or $CEA_{576-669}$ at a cell concentration of $2 \times 10^6$/mL for 4 hours and then washed. HLA-A2.1/$K^b$ transgenic mice were s.c. immunized thrice at 1-week interval with $1 \times 10^6$ protein-pulsed bone marrow–derived dendritic cells per mouse. A control group was set simultaneously to receive PBS-treated bone marrow–derived dendritic cells.

**Generation of CTLs in HLA-A2.1/$K^b$ transgenic mice.** Seven days after the last immunization as described above, splenocytes from mice were adjusted to $2 \times 10^7$ cells per well in six-well plates and stimulated with 10 μg/mL $CEA_{576-669}$ for 7 days *in vitro*. Then, cells were harvested and tested by cytotoxicity and tetramer staining assays.

**ELISPOT assay for IFN-γ production.** For human IFN-γ production assay by ELISPOT kit (R&D Systems), SW480, LoVo, and peptide-pulsed or unpulsed T2 cells were used as stimulator cells and purified $CD8^+$ T cells as effector cells. Stimulator cells ($5 \times 10^5$) and effector cells ($5 \times 10^5$) were seeded into 96-well polyvinylidene difluoride–backed microplates coated with anti-human IFN-γ mAb. After incubation at 37°C for 24 hours, cells were removed and the plates were processed following the manufacturer's procedures. For mouse IFN-γ production, the splenocytes from each group of immunized HLA-A2.1/$K^b$ transgenic mice were isolated and cultured in six-well plates at a density of $2 \times 10^7$ cells per well in the presence of stimuli, including 10 μg/mL $CEA_{576-669}$, CAP-1, $Tyr_{368-376}$, 5 μg/mL concanavalin A (Sigma), or PBS, for 72 hours and then transferred to polyvinylidene difluoride–backed microplates coated with anti-mouse IFN-γ mAb. Resulting spots were counted with a stereomicroscope (Carl Zeiss, Thornwood, NY) under magnifications of ×20 to ×40. Only black spots with fuzzy borders around were scored as spot-forming cells (SFC).

**Tetramer staining.** CAP-1-MHC tetramer-PE (ProImmune, Oxford, United Kingdom) was used for the flow cytometric analysis of CAP-1-specific T cells according to the manufacturer's protocol. Briefly, $1 \times 10^6$ cells were incubated for 30 minutes at 37°C with 2 μL PE-labeled CAP-1-MHC tetramer; then, FITC-labeled anti-human/anti-mouse CD8 mAb (PharMingen) was added at the recommended concentrations for an additional 30 minutes at 4°C. After washing with PBS, cells were fixed with 0.5% paraformaldehyde and analyzed by flow cytometry (27).

**Cytotoxicity assays.** Cytotoxicity assays were done using a standard 4-hour $^{51}$Cr-release assay. T2 cells were pulsed with 10 μg/mL CAP-1 or $Tyr_{368-376}$ peptide at 37°C for 1 hour. Peptide-pulsed or unpulsed T2 cells and $CEA^+$ tumor cell lines, as target cells, were labeled with $^{51}$Cr sodium chromate (100 μCi/$10^6$ cells) for 90 minutes at 37°C and then washed and mixed with effectors at series ratios in 96-well round-bottomed plates. After incubation at 37°C for 4 hours, the supernatant (100 μL) was collected from each well and the radioactivity was counted with a gamma counter. Percent specific lysis was calculated by the following formula: Percent specific lysis = [(Mean experimental release − Mean spontaneous release) / (Mean maximum release − Mean spontaneous release)] × 100%. Spontaneous and maximum releases were determined by incubating the labeled target cells with medium alone or 1% Triton X-100, respectively. Spontaneous release was always <15% of maximum release. The SD of triplicate wells was <15%.

**Adoptive transfer of splenocytes from immunized HLA-A2.1/$K^b$ transgenic mice to C57BL/$6^{nu/nu}$ mice bearing human colorectal carcinoma.** Splenocytes from each group of immunized HLA-A2.1/$K^b$ transgenic mice were stimulated with 10 μg/mL $CEA_{576-669}$ for 7 days as described in the cytotoxicity assays. C57BL/$6^{nu/nu}$ mice were inoculated s.c. with 5 × $10^6$ SW480 tumor cells in the left flank area and 3 days later injected i.v. with 1 × $10^8$ per mouse stimulated splenocytes derived from immunized HLA-A2.1/$K^b$ transgenic mice. This adoptive transfer was done twice at 1-week interval followed by i.p. injection of 2,000 IU/mouse IL-2 every 2 days. Control mice received splenocytes from HLA-A2.1/$K^b$ transgenic mice immunized with unpulsed dendritic cells or were administrated with IL-2 only.

**Statistical analysis.** The differences in the growth of SW480 tumors that were reflected in the tumor diameters within each group were compared using the Mann-Whitney *U* test. To compare the mouse survival between treatment and control groups, the statistical analysis was done using Kaplan-Meier test. All other statistical analyses were based on Student's *t* test.

## Results

**Maturation induction and activation of dendritic cells by recombinant $CEA_{576-669}$-Hsp70L1 fusion protein.** $CEA_{576-669}$-Hsp70L1, Hsp70L1, and LPS all could promote up-regulation of CD80, CD83, CD86, CD40, and HLA-DR expression on dendritic cells, but $CEA_{576-669}$ could not (Fig. 1*A*). We observed an increase in IL-12p40, TNF-α, and IL-1β secretions by $CEA_{576-669}$-Hsp70L1-stimulated or Hsp70L1-stimulated dendritic cells but not $CEA_{576-669}$-stimulated dendritic cells ($P < 0.05$; Fig. 1*B*). Polymyxin B, a LPS inhibitor, did not affect the cytokine secretion induced by $CEA_{576-669}$-Hsp70L1 but blocked the LPS-induced cytokine secretion, thus excluding the possibility of endotoxin contamination in $CEA_{576-669}$-Hsp70L1. The observed cytokine profile suggested that dendritic cells stimulated with $CEA_{576-669}$-Hsp70L1 could secrete high-level Th1-type cytokines, such as IL-12. After stimulation with $CEA_{576-669}$-Hsp70L1, human dendritic cells secreted more MIP-1α, MIP-1β, and RANTES. As shown in Fig. 1*B*, $CEA_{576-669}$-Hsp70L1 and Hsp70L1 could induce dendritic cells to secrete MIP-1α, MIP-1β, or RANTES more significantly, whereas the control protein $CEA_{576-669}$ could not ($P < 0.01$). Additionally, the differences between levels of MIP-1α, MIP-1β, or RANTES production of dendritic cells induced by $CEA_{576-669}$-Hsp70L1 or Hsp70L1 were not remarkable. In the presence of 50 μg/mL polymyxin B, $CEA_{576-669}$-Hsp70L1 almost elicited the same level of chemokine secretions as that without polymyxin B, indicating that it was $CEA_{576-669}$-Hsp70L1, not LPS contamination, which induces chemokine production.

In MLR, dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 or Hsp70L1, like dendritic cells stimulated with LPS, could induce T-cell proliferation more significantly ($P < 0.05$ compared with

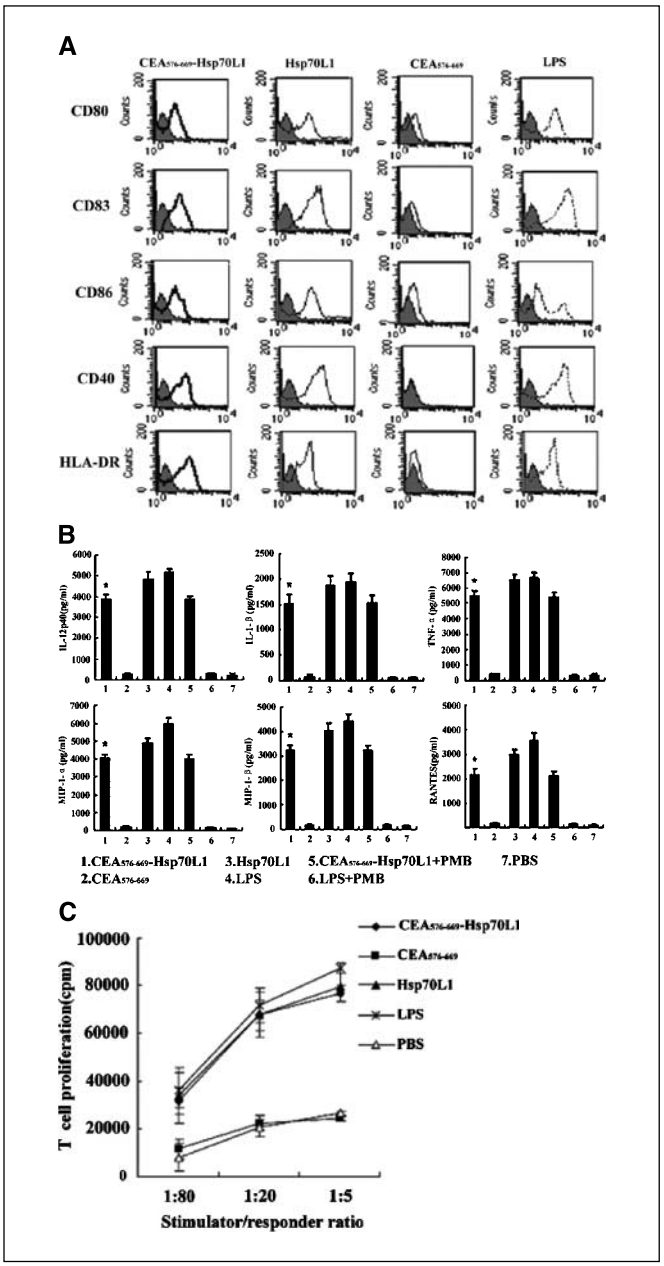


**Figure 1.** Induction of phenotypic and functional maturation and activation of dendritic cells by recombinant $CEA_{576-669}$-Hsp70L1 fusion protein. *A,* phenotypic maturation of dendritic cells induced by $CEA_{576-669}$-Hsp70L1. Dendritic cells were treated with 10 μg/mL $CEA_{576-669}$-Hsp70L1, $CEA_{576-669}$, Hsp70L1, and 1 μg/mL LPS for 48 hours and then collected for fluorescence-activated cell sorting analysis of CD80, CD83, CD86, CD40, and HLA-DR expression. *Gray histograms,* untreated dendritic cells; *white histograms,* dendritic cells stimulated with $CEA_{576-669}$-Hsp70L1, Hsp70L1, $CEA_{576-669}$, or LPS. *B,* cytokine and chemokine productions of dendritic cells stimulated with $CEA_{576-669}$-Hsp70L1. Dendritic cells cultured for 5 days were stimulated with 10 μg/mL $CEA_{576-669}$-Hsp70L1, $CEA_{576-669}$, Hsp70L1, 1 μg/mL LPS, $CEA_{576-669}$-Hsp70L1 + polymyxin B, and LPS + polymyxin B, respectively, for 48 hours. The levels of IL-12p40, TNF-α, IL-1β and MIP-1α, MIP-1β, and RANTES in supernatants were measured by ELISA. *Columns,* mean cytokine or chemokine concentration (pg/mL); *bars,* SE. *, $P < 0.05$ for cytokines production between $CEA_{576-669}$-Hsp70L1 and $CEA_{576-669}$; *, $P < 0.01$ for chemokines between $CEA_{576-669}$-Hsp70L1 and $CEA_{576-669}$. *C,* functional dendritic cell maturation as assessed by MLR. Dendritic cells pretreated with the indicated stimuli, including $CEA_{576-669}$-Hsp70L1, $CEA_{576-669}$, Hsp70L1, LPS, or PBS, were irradiated and used as stimulators, with T cells from another donor as responders. T-cell proliferation was measured by [$^3$H]thymidine incorporation. Experiments were repeated thrice. *Points,* mean; *bars,* SE.

$CEA_{576-669}$-stimulated or PBS-treated dendritic cells; Fig. 1*C*). The results indicated that $CEA_{576-669}$-Hsp70L1, like Hsp70L1, could efficiently induce phenotypic and functional maturation of dendritic cells and activate dendritic cells to secret more Th1 cytokines and chemokines that could recruit many kinds of immune cells to be in favor of immune response induction. Therefore, like Hsp70L1, Hsp70L1 in the form of $CEA_{576-669}$-Hsp70L1 fusion protein can act as Th1 adjuvant.

**More efficient induction of epitope-specific $CD8^+$ CTLs by human dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 fusion protein.** We incubated PBLs from $CEA^+$/HLA-A2.1$^+$ colon carcinoma patients and autologous dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 or other control proteins to determine whether CEA-specific CTLs could be elicited more significantly by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1. As shown in Fig. 2*A*, CEA-specific $CD8^+$ CTLs induced by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 were generated from all $CEA^+$/HLA-A2.1$^+$ colon carcinoma patients in this study and exhibited a CEA-specific killing against SW480 cells ($CEA^+$, HLA-A2.1$^+$) and T2 cells pulsed with CAP-1 but failed to lyse LoVo cells ($CEA^+$, HLA-A2.1$^-$; $P < 0.05$). Lysis of T2 cells pulsed with an irrelevant MHC-I-binding peptide $Tyr_{368-376}$ or unpulsed T2 cells were not observed. The above results indicated that these CTLs were CEA specific and HLA-A2.1 restrictive. Furthermore, the cytotoxicity of CTLs induced by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 against target cells was more potent than that of CTLs induced by dendritic cells pulsed with $CEA_{576-669}$ alone or by dendritic cells pulsed with the simple mixture of $CEA_{576-669}$ and Hsp70L1 ($P < 0.05$). In comparison, no specific lysis was observed for T cells cocultured with dendritic cells pulsed with Hsp70L1. Thus, dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 could induce CEA-specific CTLs more significantly than dendritic cells pulsed with $CEA_{576-669}$, further confirming the adjuvant activity of Hsp70L1 in the fusion protein. Hsp70L1, as the immunostimulatory component in the fusion protein, could not be separated from the antigenic peptide-binding component.

In ELISPOT assay, the target cells in cytotoxicity assay were used as stimulators. As shown in Fig. 2*B*, $CD8^+$ T cells induced by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 elicited significant IFN-γ production once received *in vitro* stimulation of SW480 or CAP-1-pulsed T2 cells, and less IFN-γ was produced by those $CD8^+$ T cells induced by dendritic cells pulsed with $CEA_{576-669}$ ($P < 0.05$). Few IFN-γ spots were detected on stimulation of LoVo or irrelevant $Tyr_{368-376}$ peptide-pulsed T2 cells in all groups. T cells cultured with autologous dendritic cells only pulsed with Hsp70L1 did not show significant IFN-γ production on stimulation of any cell lines we used. IFN-γ production of T cells induced by dendritic cells pulsed with the mixture of $CEA_{576-669}$ and Hsp70L1 seemed to have no difference from those induced by dendritic cells pulsed with $CEA_{576-669}$ (data not shown). Together with the above data, the results further showed that with the powerful adjuvant effects of Hsp70L1, dendritic cells could be induced more matured and activated by pulsing with $CEA_{576-669}$-Hsp70L1 and then could present antigen bound to Hsp70L1 more efficiently to T cells and trigger CEA-specific $CD8^+$ T cell response more potently.

**More efficient induction of CEA-specific CTLs in HLA-A2.1/$K^b$ transgenic mice immunized by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 fusion protein.** To assess whether dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 were capable of inducing CEA-specific $CD8^+$ T cell immunity *in vivo*, we immunized HLA-A2.1/$K^b$ transgenic mice with syngeneic dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 or other control proteins and used PBS-treated dendritic cells as the blank control. After three rounds of immunization *in vivo*, splenocytes were isolated, stimulated, and assayed for IFN-γ production and CTL cytotoxicity. As shown in Fig. 3*A*, splenocytes from mice immunized with dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 and $CEA_{576-669}$ were both able to lyse SW480 and CAP-1-pulsed T2 cells but were unable to lyse LoVo, $Tyr_{368-376}$-pulsed, or unpulsed T2 cells, whereas effector cells

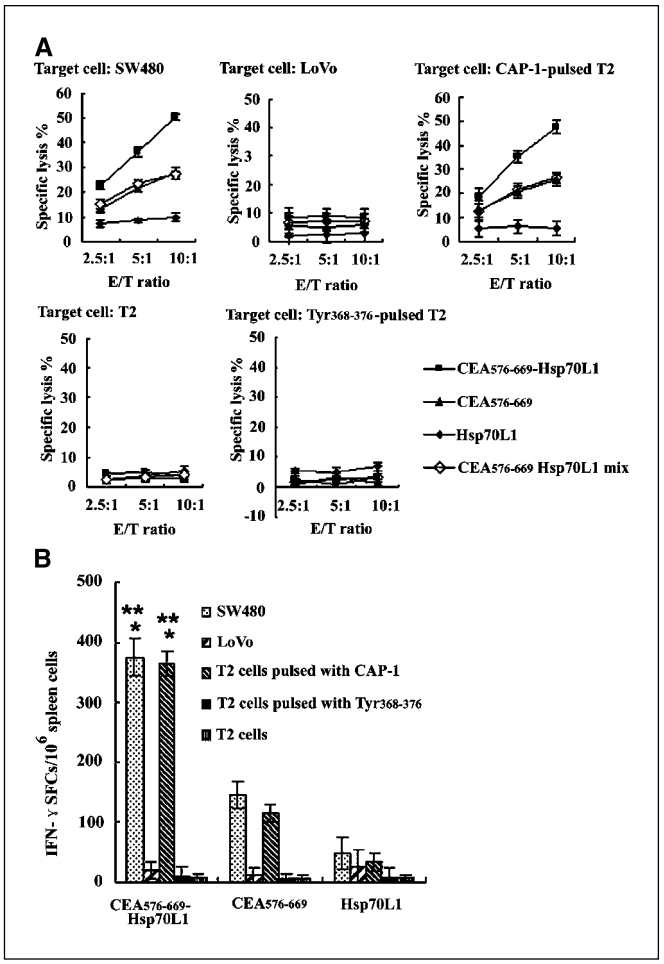


**Figure 2.** Induction of human CEA-specific CTL responses *in vitro* by $CEA_{576-669}$-Hsp70L1-pulsed dendritic cells. PBLs from $CEA^+$/HLA-A2.1$^+$ colon carcinoma patients were stimulated with autologous dendritic cells prepulsed with $CEA_{576-669}$-Hsp70L1, $CEA_{576-669}$, Hsp70L1, or the mixture of $CEA_{576-669}$ and Hsp70L1 to assess CEA-specific immune responses by CTL assay (*A*) and IFN-γ ELISPOT (*B*). *A,* assessment of HLA-A2.1-restricted CEA-specific CTL cytotoxic activity. The experiment was a standard $^{51}Cr$-release assay. $CD8^+$ CTLs purified from the PBLs stimulated three rounds by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 or control proteins were used as effector cells (*E*), SW480 cells ($CEA^+$, HLA-A2.1$^+$), and T2 cells pulsed with CAP-1 as target cells (*T*). Control targets included LoVo cells ($CEA^+$, HLA-A2.1$^-$) and irrelevant $Tyr_{368-376}$ peptide-pulsed or unpulsed T2 cells. Various E/T ratios were tested as indicated. *Points,* mean of three independent experiments; *bars,* SE. *B,* for human IFN-γ ELISPOT assay, SW480, LoVo, peptide-pulsed, and unpulsed T2 cells were used as stimulator cells and incubated for 24 hours with purified $CD8^+$ T cells induced by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 or other control proteins as effector cells. ELISPOT results are indicated by the number of IFN-γ-positive SFCs/$10^6$ $CD8^+$ T cells. *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.05$, for the number of IFN-γ-positive SFCs/$10^6$ $CD8^+$ T cells induced by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 or $CEA_{576-669}$ with the same stimulator cells; **, $P < 0.05$, for the number of IFN-γ-positive SFCs/$10^6$ $CD8^+$ T cells compared between SW480 and LoVo stimulator cells and between CAP-1-pulsed and unpulsed, irrelevant $Tyr_{368-376}$ peptide-pulsed T2 stimulator cells.

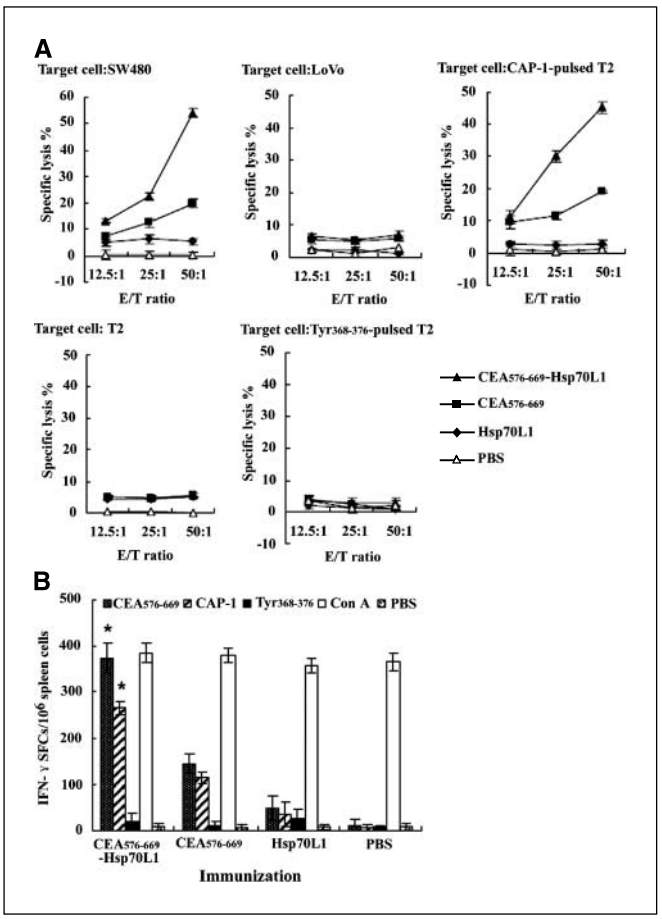


**Figure 3.** Immunization of HLA-A2.1/$K^b$ transgenic mice with $CEA_{576-669}$-Hsp70L1-pulsed dendritic cells induces CEA-specific Th1 responses and CTLs *in vivo*. Splenocytes from immunized HLA-A2.1/$K^b$ transgenic mice were used to assess CEA-specific immune responses by CTL assay and IFN-γ ELISPOT. *A,* CTL assay. The splenocytes were stimulated with $CEA_{576-669}$ and then used as effector cells, SW480 ($CEA^+$, HLA-A2.1$^+$) cells and CAP-1 peptide pulsed T2 cells were used as CAP-1-specific and HLA-A2.1-restricted targets, and LoVo, T2, or $Tyr_{368-376}$ peptide-pulsed T2 cells as control targets. Various E/T ratios were tested as indicated. *Points,* mean of three independent experiments; *bars,* SE. *B,* IFN-γ ELISPOT assay. To induce CAP-1 peptide-specific IFN-γ-producing cells, the splenocytes were restimulated with $CEA_{576-669}$, CAP-1, unrelated control peptide $Tyr_{368-376}$, concanavalin A, and PBS. ELISPOT results are indicated by the number of IFN-γ-positive-SFCs/$10^6$ splenocytes. *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.05$, for the number of IFN-γ-SFCs/$10^6$ splenocytes from HLA-A2.1 transgenic mice immunized with dendritic cells pulsed with $CEA_{576-669}$ under the same stimulus.

from mice immunized with Hsp70L1-pulsed dendritic cells or PBS-treated dendritic cells showed almost no cytotoxicity to target cells. In addition, as the same procedure was done to induce CTLs, more potent antigen-specific lysis of SW480 and CAP-1-pulsed T2 cells were observed for splenocytes from mice immunized with dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 than for those from mice immunized with dendritic cells pulsed with $CEA_{576-669}$ ($P < 0.05$). Results of ELISPOT assay in Fig. 3*B* showed that once restimulated *in vitro* with same stimuli, including $CEA_{576-669}$ or CAP-1, IFN-γ production of splenocytes from mice immunized by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 was more significant than that of splenocytes from mice immunized by dendritic cells pulsed with $CEA_{576-669}$ alone ($P < 0.05$). Splenocytes from mice immunized by dendritic cells pulsed with Hsp70L1-treated or PBS-treated dendritic cells did not show significant IFN-γ production on any stimuli. Few IFN-γ spots were detected on stimulation with irrelevant $Tyr_{368-376}$ peptide in all groups. Overall, the results indicated that immunization with dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 could induce CEA-specific CTLs more efficiently than immunization with dendritic cells pulsed with $CEA_{576-669}$ *in vivo* and that dendritic cells pulsed with only Hsp70L1 without CEA information could not induce CEA-specific CTLs.

**Increased frequencies of CAP-1–specific $CD8^+$ T cells induced by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 fusion protein.** To ulteriorly visualize and enumerate CAP-1-specific $CD8^+$ T cells from among lymphocytes induced either *in vitro* from $CEA^+$/HLA-A2.1$^+$ colon carcinoma patients' PBLs or *in vivo* in HLA-A2.1/$K^b$ transgenic mice, we used PE-labeled HLA-A2.1-CAP-1 tetramer, which was designed to specifically bind to CAP-1-reactive T-cell clones. Irrelevant PE-labeled HLA-A2.1 tetramers were prepared as a negative control containing the SSp-1 peptide derived from SARS-associated coronavirus as described previously (27). Cells were simultaneously labeled with FITC-labeled anti-CD8 mAb to distinguish $CD8^+$ T cells. As shown in Fig. 4*A*, the percentage of CAP-1-specific $CD8^+$ T cells induced from $CEA^+$/HLA-A2.1$^+$ colon carcinoma patients' PBLs stimulated by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 was 11.04%, significantly higher than that of T cells stimulated by dendritic cells pulsed with $CEA_{576-669}$ (3.84%) or Hsp70L1 (0.36%). No remarkable difference was observed in the percentage of CAP-1-specific $CD8^+$ T cells induced by $CEA_{576-669}$-pulsed dendritic cells and by the mixture of $CEA_{576-669}$-pulsed and Hsp70L1-pulsed dendritic cells (data not shown). Results in Fig. 4*B* showed that CAP-1-specific $CD8^+$ T cells from HLA-A2.1/$K^b$ transgenic mice immunized with dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 made up 9.98% of the stained splenocytes, higher than those from mice immunized with dendritic cells pulsed with $CEA_{576-669}$ (3.02%) or Hsp70L1 (0.09%). Tetramer staining was specific for the containing peptides; therefore, HLA-A2.1 tetramers bearing irrelevant HLA-A2.1-binding peptide, such as SSp-1, failed to stain specific $CD8^+$ T cells in the experiments. Virtually no specific tetramer staining could be detected in splenocytes from PBS-treated dendritic cells immunized HLA-A2.1/$K^b$ transgenic mice (data not shown). These results powerfully confirmed that induction of $CD8^+$ T cells by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 is stronger than that by dendritic cells pulsed with $CEA_{576-669}$, further demonstrating the potent adjuvant effect of Hsp70L1 and the increased immunogenicity of $CEA_{576-669}$ in the $CEA_{576-669}$-Hsp70L1 fusion protein.

**More potent antitumor effect of adoptive transfer of splenocytes from HLA-A2.1/$K^b$ transgenic mice immunized with dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 fusion protein.** C57BL/$6^{nu/nu}$ mice were inoculated with SW480, a human $CEA^+$/HLA-A2.1$^+$ colon carcinoma cell line, and 3 days later were injected i.v. with *in vitro* $CEA_{576-669}$-stimulated splenocytes derived from immunized HLA-A2.1/$K^b$ transgenic mice followed by i.p. injection of 2,000 IU/mouse IL-2 every 2 days. As shown in Fig. 5*A*, adoptive transfer of splenocytes from HLA-A2.1/$K^b$ transgenic mice immunized with dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 was able to significantly inhibit SW480 growth in nude mice; however, no marked tumor growth inhibition or survival improvement was observed in other groups. All the control mice developed palpable tumor 7 days after tumor inoculation, whereas no tumors were observed in nude mice injected with splenocytes from mice immunized by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 until 14 days. In this group, three of the eight animals

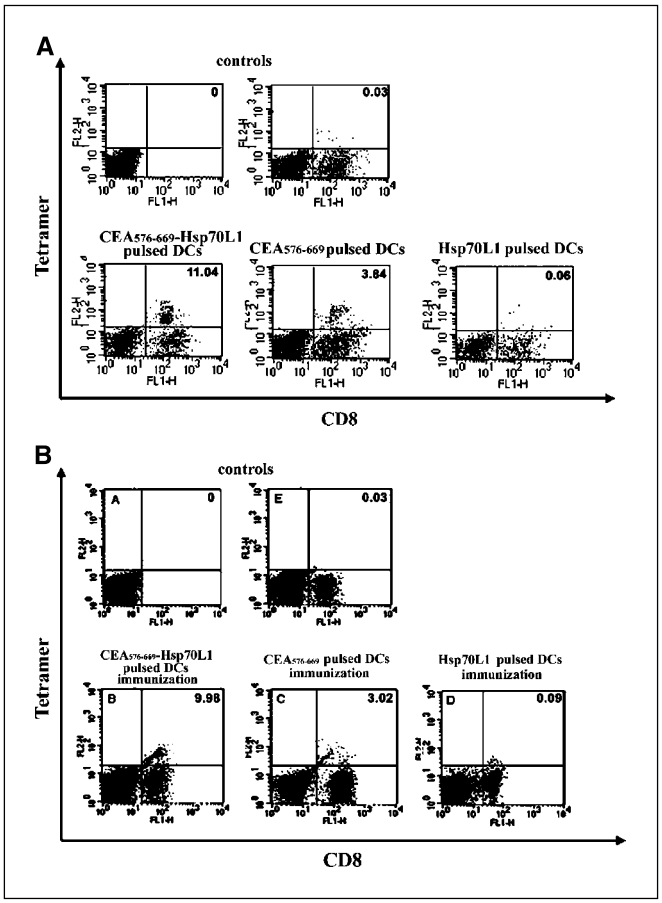


**Figure 4.** Frequency of CAP-1-specific CD8$^+$ T cells determined by HLA-A2.1-CAP-1 tetramer. *A,* CAP-1-specific CD8$^+$ T cell frequency among dendritic cell–induced human CTLs. T cells generated from PBLs of CEA$^+$/HLA-A2.1$^+$ colon carcinoma patients incubated with autologous dendritic cells prepulsed with $CEA_{576-669}$-Hsp70L1, $CEA_{576-669}$, and Hsp70L1 were stained with HLA-A2.1-CAP-1 tetramer to detect the presence of CAP-1-specific CTLs, and stained with HLA-A2.1-SSp-1 tetramer for control. *B,* CAP-1-specific CD8$^+$ T cell frequency among splenocytes from dendritic cell vaccine immunized HLA-A2.1/K$^b$ transgenic mice. Splenocytes from HLA-A2.1/K$^b$ transgenic mice immunized with $CEA_{576-669}$-Hsp70L1-pulsed dendritic cells, $CEA_{576-669}$-pulsed dendritic cells, or Hsp70L1-pulsed dendritic cells were stimulated with $CEA_{576-669}$ for 7 days *in vitro* and then stained with HLA-A2.1-CAP-1 tetramer and control HLA-A2.1-SSp-1 tetramer. *A* and *B,* tetramer-binding CD8$^+$ T cells are in the *top right quadrant*, which are labeled with the percentage of total CD8$^+$ T cells. Controls are in *top row*, the *top left* for blank and the *top right* for HLA-A2.1-SSp-1 tetramer staining. Representative of three independent experiments.

were tumor free ever since, and 37.5% of the mice survived for longer than 90 days after SW480 tumor inoculation. In contrast, all mice in control groups died between days 27 and 44 after tumor inoculation (Fig. 5*B*). A protective capacity of splenocytes derived from HLA-A2.1/K$^b$ transgenic mice immunized with $CEA_{576-669}$-Hsp70L1-pulsed dendritic cells also occurred on adoptive transfer in the absence of IL-2 administration; however, tumors were not completely rejected (data not shown). The group receiving low dose of IL-2 alone showed no significant difference in tumor growth from the control group receiving splenocytes from HLA-A2.1/K$^b$ transgenic mice immunized with PBS-treated dendritic cells. We failed to observe any sign of tumor growth inhibition or improvement in survival in tumor-bearing nude mice receiving *in vitro* restimulated splenocytes from HLA-A2.1/K$^b$ transgenic mice immunized with Hsp70L1-pulsed dendritic cells. The data above showed that adoptive transfer of splenocytes from HLA-A2.1/K$^b$ transgenic mice immunized by dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 could result in more potent protective immune response against CEA$^+$/HLA-A2.1$^+$ tumor cells than that of splenocytes from HLA-A2.1/K$^b$ transgenic mice immunized by $CEA_{576-669}$-pulsed or Hsp70L1-pulsed dendritic cells, intensively suggesting that pulsing of dendritic cell with $CEA_{576-669}$-Hsp70L1 was an effective immunotherapeutic approach for colon cancer expressing CEA and HLA-A2.1.

## Discussion

It has been well known that HSPs have adjuvant effect in peptide immunization to initiate specific cellular immune responses against associated antigens. To facilitate the presentation of antigenic peptides, the antigen or peptide must be associated with HSPs in the form of covalent HSP-peptide hybrid, fusion protein, or naturally binding in cells (1, 2, 8–11). Among

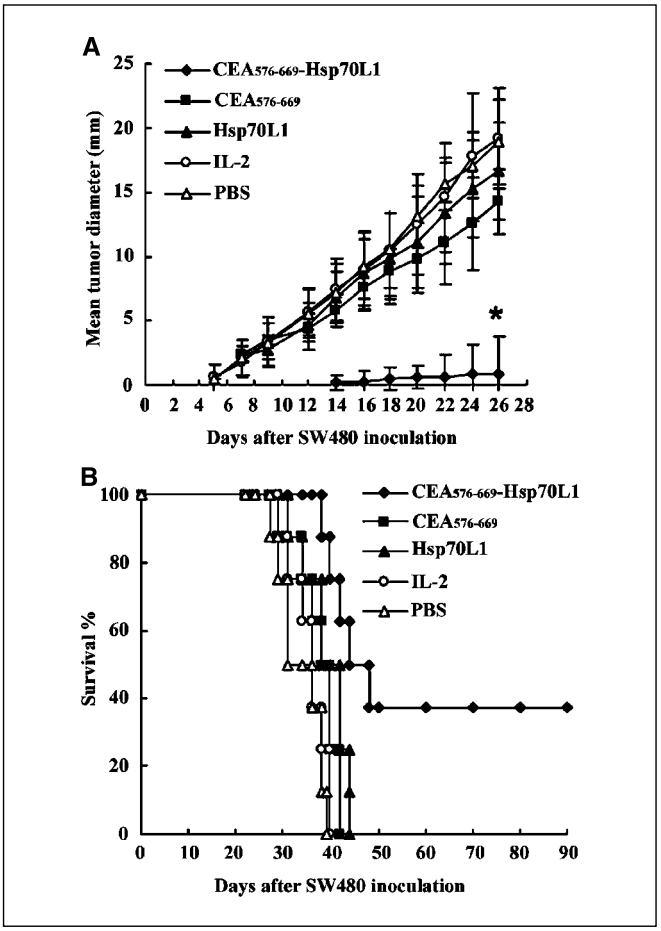


**Figure 5.** Adoptively transferred splenocytes from immunized HLA-A2.1/K$^b$ transgenic mice inhibit growth of tumor expressing CEA and HLA-A2.1. C57BL/6$^{nu/nu}$ mice were injected s.c. with $5 \times 10^6$ SW480 cells. Three days later, $1 \times 10^8$ per mouse $CEA_{576-669}$-restimulated splenocytes derived from each group of immunized HLA-A2.1/K$^b$ transgenic mice were transferred by i.v. injection. Additionally, mice received $2 \times 10^3$ IU IL-2 i.p. at a volume of 0.5 mL every 2 days. Control groups received IL-2 administration alone or no treatment. *A,* tumor growth curves. Following SW480 tumor inoculation, tumor growth was monitored by measuring the diameter of the tumor every 2 days and recorded as the average tumor diameter. *, $P < 0.05$, for the tumor diameter with other groups. *B,* survival of adoptively transferred mice after SW480 tumor inoculation. Each group contained eight mice.

these methods, fusion protein strategy, in some cases shown to be successful, is simple, feasible, and steady (8, 28–30). As a new HSP member similar to its homologue Hsp70, Hsp70L1 has been shown to have potent Th1 adjuvant effect; therefore, Hsp70L1 should be further used in fusion protein applications to explore its future clinical values.

In this study, compared with $CEA_{576-669}$-pulsed dendritic cell vaccine, the $CEA_{576-669}$-Hsp70L1-pulsed dendritic cell vaccine induced CEA-specific CTL responses and antitumor immunity more predominantly both *in vitro* and *in vivo*. CEA is a self-antigen with poor immunogenicity, which is one of the reasons for failure in some clinical trials (14). The validity of dendritic cell–based strategy to enhance the immunogenicity of CEA has been evidenced by the fact that CAP-1-pulsed dendritic cell vaccine induces CAP-1-specific CTLs in patients with $CEA^+$ tumor (21, 31). Eventually, Hsp70L1 may serve as a good adjuvant to further overcome the drawback of CEA as a weak antigen and should be able to increasingly harness the induced CTLs to kill $CEA^+$ tumor cells. Our observations in this work showed that Hsp70L1 fusion protein–based dendritic cell vaccine had been more effective than $CEA_{576-669}$-pulsed dendritic cell vaccine. CTL plays a key role in tumor regression. In all $CEA^+$/HLA-$A2.1^+$ colon carcinoma patients we tested, CTLs were generated by stimulation of dendritic cells pulsed with $CEA_{576-669}$-Hsp70L1 and exemplified by their ability to lyse SW480 cells and CAP-1-pulsed T2 cells. Moreover, this cytotoxicity was shown to be more potent than that of CTLs induced by dendritic cells pulsed with $CEA_{576-669}$ or dendritic cells pulsed with the simple mixture of $CEA_{576-669}$ and Hsp70L1. These results indicated that CAP-1, when associated with the particular adjuvant Hsp70L1, could be more efficiently processed and presented by dendritic cells. These findings were additionally confirmed by measuring IFN-γ secretion from T cells in contact with SW480 cells and by MHC-peptide tetramer staining. Our tetramer staining results showed that the percentage of CAP-1-specific $CD8^+$ T cells detected in HLA-$A2.1/K^b$ transgenic mice immunized with $CEA_{576-669}$-Hsp70L1-pulsed dendritic cells was 9.98%, which was significantly higher than control groups. Consequentially, the potent adjuvant effect of Hsp70L1 in cellular immunity enhancement was clearly shown with the above results.

We observed a remarkable increase of IL-12p40 secretion by dendritic cells stimulated with $CEA_{576-669}$-Hsp70L1 and Hsp70L1 but not by dendritic cells stimulated with $CEA_{576-669}$ ($P < 0.05$). IL-12 from dendritic cells is obligatory and dominant in directing the development of Th1 lymphocytes to secrete high amount of IFN-γ *in vitro* and *in vivo* (32, 33). The production of IL-12p40, synthesized only in certain cells, such as macrophages, granulocytes, and dendritic cells, directly leads to high levels of bioactive IL-12 secretion by dendritic cells when induced primarily by Th1-promoting innate stimulus (34–36) and engages p19 subunit to form a cytokine IL-23, which triggers IFN-γ production of both naive and memory human T cells (37). High quantity of IL-12p40 stimulated by $CEA_{576-669}$-Hsp70L1 and Hsp70L1 in our study may suggest the induction of both naive T lymphocytes and proliferation of memory T cells, which are increasingly important in the vaccine approach to evoke strong and continued antitumor immune response. $CEA_{576-669}$-Hsp70L1 also increased the production of IL-1β and TNF-α, autocrine or paracrine secretion of which could stimulate dendritic cell maturation and T-cell activation (32, 38). The maturation status of dendritic cells is critical to their ability to process and present antigens to T cells and to naive T cell activation (39, 40). In this study, we found that $CEA_{576-669}$-Hsp70L1 could enhance the expression of CD40, CD80, CD83, CD86, and HLA-DR on dendritic cells and these dendritic cells were able to elicit primary allogeneic T-cell proliferation more efficiently. Unlike some dendritic cell–based vaccine protocols in which dendritic cells should be stimulated to maturation by TNF-α or CD40 ligand (27, 31, 41), $CEA_{576-669}$-Hsp70L1 could act on immature dendritic cells to induce its phenotypic and functional maturation by the adjuvant effect of Hsp70L1 fragment. The secretion of chemokines by dendritic cells is a specifically induced phenomenon that regulates recruitment, maturation, migration, and activation of these potent antigen-presenting cells, and mature dendritic cell secrets large amounts of chemokines to attract immature dendritic cells, monocytes, T cells, and B cells, thereby increasing the efficiency of an immune response (42). MIP-1β tends to attract $CD4^+$ T lymphocytes, with some preference for T cells of the naive phenotype (43). MIP-1α is a more potent lymphocyte chemokine, compared with MIP-1β, with a broader range of chemoattractant specificities for migration of $CD4^+$ T cells or B cells, predominantly $CD8^+$ T cells (43–45). RANTES, belonging to the C-C groups of the chemokine superfamily, which also includes MIP-1α and MIP-1β, has more potent chemotaxis for its effect on resting human T cell with activity even at 1 ng/mL, whereas MIP-1α and MIP-1β have not (44, 46). In our study, MIP-1α, MIP-1β, and RANTES secreted from $CEA_{576-669}$-Hsp70L1-stimulated dendritic cells were expected to exhibit strong T cells chemoattractant effects, thus resulting in improving immune responses.

Recombinant HSP fusion proteins, in which specific peptides or proteins of interest are linked to the HSPs, would provide a well-characterized polypeptide, a large protein fragment to be an especially rich source of many different naturally processed peptides, particularly epitopes (28, 29). In another words, HSPs can serve as vehicles for antigen delivery into the MHC-I presentation pathway (29). Whereas single peptide–based approaches have the disadvantage of a certain HLA-restricted CTL response induced by only one epitope, polypeptides, however, like $CEA_{576-669}$, derived from specific antigens of interest, are suitable for forming more than one intracellular peptide-MHC complexes corresponding to multiple HLA alleles. Although we used $CEA_{576-669}$ as a model antigen to confirm the adjuvant effects of Hsp70L1 in the form of fusion protein in this study, it is reasonable to predict that Hsp70L1 can be fused with a variety of viral antigenic peptides or tumor-specific antigens, conferring special adjuvant properties to them. The application of Hsp70L1 in immunotherapeutic strategies, as a new and potent adjuvant of mammalian origin, may be greatly extended to a wider range of cancers as well as infectious diseases, such as hepatitis B virus, human papillomavirus, and HIV infection, to elicit and enhance immune responses.

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