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Targeted Immunotherapy Using Reconstituted Chaperone Complexes of Heat Shock Protein 110 and Melanoma-associated Antigen gp100[1]

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ABSTRACT

This report defines a novel approach to heat shock protein vaccine formulation that takes advantage of the chaperoning property of heat shock protein hsp110 to efficiently bind a large protein substrate (specifically, human melanoma-associated antigen gp100) during heat shock. We demonstrate that hsp110 can form chaperone complexes with gp100 and prevent heat-induced aggregation of gp100. The resultant natural hsp110-gp100 complexes are strongly immunogenic as determined by their ability to elicit an antigen-specific IFN-γ production and a cytotoxic T-cell response. Immunization with the hsp110-gp100 complex protected mice against subsequent challenge with human gp100-transduced B16 melanoma, which involves both $CD4^+$ and $CD8^+$ T-cell populations. Administration of the hsp110-gp100 vaccine also significantly suppressed the growth of established tumors in a therapeutic model. Furthermore, the hsp110-gp100 chaperone complex exhibited inhibitory effects on the progression of wild-type B16 tumor, suggesting that the induced immune response by human gp100 cross-reacts with mouse gp100. More importantly, the antitumor response obtained with the hsp110-gp100 complex is more potent than that obtained using Complete Freund's Adjuvant with gp100, whereas no response was observed against mouse hsp110 itself. Thus, the use of hsp110 to form natural chaperone complexes with tumor protein antigens such as gp100 represents a powerful approach to therapeutic vaccine formulation with significant potential for clinical application.

INTRODUCTION

Studies over many years have shown that HSPs[3] act as essential intracellular chaperones in numerous interrelated functions such as folding and translocation of newly synthesized polypeptides, assembly and disassembly of multiunit protein complexes, and refolding of misfolded proteins (1, 2). hsp110 is a major HSP of mammals and of eukaryotic cells in general. Unlike other major HSPs, which have been intensively studied for many years, hsp110 has only been cloned and studied within the last few years. The hsp110 family has been found to be distantly related to the hsp70 family in sequence (3, 4), and hsp110 has been observed to express functional similarities as well as differences compared with the hsp70 family. One important property of hsp110 is its ability to bind to and chaperone full-length proteins during heat shock with high efficiency (5, 6).

In the last several years, studies have revealed that certain HSPs also exhibit important extracellular properties in their interactions with the immune system. Increasing evidence indicates that some HSPs (*e.g.*, grp94/gp96, hsp90, hsp70, calreticulin, and hsp110) purified from a tumor elicit specific immunity against the same tumor (7–11). This immunogenicity is due to antigenic peptides that are associated with these HSPs within the tumor cell (12, 13), presumably as a result of a functional role for HSPs in peptide transport and processing (14–16). It has also been shown that hsp70 and grp94 (also called gp96) family proteins can bind to receptors on antigen-presenting cells, leading to HSP-peptide uptake (17, 18), secretion of proinflammatory cytokines (19, 20), and maturation of dendritic cells (21, 22). Thus, the adjuvant activity of some HSPs is multifold in that they induce both innate and adaptive immunity by stimulation of proinflammatory responses and cross-priming of T cells.

Melanoma-associated antigen gp100 is highly expressed in about 80% of HLA-A2-positive malignant melanomas (23, 24). It can be specifically recognized by CTLs as well as antibodies derived from melanoma patients (25, 26). The human *gp100* gene, which is about 75% identical to its mouse homologue, has been shown to induce protective immunity in mice after immunization using adenovirus-mediated gene transfer (27). The adoptive transfer of gp100-reactive tumor-infiltrating lymphocytes or gp100-derived peptide vaccines can elicit an antitumor response in some melanoma patients, implicating gp100 as a tumor rejection antigen (28, 29). Thus, gp100 is an attractive candidate for cancer vaccine development and was therefore chosen as the chaperone partner for hsp110 in the present study.

We have recently proposed a HSP-based vaccine strategy, which utilizes the chaperoning function of hsp110 to generate a natural noncovalent complex with a protein antigen *in vitro* (30). In this earlier study (30), we demonstrated that a heat-induced chaperone complex between hsp110 and the ICD of Her-2/neu induced antigen-specific immune responses when measured *in vitro*. However, this initial report did not examine critical issues such as *in vivo* antitumor activity of the chaperone vaccine and the necessity of using the *chaperone complex* itself (and not a mixture of its components) for the induction of protective immunity. To address these questions, we here characterize the molecular interaction between chaperone hsp110 and melanoma differentiation antigen gp100 during heat shock and fully define its *in vivo* antitumor efficacy and immunological activities when used as a therapeutic vaccine.

In the present study, we show that a natural hsp110-gp100 chaperone complex elicits gp100-specific immune responses, which lead to a potent antitumor immunity in both prophylactic and therapeutic settings. Importantly, the hsp110-gp100 vaccine is more efficient in inhibiting tumor growth than is gp100 used in combination with CFA. These observations provide strong evidence for the potential use of chaperone vaccines in clinical cancer treatment.

MATERIALS AND METHODS

Mice and Cell Lines. Female 8–12-week-old C57BL/6 mice purchased from Taconic (Germantown, NY) were housed under pathogen-free conditions. Human gp100-transduced B16 cells (B16-gp100) and parental B16 melanoma cells were kindly provided by Dr. Alexander Rakhmilevich (University of Wisconsin-Madison; Ref. 31). All cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc., Grand Island, NY), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Expression and Purification of Recombinant Proteins. Recombinant mouse hsp110 cDNA was subcloned into pBacPAK-his vector and expressed

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[3] The abbreviations used are: HSP, heat shock protein; ICD, intracellular domain; ELISPOT, enzyme-linked immunospot; CFA, Complete Freund's Adjuvant; WT, wild-type; OVA, ovalbumin.

using a BacPAK baculovirous expression system (BD Biosciences Clontech, Palo Alto, CA). Human gp100 cDNA provided by Dr. Nicholas Restifo (National Cancer Institute, Bethesda, MD) was subcloned into *Spe*I/*Xba*I sites of pRSETA vector (Invitrogen, Carlsbad, CA**)**. Plasmid was transformed into *Escherichia coli* JM109 (DE3) cells, and protein was purified using a nickel nitriloacetic acid-agarose (Qiagen, Valencia, CA) column. Protein purity was assessed using SDS-PAGE stained with Coomassie Blue. Protein concentration was determined using BCA protein assay kit (Pierce, Rockford, IL). Endotoxin levels in recombinant proteins (~50–70 endotoxin unit/mg protein) were measured using a Limulus Amebocyte lysate kit (Biowhittaker, Walkersville, MD).

Thermal Aggregation Experiment. gp100 (150 nM), alone or in the presence of a 1:1 molar ratio of OVA, hsp110, or hsp70, was equilibrated to room temperature in 25 mM HEPES (pH 7.4), 5 mM magnesium acetate, 50 mM KCl, and 5 mM β-mercaptoethanol followed by incubation at the indicated temperatures in a thermostated cuvette. Light scattering by protein aggregation was determined by measuring the increase of absorbance at 320 nm with a spectrophotometer. Samples were then transferred to microcentrifuge tubes and centrifuged for 15 min at 16,000 $\times$ *g* at 4°C. Supernatant and pellet were resolved into SDS-PAGE, followed by immunoblot with anti-gp100 antibody HMB45 (NeoMarkers, Fremont, CA; Ref. 32).

hsp110-Antigen Binding. hsp110 and gp100 were mixed in a 1:1 molar ratio and incubated for 30 min at the indicated temperatures in PBS. Samples were then incubated for 30 min at room temperature. The binding was evaluated by coimmunoprecipitation as described previously (30).

ELISPOT. The ELISPOT assay was used to determine antigen-specific IFN-γ-secreting T cells as described previously (30). Briefly, filtration plates (Millipore, Bedford, MA) were coated with 10 μg/ml rat antimouse IFN-γ (clone R4-6A2; PharMingen, San Diego, CA) at 4°C overnight. Plates were then washed and blocked with culture medium containing 10% FBS. Splenocytes (5×10^5 splenocytes/well) were incubated with the gp100 (20 μg/ml) or hsp110 (20 μg/ml) at 37°C for 24 h. Plates were then extensively washed and incubated with 5 μg/ml biotinylated IFN-γ antibody (clone XMG1.2; PharMingen) at 4°C overnight. After washes, 0.2 unit/ml avidin-alkaline phosphatase D (Vector Laboratories, Burlingame, CA) was added and incubated for 2 h at room temperature. Spots were developed by adding 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Boehringer Mannheim, Indianapolis, IN) followed by incubation at room temperature for 20 min.

^{51}Cr Release Assay. Splenocytes were harvested 2 weeks after immunization and stimulated *in vitro* with irradiated B16-gp100 cells for 5 days. Splenocytes were then serially diluted in 96-well plates containing ^{51}Cr-labeled tumor cells (1×10^4 cells/well) in triplicate with varying E:T ratios. After 5 h of incubation at 37°C, supernatant was analyzed for radioactivity using a gamma counter (Packard, Downers Grove, IL). In some experiments, the restimulated effector cells were incubated with anti-CD8 antibodies (20 μg/ml) at 4°C for 30 min before cytotoxicity assay.

Tumor Challenge. Mice (at least 5 mice/group) were immunized i.p. with 30 μg of hsp110 alone, 30 μg of gp100 alone, or the hsp110-gp100 complex formed by incubating 30 μg of hsp110 with 30 μg of gp100 under heat shock conditions on days -28 and -14. A group of mice was immunized s.c. with 30 μg of gp100 together with CFA and boosted together with Incomplete Freund's Adjuvant (Sigma, St. Louis, MO). Two weeks after the second immunization, mice received intradermal injection with 1×10^5 B16-gp100 cells in 50 μl of PBS on day 0. For therapeutic treatment of tumor-bearing animals, mice were first inoculated intradermally with 5×10^4 B16-gp100 tumor cells. The vaccines were then administered i.p. on days 4, 9, and 14 after tumor implantation. Tumor growth was monitored every 2 days by measuring perpendicular tumor diameters using an electronic digital caliper. The tumor volume is calculated using the formula V = (the shortest diameter^2 $\times$ the longest diameter)/2. All experiments have been repeated three times. Student's unpaired *t* test was performed for statistical analysis, and values of $P < 0.05$ were considered significant.

***In Vivo* Antibody Depletion.** Anti-CD4 hybridoma (GK1.5) and anti-CD8 hybridoma (2.43) were kindly provided by Dr. Drew Pardoll (Johns Hopkins University, Baltimore, MD). Antibodies were partially purified by ammonium sulfate precipitation from ascites of severe combined immunodeficient (SCID) mice injected i.p. with GK1.5 and 2.43 cells. Depletion of CD4$^+$ and CD8$^+$ T-cell subsets was accomplished by i.p. injection of 200 μg of GK1.5 and 2.43 mAb, respectively, given every other day for 6 days. Effective depletion of cell subsets was confirmed by fluorescence-activated cell-sorting analysis of splenocytes 1 day before vaccination or tumor challenge and maintained by the antibody injections once a week for the duration of tumor challenge experiment. Isotype-matched antibodies were also used as control, and no effect on tumor growth was observed.

RESULTS

Characterization of *in Vitro* "Natural Chaperone Complexes" of hsp110 and gp100. To characterize the molecular chaperoning function of hsp110, which is necessary to generate complexes of hsp110 and gp100 protein, we first determined the "melting temperature" of gp100 antigen using an *in vitro* aggregation assay. Recombinant human gp100 protein was incubated for 30 min at 25°C, 43°C, 50°C, 55°C, or 60°C in a thermostated cuvette. Light scattering at 320 nm by protein aggregation was measured using a spectrophotometer (Fig. 1*A*). Changes in the absorbance indicated that the melting temperature of this antigen occurred at approximately 50°C. Furthermore, after incubation at different temperatures, samples were separated into supernatant (soluble) and pellet (insoluble) fractions by centrifugation. Both fractions were resolved by SDS-PAGE and analyzed by immunoblot with anti-gp100 antibody (Fig. 1*B*). It was observed that gp100 protein became insoluble in a temperature-dependent manner and that the amount of gp100 protein in the insoluble fraction reached maximum around 50°C, which was consistent with light scattering measurements. Thus, 50°C was used in this model as an optimal temperature to characterize the chaperoning function of hsp110.

To determine whether chaperone hsp110 can protect gp100 from

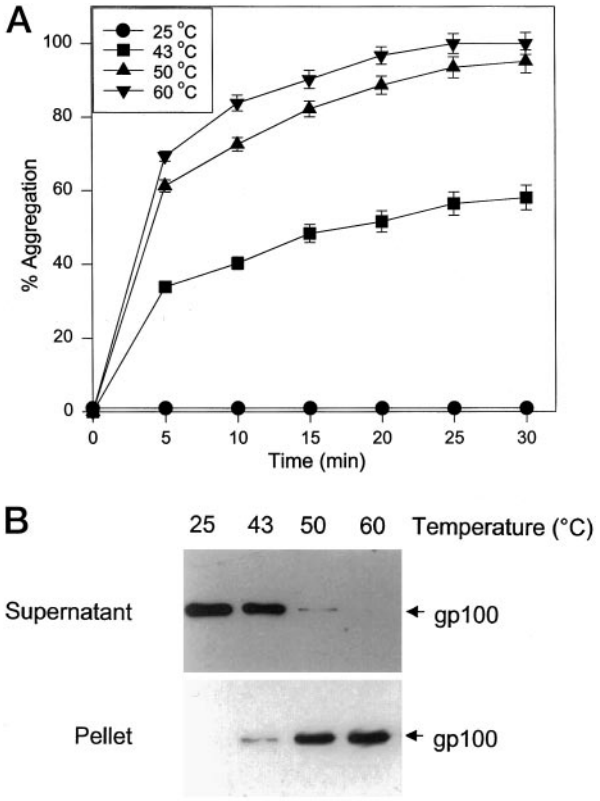

Fig. 1. Aggregation of gp100 protein induced by heat shock at different temperatures. *A*, recombinant human gp100 protein (150 nM) was incubated for 30 min at 25°C, 43°C, 50°C, and 60°C in a thermostated cuvette. Absorbance changes resulting from protein aggregation were measured at 320 nm using a spectrophotometer. *B*, samples after incubations at the indicated temperatures were separated into supernatant (soluble) and pellet (insoluble) fractions by centrifugation. Both fractions were resolved by SDS-PAGE and analyzed by immunoblot with anti-gp100 antibody.

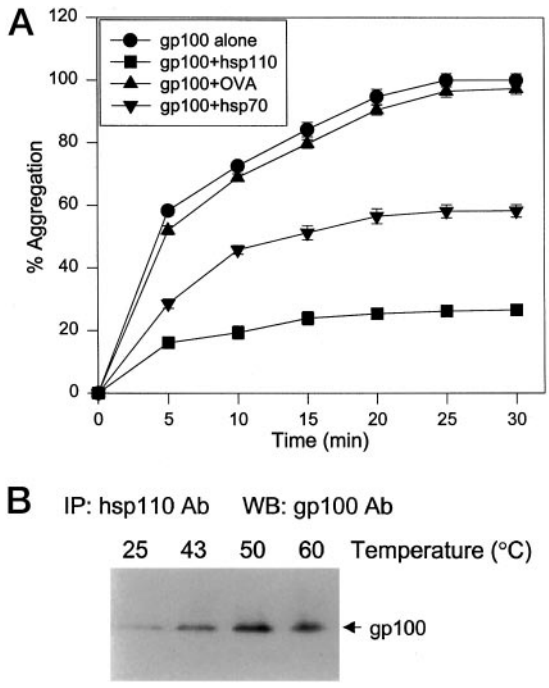

Fig. 2. hsp110 protects gp100 from heat shock-induced aggregation by forming chaperone complexes with gp100. *A*, inhibition of heat-induced gp100 aggregation by hsp110. Recombinant hsp110 and gp100 protein (1:1 molar ratio) were incubated at 50°C, and absorbance changes were measured at 320 nm using a spectrophotometer. *B*, coimmunoprecipitation analysis of gp100 binding to hsp110. The hsp110-gp100 complexes formed at the indicated temperatures were immunoprecipitated by anti-hsp110 serum (1:100). The immunocomplexes were subjected to Western blot analysis using gp100 antibody.

heat-induced aggregation, additional studies were performed at 50°C (Fig. 2*A*). It was seen that, when presented in a 1:1 molar ratio, hsp110 was highly efficient in inhibiting (*i.e.*, 80% inhibition) the heat-induced aggregation of gp100 *in vitro*. However, gp100 aggregation was not prevented in the presence of OVA. For comparative purposes, the chaperoning function of hsp70 was also examined in parallel because hsp110 shares sequence similarities with the hsp70 family (4). We found that although hsp70, as a molecular chaperone, was also capable of inhibiting gp100 aggregation, it was significantly less efficient than hsp110 in holding gp100 (Fig. 2*A*).

Coimmunoprecipitation was then carried out to demonstrate that hsp110 was indeed in a molecular chaperone complex with gp100. hsp110 and gp100 mixtures (1:1) were incubated at different temperatures for 30 min as documented in "Materials and Methods," after which anti-hsp110 antibody was added to precipitate hsp110 using protein A-Sepharose beads. It was observed that gp100 coprecipitated with hsp110, indicating that the protective effect of hsp110 was due to its direct interaction with gp100. Furthermore, the gp100 protein was seen to associate with hsp110 in a temperature-dependent manner with optimal binding at 50°C (Fig. 2*B*). Thus, 50°C was used to generate the hsp110-gp100 complex *in vitro* for vaccine use. These data, in combination with the aggregation study shown in Fig. 1, demonstrate that most gp100 is in complex with hsp110 when the vaccine is prepared in this way.

The hsp110-gp100 Complex Induces IFN-γ Production and CTL Response. Immunogenicity of the hsp110-gp100 chaperone complex reconstituted *in vitro* was first examined using the ELISPOT assay for IFN-γ production. Mice were immunized twice with hsp110 alone, gp100 alone, or the hsp110-gp100 complex at 2-week intervals. Two weeks after the second immunization, splenocytes were isolated and stimulated with gp100 *in vitro*. It was found that splenocytes derived from animals immunized with the hsp110-gp100 complex showed significant IFN-γ production upon gp100 stimulation, whereas those derived from hsp110- or gp100-immunized animals did not (Fig. 3*A*). Most notably, splenocytes from all groups did not generate IFN-γ spots when stimulated with hsp110 or OVA as a control (data not shown).

To determine the ability of hsp110-gp100 vaccine to elicit a CTL response, a chromium release assay was carried out after vaccination. Splenocytes obtained from mice immunized with the hsp110-gp100 complex demonstrated a significant cytolytic activity against the human gp100-transduced B16 cells (B16-gp100), whereas cells from mice immunized with hsp110 alone or gp100 alone revealed no cytolytic activity. This specific killing was completely abrogated by blocking of $CD8^+$ T cells with an anti-CD8 antibody, indicating that $CD8^+$ T cells were responsible for the observed CTL response (Fig. 3*B*). Rat IgG was also used as a control, which failed to inhibit the cytolytic activity of effector cells (data not shown). In addition, sera from mice before and after immunizations were also examined for an antigen-specific antibody response. It was seen that gp100-specific IgG levels were remarkably elevated in mice immunized with the

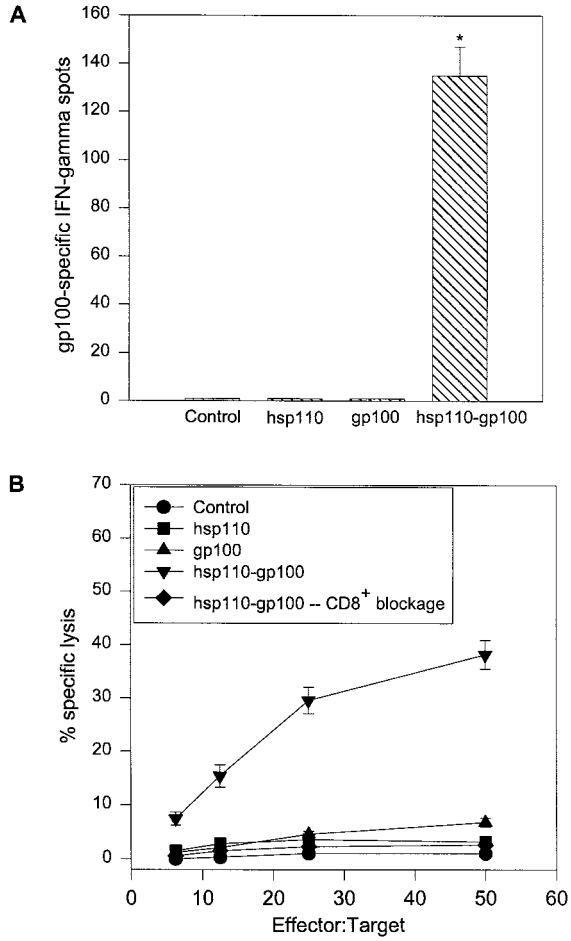

Fig. 3. Immunization with the hsp110-gp100 complex elicits gp100-specific immune responses. C57BL/6 mice (5 mice/group) were immunized i.p. with 30 μg of the hsp110-gp100 complex, hsp110 alone, gp100 alone, or left untreated. The vaccinations were repeated 2 weeks later. Two weeks after the second immunization, splenocytes (*A*; 5×10^5 cells/well) were cultured *in vitro* with gp100 (20 μg/ml) overnight, and IFN-γ secretion was detected using ELISPOT assay. *, $P < 0.005$ compared with splenocytes from naïve mice by Student's *t* test. *B*, splenocytes were isolated as effector cells and restimulated with irradiated B16-gp100 cells *in vitro* for 5 days. The effector cells were analyzed for cytotoxic activity using ^{51}Cr-labeled B16-gp100 cells as targets. For $CD8^+$ T-cell blocking, effector cells were preincubated for 30 min with mAb 2.43 (20 μg/ml). Similar results were obtained from three separate experiments.

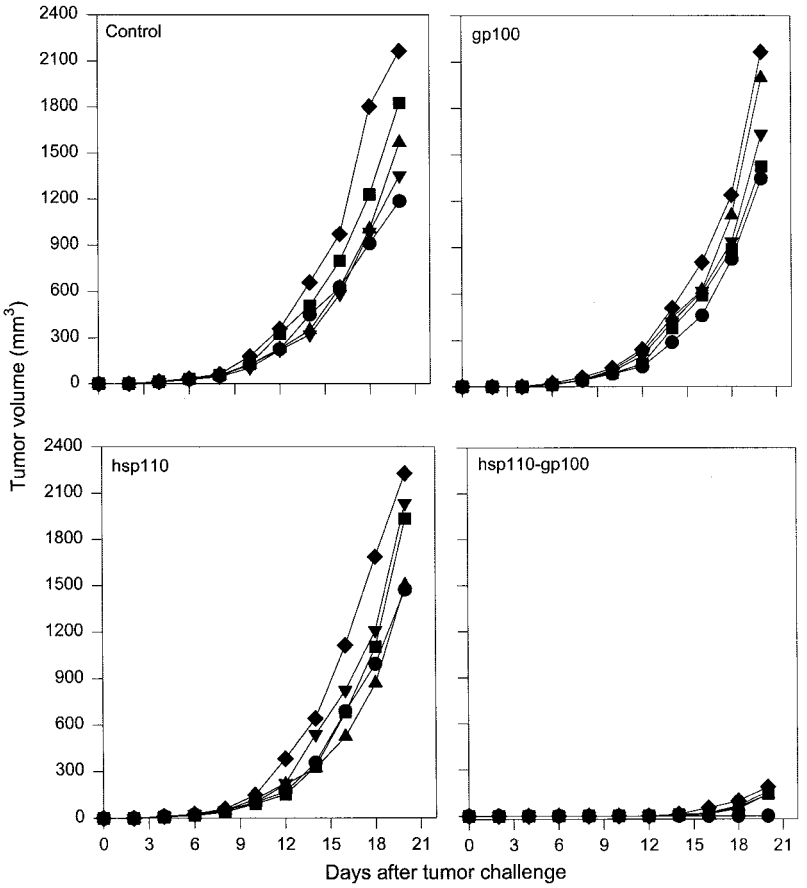

Fig. 4. Immunization with the hsp110-gp100 complex protects mice against subsequent tumor challenge. Mice (5 mice/group) were immunized on days −28 and −14 with 30 μg of the hsp110-gp100 complex, hsp110 alone, gp100 alone, or left untreated. Two weeks after the second immunization, mice were challenged on day 0 with 1×10^5 B16 cells transduced with human gp100 (B16-gp100). Tumor growth was followed three times a week. Each *line* represents data from one individual mouse. Similar results were obtained from three separate experiments.

hsp110-gp100 complex. However, sera obtained from the experimental animals did not recognize hsp110, regardless of the vaccine formulations used (data not shown).

The hsp110-gp100 Complex Elicits Protective Immunity against the B16-gp100 Tumor *in Vivo*. A tumor challenge assay was used to determine the capacity of the hsp110-gp100 complex to induce protective antitumor immunity. C57BL/6 mice were immunized twice with hsp110 alone, gp100 alone, hsp110-gp100 complexes, or left untreated. Two weeks after the second immunization, mice were challenged intradermally with 1×10^5 B16 murine melanoma cells transduced with human gp100 cDNA (B16-gp100). It was observed that naïve mice and mice receiving only hsp110 or gp100 exhibited no protection from tumor challenge, and all of these mice developed aggressively growing tumors. However, mice immunized with the hsp110-gp100 complex were protected from subsequent challenge with the B16-gp100 tumor (Fig. 4), and 20% of these mice remained tumor free for at least 40 days. Among animals that developed tumors, tumor volumes in mice immunized with the hsp110-gp100 complex were significantly smaller than those in mice immunized with hsp110 or gp100 alone.

To further characterize the immunogenicity of the hsp110-gp100 complex, different vaccine formulations were used in tumor challenge studies. Mice were immunized twice as described above with different vaccine formulations: OVA plus gp100 treated with heat shock; hsp110 plus gp100 without heat shock; hsp110 plus previously heat-denatured gp100; CFA plus gp100; or the hsp110-gp100 complex. Two weeks after a booster vaccination, mice were challenged with 1×10^5 B16-gp100 tumor cells (Fig. 5*A*). It was observed that only the hsp110-gp100 complex vaccine elicited a strong antitumor immunity, indicating that the immunogenicity of the chaperone vaccine depends on the formation of a complex between gp100 and hsp110 by heat shock. Although addition of CFA to gp100 could inhibit tumor growth, it was significantly less efficient than the hsp110-gp100 complex.

To evaluate this vaccination strategy in a model that is more analogous to the clinical setting, we also examined the therapeutic efficacy of the hsp110-gp100 complex in mice bearing established tumors. Mice were first inoculated with 5×10^4 B16-gp100 tumor cells on day 0. hsp110, gp100, or the hsp110-gp100 complex was administered i.p. on days 4, 9, and 14. It was found that treatment with the hsp110-gp100 chaperone complex significantly suppressed the growth of established tumor, whereas treatment with hsp110 or gp100 alone did not generate any antitumor response (Fig. 5*B*). The effect of therapeutic treatment against the established tumor was further confirmed by evaluating the survival time of mice, which was calculated based on the time it took for tumors to reach a diameter of 15 mm. It was found that tumor-bearing mice without treatment and mice treated with hsp110 or gp100 showed a mean survival time of 21.8 ± 0.86, 22.5 ± 1.12, and 23.6 ± 1.32 days, respectively, whereas the hsp110-gp100 complex-treated mice showed a survival time of 38.2 ± 4.58 days ($P < 0.005$).

Both $CD4^+$ and $CD8^+$ T Cells Are Required for Antitumor Immunity. To evaluate the contribution of T-cell subsets to protective immunity mediated by the hsp110-gp100 chaperone vaccine, *in vivo* antibody depletion was performed as described in "Materials and Methods." Mice were first depleted of the $CD4^+$ T-cell subset, the $CD8^+$ T-cell subset, or both the $CD4^+$ and $CD8^+$ T-cell subsets before vaccination by injecting corresponding antibodies on days −34, −32, and −30 (*i.e.*, depletion occurred in the priming phase). The depletion of T-cell subsets was confirmed on day −29 and maintained by antibody injections

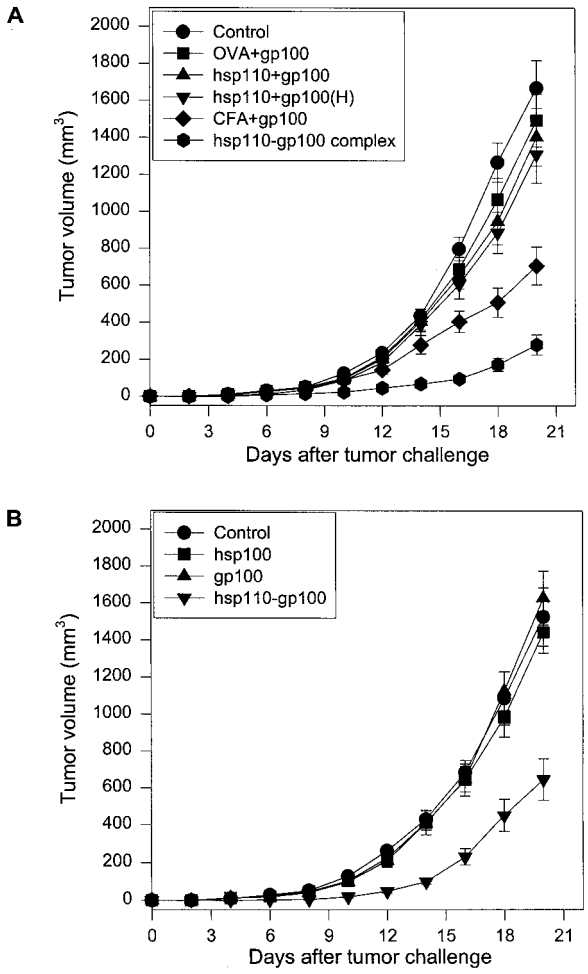

Fig. 5. The hsp110-gp100 vaccine-generated antitumor immunity depends on the formation of chaperone complex between hsp110 and gp100. *A*, mice were immunized on days −28 and −14 with different vaccine formulations: OVA plus gp100 treated with heat shock; hsp110 and gp100 mixture without heat shock; hsp110 plus previously heat-denatured gp100; CFA plus gp100; or the hsp110-gp100 complex. Two weeks after the second immunization, mice were challenged intradermally with 1×10^5 B16-gp100 tumor cells on day 0. *B*, administration of the hsp110-gp100 chaperone vaccine suppresses the growth of established tumor. Mice were first inoculated intradermally with 5×10^4 B16-gp100 tumor cells on day 0. hsp110, gp100, or the hsp110-gp100 complex was administered i.p. on days 4, 9, and 14 after tumor implantation.

weekly. Mice were then immunized on day −28 and boosted on day −14 with the hsp110-gp100 complex. Two weeks after the booster, mice were challenged with the B16-gp100 tumor cells on day 0 (Fig. 6*A*). It was observed that all naïve mice and mice depleted of $CD4^+$ T cells, $CD8^+$ T cells, or both $CD4^+$ and $CD8^+$ T cells developed aggressively growing tumors after the challenge.

To further examine the role of these two T-cell populations, antibody depletion of the subsets was carried out during the challenge phase. Mice were first primed on day −28 and boosted on day −14 with the hsp110-gp100 complex. $CD4^+$ or $CD8^+$ T-cells subsets were then depleted before tumor challenge by injecting antibodies on days −6, −4, and−2 (*i.e.*, depletion occurred in the effector phase; Fig. 6*B*). Mice were then challenged with B16-gp100 tumor cells on day 0. Injections of depleting antibodies were repeated every week after the tumor challenge until the experiment was terminated. It was seen that depletion of $CD8^+$ T cells or both $CD4^+$ and $CD8^+$ T cells abrogated the antitumor effect of vaccination. In contrast, when mice were depleted of $CD4^+$ T cells at the challenge phase, tumor immunity elicited by the hsp110-gp110 complex was intact. These data suggest that $CD8^+$ T cells are the primary effectors mediating tumor killing, whereas $CD4^+$ T cells are required for priming the effective antitumor responses.

The hsp110-gp100 Complex Inhibits the Growth of WT B16 Tumor. Additional studies were undertaken to determine whether immunization of mice with human gp100 chaperoned by hsp110 could break tolerance against mouse gp100 and protect mice against the WT B16 tumor. Two immunization schedules were tested. One group of mice was immunized with the hsp110-gp100 complex on days −28 and −14; another group was immunized on days −30, −20, and −10. Mice were then challenged with the WT B16 tumor on day 0. Although two immunizations with the hsp110-gp100 complex revealed marginal inhibition of the WT B16 tumor cells, three vaccinations with the same regimen induced a statistically significant suppression of the WT B16 tumor (Fig. 7*A*). Furthermore, a CTL assay using splenocytes from vaccinated or nonvaccinated animals also indicated that three immunizations with the hsp110-gp100 complex resulted in an enhanced cytolytic activity against the WT B16 tumor cells compared with the two-dose immunization protocol (Fig. 7*B*).

DISCUSSION

In the current study we characterize a different approach to HSP-based vaccine formulation, which utilizes the strong molecular chaperoning ability of hsp110 to form natural chaperone complexes with

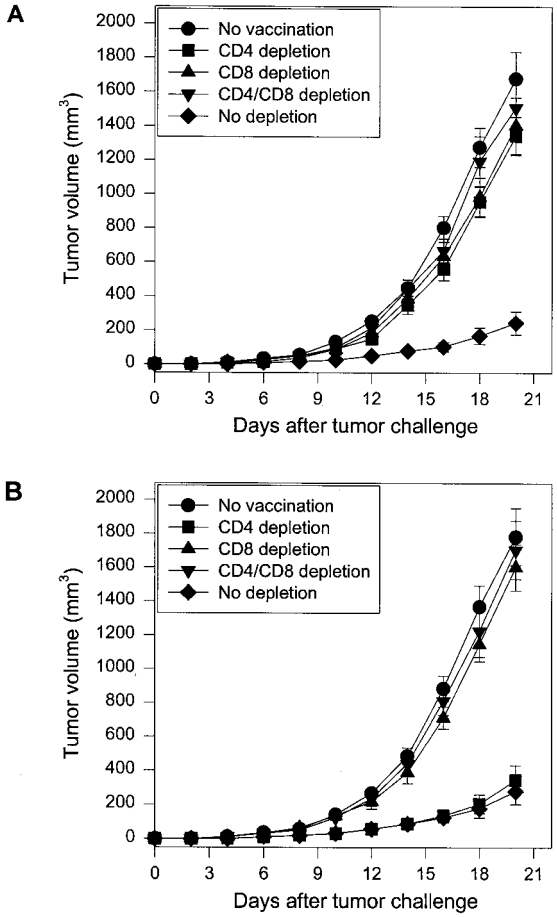

Fig. 6. Both $CD4^+$ and $CD8^+$ T cells are involved in the antitumor immunity elicited by the hsp110-gp100 chaperone vaccine. *A*, mice were depleted of $CD4^+$, $CD8^+$, or both $CD4^+$ and $CD8^+$ T cells before immunization by injections of anti-CD4 antibody (GK1.5) and anti-CD8 antibody (2.43) on days −34, −32, and−30. Mice were then primed on day −28 and boosted on day −14 with the hsp110-gp100 complex. Two weeks after the second immunization, mice were challenged with 1×10^5 B16-gp100 tumor cells on day 0. *B*, mice were primed on day −28 and boosted on day −14 with the hsp110-gp100 complex. $CD4^+$ or $CD8^+$ T-cell subsets were then depleted before tumor challenge by injections of depletion antibodies on days −6, −4, and−2. Mice were challenged with B16-gp100 tumor cells on day 0 and followed for tumor development.

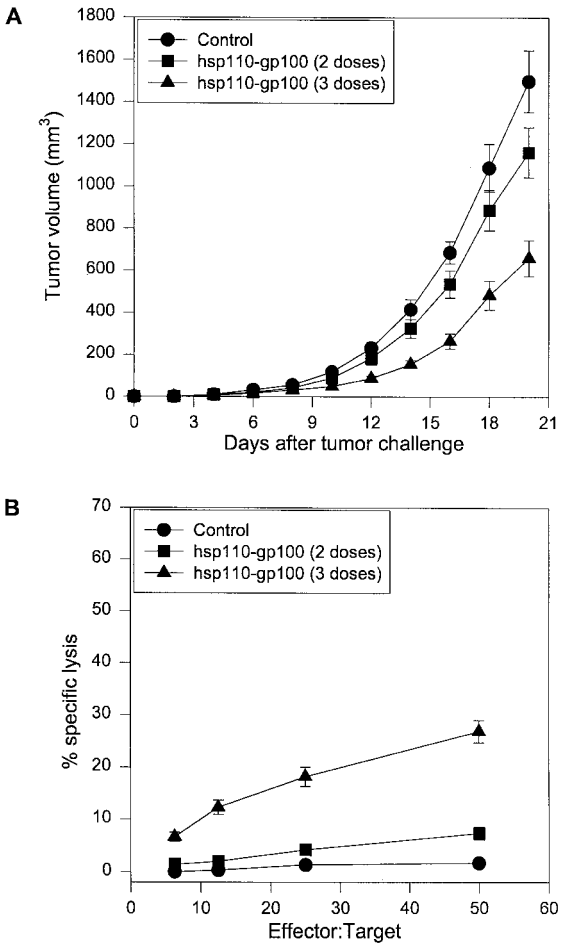

Fig. 7. Immunization with the hsp110-gp100 chaperone complex inhibits the growth of WT B16 tumor. *A*, two vaccination protocols were used to treat mice: the first group of mice was immunized with the hsp110-gp100 complex on days −28 and −14; the second group was immunized on days −30, −20, and −10. All immunized mice and naïve mice were challenged intradermally with 5×10^4 WT B16 tumor cells on day 0. *B*, splenocytes were isolated from naïve mice and mice treated with those two vaccination protocols and restimulated with irradiated WT B16 cells *in vitro* for 5 days. The effector cells were then analyzed for cytotoxic activity using ^{51}Cr-labeled WT B16 cells as targets.

large protein substrates during heat shock (5, 6). Because several studies have demonstrated that HSPs can serve as potent immune adjuvants, a study in which we use a tumor protein antigen as substrate, specifically melanoma-associated antigen gp100, was undertaken to determine whether a natural chaperone complex between hsp110 and gp100 could function as a cancer vaccine. The present study (*a*) defines the *in vitro* and *in vivo* antitumor responses induced by a heat-reconstituted hsp100-gp100 chaperone complex, (*b*) demonstrates that it is the chaperone complex that is required for the antitumor response, and (*c*) shows that using hsp110 in this way is significantly more effective than using CFA.

We were able to show here that immunization with gp100 chaperoned by hsp110 elicited a strong immune response against gp100, which is demonstrated by antigen-specific IFN-γ production and cytotoxic T-cell activity. These results are in agreement with our initial investigation of this vaccine approach using the ICD of Her-2/neu as the antigen (30). In those studies, immunization with the hsp110-ICD complexes induced both $CD8^+$ and $CD4^+$ T cells to produce IFN-γ, whereas no response was obtained when mice were immunized with ICD or hsp110 alone. The present work with gp100 goes significantly further than did the initial report with ICD, in that studies here demonstrate for the first time that the hsp110-gp100 chaperone complex is highly effective in inhibiting the *in vivo* tumor growth of human gp100-transduced B16 tumor cells in both prophylactic and therapeutic settings (Figs. 4 and 5). Surprisingly, it was found that, in terms of antitumor efficacy, the hsp110-gp100 chaperone complex is more potent than gp100 mixed with CFA (Fig. 5*A*). Indeed, it is particularly striking that a "self" protein, mouse hsp110 in mouse, acts as a strong adjuvant when in a chaperone complex with antigen as compared with CFA. More importantly, the hsp110-gp100 complex can generate cell-mediated and humoral responses without invoking an immune response against hsp110 itself. This is in contrast to other adjuvants that are not highly effective in stimulating cell-mediated immunity and against which immune responses can be obtained (33).

It was also demonstrated in this study that three immunizations with the hsp110-gp100 complex induced a significant antitumor immunity against WT B16 melanoma (Fig. 7), suggesting that induction of human gp100-specific immune response can break tolerance to the endogenous mouse gp100 expressed by the B16 tumor cells. A similar cross-reactivity between the human and mouse gp100 was also observed after immunization with recombinant vaccinia virus encoding human gp100 (34), although others reported that CTLs generated from human gp100 immunization specifically recognized human gp100, but not mouse gp100 (35). The discrepancy observed here might be due to the different immunization approaches and vaccine formulations used.

Human gp100 and mouse gp100 share 76% homology in the amino acid sequence, allowing human gp100-reactive T cells to be cross-reactive with the mouse gp100. At least one epitope from human gp100, KVPRNQDWL ($gp100_{25-33}$), has been shown to have a high affinity for binding to MHC class I, *H2-D*^{b} molecules. In this case, gp100-reactive T cells with high avidity for this epitope have been demonstrated (28). However, in addition to this epitope, there is a distinct possibility that other peptide epitopes may also be involved in the antitumor response observed in our studies. The determination of this is the next step in the analysis of this vaccine approach, and these studies are being planned.

In vivo depletion studies clearly indicate that both $CD4^+$ and $CD8^+$ T cells contribute to the protective immunity elicited by the hsp110-gp100 complex (Fig. 6). It is tempting to postulate that antigen presentation of gp100 is mediated through cross-priming, where the hsp110-gp100 complexes are internalized through HSP receptor (*e.g.*, CD91)-mediated endocytosis and processed by antigen-presenting cells, which eventually present both $CD8^+$ and $CD4^+$ T-cell epitopes of gp100 (17, 18). Although the intracellular trafficking of internalized HSP still remains unclear, observations presented here provide strong evidence that hsp110 is capable of routing exogenous antigens into an endogenous processing pathway for presentation by MHC class I molecules (36, 37). Involvement of both of the T-cell populations observed here is consistent with a previous study showing that depletion of either $CD4^+$ or $CD8^+$ T cells abrogated the antitumor effect of tumor-derived gp96 (8). In our system using gp100 as an antigen, $CD8^+$ T cells are most likely to be the primary effector cells responsible for direct tumor killing, as shown by the *in vitro* CTL assay in this study. However, induction of effective antitumor immunity by hsp110-gp100 vaccination requires $CD4^+$ T cells in the priming phase, which may play an important role in initiating and maintaining immune responses (34, 38). In addition, we have shown in this study that a hsp110-based vaccine formulation also elicits a strong humoral response specific for the chaperoned antigen gp100. Whether this antibody response is also involved in the observed antitumor immunity remains to be determined. In addition to adaptive immunity observed in this study, it is quite possible that the innate immune response may also be a factor. In our earlier studies of hsp110

purified from tumor tissue and then applied as a vaccine, we found that antitumor immunity depended on $CD8^+$ T cells as well as natural killer cells.

The present work also evaluates the vaccine activities of different hsp110/gp100 formulations to determine if, indeed, it is the heat shock-induced chaperone complex that is essential for the observed strong antitumor immunity. The vaccine formulations include hsp110-gp100 mixture without heat shock (not in a chaperone complex), gp100 heated and then mixed with hsp110 at room temperature (again, no complex), or a formulation that replaces hsp110 with OVA as a control protein. However, only the hsp110-gp100 complex has been found to be immunogenic (Fig. 4*A*), which distinctly demonstrates that formation of a chaperone complex between gp100 and hsp110 is critically important for eliciting antitumor activity.

The chaperone component of the present study is important to note. We have shown here that gp100 "melts" (as determined by aggregation) at approximately 50°C and that this is the optimal temperature for the formation of chaperone complexes with hsp110. It is necessary to obtain such information for every protein to be studied, and the "unfolding" properties of different proteins can differ significantly. It is essential to understand these properties and then to directly verify that a chaperone complex exists before applying this vaccine approach.

The recombinant hsp110-protein vaccine approach described here provides a highly concentrated vaccine (*i.e.*, one full-length gp100 protein to one hsp110) that targets a specific antigen. The entire natural antigen used in this approach contains multiple MHC class I/II epitopes and thereby allows the individual's own MHC alleles to select the appropriate epitope for presentation. Thus, vaccination with whole protein chaperone complexes increases the chance of poly-epitope-directed T- and B-cell responses. This approach would therefore circumvent HLA restriction and would not be an individual specific vaccine, as are the tumor-derived HSPs (39), and could be applied to any patient with a tumor expressing that antigen. Lastly, this vaccine can be generated in unlimited quantity and is less time-consuming to prepare compared with purification of tumor-derived HSP vaccines. Most importantly, a tumor specimen is not required for vaccine preparation. The vaccine approach defined here optimizes the presentation of HSP-protein complexes by using (perhaps) the most potent protein-chaperoning HSP, *i.e.*, hsp110, in an *in vitro*-generated complex with a protein antigen, *i.e.*, gp100. These natural chaperone hsp110-gp100 complexes exhibit an active immunological activity (*e.g.*, stimulation of a strong CTL response), which confers significant protection against tumor challenge in both prophylactic and therapeutic models. Thus, hsp110-based chaperone vaccines targeting specific tumor protein antigens represent a potentially powerful and novel approach for use in the immunotherapy of cancer.

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