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Cell surface expression of heat shock protein gp96 enhances cross-presentation of cellular antigens and the generation of tumor-specific T cell memory

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Abstract

Gp96 is an endoplasmic reticular heat shock protein (HSP). We have shown previously that surface expression of gp96 (96tm) on tumor cells led to the activation of dendritic cells and increased anti-tumor immunity. In this report, we have found that protective immunity elicited by 96tm+ tumor cells was tumor-specific and long-lasting. Both CD4+ and CD8+ T cell memory were elicited. By immunizing with tumor cells loaded with the chicken ovalbumin (ova) model antigen, we demonstrated that the priming of adoptively transferred ova-specific CD8+ T cells could occur across MHC haplotypes. The efficiency of this cross priming can be significantly increased when mice were immunized with whole cells that express both ova and cell surface gp96 (ova+96tm+). Mere mixture of soluble ova with 96tm-expressing tumor cells (ova-96tm+) was insufficient, arguing for further processing of ova and perhaps the participation of 96tm-ova complexes in this process. We further compared the relative efficiency of two whole cell vaccines based on the manipulation of gp96 expression in one system: 96tm+ whole cells and cells that secrete the gp96-Ig fusion protein. We found that both vaccines are effective in a prophylactic model against tumors. Our study has reinforced the notion that the manipulation of the site of expression of HSPs may be an effective approach for cancer immunotherapy.

Introduction

HSPs are known conventionally as stress proteins or protein chaperones which play fundamental housekeeping roles in all cellular events involving protein folding and unfolding (1, 2, 3). It is increasingly appreciated that HSPs also play important roles in both innate and adaptive immunity, based on the following attributes (see 4 for a review): First, a number of mammalian HSPs, including the cytosolic hsp70 and hsp90, as well as the endoplasmic reticular HSPs gp96, calreticulin, hsp110 and grp170, have been found to be peptide chaperones. Soluble HSP-peptide complexes can be purified from tumors or viral infected cells and used to immunize against

the respective tumors or viruses (peptide-specific immunity). Second, HSPs interact and activate professional antigen presenting cells (APCs), including dendritic cells (DCs), in a receptor-dependent manner, for the efficient priming of T cells. Third, exogenous antigens chaperoned by HSPs can be channeled or cross-presented into the MHC class I pathway.

Gp96 is a constitutively expressed HSP that normally resides in the lumen of the endoplasmic reticulum (5). The presence of gp96 receptors CD91 (6, 7) and Toll-like receptors (TLRs) (8) on the surface of APCs argues strongly that extracellular export of gp96 may have immunological consequences. Indeed, we showed previously that tumor cells engineered to express gp96 on the cell surface induced DC activation, T cell priming and efficient tumor rejection (9). Similarly, tumor cells engineered to secrete gp96 fused with the hinge, CH2 and CH3 domains of murine IgG1 (gp96-Ig) have increased immunogenicity (10). In this report, we have further studied the molecular and cellular requirements for protective immunity elicited by 96tm+ tumor cells. We found that 96tm+ tumor cells can lead to increased cross-presentation of MHC I antigens to CD8+ T lymphocytes, and induce both CD4+ and CD8+ T cell memory against tumors.

Results

Cell surface expression of gp96 on tumor cells leads to an increased immunogenicity and anti-tumor memory

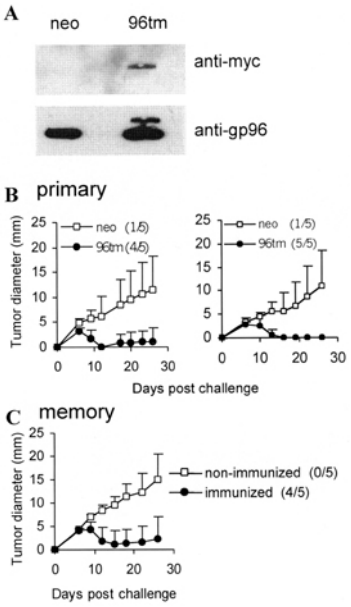

Figure 1. Cell surface expression of gp96 on tumor cells confers increased immunogenicity and anti-tumor memory. (A) Expression of cell surface gp96 by Meth A-neo and Meth A-96tm cells was analyzed by immunoprecipitation of biotinylated cell surface proteins with avidin, followed by immunoblotting with anti-myc Ab (top panel). As a control, the total cell lysate was blotted for gp96 with anti-gp96 Ab (bottom panel). (B) Efficient rejection of CT-26-96tm (left panel) and Meth A-96tm (right panel) cells, but not of control vector-transfected cells, by wild type syngeneic BALB/c mice. (C) Meth A-96tm immunized mice were re-challenged 90 days later with wild type Meth A. The kinetics of tumor growth were then measured. Each line represents the average growth kinetics of 5 mice. The numbers in parenthesis indicate the number of tumor-free mice in each group at the end of the experiment shown. Two independent experiments yielded similar results.

Gp96 resides mainly in the endoplasmic reticulum due to an ER retention signal (KDEL) at the C-terminus (5). To understand the possible impacts of extracellular expression of gp96 on tumor immunity, we transfected the Meth A (fibrosarcoma) and CT-26 (colon cancer) cell lines with an expression vector expressing KDEL-minus gp96 fused with a 10-mer myc epitope, followed by a transmembrane domain at the carboxyl terminus (96tm). The cell surface expression of 96tm was confirmed by precipitation of biotinylated cell surface proteins with Avidin-Agarose, followed by immunoblotting with anti-myc antibody (Figure 1A, top panel). Expression of 96tm did not affect the expression of the endogenous gp96 (Figure 1A, bottom panel).

To study the role of cell surface gp96 in tumor immunity, we compared the growth kinetics of Meth A-96tm and CT-26tm with that of cell lines transfected with empty vector only (Meth A-neo and CT-26-neo respectively) in immunocompetent syngeneic BALB/c mice. We found that cell surface expression of gp96 converted both tumor models from a progressive to a regressive phenotype. Mice efficiently rejected inoculations of as many as 5 x 10^5 Meth A-96tm or CT-26-96tm cells intradermally, whereas injections of 1 x 10^5 wild type tumor cells or control transfectants were lethal (Figure 1B). Moreover, by re-challenging Meth A-96tm immunized mice with wild type Meth A, we found that anti-tumor immunity could last for at least 90 days (Figure 1C).

Cell surface expression of gp96 leads to the priming and maintenance of both CD4+ and CD8+ T cell immunity against tumors

To study the cellular requirement for tumor-specific immunity in our model, we injected Abs to deplete CD4+ or CD8+ T cells from the mice, or carrageenan to deplete phagocytic cells, one day prior to the challenge with 5 x 10^5 Meth A-96tm cells. Depletions were more than 90% complete as assessed by FACS analysis of the corresponding splenic populations (data not shown). Carrageenan is thought to functionally deplete phagocytic cells (11), although we also saw disappearance of CD11b+ cells in the spleen (data not shown). We found that both CD4+ and CD8+ T cells, but not phagocytic cells, were required for tumor rejection in a primary tumor rejection experiment (Figure 2A). The rejection of primary tumors generally took approximately 12 days to complete, during which time T cells were most likely primed, activated and migrating to the tumors to mediate tumor rejection. Indeed, when day 6 tumors were examined histologically, we observed significant infiltration of both CD4+ and CD8+ T cells to 96tm+ tumors, but not to control tumors that did not express gp96 on the cell surface (Figure 3). There were no background stains with isotype-controlled Ab in either tumor (data not shown). To study if CD4+ or CD8+ T cell memory responses were generated, we performed a re-challenge experiment after T cell depletion. Mice were immunized with live Meth A-96tm. Thirty days later, when Meth A-96tm was already rejected, the mice were depleted of T cells and re-challenged with 5 x 10^5 live wild type Meth A cells 24 hours later. As shown in Figure 2B, depletion of either CD4+ or CD8+ T cells abrogated tumor protective immunity, suggesting that both CD4+ and CD8+ T cells are required for tumor rejection in the memory phase.

Cell surface gp96 potentiates cross-presentation of intracellular antigens to MHC I for activation of CD8+ T cells

To confirm that Meth A-96tm potentiated cross-priming of antigen-specific T cells, we took advantage of a well-described OT-I transgenic mouse model that expresses the T cell receptor (TCR) for chicken ovalbumin (ova) (12). A majority of CD8+ T cells in these mice express a single Valpha2+Vbeta5+ TCR that recognizes an ova-derived peptide, SIINFEKL, in association with a K^b molecule. Meth A-neo or Meth A-96tm ($H\text{-}2^d$) were osmotically loaded with ova (Figure 4A) and then used to prime adoptively transferred OT-I cells in C57BL/6 ($H\text{-}2^b$) recipient mice. Prior to transfer, OT-I cells were marked with the fluorescent dye CFSE. The fluorescence intensity of CFSE diminishes by 50% with each cell division, enabling ova-specific T cell proliferation *in vivo* to be measured in a convenient way. Immunization with 100 ng soluble ova did not lead to OT-I proliferation, whereas CD8+ OT-I T cells proliferated significantly after immunization with high dose ova (1 mg), reflected in the conversion of the high CFSE fluorescence population to a population with low/intermediate CFSE fluorescence (Figure 4B). Immunization with Meth A-96tm cells loaded with only 100 ng ova (10,000-fold less protein) also led

to efficient proliferation of OT-I cells (Figure 4B). By contrast, immunization with Meth A-neo cells loaded with the same amount of ova was less effective, even though concurrent helper T cell response against H-2^d alloantigens was possible. Moreover, mere mixing of soluble ova with 96tm-expressing tumor cells was insufficient, arguing for further processing of ova and perhaps the participation of 96tm-ova complexes in this process.

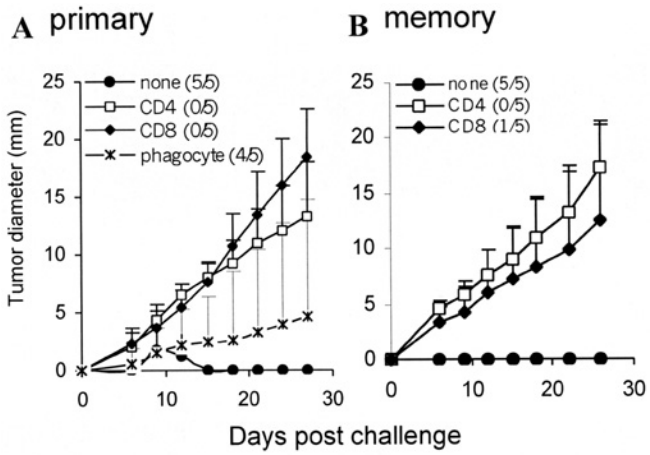

Figure 2. Cellular mechanism for tumor rejection mediated by cell surface targeting of gp96. (A) Cellular requirement for eliciting effective primary anti-tumor immune responses by Meth A-96tm. Wild type syngeneic BALB/c mice were depleted of CD4+, CD8+ cells or phagocytes on day -1 and then injected with 5 x 10^5 Meth A-96tm cells on day 0. The average growth kinetics of 5 mice per group is plotted. The numbers in parenthesis indicate the number of tumor-free mice in each group at the end of the experiment. (B) Cellular requirement for eliciting anti-tumor memory immune responses by cell surface gp96. Mice were immunized with 5 x 10^5 live Meth A-96tm cells on day -30, depleted of various subsets of cells on day -1, and re-challenged with 5 x 10^5 wild type Meth A cells on day 0. These results were replicated three times.

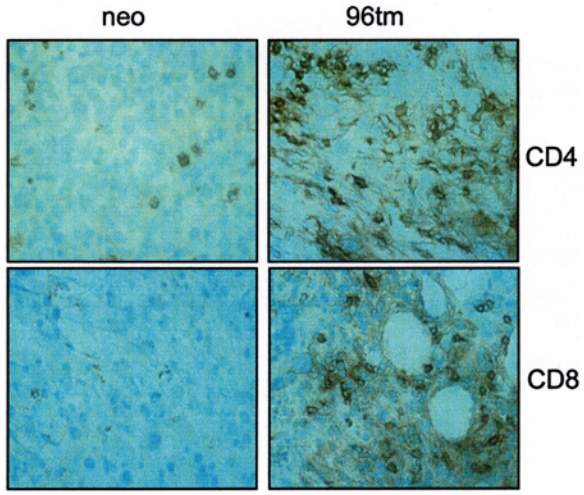

Figure 3. Expression of gp96 on the surface of tumor cells leads to increased infiltration by both CD4+ and CD8+ T cells. A total of 5 x 10^5 Meth A-96tm or Meth A-neo cells were injected intradermally into BALB/c mice, followed by surgical excision of the palpable tumors which were snap-frozen in liquid nitrogen on day +6. Ten-micron cryostat sections were fixed in acetone and immunostained with antibodies against CD4, CD8, sequentially incubated with biotinylated secondary antibody and streptavidin-HRP, and developed with H_2O_2 and diaminobenzidine. An image of a representative section, taken at 100x magnification, is shown.

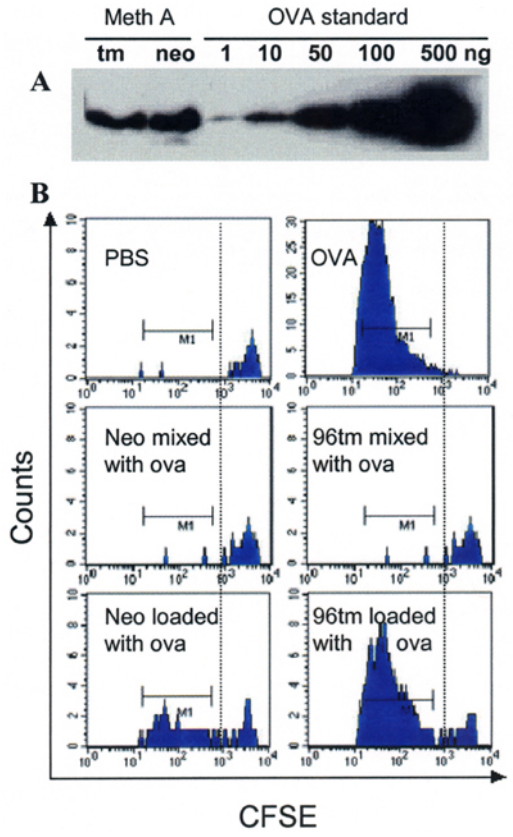

Figure 4. Cross-presentation of ova to ova-specific CD8+ T cells *in vivo* by ova-loaded gp96 surface-expressing tumor cells. (A) Meth A-neo or Meth A-96tm cells were osmotically loaded with chicken ovalbumin. To quantify the loading efficiency, 2.5 x 10^5 cells from each cell type were lysed in 20 µl PBS containing 0.5% NP-40. The supernatant, along with purified ovalbumin standard, were then subjected to 10% SDS-PAGE, followed by immunoblotting with ova-specific Ab. In this experiment, approximately 100 ng of ovalbumin was loaded into 5 x 10^5 cells. The loading efficiency of Meth A-96tm and Meth A-neo cells appeared to be equivalent. (B) 2 x 10^6 purified CD8+Valpha2+ OT-I cells were labeled with CFSE and adoptively transferred into C57BL/6 (H-2^b) mice by tail vein injections. Two days later, the mice were immunized i.p. with PBS, 1 mg ova, 5 x 10^5 Meth A-neo or Meth A-96tm cells mixed with 100 ng ova, 5 x 10^5 Meth A-neo or Meth A-96tm cells loaded with 100 ng ova intracellularly. Three days after immunization, splenocytes from each group of mice were analyzed by FACS after staining with propidium iodine (PI) and PE-labeled anti-Valpha2 mAb (Pharmingen). Cells were gated on PI-Valpha2+ populations. Shown is a representative result from three separate experiments.

Comparison of the efficiency of vaccination with tumor cells expressing gp96 on the cell surface as compared to tumor cells secreting gp96-Ig

Tumor cells secreting gp96-Ig were also found to have increased immunogenicity (10). To compare the relative activity of these two cell-based vaccines (96tm+ and 96Ig+ tumor cells) in one tumor system, we made a stable cell line, Meth A-96-Ig, that secretes gp96-Ig constitutively (Figure 5A). Mice were immunized with PBS, 1 x 10^5 live Meth A-96tm or Meth A-96-Ig cells (day 0) intradermally. On day 14, all mice were challenged with 5 x 10^5 wild type (WT) Meth A cells. In this experiment, we challenged with 5-fold more Meth A cells than reported in the literature so as to compare the relative efficiency of these two vaccines (13, 14). We found that both vaccines were effective in rejecting the growth of tumors, although 96tm+ whole cell vaccines appear to be slightly better

(Figure 5B). Immunizations with tumor-derived soluble gp96 have been shown to be tumor protective because gp96 presumably chaperones the total cellular peptide repertoire (15, reviewed in 16). In our hands, however, whole cell immunizations with engineered gp96 consistently led to tumor rejection, whereas the protective effect of soluble gp96 vaccinations in this system appeared to be more variable (data not shown, see discussion).

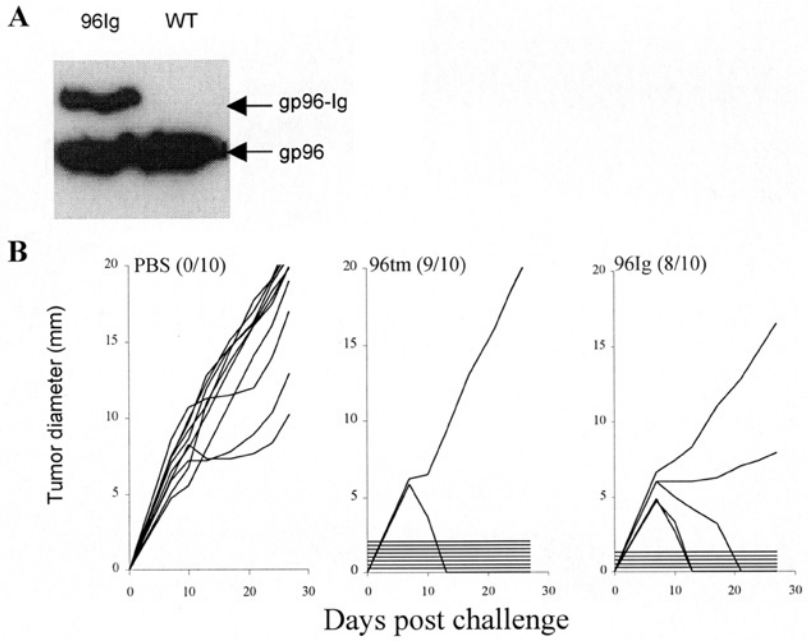

Figure 5. Comparison of the efficiency of vaccination with tumor cells that secrete gp96 as compared to tumor cells that express gp96 on the cell surface. (A) Western blot analysis of gp96Ig expression in the total cell lysate of wild type Meth A (WT) and Meth A-96-Ig cells. (B) Tumor growth kinetics of WT Meth A in mice previously immunized with PBS, Meth A-96tm or Meth A-96-Ig cells. Each line represents the tumor growth kinetics for one mouse; data from two separate experiments were pooled. The number in the parenthesis indicates the number of tumor-free mice in each group at the end of the experiment.

Discussion

We have studied the impact of cell surface expression of the HSP gp96 on tumor-specific immunity. We found that cell surface expression of gp96 induced long-lasting CD4+ and CD8+ T cell memory response. Moreover, expression of gp96 on the surface of target cells enhanced the cross-presentation of intracellular antigens from these target cells by APCs of different MHC I haplotype. These findings once again reinforce the notion that mere manipulation of the site of expression of HSPs may be an effective approach for cancer immunotherapy.

Gp96 is an ER protein normally involved in controlling protein homeostasis in the ER (see 17 for a review). We have long hypothesized that gp96 is involved in direct presentation of peptides to MHC class I (18, 19). This idea remains to be proven. Meanwhile, we have witnessed that extracellular gp96-peptide complexes can interact with the immune system on multiple levels. First of all, gp96 can bind to CD91 or other receptors on the surface of APCs. Interaction of gp96-peptide complexes with CD91 (6, 7) or other receptors (20, 21) on APCs can lead to re-presentation of peptides to MHC I molecules of APCs. Second, gp96 may stimulate and activate DCs through TLR2 and TLR4 (8), resulting in secretion of pro-inflammatory cytokines and upregulation of surface expression of co-stimulatory molecules such as CD80, CD86 and CD40.

However, gp96 is normally an exclusively intracellular molecule. Precisely how gp96 is released, or in what form, during an active immune response *in vivo* is not clear. Srivastava and colleagues have proposed and shown that gp96, along with other HSPs, were only released when cells undergo necrosis, but not apoptosis *in vitro*, suggesting that gp96 could be one of the sensors for "dangerous" conditions when necrosis is induced (6, 22, 23). It has also been found that gp96 can be both secreted and expressed on the cell surface during stressful conditions, arguing perhaps for the significance of extracellular trafficking that precedes cell death in triggering an immune response (9, 17). Although the mechanisms or conditions of gp96 release *in vivo* are unknown, the work presented here and that by others clearly shows that both soluble gp96 and membrane-bound gp96 can modulate immune responses.

By depletion experiments we showed that the tumor-specific immunity elicited by Meth A-96tm was dependent on both CD4+ and CD8+ T cells, but not on phagocytic cells (Figure 2). Anti-Meth A immunity induced by soluble gp96, on the other hand, was previously shown to be dependent on CD8+ and phagocytic cells, not dependent on CD4+ T cells in the priming phase, but dependent on all these cellular components in the effector phase (11). It can be argued that this difference in cellular requirement for tumor rejection could not so much be due to a difference in the fundamental mechanisms, but to a difference in immunization protocols. For example, in the case of Meth A-96tm vaccination, live tumor cells were used to immunize, during which time the priming of T cells must also be coupled with effector mechanisms for mediating tumor destruction. Nevertheless, the fact that Meth A-96tm immunization does not require phagocytic cells in the primary challenge, whereas immunization with soluble gp96 requires phagocytes in both the priming and effector phases, is clearly striking. We postulate that cell surface gp96 may chaperone peptides directly from tumor cells to MHC I on the surface of APCs without the need for phagocytosis. We are currently testing this idea.

By adoptive transfer of K^b-restricted ova-specific T cells into C57BL/6 mice (H-2^b), and immunization with ova-loaded Meth A-96tm (H-2^d) cells, we have shown clearly that cell surface expression of gp96 results in enhanced cross-presentation of ova across MHC haplotypes (from H-2^d to H-2^b) (Figure 4). This finding has two implications. First, it shows that cross-presentation of the intracellular antigens in tumor cells can definitely occur. Second, these data, in conjunction with the increased tumor immunity created by cell surface expression of gp96, argue strongly that the mechanism by which cell surface gp96 augments T cell immunity is by enhancing cross-priming.

Finally, we have compared the relative efficiency of gp96-engineered whole cell vaccines against one tumor model, Meth A, in a prophylactic experiment. We found that both surface-expressing and gp96-secreting tumor cells gave consistent and rapid tumor protection in a re-challenge experiment with high doses (5 x 10^5) of WT tumor cells (Figure 5). In contrast, soluble gp96 was previously found to have a narrow therapeutic window (24) against challenge with 1 x 10^5 Meth A cells. In fact, administration of a higher than optimal dosage of gp96 can even lead to immune suppression (25). Indeed, we found that the effect of soluble gp96 was much more variable, depending upon the purity, integrity, dosage and route of administration of gp96 vaccines. One may argue that it is difficult, if not impossible, to have a fair comparison between protein and cell-based vaccines, since the nature of the vaccines is so different. One obvious reason for such a difference is the presence of other HSPs and antigens in tumor cells that may also contribute to the generation of anti-tumor immunity. In a pragmatic sense, however, we believe that the manipulation of the site of gp96 expression is a reasonable option for cancer immunotherapy in the future. We have already constructed an adenovirus-based expression vector for targeting gp96 expression on the cell surface *in vivo*. We are actively testing if direct targeting of gp96 onto the surface of tumor cells *in situ* is therapeutic against established cancer and whether this approach can circumvent the current need for surgery to purify gp96 or other HSPs from tumors to prepare soluble HSP vaccines (26).

Abbreviations

HSP, heat shock protein; ova, ovalbumin; gp96-Ig, gp96 fused to the hinge, CH2 and CH3 domains of murine IgG1; 96tm, transmembrane form of gp96

Acknowledgements

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Materials and methods

Mice

Wild type BALB/c and C57BL/6 mice were all from The Jackson Laboratory (Bar Harbor, ME) and were maintained by the Center for Laboratory Animal Care at the University of Connecticut Health Center using standard guidelines. OT-I mice were kindly provided by L. Lefrancois (University of Connecticut, Farmington, CT). Animal experiments were carried out using a protocol approved by the Animal Care Committee at the University of Connecticut.

Reagents

Reagents, including carrageenan and chicken ovalbumin were obtained from Sigma (St. Louis, MO) unless otherwise specified. CFSE (carboxyfluorescein diacetate-succinimidyl ester) was obtained from Molecular Probes (Eugene, OR).

Cell lines and transfection

The parental Meth A (fibrosarcoma) and CT-26 (colon carcinoma) tumor cell lines were obtained from P. Srivastava (University of Connecticut, Farmington, CT). Meth A and CT-26 cell lines expressing gp96 at the cell surface were established as described previously (9). The eukaryotic expression vector for gp96-Ig fusion protein (10) was transfected into Meth A cells by electroporation to generate Meth A-96-Ig.

Antibodies

All antibodies used for FACS and immunohistochemistry were from Pharmingen (San Diego, CA). The other antibodies used in this study were specific against: gp96 (SPA-850, clone 9G10, Stressgen, Victoria, BC, Canada), myc (Invitrogen, Carlsbad, CA), ovalbumin (OVA-14, Sigma, St Louis, MO), CD4 and CD8 (clones GK1.5 and YTS169.4 respectively, kindly provided by P. Srivastava, University of Connecticut, Farmington, CT).

Cell surface biotinylation, immunoprecipitation and Western blotting

1 x 10^7 Meth A-96tm or Meth A-neo cells were washed 3 times with ice-cold PBS (pH 7.4), and then incubated with 1 ml PBS containing 0.5 mg of Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) for 30 minutes at room temperature. Cells were washed 3 times with ice-cold PBS before lysis with 0.5 ml lysis buffer that contained 0.5% NP-40. Nucleus-free supernatants were collected and incubated with 100 µl NeutrAvidin beads (Pierce, Rockford, IL) for 1 hour at 4°C. The beads were then washed 5 times with lysis buffer and incubated with 25 µl 2x loading buffer for 5 minutes at 100°C, and the supernatant subjected to 10% reducing SDS-PAGE. Western blotting was performed using a standard protocol, and the blot developed using a horseradish peroxidase (HRP)-conjugated secondary Ab and an enhanced Lumi-light substrate (Roche Molecular Biochemicals, Indianopolis, IN).

In vivo depletion of CD4+ T cells, CD8+ T cells and macrophages

Ascites containing anti-CD4 (GK1.5) or anti-CD8 (YTS169.4) mAb (25 µl) were injected in the tail veins as described previously (11, 27). For depletion of phagocytic cells, 1 mg of carrageenan (type II, Sigma) was injected i.p. per mouse. The amount of Ab and carrageenan used for depletion resulted in over 90% depletion of the corresponding cellular population in the spleen as analyzed by FACS (data not shown).

Tumor rejection assays

Tumor rejection assays were typically performed using 5 mice in each group. Mice were challenged/immunized intradermally with live tumor cells that either secrete gp96 or express gp96 on the cell surface. At various times after immunization, mice were challenged with wild type tumor cells intradermally. Tumor growth was monitored twice a week using vernier calipers to measure both the longitudinal and transverse diameters. Average diameters (mm) of the two axes are plotted.

Immunohistochemistry

BALB/c mice were challenged with 5 x 10^5 Meth A-96tm or Meth A-neo cells. At day 6, the mice were sacrificed and the tumors excised and snap-frozen in liquid nitrogen. Ten-micron cryostat sections were fixed in acetone, immunostained with antibodies against CD4 (L3T4, clone RM4-5) or CD8 (Ly-2, clone 53-6.7) and then sequentially incubated with biotinylated secondary antibodies and streptavidin-HRP, developed with H_2O_2 and diaminobenzidine (Sigma, St Louis, MO), and analyzed by light microscopy.

Osmotic loading of Meth A-96tm or Meth A-neo cells with ovalbumin

1×10^7 cells were incubated in 1 ml of hypertonic medium (0.5 M sucrose, 10% wt/vol polyethylene glycol 1000, and 10 mM Hepes in RPMI 1640, pH 7.2) containing 10 mg/ml ova for 10 min at 37°C. 13 ml of prewarmed hypotonic medium (40% H_2O, 60% RPMI 1640) was added and the cells were incubated for an additional 2 min at 37°C. The cells were then centrifuged and washed twice with cold PBS immediately before immunizations.

Purification of OT-I CD8+ T cells, CFSE labeling, adoptive transfer and FACS analysis of OT-I cells *ex vivo*

After lysis of red blood cells, lymphocytes from the spleens and lymph nodes of OT-I mice were washed with PBS. Approximately 1×10^7 CD8+ T cells were incubated with 20 µl of mouse CD8alpha (Ly-2) microbeads (Miltenyi Biotec, Auburn, CA) for 15 minutes at 4°C, and then eluted according to the manufacturer's protocol. Purity was about 95% as confirmed by FACS. 1×10^7/ml CD8+ T cells were then labeled with 5 µM CFSE for 5 minutes at room temperature, followed by 3 washes in cold PBS. 2×10^6 CD8+Valpha2+ OT-I cells in 200 µl PBS were injected into the tail veins of wild-type C57BL/6 mice on day 0. At day 2, mice were immunized i.p. with 5×10^5 Meth A-96tm or Meth A-neo cells mixed or loaded with ovalbumin. At day 5, the mice were sacrificed, and the CD8+ and Valpha2+ T lymphocytes analyzed by a FACScan (Becton-Dickinson, San Jose, CA) to determine their CFSE staining profile. The data was analyzed using CellQuest software.

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