TAT-mediated gp96 transduction to APCs enhances gp96-induced antiviral and antitumor T cell responses

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ABSTRACT

The heat shock protein gp96 is an adjuvant that can elicit T cell responses against cancer and infectious diseases, via antigen presentation, in both rodent models and clinical trials. Its uptake and internalization into antigen presenting cells (APCs) is a critical step in gp96-mediated immune responses. This study examined strategies to improve the cell internalization and T cell activation of gp96. It was found that recombinant fusion with the cell-penetrating peptide TAT (trans-activator of transcription) slightly decreased the aggregation level of gp96 and significantly increased its internalization into macrophages. Furthermore, immunization with the TAT-gp96 fusion dramatically enhanced gp96-mediated hepatitis B virus (HBV)-specific T cell responses and its antiviral efficiency in HBV transgenic mice compared to rgp96. In addition, the inclusion of TAT significantly improved the antitumor T cell immune response to a gp96 vaccine in the B16 melanoma model. These results provide evidence that the efficient transduction of gp96 into APCs can significantly enhance the outcome of gp96-based immunotherapy, and therefore provide a basis for more efficient approaches to improving the immunoregulatory and adjuvant functions of this unique T cell adjuvant.

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1. Introduction

As a member of the HSP90 family, the heat shock protein (HSP) gp96 (glucose-regulated protein, GRP94) is one of the most abundant proteins in the endoplasmic reticulum. There is now compelling evidence that gp96 plays important roles in both innate and adaptive immunity. Rodent models and clinical trials have shown that gp96 can act as an adjuvant to stimulate both T cell and antibody immunity against viruses and tumors, validating its potential for the development of therapeutic vaccines against cancer or infectious diseases [1,2].

It has been shown that gp96 interacts with the immune system in a number of ways, all of which rely on its interaction with the cell surface receptors of antigen presenting cells (APCs), T cells or other lymphocytes. Firstly, gp96 interacts with toll-like receptors (e.g. TLR-2, TLR-4 and TLR-9), which leads to the activation of APCs, including macrophages and dendritic cells, resulting in the secretion of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-12) and induction of innate immunity [3–6]. Secondly, gp96 has the unique ability to bind antigenic peptides derived from tumors, viruses and intracellular bacteria. The interaction of gp96 with the cell surface receptor CD91 or scavenger receptor-A (SR-A) leads to internalization of the gp96-peptide complexes and the subsequent cross-presentation of the gp96-associated peptides to MHC class I and II molecules, which activate peptide-specific cytotoxic T lymphocyte (CTL)-mediated immune responses [7–12]. In addition, a recent study has shown that the gp96–CD91 interaction can lead to the phosphorylation of CD91, which then triggers signaling cascades that activate nuclear factor-kappa B [13]. Despite these achievements in studies of gp96-mediated immunity, the molecular mechanisms underlying the interactions of gp96 with cell surface receptors and the details of its internalization into APCs remain elusive. Recent studies have suggested that the internalization of gp96–peptide complexes, which is essential for cross-presentation of antigenic peptides and T cell activation, may be CD91 independent, and that heparin sulfate proteoglycans play an important role in the surface binding of gp96 [14–16]. Moreover, it has been found that some tumor cells constitutively express receptor associated protein (RAP) that can bind CD91 with high affinity and thereby competitively block its association with gp96 [17]. Given the apparent dependence of gp96-mediated immune responses on the uptake and internalization of gp96 into APCs, it is critical to explore ways to improve these processes and enhance the capacity of gp96 in antigen presentation.

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The TAT protein transduction domain (PTD), derived from the HIV-1 trans-activator of transcription (TAT) protein, is a short basic region comprising residues 49–57 [RRKKRRQRRR], which has been shown to mediate protein transduction in both mice and cultured cells [18,19]. Many TAT fusion proteins have been generated to deliver a wide variety of size-independent molecules into cells, including peptides, proteins, antisense oligonucleotides, large iron beads and liposomes [20–23]. Furthermore, recent research has shown that the basic region of TAT can promote the expression of heterologous proteins in Escherichia coli, and improve both their yield and solubility [24].

Despite the unique immunostimulatory properties of gp96, the efficacy of gp96-based antitumor and antiviral therapeutic vaccines is still limited and requires further improvement [1,2,25]. The mechanisms that restrict the immune activity of gp96 are still not fully understood. Our previous studies have shown that gp96 complexed with hepatitis B virus (HBV) antigens can elicit virus–specific CTL and exhibit significant antiviral effects [26,27]. The aim of the current study was to determine whether fusion with TAT could increase the internalization of gp96 into APCs, and thereby enhance the adjuvancy of gp96 and its antiviral capacity. The results demonstrated that the conjugation of gp96 and the HIV-Tat49–57 peptide produced a dramatic increase in the gp96-induced MHC I restrictive CD8+ CTL response and antiviral effects, which indicates the potential of this strategy to enhance the immune activity of gp96.

2. Materials and methods

2.1. Peptides and cell culture

Three Kd-restricted epitopes, HBc87–95 (SYVNTNMGL), HBs362–371 (WYWGPSLYSI), HBV pol140–148 (HYFQTRHYL) and one control peptide HBC18–27 (FLPSDFFPSV) were used in this study. All the peptides were synthesized by GL Biochem Ltd. (Shanghai, China). The purity (>95%) was confirmed by high-performance liquid chromatography (HPLC) and mass spectrometry. The peptides were dissolved in dimethylsulfoxide (DMSO) at a concentration of 30 mg/ml and stored in aliquots at −20 °C.

B16 F10 melanoma cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 25 μg/ml streptomycin and 100 IU/ml penicillin. The mouse mastocytoma P815 cell line was used as the source of the Kd class I molecules. Both the P815 and RAW 264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO) supplemented with 10% fetal calf serum (FCS) and 25 μg/ml streptomycin and 100 IU/ml penicillin.

2.2. Expression of recombinant TAT-gp96 and recombinant gp96 in E. coli and purification of gp96 from mouse liver tissue

An expression vector (pET-TAT) was constructed for the expression of recombinant TAT-gp96 (rTAT-gp96). A double-stranded DNA Oligonucleotide encoding the 11 amino acids TAT-PTD (YGRKKRRQRRR) flanked by Gly residues was ligated in frame with the six histidine (6x-His) tag of the pET28a (+) expression vector (Novagen, Madison, WI, USA) as an Ncol/BamHI fragment. The cDNA encoding the mature mouse gp96 (aa 22–802) was subcloned into the pET-TAT vector as a BamHI/XhoI fragment. The DNA sequence encoding the 21 amino acids N-terminal signal peptide of the gp96 cDNA has been removed to avoid cotranslational cleavage. The vector for expressing the recombinant gp96 (rgp96) was created in a similar way by cloning the mouse gp96 (aa 22–802) cDNA into the pET28a (+) expression vector (Novagen, Madison, WI, USA) as a BamHI/XhoI fragment. The resulting vectors were used to express rTAT-gp96 and rgp96 in the E. coli strain BL21 (DE3) plysS (Invitrogen, Madison, WI, USA). Soluble recombinant proteins were isolated as follows. Bacterial cells were lysed using the binding buffer (20 mM Na2PO4, 500 mM NaCl, pH 7.8), the cell debris were removed by centrifugation and the cell extracts were loaded on Ni-Septarose column (GE Healthcare, GE, USA). After washing with washing buffer (20 mM Na2PO4, 500 mM NaCl, 30 mM imidazole, pH 6.0), the His-tagged proteins were eluted by elution buffer (20 mM Na2PO4, 500 mM NaCl, 300 mM imidazole, pH 6.0) and further purified using a Hitrap Q HP column (GE Healthcare, GE, USA). The purified rgp96 and rTAT-gp96 proteins were concentrated and desalted using an Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-50 membrane (Millipore), and stored as aliquots in PBS buffer (2 mg/ml) at −70 °C. The native gp96 (mpgp96) was obtained from healthy mouse livers as described previously [28].

2.3. Western blot analysis

Cytoplasmic extracts were prepared from RAW264.7 cells treated with rTAT-gp96 or rgp96 (100 μg/ml) for 2 h, according to a protocol described previously [29]. Briefly, trypsin (2.5 mg/ml) was applied for 5 min to degrade unincorporated protein before cells were lysed using RIPA buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% Deoxycholate, 5 mM EDTA and a protease inhibitor cocktail) (Roche applied science). Anti-gp94 rat antibody (Santa Cruz Biotechnology, CA, USA, 1:1000) and His-probe antibody (H-3) HRP (Santa Cruz Biotechnology, CA, USA, 1:1000) were used in the immunoblotting to detect the total gp96 and His-tagged rTAT-gp96 or rgp96, respectively.

2.4. Dynamic light scattering (DLS) assay

Dynamic light scattering (DLS) assay was performed as described previously [45].

2.5. Immunofluorescence staining

RAW 264.7 cells were incubated with either rTAT-gp96 or rgp96 (50 μg/ml) for 2 h at 37 °C. The cells were then washed three times with PBS and fixed with 4% Paraformaldehyde for 30 min. After washing three times with PBS–0.1% Tween-20 (PBST), and blocking with PBS containing 5% BSA for 30 min for cell permeabilization, the diluted (1:10) anti-His-FITC antibody (Milenyi Biotec, Germany) was added. After incubation at room temperature for 1 h, the cells were washed three times with PBST, and coverslips were mounted cell-side down using fluorescence-free glycerol-based mounting medium. Cells were viewed using a Laser Scanning Spectral Confocal Microscope (Leica TCS SP2).

2.6. Immunization of mice

Female C57BL/6 mice (6–8 weeks old) were purchased from Peking University Experimental Animal Center. Female HBV transgenic BALB/c mice (6–8 weeks old) were purchased from Transgenic Engineering Lab, Infectious Disease Center, Guangzhou, China. The various gp96–peptide complexes were prepared by incubating mgp96, rTAT-gp96, or rgp96 protein (20 μg) with HBc87–95, HBs362–371 and HBV pol140–148 peptides (50 μg each), first at 50 °C for 10 min, followed by 30 min at room temperature. The mice were immunized s.c. with HBc87–95, HBs362–371 and HBV pol140–148 peptides (50 μg each/mouse) with or without rgp96, rTAT-gp96 or mgp96 as adjuvant (20 μg gp96 protein/mouse) at weeks 1, 2 and 4, respectively. Equal quantity of recombinant TAT-fused albumin (TAT-BSA) expressed in E. coli was used as the negative control for the immunization. Mice were sacrificed at week 8, and splenocytes were dispersed with a syringe plunger.
Splenocytes were cultured in RPMI 1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), 25 μg/ml streptomycin and 100 IU/ml penicillin, in a humidified atmosphere containing 5% CO2 at 37 °C.

2.7. IFN-γ ELISPOT analysis

ELISPOT assay was performed according to the protocol of the manufacturer. In brief, isolated splenocytes (5–10 x 10^5 cells/well) and sample peptides or control peptide (10 μg/ml), or tumor Ag or BSA as control (20 μg/ml) were added to the well in triplicate and incubated at 37 °C for 48 h. The spots were counted and analyzed with the ELISPOT Reader (Biosys, Germany).

2.8. Flow cytometry and intracellular cytokine staining

Splenocytes (10^6 cells) were incubated with either rTAT-gp96 or rgp96 (100 μg/ml) for 30, 60 or 120 min, washed three times with PBS, blocked with PBS containing 5% BSA, and then stained with PE-conjugated anti-mouse CD11b antibody (eBioscience). After permeabilization using the Cytofix/Cytoperm kit (BD Pharmingen), cells were stained with anti-His-FITC antibody (Miltenyi Biotec, Germany, 1:10). Intracellular IFN-γ staining was performed using the Cytofix/Cytoperm kit (BD Pharmingen). Splenocytes were stained with various fluorochrome-conjugated anti-bodies against the surface markers of interest, including PerCP-Cy5.5-conjugated anti-mouse CD3, FITC-conjugated anti-mouse CD8, PE-conjugated anti-mouse CD4 and CD69, and APC-conjugated anti-mouse IFN-γ (eBioscience), and analyzed using a FACS-Calibur flow cytometer and CellQuest Software (BD Biosciences).

2.9. Cytotoxicity assay

5-(6)-Carboxy-fluorescein succinimidyl ester (CFSE)-based cytolyis assay was performed as described previously using HBc87–95, HBS362–371 or HBV Pol140–148 peptide-pulsed P815 cells or B16.F10 melanoma cells as the target cells [25].

2.10. Immunohistochemistry

The detection of HBcAg expression in the liver of mice by immunohistochemistry was performed as described [27].

2.11. Virology assessment

Serum HBsAg in HBV transgenic mice was determined by enzyme-linked immunosorbent assay (ELISA) using enzyme immunoassay kits. Serum HBV DNA levels were detected using real-time PCR and the HBV Fluorescent Quantitative PCR Diagnostic Kit [27].

2.12. Statistical analysis

The statistical significance of the difference between two groups were determined by the two-tailed Student’s t test and was set to P<0.05.

3. Results

3.1. TAT PTD dramatically increases the uptake of gp96 by APCs

The binding and internalization of recombinant gp96 (rgp96) and TAT-gp96 fusion protein (rTAT-gp96) into APCs, was investigated to determine whether TAT could enhance the penetration of gp96 into cells. Preparations of rgp96 and rTAT-gp96 were analyzed by Coomassie Blue-staining and Western blotting with an anti-gp96 antibody (Fig. 1A). Isolated mouse splenocytes were incubated with either His-tagged rgp96 or rTAT-gp96 for periods ranging from 30 to 120 min. FACS analysis revealed the number of anti-His-FITC+ CD11b+ cells was significantly higher for the rTAT-gp96 treatment compared to rgp96 (rTAT-gp96 vs. rgp96, 29.97±1.39 vs. 5.81±0.39, P<0.01, at 120 min) (Fig. 1B), indicating that gp96 was more efficiently delivered into APCs when fused with TAT. The mouse macrophage cell line RAW 264.7 was used to further confirm the enhanced internalization of the TAT-gp96 fusion. Western blot analysis showed that incubation with rTAT-gp96 resulted in a significantly higher level of recombinant gp96 in the cytoplasm of the cells (>3-fold), relative to cells incubated with rgp96 (Fig. 1C). Immunofluorescence staining also showed more intense plasma staining in rTAT-gp96-treated cells than in rgp96-treated cells, indicating higher level of intracellular gp96 accumulation as a result of the TAT-mediated delivery (Fig. 1D).

Since the conformational properties of gp96 might play an important role in its immune function, the self-assembly and aggregation properties of rgp96 and rTAT-gp96 were examined using dynamic light scattering (DLS) (Fig. 1E). The lower mean molecular weight of rTAT-gp96 (506 kDa) relative to rgp96 (621 kDa) indicated that the TAT fusion slightly decreased level of gp96 aggregation.

3.2. HBV peptide specific T cell responses in HBV transgenic mice induced by rTAT-gp96

Three well-characterized Kd-restricted CTL epitopes derived from the main antigens (HBsAg, HBeAg and HBV polymerase) of HBV, HBc87–95, HBS362–371, and HBV Pol140–148 were selected for testing [30–32]. These antigenic peptides, which are known to bind gp96 effectively [26], were used to immunize HBV transgenic BALB/c mice with or without rTAT-gp96 or rgp96 as an adjuvant for 3 times at weeks 1, 2 and 4, respectively. Splenocytes were isolated from mice at week 8. Native mouse gp96 (mgp96) and recombinant TAT-fused albumin (TAT-BSA) expressed in E. coli were used as positive and negative control, respectively. The results of the ELISPOT assay (Fig. 2A), indicated that both rTAT-gp96 and mgp96 were able to effectively elicit specific T cell responses, while no significant enhancement of CTL activity was observed for rgp96 relative to negative control or peptides only treatment. Detection of the T cell activation marker CD69 by FACS showed that mice vaccinated with the rTAT-gp96–peptide or mgp96–peptide complexes exhibited significant increases of CD69â€“CD8+ by 65% or 26% (both P<0.01), respectively (Fig. 2B) and also of CD69+CD4+ by 63% or 70% (both P<0.01), respectively (Fig. 2C) compared to rgp96–peptide complexes. Similar results were obtained for the T cells assay of the IFN-γ-secreting CD8+ with a 15– or 18-fold increase, respectively (Fig. 2D) and of CD4+ (6– or 11-fold increase, respectively) T cells (Fig. 2E). Moreover, a killing assay using CFSE and propidium iodide (PI) double staining (Fig. 2F) revealed significant difference in the peptide-specific cytotoxicity of CTLs from mice treated with the rTAT-gp96–peptide or gp96–peptide complexes. In addition, the number of liver-infiltrating IFN-γ+CD8+ T cells observed in mice immunized with rTAT-gp96 or mgp96 was significantly higher than those immunized with rgp96 (rTAT-gp96 vs. rgp96, 1.93±0.17 vs. 1.15±0.1, P<0.01; mgp96 vs. rgp96, 2.18±0.21 vs. 1.15±0.1, P<0.01) (Fig. 2G). These results indicate that similar to the native mouse gp96, the recombinant TAT-fused gp96 protein has the ability to induce antigen-specific T cell responses, making rTAT-gp96 a powerful adjuvant for augmenting T cell responses.

3.3. Therapeutic effect against HBV by immunization with rTAT-gp96 adjuvant vaccine

Immunization of HBV transgenic mice with the rTAT-gp96–peptide complexes led to a greater than 7-fold decrease...
in HBV DNA levels at week 8 compared to immunization with the rgp96–peptide complexes. Similar results were also found in the HBsAg assay (Fig. 3B). Consistent with the results of the serum HBsAg and HBV-DNA analyses, mice immunized with rTAT-gp96–peptide complexes exhibited a dramatic decrease in HBcAg expression in their hepatocytes by 73% or 67% relative to the rgp96–peptide complexes or control treatment (Fig. 3C). With regard to the serum alanine transaminase (ALT), its levels began to rise at week 4, peaked at week 5, and returned to normal levels by week 8, in mice immunized with rTAT-gp96 or mgp96, whereas no significant change in ALT levels was observed in mice immunized with rgp96 or in the control mice (Fig. 3D). These results suggest that the T cell responses induced by rTAT-gp96 can significantly inhibit HBV replication, and may also cause transient and mild hepatic inflammation as well.

### 3.4. rTAT-gp96 induces anti-tumor T cell response

The effect of rTAT-gp96 on tumor rejection was determined from a direct in vivo read-out of the CTL activity mediated by rTAT-gp96. It was found that C57BL/6 mice immunized with the rTAT-gp96–Ag complexes dramatically slowed tumor growth, by as much as 70% or 75% (both P<0.01), at day 22 (Fig. 4A) and reduced tumor burdens of 64% or 65% (both P<0.01) (Fig. 4B), relative to the mice immunized with the rgp96–Ag complexes or the control mice. In addition, treatment with the rTAT-gp96 vaccine significantly enhanced the survival of tumor challenged mice relative to the rgp96 treatment or the control (Fig. 4C). Furthermore, mice immunized with the rTAT-gp96–Ag complexes showed a significant increase in IFN-γ-producing CD8+ T cells (approximately 1-fold) compared to the mice immunized with the rgp96–Ag complexes (Fig. 4D). Similar results were also observed in the ELISPOT assay (Fig. 4E). A killing assay was used to measure the cytolytic activity of T effector cells of immunized mice (Fig. 4F). It was found that immunization with rTAT-gp96 effectively induced CTL with high tumor specificity and high cytotoxicity, whereas only a low cytotoxicity T cell response against B16 tumors could be detected in mice immunized with rgp96. This result suggests that TAT dramatically increases the antitumor T cell activation function of gp96.
Fig. 2. rTAT-gp96 induces HBV-specific T cell response in HBV transgenic mice. HBV transgenic mice were immunized with rgp96, rTAT-gp96, native gp96 (mgp96) or TAT-BSA complexed with HBc87–95, HBs362–371 and HBV Pol140–148 peptides for three times at weeks 1, 2 and 4, respectively, or with the peptides alone as control. The mice from each group were sacrificed at week 8 and splenocytes or liver-infiltrating lymphocytes were isolated. (A) Peptide specific CTLs were detected by IFN-γ ELISPOT assay. Splenocytes (5 × 10^5 cells/well) were stimulated with 10 μg/ml HBc87–95, HBs362–371 or HBV Pol140–148 peptide, or control peptide HBc18–27 for background evaluation. (B–E) FACS analysis to quantify CD69+CD8+ (B), CD69+CD4+ (C), IFN-γ+CD8+ (D), and IFN-γ+CD4+ (E) T cell populations in mouse spleens. (F) Cytotoxicity assay. The peptide-stimulated splenocytes were incubated with P815 cells labeled with CFSE and pulsed with HBc87–95, HBs362–371 or HBV Pol140–148 peptide. PI staining was then used to measure apoptosis by FACS analysis. CTL activity is indicated as the mean percentage of specific lysis (± SD) at different effector:target (E:T) ratios. (G) IFN-γ+CD8+ T cell infiltration in the liver of mice was detected by flow cytometric analysis. The data show means ± SD of five mice. *P<0.05, **P<0.01 compared with rgp96 immunization or control. The data are representative of two independent experiments.

4. Discussion

A number of studies have shown that the immune-regulatory and adjuvant activities of HSP gp96 which induces both innate and adaptive immunity [33–36]. However, the effectiveness of gp96-based immunotherapy has been limited [2,36]. The mechanisms of gp96 activity are still not fully understood, and evidence from different sources has indicated several alternative mechanisms for
gp96-induced T cell responses [15,16]. This study demonstrated that the fusion of gp96 with TAT peptide can significantly improve the internalization of gp96 into macrophages and produce dramatic increases of gp96-mediated HBV-specific CTL responses and antiviral efficiency in HBV transgenic mice. Furthermore, the addition of TAT also enhanced gp96-induced antitumor T cell immunity in the B16 melanoma model, supporting the hypothesis that efficient transduction and internalization of gp96–peptide complexes into APCs determine the outcome of gp96-based immunotherapy. Such enhancement of gp96 internalization and its capacity for antigen presentation could promote gp96-mediated CTL responses. The current work may therefore help to elucidate the T cell activation mechanism of gp96, and to design a more efficient approach to improve the immune activity for this unique T cell adjuvant.

It has been suggested that (unlike other HSPs) gp96 might be directly involved in the assembly of MHC class I and class II-peptide complexes as a consequence of its location within the endoplasmic reticulum [1,2]. Moreover, its broad peptide-binding properties make gp96 a universal and powerful adjuvant for CTL responses [37,38]. However, despite the unique immunostimulatory properties of gp96, the efficacy of gp96-based therapeutic vaccines for the treatment of cancer has been modest in both rodent models and clinical trials [2]. One of our previous studies has shown that the efficiency of gp96-induced antitumor activity is dependent on balance between the effector T cells and Tregs [25]. In particular, it was found that RAP expressed in tumor cells was able to abrogate the interaction between gp96 and CD91 [17]. There have been a number of studies demonstrating that TAT-based delivery systems can be utilized for the efficient transduction of proteins into cultured cells and into live tissues in vivo, for cancer therapy, enzyme replacement therapy, and therapeutic vaccinations [21,39,40]. In contrast to the internalization of gp96 into APCs, which relies heavily on specific receptors (e.g. CD91 or SR-A), many of which can be disrupted by receptor competitors [7–13,17], TAT is able to deliver proteins into all cell types by directly binding to the cell surface inducing macropinocytosis and cellular transduction to the cytoplasm [41]. In this study, it was observed that vaccination with rTAT-gp96 complexed with tumor Ag induced a prominent anti-tumor immunity against B16 melanoma. As tumor cells have been shown to constitutively express RAP, it is possible that TAT may abrogate RAP-mediated blockade of binding of CD91 by gp96. In contrast, treatment with rgp96 hardly affected antigen–specific T cell responses and antiviral or antitumor activities, which was probably due to its limited internalization into APCs (Fig. 1B–D), and the vulnerability of the E. coli expressed gp96 to degradation and aggregation [42–45]. Taken together, the results from the present study indicate that the fusion of gp96 with TAT may be able to subjugate the tumor-induced suppression of gp96 internalization, and thus enhance gp96-induced antitumor T cell immune responses.

In this study, we observed the potential of using TAT-fusion as an effective strategy for improving gp96-induced viral specific cellular immunity and anti-HBV efficiency in HBV transgenic mice. However, as rTAT-gp96 immunization also resulted in the increased serum ALT levels in mice (Fig. 3D), it is likely that rTAT-gp96-induced T cells contribute to immune-mediated liver damage during viral hepatitis. The enhanced anti-HBV efficiency of the TAT-gp96 therefore occurs at the cost of triggering liver inflammation in the HBV mouse model. Thus, rTAT-gp96 complexed with HBV peptides apparently has therapeutic application against chronic HBV infection whereas its side effect of promoting immune-mediated liver damage needs to be addressed in future studies. Conceivably, it is possible that the co-administration of rTAT-gp96–based vaccines, along with antiviral therapy (e.g. nucleos(t)ide analog therapies) to reduce HBV loads, may be an effective strategy to reduce the risk of liver immunopathology.

Despite the instability feature of recombinant gp96 proteins expressed in E. coli, rTAT–gp96 was found to exhibit similar immune-activity to naturally purified gp96 (see Figs. 2–4). This suggests that besides increased internalization, TAT fusion may also affect the stability and solubility of the gp96 protein [24]. Indeed, the results of the DLS analysis showed that TAT-fusion slightly decreased the aggregation level of gp96. It is possible that TAT-induced change to the self-assembly of gp96 may also contribute to the enhancement of rTAT-gp96-induced T cell immunity. Conceivably, it is possible that the stable recombinant gp96 expressed by the yeast [45], once fused to the TAT peptide, may have even higher capability to induce T cell responses than the native gp96. This possibility is currently under investigation.

In conclusion, this study has made significant advances toward the application of TAT-gp96 fusion proteins as adjuvants for both prophylactic and therapeutic treatments. The study demonstrated that a TAT-based gp96 delivery system dramatically increased
gp96-mediated virus or tumor-specific T cell responses, and induced a corresponding inhibitory effect on viral replication and tumor growth. These results provide further evidence that the increased internalization of gp96 into APCs is a powerful strategy to enhance gp96-mediated T cell responses by overcoming the bioavailability barrier, and could help to optimize the efficiency of gp96-based vaccines against cancer and infectious diseases.

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