Published in final edited form as:

Vaccine. 2012 January 17; 30(4): 784–793. doi:10.1016/j.vaccine.2011.11.056.

LAH4 enhances CD8+ T cell immunity of protein/peptide-based vaccines

Tong Tong Zhang^{*,1}, Tae Heung Kang^{*,1}, Barbara Ma^{*}, Yijie Xu^{*}, Chien-Fu Hung^{*,§}, and T.-C. Wu^{*,†,‡,§}

^{*}Department of Pathology, Johns Hopkins University, Baltimore, MD 21205, USA

[†]Department of Obstetrics and Gynecology, Johns Hopkins University, Baltimore, MD 21205, USA

[‡]Department of Molecular Microbiology and Immunology, Johns Hopkins University, Baltimore, MD 21205, USA

[§]Department of Oncology, Johns Hopkins University, Baltimore, MD 21205, USA

Abstract

It is now clear that CD8+ T cells are crucial for therapeutic immunity against chronic viral infections and/or tumors. We reason that a strategy capable of improving CD8+ T cell activation would improve the efficacy of protein-based vaccines, which predominantly generate CD4+ T cell-mediated responses. Herein, we explore the ability of a novel cell-penetrating peptide (CPP), LAH4, to facilitate intracellular delivery of protein-based vaccines adjuvanted with Toll-like receptor 9 agonist CpG oligonucleotide (CpG) to generate enhanced CD8+ T cell immune responses and antitumor effects. LAH4 was found to mediate the intracellular delivery of both protein and nucleotide cargo and facilitate protein internalization using mechanisms involving endosomal acidification and processing through the proteasome pathway, leading to enhanced cross presentation of protein antigen by dendritic cells to CD8+ T cells. LAH4 also improved the internalization of CpG, resulting in NFκB activation, thus potentiating the adjuvant effect of CpG. We found that protein-based vaccine comprised of LAH4 mixed with model antigen and CpG generated significantly improved antigen-specific CD8+ T cell immune responses and/or antitumor effects. Furthermore, we found that LAH4 was able to enhance the ability of a tyrosinase-related protein 2 (TRP-2) peptide-based vaccine to generate TRP2-specific CD8+ T cells and antitumor effects against TRP2-expressing tumors. Thus, our results suggest that CPP technology using LAH4 is able to enhance both protein-based and peptide-based vaccine potency to generate antigen-specific CD8+ T cells and antitumor effects. Our findings serve as an important foundation for future clinical applications of CPP technology to improve protein/peptide-based vaccine potency.

© 2011 Elsevier Ltd. All rights reserved.

Address correspondence to: Dr. T.-C. Wu, Department of Pathology, School of Medicine, Johns Hopkins University, CRB II Room 309, 1550 Orleans Street, Baltimore, Maryland, 21231, USA. Phone: (410) 614-3899, Fax: (443) 287-4295. wutc@jhmi.edu.

¹Tong Tong Zhang and Tae Heung Kang contributed equally to the work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

cell penetrating peptide; protein-based vaccine; peptide-based vaccine; LAH4; protein delivery; cross-presentation

Introduction

Protein-based vaccines have several advantages for antigen-specific immunotherapy, including improved safety over live-vector based vaccines and the ability to circumvent the MHC-restriction limitation associated with peptide vaccines (for review, see [1]). However, they suffer from low immunogenicity and generally require adjuvants. In addition, protein-based vaccines are processed through the endocytic pathway and are mainly presented via the MHC class II pathway, generating predominantly CD4⁺ rather than cytotoxic CD8⁺ T cell lymphocyte (CTL), responses. Thus, protein-based vaccines require strategies that predispose the processing of protein antigen through the MHC class I presentation pathway in order to generate CD8⁺ CTL immune responses. This process of cross presentation, where exogenous antigens are processed and presented onto the MHC class I molecules of antigen-presenting cells in order to stimulate CD8⁺ CTLs, may significantly enhance protein-based vaccine potency for the control of tumors and/or chronic viral infections (for review see, [2]).

Among several strategies designed to improve cross presentation of protein antigen, one promising approach is the use of cell penetrating peptides (CPPs). CPPs are small, amphipathic molecules that are used for the delivery of macromolecules directly into cells. They have been employed in a variety of biological applications, including drug delivery [3] and as DNA transfection reagents [4]. Because of their ability to transport whole protein antigen [5–7], CPPs have emerged as a potential tool in tumor immunotherapy [8]. While the mechanism of CPP-mediated protein transduction varies between different carrier peptides, they are largely used because they can potentially facilitate cross presentation [2].

In the current study, we explore LAH4 as a novel CPP for delivering protein cargo without prior covalent linkage. LAH4 is a synthetic histidine-rich amphipathic peptide, originally designed as a tool for studying membrane-protein dynamics [9, 10], and has been used as a DNA transfection agent [11] as well as an siRNA delivery agent [12], but never as a carrier for protein. It has been proposed that the cellular uptake of LAH4/DNA complexes is through the endocytosis pathway. According to this model, after cellular uptake of LAH4/cargo and upon acidification of the endosome, LAH4 undergoes a conformational change that leads to the release of its cargo and insertion of LAH4 into the endosomal membrane, resulting in endosomal lysis and delivery of the cargo into the cytosol [13]. This feature makes LAH4 a very desirable candidate in promoting tumor antigen delivery into the cytosol of the cell to facilitate cross priming.

Herein, we characterize the ability of LAH4 to facilitate efficient cellular internalization of protein cargo and CpG oligonucleotides adjuvant (CpG). We further characterize the ability of LAH4 to mediate cross presentation of protein antigen for enhanced antitumor immunity. We also evaluate the biological applications of LAH4 in protein-based vaccines adjuvanted with CpG. We further evaluate whether LAH4 can enhance the ability of a peptide-based vaccine derived from a self-antigen, TRP-2, to generate TRP-2 peptide-specific CD8⁺ T cell immune responses and antitumor effects against TRP2-expressing tumor. Our results suggest that CPP technology using LAH4 is able to enhance the potency of both protein-based vaccine and peptide-based vaccine in generating antigen-specific CD8⁺ T cells and

antitumor effects. Our findings serve as an important foundation for future clinical translation.

Materials and Methods

Cell Lines

The immortalized dendritic cell (DC) cell line, DC2.4, which was derived from C57BL/6 mice, was kindly provided by Dr. Kenneth Rock (University of Massachusetts, Worcester, MA) [14]. The wild type human embryonic kidney 293 cell line was purchased from ATCC and the human-TLR9 expressing cell line 293XL-hTLR9 was purchased from Invivogen. 293, 293XL-hTLR9, and DC2.4 were all cultured in RPMI 1640 (Gibco) medium containing 10% fetal bovine serum, 1× nonessential amino acid, l-glutamine, sodium pyruvate, and penicillin–streptomycin, and 0.1% β-mercaptoethanol. All cells were cultured in a 37°C incubator with 5% CO₂.

Preparation of Co-mixture with LAH4

PE-labeled donkey anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch Laboratories and was used throughout the study as PE-labeled protein, unless specified otherwise. 25 pM PE-labeled protein was incubated with 0.5 μM LAH4 in PBS for 30 min at room temperature (25°C) in eppendorf tubes. 2×10⁵ DC2.4 cells were resuspended with the Protein/LAH4 co-mixture for 1 hr at 37°C. Cells were then washed twice with fresh PBS and analyzed. Peptide/LAH4 co-mixtures were prepared with methods as described for Protein/LAH4.

Confocal Fluorescence Microscopy Examination

25 pM PE-labeled protein and/or 3 μM FITC-CpG ODN (Invivogen) were co-mixed with or without 7.2 μM LAH4 before addition to 293 cells or DC2.4 cells for 1.5 hours at 37°C. Cells were then washed and plated on to poly-l-lysine coated microscope slides (Polysciences) for 30 min at 37°C. The cells were gently washed once with PBS and then fixed in 4% paraformaldehyde for 20 min at room temperature followed by three PBS washes. The glass slides were mounted with ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Images were collected using a Nikon TE-2000 microscope or imaged at the Johns Hopkins University School of Medicine Microscope Facility using Zeiss 510 Meta confocal microscope.

In vitro bioluminescence assay to compare LAH4 with various CPPs

LAH4 (sequence: KKALLALALHHLAHLALHLALALKKA) and Pep-1 (KETWWETWWTEWSQPKKKRKV) was synthesized by Genscript Corporation. The cell penetrating peptides, HIV-TAT, Antennapedia, and Membrane Penetrating Sequence (MPS) were all purchased from AnaSpec. Poly-arginine was purchased from Sigma-Aldrich. For formation of co-mixtures of recombinant luciferase protein with each of the various tested CPPs, 1 μM QuantiLum Recombinant Luciferase (Promega) was co-mixed with 35 μM of CPP (Antennapedia, HIV-TAT, Pep-1, MPS, Poly-arginine, or LAH4) for 30 min in PBS before addition to 1×10⁶ DC2.4 for 1 hr at 37°C. The cells were washed and resuspended in 150 mg/ml of D-luciferin (Xenogen) before plating on to black 96-well plates. 10 min later, bioluminescence was measured by IVIS-200 system (Xenogen) and analyzed with Living Image software (Xenogen).

Characterization of cross presentation of protein antigen in DCs

OVA-specific CD8⁺ T cell line (OT1) was previously generated in our lab by harvesting splenocytes from OT-1 transgenic RAG^{−/−} mice and stimulating them with irradiated OVA

peptide (SIINFEKL) peptide pulsed TC-1 cells in the presence of murine IL-2 (20 IU/ml) [15]. For cross presentation of OVA protein by DC2.4 cells, 1×10^5 DC2.4 cells were incubated with 4.4 μ M OVA protein pre-mixed with each of 35 μ M CPPs (Antennapedia, HIV-TAT, Pep-1, MPS, Poly-arginine, or LAH4) for 1 hr. For inhibition of cross presentation of protein antigen in DC2.4 cells by NH_4Cl or bortezomib, DC2.4 cells were pre-incubated in serum-free RPMI, with 20 mM NH_4Cl (Sigma-Aldrich), or with 7 μ M Bortezomib (Millennium Pharmaceuticals) for 1 hr before the addition of OVA protein pre-mixed with LAH4 for 1 hr. Cells were then washed and transferred to 96-well U-bottom plates and left for overnight incubation at 37°C. The next day, OT-1 were added to DC cultures for 18 hours at 2:1 E:T ratio in the presence of 1 μ g/ml GolgiPlug (BD Pharmingen). The activation of OT1 cells by DC2.4 cells was characterized by intracellular cytokine staining for IFN- γ followed by flow cytometry analysis using methods as described previously [15]. Where indicated, DCs pulsed with 1 mg/ml SIINFEKL (OVA class I peptide) was added as positive controls.

Luciferase Reporter Assay to characterize NF κ B activation

CpG-ODN 1826 (CpG) and control oligonucleotide (GpC-ODN 1928) were synthesized by Invitrogen. 5×10^4 293-hTLR9-NF κ B cells were plated in triplicates on a 96-well plate overnight. The next day, 0.01 mg, 0.1 mg, or 1 mg of LAH4 were co-mixed with 0.1 mg of CpG or negative control GpC for 30 min in 10 ml of PBS before addition to seeded cells for 18 hrs. Cells were lysed using the luciferase assay system lysis buffer from Promega. Luminescence was determined on a Wallac 1420 Victor 2 plate reader using a working solution containing 6 mM MgSO_4 , 2mM ATP and 0.6mM D-luciferin.

Immunization of Mice

All animal experimental work was done in accordance with Johns Hopkins Medical Institutions Animal Care and Use Committee guidelines. 6–8 week old C57BL/6 mice (National Cancer Institute, 5 per group) were immunized by subcutaneous injection with 10 μ g OVA protein or TRP-2 peptide (aa 180–188, SVYDFVWL) alone and/or 1 μ g CpG-ODN with or without 10 μ g LAH4 in 50 μ l of PBS. Boost injections were given at the same dose and regimen on days 7 and 14 after initial vaccination.

Characterization of Antigen-specific T cell-mediated Immune Responses in Vaccinated Mice

Splenocytes or peripheral blood mononuclear cells (PBMCs) were harvested from vaccinated mice 1 week after last vaccination (or 7 weeks for long-term immunity). Before intracellular cytokine staining, 5×10^5 pooled splenocytes or PBMCs from each vaccination group were incubated overnight with 1 μ g/ml of MHC class I OVA peptide (SIINFEKL), class II OVA peptide (aa 323–339, LSQAVHAAHAEINEAGR) or TRP2 peptide (aa 180–188, SVYDFVWL) in the presence of 1 μ l/ml GolgiPlug for 18 hrs. Cell surface marker staining of CD8 and intracellular cytokine staining for IFN- γ using Phycoerythrin-conjugated monoclonal rat anti-mouse CD8 antibody (BD Pharmingen) and FITC-conjugated anti-mouse IFN- γ antibodies (BD Pharmingen) as well as flow cytometry analysis, were performed using conditions described previously. Antigen-specific T cell immune responses were analyzed by intracellular cytokine staining for IFN- γ followed by flow cytometry analysis as described previously [16]. After staining, flow cytometry analysis was performed using FACScalibur with CellQuest software. All of the analyses shown were carried out on a gated lymphocyte population.

***In vivo* tumor experiments**

The OVA-expressing B16 murine tumor model has been described previously [17]. For *in vivo* tumor protection experiments, C57BL/6 mice were vaccinated with OVA protein, OVA/LAH4, OVA/CpG, or OVA/CpG/LAH4 as described above. One week after the last vaccination, C57BL/6 mice were challenged with 1×10^5 cells/mouse of B16/OVA tumor cells in the right leg. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week and sacrificed at day 60 after last vaccination. For *in vivo* tumor treatment experiments, mice were challenged with 1×10^5 B16/OVA tumor cells subcutaneously. Three days after tumor challenge, mice were vaccinated with 3 weekly vaccinations as described. Mice were monitored twice a week and sacrificed on day 30 after the last vaccination. Antibody depletion experiments were performed as described previously [18]. For *in vivo* tumor protection experiments in the TRP-2 peptide-based vaccine, C57BL/6 mice were vaccinated with TRP2 peptide (aa 180–188), TRP2/LAH4, TRP2/CpG, or TRP2/CpG/LAH4 similar to what is described above. Vaccinated mice were challenged subcutaneously with 1×10^5 cells/mouse of B16 tumor one week after the last vaccination. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week and sacrificed at day 60 after last vaccination.

Statistical analysis

All data expressed as means $\pm$ standard deviation (SD) are representative of at least two different experiments. Comparisons between individual data points were made using a Student's t-test. Tumor sizes were calculated using the following equation: (tumor length $\times$ width $\times$ height) / 2. For statistical analysis of the tumor protection and antibody depletion experiments, we used Kaplan-Meier analysis. The value of $p < 0.05$ was considered significant.

Results

LAH4 facilitates protein internalization and cross-presentation

We initially characterized the ability of LAH4 to facilitate binding of protein cargo to the cell. Our data show that co-mixture of LAH4 and protein can bind to DC2.4 dendritic cells in a concentration-dependent manner and that prior co-mixing of LAH4 and protein is critical for the efficient binding of protein to the cell (Supplementary Figure 1). We next examined whether the LAH4-mediated binding of protein cargo to cells could lead to internalization of the bound protein. Cells incubated with PE-labeled protein alone were visualized by confocal microscopy and showed no fluorescence signal while cells incubated with PE-labeled protein/LAH4 co-mixtures showed fluorescence signal consistent with cellular internalization as demonstrated by its punctate staining pattern (Figure 1A). To directly measure the ability of LAH4 to facilitate protein internalization in comparison to other well-known CPPs, luciferase protein was co-mixed with LAH4 or each of 5 well-studied CPPs (Antennapedia, HIV-TAT, Pep-1, Membrane Penetrating Sequence (MPS), and Polyarginine) before addition to DC2.4 cells. Because luciferase enzymatic activity requires ATP [19], which is only found intracellularly in our system, luciferase activity was used as an indicator of protein internalization. Cells incubated with luciferase protein co-mixed with LAH4 led to significantly improved luciferase activity over recombinant luciferase alone or luciferase co-mixed with the other CPPs (Figure 1B). Furthermore, since internalization by many CPPs involves direct membrane disruption, we checked for the cytotoxicity of LAH4. At concentrations of 1 μ g of LAH4/50 μ L of PBS, we found no effect on cell death after overnight incubation with LAH4 (Supplementary Figure 2). While there was a slight increase in cell toxicity at 10 μ g of LAH4, this increase in cytotoxicity was marginal as compared to the overall cytotoxicity of another CPP, polyarginine. Thus, LAH4 is able to mediate the internalization of noncovalently bound proteins with minimal toxicity.

The ability of LAH4 to internalize proteins may have important implications in improving protein presentation through the MHC class I pathway within dendritic cells (DCs). We examined the contribution of LAH4 to the cross presentation of OVA protein by adding co-mixed LAH4 and OVA to DC2.4 cells. These treated DCs were then used to stimulate OVA-specific CD8⁺ T cells (OT1 cells) overnight. T cell activation was measured using flow cytometry analysis of intracellular staining for IFN- γ to determine the percentage of IFN- γ -producing T cells. DCs incubated with OVA+LAH4 complexes demonstrated a significant improvement of T cell activation as compared to DCs incubated with OVA protein alone (Figure 1C). Control DCs incubated with LAH4 alone did not generate any appreciable T cell activation. We also compared the ability of LAH4 to improve cross presentation with other widely used CPPs such as Antennapedia, MPS, Pep-1, Poly-arginine, and HIV-TAT. LAH4 was able to best enhance cross presentation of bound OVA protein to activate OT1 T cells as compared to other CPPs (Figure 1D). The other tested CPPs, when co-mixed with OVA protein in the same molar ratio as LAH4, were unable to significantly improve cross presentation over OVA protein alone. While previous groups have demonstrated these widely used CPPs to be effective in improving cross presentation, most required physical conjugation to its protein cargo or required higher CPP+protein concentrations ([5, 20], for review, see [21]). Our results indicate that LAH4 is able to enhance the cross presentation of noncovalently-bound protein in DCs compared to other tested CPPs.

Endosomal acidification and the proteasome pathway are important for the cross priming of the protein antigen mediated by LAH4

The binding of protein cargo to the cell is the first step for cross presentation to occur. We found that cell surface proteoglycan content contributes to the cell binding of proteins mediated by LAH4 (Supplementary Figure 3). After cellular uptake of LAH4/protein cargo, the next potentially important steps in cross presentation may involve the release of the LAH4/protein complex from the endosomal/lysosomal compartment into the cytosol as well as the subsequent processing by the proteasome degradation pathway. We therefore used ammonium chloride (NH₄Cl) [22] and bortezomib to study the involvement of endosomal acidification as well as the proteasome pathway, respectively, to further characterize the mechanism of cross presentation of protein antigen mediated by LAH4. We examined the effect of inhibitors on the ability of dendritic cells, which have been pulsed with LAH4 and OVA protein, to activate OT1 cells as measured by flow cytometry analysis of intracellular staining for the percentage of IFN- γ producing T cells. DC2.4 cells incubated with co-mixture of OVA protein and LAH4 in the presence of NH₄Cl showed significantly decreased ability to activate OT1 cells as compared to co-mixture of OVA protein and LAH4 in the absence of NH₄Cl (Figure 2). In addition, DC2.4 cells that were pre-incubated with 7 μ M Bortezomib and then pulsed with co-mixed LAH4 and OVA, demonstrated significantly lower ability to activate OT1 cells as compared to DCs incubated without inhibitor (Figure 2). These results indicate that both endosomal acidification and the proteasome pathway are important in the LAH4-mediated improvement of MHC class I presentation of protein in dendritic cells. As a control, we examined whether the endosomal acidification inhibitor or proteasome inhibitor would influence the ability of DCs pulsed with OVA MHC class I CTL epitope (SIINFEKL), which does not require internalization or processing, to activate OT1 T cells. We did not observe a significant difference between SIINFEKL-pulsed DCs with or without inhibitor (Figure 2). Taken together, these results support that endosomal acidification and the proteasome pathway are required for the enhanced cross presentation of protein in dendritic cells mediated by LAH4.

LAH4 mediates the concurrent internalization of protein cargo and CpG

It is now clear that most protein-based vaccines require the usage of adjuvant to enhance vaccine potency (for reviews, see [1, 23]). Thus, it is important to determine whether LAH4 can mediate the intracellular delivery of both protein of interest and intracellular Toll-like receptor ligand adjuvant such as CpG oligonucleotide adjuvant (CpG). While other investigators have previously shown LAH4 as an effective tool for nucleotide transfection [4, 12], we sought to use LAH4 as a reagent to simultaneously internalize CpG and protein antigen. We used confocal microscopy to determine the cellular internalization of LAH4 co-mixed with FITC-labeled CpG and PE-labeled protein together in DC2.4 cells. As shown in Figure 3A, CpG and Protein co-mixed with LAH4 demonstrated intracellular colocalization. Likewise, in another cell line, 293, co-mixture of LAH4 with CpG-FITC or PE-labeled protein also showed obvious internalization in 293 cells compared to co-mixtures without LAH4 (Supplementary Figure 4). These data support the ability of LAH4 to mediate delivery of protein cargo and CpG in different cell lines.

We further determined whether the LAH4-mediated internalization of the intracellular TLR ligand CpG would lead to enhanced activation of NF κ B signaling pathway. When CpG was mixed with different concentrations of LAH4 and added to human-TLR9 expressing 293 cells stably transfected with pGreenfire-NF κ B luciferase reporter construct, cells incubated with 0.1 μ g CpG and 1 μ g LAH4 were able to significantly improve the NF κ B activation pathway when compared to 0.1 μ g CpG alone (Figure 3B). This induction was, in fact, not significantly different from the induction with the higher 1 μ g concentration of CpG. In comparison, a reversed CpG sequence, GpC, used as a negative control, was unable to induce the same level of activation. Our data showed that the internalization of CpG mediated by LAH4 resulted in activation of the NF κ B signaling pathway.

Co-mixture of LAH4 with protein and CpG leads to increased retention and migration of protein *in vivo*

Because antigen-presenting cells (APCs) such as DCs are capable of engulfing antigen and migrating to the draining lymph nodes, we tested the ability of LAH4 to enhance the uptake of protein antigen by APCs at the injection site, which would lead to the migration of the antigen-loaded DCs to the draining lymph nodes to activate antigen-specific CD8⁺ T cells. To investigate the effect of LAH4 on protein retention and internalization *in vivo*, mice were injected subcutaneously with co-mixed LAH4 with CpG and fluorescently-labeled APC-Alexa fluor-750 streptavidin protein (protein+LAH4+CpG), or co-mixed protein and LAH4 (protein+LAH4), co-mixed protein and CpG (protein+CpG), or protein alone, and followed for the retention of protein at the site of injection as well as the migration of the protein to lymph nodes. Both protein+LAH4 and protein+LAH4+CpG co-mixtures demonstrated similar retention at the site of injection over time, and significant improvement in retention over protein+CpG or protein alone (Figure 4A). In addition, draining inguinal lymph nodes harvested from mice injected with protein+CpG or protein+CpG+LAH4 co-mixture demonstrated significantly greater fluorescence intensity on day 2 compared to mice injected with protein only or protein+LAH4. However, the only group injected with protein+CpG+LAH4 had strong fluorescence intensity on day 5 (Figure 4B). Similarly, when mice were injected with mixtures of LAH4 and CpG with OVA protein and examined for the ability of lymph node cells to activate OT1 cells *ex vivo*, lymph nodes harvested from mice injected with OVA+CpG+LAH4 co-mixtures were able to demonstrate significantly improved activation of CD8⁺ T cells over mice injected with OVA protein alone, OVA+LAH4, or OVA+CpG (Figure 4C). Taken together, these data suggest the combination of LAH4 and CpG with protein is able to facilitate the retention of protein at the site of injection, lead to increased accumulation of the protein in the draining lymph nodes, and result in improved activation of protein antigen-specific CD8⁺ T cells.

Vaccination of mice with OVA/CpG/LAH4 protein-based vaccine generates enhanced antigen-specific CD4⁺ and CD8⁺ T cell immune responses

To evaluate the ability of a protein-based vaccine comprised of OVA and CpG mixed with LAH4 (OVA/CpG/LAH4) to generate an OVA antigen-specific immune response, we vaccinated C57BL/6 mice with injections of OVA protein alone, OVA/LAH4, OVA/CpG, or OVA/CpG/LAH4. One week after the last injection, splenocytes from vaccinated mice were harvested and characterized for the number of OVA-specific CD8⁺ T cells using intracellular cytokine staining for IFN- γ followed by flow cytometry analysis. Mice injected with OVA/CpG/LAH4 were able to demonstrate the largest number of OVA-specific CD8⁺ T cells, with approximately three times more OVA-specific CD8⁺ T cells than mice injected with OVA/CpG (Figure 5A). These OVA-specific CD8⁺ T cell immune responses were maintained long term at 7 weeks post injection (Figure 5B). Furthermore, significant enhancement of OVA-specific IFN- γ +CD4⁺ T cell immune responses was observed in mice vaccinated with OVA/CpG/LAH4 compared to mice vaccinated with OVA protein alone (Figure 5C).

Vaccination of mice with OVA/CpG/LAH4 protein-based vaccine generates enhanced protective and therapeutic antitumor effects, primarily through CD8⁺ T cell immune responses

To determine if the enhanced OVA-specific CD8⁺ and CD4⁺ T cell immune responses generated by OVA/CpG/LAH4 protein-based vaccine could translate to potent antitumor effects, we performed *in vivo* tumor protection and tumor treatment experiments. Mice vaccinated with OVA/CpG/LAH4 or OVA/CpG demonstrated significantly prolonged survival as compared to mice vaccinated with OVA/LAH4 or OVA alone upon challenge with B16/OVA tumor cells (Figure 6A). To distinguish the antitumor effects between OVA/CpG/LAH4 and OVA/CpG, we further characterized the therapeutic antitumor effects of the various vaccinations. C57BL/6 mice were inoculated subcutaneously with B16/OVA tumor cells and treated with the various vaccination groups three days later. Tumor-bearing mice treated with OVA/CpG/LAH4 generated significantly better therapeutic antitumor effects over all other treatment groups, including OVA/CpG (Figure 6B). We also noted that tumor-bearing mice treated with irrelevant protein E7 mixed with CpG and LAH4 did not generate appreciable antitumor effects (data not shown). We further investigated the critical immune cell subsets responsible for the antitumor effects conferred by our OVA/CpG/LAH4 protein-based vaccine using antibody depletion of CD4, CD8, and NK cells. We found that vaccinated mice with depletion of CD8⁺ T cells demonstrated significantly reduced survival compared to vaccinated mice with no depletion, CD4 cell depletion, or NK cell depletion (Figure 6C). Thus our data suggest that CD8⁺ T cells are essential for the antitumor effects generated by OVA/CpG/LAH4 protein-based vaccine.

Vaccination with TRP2/CpG/LAH4 peptide-based vaccine generates enhanced TRP2-specific CD8⁺ T cell immune responses and potent antitumor effects

The application of LAH4 in a clinically relevant antigenic system serves as an important foundation for future clinical translation. Tyrosinase-related protein 2 (TRP-2) is a melanosomal enzyme that has been widely used as a target antigen for the control of melanoma. As TRP-2 is a self antigen, it is less immunogenic and represents a more stringent system for testing the LAH4 strategy. Thus, by using TRP-2 as a model self-antigen, we generated a peptide-based vaccine using TRP-2 (aa 181–188) adjuvanted with CpG, and co-mixed with LAH4 (TRP2/CpG/LAH4). C57BL/6 mice were vaccinated with TRP2 alone, TRP2/LAH4, TRP2/CpG, or with TRP2/CpG/LAH4. One week after the last vaccination, PBMCs from vaccinated mice were harvested and the TRP2-specific T cell immune responses were characterized using intracellular cytokine staining for IFN- γ followed by flow cytometry analysis. Mice vaccinated with TRP2/CpG/LAH4 demonstrated

significantly higher TRP2-specific CD8⁺ T cell immune responses as compared to mice vaccinated with TRP2 alone or TRP2 with CpG (Figure 7A). To determine if the enhanced TRP2-specific CD8⁺ T cell immune responses generated by TRP2/CpG/LAH4 peptide-based vaccine could translate to potent antitumor effects, we performed *in vivo* tumor protection experiments. Mice vaccinated with TRP2/CpG/LAH4 demonstrated significantly prolonged survival as compared to mice vaccinated with any of the other groups upon challenge with B16 tumor cells (Figure 7B). Thus, our data suggest that co-administration of LAH4 is also able to enhance the antigen-specific CD8⁺ T cell-mediated immune responses and generate potent antitumor effects in a clinically relevant model.

Discussion

Our study demonstrates for the first time that LAH4 can be used to facilitate the delivery of protein cargo into cells. We demonstrated that incubation of LAH4 with protein led to the formation of noncovalently linked (co-mixed) LAH4/protein complexes in a concentration-dependent manner. Additionally, these LAH4/protein complexes could be internalized into multiple cell types and lead to enhanced cross presentation of protein antigens in dendritic cells through a mechanism that requires endosomal acidification and the proteasome pathway. We also found that LAH4 is able to mediate the concurrent delivery of protein cargo and CpG oligonucleotide into cells. LAH4 co-mixed with CpG and protein led to increased retention at the local injection site and increased accumulation of protein in the draining lymph nodes in vaccinated mice. In addition, our data suggest that LAH4 improves both DC uptake of proteins at the local injection site as well as DC maturation by CpG adjuvant, which likely results in increased migration to the draining lymph nodes and activation of antigen-specific T cells. We also observed that vaccination of mice with protein-based vaccine comprised of co-mixed Protein/CpG/LAH4 generates enhanced antigen-specific CD4⁺ and CD8⁺ T cell immune responses and enhanced protective and therapeutic antitumor effects, primarily through CD8⁺ T cells. Furthermore, we found that LAH4 was able to enhance the ability of a peptide-based vaccine to generate antigen-specific CD8⁺ T cell immune responses and antitumor effects. Thus, our findings suggest that LAH4 represents a novel CPP that can serve as an effective reagent for improving both protein-based and peptide-based vaccine potency for cancer immunotherapy.

Several factors indicate that LAH4 may represent a more desirable reagent for enhancing protein-based vaccines as compared to other well-known CPPs. First, LAH4 can deliver proteins intracellularly without covalent linkage to its protein cargo, therefore providing the potential to transduce a greater range of tumor antigens with increased ease and efficiency. In the past, the employment of CPPs for enhancing the delivery of protein has been hindered by the need to covalently link the individual cargo proteins to the carrier peptides. Our study shows that LAH4 facilitates the internalization of protein without covalent linkage in a variety of cell types and does so at a higher efficiency than other CPPs (TAT, Polyarginine, Antennapedia, etc) when co-mixed. Though other CPPs, such as Pep-1, have in previous studies shown the ability to transduce protein without covalent linkage [6], we observed that these CPPs were unable to confer a high amount of protein transduction as compared to LAH4 when used at similar molar ratios (Figure 1D). Such noncovalent linkage also allows for exploration of the optimal ratio of carrier: cargo for intracellular delivery of protein cargo. Second, LAH4 is relatively safe. We observed that LAH4 generates far minimal cytotoxicity as compared to another CPP such as polyarginine (Supplementary Figure 2). This suggests that LAH4 may be suitable for future clinical translation. In addition, while most CPPs are cytotoxic at high concentrations due to their membrane disruptive properties, the concentrations of LAH4 used in this study did not inhibit protein internalization either *in vitro* or *in vivo*. Third, we have shown LAH4 to significantly enhance the cross presentation of protein antigen in DCs over other co-mixed CPPs. Finally, the ability of LAH4 to co-

deliver protein cargo with intracellular Toll-like receptor agonist makes it quite desirable to further enhance protein-based vaccines compared to other CPPs. Taken together, our results have shown LAH4 to exhibit several advantages over other tested CPPs in such areas as convenience in preparation, improved safety, and greater efficiency in mediating cross presentation of protein cargo.

In summary, the enhanced intracellular delivery of protein and CpG mediated by LAH4 may serve as an important strategy to improve protein-based cancer vaccine potency. In addition, we showed that LAH4 could enhance the antigen-specific CD8⁺ T cells and antitumor effects generated by a peptide-based vaccine derived from a clinically relevant self-antigen. Thus, LAH4 has been tested in two different antigenic systems and can potentially serve as a platform for applications to other clinically relevant antigenic systems to enhance CD8⁺ T cell immune responses for the control of chronic infectious disease and/or cancer.

Highlights

>LAH4 facilitates protein internalization and cross-presentation. >Co-administration of LAH4 increases retention/migration of protein *in vivo*. >OVA/CpG/LAH4 vaccine enhances antigen-specific cell mediated immune responses. >OVA/CpG/LAH4 vaccine generates enhanced protective and therapeutic antitumor effects. >TRP2/CpG/LAH4 peptide-based vaccine generates potent antitumor effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

CPP	cell-penetrating peptide
CpG	CpG oligonucleotide
DC	dendritic cell

Acknowledgments

We would like to thank Elizabeth M. Jaffee, James Eshleman, Linzhao Cheng, and Joel Pomerantz for helpful discussions. We would also like to thank Janson Trieu and Ellen M. Axenfeld for critical review of the manuscript. This work was supported by the National Cancer Institute SPORE in Cervical Cancer P50 CA098252 and the 1 RO1 CA114425-01.

References

1. Su JH, Wu A, Scotney E, Ma B, Monie A, Hung CF, et al. Immunotherapy for cervical cancer: Research status and clinical potential. *BioDrugs*. 2010 Apr 1; 24(2):109–129. [PubMed: 20199126]
2. Ackerman AL, Cresswell P. Cellular mechanisms governing cross-presentation of exogenous antigens. *Nat Immunol*. 2004 Jul; 5(7):678–684. [PubMed: 15224093]
3. Sugahara KN, Teesalu T, Karmali PP, Kotamraju VR, Agemy L, Greenwald DR, et al. Coadministration of a tumor-penetrating peptide enhances the efficacy of cancer drugs. *Science*. 2010 May 21; 328(5981):1031–1035. [PubMed: 20378772]
4. Kichler A, Leborgne C, Marz J, Danos O, Bechinger B. Histidine-rich amphipathic peptide antibiotics promote efficient delivery of DNA into mammalian cells. *Proc Natl Acad Sci U S A*. 2003 Feb 18; 100(4):1564–1568. [PubMed: 12563034]

5. Gros E, Deshayes S, Morris MC, Aldrian-Herrada G, Depollier J, Heitz F, et al. A non-covalent peptide-based strategy for protein and peptide nucleic acid transduction. *Biochim Biophys Acta*. 2006 Mar; 1758(3):384–393. [PubMed: 16545342]
6. Morris MC, Depollier J, Mery J, Heitz F, Divita G. A peptide carrier for the delivery of biologically active proteins into mammalian cells. *Nat Biotechnol*. 2001 Dec 19;(12):1173–1176. [PubMed: 11731788]
7. Stewart KM, Horton KL, Kelley SO. Cell-penetrating peptides as delivery vehicles for biology and medicine. *Org Biomol Chem*. 2008 Jul 7; 6(13):2242–2255. [PubMed: 18563254]
8. Batchu RB, Moreno AM, Szmania SM, Bennett G, Spagnoli GC, Ponnazhagan S, et al. Protein transduction of dendritic cells for NY-ESO-1-based immunotherapy of myeloma. *Cancer Res*. 2005 Nov 1; 65(21):10041–10049. [PubMed: 16267030]
9. Bechinger B. Towards membrane protein design: pH-sensitive topology of histidine-containing polypeptides. *J Mol Biol*. 1996 Nov 15; 263(5):768–775. [PubMed: 8947574]
10. Bechinger B, Ruyschaert JM, Goormaghtigh E. Membrane helix orientation from linear dichroism of infrared attenuated total reflection spectra. *Biophys J*. 1999 Jan; 76(1 Pt 1):552–563. [PubMed: 9876168]
11. Kichler A, Leborgne C, Savage PB, Danos O. Cationic steroid antibiotics demonstrate DNA delivery properties. *J Control Release*. 2005 Sep 20; 107(1):174–182. [PubMed: 16126297]
12. Langlet-Bertin B, Leborgne C, Scherman D, Bechinger B, Mason AJ, Kichler A. Design and evaluation of histidine-rich amphipathic peptides for siRNA delivery. *Pharm Res*. 2010 Jul; 27(7):1426–1436. [PubMed: 20393870]
13. Prongidi-Fix L, Sugawara M, Bertani P, Raya J, Leborgne C, Kichler A, et al. Self-promoted cellular uptake of peptide/DNA transfection complexes. *Biochemistry*. 2007 Oct 9; 46(40):11253–11262. [PubMed: 17848098]
14. Shen Z, Reznikoff G, Dranoff G, Rock KL. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. *J Immunol*. 1997 Mar 15; 158(6):2723–2730. [PubMed: 9058806]
15. Peng S, Monie A, Kang TH, Hung CF, Roden R, Wu TC. Efficient delivery of DNA vaccines using human papillomavirus pseudovirions. *Gene Ther*. 2010 Dec; 17(12):1453–1464. [PubMed: 20668481]
16. Kim TW, Hung CF, Boyd DA, He L, Lin CT, Kaiserman D, et al. Enhancement of DNA vaccine potency by coadministration of a tumor antigen gene and DNA encoding serine protease inhibitor-6. *Cancer Res*. 2004 Jan 1; 64(1):400–405. [PubMed: 14729651]
17. Kang TH, Lee JH, Song CK, Han HD, Shin BC, Pai SI, et al. Epigallocatechin-3-gallate enhances CD8+ T cell-mediated antitumor immunity induced by DNA vaccination. *Cancer Res*. 2007 Jan 15; 67(2):802–811. [PubMed: 17234792]
18. Kang TH, Monie A, Wu LS, Pang X, Hung CF, Wu TC. Enhancement of protein vaccine potency by in vivo electroporation mediated intramuscular injection. *Vaccine*. 2010 Dec 4.
19. Kimmich GA, Randles J, Brand JS. Assay of picomole amounts of ATP, ADP, and AMP using the luciferase enzyme system. *Anal Biochem*. 1975 Nov; 69(1):187–206. [PubMed: 2029]
20. Saleh AF, Aojula H, Arthanari Y, Offerman S, Alkotaji M, Pluen A. Improved Tat-mediated plasmid DNA transfer by fusion to LK15 peptide. *J Control Release*. 2010 Apr 19; 143(2):233–242. [PubMed: 20060860]
21. Morris MC, Deshayes S, Heitz F, Divita G. Cell-penetrating peptides: from molecular mechanisms to therapeutics. *Biol Cell*. 2008 Apr; 100(4):201–217. [PubMed: 18341479]
22. Chen GL, Sutrina SL, Frayer KL, Chen WW. Effects of lysosomotropic agents on lipogenesis. *Arch Biochem Biophys*. 1986 Feb 15; 245(1):66–75. [PubMed: 3947102]
23. Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. *Immunity*. 2010 Oct 29; 33(4):492–503. [PubMed: 21029960]

Figure 1. Characterization of internalization and cross presentation of protein mediated by LAH4

(a). Confocal fluorescence microscopic examination of 293 cells incubated with PE-labeled protein (red) alone (*left panel*) or PE-labeled protein pre-mixed with LAH4 (*right panel*). Slides were counterstained with DAPI (blue) for nuclear staining before visualization with confocal microscopy. (b). Characterization of luciferase activity by luminescent imaging of DC2.4 cells incubated with recombinant luciferase protein (r-luciferase) pre-mixed with each of the various cell-penetrating peptides (Antennapedia, HIV-TAT, Pep-1, MPS, Poly-arginine, or LAH4) at equal molar ratios. (c). Flow cytometry analysis to characterize activation of OVA-specific CD8+ T Cells (OT1) by DC2.4 cells pulsed with OVA protein

with or without LAH4. **(d)**. Representative bar graph to demonstrate activation of OT1 cells by DCs incubated with OVA protein alone or pre-mixed with each of the various CPPs. Data shown are representative of 2 experiments performed. NS denotes a $p > 0.5$.

Figure 2. Characterization of the mechanisms involved in cross presentation of protein antigen mediated by LAH4

(a). Representative figure of flow cytometry analysis of intracellular cytokine staining for IFN- γ to characterize the activation of OT1 T cells by DC2.4 cells. DC2.4 cells were pre-incubated with or without 20 mM NH₄Cl or 7 M Bortezomib for 1 hr before addition of OVA protein alone or OVA pre-mixed with LAH4. NH₄Cl is a lysosomotropic inhibitor of endosomal/lysosomal acidification. Bortezomib is a specific inhibitor of the 26S proteasome. LAH4 was included as a negative control. MHC class I-restricted OVA-specific CTL epitope, SIINFEKL, was included for comparison. (b). Bar graph depicting percentage of IFN- γ -expressing OVA-specific CD8⁺ T cells out of total CD8⁺ T cells activated by

DC2.4 cells pre-incubated with OVA protein alone or OVA pre-mixed with LAH4 (mean $\pm$ SD). Data shown are representative of 2 experiments performed.

A **B**

Figure 3. Characterization of the intracellular localization of protein and CpG mixed with LAH4 and characterization of NFkB activation by CpG with or without LAH4

(a). Confocal fluorescence microscopic examination of DC2.4 cells incubated with PE-labeled protein and FITC-labeled-CpG-ODN pre-mixed with LAH4. Staining of DAPI was used for nuclear staining. (b). Representative bar graph to demonstrate NFkB activity by luciferase reporter assay of 293-hTLR9-NFkB reporter cells incubated with CpG-ODN 1826 (CpG) alone, GpC-ODN 1928 (GpC) alone, LAH4 alone, or CpG or GpC co-mixed with LAH4 at various concentrations. Data shown are representative of 3 experiments performed.

Figure 4. Characterization of the subcutaneous retention and lymph node migration of protein antigen and CpG pre-mixed with LAH4 (Protein+CpG+LAH4) in vivo

(a). Characterization of fluorescence activity in C57BL/6 mice (5 per group) injected subcutaneously with 1 μ g Alexa Fluor 750 APC-streptavidin (Protein) alone, 1 μ g protein pre-mixed with 1 μ g LAH4 (protein+LAH4), 1 μ g protein and 0.1 μ g CpG-ODN 1826 (protein+CpG), or 1 μ g protein and 0.1 μ g CpG pre-mixed with 1 μ g LAH4 (protein+CpG+LAH4) in a final volume of 50 μ l PBS. Fluorescence activity was measured immediately after injection (Day 0), 1 day, or 2 days post-injection. Imaging was conducted on IVIS system (Xenogen/Caliper Life Sciences) with an acquisition time of 1 minute. Representative imaging is depicted in upper panel. Representative line graph is depicted in lower panel. (b). Characterization of fluorescence activity of harvested inguinal lymph nodes from mice injected subcutaneously with protein alone, protein+LAH4, protein+CpG, or with protein+LAH4+CpG as described above. Inguinal lymph nodes were harvested from different groups of mice and imaged at 2 days or 5 days after injection. Bar graph depicts difference in fluorescence on day 2 (black bars) and day 5 (white bars) after injection (mean $\pm$ SD). (c). Representative figure of flow cytometry analysis to demonstrate activation of OT1 cells by cells derived from lymph node isolated 5 days post-injection from mice that were untreated or subcutaneously injected with OVA protein, OVA+LAH4, OVA+CpG, or OVA+CpG+LAH4. Inguinal lymph nodes were harvested from mice 5 days after subcutaneous injection with 10 μ g of OVA protein alone (OVA), or 10 μ g of OVA pre-

mixed with 10 µg of LAH4 alone (OVA+LAH4), 10 µg of OVA and 1 µg CpG-ODN alone (OVA+CpG), or 10 µg of OVA and CpG-ODN pre-mixed with 10 µg of LAH4 (OVA+CpG+LAH4) in a final volume of 50 µl of PBS. Lymph node cells were lysed for red blood cells and then incubated with OT1 T cells at 1:10 T cell:lymphocytes ratio for 18 hrs in the presence of GolgiPlug. Activation of OT1 T cells was measured by intracellular cytokine staining for IFN γ followed by flow cytometry analysis. Data shown are representative of 2 experiments performed.

Figure 5. Characterization of antigen-specific T cell-mediated immune responses in mice vaccinated with OVA and CpG mixed with LAH4 (OVA/CpG/LAH4)
C57BL/6 mice (5 per group) were subcutaneously injected with OVA protein, OVA/LAH4, OVA/CpG, or OVA/CpG/LAH4 for 3 weeks at 1-week interval. (a). Intracellular cytokine staining for IFN- γ followed by flow cytometry analysis to characterize OVA-specific CD8+ T cells expressing MHC class I OVA peptide (SIINFEKL) in mice vaccinated with the various OVA protein-based vaccines. (b). Intracellular cytokine staining for IFN- γ followed by flow cytometry analysis to characterize long term (7 weeks) OVA-specific CD8+ T cell immune responses in mice vaccinated with the various OVA protein-based vaccines. (c). Intracellular cytokine staining for IFN- γ followed by flow cytometry analysis to characterize OVA-specific CD4+ T cells expressing class II OVA peptide (aa 323–339, LSQAVHAAHAEINEAGR) in mice vaccinated with the various OVA protein-based vaccines. Data shown are representative of 3 experiments performed.

Figure 6. Characterization of the antitumor effects in mice vaccinated with OVA/CpG/LAH4
 C57BL/6 mice (5 per group) were subcutaneously injected with OVA protein, OVA/LAH4, OVA/CpG, or OVA/CpG/LAH4 for 3 weeks at 1-week interval. **(a).** Kaplan & Meier survival analysis of C57BL/6 mice left untreated (control) or vaccinated with the various OVA protein-based vaccines and then challenged with 1×10^5 B16/OVA tumor cells/mouse one week after the last vaccination. **(b).** Tumor treatment experiment of C57BL/6 mice challenged with 1×10^5 B16/OVA tumor cells subcutaneously, with immunizations initiated three days after tumor challenge. Mice were boosted twice with the various OVA protein-based vaccines at the same dose and regimen. **(c).** Kaplan & Meier survival analysis of the vaccinated mice depleted of subsets of lymphocytes. Mice were immunized as described above and then challenged with B16/OVA tumors one week after the last vaccination. CD4, CD8, or NK1.1 depletions were initiated 1 week before tumor challenge. Data shown are representative of 3 experiments performed.

Figure 7. Characterization of the TRP2-specific CD8+ T cell immune responses and antitumor effects generated by mice vaccinated with TRP2/CpG/LAH4

(a) C57BL/6 mice (5 per group) were left untreated (control) or vaccinated with TRP2 peptide (aa 180–188) alone, TRP2/CpG, TRP2/LAH4, or TRP2/CpG/LAH4. PBMCs were collected 1 week after last immunization and the TRP2 peptide-specific CD8+ T cell immune responses were characterized using intracellular cytokine staining for IFN- γ followed by flow cytometry analysis. *Left panel*, Representative flow cytometry data depicts the number of TRP2 peptide-specific CD8+ T cells. *Right panel*, Bar graph represents the number of TRP2-specific CD8+ T cells/ 3×10^5 splenocytes (mean $\pm$ SD) (b) Kaplan & Meier survival analysis of vaccinated mice challenged with B16 tumors. C57BL/6 mice (5 per group) were left untreated (control) or immunized with TRP2, TRP2/LAH4, TRP2/CpG, or TRP2/CpG/LAH4 and then challenged with B16 tumor one week after the last vaccination. Mice were monitored for evidence of tumor growth by palpation and inspection. Line graph depicts the percentages of mice surviving per group. Data shown are representative of 2 experiments performed.