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Viruslike Particles Encapsidating Respiratory Syncytial Virus M and M2 Proteins Induce Robust T Cell Responses

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Abstract

Subunit vaccines provide a safe, focused alternative to conventional vaccines. However, these vaccines often require significant adjuvants and are particularly hard to target toward cytotoxic T lymphocyte (CTL) immunity. Viruslike particles (VLPs) provide biomaterial scaffolds with pathogen-like polyvalent structures making them useful platforms for biomimetic antigen delivery to the immune system. Encapsulation of antigens within VLPs has been shown to enhance antigen availability for CD8 T cell responses. Here, we examine the potential to generate complex responses to multiple subunit antigens localized within the same VLP particle. Two proteins of respiratory syncytial virus (RSV) with well-characterized CD8 T cell responses, the matrix (M) and matrix 2 (M2) proteins, were successfully coencapsidated within the P22 VLP. Upon intranasal administration in mice, the particles stimulated CD8 T cell memory responses against both antigens. In addition, vaccination elicited tissue-resident T cell populations. Upon subsequent RSV challenge, P22-M/M2-treated mice displayed significantly reduced lung viral titers. This demonstrates the utility of the P22 VLP in directing immune responses to multiple encapsidated

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Author Contributions

T.D., B.S.G., and D.P.P. conceived initial P22-M/M2 design. B.S., T.D., and H.M.M. optimized final P22-M/M2 design. H.M.M. performed molecular biology. B.S. and J.A. produced and characterized all VLP constructs. K.M.M., T.J.R., and B.S.G. designed and performed all murine studies. B.S., K.M.M., T.J.R., T.D., and B.S.G. compiled and discussed results and wrote manuscript.

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Supporting Information

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viral antigens, demonstrating the potential of this technology to facilitate immunity to multiple targets simultaneously.

Graphical Abstract

Keywords

subunit vaccine; viruslike particle; respiratory syncytial virus; CD8+ T cell immunity; tissue-resident memory T cells

INTRODUCTION

Subunit vaccines composed of isolated proteins offer a means of focusing the immune response on a specific antigen of a pathogen.¹ In addition, subunit vaccines offer safety, ease of production, and precise product control compared to other vaccination strategies.² However, subunit protein vaccine strategies are often limited by relatively low immunogenicity and are generally restricted to the induction of CD4 T cell and antibody responses.^{3,4} Although eliciting humoral protection is a major goal for antiviral vaccines, induction of appropriate T cell responses, particularly cytotoxic T lymphocytes (CTL), can improve the breadth and efficacy of vaccine-induced immunity. CTL immunity remains largely inaccessible to isolated proteins without significant formulation with adjuvants.⁵ Stimulation of effective CTL responses against a specific protein subunit has typically only been pursued through DNA and viral vectors, which allow for the endogenous production of the target subunit.⁶ Here, we demonstrate the effective, exogenous delivery of multiple CTL-directed antigens from respiratory syncytial virus (RSV) through controlled encapsidation of a poly antigen within the viruslike particle (VLP) derived from the bacteriophage P22.

VLPs are noninfectious nanoparticle architectures derived from viral sources or other naturally occurring cage architectures. Pathogen-derived VLPs have been shown to promote immunity by maintaining the repeated subunit structure of the native pathogen, thereby enhancing antigen delivery and recognition.^{7–11} Toward the goal of generating CTL immunity, pathogen-derived VLPs have been shown to promote cross-presentation of exogenous antigens via MHC-I, particularly in dendritic cells.^{4,12,13} Studies focusing on the human papilloma virus (HPV) VLP have demonstrated the utility of this pathogen-associated VLP for the delivery of CTL-directed HPV antigens not typically part of the cage through genetic fusion to cage proteins.¹⁴ Additionally HPV-encapsidated DNA plasmids have shown that delivery with a pathogen-associated VLP can promote introduction of the vector and subsequent CTL stimulation.⁶ Nonpathogen associated protein-based VLPs have

been shown to nonspecifically stimulate the formation of secondary lymphoid tissue in the lung, suggesting that they could be repurposed to deliver CTL antigens.^{15,16} These nonpathogen associated VLPs offer platforms for the versatile conjugation of pathogen subunits that do not naturally form VLPs or form unstable VLPs.^{17,18}

Previous efforts to design VLPs as versatile antigen-presentation platforms have focused primarily on the generation of humoral protection through presentation of antigens on the cage surface.^{3,18–22} Membrane enveloped VLPs produced in a variety of hosts have been used to display membrane-anchored surface antigens.^{23,24} Efforts have also taken advantage of the ability to genetically conjugate antigens and other immune targets to a wide variety of VLP structural proteins generating chimeric protein-based VLPs.^{18,19,25–27} These chimeric protein VLPs offer an unmatched level of particle homogeneity, spatial control, and ease of production making them appealing platforms for the development of polyvalent, nanoparticle preventive vaccines.

The VLP derived from the *Salmonella typhimurium* bacteriophage P22 has been shown to be amenable to genetic modification enabling the redesign of this particle for the delivery of protein cargo. The infectious P22 bacteriophage is a short-tailed phage with a dsDNA genome. The viral capsid assembles from 415 copies of a 46 kDa coat protein (CP) template by as many as 300 copies of a 34 kDa scaffold protein (SP), which are encapsidated within the immature procapsid. A portal protein complex occupies one of the 5-fold axes of the icosahedral procapsid particle and pumps the genome into the particle leading to expulsion of the SP.

A noninfectious VLP can be formed by coexpression of the CP and SP resulting in a ~ 60 nm diameter cage with $T=7$ icosahedral symmetry wherein the encapsidated SP is surrounded by the CP shell in the assembled particle.²⁸ Target protein encapsidation within the VLP can be directed by fusion of the target gene to the scaffolding domain of SP and subsequent coexpression with CP.²⁹ The utility of the P22 system in delivering CTL-directed antigens has been demonstrated with the encapsidation and administration of influenza nucleoprotein. Intranasal administration of the encapsidated nucleoprotein led to CTL-mediated protection against multiple serotypes of influenza.¹⁷

By avoiding direct fusion of targets to the coat protein, P22 overcomes a key design hurdle in VLP delivery of complex protein cargo. With complex protein targets, the likelihood of interrupting the CP structure, and thus the structure of the capsid, upon fusion increases.⁴ The modularity of the P22 system leaves the CP unmodified giving it the potential to tolerate large and complex cargo. Previously, we have demonstrated the encapsidation of multiple whole proteins with precise relative stoichiometry by fusing the targets together as an extended concatenated fusion terminating in the SP domain.^{30,31}

Application of this strategy in the delivery of multiple antigens may elicit multiple adaptive immune responses that distribute selective pressures across multiple epitopes instead of concentrating escape pressure on a single dominant epitope.³² Design of vaccines that can induce immunity toward multiple select antigens may be especially important for designing

vaccines against pathogens such as human immunodeficiency virus, hepatitis C virus, *Plasmodium falciparum* and *Mycobacterium tuberculosis*.^{32–36}

RSV is a common lung pathogen for which there is no licensed vaccine. RSV is a primary cause of respiratory complications in infants leading to a high global rate of mortality in the first year of life.³⁷ Several factors make vaccination against RSV difficult. These include the early age of severe disease, viral evasion of innate immunity, maternally transferred humoral immunity, and the relatively short persistence of adaptive memory populations.^{38,39} A diverse range of antigen delivery strategies have been employed including enveloped VLPs, gene-based vectors, live-attenuated viruses, inactivated viruses, and subunit vaccines all with varying degrees of success.^{6,24,40}

The well-studied CD8 T cell epitope hierarchy following RSV infection of CB6F1 mice makes it an interesting model for examining the ability of the P22 VLP system to deliver multiple antigens for the induction of CD8 T cells. Here, we employ the P22 VLP as an antigen delivery platform for a poly antigen of RSV matrix (M) and matrix 2 (M2) proteins. This M/M2 poly antigen has been previously used in the form of a DNA vector to elicit T cell responses against the D^bM₁₈₇₋₁₉₅ (M) and K^dM₂₈₂₋₉₀ (M2) T cell epitopes.^{6,41} Through encapsidation of M/M2 within the interior of the P22 particle, we aim to deliver the poly antigen in order to stimulate CTL-dependent immunity without the need for intracellular production or additional adjuvants. Successful delivery of M/M2 should result in a native hierarchy between the dominant M2 epitope and the subdominant M epitope.

A P22 construct for the encapsidation of M/M2 was successfully optimized to produce well-formed particles containing 127–157 copies of the poly antigen per particle. A clinically relevant administration schedule utilizing a three-week boost was developed and resulted in CD8 T cell responses to both the M2 and M epitopes. The native dominance of M2 over M was observed in P22-M/M2 immunized mice. Immunity to RSV challenge was evident in lower viral titers in the lung compared to unvaccinated or control vaccinated mice. In addition, tissue-resident memory T cell populations were elicited by vaccination suggesting that this delivery platform may be efficacious in stimulating tissue-specific long-term immune memory.

MATERIALS AND METHODS

Materials

DNA modifying enzymes were purchased from New England Biolabs and Promega. DNA primers were purchased from Eurofins MWG Operon and Integrated DNA Technologies. *E. coli*, electro competent ClearColi, cells were purchased from Lucigen. QIAquick gel extraction kit and QIAprep Spin Miniprep kit were purchased from Qiagen. TEM grids were purchased from Electron Microscopy Sciences. All other chemical reagents were purchased from Fisher Scientific.

Plasmid Construction

The M/M2 cassette was amplified with Q5 High-Fidelity DNA polymerase (New England Biolabs) from p8400 VRC CMV/R plasmid using a forward primer containing a *Nco*I-site

and a reverse primer containing a *Bam*HI-site.³³ The PCR product was gel purified using the Isolate II PCR and Gel Kit (Bioline), digested with *Nco*I and *Bam*HI, column purified using a DNA Clean & Concentrator column (Zymo Research), and ligated into the corresponding sites of linearized pRSFDuet1 SP. CP was inserted into the *Nde*I/*Xho*I site in MCS2. The M134A mutation was created by PCR using the Q5 Site-directed Mutagenesis Kit (New England Biolabs) with pRSFDuet1M/M2 SP CP as template, and back-to-back primers with a 3-nucleotide substitution located in the middle of the forward primer. All plasmids were transformed into homemade electrocompetent *E. coli* TOP10. Plasmids were isolated from *E. coli* by the alkaline lysis method. Larger scale plasmid preparations were further purified using DNA Clean & Concentrator columns (Zymo Research). The plasmid inserts were confirmed by DNA sequencing (GenScript and Eurofins MWG Operon).

Expression and Lysis

All constructs were transformed into Clear Coli, a nonimmunostimulatory LPS generating *E. coli*. Transformed *E. coli* strains were grown on LB medium at 37 °C in the presence of ampicillin or kanamycin. Expression of the genes was induced once the culture reached mid log phase ($OD_{600} = 0.6$) by addition of isopropyl β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Cultures were grown for 4 h after addition of IPTG, then the cells were harvested by centrifugation and cell pellets stored at -20 °C overnight.

Cell pellets were resuspended in Lysis buffer (10 mM sodium phosphate, 125 mM sodium chloride, pH 7.4) with lysozyme and RNase added and incubated at room temperature for 30 min. The cell suspension was lysed by sonication and cell debris was removed by centrifugation at 12,000 *g* for 45 min at 4 °C. P22 particles were purified from the supernatant by ultracentrifugation through a 35% (w/v) sucrose cushion. The resulting viral pellet was resuspended in PBS (10 mM sodium phosphate, 175 mM sodium chloride, pH 7.4) and then purified over an S-500 Sephadex size exclusion column using a Biorad Biologic Duoflow FLPC. Flow rate for SEC purification was 1 mL/min. Fractions taken from SEC containing P22 were concentrated by ultracentrifugation and the resulting viral pellet was resuspended in an adequate volume of PBS.

SDS-PAGE

Protein samples (total protein ~10 μ g each) were combined with 4X loading buffer containing 100 mM DTT, heated in a boiling water bath. Samples were separated on a 15% acrylamide gel at a constant current of 35 mA. Gels were developed with Coomassie blue stain. Images were recorded on a UVP MultDoc-IT Digital Imaging System. A 10–180 kDa PageRuler prestained ladder was used for reference.

Multiangle Light Scattering

Samples were separated over a WTC-200S5 (Wyatt Technologies) size exclusion column and an Agilent 1200 HPLC at 0.7 mL/min of 50 mM phosphate, 100 mM sodium chloride and 200 ppm sodium azide pH 7.4. Samples were injected in 25 μ L volumes of 1 mg/mL total protein. Sample elution was detected using a UV-vis detector (Agilent), a Wyatt HELEOS Multi Angle Laser Light Scattering (MALS) and detector, and an Optilab rEX differential refractometer (Wyatt Technology Corporation). The number-average particle

molecular weight, Mn, was calculated across each peak half max with Astra 6 software (Wyatt Technology Corporation) using a previously calculated dn/dc value of 0.185 mL/g.

Transmission Electron Microscopy

Samples (5 μ L, 0.1 mg/mL total protein) were applied to carbon-coated grids and incubated for 30 s. After liquid was wicked away, grids were washed with 5 μ L of distilled water. Grids were stained with 5 μ L 2% Uranyl acetate for 20 s and excess stain was wicked away with filter paper. Images were taken on a JEOL 1010 transmission electron microscope at accelerating voltage of 80 kV.

PCR for M/M2 Gene Detection

To probe for the presence of the M/M2 gene, we designed primers that amplified the region of the M/ M2 gene from base pair 201-1115.

MM2 201 fwd: ccttcctgagagtgatgatta

MM2 1115 rev: cctcggtatcccgcagcttc

A OneTaq reaction containing ~0.25 mg total protein of P22-M/ M2 was used to probe for the presence of any M/M2 encoding nucleic acid in the VLP sample. A reaction containing 50 ng of the P22-M/M2 expression vector was used as a positive control and a buffer only sample assessed for background signal.

Mice

Age-matched 6–10 weeks old female CB6F1/J mice (Jackson Laboratories Bar Harbor, ME) were used for all experiments. Mice were housed in the animal care facility at the National Institute of Allergy and Infectious Diseases under specific-pathogen-free conditions and maintained on standard rodent chow and water supplied ad libitum. All studies were reviewed and approved by the NIH Animal Care and Use Committee.

Vaccination and Challenge

Mice were vaccinated intranasally with 100 μ g of P22-Ctrl or P22-M/M2 in 50 μ L. RSV stocks were generated from the A2 strain of RSV by sonication of infected HEp-2 monolayers as previously described.⁴² Mice were challenged following anesthesia with isoflurane (3%) with 2×10^6 pfu of RSV intranasally in 100 μ L of 10% MEM. All intranasal inoculations were performed under anesthesia with isoflurane (3%). Mice were euthanized by lethal overdose of pentobarbital (250 mg/kg).

Flow Cytometry

Following euthanasia, lungs and spleen were harvested. Lymphocytes were isolated by manual disruption of tissue using a GentleMACs machine (Miltenyi Biotec, San Diego, CA) followed by separation of lymphocytes using Fico-LITE density gradient. Isolated mononuclear cells were washed with PBS and resuspended in FACS staining buffer (PBS + 1%FBS+0.05% sodium azide). Cells were stained with fluorochrome-labeled antibodies for lineage markers CD3 (145-2C11) and CD8 (53-6.7) and tissue-resident memory markers CD69 (H1.2F3) and CD103 (M290) from BD Biosciences or Biolegend (San Diego, CA). A

viability dye, AquaBlue Amino stain (Invitrogen) was used to identify dead cells. CD8 T cell receptor specificity was determined by peptide-MHC tetramers consisting of D^bM₁₈₇₋₁₉₅ conjugated to APC and K^dM₂₈₂₋₉₀ conjugated to PE (MBL, Woburn, MA). CD4 T cell receptor specificity was determined by peptide-MHC tetramers consisting of IA(b)M₂₀₉₋₂₂₃ kindly provided by the NIH Tetramer core. All samples were stained with antibody cocktail for 20 min at 4 °C and run on an LSR II (Becton Dickinson, San Jose, CA) and analyzed using FlowJo software (TreeStar, San Carlos, CA).

Plaque Assay and Cytokine Analysis

Plaque assays were performed as previously described.⁴³ Briefly, the left lobe of the lungs was harvested, weighed, and quick frozen in 10% MEM, 2 mM glutamine, 10 U/mL penicillin and 10 µg/mL streptomycin. After thawing, lung tissue was dissociated using the GentleMACs machine on program lung_02 (Miltenyi). Cell suspensions were pelleted to remove cellular debris. Supernatants were serially diluted and inoculated on 80% confluent HEp-2 cell monolayers in triplicate. Following a 1 h incubation at room temperature, a 0.8% methyl cellulose in 10% MEM was overlaid. After 4 days at 37 °C, cells were fixed with 10% buffered formalin and stained with hematoxylin and eosin. Plaques were counted and expressed as Log₁₀ PFU/gram of lung tissue. Limit of detection is 1.8 Log₁₀ PFU/gram. Supernatants were analyzed by bead-based multiplex assays for cytokines by AssayGate, Inc. (Ijamsville, MD).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism using a one-way or two-way ANOVA as appropriate for multiple comparisons.

RESULTS

The P22 VLP Successfully Encapsidates the M/M2 Poly Antigen

Building on previous successes utilizing the SP to direct the encapsidation of protein cargo, the C-terminal 162 residues of SP were genetically fused to the C-terminus of an RSV M/M2 protein chimera forming a nonoptimized M/M2-SP fusion gene (pre-M/M2-SP). Although coexpression of separate fusions of each cargo gene to a SP domain has been previously shown to effectively direct coencapsulation, a single concatenated gene was used here to ensure a predictable and reproducible ratio of the M and M2 proteins in the final particles. This strategy had the potential to generate more uniform particles but required that the quaternary structure of each protein be accommodated in the expression and encapsidation of the cargo. Figure 1 shows a potential orientation of the M/M2-SP cargo that accommodates the quaternary structures of both the M and M2 proteins as well as the necessity of the SP domain to interact with the CP during the assembly of the final VLP.

The pre-M/M2-SP gene was coexpressed with CP resulting in the assembly of P22-M/M2 particles. All VLPs were produced in Clear Coli to avoid the introduction of TLR4-stimulating lipopolysaccharide into the samples. VLPs were purified by ultracentrifugation followed by size exclusion chromatography (SEC) and initially assessed by SDS-PAGE for the presence of cargo. In addition to the expected CP band at ~46 kDa and the expected

M/M2-SP band at ~70 kDa, a third band was observed at ~60 kDa (Figure S1A). Western blot analysis using either anti-M or anti-M2 antibodies reveal that the ~60 kDa band is bound by anti-M2 but not anti-M (Figure S1B). Lack of anti-M binding coupled with the retained ability of the secondary product to be encapsidated suggested that this band was an N-terminally truncated version of the pre-M/M2-SP gene with an intact SP region and likely part of the M protein missing.

A putative alternative start codon and accompanying ribosome-binding site, characterized by a canonical Shine-Dalgarno sequence, were identified in the pre-M/M2-SP gene at amino acid position 134 (Figure S1C). Site-directed mutagenesis generating an M134A mutant successfully terminated the alternative product resulting in only the expected CP and M/M2-SP bands by SDS-PAGE (Figure 2A). This finalized M/M2-SP gene was used in all subsequent experiments.

By transmission electron microscopy (TEM), P22-M/M2 particles appeared well-formed with the majority of the particles displaying an uninterrupted ring of density (Figure 2B). Assessment by size exclusion chromatography (SEC) monitored by multiangle and quasi-elastic light scattering also suggested well-formed particles of the expected size for P22 VLPs.⁴⁴ Samples showed a single peak by SEC, which migrated with the same retention time as an unmodified P22 VLP, with an average particle molecular weight of 29.5 ± 1.1 MDa by multiangle light scattering corresponding to 142 ± 15 M/M2-SP per capsid (Figure 2C). The hydrodynamic radius across the same peak was 26.6 ± 0.8 nm, which was indistinguishable from an unmodified P22 VLP (Figure 2D).

Previous studies have shown that nucleic acid vectors carrying the M/M2 poly antigen have successfully stimulated antigen production and T cell responses to both antigens.^{6,41} It is conceivable that the P22-M/M2 particles could contain nucleic acid encoding the M/M2 sequence left over from expression and purification despite treatment with RNase and DNase. To examine this possibility, PCR was performed on the purified P22-M/M2 materials using primers targeted to a section from the central region of the M/M2 gene (base pair 201-1115) as a reporter. No band corresponding to an amplification product was observed suggesting the absence of potentially productive nucleic acid in the VLP samples (Figure S2).

Vaccination with P22-M/M2 Elicits RSV-Specific CD8 and CD4 T Cells

To evaluate the immune response to P22-M/M2, we vaccinated mice intranasally using a prime/boost schedule with 100 μ g of an empty P22 nanoparticle (P22-Ctrl) or a P22 nanoparticle containing the M/M2 protein (P22-M/ M2) at week 0 and boosted with the same dose 3 weeks later (Figure 3A). At week 7, mice were challenged intranasally with RSV. At weeks 1, 4, and 7 the magnitude of the CD8 T cell response was evaluated using tetramers comprised of K^dM2₈₂₋₉₀ and D^bM₁₈₇₋₁₉₅ to identify RSV epitope-specific CD8 T cells in the lungs of vaccinated mice. Vaccination with P22-M/M2 generated M2 and M-specific CD8 T cell responses after a single administration, which were boosted by a second vaccination at week 3 (Figure 3B, C). By week 7, the M2-specific and M-specific CD8 T cell populations contracted, but remained detectable above background. Mice vaccinated with P22-Ctrl had no detectable M or M2-specific CD8 T cell responses (Figure S3A). CD8

T cell responses to RSV have previously been demonstrated to form a reproducible epitope hierarchy with the M2-specific response dominating the M-specific response.⁴⁵ This hierarchy was recapitulated using the P22-M/M2 vector at all time points. At week 4 postvaccination, CD4 T cell responses to a previously identified epitope within the M protein were measured using a tetramer composed of IA^bM₂₀₉₋₂₂₃.⁴¹ P22-M/M2 induced a significant response to this CD4 epitope (Figure 3D). These data demonstrate that P22 nanoparticles can be utilized to generate CD8 T cell responses to multiple antigens as well as elicit CD4 T cell responses.

P22-M/M2 Immunization Generates Tissue-Resident Memory CD8 T Cells in the Lungs

Tissue-resident memory CD8 T cells have been shown to be able to respond quickly to antigen re-exposure and are an ideal population to elicit through vaccination. We determined the number of tissue-resident memory CD8 T cells generated by vaccination with P22-M/M2 at week 4 and week 8 by flow cytometry using the tissue-resident memory markers CD69 and CD103. Tissue-resident memory cells were identified by expression of both CD69 and CD103 (Figure 4A). P22-M/M2 vaccination generates both M2 and M-specific tissue-resident memory cells at week 4, which are sustained through week 8 (Figure 4B, C). Mice vaccinated with P22-Ctrl had no detectable M or M2-specific tissue-resident memory CD8 T cells (Figure S3B).

P22-M/M2 Vaccination Results in Increased CD8 T Cell Responses and Decreased Lung Viral Titers Following RSV Challenge

To determine the effect of vaccination on subsequent RSV infection, we challenged mice with RSV 3 weeks postboost and harvested lungs for CD8 T cell quantification and plaque assay 5 days postchallenge. Mice vaccinated with P22-M/M2 had increased frequency and numbers of M2-specific and M-specific CD8 T cells in their lungs than control mice on day 5 postchallenge (Figure 5A, B, $p > 0.001$) and significantly higher expression of interferon gamma (IFN γ) ($p > 0.001$) and interferon alpha (IFN α) ($p > 0.0001$) on day 3 compared to both unvaccinated and P22-Ctrl-vaccinated mice (Figure S4). In addition to increased CD8 T cell responses, P22-M/M2 vaccination led to a nearly 1000-fold reduction in RSV viral load (Figure 5C, $p > 0.0001$). There was no significant difference in viral loads between unvaccinated mice and P22-Ctrl vaccinated mice. Together, these data indicate that P22-M/M2 vaccination leads to early CD8 T cell responses and concordantly reduced viral loads after RSV challenge.

DISCUSSION

The P22 VLP encapsidation system can be used to successfully sequester and deliver multiple CTL antigens within the same particle offering the potential for complex multiantigen-directed immune responses. Stimulation of immunity to multiple antigens has the potential to distribute selective pressure toward the target pathogen and allow for the design of complex immune responses. Herein we utilize the P22 particles expressing RSV antigens to induce multiple antigen-specific CD8 T cell responses in the CB6F1 murine model.

The P22 VLP SP-directed encapsidation strategy was successfully adopted to sequester the M/M2 poly antigen. Expression resulted in the packaging of high numbers (142 ± 15) of M/M2-SP per capsid, resulting in well-formed particles of the expected dimensions and morphology despite the known complex quaternary structure of both the M and M2 proteins. Previous results have shown that multiple proteins with nontrivial quaternary structure can be fused as a single concatenated gene product to the SP domain for directed encapsidation.³⁰ However, the order of the cargo fusion was shown to be important in capsid assembly and cargo activity presumably due to structural conflicts. The M/M2 fusion presented a similar challenge, as the M2 protein was isolated as a tetramer in previous crystallographic studies and the M protein has been shown to dimerize (Figure 1A).^{46,47} Although it is likely not essential to keep internal antigens targeted to CTL type immunity in native conformations within the capsid, the successful structural assembly of the antigen-loaded VLPs likely requires all components to remain partially soluble. From the successful assembly of P22-M/M2, we infer that VLP assembly accommodated the structurally complex cargo. One potential orientation of the cargo is represented in Figure 1B.

The concatenated fusion M/M2-SP gene used in this experiment ensures a one to one encapsulation ratio between the two target antigens. In this study, this strategy allowed for the production of a well-characterized product with a known loading of cargo. Alternatively, we have previously demonstrated that encapsulation of two different protein targets can be directed using a multi-plasmid system in which each target gene is fused to a separate SP domain.⁴⁸ In future studies, this design strategy may be useful for extending the flexibility of this system beyond two antigens or to examine the effects of variable antigen ratios on immune responses.

Upon administration, P22-M/M2 elicited a significant response to both the M and M2 CD8 T cell epitopes. The expected hierarchy of these epitopes was established following P22-M/M2 immunization and confirmed that the P22 VLP was able to effectively deliver both of these antigens to the MHC class I processing pathway.⁴⁵ The effective delivery and processing of M/M2 via MHC class I pathways supports the idea that these VLPs can function as both carriers, increasing the dose of antigen upon a particle reaching a cell, and adjuvants, stimulating the immune system and facilitating uptake. The full mechanism by which VLPs potentially stimulate adaptive responses is still unclear but potential contributing factors have been identified including size, polyvalency, and an apparent disposition toward dendritic cell populations.^{4,49,50}

Following RSV challenge, P22-M/M2 vaccinated mice had an early expansion of M and M2-specific CD8 T cells. Responses generated by P22-M/M2 led to significantly decreased viral lung titers after RSV challenge. The number and type of CD8 T cells present in RSV infection have been shown to be essential in curbing the potential for vaccine-induced eosinophilia.⁵¹ Many 1CD8 T cells elicited through intranasal vaccination assumed a tissue-resident memory phenotype. Tissue-resident memory cells have been shown to act as immune sentinels with rapid cytokine and antiviral responses upon antigen re-exposure, which recruit innate and adaptive immune cells to the infected tissue.^{52,53} This suggests that tissue-localized administration of P22 vaccines can generate CD8 T cell populations that are

able to respond more quickly to subsequent infection than vaccinations that generate systemic immune responses.

In summary, P22 provides a versatile platform for the encapsidation and delivery of complex protein subunit antigens allowing for the stimulation of CTL-dependent immunity without the need for adjuvants or endogenous production of target antigens. Further engineering of P22 may allow for both the encapsidation of antigens designed to induce the appropriate T cell responses and the addition of antigens to the surface of the P22 particle to elicit neutralizing antibodies. Thus, the P22 VLP system represents a modular vaccine platform with the potential for inducing both cellular and humoral immunity against multiple antigens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

M/M2 fusion protein has a nontrivial quaternary structure that must be accommodated during encapsidation. (A) M2 protein is arranged as a C4 parallel tetramer (red) localizing both the N- (blue) and C-termini (purple) on one face of the molecule. M protein has been crystallized as a monomer (shown in blue) but also observed to form higher order quaternary structures. The C-terminus of M protein is displayed in red. (B) One possible orientation of the M/M2-SP chimera is shown with M protein (blue), M2 protein (red) and SP (purple). (C) Following expression, the SP directs encapsidation of the M/M2-SP through association with the coat protein (CP) (gray) during capsid self-assembly, resulting in a P22 VLP encapsidated M/M2-SP. All images were generated using UCSF Chimera fusing reported protein structures from the PDB. (PDB: 2XYY, 2VQP, 4C3B, and 2GP8).

Figure 2.

P22-M/M2 is produced as well-formed particles with high copy number of M/M2-SP cargo. (A) SDS-PAGE shows only the expected M/M2-SP (70 kDa) and CP (46 kDa) bands after a M134A mutation is introduced to eliminate the alternative gene product. (B) Negatively stained TEM micrographs of P22-M/M2 show well-formed particles of ~60 nm diameter. Scale bar is 500 nm. (C) Size exclusion chromatography of the P22-M/M2 particles monitored by multiangle light scattering results in a single peak with an average molecular weight of 29.5 ± 1.1 MDa (red). A representative chromatogram and molecular weight trace (19.1 ± 0.7 MDa) of empty P22 particles is displayed for reference (black). The difference in molecular weight between the P22-M/M2 particles and the empty P22 corresponds to 142 ± 15 copies of the M/M2-SP chimera per particle. (D) The average radius of hydration over

the same peak measured by quasi-elastic light scattering is 26.6 ± 0.8 nm (blue) and does not vary from the radius of hydration of the empty P22 control (26.3 ± 0.3) displayed below (black).

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Figure 3.

Vaccination with P22-M/M2 induces RSV-specific CD8 and CD4 T cells. (A) Vaccination and challenge schedule. (B) Percent and (C) number of M2 and M-specific CD8 T cells in the lungs of mice vaccinated with P22-M/M2 at weeks 1, 4, and 7. Background subtracted based on P22-Ctrl. (D) Percent of I-A^bM209-specific CD4 T cells in the lungs of mice vaccinated with P22-M/M2 at week 4. (E, F) Representative flow cytometry plot gated on CD3+CD8+ T cells showing staining with (E) M2-specific and (F) M-specific tetramers. Numbers reflect percent of positive CD8 T cells. Bars represent mean $\pm$ SEM with 5 mice per group. **** $p < 0.0001$, ** $p < 0.01$ by 2-way ANOVA.

Figure 4. P22-M/M2 vaccination induces tissue-resident memory CD8 T cells. (A) Gating of CD69+CD103+ tissue-resident memory cells. (B) Percent of M2-specific and M-specific CD8 T cells with a tissue-resident memory phenotype. (C) Number of M2-specific and M-specific CD8 T cells with a tissue-resident memory phenotype. Bars represent mean $\pm$ SEM with 5 mice per group. * $p < 0.05$ by 2-way ANOVA.

Figure 5. P22-M/M2 vaccination leads to increased CD8 T cell responses and decreased viral loads following RSV challenge. (A) Percent and (B) number of M2-specific and M-specific CD8 T cells in the lungs of mice 5 days postchallenge. (C) RSV replication in lung was determined by plaque assay on day 5 postchallenge. Bars represent mean $\pm$ SEM with 5 mice per group. **** $p > 0.0001$, *** $p > 0.001$ by (C) 1-way or 2-way ANOVA.