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Preliminary evidence that the novel host-derived immunostimulant EP67 can act as a mucosal adjuvant

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Abstract

EP67 is a complement component 5a (C5a)-derived peptide agonist of the C5a receptor (CD88) that selectively activates DCs over neutrophils. Systemic administration of EP67 covalently attached to peptides, proteins, or attenuated pathogens generates TH1-biased immunogen-specific humoral and cellular immune responses with little inflammation. Furthermore, intranasal administration of EP67 alone increases the proportion of activated APCs in the airways. As such, we hypothesized that EP67 can act as a mucosal adjuvant. Intranasal immunization with an EP67-conjugated CTL peptide vaccine against protective MCMV epitopes M84 and pp89 increased protection of naïve female BALB/c mice against primary respiratory infection with salivary gland-derived MCMV and generated higher proportions of epitope responsive and long-lived memory precursor effector cells (MPEC) in the lungs and spleen compared to an inactive, scrambled EP67-conjugated CTL peptide vaccine and vehicle alone. Thus, EP67 may be an effective adjuvant for mucosal vaccines and warrants further study.

Keywords

mucosal adjuvant; host-derived adjuvant

1. Introduction

Mucosal immunization is the generation of mucosal and systemic adaptive immune responses by administering a vaccine to mucosal tissues through the oral, nasal, sublingual, buccal, pulmonary, rectal, or vaginal routes [1]. It potentially provides several advantages over systemic immunization including (i.) the generation of long-term mucosal and systemic

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immune responses to protect against both early and late stages of infection (ii.) the generation of long-term immune responses in mucosal tissues distal to the site of vaccine administration (iii.) a high level of “vaccine take” (immunogenicity) even with pre-existing systemic immunity (iv.) simple, relatively painless administration that requires little medical training, increases patient compliance, and has no risk of spreading blood-borne infections through needles (v.) the possibility of frequent boosting and (vi.) relatively lower production costs and regulatory considerations [1–5]. As such, there is great interest in developing mucosal vaccines.

Licensed mucosal vaccines are predominantly oral vaccines composed of live attenuated pathogens that generate long-term protective mucosal and systemic immune responses without the associated pathogenicity [2]. Live attenuated vaccines, however, require a long time to develop, seldom produce both a safe and stable vaccine, and have the potential for pathogenic reversion [2, 4, 6].

Mucosal vaccines composed of killed (bacteria) or inactivated (virus) pathogens (killed/inactivated vaccines) or protective fragments of the pathogen (subunit vaccines) can potentially overcome problems associated with live attenuated vaccines but require the addition of an adjuvant because they are much less immunogenic and incapable of generating cellular immune responses. Cholera toxin subunit B (CTB) is currently the only adjuvant included as part of a licensed mucosal vaccine (Dukoral: oral, killed vaccine) [7, 8]. Inclusion of a similar enterotoxin, *E. coli* heat-labile toxin (HLT) [9], or a “detoxified” HLT [10] with live attenuated intranasal vaccines against influenza, however, caused Bell’s palsy in a number of participants during early clinical trials. Thus, numerous experimental adjuvants are being developed for clinical use with all routes of mucosal immunization.

The majority of experimental mucosal adjuvants to date are based on pathogen-associated molecular pattern (PAMP) agonists that stimulate innate immune responses through pattern recognition receptors (PRRs) [11–13]. These and other adjuvants [14] increase the generation of mucosal and systemic adaptive immune responses in clinical trials [15] but their relative ability to generate both humoral and cellular immune responses is varied or associated with high levels of inflammation and/or toxicity [2, 4]. Thus, there continues to be a great need to develop mucosal adjuvants that generate predictable humoral and cellular immune responses through distinct cellular targets and signal transduction pathways, are minimally pro-inflammatory, and are safe for massimmunization [2, 3, 16].

In contrast to the vast majority of experimental mucosal adjuvants to date that are pathogen-derived, we previously developed a novel host-derived adjuvant, EP67, based on the C-terminal of human complement component 5a (C5a) from the innate immune system [17, 18]. EP67 is a conformationally-biased 10 amino acid peptide [18] that acts as an immunostimulant [19–21] and an adjuvant [22–24] presumably, in large part, by activating and increasing subsequent processing and presentation of conjugated immunogens by DCs through the binding and activation of the C5a receptor (C5aR/CD88) [19, 22]. EP67, or the previous analog EP54, selectively activate murine and human monocyte-derived DCs (moDCs) over neutrophils through the selective binding and activation of the C5a receptor (C5aR/CD88) on the surface of DCs [17, 18], present conjugated epitopes in MHC I and

MHC II of human moDCs [25], and generate TH1-biased humoral and cellular immune responses in mice against conjugated peptides, intact proteins, or attenuated pathogens after systemic administration with significantly less direct activation of neutrophils than C5a [20, 26–29]. Furthermore, intranasal (IN) administration of EP67 alone increases the proportion of activated APCs in the airways of C57BL/6 mice [18]. Thus, we hypothesized that EP67 can act as a mucosal adjuvant. To test this hypothesis, we individually conjugated protective MCMV CTL epitopes (M84 and pp89) to the N-terminal of EP67 or inactive scrambled EP67 (scEP67) through a protease-labile double arginine linker. We then compared the extent to which IN immunization with M84-RR-EP67 / pp89-RR-EP67 (CMV-EP67), inactive M84-RR-scEP67 / pp89-RR-scEP67 (CMV-scEP67), or vehicle (PBS) alone protects naïve female BALB/c mice against primary respiratory challenge with salivary gland-derived murine cytomegalovirus (SGV) and affects the generation of mucosal and systemic epitope-specific CD8⁺ T cells by the day of challenge with SGV.

2. Materials and methods

2.1 Peptides

The protective MCMV CTL epitope from M84 (²⁹⁷AYAGLFTPL³⁰⁵) [30] or pp89 (⁶⁸YPHFMPNTL⁷⁶) [31] was synthesized and purified alone or after being covalently attached to the N-terminal of EP67 (YSFKDMP[MeL]aR) [18] or inactive scrambled scEP67 ([MeL]RMYKPaFDS) [22] during solid-phase synthesis via a double-arginine (RR) linker [28]. The molecular mass (M⁺H)⁺ of each peptide was confirmed by MALDI/TOF or electrospray ionization mass spectrometry.

2.2 Animals

All animal procedures were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Mice (Female BALB/c AnNHsd [H-2^d haplotype], 3 weeks old, Harlan Laboratories) were acclimatized in an ABSL-2 facility under pathogen-free conditions at least one week before experiments.

2.3 NIH/3T3 cells and salivary gland-derived MCMV (SGV)

NIH/3T3 cells (ATCC: CRL-1658) were incubated (37°C/10% CO₂) in Growth Media (DMEM containing glucose [4.5 g / L], sodium bicarbonate [3.7 g/L], heat-inactivated newborn calf serum [HINCS, 10% v/v, Thermo-Scientific HyClone New Zealand], L-glutamine [2 mM], sodium pyruvate [1 mM], penicillin [100 U/mL], streptomycin G [100 µg/mL]) and passaged at 75% confluence. Salivary gland-derived MCMV (SGV) was obtained by serially passaging the Smith strain of MCMV (ATCC: VR-1399) twice in female BALB/c mice (4–5 weeks old) by the intraperitoneal (IP) route (5 × 10⁴ PFU in 0.2 mL PBS, 25G needle), euthanizing by CO₂ asphyxiation/cervical dislocation two weeks after infection, suspending isolated salivary glands in Freezing Media (DMEM containing glucose [4.5 g / L], sodium bicarbonate [3.7 g/L], HI-NCS [10% v/v], DMSO [cell culture grade, 10% v/v]), homogenizing (Fisher PowerGen 500: 6500 rpm, 45 to 60 sec; drive shaft initially rinsed / wiped sequentially with DuPont broad-spectrum disinfectant / 70% ethanol / sterile PBS, then with 70% ethanol / sterile PBS between treatment groups), and

storing aliquots (0.3 mL) of homogenate supernatants (840 RCF, 3 min) at -80°C . SGV titers were determined by plaque assay (Section 2.6).

2.4 Intranasal immunization with CMV-EP67 and respiratory challenge with MCMV

Naïve female BALB/c mice (4-weeks old) were immunized with sterile PBS alone (15 μL) or sterile PBS containing inactive CMV-scEP67 (mixture of pp89-RR-scEP67 and M84-RR-scEP67, 50 μg each) or active CMV-EP67 (mixture of pp89-RR-EP67 and M84-RR-EP67, 50 μg each) (15 μL) on days 0, 7, 14 by anesthetizing with isoflurane in a drop jar, holding upright, and alternating drops between nares with a 20 μL pipette. A volume of 15 μL is expected to deposit vaccine primarily in the nasal cavity [32]. Fourteen days after the final immunization (Day 28), mice were challenged with a sublethal amount of SGV (5×10^3 PFU) by IN administration as described for administering vaccines but in a volume of sterile PBS (50 μL with a 200 μL pipette) expected to deposit MCMV in the nasal cavity and the lungs (i.e., respiratory challenge) [32].

2.5 Extraction of MCMV from tropic organs

MCMV was extracted from tropic organs by homogenization and centrifugation [33]. Mice were sacrificed by CO_2 asphyxiation/cervical dislocation 6 days (Day 34: lungs, liver, spleen) and 14 days post-challenge with SGV (Day 42: salivary glands) ($n = 3$ mice per time point), the lungs and liver were perfused by injecting ice-cold sterile DPBS (5 mL, 25G needle) through the right ventricle of the heart, and the indicated tropic organs were isolated, weighed, suspended in Freezing Media (Section 2.3) at 10% w/v (lungs, spleen, salivary glands) or 20% w/v (liver) then stored at -20°C . MCMV was extracted from thawed organs and stored (Section 2.3) before determining titers by plaque assay (Section 2.6).

2.6 MCMV titers

Titers of MCMV were determined by plaque assay [33, 34]. NIH/3T3 cells were grown (Section 2.3) in 24-well plates (18,000 cells/well in 0.6 mL Growth Media) for 2 days until ~40 to 50% confluent, infected with MCMV by replacing aspirated Growth Media (8 wells at a time) with 10-fold serial dilutions (10^2 to 10^6) of thawed organ MCMV extracts diluted in Infection Media (DMEM containing glucose [4.5 g / L], sodium bicarbonate [3.7 g/L], and HI-NCS [2% v/v]; 0.2 mL/well) pipetted down the side of each well ($n = 3$ wells / dilution factor) and incubating ($37^{\circ}\text{C}/10\% \text{ CO}_2$) for 4 h. Overlay Media was prepared during MCMV infection by mixing an equal volume of a pre-warmed (41°C for 10 min.) agarose solution (Seakem ME Agarose [1% w/v] in dH_2O) with a pre-warmed (41°C for 10 min.) $2\times$ Growth Media solution (HI-NCS [10% v/v] in $2\times$ DMEM). After infection, Growth Media was quickly replaced with Overlay Media (1 mL down the side of each well) one plate at a time and plates were incubated ($37^{\circ}\text{C}/10\% \text{ CO}_2$) for 5 days. MCMV plaques were fixed by adding Fixing Solution (Formalin [10% w/v] in sterile DPBS; 0.5 mL/well), tightly sealing stacked plates in a Ziploc[®] bag, and incubating at r.t. overnight. Fixing Solution was aspirated, agarose plugs were carefully removed with a steel spatula, and remaining agarose was removed by submerging plates in cold water as needed. Plaques were visualized by incubating each well with Staining Solution (crystal violet [0.25% w/v] and ethanol [2% v/v] in dH_2O ; 0.3 mL/well) at r.t. for up to 15 min, rinsing with the wells with cold water

(2×), and allowing to air dry before counting by eye. Average MCMV plaque forming units (PFU) / mL ± SD (n = 3 wells) was calculated by:

$$\frac{MCMVPFU}{mL} = \text{number of plaques} \times DF \times \frac{1}{\text{infection volume}(mL)}$$

where *number of plaques* was taken from the organ homogenate dilution factor where 5 to 50 plaques were observed, *DF* was the selected dilution factor of the organ homogenate where 5 to 50 plaques were observed, and *infection volume* was the volume used to infect NIH/3T3 (0.2 mL). MCMV PFU / g tissue was then calculated from MCMV PFU / mL by:

$$\frac{MCMVPFU}{g\text{ tissue}} = \frac{MCMVPFU}{mL} \times \text{extraction volume}(mL) \times \frac{1}{\text{mass of tropic or gan}(g)}$$

where *extraction volume* was the volume of Freezing Media used for MCMV extraction (1 mL: lungs, spleen, salivary glands; 2 mL: liver) and *mass of tropic organ* was the mass of the isolated organ used in the extraction. Average MCMV PFU /g ± SD (n=3 mice) were compared to PBS by one-way ANOVA with Dunnett's post-test. Plaque assays were repeated at least once for accuracy.

2.7 Isolation of lymphocytes (lungs) and splenocytes

Mice were sacrificed by CO₂ asphyxiation/cervical dislocation on the same day as primary respiratory challenge with SGV (14 days post-immunization), lungs were perfused by injecting ice-cold sterile DPBS (5 mL, 25G needle) through the right ventricle of the heart, and the lungs and spleen were isolated and stored on ice in sterile 15-mL conical tubes containing complete RPMI Media (cRPMI: RPMI 1640 containing FBS [10% v/v], PEN [100 U/mL]/STREP [100 µg/mL], NEAA [1% w/v], Vitamins [1% w/v], Sodium Pyruvate [1% w/v], β-mercaptoethanol [50 µM]) until further processing.

To isolate lymphocytes, lungs were transferred into a sterile 6-well cell culture plate (one lung/well), minced into small pieces using a sterile scalpel (#15, Bard-Parker), and incubated with Collagenase IV (2 mg/mL; Worthington Enzymes) in cRPMI (6 mL) at 37°C for 1 hr with shaking (Vortemp 56 shaking incubator, 200 RPM). Digested lungs were triturated (18G needle) (3×) and filtered through a sterile 70-µm cell strainer into a sterile 15-mL conical tube. Filtered cells were pelleted (400 RCF, 4°C, 5 min.), resuspended in RPMI 1460 (5 mL), layered onto Lympholyte-M (Cedarlane Labs; 5 mL) in a sterile 15-mL conical tube using a sterile Pasteur pipette, and centrifuged (1500 RCF w/o brakes, 4°C, 20 min). Lymphocytes were collected from the interphase with a sterile Pasteur pipette, transferred to a sterile 15-mL conical tube, resuspended in sterile DPBS (10 mL), pelleted (500 RCF, 4°C, 5 min.), resuspended in cRPMI (1 mL), and stored on ice for later use.

To isolate splenocytes, spleens were transferred to a sterile 70-µm strainer inserted into a sterile 50-mL conical tube, cut into small pieces with a sterile scalpel (#15, Bard-Parker), then gently pushed through the cell strainer with the rubber end of a sterile syringe plunger while adding RPMI 1640 (30 mL). The strainer was rinsed with additional RPMI (10 mL),

filtered cells were diluted to 50 mL with sterile DPBS, pelleted (500 RCF, 4°C, 10 min.), resuspended in RBC lysis buffer (ACK Lysing Buffer; 4 mL), and incubated at r.t. for 7 min. cRPMI (10 mL) was added, the entire solution was triturated with a sterile 5-mL pipette to obtain a single cell suspension, and passed through a 40-µm cell strainer into a sterile 50-mL conical tube. Filtered cells were then diluted to 50 mL with sterile DPBS and pelleted (500 RCF, 4°C, 10 min.) (2×), resuspended in cRPMI (3 mL), then stored on ice for later use.

2.8 Epitope-responsiveness of CD8⁺ T cells

The proportion of epitope-responsive CD8⁺ T cells generated in the lungs and spleen on the same day as primary mucosal challenge with MCMV was compared by intracellular cytokine staining (ICS) (BD Cytofix Manual) [35] and ELISPOT (Ready-SET-Go!® kit; eBioscience) [36, 37] according to manufacturer's instructions. For the ICS assay, pooled lymphocytes from the lungs (n=3 mice) or splenocytes from individual mice (n=3) were incubated with cRPMI or cRPMI containing of M84 or pp89 (2 µM) and Brefeldin A (10 µg/mL) for 6 hours. An additional single well was plated from PBS immunizations as an unstained FACS control. Cells were incubated with BD Fc Block and stained with FITC Anti-Mouse CD8a [Clone 53-6.] (0.25 µg / 10⁶ cells; eBioscience). Cells were then fixed and permeabilized by adding BD Stain Buffer (0.15 mL/well), pelleting the cells (400 RCF, 4°C, 5 min.), resuspending cells in BD CytoFix/Cytoperm Buffer (0.1 mL/well), and incubating on ice for 20 min. Intracellular cytokines were stained with APC Anti-Mouse IFN-γ [Clone XMG-1.2] (0.06 µg / 10⁶ cells; BD Biosciences) and PE Anti-Mouse TNF-α [Clone MP6-XT22] (0.25 µg / 10⁶ cells; BD Biosciences) and analyzed by flow cytometry (Section 2.8). Average SFU/10⁶ splenocytes ±SD (n=3 mice) were compared to PBS by nonparametric (Kruskal-Wallis) one-way ANOVA with Dunn's post-test.

For the ELISPOT assay, splenocytes from individual mice (n=3) (2.5 × 10⁵ cells/well or 5 × 10⁵ cells/well) were incubated with cRPMI or cRPMI containing M84 or pp89 (10 µg/mL epitope) for 48 hrs (37°C/10% CO₂) and IFN-γ detected (Biotinylated Rat Anti-mouse IFN-γ [Clone R4-6A2]; eBioscience). Spot forming units (SFU) were counted (surgical dissecting microscope) and normalized for each epitope by subtracting the SFU from the same immunization group treated with media alone. SFU/10⁶ splenocytes were then calculated by:

$$\frac{SFU}{10^6 \text{ splenocytes}} = \frac{(SFU_{\text{Epitope}} - SFU_{\text{Media}})}{\# \text{ treated splenocytes}} \times \left(\frac{\frac{10^6 \text{ splenocytes}}{\# \text{ treated splenocytes}} \times \# \text{ treated splenocytes}}{10^6 \text{ splenocytes}} \right)$$

where SFU_{Epitope} was the SFU from epitope-treated wells, SFU_{Media} was the SFU from media-treated wells at the same cell density, and # (number) of treated splenocytes was 2 × 10⁵ or 5 × 10⁵. Responses were considered positive when SFU/10⁶ splenocytes obtained with growth media (>55 SFU/10⁶ splenocytes for the current study) [38]. Average SFU/10⁶ splenocytes ±SD (n=3 mice) were compared to PBS by nonparametric (Kruskal-Wallis) one-way ANOVA with Dunn's post-test.

2.9 Surface phenotype of epitope-specific CD8⁺ cells

The surface phenotype of epitope-specific CD8⁺ cells was determined by flow cytometry (BD Cytotfix/Cytoperm manual). Pooled lymphocytes from the lungs (n=3 mice) or splenocytes from individual mice (n=3) or an additional single well from PBS immunizations (unstained control) were treated with BD Fc Block and stained with Alexa680 H-2K^d M84 or Alexa 680 H-2L^d pp89-tetramers (1 µg / 10⁶ cells; NIAID tetramer core facility at Emory University, Atlanta, Georgia, USA; r.t. in the dark for 30 min) then stained with half manufacturer's (eBioscience) suggested amount of FITC Anti-Mouse CD8a [Clone 53-6.7], PE Anti-Mouse CD127 [Clone A7R34], and APC Anti-Mouse KLRG1 [Clone 2F1] on ice in the dark for 30 min. Cells were fixed (Fixation Buffer; BioLegend) then analyzed on a BD LSR II flow cytometer (Becton and Dickinson, La Jolla, CA). Maximum number of events from each sample (at least 15,000 CD8a⁺ cells from the lungs and 10,000 CD8a⁺ cells from the spleen) were acquired and analyzed by FlowJo software (Tree Star, Ashland, OR, USA). Average proportions of cell populations and geometric mean fluorescence intensities (gMFI) of the indicated staining antibodies from the spleen ± SD (n=3 mice) were compared to PBS treatment by one-way ANOVA with Dunnett's post-test.

3. Results

3.1 Mucosal immunization with an EP67-conjugated CTL peptide vaccine increases protection against primary respiratory infection with MCMV

CD8⁺ T cells are primarily responsible for controlling CMV infection in mice [39] and humans [40]. Furthermore, adoptive transfer of CD8⁺ T cells specific for the H-2K^d-restricted epitope from the MCMV tegument protein M84 (²⁹⁷AYAGLFTPL³⁰⁵) [30] or for the H-2L^d-restricted epitope from the MCMV immediate early protein (IE) pp89 (⁶⁸YPHFMPNTL⁷⁶) [31] increases protection of BALB/c mice against primary systemic infection with MCMV [41, 42]. Thus, mucosal immunization of BALB/c mice with an EP67-conjugated vaccine against the protective M84 and pp89 CTL epitopes is expected to increase protection against primary mucosal infection with MCMV and provide an initial indication that EP67 can act as a mucosal adjuvant.

To determine if mucosal immunization with an EP67-conjugated CTL peptide vaccine increases protection against primary mucosal infection with MCMV, we attached M84 or pp89 to the N-terminal of EP67 or inactive scrambled EP67 (scEP67) through a subtilisin-labile double arginine linker (RR) that increases the generation of epitope-specific CTL by presumably increasing the release of CTL epitopes from EP67 within the endosomes of DCs during immunogen processing [28]. We then immunized naïve female BALB/c mice intranasally with M84-RR-EP67 / pp89-RR-EP67 (CMV-EP67), inactive M84-RR-scEP67 / pp89-RR-scEP67 (CMV-scEP67), or PBS alone (vehicle), challenged with salivary gland-derived MCMV (SGV) 14 days post-immunization by respiratory administration, then compared peak titers of productive MCMV in the lungs, liver, spleen, and salivary glands by plaque assay (Fig. 1A). Mice from all treatment groups survived at least one month post-challenge during initial studies (not shown).

CMV-EP67 (Fig. 1B, black bars) decreased peak titers of productive MCMV below PBS alone (Fig. 1B, white bars) ~4-fold in the lungs (6 days post-challenge) [$1 \pm 0.8 \times 10^4$ (SD) vs. $4 \pm 1 \times 10^4$ PFU/g tissue, $P = 0.0085$] and ~9-fold in the salivary glands (14 days post-challenge) [$1.1 \pm 0.4 \times 10^7$ (SD) vs. $10 \pm 4 \times 10^7$ PFU/g tissue, $P = 0.0202$]. In contrast, inactive CMV-scEP67 (Fig. 1B, grey bars) did not affect peak titers of productive MCMV compared to PBS alone (Fig. 1B, white bars) in the lungs [$4.3 \pm 0.4 \times 10^4$ (SD) vs. $4 \pm 1 \times 10^4$ PFU/g tissue, $P = 0.9716$] or the salivary glands [$8 \pm 3 \times 10^7$ (SD) vs. $10 \pm 4 \times 10^7$ PFU/g tissue, $P = 0.6960$]. Peak titers of productive MCMV in the liver and spleen (6 days post-infection) were below our limit of detection (250 PFU/mL) for all treatment groups (not shown) as reported for primary respiratory challenge with a 100-fold higher titer of cell culture-derived MCMV [33] (5×10^5 PFU vs. 5×10^3 PFU SGV in our study). Thus, mucosal immunization with an EP67-conjugated CTL peptide vaccine increases protection against primary mucosal infection with MCMV.

3.2 Mucosal immunization with an EP67-conjugated CTL peptide vaccine increases the proportion of epitope-responsive mucosal and systemic CD8⁺ T cells by the day of primary challenge

A decrease in the titers of productive MCMV in tropic organs [41] and survival against lethal primary systemic challenge with MCMV [42] are proportional to the number of M84-specific or pp89-specific CD8⁺ T cells adoptively transferred to BALB/c mice, respectively. Thus, CMV-EP67 is expected to generate the highest proportions of epitope-responsive mucosal and systemic CD8⁺ T cells compared to inactive CMV-scEP67 and vehicle alone if protection against primary respiratory infection with MCMV is mediated through CD8⁺ T cells.

To determine if mucosal immunization with CMV-EP67 increases the proportion of epitope-responsive mucosal and systemic CD8⁺ T cells, we immunized naïve female BALB/c mice IN with CMV-EP67, inactive CMV-scEP67, or PBS alone under the same dosage regimen (Fig. 1A) and compared the proportion of M84- and pp89-responsive CD8⁺ cells present in the lungs and spleen by intracellular cytokine staining (ICS) on the same day as primary respiratory challenge with SGV (14 days post-immunization) (Fig. 2A). CMV-EP67 generated higher proportions of M84- and pp89-responsive CD8⁺IFN- γ ⁺ (Fig. 2B, white bars) and CD8⁺TNF- α ⁺ (Fig. 2B, grey bars) cells in the lungs than inactive CMV-scEP67 or PBS, whereas multifunctional M84- or pp89-specific CD8⁺IFN- γ ⁺TNF- α ⁺ cells in all treatment groups were undetectable over background staining after incubation with media alone (not shown). In contrast to the lungs, no differences in staining between treatment groups were observed by ICS after incubating splenocytes with M84, pp89, or media alone (not shown).

We additionally compared the proportion of M84- and pp89-responsive splenocytes that secrete IFN- γ by the potentially more sensitive ELISPOT assay [43] on the same day as primary respiratory challenge with SGV (Fig. 2C). CMV-EP67 (Fig. 2D, black bars) generated a higher proportion of M84- and pp89-responsive splenocytes that secrete IFN- γ than PBS alone (Fig. 2D, white bars) [M84: 94 ± 15 (SD) vs. 6 ± 4 SFU/ 10^6 splenocytes, $P = 0.0141$; pp89: 60 ± 10 vs. 3 ± 3 SFU/ 10^6 splenocytes, $P = 0.0146$], whereas CMV-scEP67

(Fig. 2D, grey bars) generated lower proportions than CMV-EP67 that were statistically similar to PBS alone (Fig. 2D, white bars) [M84: 43 ± 7 (SD) vs. 6 ± 4 SFU/ 10^6 splenocytes, $P = 0.3558$; pp89: 17 ± 7 vs. 3 ± 3 SFU/ 10^6 splenocytes, $P = 0.3594$]. Given that APCs potentially secrete IFN- γ after incubation with CTL epitopes, whereas CD4 $^+$ T cells do not [44] and very few IFN- γ SFU were observed after incubating splenocytes from PBS-treated mice with M84 or pp89 (Fig. 2D, white bars), an increase in IFN- γ SFU in the splenocytes is most likely due primarily to an increase in the number of epitope-responsive IFN-gamma secreting CD8 $^+$ T cells. Thus, consistent with increased protection against primary mucosal infection with MCMV, mucosal immunization with an EP67-conjugated CTL peptide vaccine increases the proportion of epitope-responsive mucosal and systemic CD8 $^+$ T cells.

3.3 Mucosal immunization with an EP67-conjugated CTL peptide vaccine increases the proportion of memory precursor effector cells (MPEC)

Immunization provides long-term protection by generating protective memory cells that survive well beyond the contraction phase and rapidly expand in response to subsequent encounters with the same pathogen (i.e., recall response) [45]. Thus, although mucosal immunization with CMV-EP67 decreased peak titers of productive MCMV in the lungs and salivary glands after primary respiratory challenge with SGV two weeks after immunization (Fig. 1B) and increased the proportion of epitope-responsive mucosal and systemic CD8 $^+$ T cells in the lungs and spleen by the day of challenge (Fig. 2B & C), it remained unclear whether an EP67-conjugated mucosal vaccine was likely to provide long-term protection against mucosal infection.

To first determine if mucosal immunization with CMV-EP67 generates mucosal and systemic memory CD8 $^+$ T cells that recognize M84 or pp89 bound to MHC I, we administered CMV-EP67, inactive CMV-scEP67, or PBS alone under the same dosage regimen (Fig. 1A) and compared the proportion of CD8a $^+$ cells present in the lungs and spleen on the same day as primary respiratory challenge with SGV (14 days post-immunization) that bind to tetramer-M84 or tetramer-pp89 by flow cytometry (Fig. 3A). CMV-EP67 (Fig. 3B & E, black bars) increased the proportions of CD8a $^+$ Tet-M84 $^+$ cells [0.5% in the lungs; 0.8% in the spleen] and CD8a $^+$ Tet-pp89 $^+$ cells [0.2% in the lungs; 0.7% in the spleen] over PBS (Fig. 3B & E, white bars) by the day of primary respiratory challenge with SGV, whereas inactive CMV-scEP67 (Fig. 3B & E, grey bars) generated lower proportions than CMV-EP67 that were statistically similar to PBS (Fig. 3B & E, white bars). Thus, consistent with increased protection against primary mucosal infection with MCMV, mucosal immunization with CMV-EP67 increases the proportion of mucosal and systemic memory CD8 $^+$ T cells that recognize M84 or pp89 bound to MHC I.

Epitope-specific CD8 $^+$ T cells with a CD127 $^+$ KLRG1 $^-$ cell surface phenotype differentiate into long-lived memory CD8 $^+$ T cells (MPEC: memory precursor effector cells) [46, 47] by immunogen-independent homeostatic proliferation and maintenance after clearance of an infection or vaccine [48]. Thus, to next determine if mucosal immunization with an EP67-conjugated CTL peptide vaccine is likely to generate long-lived mucosal and systemic memory cells, we further compared the proportion of CD8a $^+$ Tet $^+$ cells present in the lungs

and spleen on the same day as primary respiratory challenge with SGV that were CD127⁺KLRG1⁻ (Fig. 3C & F).

With the exception of CD8a⁺Tet-M84⁺ cells in the spleen [$P = 0.1140$], CMV-EP67 (Fig. 3C & F, black bars) increased the proportion of CD8a⁺Tet-M84⁺ (15% in the lungs) and CD8a⁺Tet-pp89⁺ cells (55% in lungs; 28% in the spleen) over PBS that were CD127⁺KLRG1⁻ (Fig. 3C & F, white bars), whereas inactive CMV-scEP67 (Fig. 3C & F, grey bars) generated proportions that were similar or below PBS (Fig. 3C, white bars). Furthermore, CMV-EP67 (Fig. 3D & G, black bars) increased the gMFI of anti-CD127 staining of both CD8a⁺Tet-M84⁺ and CD8a⁺Tet-pp89⁺ cells over PBS in the lungs and spleen (Fig. 3D & G, white bars), whereas the gMFI of anti-CD127 staining from CMV-scEP67 (Fig. 3D & G, grey bars) was similar to PBS, indicating that CMV-EP67 also increased the cell surface expression of CD127. Thus, mucosal immunization with an EP67-conjugated CTL peptide vaccine likely generates long-lived mucosal and systemic memory CD8⁺ T cells. As such, an EP67-conjugated mucosal vaccine is expected to provide long-term protection against primary mucosal infections.

4. Discussion

This study provides evidence that mucosal immunization with an EP67-conjugated CTL peptide vaccine generates mucosal and systemic memory CD8⁺ T cells that increase protection against primary mucosal infection with MCMV. We found that intranasal immunization with a peptide vaccine composed of two protective MCMV CTL epitopes, M84 and pp89, covalently attached to the N-terminal of EP67 through a protease-labile double arginine linker (CMV-EP67) (i.) decreased peak titers of productive MCMV in the lungs and salivary glands of naïve female BALB/c mice after primary respiratory challenge with MCMV below inactive CMV-scEP67 and PBS (Fig. 1B) and (ii.) generated a higher proportion of M84- and pp89-responsive CD8⁺ T cells in the lungs and spleen than inactive CMV-scEP67 or PBS (Fig. 2). Although IN administration of EP67 alone to C57BL/6 mice recruits activated neutrophils and NK cells to the airways [21] that can potentially increase protection against MCMV [49–51], both cell populations return to baseline levels within ~7 days of administration even at a relatively higher dose of EP67 alone (individual dose of 240 µg EP67 vs. final individual dose of 50 µg M84-RR-EP67 and 50 µg pp89-RR-EP67 in this study) [21]. In contrast, our CMV-EP67 CTL peptide vaccine increased protection against primary respiratory challenge with SGV 14 days post-immunization (Fig. 1B). Thus, it is unlikely that the activation of innate immunity by EP67 directly contributes to protection against primary respiratory challenge with MCMV in this study.

This study also provides evidence that an EP67-conjugated mucosal vaccine is likely to provide long-term protection against primary mucosal infection. We found that respiratory immunization with CMV-EP67 increased the proportion of mucosal (lungs) and systemic (spleen) epitope-specific CD8a⁺ cells with a cell surface phenotype found on long-lived memory CD8⁺ T cells (CD127⁺KLRG1⁻) over inactive CMV-scEP67 and PBS (Fig. 3C & F).

4.1 Role of EP67 in generating mucosal and systemic memory CD8⁺ T cells by CTL peptide vaccines

The volume used for IN administration in this study (15 µL) is expected to deposit vaccine primarily in the nasal cavity [32]. Thus, given that EP67 has increased affinity for APCs that express C5aR [18], it is possible that EP67 generates mucosal and systemic memory CD8⁺T cells against covalently attached CTL epitopes, in part, by first increasing M-cell transcytosis of the vaccine into nasal-associated lymphoid tissue (NALT) through EP67's affinity for the C5a receptor (C5aR/CD88) that is likely expressed on the surface of NALT M-cells as it is on the surface of gut-associated lymphoid tissue (GALT) M-cells [52]. EP67 then likely generates mucosal adaptive immune responses by (i.) binding and activating NALT DCs through interactions with C5aR [22, 25] to increase processing and presentation of attached epitopes and subsequent DC migration to draining lymph nodes and (ii.) increasing the recruitment of monocytes, macrophages, neutrophils, and DCs into the airways through the release of cytokines and chemokines from resident and recruited DCs as observed after IN administration of EP67 alone [21] to promote vaccine uptake and subsequent generation of CD8⁺ T cell responses. A portion of the vaccine in the NALT is also likely carried by DCs and/or transported by lymphatic drainage to the spleen where it generates systemic cellular immune responses by activating APCs in the spleen [16].

4.2 Role of EP67 in generating long-lived memory MPEC by CTL peptide vaccines

DCs shape CD8⁺ T cell differentiation through three major signals [53]: The recognition of the CTL epitope-MHCI complex on the surface of DCs by naïve epitope-specific CD8⁺ T cells (Signal 1), the formation of an immunological synapse between DCs and naïve epitope-specific CD8⁺ T cells through interactions between leukocyte function associated antigen (LFA) on CD8⁺ T cells and ICAM-1 (CD54) on the surface of DCs (Signal 2), and the localized cytokine milieu within the DC-CD8⁺ T cell interacting region (Signal 3). When the concentration of immunogen is limited (e.g., peptide vaccines), the expression of ICAM-1 (CD54) on the surface of activated DCs during Signal 2 is essential for generating long-lived memory precursor effector cells (MPEC: CD8⁺CD127⁺KLRG1⁻ T cells) by increasing the recruitment of naïve T cells, prolonging DC-CD8⁺ T cell interactions, and facilitating cytokine signaling [54]. Treatment of human monocyte-derived DCs (moDCs) with C5a (from which EP67 is derived) or EP67 alone (not shown) increases the surface expression of ICAM-1 [55]. Thus, it is possible that EP67 increases the generation of long-lived CD8⁺ T cells by CTL peptide vaccines, in part, by increasing the expression of ICAM-1 on the surface of DCs during Signal 2. Furthermore, high levels of inflammation during DC-CD8⁺ T cell interactions (Signal 3), especially increased levels of IL-12, decrease the generation of long-lived MPEC [56–58], whereas neutralizing IL-12 or using adjuvants that generate low levels of inflammation increases the generation of long-lived MPEC [58]. Thus, given that EP67 activates C5aR-expressing DCs with minimal activation of neutrophil-mediated inflammation [18] and treating human moDCs with EP67 alone induces low levels of IL-12 *in vitro* (not shown), it is also possible that EP67 increases the generation of long-lived CD8⁺ T cells by CTL peptide vaccines, in part, by limiting neutrophil activation during APC-T cell interactions and inducing only low levels of IL-12 from DCs during CD8⁺ T cell differentiation.

5. Conclusions

In summary, our results indicate that mucosal immunization with an EP67-conjugated CTL peptide vaccine generates epitope-responsive, long-lived mucosal and systemic memory CD8⁺ T cells that increase protection against primary mucosal infection with MCMV. Thus, EP67 may be an effective adjuvant for mucosal vaccines and warrants further study.

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REFERENCES

1. Czerkinsky, C.; Holmgren, J. Mucosal Delivery Routes for Optimal Immunization: Targeting Immunity to the Right Tissues. In: Kozlowski, PA., editor. Mucosal vaccines : modern concepts, strategies, and challenges. Heidelberg: Springer; 2012. p. 1-18.
2. Lycke N. Recent progress in mucosal vaccine development: potential and limitations. Nature reviews. Immunology. 2012; 12:592–605.
3. Ebsensen T, Guzman CA. Immune modulators with defined molecular targets: cornerstone to optimize rational vaccine design. Hum Vaccin. 2008; 4:13–22. [PubMed: 18376145]
4. Kraehenbuhl JP, Neutra MR. Mucosal vaccines: where do we stand? Curr Top Med Chem. 2013; 13:2609–2628. [PubMed: 24066889]
5. Woodrow KA, Bennett KM, Lo DD. Mucosal vaccine design and delivery. Annual review of biomedical engineering. 2012; 14:17–46.
6. Levine MM. Immunogenicity and efficacy of oral vaccines in developing countries: lessons from a live cholera vaccine. BMC biology. 2010; 8:129. [PubMed: 20920375]
7. Czerkinsky C, Holmgren J. Enteric vaccines for the developing world: a challenge for mucosal immunology. Mucosal immunology. 2009; 2:284–287. [PubMed: 19421181]
8. Holmgren J. Actions of cholera toxin and the prevention and treatment of cholera. Nature. 1981; 292:413–417. [PubMed: 7019725]
9. Gluck U, Gebbers JO, Gluck R. Phase 1 evaluation of intranasal virosomal influenza vaccine with and without Escherichia coli heat-labile toxin in adult volunteers. Journal of virology. 1999; 73:7780–7786. [PubMed: 10438868]
10. Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, Spyr C, Steffen R. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med. 2004; 350:896–903. [PubMed: 14985487]
11. Lawson LB, Norton EB, Clements JD. Defending the mucosa: adjuvant and carrier formulations for mucosal immunity. Current opinion in immunology. 2011; 23:414–420. [PubMed: 21511452]
12. Chadwick S, Kriegel C, Amiji M. Nanotechnology solutions for mucosal immunization. Advanced drug delivery reviews. 2010; 62:394–407. [PubMed: 19931581]
13. Rhee JH, Lee SE, Kim SY. Mucosal vaccine adjuvants update. Clinical and experimental vaccine research. 2012; 1:50–63. [PubMed: 23596577]
14. Ramirez K, Wahid R, Richardson C, Bargatze RF, El-Kamary SS, Szein MB, Pasetti MF. Intranasal vaccination with an adjuvanted Norwalk virus-like particle vaccine elicits antigen-specific B memory responses in human adult volunteers. Clinical immunology. 2012; 144:98–108. [PubMed: 22710446]

15. Newsted D, Fallahi F, Golshani A, Azizi A. Advances and challenges in mucosal adjuvant technology. *Vaccine*. 2015; 33:2399–2405. [PubMed: 25865473]
16. Borges O, Lebre F, Bento D, Borchard G, Junginger HE. Mucosal vaccines: recent progress in understanding the natural barriers. *Pharmaceutical research*. 2010; 27:211–223. [PubMed: 19953309]
17. Taylor SM, Sherman SA, Kirnarsky L, Sanderson SD. Development of response-selective agonists of human C5a anaphylatoxin: conformational, biological, and therapeutic considerations. *Curr Med Chem*. 2001; 8:675–684. [PubMed: 11281848]
18. Vogen SM, Paczkowski NJ, Kirnarsky L, Short A, Whitmore JB, Sherman SA, Taylor SM, Sanderson SD. Differential activities of decapeptide agonists of human C5a: the conformational effects of backbone N-methylation. *Int Immunopharmacol*. 2001; 1:2151–2162. [PubMed: 11710544]
19. Sheen TR, Cavaco CK, Ebrahimi CM, Thoman ML, Sanderson SD, Morgan EL, Doran KS. Control of methicillin resistant *Staphylococcus aureus* infection utilizing a novel immunostimulatory peptide. *Vaccine*. 2011; 30:9–13. [PubMed: 22044742]
20. Hung CY, Hurtgen BJ, Bellecourt M, Sanderson SD, Morgan EL, Cole GT. An agonist of human complement fragment C5a enhances vaccine immunity against *Coccidioides* infection. *Vaccine*. 2012; 30:4681–4690. [PubMed: 22575167]
21. Sanderson SD, Thoman ML, Kis K, Virts EL, Herrera EB, Widmann S, Sepulveda H, Phillips JA. Innate immune induction and influenza protection elicited by a response-selective agonist of human C5a. *PLoS one*. 2012; 7:e40303. [PubMed: 22792270]
22. Morgan EL, Morgan BN, Stein EA, Virts EL, Thoman ML, Sanderson SD, Phillips JA. Enhancement of in vivo and in vitro immune functions by a conformationally biased, response-selective agonist of human C5a: implications for a novel adjuvant in vaccine design. *Vaccine*. 2009; 28:463–469. [PubMed: 19836478]
23. Phillips JA, Morgan EL, Dong Y, Cole GT, McMahan C, Hung CY, Sanderson SD. Single-step conjugation of bioactive peptides to proteins via a self-contained succinimidyl bis-arylhydrazone. *Bioconjug Chem*. 2009; 20:1950–1957. [PubMed: 19788175]
24. Morgan EL, Thoman ML, Sanderson SD, Phillips JA. A novel adjuvant for vaccine development in the aged. *Vaccine*. 2010; 28:8275–8279. [PubMed: 20965299]
25. Hegde GV, Meyers-Clark E, Joshi SS, Sanderson SD. A conformationally-biased, response-selective agonist of C5a acts as a molecular adjuvant by modulating antigen processing and presentation activities of human dendritic cells. *Int Immunopharmacol*. 2008; 8:819–827. [PubMed: 18442785]
26. Buchner RR, Vogen SM, Fischer W, Thoman ML, Sanderson SD, Morgan EL. Anti-human kappa opioid receptor antibodies: characterization of site-directed neutralizing antibodies specific for a peptide kappa R(33–52) derived from the predicted amino terminal region of the human kappa receptor. *Journal of immunology*. 1997; 158:1670–1680.
27. Tempero RM, Hollingsworth MA, Burdick MD, Finch AM, Taylor SM, Vogen SM, Morgan EL, Sanderson SD. Molecular adjuvant effects of a conformationally biased agonist of human C5a anaphylatoxin. *Journal of immunology*. 1997; 158:1377–1382.
28. Ulrich JT, Cieplak W, Paczkowski NJ, Taylor SM, Sanderson SD. Induction of an antigen-specific CTL response by a conformationally biased agonist of human C5a anaphylatoxin as a molecular adjuvant. *Journal of immunology*. 2000; 164:5492–5498.
29. Sanderson SD, Cheruku SR, Padmanilayam MP, Vennerstrom JL, Thiele GM, Palmatier MI, Bevins RA. Immunization to nicotine with a peptide-based vaccine composed of a conformationally biased agonist of C5a as a molecular adjuvant. *Int Immunopharmacol*. 2003; 3:137–146. [PubMed: 12538044]
30. Holtappels R, Thomas D, Reddehase MJ. Identification of a K(d)-restricted antigenic peptide encoded by murine cytomegalovirus early gene M84. *J Gen Virol*. 2000; 81:3037–3042. [PubMed: 11086134]
31. Reddehase MJ, Rothbard JB, Koszinowski UH. A pentapeptide as minimal antigenic determinant for MHC class I-restricted T lymphocytes. *Nature*. 1989; 337:651–653. [PubMed: 2465495]

32. Southam DS, Dolovich M, O'Byrne PM, Inman MD. Distribution of intranasal instillations in mice: effects of volume, time, body position, and anesthesia. *American journal of physiology. Lung cellular and molecular physiology*. 2002; 282:L833–L839. [PubMed: 11880310]
33. Morello CS, Ye M, Hung S, Kelley LA, Spector DH. Systemic priming-boosting immunization with a trivalent plasmid DNA and inactivated murine cytomegalovirus (MCMV) vaccine provides long-term protection against viral replication following systemic or mucosal MCMV challenge. *Journal of virology*. 2005; 79:159–175. [PubMed: 15596812]
34. Gonzalez Armas JC, Morello CS, Cranmer LD, Spector DH. DNA immunization confers protection against murine cytomegalovirus infection. *Journal of virology*. 1996; 70:7921–7928. [PubMed: 8892915]
35. Ye M, Morello CS, Spector DH. Strong CD8 T-cell responses following coimmunization with plasmids expressing the dominant pp89 and subdominant M84 antigens of murine cytomegalovirus correlate with long-term protection against subsequent viral challenge. *Journal of virology*. 2002; 76:2100–2112. [PubMed: 11836387]
36. Gopal IN, Quinn A, Henry SC, Hamilton JD, Staats HF, Frothingham R. Nasal peptide vaccination elicits CD8 responses and reduces viral burden after challenge with virulent murine cytomegalovirus. *Microbiology and immunology*. 2005; 49:113–119. [PubMed: 15722596]
37. Streeck H, Frahm N, Walker BD. The role of IFN- γ ELISPOT assay in HIV vaccine research. *Nature protocols*. 2009; 4:461–469. [PubMed: 19282851]
38. Saletti G, Cuburu N, Yang JS, Dey A, Czerkinsky C. Enzyme-linked immunospot assays for direct ex vivo measurement of vaccine-induced human humoral immune responses in blood. *Nature protocols*. 2013; 8:1073–1087. [PubMed: 23660756]
39. Krmpotic A, Bubic I, Polic B, Lucin P, Jonjic S. Pathogenesis of murine cytomegalovirus infection. *Microbes Infect*. 2003; 5:1263–1277. [PubMed: 14623023]
40. Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science*. 1992; 257:238–241. [PubMed: 1352912]
41. Holtappels R, Podlech J, Grzimek NK, Thomas D, Pahl-Seibert MF, Reddehase MJ. Experimental preemptive immunotherapy of murine cytomegalovirus disease with CD8 T-cell lines specific for ppM83 and pM84, the two homologs of human cytomegalovirus tegument protein ppUL83 (pp65). *Journal of virology*. 2001; 75:6584–6600. [PubMed: 11413326]
42. Fernandez JA, Zavala F, Tsuji M. Phenotypic and functional characterization of CD8(+) T cell clones specific for a mouse cytomegalovirus epitope. *Virology*. 1999; 255:40–49. [PubMed: 10049820]
43. Karlsson AC, Martin JN, Younger SR, Brecht BM, Epling L, Ronquillo R, Varma A, Deeks SG, McCune JM, Nixon DF, Sinclair E. Comparison of the ELISPOT and cytokine flow cytometry assays for the enumeration of antigen-specific T cells. *Journal of immunological methods*. 2003; 283:141–153. [PubMed: 14659906]
44. Pearce EL, Shedlock DJ, Shen H. Functional characterization of MHC class II-restricted CD8+CD4– and CD8–CD4– T cell responses to infection in CD4–/– mice. *Journal of immunology*. 2004; 173:2494–2499.
45. Ahmed R, Gray D. Immunological memory and protective immunity: understanding their relation. *Science*. 1996; 272:54–60. [PubMed: 8600537]
46. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nature immunology*. 2003; 4:1191–1198. [PubMed: 14625547]
47. Schluns KS, Kieper WC, Jameson SC, Lefrancois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nature immunology*. 2000; 1:426–432. [PubMed: 11062503]
48. Sallusto F, Lanzavecchia A, Araki K, Ahmed R. From vaccines to memory and back. *Immunity*. 2010; 33:451–463. [PubMed: 21029957]
49. Stacey MA, Marsden M, Pham NT, Clare S, Dolton G, Stack G, Jones E, Klenerman P, Gallimore AM, Taylor PR, Snelgrove RJ, Lawley TD, Dougan G, Benedict CA, Jones SA, Wilkinson GW,

- Humphreys IR. Neutrophils recruited by IL-22 in peripheral tissues function as TRAIL-dependent antiviral effectors against MCMV. *Cell host & microbe*. 2014; 15:471–483. [PubMed: 24721575]
50. Bancroft GJ, Shellam GR, Chalmer JE. Genetic influences on the augmentation of natural killer (NK) cells during murine cytomegalovirus infection: correlation with patterns of resistance. *Journal of immunology*. 1981; 126:988–994.
51. Bukowski JF, Woda BA, Welsh RM. Pathogenesis of murine cytomegalovirus infection in natural killer cell-depleted mice. *Journal of virology*. 1984; 52:119–128. [PubMed: 6207307]
52. Kim SH, Jung DI, Yang IY, Kim J, Lee KY, Nochi T, Kiyono H, Jang YS. M cells expressing the complement C5a receptor are efficient targets for mucosal vaccine delivery. *Eur J Immunol*. 2011; 41:3219–3229. [PubMed: 21887786]
53. Cui W, Kaech SM. Generation of effector CD8+ T cells and their conversion to memory T cells. *Immunological reviews*. 2010; 236:151–166. [PubMed: 20636815]
54. Cox MA, Barnum SR, Bullard DC, Zajac AJ. ICAM-1-dependent tuning of memory CD8 T-cell responses following acute infection. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:1416–1421. [PubMed: 23297203]
55. Li K, Fazekasova H, Wang N, Peng Q, Sacks SH, Lombardi G, Zhou W. Functional modulation of human monocytes derived DCs by anaphylatoxins C3a and C5a. *Immunobiology*. 2012; 217:65–73. [PubMed: 21855168]
56. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, Gapin L, Kaech SM. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity*. 2007; 27:281–295. [PubMed: 17723218]
57. Kaech SM, Cui W. Transcriptional control of effector and memory CD8+ T cell differentiation. *Nature reviews. Immunology*. 2012; 12:749–761.
58. Slutter B, Pewe LL, Kaech SM, Harty JT. Lung airway-surveilling CXCR3(hi) memory CD8(+) T cells are critical for protection against influenza A virus. *Immunity*. 2013; 39:939–948. [PubMed: 24238342]

Highlights

- An EP67-conjugated MCMV CTL peptide vaccine (CMV-EP67) was administered IN
- CMV-EP67 partially protected against respiratory challenge with MCMV
- CMV-EP67 increased epitope-responsive CD8⁺ T cells in the lungs and spleen
- CMV-EP67 increased long-lived CD127⁺/KLRG1⁻ CD8⁺ T cells in the lungs and spleen
- EP67 may be an effective adjuvant for mucosal vaccines

Figure 1.

Effect of intranasal immunization with an EP67-conjugated CTL peptide vaccine on peak titers of productive murine cytomegalovirus (MCMV) in tropic tissues after primary respiratory challenge with salivary gland-derived MCMV (SGV). (A) Sterile PBS vehicle (white bars, 15 μ L), inactive CMV-scEP67 (M84-RR-scEP67/pp89-RR-scEP67, 50 μ g each) (grey bars), or CMV-EP67 (M84-RR-EP67/pp89-RR-EP67, 50 μ g each) (black bars) was administered intranasally (IN) to 4-week-old naïve female BALB/c mice on days 0, 7, and 14. On day 28 (14 days post-immunization), SGV was administered IN (5×10^3 PFU in 50 μ L) and average titers of productive MCMV (MCMV PFU/g tissue) in the lungs, and salivary glands were compared on the indicated day of peak MCMV infection. (B) Average MCMV plaque forming units (PFU) / g tissue $\pm$ SD (n=3 mice) in MCMV-tropic tissues on the indicated day of peak MCMV infection were determined by plaque assay against NIH/3T3 cells and compared to PBS by one-way ANOVA with Dunnet's post-test. Data are representative of at least two independent experiments.

Figure 2.

Effect of intranasal immunization with an EP67-conjugated CTL peptide vaccine on the generation of epitope-responsive mucosal and systemic CD8⁺ T cells by the day of primary respiratory challenge with salivary gland-derived MCMV (SGV). Epitope-responsive lymphocytes (lungs) and splenocytes were compared 14 days post-immunization by ICS (A & B) and ELISPOT (C & D), respectively. (A) Representative ICS data of gated CD8a⁺ cells showing the percent of total CD8a⁺ cells in the lungs that were IFN-γ⁺ (upper left quadrant), TNF-α⁺ (lower right quadrant), or both IFN-γ⁺ and TNF-α⁺ (upper right quadrant) after stimulating pooled lymphocytes (n=3 mice) from each treatment group with the indicated CTL epitope (M84 or pp89) or media alone (not shown). (B) The number of M84- or pp89-responsive CD8a⁺ cells / 10⁶ CD8a⁺ cells from A. that secrete IFN-γ (white bars) or TNF-α (grey bars) was determined by subtracting background staining from unstimulated cells. (C) Representative ELISPOT data showing the number of IFN-γ spots after incubating splenocytes with media alone, M84, or pp89 for 48 hrs. (D) The average number of epitope-responsive IFN-γ spot-forming units (SFU) / 10⁶ splenocytes ± SD (n=3

mice) from **C.** was determined by subtracting spots from splenocytes treated with media alone, then compared to PBS (D, white bars) by nonparametric one-way ANOVA with Dunn's post-test. Data are representative of at least two independent experiments.

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Figure 3.

Effect of intranasal immunization with an EP67-conjugated CTL vaccine on the proportion and cell surface phenotype of mucosal and systemic epitope-specific CD8⁺ T cells. The surface phenotype of mucosal (lungs) and systemic (spleen) epitope-specific CD8a⁺ cells was determined 14 days post-immunization by flow cytometry after staining pooled lymphocytes from the lungs (n=3) or splenocytes from individual animals (n=3) for CD8a, CD44, CD127, KLRG1, and Tetramer-M84 or Tetramer-pp89. (A) Representative FACS data of CD8a⁺ gated lymphocytes from the lungs and spleen that were Tet-M84⁺ or Tet-

pp89⁺. (B) Percent of total CD8a⁺ cells in the lungs from **A.** that were Tet-M84⁺ or Tet-pp89⁺. (C) Percent of total CD8a⁺Tet-M84⁺ or CD8a⁺Tet-pp89⁺ cells in the lungs from **B.** that were CD127⁺KLRG1⁻. (D) Geometric mean fluorescence intensity (gMFI) of anti-CD127 cell surface staining from **B.** (E) Average percent of total CD8a⁺ cells in the spleen $\pm$ SD from **A.** that were Tet-M84⁺ or Tet-pp89⁺. (F) Average percent of total CD8a⁺Tet-M84⁺ or CD8a⁺Tet-pp89⁺ cells in the spleen $\pm$ SD from **E.** that were CD127⁺KLRG1⁻ (G) Average gMFI of anti-CD127 cell surface staining $\pm$ SD from **E.** Average values $\pm$ SD (n=3) in the spleen were compared to PBS treatment by one-way ANOVA with Dunnett's post-test. Data are representative of at least two independent experiments.