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Surface conjugation of EP67 to biodegradable nanoparticles increases the generation of long-lived mucosal and systemic memory T-cells by encapsulated protein vaccine after respiratory immunization and subsequent T-cell-mediated protection against respiratory infection

Shailendra B. Tallapaka¹, Bala V. K. Karuturi², Pravin Yeapuri⁴, Stephen M. Curran⁴, Yogesh A. Sonawane⁵, Joy A. Phillips⁶, D. David Smith⁷, Sam D. Sanderson⁴, Joseph A. Vetro^{3,4,*}

¹Current address: DILIsym Services Inc., Six Davis Drive, PO Box 12317, Research Triangle Park, NC 27709, USA

²Current address: Mylan Pharmaceuticals Inc., 781 Chestnut Ridge Road, Morgantown, WV 26505, USA

³Center for Drug Delivery and Nanomedicine, University of Nebraska Medical Center, 986025 Nebraska Medical Center, Omaha, Nebraska 68198-6025, USA

⁴Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, 986025 Nebraska Medical Center, Omaha, Nebraska 68198-6025, USA

⁵Eppley Institute for Research in Cancer and Allied Diseases, Omaha, NE 68022, USA.

⁶Donald P. Shiley BioScience Center, San Diego State University, San Diego, CA 92115, USA

⁷Department of Biomedical Sciences, Creighton University, 2500 California Plaza, Omaha, NE 68178, USA

Abstract

Encapsulation of protein vaccines in biodegradable nanoparticles (NP) increases T-cell expansion after mucosal immunization but requires incorporating a suitable immunostimulant to increase long-lived memory T-cells. EP67 is a clinically viable, host-derived peptide agonist of the C5a receptor that selectively activates antigen presenting cells over neutrophils. We previously found that encapsulating EP67-conjugated CTL peptide vaccines in NP increases long-lived memory subsets of CTL after respiratory immunization. Thus, we hypothesized that alternatively conjugating EP67 to the NP surface can increase long-lived mucosal and systemic memory T-cells generated by encapsulated protein vaccines. We found that respiratory immunization of naïve female C57BL/6 mice with LPS-free ovalbumin (OVA) encapsulated in PLGA 50:50 NP (~380 nm diameter) surface-conjugated with ~0.1 wt% EP67 through 2 kDa PEG linkers (i.) increased T-cell expansion and long-lived memory subsets of OVA₃₂₃₋₃₃₉-specific CD4⁺ and OVA₂₅₇₋₂₆₄-

* Corresponding Author: Phone: 1-(402)-559-9359; jvetro@unmc.edu.

Declaration of Competing Interests

Authors have no competing interests to declare.

specific CD8a⁺ T-cells in the lungs (CD44^{HI}/CD127/KLRG1) and spleen (CD44^{HI}/CD127/KLRG1/CD62L) and (ii.) decreased peak CFU of OVA-expressing *L. monocytogenes* (LM-OVA) in the lungs, liver, and spleen after respiratory challenge vs. encapsulation in unmodified NP. Thus, conjugating EP67 to the NP surface is one approach to increase the generation of long-lived mucosal and systemic memory T-cells by encapsulated protein vaccines after respiratory immunization.

Keywords

mucosal vaccine; vaccine delivery; nanoparticle; targeted vaccines; dendritic cell targeting; CD88; host-derived immunostimulant; complement-derived immunostimulant

1. Introduction

To establish long-term immunity, prophylactic vaccines must generate long-lived, pathogen-specific adaptive immune responses within the host that reduce, clear, control, and, ideally, prevent infection with the pathogen (i.e., protective immune responses) at levels that correlate with preventing or greatly reducing the incidence of infectious disease in a specific population (Nguipdop Djomo et al., 2013; Plotkin and Gilbert, 2018). Most licensed vaccines protect against infectious disease by generating one or more subsets of long-lived memory B-cells (Ionescu and Urschel, 2019) after intramuscular (IM), subcutaneous (SQ), or percutaneous administration (systemic immunization) that maintain or quickly establish sufficient levels of protective antibodies (humoral immune responses) within the bloodstream during subsequent infections (Rappuoli, 2007; Thakur et al., 2012). Although generating protective antibodies by systemic immunization is relatively effective against many extracellular pathogens that infect the blood, lymph, epithelial surfaces, or interstitial spaces, there is preclinical and clinical evidence that additionally generating long-lived protective CD4⁺ and/or CD8⁺ memory T-cells (cellular immune responses) is required for protection against pathogens without licensed vaccines that have a relatively short extracellular phase (intracellular pathogens) and/or have hypermutated surface antigens (Amanua and Slifka, 2011). Furthermore, the majority of pathogens that cause infectious disease invade the host through digestive, respiratory, and urogenital mucosal surfaces (Kraehenbuhl and Neutra, 2013). Thus, additionally generating long-lived protective memory T-cells after oral, sublingual, buccal, nasal, pulmonary, rectal, or vaginal administration (mucosal immunization) has several potential advantages over generating protective antibodies alone by systemic immunization including (i.) establishing long-lived mucosal and systemic immune responses that protect against mucosally-acquired pathogens, pathogens that bypass/quickly bypass epithelial barriers, and antibody-resistant pathogens (ii.) establishing long-lived immune responses in mucosal tissues distal to the site of vaccine administration (iii.) establishing long-lived immune responses despite pre-existing systemic immunity (iv.) simple, painless administration that increases patient compliance, requires little medical training, and will not spread blood-borne infections (v.) the option of frequent boosting and (vi.) lower production costs and regulatory considerations (Czerkinsky and Holmgren, 2012; Ebensen and Guzman, 2008; Kraehenbuhl and Neutra, 2013; Lycke, 2012; Woodrow et al., 2012).

Currently licensed mucosal vaccines are composed of live pathogens (live vaccine) or attenuated strains of live pathogens (live-attenuated vaccine) that generate sufficient levels of protective antibodies. These vaccines are also the most likely to generate long-lived protective memory T-cells but are limited to pathogens that can be grown in culture, take a long time to develop, don't always cross-protect against different pathogenic strains, potentially revert to wild-type virulence, and are rarely safe and stable (Lycke, 2012). Mucosal vaccines alternatively composed of proteins from the pathogen that generate protective humoral and cellular immune responses after infection ("recombinant protein vaccines") can potentially overcome the limitations of live and live-attenuated vaccines. Most protein vaccines, however, do not generate sufficient levels of long-lived mucosal and systemic adaptive immune responses because they are rapidly degraded and cleared from mucosal tissues, do not sufficiently localize to mucosa-associated lymphoid tissue (MALT), are not efficiently internalized by antigen-presenting cells (APC), and lack immunostimulatory moieties from the original pathogen to sufficiently activate APC (Moyle and Toth, 2013; Purcell et al., 2007).

Encapsulation of protein vaccines in nanoscale-sized particles composed of biodegradable polymers is a promising approach to increase the generation of long-lived memory T-cells after mucosal immunization but requires the inclusion of suitable mucosal immunostimulants (Lycke, 2012). Cholera toxin subunit B (CTB), a non-toxic protein with high affinity for GM1 gangliosides on the surface of mammalian cells, is currently the only immunostimulant included as part of a licensed mucosal vaccine (Dukoral: oral, killed vaccine) (Czerkinsky and Holmgren, 2009; Holmgren, 1981). Intranasal (IN) administration of a similar enterotoxin, *E. coli* heat-labile toxin (HLT) (Gluck et al., 1999), or a less toxic form of HLT (Mutsch et al., 2004) with live attenuated influenza vaccines, however, caused Bell's palsy in several participants during Phase I clinical trials. Thus, numerous experimental mucosal immunostimulants are being developed that may be more suitable for all mucosal routes.

Most experimental mucosal immunostimulants are derived from pathogen-associated molecular pattern (PAMP) agonists that stimulate innate immune responses through pattern recognition receptors (PRRs) on APC and other immune sensor cells (Chadwick et al., 2010; Lawson et al., 2011; Rhee et al., 2012). Although PAMP-based immunostimulants increase the generation of mucosal and systemic adaptive immune responses in clinical trials, levels of humoral and cellular immune responses are variable or associated with high levels of inflammation and/or toxicity and stable formulations are difficult to establish (Kraehenbuhl and Neutra, 2013; Lycke, 2012; Newsted et al., 2015).

In contrast to the majority of current mucosal immunostimulants, we previously developed EP67 (Vogen et al., 2001), a novel, host-derived 10-amino acid peptide agonist of C5a receptor 1 (C5aR1/CD88) (Morgan et al., 2009; Sheen et al., 2011) based on the C-terminal of human C5a that acts as an immunostimulant (Sanderson et al., 2012; Sheen et al., 2011) and an adjuvant (Taylor et al., 2001) while minimizing the inflammatory side effects of C5a by selectively activating APC over neutrophils. Systemic immunization with EP67 covalently conjugated to chemical moieties, peptides, intact proteins, or attenuated pathogens generates Th1-biased humoral and cellular immune responses in mice (Buchner et

al., 1997; Hung et al., 2012; Sanderson et al., 2003; Taylor et al., 2001; Tempero et al., 1997; Ulrich et al., 2000). EP67 also increases presentation of conjugated epitopes in MHC I and MHC II of human DC (Hegde et al., 2008) and generates adaptive immune responses with minimal inflammation during immunization (Taylor et al., 2001), increasing the likelihood of generating a larger pool of long-lived memory T-cells (Badovinac et al., 2004; Mueller et al., 2013) while decreasing the possibility of toxicity in humans.

We previously found that EP67-conjugated CTL peptide vaccines generate long-lived memory subsets of CTL after respiratory immunization (Karuturi et al., 2015) that can be increased by encapsulation in biodegradable PLGA 50:50 nanoparticles (NP) and microparticles (MP) (Karuturi et al., 2017). These results indicate that co-encapsulation with conjugated and likely unconjugated EP67 is one strategy to increase the generation of long-lived memory T-cells by encapsulated peptides and proteins. Given that increasing affinity for C5aR1 and other proteins on the surface of M cells increases the efficacy of oral vaccines (Islam et al., 2019; Kim et al., 2011) and that EP67 increases affinity for C5aR1 on rat macrophages (Vogen et al., 2001) and possibly M cells, we hypothesized that alternatively conjugating EP67 to the surface of biodegradable nanoparticles can increase the generation of long-lived memory T-cells by encapsulated protein vaccines after respiratory immunization. To test this hypothesis, we encapsulated an LPS-free model protein, ovalbumin (OVA), in biodegradable PLGA 50:50 nanoparticles (NP) or NP with EP67 surface-conjugated through 2 kDa PEG linkers (EP67-NP) at ~0.1 wt%. We then compared the extent to which NP or EP67-NP affects (i.) the activation and rate of NP internalization in immature murine bone marrow derived dendritic cells (BMDC) (ii.) total expansion and long-lived memory subsets of T-cells specific for encapsulated OVA in the lungs (mucosal) and spleen (systemic) of naïve female C57BL/6 mice after respiratory immunization and (iii.) the extent to which respiratory immunization increases T-cell-mediated protection of naïve female C57BL/6 mice against primary respiratory challenge with recombinant *Listeria monocytogenes* ectopically expressing soluble OVA (LM-OVA).

2. Materials and Methods

2.1 LPS removal from ovalbumin (OVA)

LPS was removed from Grade V hen egg white ovalbumin (OVA: 385 amino acids, MW: 44,287 Da, Sigma) [100 mg] by dissolving in sterile PBS (Dulbecco's "PBS" without Ca^{2+} or Mg^{2+} , GE Healthcare Life Sciences) [1 mL], loading onto a prepacked Detoxi-Gel™ column as instructed (Thermo Scientific), eluting with sterile PBS [1 mL] into pyrogen-free centrifuge tubes, then storing at 4°C.

2.2 Fluorescein conjugation to LPS-free ovalbumin

Isothiocyanate-activated fluorescein (FITC; Fisher Scientific: AC119252500) [5 mg] was dissolved in DMSO (99.5%; Sigma) [0.1 mL] then added to Carbonate Buffer (anhydrous Na_2CO_3 [0.1 M], anhydrous NaHCO_3 [0.1 M] in dH_2O , pH 9.0) [1 mL] containing LPS-free OVA [100 mg] in an LPS-free amber microcentrifuge tube and incubated [25°C] for 2 h with stirring (VorTemp Shaking Incubator) [1000 RPM]. Fluorescein-conjugated OVA (F-OVA) was purified from unconjugated FITC by a PD MidiTrap G-10 column (GE Healthcare Life

Science) as directed under minimal light conditions, eluted with PBS [1.8 mL] into an amber LPS-free microcentrifuge tube then stored at 4°C. The molar ratio of fluorescein to OVA conjugation was determined by measuring the absorbance of F-OVA [1/100 dilution of eluted F-OVA in PBS: ~55.6 mg/mL] at 280 nm and 495 nm:

$$\text{Molar } \frac{F}{P} = \frac{A_{495} / \epsilon_{\text{FITC}}}{(A_{280} - (0.35 \times A_{495})) / \epsilon_{\text{OVA}}}$$

where A_{495} and A_{280} are the respective absorbances at 495 nm and 280 nm, ϵ_{FITC} and ϵ_{OVA} are the respective molar absorptivities of FITC ($68,000 \text{ cm}^{-1} \text{ M}^{-1}$) and OVA ($30,590 \text{ cm}^{-1} \text{ M}^{-1}$) at pH 7.0, and 0.35 is a correction factor for A_{280} of OVA with fluorescein labeling. The F/P molar ratio of OVA fluorescein conjugation was ~1.2. ($A_{280} = 0.797 \pm 0.001$ and $A_{495} = 1.106 \pm 0.002$ vs PBS blank).

2.3 Peptide Synthesis

EP67 (YSFKDMP[MeL]aR where “MeL” is *N*-methyl leucine and “a” is *D*-alanine) (Vogen et al., 2001) or inactive scrambled scEP67 ([MeL]RMYKPaFDS) was activated with sulfhydryl groups by introducing N-terminal cysteine during solid-phase synthesis through a glycine double arginine linker (GRR) and characterized as previously described (Sanderson et al., 1994).

2.4 Encapsulation of ovalbumin in biodegradable nanoparticles surface conjugated with EP67

OVA or F-OVA was encapsulated in PLGA 50:50 nanoparticles (NP) at 10 wt% theoretical loading (mass OVA / mass of NP-OVA) by the emulsification solvent extraction method (ESE) and the NP surface was modified during encapsulation with EP67 or inactive scrambled EP67 through 2 kDa PEG linkers by interfacial activity-assisted surface functionalization (IAASF) (Patil et al., 2009; Toti et al., 2010). A primary water-oil emulsion (W_1/O) was formed by adding an LPS-free “water” solution of LPS-free OVA or F-OVA in PBS [50 mg OVA/mL; 0.2mL] dropwise to a vortexing [500 RPM] dichloromethane (DCM) “oil” solution of ester-terminated poly D,L-lactic-co-glycolic acid (PLGA 50:50; research grade; inherent viscosity 0.58 dL/g; Lactel Pelham, AL) [50 mg PLGA/mL; 2 mL] in an 8-mL glass vial and sonicated on ice for 30 secs total (10 sec pulse/ 2 sec intervals) at 70% amplitude (Misonix Sonicator 3000 w/ model 419 tapered microtip horn with 0.125 inch diameter tip). The secondary water-oil-water emulsion ($W_1/O/W_2$) was formed by immediately transferring the primary W_1/O emulsion dropwise (Pasteur pipette wetted by filling/emptying W_1/O emulsion 10 to 15 times before transfer) into a 20-mL glass scintillation vial containing a vortexed (500 RPM) polyvinyl alcohol (PVA) solution (87-90% hydrolyzed; 30,000 –70,000 Da; Sigma-Aldrich: P8136) [5% v/v in dH₂O; 8 mL] then sonicating again on ice for 30 secs total but at 60% amplitude. The secondary emulsion was transferred to a fresh 20-mL scintillation glass vial and a solution of DCM alone [0.200 mL] or containing poly(L-lactide)(10 kDa)-b-poly(ethylene glycol)(2 kDa)-maleimide (PLLA(10K)-PEG(2K)-MAL, M_n : 14,627 Da, Polysciotech) [20 mg/mL; 0.200 mL] was

added before stirring (cross-shaped 9.5 × 4.7 mm, Teflon-coated, smooth stirring bar) [1000 RPM] for 18 h to fully remove DCM.

Hardened NP were pelleted [25,000 RCF, 4°C, 20 min] in a 50-mL Nalgene centrifuge vial then resuspended in dH₂O [50 mL] and pelleted again [3X] to remove residual PVA. Washed NP were resuspended in dH₂O [10 mL] in a pre-weighed 20-mL scintillation vial. The suspension was flash frozen by submerging the capped vial in liquid N₂, stored at –80°C for 1 h, lyophilized for 48 h, and stored at –20°C. EP67 or inactive scrambled EP67 (scEP67) was then conjugated to the NP surface through the physically incorporated MAL-activated PEG linkers by adding a solution of CGRR-EP67 or CGRR-scEP67 in PBS [1.4 mg peptide/mL; 0.5 mL] to a suspension of maleimide-activated NP in PBS [2 mg/mL; 5 mL] in an 8-mL scintillation glass vial and stirring (cross-shaped 9.5 × 4.7 mm stirring bar) [1000 rpm, r.t., 12 h]. EP67 or scEP67-modified NP were then pelleted [25,000 RCF, 4°C, 20 min.] and resuspended in dH₂O [50 mL] [2X], then pelleted [25,000 RCF, 4°C, 20 min] and resuspended in dH₂O [10 mL], transferred to a pre-weighed capped 20-mL glass scintillation vial, flash frozen as described above, lyophilized for 48 h, and stored at –20°C after sealing with parafilm.

2.5 Diameter and zeta potential of biodegradable particles

Average hydrodynamic diameters (nm) and zeta-potentials (mV) ± SD (n=3 samples from at least two batches) of the particles were determined in dH₂O [0.5 mg/mL] at 25°C using a ZetaSizer Nano ZA (Malvern Instruments, Malvern, UK) equipped with a He-Ne laser (λ = 633 nm) as the incident beam.

2.6 Quantitation of PLL-PEG-MAL incorporation and EP67 surface conjugation to biodegradable nanoparticles

Average wt% (n=2 from at least two batches) of PLLA(10K)-PEG(2K)-MAL that was physically incorporated into NP loaded with OVA or F-OVA was determined by ¹H-NMR (500 MHz, CDCl₃, 1 mg/mL) using unique proton peaks for PLGA 50:50 (polyglycolic acid [PGA]: 4.8 ppm) and PLLA(10K)-PEG(2K)-MAL (PEG: 3.6 ppm) (in bold):

- i. *PLGA 50:50*: [δ 5.28 – 5.19 (m, 2H), 5.17 (dd, *J* = 15.0, 7.2 Hz, 2H), **4.87 (dd, *J* = 18.2, 4.6 Hz, 1H)**, 4.82 (s, 2H), 4.80 (s, 1H), 4.72 (ddd, *J* = 29.5, 16.6, 6.2 Hz, 2H), 1.58 (dt, *J* = 11.0, 4.9 Hz, 11H)]
- ii. *PLLA(10K)-PEG(2K)-MAL*: [δ 5.16 (q, *J* = 7.1 Hz, 1H), **3.64 (s, 1H)**, 1.58 (d, *J* = 7.1 Hz, 3H)]
- iii. *NP*: [δ 5.20 (th, *J* = 17.9, 6.0, 5.5 Hz, 2H), 4.91 – 4.78 (m, 2H), 4.80 – 4.63 (m, 1H), 1.57 (dq, *J* = 14.5, 6.9, 4.9 Hz, 6H)]
- iv. *NP (OVA)*: [(tq, *J* = 23.5, 6.7 Hz, 1H), 4.91 – 4.64 (m, 2H), 1.62 – 1.53 (m, 3H)]
- v. *NP (F-OVA)*: [δ 5.26 (dd, *J* = 13.7, 6.6 Hz, 2H), 5.25 – 5.12 (m, 3H), 4.92 – 4.84 (m, 1H), 4.82 (d, *J* = 5.2 Hz, 4H), 4.82 – 4.75 (m, 1H), 4.75 – 4.65 (m, 1H), 1.58 (ddt, *J* = 10.8, 6.8, 3.6 Hz, 16H), 1.26 (d, *J* = 7.4 Hz, 1H), 0.85 – 0.79 (m, 1H)]

The mass of PLLA(10K)-PEG(2K)-MAL in the NP formulation was calculated as

$$Mass_{PLLA-PEG-MAL} = \frac{(Mass_{NP\ formulation} - Mass_{OVA})}{1 + \left(\frac{moles_{PLLA-PEG-MAL}}{moles_{PLGA}} \right) \left(\frac{M.W_{PLLA-PEG-MAL}(14627\ Da)}{M.W_{PLGA}(\sim 40\ kDa)} \right)}$$

where $Mass_{NP\ formulation}$ was known, $Mass_{OVA}$ in the NP formulation was determined by BCA assay as described (Section 2.7), and, given that $moles_{PEG} = moles_{PLLA-PEG-MAL}$ and $moles_{PGA} = moles_{PLGA}$,

$$\frac{moles_{PLLA-PEG-MAL}}{moles_{PLGA}} = \frac{\left(\frac{Area_{PEG}(3.6\ ppm)}{\#repeating\ units_{PEG}(46\ units) \times nucleides\ per\ repeating\ unit_{PEG}(4\ units)} \right)}{\left(\frac{Area_{PGA}(4.8\ ppm)}{\#repeating\ units_{PGA}(132\ units) \times nucleides\ per\ repeating\ unit_{PGA}(2\ units)} \right)}$$

The wt% of PLLA(10K)-PEG(2K)-MAL in the total NP formulation was then calculated as

$$wt\%_{PLLA-PEG-MAL} = \frac{Mass_{PLLA-PEG-MAL}}{Mass_{NP\ formulation}} \times 100$$

Average mass ratio of surface conjugated EP67 to total NP mass $\pm$ SD (n=2 samples from at least two batches) was determined by amino-acid analysis using norleucine as an internal standard. NP were dissolved in acetone [~ 21 mg/mL] and a small volume [0.05 to 0.1 mL] was spiked with norleucine, dried by Speedvac, hydrolyzed in HCl vapor under argon atmosphere for 21 hours at 110°C , dissolved in 0.02N HCl [0.100 mL], then loaded [50 μL] onto a Hitachi L-8800 Amino Acid Analyzer.

2.7 OVA loading and burst release from biodegradable nanoparticles

Average OVA loading in the NP formulation $\pm$ SD (n=3 from at least two independent batches) was determined as described with modification (Sah, 1997). Particles [~ 10 mg] were equilibrated to r.t., added to DMSO [0.5 mL] in an 8-mL glass vial, and incubated [r.t.] for 1 h with constant shaking. Digestion Solution (NaOH [0.05 M]/ SDS [0.5% w/w] in dH_2O) [5 mL] was added and the entire solution was stirred [400 RPM] in a capped vial overnight. Undissolved polymer was pelleted [10,000 RCF, 10 min] and average OVA concentration in the supernatant was determined by Pierce Micro BCA assay (Thermo Scientific) using LPS-free OVA as the standard and DMSO [0.9% v/v], NaOH [0.05 M] / SDS [0.05% w/v] as the diluent. For scEP67/EP67 surface-conjugated NP, loading was determined in MAL-activated NP before EP67 or scEP67 conjugation. OVA loading was calculated as

$$OVA\ Loading = \frac{\frac{Assay\ \mu g\ OVA}{mL} \times Assay\ Sample\ Volume\ [5.5\ mL]}{Mass_{NP\ formulation}} \times 100$$

and percent encapsulation efficiency (EE%) was calculated as

$$EE \%_{OVA} = \frac{\text{Assayed } \mu\text{g OVA} / \text{mg particles}}{\text{Theoretical } \mu\text{g OVA} / \text{mg particles}} \times 100$$

Average percent of total OVA released from lyophilized NP 24 hours after resuspension (“burst release”) $\pm$ SD (n=3 from at least two independent batches) was determined as described with modification (Karuturi et al., 2017). PLGA particles [~10 mg] were added to a centrifuge tube [2 mL], resuspended in PBST (PBS and Tween-20 [0.05% v/v]) [1 mL], vortexed [20 s], and incubated [37°C] with shaking [200 rpm/min] (Vortemp 56 Shaking Incubator). Supernatants [10,000 RCF, 5 min] were collected after 24 h, stored [–20°C], and the concentration of OVA determined as described above using PBST as the diluent. Percent OVA released from the particles was calculated as

$$\% \text{ OVA released} = \left[\frac{OVA[\mu\text{g} / \text{mL}]_{\text{Supernatant}} \times \text{Sample Volume}[1 \text{ mL}]}{\frac{\text{mass OVA}[\mu\text{g}]}{\text{mass of particles}(\text{mg})} \times \text{mass of particles}[10 \text{ mg}]} \right] \times 100$$

2.8 Animals

All animal procedures were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Naïve mice (C57BL/6NCrl, Charles River Laboratories) were acclimatized in an ABSL-1 (BMDC isolation) or ABSL-2 facility (LM-OVA challenge) under pathogen-free conditions at least one week before experiments.

2.9 Generation and culture of immature bone marrow-derived dendritic cells (BMDC)

BMDC were generated from male C57BL/6 mice [6 to 7 weeks old] as described (Lutz et al., 1999). Femurs and tibiae were harvested from the hind limbs and transferred to a 100-mm petri dish. Bones were cleaned by scraping the muscle tissue using a sterile surgical scalpel (#15, Aspen Surgical, Caledonia, MI), transferring to another 100-mm petri dish and incubating in 70% ethanol [10 mL] for 5 min, then rinsing with RPMI-1640 in another 100-mm petri dish [10 mL] [2x]. Bone marrow was exposed by cutting off the ends with a sterile scissors and flushed out with complete RPMI (cRPMI: HI-FBS (Atlanta Biologicals) [10% v/v], RPMI (Hyclone), L-glutamine (GIBCO) [2 mM], sodium pyruvate (Gibco) [1 mM], non-essential amino acids (Hyclone) [0.1 mM], MEM vitamin solution (Hyclone) [1X], penicillin G / streptomycin sulfate (Gibco) [100 U/mL / 100 µg/mL], β-mercaptoethanol (Sigma) [50 µM]) [5 mL] using a 25-gauge needle. Bone marrow pieces were dispersed in flushed media by trituration (1 mL pipette) then passed through a 70 µm cell strainer (Becton Dickinson, Franklin Lakes, NJ). Cells were pelleted [400 RCF, 4°C, 10 min.] and erythrocytes lysed by resuspending pellet in RBC Lysis Buffer (NH₄Cl (Thermo Fisher) [155 mM], KHCO₃ [10 mM], Tris-EDTA (Fluka) [0.1 M]) [5 mL] and incubating [r.t., 5 min]. Bone marrow cells were pelleted [400 RCF, 4°C, 10 min.] and resuspended in RPMI-1640 [5 mL] [2x] then resuspended in BMDC Media (cRPMI supplemented with rmGM-CSF (Peprotech) [20 ng/mL]) at 4×10^5 cells/mL and plated [10 mL] in 100 mm petri dishes [4×10^6 cells/dish]. Fresh BMDC Media [10 mL] was added to the wells on day 3. BMDC media was collected from each well on day 6, cells were pelleted [400 RCF, 4°C,

10 min.], resuspended in fresh BMDC media [10 mL], and added back to the same wells. BMDC were collected by scraping on day 8 for subsequent experiments.

2.10 Expression of surface activation markers on BMDC after treatment with biodegradable nanoparticles

Day 8 BMDC (Section 2.9) were scraped into a 50 mL conical tube, pelleted [500 RCF, 4°C, 10 min], resuspended in cRPMI [3 mL], counted, resuspended in cRPMI [4×10^6 cells/mL], plated [0.1 mL] in 24-well plates [4×10^5 cells/well], then incubated [4°C or 37°C, 1 h]. Media was aspirated and cRPMI alone [0.5 mL] or cRPMI containing LPS-free OVA [7.8 µg total] encapsulated at 7.8 wt% in the indicated NP [100 µg NP] was added to the wells for 24 h at 37°C. Media and scraped cells [0.5 mL PBS] from each well were combined in the same tube, pelleted [500 RCF, 4°C, 5 min.] and resuspended in PBS [1 mL] [3x], resuspended in PBS [0.1 mL] containing Zombie Yellow (BioLegend) [1 µL], then incubated [r.t.] in the dark [20 min]. FACS Stain Buffer (BD Biosciences) [0.1 mL] was added and cells were pelleted [500 RCF, 4°C, 5 min.]. Supernatants were aspirated and Fc receptors were blocked by resuspending cells in FACS Stain Buffer [0.1 mL/well] containing mouse BD Fc Block [1 µg / 10^6 cells], and incubating on ice for 20 min. FACS Stain Buffer [0.1 mL] was added and cells were pelleted [500 RCF, 4°C, 5 min.]. Cell surfaces were stained for activation markers by resuspending the cells in FACS Stain Buffer [50 µL] containing half the manufacturer's suggested amount of PE-Cy5 Anti-Mouse CD11c (Clone N418, eBioscience), PE Anti-Mouse MHC-II (Clone M5/114.15.2, Miltenyi), FITC Anti-Mouse CD80 (Clone 16-10A1, Miltenyi), APC Anti-Mouse CD86 [Clone PO3.3] (Miltenyi), then incubating on ice in the dark for 30 min. FACS Stain Buffer [0.15 mL] was then added and cells were pelleted [500 RCF, 4°C, 5 min.], resuspended in Fixation Buffer (BioLegend) [0.1 mL/well], and incubated on ice [20 min]. Cells were then prepared for flow cytometry by pelleting [500 RCF, 4°C, 5 min.] and resuspending in FACS Stain Buffer [0.2 mL] [3x]. Stained cells were then analyzed on a BD LSR II flow cytometer (Becton and Dickinson, La Jolla, CA) with a BD High Throughput Sampler using single stained cells for compensation and the maximum number of events were acquired and analyzed by FlowJo software (Tree Star, Ashland, OR, USA). Average mean fluorescence intensities (MFI) of staining live CD11c⁺ cells with the indicated antibody $\pm$ SD (n=2 wells) were compared by one-way ANOVA with Tukey's post-test.

2.11 Biodegradable nanoparticle internalization in BMDC

Day 8 BMDC (Section 2.9) were plated in 24-well plates [4×10^5 cells/well] as described (Section 2.10) but incubated at 4°C or 37°C for 1 h. Media was aspirated and cRPMI [0.5 mL] or cRPMI containing F-OVA [1.6 µg total] encapsulated at 7.8 wt% in the indicated NP [20 µg NP] was added to the wells and incubated [4°C or 37°C, 2 h]. Cells were washed with PBS, stained with Zombie Yellow (BioLegend) and anti-mouse CD11c-PE Cy5 (clone N418, eBioscience, San Diego, CA), then analyzed by flow cytometry gating by forward and side light scattering, zombie yellow exclusion, PE, and fluorescein using unstained cells as background as described (Section 2.10). Average percent of total viable CD11c⁺ cells with F-OVA staining and average median fluorescence intensities (MFI) of F-OVA staining $\pm$ SD (n=2 wells) were compared by one-way ANOVA with Tukey's post-test.

2.12 BMDC activation of T-cell hybridomas specific for encapsulated OVA protein

Day 8 BMDC were plated in 24-well plates [4×10^5 cells/well] as described (Section 2.10). Media was aspirated, cRPMI alone [0.5 mL] or cRPMI containing LPS-free OVA [7.8 μ g] encapsulated in the indicated biodegradable particles [100 μ g] was added to the wells, and incubated [37°C, 2 h]. OVA_{323–339}-specific CD4⁺ (DOBW) or OVA_{257–264}-specific CD8⁺ T-cell hybridomas were grown as described (Harding et al., 1991; Pfeifer et al., 1993), collected into 50-mL centrifuge tubes, pelleted [500 RCF, 4°C, 10 min], resuspended in D10F Media [DMEM/High Glucose (Hyclone, Logan, UT), HI-FBS [10% v/v], penicillin/streptomycin [100 U/mL], sodium pyruvate [1 mM], HEPES [10 mM], and 2-mercaptoethanol [0.5 mM]] [3 mL], counted, resuspended in D10F at 5×10^5 cells/mL, then plated [0.1 mL] in a 96-well plate [5×10^4 cells/well]. BMDC were scraped into 15-mL centrifuge tubes, pelleted [500 RCF, 4°C, 10 min] and resuspended in PBS [15 mL] [2x], resuspended in D10F [5×10^5 cells/mL], added [0.1 mL] to the 96-well plate containing the T-cell hybridomas [1:1 BMDC:T-cell hybridoma], then incubated [37°C, 18 h]. Supernatants were pelleted [1000 RCF, 4°C, 10 min.], stored at –80°C, and IL-2 concentrations released from the T-cell hybridomas were determined by ELISA as instructed (ELISA MAXTM Standard, BioLegend). Average concentrations of IL-2 released into the media $\pm$ SD (n=2 wells) were compared by one-way ANOVA with Tukey's post-test.

2.13 Respiratory immunization

Vehicle alone (sterile PBS) [50 μ L] or vehicle containing LPS-free OVA encapsulated in the indicated NP [25 μ g LPS-free OVA in ~320.5 μ g NP total] was intranasally administered to naïve female C57BL/6 mice (~6-weeks old; n=7) on days 0 and 7 by administering ketamine/xylazine [105/5 mg/kg] I.P., holding upright, and alternating drops between nares with a 200 μ L pipette. Intranasal administration of 50 μ L is expected to deposit vaccine primarily in the nasal cavity and lungs (i.e. respiratory immunization) (Southam et al., 2002).

2.14 Preparation of lymphocytes (lungs) and splenocytes

Mice were sacrificed by CO₂ asphyxiation/cervical dislocation on the same day as primary respiratory challenge with LM-OVA (14 days post-immunization). The lungs were then perfused by injecting ice-cold PBS [5 mL] with a 25G needle through the right ventricle of the heart. Both the lungs and spleen were isolated and immediately stored on ice in sterile 15-mL conical tubes containing 5 mL of ice-cold complete RPMI Media (cRPMI: RPMI 1640 containing heat-inactivated FBS [10% v/v; < 0.3 EU LPS], PEN [100 U/mL]/STREP [100 μ g/mL], NEAA [1% w/v], Vitamins [1% w/v], Sodium Pyruvate [1% w/v], β -mercaptoethanol [50 μ M added fresh on the day of isolation]). To isolate lung lymphocytes, lungs were transferred into a sterile 6-well cell culture plate [one lung/well], minced into small pieces with a sterile scalpel (#15, Bard-Parker), and transferred to a sterile C-Tube (Miltenyi) containing cRPMI [5 mL] with collagenase IV (Worthington Enzymes) [2 mg/mL]. Lung fragments were homogenized with a gentleMACSTM Tissue Dissociator (Miltenyi) using the “m_lung_01” setting) then incubated [37°C, 1 h] in a shaking incubator (Vortem) [200 RPM]. Digested lung fragments were homogenized again with the Tissue Dissociator cells were passed through a sterile 40 μ m cell strainer. Cells were then pelleted

[400 RCF, 4°C, 5 min] and resuspended in cRPMI [5 mL] in a sterile 15 mL conical tube (BD Falcon). Lympholyte-M solution (Cedarlane Labs) [5 mL] was under-laid below the cell suspension using a sterile Pasteur pipette and the entire solution was centrifuged [1500 RCF, 20 mins, RT, no brakes]. Lymphocytes were collected from the interphase with a sterile Pasteur pipette, transferred to a sterile 15-mL conical tube, resuspended in PBS [10 mL], pelleted [500 RCF, 4°C, 5 min.], resuspended in cRPMI [1 mL], cell count and viability determined by trypan blue exclusion (Cellometer Auto T4, Nexcelom Biosciences), 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 at least 3 to 4 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 PBS, pelleted [500 RCF, 4°C, 10 min], resuspended in RBC lysis buffer (ACK Lysing Buffer) [4 mL], and incubated at r.t. for 5–7 min. After incubation, 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 with PBS to 50 mL and pelleted [500 RCF, 4°C, 10 min.] [2X], resuspended in cRPMI [3 mL], cell count and viability determined by trypan blue exclusion (Cellometer Auto T4, Nexcelom Biosciences), then stored on ice for later use.

2.15 FACS Analysis of OVA-specific CD4⁺ and CD8⁺ T-cells

The surface phenotype of epitope-specific CD8⁺ cells was determined 14 days post-immunization (Day 21) by flow cytometry. Lymphocytes (lungs) and splenocytes were isolated (Section 2.14), diluted in cRPMI [10^7 cells / mL], and plated [0.1 mL] in a sterile 96-well plate [1×10^6 cells/well]. An additional single well was plated [0.1 mL] from PBS immunizations for an unstained FACS control. Cells were pelleted [400 RCF, 5°C, 5 min] and resuspended in PBS [0.1 mL] [2x], resuspended in PBS [0.1 mL] containing Zombie NIR dye (BioLegend) [1 µL], incubated at r.t. for 20 min in the dark, resuspended in BD Stain Buffer [0.1 mL], resuspended in BD Stain Buffer [0.1 mL] containing mouse BD Fc Block [1µg / 10^6 cells], and incubated on ice [20 min]. Cells were resuspended in BD Stain Buffer [0.1 mL], then resuspended in BD Stain Buffer alone [50 µL] [unstained cells; single well] or BD Stain Buffer [50 µL] containing (MHC Class-I Tetramers-BV421 (H-2Kb / SIINFEKL) or MHC Class-II Tetramers-BV421 (I-Ab / HAAHAEINEA) (NIH Tetramer Core Facility at Emory University, Atlanta, GA, USA [1 µg/ 10^6 cells], and incubated [37°C, in the dark, 30 min]. Cells were resuspended in BD Stain Buffer [0.15 mL] then resuspended in BD Stain Buffer [50 µL] containing half the manufacturer's suggested amount of FITC Anti-Mouse CD8a FITC (Clone 53–6.7; BioLegend) or Alexa fluor 488 Anti-Mouse CD4 (Clone GK1.5;BioLegend), PE Anti-Mouse CD127 (Clone A7R34;BioLegend), APC Anti-Mouse KLRG1 (Clone 2F1;BioLegend), PE/Dazzle Anti-Mouse CD62L (Clone MEL-14;BioLegend) and PE/Cy5 Anti-Mouse CD44 (Clone IM7;BioLegend) and incubated [on ice, in the dark, 30 min]. Cells were then resuspended in BD Stain Buffer [0.15 mL], resuspended in Fixation Buffer [0.1 mL], then incubated [on ice, 20 min]. Fixed cells were resuspended in BD Stain Buffer [0.2 mL] [3x] and analyzed on a BD LSR-II flow cytometer

(Becton and Dickinson, La Jolla, CA). Splenocytes from immunized animals were used as single-stains for tetramers and BD CompBeads (Anti- Rat/Hamster Igκ) were used as single-stains for all the other antibodies for compensation settings. Maximum number of events from each sample were acquired and analyzed by FLOWJo software (Tree Star, Ashland, OR, USA). The lymphocyte population was gated based on FSC (A) vs. SSC (A) plots and used for subsequent analysis (at least 15,000 CD8⁺ cells from the lungs and at least 10,000 CD8⁺ cells from the spleen). Average proportions of total CD4⁺CD44^{HI}Tet-OVA_{323–339}⁺ and CD8a⁺CD44^{HI}Tet-OVA_{257–264}⁺ T-cells in the lungs per 10⁶ CD4⁺ or 10⁶ CD8a⁺ T-cells, respectively, and proportions of CD127/KRLG1 memory subsets within CD4⁺CD44^{HI}Tet-OVA_{323–339}⁺ or CD8⁺CD8a⁺CD44^{HI}Tet-OVA_{257–264}⁺ T-cells ±SD (n=4 mice) per 10⁶ CD4⁺ or 10⁶ CD8a⁺ T-cells, respectively, were compared using Kruskal-Wallis one-way ANOVA with uncorrected Dunn's post-test

2.16 LM-OVA growth and storage

LM-OVA, a virulent strain of recombinant *Listeria monocytogenes* ectopically expressing a secreted form of ovalbumin ("rLM-OVA", kindly donated by J. Harty, University of Iowa) (Pham et al., 2010; Pope et al., 2001) was handled according to UNMC IBC regulations. LM-OVA was cultured in Tryptic soy broth (Sigma-Aldrich) containing streptomycin sulfate [50 µg/mL] at 37°C in a shaking incubator [150 RPM] for 24 h. Sterile glycerol [80% v/v in sterile dH₂O, 1 mL] was added to LM-OVA culture [9 mL], aliquoted into cryovials [1 mL], and stored at –80°C until the day of infection. LM-OVA titers of a frozen stock from the same batch were determined one day before challenge.

2.17 LM-OVA quantitation

To determine titers of LM-OVA, serial dilutions [20 µL] were pipetted onto a TSB-agar plate (100 mm; MIDSCI), dried in a sterile hood, then incubated for 24 h at 37°C. Average LM-OVA titers/mL were calculated as

$$\frac{LM-OVA\ CFU}{mL} = Number\ of\ colonies \times \frac{Dilution\ factor}{Dilution\ volume\ [mL]}$$

where *Number of colonies* was taken from the highest dilution factor where 5 to 50 colonies were observed, *Dilution factor* was the dilution factor used for colony counting, and *Dilution volume* was the volume plated onto the agar plate [0.02 mL].

2.18 LM-OVA respiratory challenge and quantitation of LM-OVA burden

Fourteen days after the final immunization (Day 21) animals were challenged with a sub-lethal dose of LM-OVA [2×10^7 CFU] in the same method as vaccines. Three days post-challenge (Day 24), animals were sacrificed, organs were harvested (lungs, liver, and spleen) and homogenized in cRPMI [1 mL] using a hand-held homogenizer, and 10-fold serial dilutions were prepared using D-PBS as diluent. Dilutions [20 µL] were pipetted onto TSB-agar plates (100 mm; MIDSCI), allowed to dry in a sterile hood, and incubated [37°C, 24 h]. The number of bacterial colonies was then counted by eye. Average LM-OVA titers/g of tissue was calculated as

$$\frac{LM - OVA CFU}{g} = \text{Number of colonies} \times \frac{\text{Dilution factor}}{\text{Dilution volume [mL]}} \times \frac{\text{Volume of homogenate (mL)}}{\text{Organ sample mass (g)}}$$

where *Number of colonies* was taken from the highest *Dilution factor* where 5 to 50 colonies were observed, *Dilution factor* was the dilution used for colony counting, *Dilution volume* was the volume plated onto the agar plate [0.02 mL], *Volume of homogenate* was the volume used for homogenizing the organ [1 mL] and *Organ sample mass* was mass of the organ sample that was homogenized. Average peak LM-OVA colony forming units (CFU) per gram of indicated tissue $\pm$ SD (n = 7 mice) were compared by Kruskal-Wallis one-way ANOVA with Dunn's uncorrected post-test.

3. Results

3.1 Conjugating EP67 to the surface of PLGA 50:50 nanoparticles

To conjugate EP67 or inactive scrambled EP67 (scEP67) to the surface of biodegradable nanoparticles (NP), we first activated the NP surface with maleimide (MAL) groups through 2 kDa PEG linkers by physically incorporating a PLLA(10K)-PEG(2K)-MAL diblock copolymer into PLGA 50:50 NP during encapsulation of LPS-free ovalbumin (OVA) or fluorescein-labeled OVA (F-OVA) at 10 wt% theoretical loading (Patil et al., 2009; Toti et al., 2010) (Fig 1A). PLLA(10K)-PEG(5K)-MAL was consistently incorporated into NP at ~3.3 wt% by ¹H-NMR (not shown). We then conjugated sulfhydryl (cysteine)-activated EP67 or inactive scrambled EP67 (scEP67) to MAL-activated NP through an N-terminal Gly-Arg-Arg linker (Fig 1B). CGRR-EP67 or CGRR-scEP67 was consistently conjugated to MAL-activated NP between 0.1 to 0.2 wt% under the same reaction conditions without background OVA by amino acid analysis. OVA and F-OVA were consistently loaded at ~7.8 wt% in unmodified or surface-conjugated NP with diameters of 340 nm to 440 nm (Table 1). Both EP67-NP and scEP67-NP were prepared from the same batch of MAL-activated NP. Thus, differences in their average NP diameters and PDIs are due, in large part, to NP lyophilization in the absence of cryoprotectants (Holzer et al., 2009) and possible effects of the surface conjugated peptides. We did not include cryoprotectants to minimize the possibility of non-specific inflammation.

3.2 Surface conjugation of EP67 to PLGA 50:50 NP increases activation markers on immature murine BMDC

The generation of long-lived memory T-cells during mucosal infections is initiated by the activation of resting (immature) intraepithelial, tissue-, lymph node (LN)-, or spleen-resident myeloid (conventional) dendritic cells (DC) through receptors for pathogen- or host-derived immunostimulant molecules as DC “capture” (endocytose) and “process” (proteolytically degrade) extracellular protein immunogens into smaller peptide epitopes that are selectively loaded into major histocompatibility complexes (MHC) and “presented” as part of MHC on the DC surface (pMHC) (Abbas et al., 2017; Murphy and Weaver, 2017). Non-protein immunogens including polysaccharides, lipids, and small metabolites are also processed and presented by DC but to a much lesser extent (Lepore et al., 2018). Activated (mature) migratory and secondary lymphoid organ (SLO)-resident DC then physically interact with

naïve T-cells (Tn) in mucosa-associated lymphoid tissues (MALT), draining lymph nodes (LN), or the spleen and “prime” Tn with T-cell receptors (TCR) that have sufficient affinity for foreign peptide epitopes presented in MHC-I (pMHC-I: CD8⁺ T-cells) or MHC-II (pMHC-II: CD4⁺ T-cells). Priming then drives clonal expansion and differentiation of epitope-specific Tn into short-lived effector T-cells that survive long enough to help clear the infection and long-lived memory T-cells that persist years after the primary infection and respond more rapidly to subsequent infections (Seder et al., 2008).

The activation of immature DC increases the levels of several cell surface proteins involved in priming epitope-specific Tn during DC-T-cell interactions (Benvenuti, 2016) including CD54 (ICAM-1), CD102 (ICAM-2), CD58 (LFA-3), CD80 (B7-1), CD86 (B7-2), MHC-I (Adiko et al., 2015; Rescigno et al., 1998), and MHC-II (Roche and Furuta, 2015) (“activation markers”). Thus, given that EP67 activates APC through the C5aR1 cell surface receptor (Morgan et al., 2009; Sheen et al., 2011), conjugating EP67 to the NP surface was expected to increase the levels of activation markers on immature DC.

To determine if conjugating EP67 to the surface of NP increases activation markers on immature DC, we incubated immature murine bone marrow-derived dendritic cells (BMDC, male C57BL/6 mice) at 37°C for 24 hours with media alone or media containing 7.8 µg LPS-free OVA encapsulated at 7.8 wt% in NP [NP(OVA)] or in NP surface-conjugated with inactive scrambled EP67 [scEP67-NP(OVA)] or EP67 [EP67-NP(OVA)] (Table 1). We then compared median fluorescence intensities (MFI) of CD80, CD86, and MHC-II staining on the surfaces of live CD11c⁺ BMDC by flow cytometry (Fig.2). These surface proteins are critical markers of DC activation because Tn primed by DC with insufficient levels of CD80 and CD86 (T-cell “co-stimulators”) potentially become permanently unresponsive (anergic) to their displayed pMHC (Valdor and Macian, 2013) and because MHC-II is expressed primarily by APC (Roche and Furuta, 2015).

Each NP construct increased the MFI of BMDC staining with CD80 (2.5 to 5.7-fold), CD86 (2.3 to 3.4-fold), and MHC-II (1.6 to 2.8-fold) over treatment with media alone (Fig.2). EP67-NP(OVA), however, also increased the MFI of BMDC staining with CD80 (2.2-fold and 1.4-fold), CD86 (1.54-fold and 1.5-fold), and MHC-II (1.7-fold and 1.3-fold) over unmodified NP(OVA) (Fig.2A, white bars vs. dark grey bars) and scEP7-NP(OVA) (Fig.2A, white bars vs. light grey bars). Thus, conjugating EP67 to surface of NP increases activation markers on immature DC.

3.3 Surface conjugation of EP67 to PLGA 50:50 NP increases the rate that encapsulated OVA protein is endocytosed by immature murine BMDC

Immature DC endocytose extracellular protein immunogens by phagocytosis, pinocytosis, and receptor-mediated endocytosis then process them into smaller peptide epitopes that are selectively presented in MHC-II (Burgdorf and Kurts, 2008; Mantegazza et al., 2013). Encapsulating peptides and proteins in nanoscale biodegradable particles, however, increases cross-presentation to MHC-I, the duration of pMHC-I and pMHC-II presentation by immature human monocyte-derived DC (MoDC) and immature murine BMDC (Audran et al., 2003; Waeckerle-Men et al., 2006), and the proportion of CD4⁺ and CD8⁺ memory T-cells generated by encapsulated protein *in vivo* (Moore et al., 1995; Schliehe et al., 2011).

Furthermore, increasing the rate that APC internalize protein immunogens increases levels of surface pMHC (Singer and Linderman, 1990). Thus, given that activated APC cannot initiate or sustain the priming of Tn below a threshold level of surface pMHC (Henrickson et al., 2008; Henrickson et al., 2013; Kimachi et al., 1997), increasing the internalization rate of encapsulated protein vaccine may increase the ability of activated DC to both initiate and sustain the priming and subsequent expansion of epitope-specific Tn.

EP67 binds and activates APC through cell surface C5aR1 receptors (Morgan et al., 2009; Sheen et al., 2011). Furthermore, activating murine BMDC or spleen-derived DC with the pathogen-derived toll-like receptor (TLR) immunostimulant LPS increases peaks rates of macropinocytosis within 30 or 45 minutes of treatment, respectively (West et al., 2004). Thus, given that DC likely endocytose solid NP with diameters ~332 nm (Table 1) primarily by macropinocytosis (Gutjahr et al., 2016; Xiang et al., 2006), conjugating EP67 to the surface of NP was expected to increase the rate that immature DC endocytose encapsulated protein.

To determine if conjugating EP67 to the surface of biodegradable nanoparticles increases the rate that encapsulated protein vaccine is endocytosed by immature DC, we pre-incubated immature murine BMDC (male C57BL/6) at 4°C or 37°C for 1 hour then incubated them with ~1.6 µg LPS-free fluorescein-modified OVA (F-OVA) encapsulated at 7.8 wt% in unmodified NP [NP(F-OVA)] or in NP surface-conjugated with scEP67 [scEP67-NP(F-OVA)] or EP67 [EP67-NP(F-OVA)] at 4°C or 37°C, respectively, for 2 hours (Table 1). We then compared proportions and median fluorescence intensities (MFIs) of F-OVA staining of live CD11c⁺ BMDC by flow cytometry (Fig.3).

EP67-NP(F-OVA) increased BMDC staining with F-OVA 1.3-fold over NP(F-OVA) (Fig.3E, white bar vs. dark grey bar) and scEP67-NP(F-OVA) (Fig.3E, white bar vs. light grey bar) at 37°C. Furthermore, EP67-NP(F-OVA) increased the MFI of BMDC staining with F-OVA 2-fold over NP(F-OVA) (Fig.3F, white bar vs. light grey bar) and 2.6-fold over scEP7-NP(F-OVA) (Fig.3F, white bar vs. dark grey bar) at 37°C. In contrast, there were no differences in F-OVA staining on BMDC between NP constructs at 4°C (Fig.3B & C), indicating that differences between F-OVA staining at 37°C are due to differences between the rates that NP are endocytosed by BMDC and not differences in NP adsorption to the BMDC surface. Thus, conjugating EP67 to the surface of biodegradable nanoparticles increases the rate that encapsulated protein vaccine is endocytosed by immature DC.

3.4 Surface conjugation of EP67 to PLGA 50:50 NP increases BMDC activation of CD4⁺ or CD8⁺ T-cell hybridomas specific for encapsulated OVA protein

Conjugating EP67 to the surface of NP increased the levels of activation markers on immature DC (Fig.2) and rate that immature DC endocytose encapsulated protein (Fig.3). Thus, surface conjugation of EP67 to NP was expected to increase DC priming of T-cells specific for encapsulated protein vaccine.

To determine if conjugating EP67 to the surface of NP increases DC priming of T-cells specific for encapsulated protein vaccine, we pre-treated immature murine BMDC (male C57BL/6) with 7.8 µg LPS-free OVA encapsulated at 7.8 wt% in unmodified NP [NP(OVA)]

or NP surface-conjugated with scEP67 [scEP67-NP(OVA)] or EP67 [EP67-NP(F-OVA)] at 37°C for 2 hours (Table 1). We then co-incubated pretreated BMDC for 18 h with T-cell hybridomas from C57BL/6 mice specific for the OVA_{323–339} CD4 epitope (DOBW) (Harding et al., 1991) or the OVA_{257–264} CD8 epitope (CD8 OVA 1.3) (Pfeifer et al., 1993) and compared concentrations of IL-2 released into the media (Fig.4). IL-2 is secreted from naïve or antigen-experienced epitope-specific CD4⁺ T-cells and, to a lesser extent, CD8⁺ T-cells within 1 to 2 hours of DC priming/activation to drive T-cell growth, survival, and differentiation (Abbas et al., 2017). Thus, levels of IL-2 released from co-incubated DOBW (CD4⁺) or CD8A OVA 1.3 (CD8⁺) T-cell hybridomas are surrogate markers for the extent that activated DC “prime/activate” CD4⁺ or CD8⁺ T-cells, respectively.

Pre-treatment of BMDC with each NP construct increased concentrations of IL-2 released from co-cultured OVA_{323–339}-specific CD4⁺ (DOBW) or OVA_{257–264}-specific CD8⁺ (CD8 OVA 1.3) T-cell hybridomas (Fig.4, dark grey, light grey, and white bars) above very low concentrations of IL-2 released from the same T-cell hybridomas co-cultured with BMDC pre-treated with media alone (Fig.4, black bars). Pre-treatment of BMDC with EP67-NP(OVA), however, increased concentrations of IL-2 released by CD4⁺ DOBW T-cell hybridomas 1.5-fold over NP(OVA) (Fig.4A, white bars vs. dark grey bars) and 1.7-fold over scEP7-NP(OVA) (Fig.4A, white bars vs. light grey bars), respectively. EP67-NP(OVA) also increased concentrations of IL-2 released by CD8⁺ CD8A OVA 1.3 T-cell hybridomas 1.6-fold over NP(OVA) (Fig.4B, white bars vs. dark grey bars) and 1.8-fold over scEP67-NP(OVA) (Fig.4B, white bars vs. light grey bars). Thus, conjugating EP67 to the surface of NP increases DC priming of T-cells specific for encapsulated protein vaccine.

3.5 Surface conjugation of EP67 to PLGA 50:50 NP increases the expansion of mucosal and systemic memory T-cell by encapsulated OVA protein after respiratory immunization of female C57BL/6 mice

Long-lived memory T-cells are generated by the proliferative clonal expansion of primed epitope-specific T_H and subsequent contraction to a 90 to 95% smaller pool of memory T-cells (~28 days after expansion begins) that survive long after the infection or vaccine has been cleared (Abbas et al., 2017; Harty and Badovinac, 2008). Several studies suggest that the magnitude of memory T-cell expansion during infection or immunization correlates with subsequent levels of long-lived memory T-cells (Panagioti et al., 2018; Panagioti et al., 2016). Thus, the magnitude of memory T-cell expansion is likely an important surrogate marker for the generation of long-lived memory T-cells. Given that conjugating EP67 to the surface of biodegradable NP increased DC priming of CD4⁺ and CD8⁺ T-cells specific for encapsulated OVA protein *in vitro* (Fig.4), it was also expected to increase the magnitude of mucosal and systemic memory T-cell expansion by encapsulated OVA protein after respiratory immunization.

To determine if conjugating EP67 to the surface of NP increases the magnitude of mucosal and systemic memory T-cell expansion generated by encapsulated protein vaccine after respiratory immunization, we intranasally administered (IN) vehicle alone [PBS] or vehicle containing 25 µg LPS-free OVA encapsulated at 7.8 wt% in unmodified NP [NP] or in NP with surface-conjugated EP67 [EP67-NP] to 6-week old female C57BL/6 mice in a volume

(50 μ L) expected to deliver vaccine to the nasal cavity and lungs (i.e., respiratory immunization) (Fig.5) (Southam et al., 2002). We then compared proportions of CD4⁺CD44^{HI}Tet-OVA_{323–339}⁺ and CD8a⁺CD44^{HI}Tet-OVA_{257–264}⁺ T-cells from perfused lungs (Fig.6A,C) and spleens (Fig.7A,C) 14 days post-immunization (T-cell contraction almost complete) by flow cytometry. Cells were also gated for CD44^{HI} staining to include immunogen-inexperienced (Marusina et al., 2017; White et al., 2017) and immunogen-experienced OVA-specific T-cells (Baaten et al., 2012). We didn't include scEP67-NP because it increased BMDC cell surface activation markers (Fig.2), rate that encapsulated OVA was endocytosed into BMDC (Fig.3), and BMDC activation of OVA-specific T-cell hybridomas (Fig.4) to a similar extent as unmodified NP.

EP67-NP increased the proportion of CD4⁺CD44^{HI}Tet-OVA_{323–339}⁺ cells above NP by 2-fold in the lungs (Fig.6A, EP67-NP vs. NP) and by 5.5-fold in the spleen (Fig.7A, EP67-NP vs. NP). EP67-NP also increased the proportion of CD8a⁺CD44^{HI}Tet-OVA_{257–264}⁺ cells above NP by 1.6-fold in the lungs (Fig.6C, EP67-NP vs. NP) and by 4.1-fold in the spleen (Fig.7C, EP67-NP vs. NP). In contrast, NP and vehicle (PBS) had similar proportions of CD4⁺CD44^{HI}Tet-OVA_{323–339}⁺ cells and CD8a⁺CD44^{HI}Tet-OVA_{257–264}⁺ cells in the lungs (Fig.6A & 6C, NP vs. PBS) and the spleen (Fig.7A & 7C, NP vs. PBS). Thus, conjugating EP67 to the surface of biodegradable nanoparticles increases the expansion of mucosal and systemic T-cell expansion by encapsulated protein vaccine after respiratory immunization.

3.6 Surface conjugation of EP67 to PLGA 50:50 NP increases long-lived CD127/KLRG1 subsets of mucosal T-cells generated by encapsulated OVA protein after respiratory immunization of female C57BL/6 mice

Although conjugating EP67 to the NP surface increased mucosal and systemic T-cell expansion by encapsulated OVA protein vaccine (Figs. 6&7, A&C), it remained unclear if it would also increase long-lived memory T-cells. T-cell memory subsets that potentially differentiate between long-lived and short-lived memory T-cells at mucosal sites are based on the cell surface expression of CD44^{HI}, CD127 (IL-7R α), and KLRG1 (Jameson and Masopust, 2018; Panagioti et al., 2018). These subsets include (i.) “early effector cells” (EEC: CD44^{HI}CD127⁺KLRG1⁺) that, in the absence of pro-inflammatory signals (Plumlee et al., 2015), potentially give rise to (ii.) long-lived memory precursor cells (MPEC: CD44^{HI}CD127⁺KLRG1⁺) (Kaech et al., 2003; Schluns et al., 2000), (iii.) short-lived effector cells (SLEC: CD44^{HI}CD127⁺KLRG1⁺) that die shortly after an infection or vaccine has been cleared (~28 days), and (iv.) “intermediate-lived” (SLEC<DPEC<MPEC) double-positive effector cells (DPEC: CD44^{HI}CD127⁺KLRG1⁺) (Ahlers and Belyakov, 2010; Obar and Lefrancois, 2010a, b, c) that may rapidly expand into EEC during subsequent infections and contract back into DPEC after infection is resolved (Obar et al., 2011).

To determine if conjugating EP67 to the surface of biodegradable nanoparticles increases long-lived CD127/KLRG1 memory subsets of mucosal T-cells generated by encapsulated protein after respiratory immunization, we compared proportions of CD127/KLRG1 subsets within CD4⁺CD44^{HI}Tet-OVA_{323–339}⁺ cells (Fig.6B) or CD8a⁺CD44^{HI}Tet-OVA_{257–264}⁺ cells (Fig.6D) from perfused lungs 14 days post-immunization (end stage of T-cell contraction) by flow cytometry. EP67-NP had 8.4-fold more intermediate-lived DPEC

(CD127⁺KLRG1⁺) and 3.1-fold more long-lived MPEC (CD127⁺KLRG1⁻) within total CD4⁺CD44^{HI}Tet-OVA₃₂₃₋₃₃₉⁺ cells than NP mice in the lungs (Fig.6B). EP67-NP also had 8.2-fold more long-lived MPEC (CD127⁺KLRG1⁻) within total CD8a⁺CD44^{HI}Tet-OVA₂₅₇₋₂₆₄⁺ cells than NP in the lungs (Fig.6D). In contrast, except for EEC subsets within CD8a⁺CD44^{HI}Tet-OVA₂₅₇₋₂₆₄⁺ cells, NP and PBS had similar levels of CD127/KLRG1 memory subsets within total CD4⁺CD44^{HI}Tet-OVA₃₂₃₋₃₃₉⁺ cells (Fig.6B) and CD8a⁺CD44^{HI}Tet-OVA₂₅₇₋₂₆₄⁺ cells (Fig.6D). Thus, conjugating EP67 to the surface of biodegradable nanoparticles increases long-lived CD127/KLRG1 memory subsets of mucosal T-cells generated by encapsulated protein after respiratory immunization.

3.7 Surface conjugation of EP67 to PLGA 50:50 NP increases long-lived CD127/KLRG1/CD62L memory subsets of systemic T-cells generated by encapsulated OVA protein after respiratory immunization of female C57BL/6 mice

Similar to mucosal T-cells, cell surface expression of CD44^{HI}, CD127 (IL-7Rα), and KLRG1 can potentially differentiate between long-lived and short-lived memory T-cell subsets at systemic sites but includes an additional subset based on the cell surface expression of the lymph node- and spleen-homing adhesion molecule, CD62L (Jameson and Masopust, 2018; Panagioti et al., 2018). These T-cell subsets include (i.) “effector cells” (TEFF: CD127⁻KLRG1⁻CD62L⁻) similar to EEC, (ii.) “intermediate-lived” (SLEC<DPEC<MPEC) double positive effector cells (DPEC: CD127⁺KLRG1⁺CD62L⁻), (iii.) long-lived “effector memory cells” (TEM: CD127⁺KLRG1⁻CD62L⁻) similar to long-lived MPEC, (iv.) long-lived “central memory cells” (TCM: CD127⁺KLRG1⁻CD62L⁺) also similar to long-lived MPEC but retained in secondary lymphoid organs (SLO), and (v.) “short-lived effector cells” (SLEC: CD127⁻KLRG1⁺CD62L⁻).

To determine if conjugating EP67 to the surface of biodegradable nanoparticles also increases long-lived CD127/KLRG1/CD62L memory subsets of systemic T-cells generated by encapsulated protein after respiratory immunization, we compared respective proportions of CD127/KLRG1/CD62L subsets within CD4⁺CD44^{HI}Tet-OVA₃₂₃₋₃₃₉⁺ cells (Fig.7B) or CD8a⁺CD44^{HI}Tet-OVA₂₅₇₋₂₆₄⁺ cells (Fig.7D) in the lungs 14 days post-immunization by flow cytometry. EP67-NP had 5.4-fold more intermediate-lived DPEC (CD127⁺KLRG1⁺CD62L⁻) and 10.8-fold more long-lived TEM (CD127⁺KLRG1⁻CD62L⁻) within total CD4⁺CD44^{HI}Tet-OVA₃₂₃₋₃₃₉⁺ cells than NP in the lungs (Fig.7B). EP67-NP also had 9.4-fold more intermediate-lived DPEC (CD127⁺KLRG1⁺CD62L⁻), 16-fold more long-lived TEM (CD127⁺KLRG1⁻CD62L⁻), and 222-fold more long-lived TCM (CD127⁺KLRG1⁻CD62L⁺) within total CD8a⁺CD44^{HI}Tet-OVA₂₅₇₋₂₆₄⁺ cells than NP in the lungs (Fig.7D). In contrast, with the exception of TCM subsets within CD4⁺CD44^{HI}Tet-OVA₂₅₇₋₂₆₄⁺ cells, NP and PBS had similar levels of CD127/KLRG1 memory subsets within total CD4⁺CD44^{HI}Tet-OVA₃₂₃₋₃₃₉⁺ cells (Fig.7B) and CD8a⁺CD44^{HI}Tet-OVA₂₅₇₋₂₆₄⁺ cells (Fig.7D). Thus, conjugating EP67 to the surface of biodegradable nanoparticles increases long-lived CD127/KLRG1/CD62L memory subsets of mucosal T-cells generated by encapsulated protein after respiratory immunization.

3.8 Surface conjugation of EP67 to NP increases T-cell-mediated efficacy of respiratory immunization with encapsulated OVA protein

Conjugating EP67 to biodegradable NP increased the expansion of mucosal and systemic CD4⁺ and CD8⁺ T-cells generated by encapsulated OVA protein vs. unmodified NP (Figs. 7&8, A&C). It remained unclear, however, if these differences correlated with increased T-cell activity *in vivo*.

To determine if conjugating EP67 to the surface of biodegradable nanoparticles increases T-cell-mediated efficacy of respiratory immunization with encapsulated protein vaccine, we intranasally administered vehicle alone [PBS] or vehicle containing 25 µg LPS-free OVA encapsulated at 7.8 wt% in unmodified NP [NP] or NP surface-conjugated with EP67 [EP67-NP]. Fourteen days post-immunization, we intranasally administered sublethal titers (2×10^7 CFU) of virulent recombinant *L. monocytogenes* ectopically expressing a secreted form of OVA (LM-OVA) in PBS (50 µL) (i.e., respiratory challenge) (Fig.5) then compared peak titers of LM-OVA in the lungs, liver, and spleen 3 days post-challenge by dilution plating (Fig.8). Protection of seronegative mice against LM-OVA, an intracellular bacterial pathogen, by an OVA protein vaccine is expected to primarily involve OVA-specific CD4⁺ and CD8⁺ memory T-cell responses (Ladel et al., 1994; Zenewicz and Shen, 2007).

EP67-NP decreased peak titers of LM-OVA ~4.6-fold in the lungs (Fig.8A, triangles vs. squares), ~52.5-fold in the liver (Fig.8B, triangles vs. squares), and ~6.9-fold in the spleen (Fig.8C, triangles vs. squares) below NP(OVA). In contrast, there were no differences between peak titers of LM-OVA in the lungs, livers, or spleens of NP and PBS (Fig.8). Thus, conjugating EP67 to the surface of biodegradable nanoparticles increases T-cell-mediated efficacy of respiratory immunization with encapsulated protein vaccine.

4. Discussion

Our study provides evidence that conjugating EP67 to the surface of biodegradable nanoparticles increases mucosal and systemic memory T-cell expansion and long-lived memory subsets generated by encapsulated protein vaccine after respiratory immunization. We found that, 14 days post-immunization, respiratory immunization of naïve female C57BL/6 mice with LPS-free OVA protein encapsulated in PLGA 50:50 NP (~380 nm diameter) surface-conjugated with EP67 through 2 kDa PEG linkers at ~0.1 wt% (NP-EP67) (i.) increased the magnitude of OVA₃₂₃₋₃₃₉-specific CD4⁺ and OVA₂₅₇₋₂₆₄-specific CD8⁺ T-cells in the lungs (Fig.6A&C) and spleen (Fig.7A&C) and (ii.) increased proportions of long-lived memory subsets of OVA₃₂₃₋₃₃₉-specific CD4⁺ and OVA₂₅₇₋₂₆₄-specific CD8⁺ T-cells in the lungs (CD44^{HI}/CD127/KLRG1) (Fig.6B&D) and spleen (CD44^{HI}/CD127/KLRG1/CD62L) (Fig.7B&D) compared to LPS-free OVA protein encapsulated in unmodified PLGA 50:50 nanoparticles. Furthermore, our study shows that conjugating EP67 to the surface of biodegradable nanoparticles increases T-cell-mediated protection by encapsulated protein vaccine after respiratory immunization. We found that respiratory immunization of naïve female C57BL/6 mice with NP-EP67 decreased peak CFU of LM-OVA in the lungs, liver, and spleen below NP after respiratory challenge with LM-OVA 14 days post-immunization (Fig.8), consistent with an increase in OVA-specific mucosal and

systemic T-cells (Figs.6&7) that are expected to increase protection of seronegative mice against infection with LM-OVA (Ladel et al., 1994; Zenewicz and Shen, 2007).

A murine model of CD8⁺ T-cell priming that closely resembles immunization with subunit vaccines suggests that T-cell expansion and memory formation occur in draining lymph nodes (LN) and likely other SLO (MALT and spleen) during three phases of interactions between naïve T-cells (Tn) and activated DC presenting foreign peptide epitopes in MHC (DC-pMHC) (Henrickson and von Andrian, 2007; Mempel et al., 2004). Phase 1 (first ~8 h after Tn enter LN) involves multiple transient interactions between Tn and activated DC-pMHC and the upregulation of T-cell surface activation markers. Phase 2 (between ~8 and 24 h after Tn enter LN) involves the formation of immunological synapses and more prolonged interactions between Tn and activated DC-pMHC (~1 h) as well as cytokine release from activated T-cells. Phase 3 (~24 h after Tn enter LN) again involves multiple transient interactions between T-cells and activated DC-pMHC but with increased T-cell motility and rapid proliferation. Similar phases have been observed with CD4⁺ T-cells and activated DC-pMHC (Miller et al., 2004).

The transition from Phase I to Phase II (i.e., formation of immunological synapses between activated DC-pMHC and Tn) requires a threshold level of pMHC on activated DC and is accelerated by increasing the number of pMHC per activated DC and/or density of antigen-presenting DCs in the LN (Henrickson et al., 2008). T-cells that do not transition to Phase II rapidly expand but have a short-lived effector (TEFF) phenotype, whereas T-cells that transition to Phase II have a long-lived phenotype (Henrickson et al., 2013). Furthermore, Tn locally primed in nasal-associated lymphoid tissue (NALT) and likely other MALT in the respiratory tract (key inductive sites within respiratory mucosa) after IN immunization localize to MALT-draining LN and the spleen (Ciabattini et al., 2011). As such, this model of T-cell priming (Henrickson and von Andrian, 2007) and other studies suggest that the generation of long-lived mucosal and systemic memory T-cells by encapsulated protein vaccine after respiratory immunization can be increased by (i.) increasing vaccine localization to NALT (Date et al., 2017) and, likely, other respiratory MALT, (ii.) increasing the rate that immunological synapses are formed between activated DC-pMHC and Tn (transition to Phase II) (iii.) increasing the duration that DC-pMHC are able to prime Tn in SLO including NALT (Ciabattini et al., 2011) and, likely, other respiratory MALT (iv.) increasing the number of activated DC-pMHC in SLO, and (v.) inducing a cytokine microenvironment within MALT-draining LN and the spleen that favors memory T-cell expansion and differentiation into long-lived memory subsets (Badovinac et al., 2004; Kim and Harty, 2014).

With the above model of T-cell priming in mind, there are one or more possible reasons that conjugating EP67 to NP increases memory T-cell expansion and long-lived memory subsets generated by encapsulated protein vaccine. The first possibility is that surface conjugated EP67 (a 10-amino acid analog of C5a) increases NP affinity for C5aR1 expressed on the surface of M cells. This would increase the rate and extent that encapsulated protein vaccine localizes to MALT within the respiratory tract by potentially increasing M cell-mediated transcytosis as is likely the case for improved oral vaccines with increased affinity for C5aR1 (Kim et al., 2011; Wang et al., 2014) or other proteins (Islam et al., 2019) expressed

on the surface of M cells. There were, however, no differences in F-OVA staining between the same sub-saturating concentrations of EP67-NP, scEP67-NP, and NP on live immature BMDC after incubation for 2 hours at 4°C (Fig.3B & C). This suggests that EP67-NP does not have a greater affinity than NP for C5aR1-bearing cells including M cells or that the nonspecific affinity of NP for C5aR1-bearing cells is higher than the affinity of EP67-NP for C5aR1. Thus, it is unclear if current levels of surface-conjugated EP67 increase C5aR1-mediated M cell transcytosis unless stimulating C5aR1 also increases the rate of M cell transcytosis. It's also possible that nonspecific affinity of NP for M cells already maximizes the rate of M cell transcytosis into MALT regardless of EP67-NP affinity for C5aR1.

The second possibility is that surface conjugated EP67, similar to the parent molecule C5a, activates immature DC in MALT by stimulating C5aR1 on the DC surface. This would increase the rate of immunological synapses formation and duration that DC prime Tn in SLO by increasing and maintaining critical activation markers (e.g., CD80 and CD86) and pMHC on the DC surface above threshold levels for Tn priming through the C5aR1-mediated upregulation of activation markers and macropinocytosis rate of encapsulated protein vaccine by individual DC. A similar effect would be expected with LN- and spleen-resident DC if EP67-NP diffuses directly to MALT-draining LN and the spleen as observed after IN administration (50 µL) of fluorescent polystyrene carboxylate microspheres (1.1 µm diameter) to BALB/c mice (Eyles et al., 2001) or oral pharyngeal aspiration of fluorescent polystyrene latex spheres (100 nm and 1 µm diameter) to F344 rats (Sarlo et al., 2009). This is supported, in part, by our findings that treating immature murine BMDC with EP67-NP increased surface levels of CD80, CD86, MHC-II (Fig.2), the rate that encapsulated F-OVA is internalized (Fig.3), and BMDC activation of OVA-specific murine CD4⁺ and CD8⁺ T-cell hybridomas (Fig.4) compared to unmodified NP and NP surface conjugated with inactive scrambled EP67 (scEP67). Activating immature MALT DC by stimulating C5aR1 may also increase the number of activated DC-pMHC in MALT-draining LN and the spleen by increasing trafficking of activated DC-pMHC from MALT (Iwasaki, 2007).

The third possibility is that surface conjugated EP67, again similar to the C5a parent molecule, activates other C5aR1-bearing cells near and within MALT that support the generation of adaptive immune responses. This would potentially increase the number of activated DC-pMHC in SLO, in part, by creating a chemokine and cytokine microenvironment that directly or indirectly helps recruit circulating DC and “inflammatory” monocytes that differentiate into DC (Iwasaki, 2007) to respiratory MALT. The EP67-induced cytokine microenvironment may also potentially (i.) increase the rate (formation of immunological synapses) and duration that DC prime Tn in SLO by reinforcing the stimulatory effects of EP67 on DC and (ii.) be favorable to memory T-cell expansion and differentiation into long-lived memory subsets. Whether differences in C5aR1 stimulation between EP67 and C5a and greatly decreased direct stimulation of recruited neutrophils by EP67 creates a more favorable cytokine environment for the formation of long-lived memory T-cells than stimulation with the C5a parent molecule remains to be determined.

5. Conclusions

In summary, our study indicates conjugating EP67 to the surface of biodegradable nanoparticles is one potential approach to increase the generation of long-lived memory T-cells by encapsulated protein vaccine after respiratory immunization and possibly other routes of mucosal immunization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Synthetic strategy for conjugating EP67 to the surface of PLGA 50:50 nanoparticles (NP). LPS-free ovalbumin alone (OVA) or labeled with fluorescein (F-OVA) was encapsulated in spherical PLGA 50:50 NPs by the W/O/W emulsification solvent extraction (ESE) method. (A) NP were modified with maleimide-activated 2 kDa PEG linkers during OVA encapsulation by interfacial activity assisted surface functionalization (IAASF) using PLLA(10 kDa)-b-PEG(2 kDa)-maleimide diblock copolymers then lyophilized. (B) EP67 was activated with sulfhydryl groups by the addition of an N-terminal Cys through a protease-labile Gly-Arg-Arg-linker then reacted with maleimide-activated NP resuspended in PBS [pH 7.4].

Fig. 2.

Conjugating EP67 to the surface of biodegradable nanoparticles increases the expression of surface activation markers on immature murine BMDCs after treatment. Immature bone marrow-derived dendritic cells (BMDCs, Day 8 GM-CSF/IL-4, male C57BL/6) were incubated with media alone [black bars, Media] or media containing 7.8 μ g LPS-free OVA encapsulated at 7.8 wt% in unmodified PLGA 50:50 nanoparticles [dark grey bars, NP(OVA)], PLGA 50:50 nanoparticles surface-modified with inactive scEP67 [grey bars, scEP67-NP(OVA)], or PLGA 50:50 nanoparticle surface-modified with EP67 [white bars, EP67-NP(OVA)] at 37°C for 24 h then analyzed by flow cytometry. Average median fluorescence intensities (MFI) $\pm$ SD ($n=2$ independent treatments) from (A) CD80, (B) CD86, or (C) MHC-II staining of live CD11c+ BMDCs were compared between treatment groups using one-way ANOVA with Tukey's post-test where ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Data are representative of at least three independent experiments.

Fig. 3.

Conjugating EP67 to the surface of biodegradable nanoparticles (NPs) increases the rate of NP internalization by immature murine BMDCs. Immature BMDCs (male C57BL/6) were pre-incubated at 4°C or 37°C for 1 h, then incubated at 4°C (**A, B, C**) or 37°C (**D, E, F**) for 2 h with ~1.6 µg of LPS-free fluorescein-modified OVA (F-OVA) encapsulated at 7.8 wt% in unmodified NPs [NP(F-OVA), dark grey bars], NPs surface-modified with inactive scEP67 [scEP67-NP(F-OVA), grey bars], or NPs surface-modified with EP67 [EP67-NP(F-OVA), white bars] as described in Fig.1. Cells were rinsed with PBS, stained with viability dye and PE-Cy5 anti-CD11c antibodies, then analyzed by flow cytometry. Representative FACS data from viable CD11c⁺ BMDCs incubated with the indicated NPs at (**A**) 4°C or (**D**) 37°C for 2 h. The average percent of total viable CD11c⁺ BMDCs at (**B**) 4°C or (**E**) 37°C that were F-OVA⁺ ± SD (n=2 independent treatments) and the average median fluorescence intensities (MFI) of F-OVA staining of viable CD11c⁺F-OVA⁺ BMDCs at (**C**) 4°C or (**F**) 37°C ± SD (n=2 independent treatments) between treatment groups were compared by one-way ANOVA with Dunnett's post-test where * $P < 0.05$ and ** $P < 0.01$. Data are representative of at least two independent experiments.

Fig. 4.

Conjugating EP67 to the surface of biodegradable nanoparticles increases BMDC activation of murine T-cell hybridomas specific for encapsulated protein. Immature murine BMDCs (male C57BL/6) were pre-treated at 37°C for 2 h with media alone [black bars, Media] or media containing 7.8 µg LPS-free OVA encapsulated at 7.8 wt% in unmodified nanoparticles [NP(OVA), dark grey bars], nanoparticles surface-modified with inactive scEP67 [scEP67-NP(OVA), grey bars], or nanoparticles surface-modified with active EP67 [EP67-NP(OVA), white bars] through 2 kDa PEG linkers (~0.3 wt% scEP67/EP67). Pre-treated BMDCs were then incubated with murine OVA₃₂₃₋₃₃₉-specific CD4⁺ (DOBW) or OVA₂₅₇₋₂₆₄-specific CD8⁺ (CD8 OVA 1.3) T-cell hybridomas for 18 h at 37°C. Average concentrations of IL-2 ± SD (n=2 independent treatments) released into the media by (A) CD4⁺ or (B) CD8⁺ T-cell hybridomas were determined by ELISA and compared between treatment groups by one-way ANOVA with Tukey's post-test where ***P* 0.01, ****P* 0.001, *****P* 0.0001. Results are representative of at least two independent experiments.

Fig. 5.

Design overview of EP67-NP immunization studies. Vehicle alone (PBS) or vehicle containing LPS-free OVA [$\sim 25 \mu\text{g}$] encapsulated at $\sim 7.8 \text{ wt\%}$ in unmodified PLGA 50:50 nanoparticles (NP) or NP surface-modified with EP67 (Table 1) was administered intranasally to naïve female C57BL/6 mice ($\sim 6 \text{ wk}$ old) on Day 0 and Day 7 using a volume [$50 \mu\text{L}$] expected to enter the nasal cavity and lungs (“respiratory immunization”). Fourteen days after respiratory immunization (Day 21), (i.) total magnitude and respective memory subsets of OVA-specific CD4^+ or CD8a^+ T-cells in the lungs (CD127/KLRG1) (Fig.6) and spleen (CD127/KLRG1/CD62L) (Fig.7) were compared by flow cytometry or (ii.) vehicle alone (PBS) [$50 \mu\text{L}$] or vehicle containing transgenic *L. monocytogenes* ectopically expressing OVA (LM-OVA) [$2 \times 10^7 \text{ CFU}$] was administered IN (“respiratory challenge”) and peak LM-OVA colony forming units (CFU) in the lungs, liver, and spleen were compared three days after primary respiratory challenge (Day 24) by dilution plating (Fig.8).

Lungs

Fig. 6.

Effect of conjugating EP67 to the surface of biodegradable nanoparticles on T-cell expansion and CD127/KLRG1 memory subsets of $CD4^+$ and $CD8^+$ T-cells in the lungs specific for encapsulated OVA protein 14 days after respiratory immunization. Vehicle alone (PBS) or vehicle containing LPS-free OVA (~25 μ g) encapsulated at 7.8 wt% in unmodified PLGA 50:50 nanoparticles (NP) or NP surface-modified with EP67 (EP67-NP) (Table 1) was administered IN to naïve female C57BL/6 mice (~6 wk old) as described in Fig.5. Fourteen days after respiratory immunization, average proportions of total (A) $CD4^+CD44^{HI}Tet-OVA_{323-339}^+$ and (C) $CD8a^+CD44^{HI}Tet-OVA_{257-264}^+$ T-cells in the lungs and proportions of CD127/KLRG1 memory subsets within (B) $CD4^+CD44^{HI}Tet-OVA_{323-339}^+$ and (D) $CD8a^+CD44^{HI}Tet-OVA_{257-264}^+$ T-cells $\pm$ SD (n=4 mice) per 10^6 $CD4^+$ or 10^6 $CD8a^+$ T-cells, respectively, were determined by flow cytometry and compared between treatment groups using Kruskal-Wallis one-way ANOVA with uncorrected Dunn's post-test where ns = not significant, * $P < 0.05$, or ** $P < 0.01$ vs. PBS, NP, respectively. N.D. = not detected. Results are representative of two independent experiments.

Fig. 7.

Effect of conjugating EP67 to the surface of biodegradable nanoparticles on T-cell expansion and CD127/KLRG1/CD62L memory subsets of $CD4^+$ and $CD8^+$ T-cells in the spleen specific for encapsulated OVA protein 14 days after respiratory immunization. Vehicle alone (PBS) or vehicle containing LPS-free OVA ($\sim 25 \mu g$) encapsulated at 7.8 wt% in unmodified PLGA 50:50 nanoparticles (NP) or NP surface-modified with EP67 (Table 1) was administered IN to naïve female C57BL/6 mice (~ 6 wk old) as described in Fig.5. Fourteen days after respiratory immunization, average proportions of total (A) $CD4^+CD44^{HI}Tet-OVA_{323-339}^+$ and (C) $CD8a^+CD44^{HI}Tet-OVA_{257-264}^+$ T-cells in the spleen and respective proportions of CD127/KLRG1/CD62L memory subsets within (B) $CD4^+CD44^{HI}Tet-OVA_{323-339}^+$ and (D) $CD8a^+CD44^{HI}Tet-OVA_{257-264}^+$ T-cells $\pm$ SD ($n=4$ mice) per 10^6 $CD4^+$ or 10^6 $CD8a^+$ T-cells, respectively, were determined by flow cytometry and compared between treatment groups using Kruskal-Wallis one-way ANOVA with uncorrected Dunn's post-test where ns = not significant, $*P < 0.05$, or $**P < 0.01$ vs PBS, NP, respectively. Results are representative of two independent experiments. The y-axis in (A) and (C) is truncated to help differentiate between stacked memory subsets.

Fig. 8.

Conjugating EP67 to the surface of biodegradable nanoparticles increases T-cell-mediated efficacy of respiratory immunization with encapsulated OVA protein against primary respiratory infection with LM-OVA. Vehicle alone (PBS) or vehicle containing LPS-free OVA (~25 μ g) encapsulated at 7.8 wt% in unmodified PLGA 50:50 nanoparticles (NP) or NP surface-modified with EP67 (Table 1) was administered IN to naïve female C57BL/6 mice (~6 wk old) as described in Fig.5. Fourteen days after respiratory immunization, *L. monocytogenes* ectopically expressing OVA (LM-OVA) (2×10^7 CFU in 50 μ L) was administered IN using 50 μ L of the vehicle (“respiratory challenge”). Three days after respiratory challenge, average peak LM-OVA colony forming units (CFU) per gram of tissue $\pm$ SD ($n = 7$ mice) in the (A) lungs, (B) liver, and (C) spleen from each treatment group were determined by dilution plating and compared by Kruskal-Wallis one-way ANOVA with Dunn’s uncorrected post-test. Data are representative of two independent experiments.

Table 1.

Representative characteristics of F-OVA and OVA-encapsulated PLGA nanoparticles.

Formulation	F-OVA / OVA Loading ($\mu\text{g}/\text{mg}$ NP $\pm$ SD)	Burst release ^a (% Loaded OVA)	EP67 Conjugation ^b ($\mu\text{g}/\text{mg}$ NP $\pm$ SD)	Diameter (nm $\pm$ SD)	Polydispersity Index (PDI $\pm$ SD)	Zeta Potential (mV $\pm$ SD)
NP	78 $\pm$ 3	3.7	N/A	332 $\pm$ 4	0.20 $\pm$ 0.02	-16.8 $\pm$ 0.5
scEP67-NP	79 $\pm$ 2	3.2	1.7 $\pm$ 0.2	432.8 $\pm$ 0.8	0.41 $\pm$ 0.03	-12.5 $\pm$ 0.6
EP67-NP	78 $\pm$ 4	3.2	1.0 $\pm$ 0.2	382 $\pm$ 7	0.171 $\pm$ 0.002	-13.3 $\pm$ 0.7

^aBurst release of F-OVA / OVA from MAL-activated NP before surface conjugation of scEP67 or EP67.

^bSurface conjugation of scEP67 or EP67 was determined under the same reaction conditions without background OVA by amino acid analysis. Results are representative of at least two independent batches.