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Development of C5AR-Targeted Nanoparticles for Delivery of Vaccines

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DEVELOPMENT OF C5AR-TARGETED NANOPARTICLES FOR DELIVERY OF VACCINES

by

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Since the early attempts of Benjamin Jesty at inducing immunity against smallpox and the pioneering work of Edward Jenner, vaccination has been, and continues to remain, the principal method of protection from diseases. However, most of the successful vaccines have been against pathogens that do not have major mechanisms to evade the immune system. So far, many life-threatening diseases like hepatitis C, HIV infection, malaria etc., have been resistant to existing vaccination strategies. Thus, there is an urgent need to develop new vaccination strategies that can generate long-lived protective immunity against such pathogens.

The purpose of this thesis is to investigate the effect of targeting PLGA nanoparticles to antigen-presenting cells using a novel immunostimulatory peptide EP67 as the targeting moiety on the immune responses generated against the encapsulating model antigen. In this study, we have shown that surface modification of PLGA nanoparticles with EP67 simultaneously targets and activates BMDCs, which results in enhanced antigen presentation to T-cells. Furthermore, we demonstrated that respiratory immunization with EP67 surface-modified OVA-encapsulated PLGA nanoparticles (i) increased protection against respiratory infection with LM-OVA by significantly reducing bacteria (ii) increased magnitudes of OVA-specific CD4+/CD8+ T-cells in lungs and spleen, (iii) increased proportions of short-lived effector cells

(SLECs), double positive effector cells (DPECs), and memory precursor effector cells (MPECs) in lungs, (iv) increased effector memory MPECs and central memory MPECs without affecting SLECs in spleen, and (v) affected the cytokine secretion profile of splenocytes responsive to MHC-II epitope of OVA. Overall, this work demonstrates the proof-of-concept that surface modification of PLGA nanoparticles with EP67 can increase the efficacy of immune responses generated against the encapsulated antigen.

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List of Abbreviations

APCs: Antigen-presenting cells

APC: Allophycocyanin

BMDC: Bone marrow-derived dendritic cell

CCL1: Chemokine ligand 1

CCL2: Chemokine ligand 2

CCL3: Chemokine ligand 3

CCL4: Chemokine ligand 4

CCL5: Chemokine ligand 5

CD62L: L-selectin

cDC1: Conventional DC 1

cDC2: Conventional DC 2

CFU: Colony forming unit

CpG: Cytosine-phosphate-guanine

CRS: Congenital rubella syndrome

CTL: Cytotoxic T lymphocytes

DC: Dendritic cell

DMEM: Dulbecco's modified eagle's medium

DMSO: Dimethyl sulfoxide

E: Early

EEC: Early effector cell

ESE: Emulsion solvent evaporation

EP: Electroporated

FP: Foot pad

GM-CSF: Granulocyte macrophage-colony stimulating factor

HCMV: Human cytomegalovirus

HI-NCS: Heat-inactivated new born calf serum

ID: Intradermal

IM: Intramuscular

IN: Intranodal

iNKT: induced Natural Killer T cell

IP: Intraperitoneal

IT: Intratumoral

IV: Intravenous

IFN-γ: Interferon gamma

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IL-2: Interleukin-2

IL-4: Interleukin-4

IL-6: Interleukin-6

IL-8: Interleukin-8

IL-10: Interleukin-10

IL-12: Interleukin-12

IL-1β: Interleukin-1beta

IL-13: Interleukin-13

IP-10: Interferon-gamma induced protein-10

KLRG1: Killer cell lectin-like receptor subfamily G member 1

L: Late

LV: Lentiviral vector

MALT: Mucosal Associated Lymphoid Tissues

M-CSF: Macrophage-colony stimulating factor

MHC II: Major histocompatibility complex class II

MCMV: Murine cytomegalovirus

MHC I: Major histocompatibility complex class I

MPLA: Monophosphoryl Lipid A

MP: Microparticle

MPEC: Memory precursor effector cell

NP: Nanoparticle

NK: Natural killer

P: Prophylactic

PAMPs: Pathogen-associated molecular patterns

PFU: Plaque forming units

PBS: Phosphate buffered saline

PLGA: Poly(D,L-lactic-co-glycolic acid)

PVA: Poly(vinyl) alcohol

RPMI 1640: Roswell Park Memorial Institute 1640

SLEC: Short-lived effector cell

SQ: Subcutaneous

Tcm: Central memory T cells

TCR T-cell receptor

Tem: Effector memory T cells

Teff: Effector T cells

Th1: T helper 1

Th2: T helper 2

Treg: Regulatory T cell

TET: Tetramer

TLR: Toll-like receptor

TNF: Tumor necrosis family

Trm: Tissue-resident memory T-cells

TRT: Total respiratory tract

U: Unknown

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Vaccination is arguably the greatest achievement of medical science, in terms of the effect it had on improving human life expectancy. So far, mass immunization programs have led to the complete eradication of smallpox and reduced the number of reported cases of many diseases, including diphtheria, polio, measles, mumps, rubella, CRS, and Haemophilus influenza, by more than 99% [1, 2]. Apart from reducing morbidity and suffering in humans, vaccination also has a significant impact on the global economy. In 2009, the routine childhood immunization program in the United States (which includes diphtheria and tetanus toxoids and acellular pertussis, *Haemophilus influenzae* type b conjugate, inactivated poliovirus, measles/mumps/rubella, hepatitis V, varicella, 7-valent pneumococcal conjugate, hepatitis A and rotavirus vaccines) has been expected to have prevented ~42,000 early deaths, 20 million cases of disease, with savings of $13.5 billion in direct costs and $68.8 billion in societal costs [3].

1.2 Conventional vaccines

Vaccination is majorly a prophylactic pharmacotherapy that is aimed at generating long-lived immune responses that can protect against infection by the corresponding microbe. Conventional vaccines utilize either a live-attenuated strain of a pathogen with low virulence or a killed pathogen to generate strong immune responses. Although both of these vaccines are effective at generating protective responses, primarily due to their resemblance to natural infection, they have many disadvantages like (i) risk of reversion to virulence, (ii) safety concerns when used in

immunocompromised patients, (iii) adverse side-effects in patients, (iv) and also a need for refrigeration to maintain efficacy, which is an issue in developing countries that may not have required facilities [4-6].

1.3 Subunit vaccines

To overcome the disadvantages of conventional vaccines, recent studies have focused on developing vaccines that contain only specific pathogenic components (subunits) that are recognized by the immune system. As these subunit vaccines do not contain any harmful microbial components they are much safer to use in humans. Subunit vaccines contain either whole protein antigens, which are purified directly from the pathogen or synthesized using recombinant DNA technology, or antigenic peptides that are chemically synthesized. However, subunit vaccines have low immunogenicity and are not capable of generating effective immune responses; this requires the addition of components known as adjuvants that can stimulate the immune system. Currently, there are not many adjuvants that are approved for human use and those approved adjuvants have limited capacity to generate cell-mediated immune responses. Therefore, there remains a great need for developing novel adjuvants and delivery systems that can improve the efficacy of vaccines [7-10].

1.4 Vaccine-induced immunity

The goal of vaccination is to generate long-lived antigen-specific immunological memory that can quickly respond to pathogenic invasion and protect against the development of disease [11]. Immunological memory is a hallmark of the adaptive

immune system which consists of two major components – humoral and cellular immunity. The success of a vaccine depends on whether it can generate the type of immune response required to protect against the corresponding pathogen. However, the type of immune response generated by a vaccine depends on its contents. For example, vaccines containing live-attenuated pathogens are very potent at generating both humoral and cellular responses. On the other hand, vaccines containing killed inactivated pathogens or antigenic proteins are weakly immunogenic and are unable to generate strong cellular responses[12]. Furthermore, chronic infectious diseases such as AIDS, tuberculosis, and herpes have multiple immune evasive strategies, and so development of vaccines against such diseases requires a thorough understanding of the immunological correlates of protection and the virulence strategies of the pathogen [13].

1.4.1 Humoral immune responses

Protection of the host from infection by humoral immune responses involves the production of antibodies by activated B-cells, which destroy extracellular pathogens and prevent the spread of intracellular pathogens. Activation of B-cells requires the binding of cognate antigen to B-cell receptors and interaction with antigen-specific CD4+ helper T-cells that recognize the peptide-MHC-II complexes derived from the antigen internalized by the B-cell. Some B-cells become plasma cells and the rest will migrate to the germinal center, where they undergo somatic hypermutation and B-cells that produce antibodies with the highest avidity are selected. Helper T-cells promote the survival of germinal center B-cells through

CD40-CD40L interactions and release various cytokines like IL-4, IL-5, IFN-γ and TGF-β which regulate the production of different antibody classes. B-cells that survive in the germinal center either differentiate into plasma cells which produce large amounts of an antibody or become memory B-cells which produce small amounts of antibodies (if any) [14].

Antibodies contribute to resistance by either directly neutralizing the pathogen, or by coating the surface of a pathogen, which increases its phagocytosis (opsonization) and activates the proteins of complement system via the classical pathway. Until the early 21st century, it was largely accepted that antibody-mediated responses provide immunity to extracellular pathogens while the immunity to intracellular pathogens rely on cell-mediated immune responses. However, persisting antibody production after immunization is considered as a sign of successful vaccination, due to the fact that antibody-mediated immunity induced by many viral vaccines confers protection against infection. This is because most pathogens are susceptible to antibody-mediated mechanisms during some part of the pathogenesis such as during replication in extracellular spaces or when spreading through extracellular fluids. The majoritymajority of the licensed vaccines produce serum antibody titers that correlate with the level of protection induced by the vaccine [15].

1.4.2 Cellular immune responses

Although intracellular pathogens are susceptible to humoral responses during the brief time they spend in the extracellular spaces, once they have entered into cells the only way to resolve infection is by destroying the infected cells. The

elimination of infected cells without affecting the healthy cells requires cell-mediated immune responses. A cell-mediated immune response is generated when naïve CD8+/CD4+ T-cells come into contact with mature, activated antigen-presenting cells (APCs) in the lymphoid organs. T-cells are activated only when the APC is presenting an antigen specific to the T-cell receptor (TCR) in the context of major histocompatibility complex (MHC) molecules and a co-stimulatory molecule. Once activated, naïve T-cells expand rapidly and differentiate into effector T-cells that can resolve the infection. Effector cytotoxic CD8+ T cells (CTLs) recognize infected cells and kill them by releasing several cytolytic molecules. Effector Th1 type CD4 T-cells activate other antigen-presenting cells (APCs) and B-cells, Th17 type effector cells recruit neutrophils to the site of infection, and Th2 type effector cells recruit eosinophils, basophils and mast cells against pathogens such as helminths. Upon resolution of infection, most of the effector T cells undergo apoptosis, while a small population differentiates into long lived memory cells that can protect from reinfection.

Currently, almost all of the licensed vaccines utilize humoral responses as a correlate of protection. However, it has become well known that humoral responses are a poor correlate for many intracellular infections, and so many studies have been conducted to better characterize cell-mediated immune responses. As of now, measurements of antigen specificity of T-cells and T-cell functional responses to *ex vivo* restimulation with antigen remain as the best correlates of protection.

1.5 Dendritic cells

Antigen processing and presentation to naïve T-cells in the context of MHC I and MHC II molecules is necessary for the generation of adaptive immune responses. This is a job that can only be carried out by APCs – dendritic cells (DCs), macrophages and B-cells. Of these, only DCs are specialized APCs while the other two cell types participate in other functions of the immune system. DCs, often referred to as the most professional APCs, can bind 30-200 times more antigen and are also up to 1000-fold more efficient at activating T-cells than macrophages and B-cells [16]. Given that DCs are the most potent APCs, the type of immune response (pro-inflammatory or tolerant) generated after an infection or immunization depends on the type of antigen, DC and the microenvironment during antigen uptake and presentation.

1.5.1 DC subsets

DCs are a heterogeneous population of cells with a distinct shape consisting of stellate cytoplasmic projections, which give them with a large surface area for antigen capture and presentation [17]. In mice and humans, all steady state DCs originate from hematopoietic precursors of myeloid or lymphoid pathways. Based on the origin, phenotype and function, DCs can be classified into five subsets: conventional DCs (cDCs 1 and 2), plasmacytoid DCs (pDCs), Langerhans cells (LCs), and monocyte-derived DCs (moDCs).

Both murine and human cDCs are CD11c+ MHC II+ cells that can be further differentiated into CD8α+ (cDC1) or CD4+ (cDC2) cells in mice and CD1c+ (cDC1) or CD141+ (cDC2) cells in humans. Both murine and human cDC1 cells express high

level proteins required for cross-presentation and have the capacity to produce type-I IFN and IL-12 (p70), which gives them the ability to activate CD8+ T cells and prime Th1 helper T-cells [18, 19]. In contrast, cDC2 cells in humans and mice polarize CD4+ T cells to Th2 and Th17 types.

The pDCs in humans and mice are broadly distributed throughout the body and express CD11c, B220, SiglecH, and BST2 in mice versus CD123, BDCA-2 and BDCA-4 in humans. pDCs have a high capacity to produce type-I IFN in response to viral stimuli and play an important role in controlling the progress of viral infections. Murine LCs express langerin (DC207) whereas human LCs are identified as langerin+ DEC205+ $CD1a^{hi}$ $CD11c^{lo}$ cells. The role of LCs in mice seems to be adaptable and is dictated by the microenvironment. In steady state, murine epidermal LCs continuously internalize and process antigens to induce tolerance, whereas during inflammation they produce IL-23, IL-6 and IL-1β, which drive Th17 polarization. In humans, LCs are known to be efficient at cross-presentation to naïve CD8 T-cells and secrete IL-15 which promotes differentiation of granzymeB+/perforin+ CTLs [20]. MoDCs are a special type of DCs that are produced from monocytes during inflammation. MoDCs predominantly drive Th1 and Th17 type immune responses by producing IL-12 or IL-23.

1.5.2 Antigen uptake

DCs are abundantly located throughout the body at strategic locations like body barriers (under the epithelium at mucosal surfaces) and organ entry ports [21, 22]. DCs continuously sample the surrounding environment for both self and non-self

antigens using their dendritic appendages and induce immune tolerance or protective responses, depending on the nature of antigen (self or non-self). Immature DCs are very efficient at internalizing antigens from the environment using various endocytic pathways, like macropinocytosis for the uptake of solutes and nutrients and phagocytosis for the uptake of pathogens, apoptotic or dead cells, and large particulate antigens. DCs also have highly specialized receptors like C-type lectin receptors (CLRs), scavenger receptors (SRs) and Fc receptors (FcRs) on the cell surface to capture and internalize antigens via receptor-mediated endocytosis. Upon internalization, the antigen is then processed and presented to T-cells in the context of MHC molecules.

1.5.3 DC Maturation and migration

The nature of the immune response generated towards an antigen depends upon the maturation status of the presenting DC. On the one hand, antigen presentation by immature DCs leads to T-cell anergy and immune tolerance [23, 24]. On the other hand, presentation by mature, activated DCs leads to T-cell activation and an inflammatory response. DCs express several pattern recognition receptors (PRRs), like Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-1-like receptors (RLRs) and CLRs, that can recognize pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs), and triggering of these receptors leads to the activation of DCs. Activation of DCs can also be caused by triggering of FcR by immune complexes or CD40 by CD40L on

CD4+ T-cells or by inflammatory cytokines like TNFα, IL-1 and PGE2, secreted as a response to pathogenic infection.

Activation induces several changes in the expression of genes in DCs, leading to secretion of chemokines and cytokines that can recruit other immune cells [25]. It also leads to cytoskeletal rearrangement, downregulation of CCR1, CCR5 and CCR6, and upregulation of CCR7, which allows their migration to lymph nodes [26]. Importantly, activation leads to redistribution of MHC and co-stimulatory molecules (CD80, CD86 and CD40) to the surface of DCs which results in improved T-cell activation [27].

1.5.4 Antigen processing and presentation

Typically, peptides derived from the degradation of cytosolic proteins by proteases are transported into the endoplasmic reticulum by transporters associated with antigen processing (TAP), where they are further processed by the ER aminopeptidase associated with antigen processing (ERAAP) [28], then loaded onto MHC I molecules and presented on the DC cell surface to CD8+ T-cells. Exogenous antigens upon internalization are translocated from the phagosomes into the cytosol, ubiquitinated and degraded into peptides by the proteasome. These peptides are then transported into the ER or back into the same phagosomes, which have acquired TAP and MHC I loading machinery by fusing with the ER, where they are loaded onto MHC I molecules, transported to the cell surface and presented to CD8+ T-cells in a process known as cross-presentation [29, 30].

In contrast to peptide loading of MHC I molecules, loading of MHC II molecules occurs only in the late stage phagolysosomes. Phagosomes containing exogenous antigens or endogenous antigens (taken up during autophagy) fuse with lysosomes containing proteases that degrade the antigens into peptides. These phagolysosomes then fuse with ER vesicles containing the MHC II loading machinery where they load the peptide onto MHC II molecules and transport them to the cell surface to be presented to CD4+ T-cells.

1.6 Targeting antigen to dendritic cells for improving vaccine efficacy

Given the important role DCs play in coordinating adaptive immune responses, targeting the antigen to DCs would potentially improve the immune responses generated. So far, researchers have tried to target antigen to DCs in two ways: (i) by administering vaccines in a way that they accumulate in DC-rich areas and (ii) by targeting the receptors on DCs to mediate internalization and increase immune responses.

1.6.1 Administration-based targeting

Simplest way of getting a vaccine to DC-rich areas is to directly inject the vaccine into lymph nodes. Intranodal immunization with lentiviral vectors encoding tumor-associated antigens or mRNA resulted in a strong CD8+ T-cell response when compared to subcutaneous delivery [31, 32]. Another way of reaching lymph nodes is to administer the vaccine near a lymph node so that the vaccine drains into the lymph node. Intradermal delivery of antigens conjugated to nanoparticles (NPs) has been shown to allow the antigens to drain efficiently into lymph nodes, resulting in an

increase in antigen-specific immune responses [33, 34]. However, care must be taken while formulating the vaccine so that the vaccine does not form a depot at the injection site, which might lead to exhausted T-cell responses and reduce vaccine efficacy [35-37].

1.6.2 Targeting DC receptors

DCs express several endocytic cell surface receptors that can be used as targets for vaccine delivery (Table 1.2). So far, numerous DC-targeting vaccines have been evaluated in pre-clinical and clinical stages (Table 1.3). Mostly, five receptor families have been evaluated for DC targeting: CLRs, integrins, Fcγ receptors, MHC II molecules and stimulatory receptors. Of these, CLRs are the most extensively studied receptors. CLRs contain carbohydrate recognition domains (CRDs) that bind to sugars in calcium-dependent manner. CLRs have the ability to internalize glycosylated antigens, resulting in antigen processing and presenting in the context of MHC molecules and making them an attractive target for vaccine delivery. Amongst CLRs, macrophage mannose receptor (CD206), DEC-205, and DC-SIGN are the most examined candidates for DC targeting.

1.6.2.1 Macrophage mannose receptor

CD206 is a type I CLR, which upon ligand binding is internalized through clathrin-coated vesicles and recycled through the early endosomes. CD206-mediated endocytosis of antigens has been shown to result in antigen presentation in the context of both MHC I and II molecules. It has been demonstrated that immunization

with mannan-conjugated MUC1 induces both Th1 and Th2 type immune responses based on the type of mannan. MUC-1 conjugated to oxidized mannan was shown to be 1000 times more efficient at generating antigen-specific CTL and Th1 responses when compared to MUC1 conjugated to reduced mannan, which generated Th2 type responses [38]. Coating of cationic liposomes containing HIV-1 DNA with mannan was also shown to induce HIV-specific CTL responses and significantly enhanced Th1 type immune responses as seen by antibody isotyping and cytokine secretions [39]. It has also been demonstrated that targeting CD206 using a fusion protein of human anti-CD206 mAb and melanoma-associated antigen pmel17 or model antigen ovalbumin (OVA) generates potent antigen-specific CTLs [40, 41].

1.6.2.2 DEC205

Unlike CD206, DEC205 (CD205) is recycled through late endosomes that are rich in MHC II molecules; because of this, antigens internalized through DEC205 mediated endocytosis are efficiently presented through MHC II molecules [42]. However, the DEC205 endocytic pathway is non-stimulatory and does not activate DCs, which results in the induction of T-cell tolerance [43, 44]. Therefore, in order to generate antigen-specific immunity, immunostimulatory adjuvants have to be included in DEC205-targeting vaccines. Thus far, DEC205-targeted vaccines have used HB290 single chain antibody (scFv) as a targeting moiety. Immunization with scFv-coated liposomes containing OVA or OVA peptide antigens were found to generate potent OVA-specific CTL responses that reduced growth of B16-OVA tumors in mice. Protection was depended on the concurrent delivery of OVA and an

adjuvant (IFN-γ or LPS) [44]. Also, immunization of mice with OVA conjugated to anti-DEC205 mAb and anti-CD40 mAb protected against mucosal challenge[45].

1.6.2.3 Dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN)

DC-SIGN is a type II CLR that is primarily expressed on immature DCs; it recognizes various pathogens such as EbolaEbola, herpes and HIV viruses. Like DEC205, DC-SIGN-mediated endocytosis is routed through late endosomes where the antigen is loaded onto MHC II molecules and presented to CD4+ T-cells [46]. Targeting antigen to DC-SIGN using anti-DC-SIGN mAb was shown to induce proliferation in antigen-specific T-cells at a 100-fold lower concentration than non-targeted antigen [47]. Additionally, glycoliposomes modified with the glycan Lewis (Le)(X), which is highly specific for DC-SIGN, were preferentially internalized by human monocyte-derived DCs and also induced antigen-specific CD8+ T-cell responses when the glycoliposomes were loaded with gp100 tumor antigen and the adjuvant MPLA [48].

1.7 Immunomodulatory properties of C5a

The complement system is an important part of innate immunity, which plays an integral role in the defense against invading pathogens. Activation of complement system in response to pathogens, immune complexes, or injured tissue results in a series of events that includes release of several biologically active peptide fragments called anaphylatoxins, C3a, C4a and C5a, that have diverse biological functions [49]. Of the three anaphylatoxins, C5a is the most potent and binds to at least two seven-

transmembrane receptors, C5aR and C5L2 that are expressed on multiple cell types especially immune cells like DCs, macrophages, neutrophils and T-cells. C5a has several immunological functions including recruitment of inflammatory cells, increase vascular permeability and induce smooth muscle contraction, mast cell and neutrophil degranulation and various cytokines (Fig 1.2)[50-56]. Apart from its immunological functions C5a has also been implicated in developmental biology, CNS development and neurodegeneration, tissue regeneration and haematopoiesis[57]. C5a has also been shown to enhance antigen-specific antibody responses, antigen-induced T-cell proliferation[58, 59], provide costimulatory and survival signals to naïve CD4+ T-cells. C5a also stimulates murine and human DCs resulting in the upregulation of MHC II, CD80, CD86, CD40 and CD54, and secretion of Th1 polarizing cytokines [60-62]. In contrast, hindering of C5aR signaling promoted Th2 type and regulatory T cell responses[63], and impaired memory CD4+ T cell generation [64].

1.8 Development of EP54 and EP67

Given the immunostimulatory properties of C5a, its use as a vaccine adjuvant has tremendous promise. However, its use in clinic is limited by the potential side effects that arise due to its pleotropic properties. Therefore, it became necessary to identify the effector fragment of C5a that has immunostimulatory properties but not inflammatory properties for clinical use[65, 66].

Human C5a is a 74-amino acid peptide that consists of two domains, first, the N-terminal core ($C5a_{1-63}$) domain that is responsible C5aR recognition and binding and second, the functional C-terminal domain ($C5a_{64-74}$) which is necessary for

inducing biological functions (fig 1.3) [67]. Peptide analogs of C-terminal region ($C5a_{65-74}$) were demonstrated to induce several C5a-like biological effects which depended on the peptide conformation[68]. This led to the hypothesis that conformationally restricted $C5a_{65-74}$ analogs would induce specific C5aR-mediated activities. Examination of a large panel of analogs based on $C5a_{65-74}$ Y65,F67 (YSKKDMQLGR) in which the backbone of MQLGR was restricted resulted in the discovery of EP54 (YSFKPMPLaR), which demonstrated significantly more potent ability to induce smooth muscle contraction of human umbilical artery [66, 69-71], however, EP54 also triggered the C5aR on PMNs inducing β-glucuronidase release. Further analoging of EP54 by introducing N-methylation generated EP67, which demonstrated ~3000 fold higher selectivity towards C5aR expressed on antigen presenting cells compared to PMNs, in contrast, EP54 was only 34 fold more selective[66]. Furthermore, these conformational features protect EP54 and EP67 from proteolytic degradation by serum carboxy-peptidases.

1.8.1 Vaccine adjuvant properties of EP54 and EP67

Because of the potent immunostimulatory properties of EP54 and EP67, several studies have investigated their ability as a vaccine adjuvant (Table 1.4) to improve antigen-specific humoral and cellular immune responses. Immunization of mice and rabbits with a B-cell epitope form MUC1 conjugated to N-terminal of EP54 generated high MUC1-specific antibody titers, with an isotype characteristic to Th1 type response in mice[67]. Also, EP54 conjugated to a T-cell epitope from hepatitis B surface antigen was shown to generate CD8+ CTL responses in mice. In the same

study, it was also shown that it necessary to conjugate the T-cell epitope to the N-terminus of EP54 using a protease labile linker to generate CTL responses[72]. Similarly, EP67-conjugated protein vaccines were also shown to generate Th1-type humoral immune responses, furthermore, EP67 was demonstrated to generate higher antibody responses and Th1-type isotype switching in aged mice which were not observed when CpG or alum were used as adjuvants [73]. Recently, we have also shown preliminary evidence that EP67 can be used as a mucosal adjuvant in mice[74]. It is important to note that EP54 and EP67 have been shown to increase uptake, processing and presentation of the conjugated antigen by interacting with the C5aRs present on APCs, while simultaneously inducing the release of Th1 type cytokines making them effective dual purpose vaccine adjuvants[75].

1.9 Summary

In summary, targeting of antigen presenting cells, especially dendritic cells, appears to be a promising strategy to improve the efficacy of vaccines against many life-threatening diseases. Despite the tremendous progress, we have made in APC targeted vaccines a successful human vaccine is yet to be developed. Therefore, there is a need to research more ways to target antigen to APCs. Unlike, most vaccine adjuvants EP67 is a host-derived dual purpose immunostimulant with the capacity to simultaneously, target and activate APCs and has shown great potential as an adjuvant. This dissertation is an effort to explore the ability of EP67 to deliver encapsulated antigen to APCs and increase immune responses generated against the encapsulated antigen.

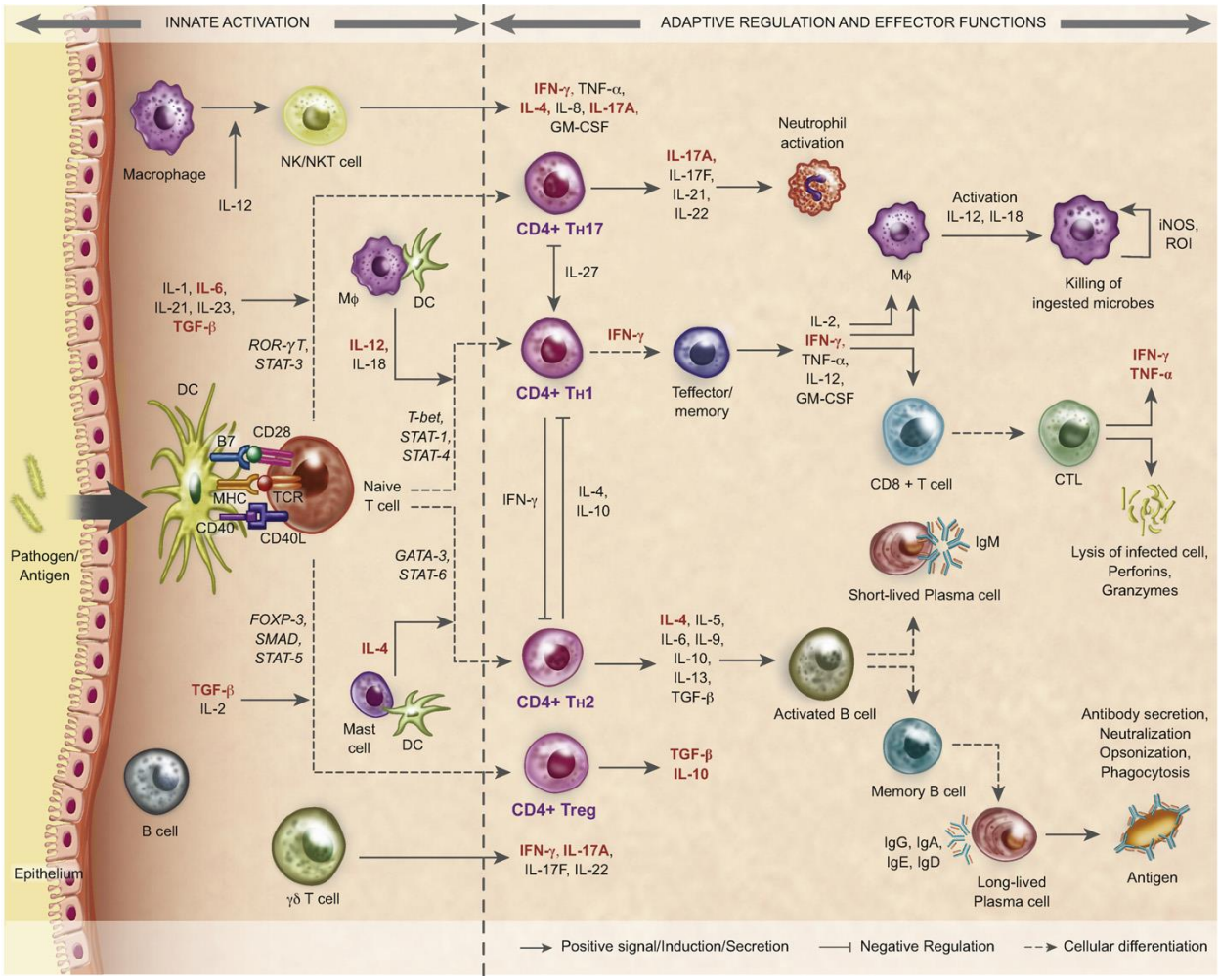

Figure1.1 Schematic representation of the different stages involved in the induction and regulation of cell-mediated and humoral immune response against pathogens.

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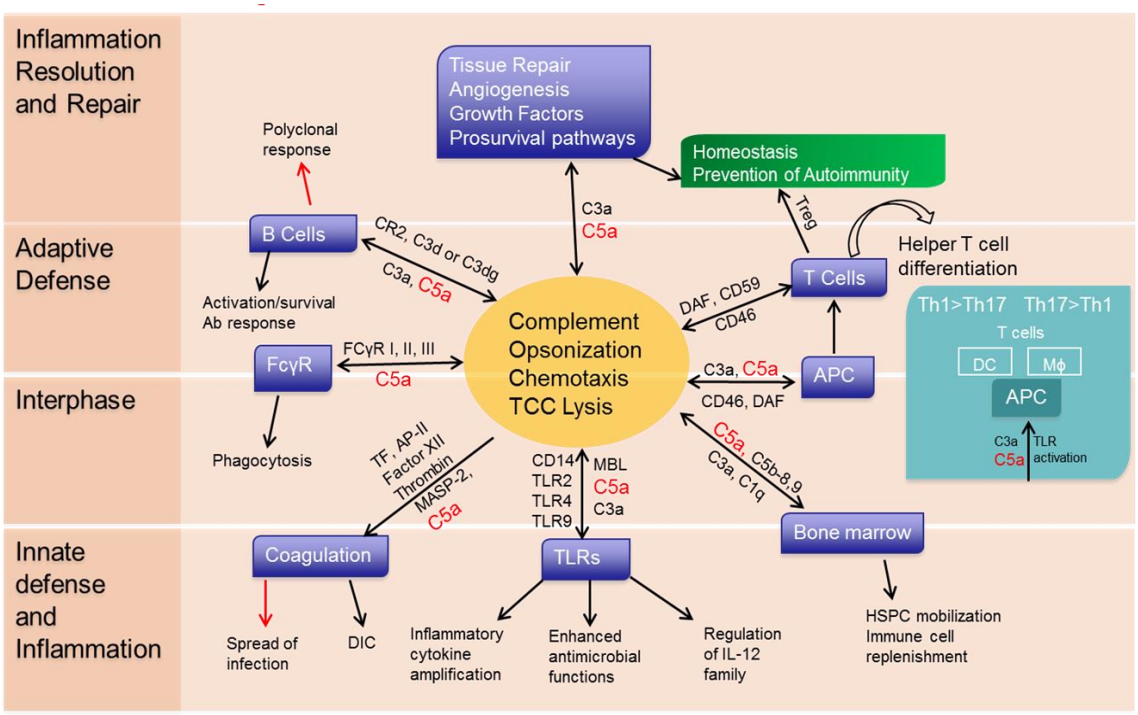

Figure 1.2 Role of Complement C5a in host defense and homeostasis.

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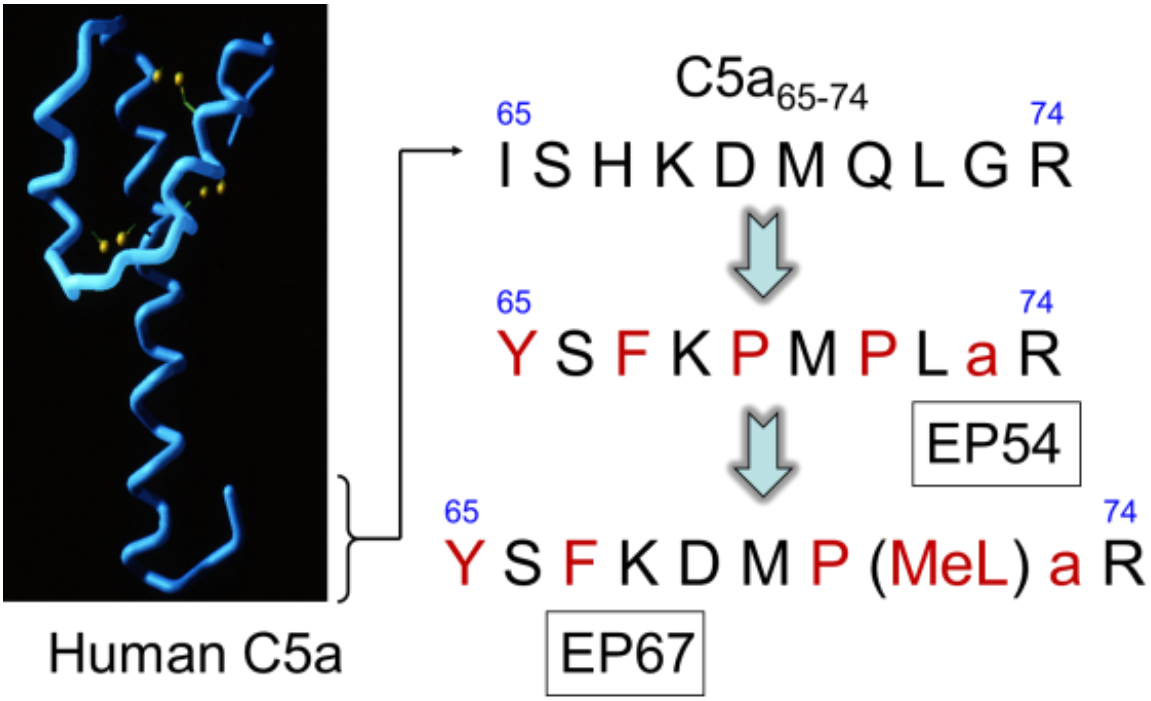

Figure 1.3. Structure of human C5a

Table 1.1 – Overview of phenotypic and functional features of DC subtypes.
Adapted with permission from [77]

	cDC1	**cDC2**	**pDC**	**LC**	**moDC**
Murine markers	CD8α+/CD103+ DEC205^{+}	CD4+ CD11b+	SiglecH+ BST2+ B220+	Langerin+	CD11b+ CD64 FcγRε and Ly6c
Human markers	CD141+ CD162^{hi} DEC205^{hi}	CD1c+ CD11b^{lo}	CD123+ BDCA-2+ BDCA-4+	Langerin+ DEC205 CD1a^{hi}	CD11b+ CD1a+ CD24+ CD206+ CD16+ DC-SIGN
Functions	T_H1 Cross-presentation	T_H2 and T_H17 Cross-presentation	IFN-α/β and IFN-λ Humoral	Adaptable MOUSE: Treg or T_H17 HUMAN: IL-15 promoting CTLs + Cross-presentation	Highly adaptable (IL-12, IL-23, TNFα, and iNOS)

Table 1.2: Summary of dendritic cell receptors targeted for vaccine development. Adapted with permission from[78]

Receptor	Designation	Function
1. Group 1 C-type lectin receptors		
1.1. Mannose receptor	CD206	Expressed on macrophages and DCs. Binds to mannan, mannose, fucose, glucose, maltose, GlcNAc, lipoarabinomannan, cell wall of yeast, viruses, and bacteria leading to phagocytosis/endocytosis. Used to target protein, peptides, DNA, dendrimers, liposomes, and anti-MR antibodies for vaccine development withTh1, Th2, CTL, and Ab responses induced. Targeting antigens to MR using mannan has been used in human clinical trials.
1.2. DEC205	CD205 Ly 75	Homologous to the mannose receptor. Expressed on DCs and thymic epithelial cells. Targeting induces an array of immune responses.
2. Group 2 C-type lectin receptors		
2.1. Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN	CD209 Clec4L	Expressed on immature DCs, macrophages endothelial vascular cells, atherosclerotic plaques, and lymphatic vessels, not on placmacytoid DCs. Binds to mannan, mannose, fucose, GlcNAc, GalNAc, yeast, lewis blood group antigens Lex, HIV-1 gp120, Ebola virus, hepatitis C virus, dengue virus, respiratory syncytial virus, measles virus, Mycobacterium tuberculosis, Leishmania amastigote, Helicobacter pylori, Leishmania mexicana, Schistosoma mansoni, Porphyromonas gingivalis, Neisseria gonorrhoeae, Candida albicans, house dust mite (Der p1), and dog allergens (Can f1). Interacts with ICAM-3 and ICAM-2. Targeting DC-SIGN using antigen linked to anti-DC-SIGN antibodies, Manalpha-6 Man, lactoside, and Lewis oligosaccharide, stimulates T-cell and/or antibody responses, and has been studied as a potential receptor for vaccine targeting. Eight murine homologs identified, SIGN-R1 (CD209b) to SIGN-R8.

Receptor	Designation	Function
2.1.1. L-SIGN or DC-SIGNR	CD299 CD209L Clec4M	Expressed on liver sinusoidal cells, lymph nodes, and endothelial vascular cells, but not on DCs. Binds to HIV gp120, Man9GlcNAc2, HIV, simian immunodeficiency virus, ebola virus, hepatitis C virus, and respiratory syncytial virus. Targeting L-SIGN with anti-L-SIGN antibodies induces T-cell responses. Targeting L-SIGN shows promise for the development of targeted vaccines.
2.1.2. Liver and lymph node sinusoidal cell type lectin (LSECtin)	Clec4G	Expressed in liver, lymph nodes, sinusoidal endothelial cells, DCs, and Kupffer cells. Binds to N-acetyl-glucosamine, fucose, ebola virus, filovirus glycoproteins, lymphocytic choriomeningitis virus, S-protein of SARS coronavirus, and to CD44, but not to mannose, HIV, and hepatitis C. Coexpressed with DC-**SIGNR and CD23.** Antibody or ligand-mediated engagement of LSECtin activates rapid internalization, indicating that LSECtin may be a suitable receptor for targeting antiges in the development of vaccination regimes.
2.1.3. C-type lectin immune receptor (CIRE) (murine homologue of DC-SIGN)	CD209	Expressed by immature CD8− splenic DCs (CD8−CD4+ and CD8−CD4−), on some CD4+ DCs, plasmacytoid pre-DCs, and not by, CD8+ DCs, macrophages, or monocytes. It is a ligand for ICAM-3 and binds to HIV. Polyanhydride nanoparticles covalently linked to dimannose and lactose matures DCs and are internalized by DCs. CIRE shows promise as an appropriate target for antigen delivery for improved vaccine development.
2.2. Langerin CD207	Clec4K	Expressed on Langerhans cells, CD103+ DCs, and splenic CD8+ DCs. Binds to mannose and internalizes mannose residues into Birbeck granules, where Langerin is expressed. Anti-Langerin antibody targeting antigens to Langerin is endocytozed in vitro and in vivo and induces Th1 and antibody responses.

Receptor	Designation	Function
2.3. MGL (human macrophage galactose- and N-acetylgalactosamine-specific C-type lectin)		Expressed on macrophages, immature DCs galactose, GalNAc, Tn antigen, filoviruses, and gonorrhea. GalNAc modified peptides to target MGL receptor expressed on murine and human DCs, which stimulates T-cell and antibody responses, and this approach could be used to design novel anticancer vaccines.
2.4. Dectin-1 or beta-glucan receptor (DC-associated C-type lectin-1)	DCAL-1 Clec7A	Expressed on myeloid DCs, CD8−CD8− DCs, dermal DCs, monocytes, macrophages, neutrophils, T cells, B cells, mast cells, eosinophils, and monocytes. Binds to beta-glucan on yeast, mycobacteria, plant cell walls, *Saccharomyces, Candida, Pneumocystis, Coccidioides, Penicillium,* and *Aspergillus*, but not *Cryptococcus* fungal species, and interacts with CD37. Anti-Dectin-1 and anti-Dectin-2 antibodies linked to proteins stimulate CD8+ and CD4+ T cells, and immunization with beta-glycan modified proteins induces CD4+ andTh17 bias responses.
2.4.1. DNGR-1 (NK lectin group receptor-1)	Clec9A	Expressed onmurine CD8+ DCs not on CD4+ DCs, on CD11c+ DCs but not by CD11c− cells (B cells, T cells, NK cells, NKT cells, macrophages, and granulocytes), on plasmacytoid DCs, and on human blood DCsBDCA-3+ DCs) and monocytes (CD14+CD16−). Highly expressed on Flt3 ligand bone marrow derived CD8+ DCs. Target for immune response induction.
2.4.2. Myeloid inhibitory C-type lectin receptor (MICL)	Clec12A	Homologous to Dectin-1 and part of Dectin-1 cluster. Also termed as CLL-1, DCAL-2, and KLRL1. Expressed on granulocytes, monocytes, macrophages, B cells, CD8+ T cells in peripheral blood, and DCs

Receptor	Designation	Function
2.4.3. C-type lectin-like receptor 2 (CLEC2)	Clec1B	Expressed on NK cells, monocytes, granulocytes, platelets, megakaryocytes, and liver sinusoidal epithelial cells. Binds to HIV-1 and facilitates HIV-1 spread to other cells and binds to snake venom rhodocytin. Not much is known regarding stimulating immune responses; however, colocalization with DC-SIGN suggests that it may have an immune stimulatory effect.
2.4.4. CLEC12B (macrophage antigen H)	Clec21B	Part of the NK gene complex/dectin-1 cluster of C-type lectin receptors. Expressed on macrophages, monocytes, and DCs. Not much is known regarding its function.
2.4.5. LOX-1 (Lectin-like receptor for oxidized density lipoprotein-1)	Clec8A	Part of the dectin-1 cluster of C-type lectin receptors and scavenger receptor family. Expressed on endothelial cells, smooth muscle cells, platelets, fibroblasts, and macrophages. Binds to Gram-positive and Gram-negative bacteria, oxidized LDL modified lipoproteins, phospholipids, apoptotic cells, C-reactive protein, and heat shock protein (HSP)-70. Targeting LOX-1 induces immune responses and is a promising target for cancer immunotherapy.
2.5. DC immunoreceptor subfamily		
2.5.1. DC immunoreceptor (DCIR)	Clec4A	Expressed on plasmacytoid DCs, immature and mature monocyte-derived DCs monocytes, macrophages, and B cells. Binds to TLR9. Targeting DCIR stimulates immune responses especially CD8+ T cells.
2.5.2. Dectin-2 (or beta-glucan receptor)	DCAL-2 Clec6A	Expressed on DCs, macrophages neutrophils, and monocytes. Binds to beta1,3 and beta1,6-linked glucans on yeast, mycobacteria, and plant cell walls. Targeting dectin-2 stimulates immune responses in mice.

Receptor	Designation	Function
2.5.3. Blood DC antigen (BDCA-2)	Clec4C	Expressed on human blood DCs. Targeting BDCA-2 suppresses IFN-alpha/beta cytokine secretion.
3. Scavenger receptors 3.1. Scavenger receptor macrosialin, mucins, and LOX-1. Targeting of scavenger receptors induces immune responses in mice.		Expressed on macrophages. Bind to modified low density lipoproteins (LDL) by oxidation (oxLDL) or acetylation (acLDL). Bind to CD68, macrosialin, mucins, and LOX-1. Targeting of scavenger receptors induces immune responses in mice.
3.1.1. Scavenger receptor class A	SR-A1 SR-A2	Expressed on macrophages as a trimer. Members include SCARA1 (MSR1), SCARA2 (MARCO), SCARA3, SCARA4 (COLEC12), and SCARA5.
3.1.2. Scavenger receptor class B	SR-B1	Consists of 2 transmembrane units. Members include SCARB1, SCARB2, and SCARB3 (CD36).
3.1.3. Scavenger receptor class C	SR-B1	Consists of a transmembrane region in which the N-terminus is located extracellularly.
3.2. DC-asialoglycoprotein receptor (DC-ASGPR)		A lectin-like scavenger receptor. Expressed on monocyte derived DCs (CD14+CD34+), tonsillar interstitial-type DCs, and granulocytes. Targeting DC-ASGPR induces suppressive responses.
4. F4/80 receptor		Expression restricted to macrophages. Murine homolog of the epidermal growth factor-like module containing mucin-like hormone receptor-1 protein encoded by the EMR1 gene.
4.1. FIRE		Expressed on CD8−CD4+ and CD8−CD4− immature DCs, and weakly on monocytes and macrophages. Targeting FIRE stimulates immune responses in mice.

Receptor	Designation	Function
5. DC-specific transmembrane protein (DC-STAMP)		Expressed on DCs and activated blood DCs. Targeting DC-STAMP results in immunosuppressive responses in some studies and in other studies stimulates strong cellular responses.
6. FcR		Links humoral and cellular immune (Fc Receptor) responses, links innate and adaptive immune responses by binding pathogens and immune complexes, and stimulates T cells. Targeting FcR is a novel vaccine strategy for stimulating immune responses.

Table 1.3: Summary of APC targeting studies. Adapted with permission from [77]**.**

Targeting moiety	Injection	Effect	Ref.
DEC205			
α-GalCer NP	fp	↑iNKT, ↓growth in B16F10, and EG7-OVA (P + T)	[79]
Selected nucleic acid aptamer	i.v.	↑CD8, ↓growth OVA-B16 tumor (T if OT-I transfer)	[80]
Anti-CD11c and DEC205 scFv coupled to NP	i.v.	↑CD8, ↓growth OVA-B16 tumor (P)	[44]
mAb fused protein	s.c.	↑CD8, ↓growth OVA-B16 (P + T)	[45]
mAb fused protein	i.p.	↑CD8, ↑CD4, ↑humoral, and ↓growth neu-expressing mammary tumor (P)	[81, 82]
mAb fused protein	i.p.	↑cross-presentation, ↑CD4, ↑humoral, and ↓growth neu-expressing mammary tumor (P)	[83]
scFV modified adenoviral vector	fp	↑T cell, ↑humoral (at low doses), ↓growth OVA-B16 (P) BUT better for untargeted vectors	[84]
mAb fused protein	fp	↑CD8, ↓growth B16 pseudo-metastasis model (P + T)	[85]
Bacteriophage displaying scFV	fp	↓growth B16F10 (Pro + Ther)	[86]
mAb fused protein	s.c.	↑CD8, ↑CD4, and ↓growth B16 melanoma (P + T)	[87]
scFV fused to DNA vaccine	i.m.	↑CD8, ↑humoral, long lasting memory ↓growth ER2/neu^{+} D2F2/E2 breast tumor + spontaneous mammary carcinomas (P + T)	[88]

Targeting moiety	Injection	Effect	Ref.
Phase I clinical trial with CDX-1401 = human mAb fused protein	i.d.	Patients with advanced malignancies: ↑cellular, ↑humoral (T)	[89]
DEC206			
Mannosylated NP	s.c.	↑T_H1 cell, ↑humoral, and ↓growth B16F10 (P + T)	[90]
mAb fused to protein	s.c.	↑T cell, ↑humoral, and ↓growth B11-OVA (P)	[41]
Mannan coupled protein	i.p.	↑CD8, ↓growth P815 mastocytoma (T)	[91]
Mannose coupled dendrimer	i.d.	↑CD8, ↑CD4, ↑humoral, ↓growth B16-OVA (P)	[92]
Mannosylated NP	s.c.	↓growth huErbB2$^+$ renal carcinoma cells (T)	[93]
Mannan coated liposome-protamine-DNA	U	↓growth E7$^+$ TC-1 (P + T)	[92]
Mannosylated and/or histidylated NP loaded with mRNA	i.v.	↑CD8, ↓growth B16F10 (P)	[94, 95]
Mannan or pullulan NP complexed with protein	U	↑CD8, ↓growth HER2$^+$ tumors (P + T)	[96]
D-mannose conjugated lipid-core peptide system	s.c.	↓growth TC-1 HPV-16 tumor (P)	[97]
Clinical trial with mannan coupled protein	s.c.	↑humoral, less ↑CD8, protection against recurrence in breast cancer patients	[98]

Targeting moiety	Injection	Effect	Ref.
Two phase I studies with CDX-1307 = hCG-β fused to mAb	i.d. or i.v.	↑humoral and T cell with clinical benefit in patients with advanced epithelial malignancies	[99]
DC-SIGN			
LV pseudotyped with point-mutated Sindbis virus glycoprotein	i.d.	↑CD8, ↑CD4, and ↓growth transgenic adenocarcinoma, E.G7-OVA and PSCA-expressing B16-F10 (P + T)	[100]
mAb coupled protein	U	↓growth human Burkitt's lymphoma cell line in humanized mice (P)	[101]
IDLV pseudotyped with engineered Sindbis virus glycoprotein	s.c.	↑CD8, ↓growth CT26 colon carcinoma cells (P + T)	[102]
LOX-1			
HSP70 fused to protein	s.c.	↑CD8 and cross-priming, ↓growth E.G7 cells (P)	[103]
CLEC9A			
mAb coupled to peptide	s.c.	↑T_H1, ↓growth MUC-1-A2K/b$^+$ MC38 (P + T)	[104]
mAb coupled to peptide	s.c.	↑CD8, ↓growth B16 lung pseudo metastases (P + T)	[105]
DCIR2			
Anti-DCIR2 or anti-DEC205 mAb coupled protein	i.p.	↑CD8, ↑CD4 (mixed T_H1/T_H2), ↑humoral, and ↓growth B16F10-OVA (P + T)	[104]

Targeting moiety	Injection	Effect	Ref.
CD11c			
Targeted lipopeptide	i.d.	↓growth for OVA: E.G7-OVA, for mWT1: mWT1–1498 cells and for tumor lysate: MHC-I^- B16D8 melanoma (T)	[106]
Tumor-derived plasma membrane vesicles engrafted with two CD11c binding peptides	i.v.	↑CD8, ↑humoral, and ↓growth of metastatic B16-OVA (T)	[107]
CD11b			
Adenylate cyclase-(CyaA-) based vector	i.p. vs. i.v. or i.d.	↓growth OVA-B16 or E.G7-OVA versus TC-1 (P + T)	[108]
Phase II study with ProCervix = CyaA-based vector	s.c.	Clinical phase I trial indicated good safety and local tolerance at the highest dose, ↑T + ↑viral clearance + controlled HPV recurrence	[109]
Fc γ receptor			
IgG1-Fc tumor cells	s.c.	↓growth E.G7 (P + T)	[110]
HER2-Fc cDNA	i.m. + EP	Mu: ↑T, ↓growth $HER2^+$ D2F2/E2 cells (P) Hu: in vitro cross-processing and ↑$CD8^+$ T cells from breast cancer patients	[111]
MHC-II			
DNA loaded dendrimer with targeting peptide	s.c.	↑CD8, ↑humoral, ↓growth, strong for B16OVA, and weak for gp70 BUT better with EP (P) ↔ B16 with Trp2 (T)	[112]

Targeting moiety	Injection	Effect	Ref.
LV pseudotyped with scFv coupled to H protein of measles virus envelope	i.v.	↑CD4, ↑cytotoxic, and memory CD8 BUT not to the same extent as broad tropism LVs	[113]
LV pseudotyped with scFV coupled to murine leukemia virus envelope	s.c.	↑CD8 mediated IFNγ secretion	[114]
DNA encoding anti-MHC II and anti-CD40 scFv or chemokines (MIP-1α, RANTES) with scFV of idiotype	i.m. or i.d. + EP	↑CD8, ↑humoral, and ↓growth Id^+ tumors (P)	[115]
BST2			
Protein fused anti-BST2 Ab	i.p.	↑CD4, ↑CD8, ↑humoral + ↓growth B16-OVA (P)	[116]
CD40			
CD40 PLGA-NP coated with mAb	s.c.	↑CD8, ↑CD4 + ↓growth B16-OVA (P + T)	[117]
CD40 targeted adenoviral vector	i.p.	↑CD8, ↓growth RM-1-PSMA model (T)	[118]
CD40L extracellular domain to adenoviral vector in mice + Clinical trial	i.d.	Mice: ↑CD8, ↓growth B16F10 (T) ↑CD8 in melanoma-draining sentinel lymph nodes	[119]

Targeting moiety	Injection	Effect	Ref.
B7			
Syngeneic epithelial cells continuously secreting CTLA-4-ErbB2 fusion vaccine	s.c.	↑CD8, ↑humoral, ↓growth ErbB2^{+} renal cell carcinoma (T)	[120]
Treml4, Ig superfamily member			
mAb against Treml4	i.p.	↑CD8, ↑CD4, ↓growth neu^{+} mammary tumor cell line NT2.5 (P)	[121]
TLRs			
TLR9 targeting protein (via DNA sequence)	i.d.	T_H - independent ↑CD8 + ↓growth E.G7-OVA (P + T)	[122]
TLR2 targeting lipid moiety + epitopes	s.c.	↑CD8, ↑humoral + ↓growth B16-OVA, and Lewis lung-OVA (P + T)	[123]
TLR5 targeted peptides (via flagellin) engrafted onto liposomes	i.v.	↑maturation of DCs, ↑CD8, ↑humoral, ↓growth B16, and P815 (P + T)	[124]
TLR4 targeting protein (via fibronectin)	i.t. or i.v.	↑CD8 with cure of established TC-1 tumors i.t.: in the absence of additional ADJ i.v.: when + ADJ or CPM + ADJ	[125]
TLR4 targeting protein (via fibronectin) + anti-CD40, TLR3 and TLR7 ligands	s.c.	↑CD8, ↓growth B16-OVA or B16.F10 (T)	[126]

Targeting moiety	Injection	Effect	Ref.
Chemokine related			
Fusion of chemokine MCP3 or IP10 to lymphoma-derived scFv as protein or DNA plasmid	s.c. or i.d.	↑humoral, ↓growth 38C-13 and A20 (P)	[127]
OVA with mAb or chemokine ligand XCL1 against XCR1	i.v.	↑CD8, ↓growth E.G7 (P)	[128]

P: prophylactic, T: therapeutic, fp: footpad, i.v.: intravenous, s.c.: subcutaneous, i.p.: intraperitoneal, i.m.: intramuscular, EP: electroporated, i.d.: intradermal, i.n.: intranodal, i.t.: intratumoral, U: unknown, CPM: cyclophosphamide iNKT: induced natural killer T cell.

Table 1.4: Vaccine adjuvant properties of EP54 and EP67

Vaccine Construct	Route	Immune Response	Ref
Peptide epitope from MUC1 glycoprotein conjugated to EP54	i.p.	↑IgG2b, IgG2c and IgM Abs	[67]
CTL peptide epitope derived from Hepatitis B surface Antigen (HBsAg) conjugated to EP54	s.c	Ag-specific CD8+ CTL responses against murine P815S target cells	[72]
Nicotine hapten conjugated to EP54	i.p.	Nicotine-specific Abs	[129]
Methamphetamine (meth) hapten conjugated to EP54	s.c /i.p.	Meth-specific Abs in sera	[130]
OVA conjugated to EP67	i.p.	OVA-specific Th1-like Ab class switch and OVA-specific proliferative responses in splenocytes	[75]
OVA conjugated to EP67	i.p.	↑ Ag-specific humoral	[73]
rPrp1,a protein from cell wall of *coccidioides* conjugated to EP67	i.p.	↑ humoral responses compared to alum and CpG	[73]
Peptide epitope derived from gp70 glycoprotein conjugated to EP54 and EP67	s.c	↑ CTL responses and ↓RAW117-H10 growth	[131]
Live spores of attenuated vaccine strain of *Coccidioides posadasii* conjugated to EP67	s.c	↑ IgG1, IgG2a, Th1, and Th17 immune responses	[132]
CTL peptide epitopes from MCMV PP89 and M84 conjugated to EP67	i.n.	↑ CTL responses ↓ MCMV titers	[74]

CHAPTER 2

Hypothesis and Specific Aims

2.1 Hypothesis

Antigen presentation by APCs to T-cells is the bridge between innate and adaptive immune systems and dendritic cells as the most professional antigen presenting cells play a central role in shaping the immune response. Therefore, many strategies have been explored to harness DCs and other APCs to improve vaccine efficacy, of these *in vivo* targeting of APCs using ligands and antibodies against several APC surface receptors has shown promise in preclinical studies. However, most of these studies have focused on PRRs, which recognize foreign antigens, as the targets for antigen delivery and have ignored the complement receptors, which respond to molecules produced by the host complement system. EP67 is a host-derived decapeptide, based on complement component 5a (C5a), that selectively binds to the C5a receptor (C5aR/CD88) on APCs, resulting in their activation and subsequent increase in processing and presentation of conjugated antigen.

The primary objective of this work is to develop C5aR-targeted PLGA nanoparticles to improve immune responses against the encapsulated antigen, using EP67 as the targeting moiety. The central hypothesis of this work is that surface modification of PLGA nanoparticles encapsulating model antigen ovalbumin with EP67 can target the nanoparticles to APCs, and generate efficacious immune responses against the encapsulated antigen. The hypothesis has been tested through the following specific aims.

2.2 Specific Aims

2.2.1 Specific Aim-1 – Develop C5aR-targeted PLGA nanoparticles by first functionalizing the surface of nanoparticles with PLLA-PEG-MAL linker and then conjugating EP67 to the particles.

2.2.2 Specific Aim -2 – To determine the effect of surface modification of PLGA nanoparticles with EP67 on antigen presentation by bone marrow-derived dendritic cells *in vitro*.

2.2.3 Specific Aim – 3 – To determine the efficacy of respiratory immunization with EP67 surface-modified nanoparticles to protect against primary respiratory infection with *Listeria monocytogenes* expressing ovalbumin and to evaluate the immune responses generated.

CHAPTER 3

Materials and Methods

3.1 Peptides

CGRR-EP67 (CGRR-YSFKDMP[MeL]aR) [70] and inactive scrambled CGRR-scEP67 (CGRR-M[MeL]RYKPaFDS) [75] were synthesized by standard Fmoc (9-fluorenyl-methoxycarbonyl) solid-phase methods on a pre-loaded Arg or Ser Wang resin, respectively, by sequential coupling of the HBTU (2-(1Hbenzotriazole-1-yl-1,1,3,3-tetramethyluronium hexafluorophosphate) esters of each amino acid as described [66]. Peptides were cleaved from the resin by acidolysis with TFA (trifluoroacetic acid) containing phenol [5% v/v], water [2% v/v], and triisopropylsilane [2.5% v/v] as scavengers. Cleaved peptides were purified by analytical and preparative reverse-phase HPLC, with C18-bonded silica columns using 0.1% TFA (dH_2O containing TFA [0.1% v/v]) as the running buffer (solvent B) and 0.1% TFA-containing acetonitrile [60% v/v] (solvent B) as the eluent, and lyophilized.

3.2 Encapsulation of ovalbumin in biodegradable surface-modified PLGA nanoparticles

Model antigen ovalbumin (OVA; Sigma-Aldrich) was purified by passing through a Detox-Gel^{TM} endotoxin removal column (Thermo Scientific) and conjugated to fluorescein isothiocyanate (FITC-OVA) before encapsulating in biodegradable maleimide-functionalized nanoparticles (NP) at 10 wt% theoretical loading using a modified double-emulsification / solvent evaporation (ESE) method [133, 134]. The primary water-oil emulsion (W_1/O) was formed by adding an endotoxin-free “water” solution of OVA in PBS (50 mg/mL; 0.2mL) drop-wise to a vortexing (500 RPM)

dichloromethane (DCM) “oil” solution containing ester-terminated 50:50 poly D,L-lactic-co-glycolic acid (PLGA 50:50; research grade; inherent viscosity 0.58 dL/g; Lactel Pelham, AL; 50 mg / mL DCM; 2 mL) in an 8 mL scintillation glass vial and sonicated on ice for 30 sec (pulse time - 10 sec; interval – 2 sec) at 70% amplitude (Misonix Sonicator 3000 w/ model 419 tapered microtip horn with 0.125 in. diameter tip). The W_1/O emulsion was immediately transferred dropwise to a vortexing PVA solution (5% v/v polyvinyl alcohol [70% hydrolyzed; 30,000 -70,000 Da; Sigma-Aldrich] in dH2O; 8 mL) in a 20-mL scintillation glass vial and sonicated to form the secondary water-oil-water emulsion (W_1/O/W_2). The secondary emulsion was then transferred to a fresh 20-mL scintillation glass vial and the NPs were surface-functionalized with maleimide by adding a DCM solution containing the diblock copolymer poly L-lactic acid – polyethylene glycol-maleimide (PLLA-PEG-MAL [10 kDa-2 kDa; Polyscitech]; 20 mg/mL; 200 µL) to the emulsion. The emulsion was stirred (1000 rpm using a cross shaped 9.5 × 4.7 mm, Teflon-coated, smooth stirring bar) for 18 h to fully remove DCM. Hardened NP was pelleted (25,000 RCF, for 15 min at 4°C) then resuspended and pelleted 3X using dH_2O (50 mL) to remove residual PVA. Washed particles were resuspended in dH_2O (10 mL) in a pre-weighed 20-mL scintillation vial, flash frozen in liquid N2, lyophilized for 48 h, and stored at -20°C until further use. Maleimide-functionalized NP were then coated with CGRR-EP67/CGRR-scEP67 by adding peptide solution in PBS (1.4 mg/mL; 0.5 mL) to a suspension of NP in PBS (2 mg/mL; 5 mL) in an 8 mL scintillation glass vial and stirring for 12 h (1000 rpm; cross-shaped 9.5 × 4.7 mm stirring bar). Surface modified NPs were then washed 3X in dH_2O (50

mL), resuspended in dH_2O (10 mL), flash frozen in liquid N2, lyophilized for 48 h, and stored at -20°C after sealing with parafilm.

3.3 OVA loading in PLGA particles

OVA loading was determined as described with modification [135]. Particles (~10 mg) were allowed to equilibrate to r.t., added to DMSO (0.5 mL) in an 8-mL glass vial, and incubated for 1 hr on with constant shaking. A solution of 0.05 M NaOH/0.5% SDS in dH_2O (5 mL) was added to the DMSO/particle solution and the entire solution was stirred (400 RPM) in the capped vial overnight. The 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) with OVA as the standard and DMSO/0.05 N NaOH (0.05% SDS) as the diluent. For scEP67/EP67 surface modified NPs, maleimide-functionalized NPs were stirred for 12 h in PBS (Section 2.1), washed, and lyophilized before determining OVA loading. Average µg OVA/ mg formulation ± SD (n=3) was calculated by multiplying OVA concentration from the assay by the volume of the sample (5.5 mL) and encapsulation efficiency (EE%) was calculated as

$$\mathrm{EE\%} = \frac{Assayed\ \mu g\ OVA\ /mg\ particles}{Theoretical\ \mu g\ OVA\ /mg\ particles}\ x\ 100$$

3.4 Diameter and zeta potential of PLGA nanoparticles

Average hydrodynamic diameters and zeta-potentials ± SD (n=3 independent samples from the same batch) of the particles were measured in dH_2O (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.

3.5 Generation and culture of bone marrow-derived dendritic cells.

Bone marrow-derived dendritic cells (BMDCs) were generated as described [136, 137]. Femurs and tibiae were harvested from the hind limbs and cleaned by scraping the muscle tissue using a sterile surgical scalpel (#15, Aspen Surgical, Caledonia, MI). Bones were sterilized by placing in 70% ethanol for 5 min and rinsed 2X with RPMI-1640 medium. Bone marrow was exposed by cutting off the ends with a sterile scissors and flushed out with complete RPMI (5 mL) (cRPMI; RPMI-1640 [Hyclone, Logan, UT] containing heat-inactivated fetal bovine serum [HI-FBS, 10% v/v, Atlanta Biologicals, Atlanta, GA], L-glutamine [2 mM], sodium pyruvate [1 mM], non-essential amino acids [0.1 mM], MEM vitamin solution [1X], penicillin G [100 U/mL], streptomycin sulfate [100 µg/mL], β-mercaptoethanol [50 µM]) using a 25-gauge needle. Bone marrow pieces were broken down by gently pipetting the media and the resulting cell suspension was passed through a 70 µm cell strainer (Becton Dickinson, Franklin Lakes, NJ). Cells were pelleted (400 RCF;10 min at 4°C), supernatant discarded, and erythrocytes lysed by resuspending in RBC lysis buffer (5 mL, 5 min at RT, RBC lysis buffer: 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Bone marrow cells were then washed 2X with RPMI-1640, and cultured in 100 mm petri dishes (4×10^6 cells/dish) containing BMDC media (cRPMI supplemented with rmGM-CSF [20 ng/mL, Peprotech, Rocky Hill, NJ]). On day 3, fresh BMDC media (10 mL) was added to the dishes. On day 6,six half of the media was collected, cells were

pelleted, resuspended in BMDC media (10 mL) and added back to the dishes. BMDCs were collected on day 8 and used for further experiments.

3.6 Internalization of nanoparticles by BMDCs

Internalization of NPs by BMDCs was measured by culturing BMDCs (4 x 10^5 cells/well; 24-well plate) in cRPMI (1 mL) containing FITC-OVA encapsulated, uncoated NP [NP(FITC-OVA); 20 µg], FITC-OVA encapsulated NP surface modified with scrambled EP67 [scEP67-NP(FITC-OVA); 20 µg] or EP67 [EP67-NP(FITC-OVA); 20 µg] for 2 h at 37 ^oC/5% CO_2. To control for adsorption of NP onto the cell surface, BMDCs were allowed to equilibrate at 4 ^oC for one h, then treated with NP and incubated for 2 h at 4 ^oC. Cells were washed 3X in PBS (1 mL), stained with Zombie Yellow, anti-mouse CD11c-PE Cy5 (clone N418, eBioscience, San Diego, CA) and analyzed by flow cytometry.

3.7 Activation of BMDCs

Activation of BMDCs by NPs was measured by culturing BMDCs (4 x 10^5 cells/well; 24-well plate) in cRPMI (1 mL) containing NP(OVA) [100 µg], scEP67-NP(OVA) [100 µg] or EP67-NP(OVA) [100 µg] for 24 h at 37 ^oC/5% CO_2. Cells were then washed 3X in PBS (1 mL), resuspended in sterile PBS (0.1 mL) containing Zombie Yellow (1 µL, BioLegend), incubated at r.t. for 20 min in the dark, FACS Stain Buffer (0.1 mL) was then added and cells were pelleted (500 RCF, 4°C, 5 min.). Supernatants were aspirated and Fc receptors were blocked by resuspending 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 then 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.). Cells were then fixed by resuspending cells in Fixation Buffer (0.1 mL/well; BioLegend), and incubating on ice for 20 min. Cells were then prepared for flow cytometry by washing in FACS Stain Buffer (0.2 ml) (3X).

Cells were analyzed on a BD LSR II flow cytometer (Becton and Dickinson, La Jolla, CA) with BD High Throughput Sampler. Flow cytometer was compensated using single-stained cells, and thea maximum number of events were acquired and analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

3.8 Detection of antigen presentation by BMDCs

Antigen presentation of OVA epitopes ($OVA_{257–264}$ /SIINFEKL – H-$2K^b$ peptide ligand; $OVA_{323-339}$ /I-A^b peptide ligand) by BMDCs was measured by T-cell hybridoma assay using OVA responsive T-cell hybridomas CD8OVA1.3 ($OVA_{257–264}$) and DOBW ($OVA_{323-339}$) (kindly donated by C.V Harding, Case Western Reserve University) [138, 139]. Day 8 BMDCs (4 x 10^5 cells/well; 24-well plate) were cultured in cRPMI (1 mL) containing NP(OVA) [100 µg], scEP67-NP(OVA) [100 µg] or EP67-NP(OVA) [100 µg] for 2 h at 37 ^oC/5% CO_2. BMDCs were then washed 2X in PBS (1 mL) and co-cultured

with equal number of CD8OVA1.3 or DOBW cells (5 x 10^4 cells/well; 96-well plate) in D10F media (0.2 mL) (DMEM/High Glucose [Hyclone, Logan, UT] containing HI-FBS [10% v/v], penicillin/streptomycin [100 U/ml], sodium pyruvate [1 mM], HEPES [10 mM], and 2-mercaptoethanol [0.5 mM]) for 18 h at 37 ^oC/5% CO_2[140]. Cells were then pelleted (2000 RCF; 10 min; 4 ^oC), and supernatants collected and stored at -80 ^oC until analyzed for secreted IL-2 by ELISA (Mouse IL-2 ELISA MAX™ Standard, BioLegend) per manufacturer's instructions.

3.9 Respiratory Immunization

All animal procedures were approved by University of Nebraska Medical Center Institutional Animal Care and Use Committee. Naïve female C57BL6/NHsd mice (4 weeks old, Envigo) were housed under pathogen-free conditions and allowed to acclimatize for at least one week before experiments. Animals were immunized with sterile PBS (50 µL) or sterile PBS containing NP-OVA or EP67 NP-OVA (25 µg OVA equivalent) on days 0 and seven by intranasal instillation, which is expected to deliver the vaccine to entire respiratory tract and lungs[141, 142]. For intranasal instillation, mice were anesthetized with Ketamine/Xylazine cocktail (105/5 mg/kg, I.P), held upright and the vaccine was administered by both nostrils using a 200 µL micropipette.

3.10 LM-OVA respiratory challenge and quantitation of bacterial burden.

Recombinant *Listeria monocytogenes* expressing ovalbumin (LM-OVA) (kindly donated by John T. Harty, University of Iowa) [143] was cultured in Tryptic soy broth (Sigma-Aldrich) containing streptomycin sulfate (50 µg/mL) at 37 ^oC in a shaking incubator (150 RPM) for 24 h. Sterile glycerol (1 mL; 80% v/v) was added to LM-OVA

culture (9 mL), aliquoted into cryovials (1 mL) and stored at -80 ^{o}C until the day of infection. LM-OVA titers in one of the frozen stocks were determined one day before immunization.

At fourteen days post-immunization (Day 21), animals were infected with a sub-lethal dose of LM-OVA (2×10^7 CFU) in the same method as vaccines. Three days post-infection (Day 24) animals were sacrificed and the organs were harvested (lungs, liver and spleen). Bacterial burden in the organs was determined by homogenizing the organs in cRPMI (1 mL) using a hand-held homogenizer and 10-fold serial dilutions were prepared using D-PBS as diluent. The dilutions (20 µL) were pipetted onto a TSB-agar plate (100 mm; MIDSCI), the plates were allowed to dry in a sterile hood and incubated for 24 h at 37 ^{o}C[144]. The number of bacterial colonies was then counted by eye. Average LM-OVA titers/g of tissue was calculated as

$$\frac{LM-OVA\ CFU}{g} = number\ of\ colonies \times DF \times \frac{volume\ of\ homogenate\ (mL)}{sample\ volume\ (mL) \times organ\ weight}$$

Where the *number of colonies* was taken from the highest dilution where 5 to 50 colonies were observed, *DF* was the selected dilution factor, the *sample volume* is the volume plated (0.02 mL) and volume of homogenate = [volume of cRPMI (1 mL) + organ weight].

3.11 Preparation of lung lymphocytes and splenocytes

Fourteen days' post-immunization (Day 21), on the same day as respiratory challenge with LM-OVA, mice were euthanized, and the lungs were perfused with sterile DPBS (5 mL) via right ventricle of the heart and harvested. Lungs were then

dissected into small pieces using a sterile scissors and transferred to a sterile C-Tube (Miltenyi) containing cRPMI (5 mL) with collagenase IV (2 mg/mL; Worthington Enzymes). Lungs were then homogenized using Miltenyi tissue dissociator ("m_lung_01" setting) and incubated for 1 h at 37° C in a shaking incubator (200 RPM, Vortemp). Lungs were homogenized again on the tissue dissociator ("m_lung_01" setting) and a single cell suspension was obtained by passing the cells through a sterile 40 µm cell strainer. Cells were then pelleted (400 RCF, 4°C, 5 min), supernatant decanted and resuspended in cRPMI (5 mL) in a sterile 15 mL conical tube (BD Falcon). Lympholyte-M solution (5 mL; Cedarlane Labs) was under-layed below the cell suspension using a sterile Pasteur pipette and centrifuged (1500 RCF, 20 mins, RT, no brakes) to separate lymphocytes. Lymphocytes at the interphase were collected, washed 1X with sterile PBS, resuspended in cRPMI and stored on ice until used.

To prepare single cell suspensions of splenocytes, spleens were first homogenized using a tissue dissociator ("m_spleen_01" setting) in cRPMI (5 mL), passed through a 70 µm cell strainer and diluted with sterile DPBS (45 mL). Cells were then pelleted (500 RCF, 4°C, 10 min), the supernatant was decanted, and the cells were resuspended in RBC lysis buffer (4 mL) and incubated for 5-7 min at RT. Cells were then passed through a 40 µm cell strainer, washed twice with D-PBS (50 mL), resuspended in cRPMI and stored on ice until used. Percent viability and total live cell counts were determined by trypan blue exclusion using a Cellometer Auto T4 cell counter (Nexcelom Biosciences).

3.12 Surface phenotyping of antigen-specific T-cells

The surface phenotype of antigen specific T-cells was determined at 14 days post-immunization (Day 21) by flow cytometry. Lung lymphocytes and splenocytes were isolated and plated in 96-well plates (10^6 cells/0.1 mL/well). Cells were washed twice by pelleting (400 RCF, 4°C, 5 min.) and resuspending in DPBS (0.2 mL). Dead cells were then stained by incubating the cells in DPBS (0.1 mL) containing Zombie NIR dye (1 µL/10^6 cells), and the cells were incubated for 20 min at RT in dark. Cells were then washed once with BD Stain Buffer (0.1 mL) and Fc receptors were blocked by incubating in BD Stain Buffer (0.1 mL) containing mouse BD Fc Block (1µg / 10^6 cells) and incubating on ice for 20 min. Cells were washed with BD Stain Buffer (0.1 mL) and stained with OVA tetramers by incubating with BD Stain Buffer (50 µL) containing MHC Class-I Tetramers-BV421 (NIH Tetramer core - H-2Kb / SIINFEKL) or MHC Class-II Tetramers-BV421 (NIH Tetramer core-I-Ab / HAAHAEINEA) (1 µg/ 10^6 cells) for 30 mins at 37 ^oC in the dark. Cells were then washed with BD Stain Buffer (0.15 mL) and stained with cell surface markers by incubating 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 flour 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) on ice for 30 min in the dark. Cells were then washed in BD Stain Buffer (0.15 mL) and fixed by incubating with Fixation Buffer (0.1 mL) on ice for 20 min. Cells were then washed twice with BD Stain Buffer (0.2 mL), resuspended in BD Stain Buffer (0.2 mL) and

analyzed on a BD LSR-II flow cytometer. 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).

3.13 Quantitation of cytokine secretion by epitope responsive splenocytes

The cytokine secretion profile of splenocytes harvested 14 days post-immunization was determined by re-stimulating cells (2×10^6 cells) *ex vivo* by incubating with cRPMI (0.4 mL) containing OVA I-A^b epitope (ISQAVHAAHAEINEAGR; 10 µg/mL) in a 48-well plate for 48 h at 37 ^oC/5% CO_2. Supernatants were collected and stored in -80 ^oC freezer until analyzed, using a multiplex assay (Mouse Th17 Magnetic Bead Panel, Millipore) per manufacturer's instructions. The concentration of cytokines was measured as median fluorescence intensity (MFI) of at least 50 beads per cytokine. Standards were measured in duplicates for seven concentrations including a blank control. Standard curves for each cytokine were plotted using a five-parameter logistic curve fit (www.myassays.com).

CHAPTER 4

RESULTS

Encapsulation of protective proteins or peptides in nanoparticles composed of biodegradable poly (lactide-co-glycolic acid) polymers is a well-established platform to improve efficacy of subunit vaccines by (i) protecting vaccines from enzymatic degradation, (ii) increasing residence time at administration site, (iii) facilitating uptake by APCs because of their particulate nature and, (iv) acting as an adjuvant that can activate APCs. Moreover, the surface of nanoparticles can also be modified with various ligands or antibodies that can target APCs to further improve vaccine efficacy. Several studies have demonstrated that modifying the surface of nanoparticles with ligands or antibodies targeting intracellular and extracellular pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), C-type lectin receptors (CRRs) and NOD-like receptors (NLRs) can induce humoral and cell-mediated immune responses against encapsulated antigens.

In our previous studies, we have demonstrated that EP67, a host-derived decapeptide based on complement component 5a (C5a) of the innate immune system, acts as an immunostimulant and an adjuvant capable of generating Th1 biased humoral and cellular immune responses in mice against a covalently conjugated peptide, protein or inactivated pathogens upon systemic administration. In our recent studies, we have also shown that respiratory immunization with an EP67-based vaccine composed of protective CD8+ T-cell epitopes from murine cytomegalovirus (MCMV) protects against primary respiratory infection with MCMV and increases the proportions of epitope-specific 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, suggesting that EP67 can act as a mucosal adjuvant.

EP67 is expected to act as an adjuvant, in large part, by selectively binding to the C5a receptor (C5aR/CD88) on APCs, resulting in their activation and subsequent increase in processing and presentation of conjugated antigen. Thus, we hypothesized that surface modification of biodegradable PLGA nanoparticles with EP67 would increase the efficacy of immune responses against the encapsulated antigen. To test this hypothesis, we encapsulated a model antigen ovalbumin (OVA) in biodegradable PLGA nanoparticles, modified the surface of nanoparticles with EP67 using the interfacial activity assisted surface functionalization (IAASF) technique. We then compared the extent to which respiratory immunization with OVA encapsulated in the EP67 surface-modified, unmodified nanoparticles or vehicle alone protects naïve female C57Bl/6 mice against primary respiratory infection with recombinant *Listeria monocytogenes* expressing soluble OVA (LM-OVA) and affects the generation of systemic and mucosal cellular responses. Additionally, we have also tested the effect that EP67 surface modification of nanoparticles has on their internalization and activation of murine bone marrow-derived dendritic cells (BMDCs) *in vitro*.

4.1 Surface modification of PLGA nanoparticles with EP67 improves internalization by BMDCs.

Presentation of exogenous antigen to T cells requires the uptake of antigen by antigen-presenting cells followed by processing and presentation in the context of

MHC molecules [145, 146]. T-cell activation and memory development have also been linked to the dose of antigen-experienced by dendritic cells [147, 148]. Therefore, internalization of antigen by dendritic cells is an important parameter that affects the efficacy of vaccines.

To determine if the surface modification of PLGA nanoparticles with EP67 affects the internalization of the NPs by murine BMDCs, we first encapsulated FITC-labeled OVA (FITC-OVA) in PLGA NPs and modified the surface with reactive maleimide groups by incorporating the diblock copolymer PLLA-PEG-MAL into the NPs using IAASF technique (Table 4.1) (Fig 4.1A)[134]. Surface modification with PLLA-PEG-MAL was confirmed by the presence of an ethylene glycol peak (3.8 ppm) in the ^{1}H-NMR NMR (Fig. 4.2). We then attached EP67 to the NPs using a Cys-Gly-Arg-Arg linker (PLGA NP-MAL-CGRR-EP67) (Fig 4.1B), and EP67 attachment to NPs was then confirmed by amino acid analysis (Fig. 4.3). We next incubated murine BMDCs (Day 8) with unmodified [NP(FITC-OVA)], scrambled EP67 (CGRR-M[MeL]RYKPaFDS)-modified [scEP67-NP(FITC-OVA)], and EP67- modified [EP67-NP(FITC-OVA)] nanoparticles for 2 h and compared the internalization by measuring the proportion of BMDCs positive for FITC-OVA using flow cytometry. Internalization of nanoparticles was significantly higher for EP67-modified (52%) when compared to unmodified (40%; $p \leq 0.05$) or scEP67-modified (38%; $p \leq 0.01$) NPs (Fig. 4.5E). The MFI of FITC in BMDCs treated at 37 ^{o}C was significantly higher for EP67-modified (4953 AU) when compared to unmodified (2472; $p \leq 0.01$) or scEP67-modified (1918 AU; $p \leq 0.01$) NPs. To rule out false positives due to surface adsorption of NPs, we also compared NP internalization and MFI of FITC in BMDCs treated with NPs at 4 ^{o}C (Figs

4.5B, C). In contrast to treatment at 37 ^{o}C, there was no difference between the groups when BMDCs were treated at 4 ^{o}C. Thus, surface modification with EP67 increases the internalization of nanoparticles by BMDCs.

4.2 Surface modification of nanoparticles with EP67 activates BMDCs and improves antigen presentation to T-cells.

Although the level of antigen uptake is an important criterion, it is not enough to induce T-cell activation. For generating effective immune responses, it is necessary that the antigen is presented to T-cells by activated antigen-presenting cells that have upregulated expression of co-stimulatory molecules [145, 149]. Antigen presentation in the absence of co-stimulation leads to T-cell anergy and induces antigen tolerance [24, 150], an undesirable outcome for vaccination.

To determine if the surface modification of nanoparticles activates BMDCs, we treated immature murine BMDCs with NP(OVA), scEP67-NP(OVA) and EP67-NP(OVA) for 24 h and compared the surface expression levels of BMDC activation markers CD80, CD86 and MHC II by flow cytometry (Fig. 4.6). The expression of activation markers, as measured by MFI, was significantly higher on BMDCs treated with EP67-NP(OVA) [CD80 – 43306 AU, CD86 – 1221 AU, MHC-II – 26491 AU] when compared to BMDCs treated with NP(OVA) [CD80 – 19441 AU ($p \leq 0.01$), CD86 – 792 AU ($p \leq 0.01$), MHC II – 15295 AU ($p \leq 0.01$)], and scEP67-NP(OVA) [CD80 – 30607 AU ($p \leq 0.001$), CD86 – 812 AU ($p \leq 0.01$), MHC-II – 20806 AU ($p \leq 0.001$)]. Thus, surface modification of nanoparticles with EP67 activates BMDCs better than unmodified and scEP67-modified nanoparticles.

To determine if the activation of BMDCs by surface-modified nanoparticles translates into increased antigen cross-presentation/presentation via the MHC I/II pathways, we co-incubated nanoparticle-treated BMDCs with OVA-responsive T-cell hybridomas CD8OVA1.3 (MHC-I/$OVA_{257–264}$) and DOBW (MHC-II/$OVA_{323-339}$) for 18 h and compared the concentrations of IL-2 in the culture supernatant by ELISA. IL-2 is secreted by T-cells upon T-cell receptor ligation by cognate peptide-MHC complexes [151] on activated APCs, and therefore can be used as a surrogate for antigen presentation [152]. The concentration of IL-2 was significantly higher in the supernatants of DOBW cells (Fig. 4.7A) when co-incubated with EP67-NP(OVA)-treated BMDCs [EP67-NP(OVA) -770.47 pg/mL; NP(OVA) -510.46 pg/mL ($p \leq 0.001$); scEP67-NP(OVA) -460.41 pg/mL ($p \leq 0.001$)]. Similarly, the IL-2 concentrations in CD8OVA1.3 cell supernatant (Fig. 4.7B) co-incubated with EP67-NP(OVA)-treated BMDCs [96.34 pg/mL] was significantly higher when compared to CD8OVA1.3 cells co-incubated with NP(OVA)- [59.90 pg/mL ($p \leq 0.001$)] and scEP67-NP(OVA)- [51.22 pg/mL ($p \leq 0.001$)] treated BMDCs. Thus, BMDCs treated with nanoparticles surface-modified with EP67 were significantly better at antigen cross-presentation/presentation when compared to unmodified or scEP67-modified nanoparticles.

4.3 Immunization with EP67 surface-modified OVA-encapsulated PLGA NPs increases protection against primary mucosal infection with LM-OVA.

In our earlier studies, we found that EP67, when conjugated to a CD8+ T-cell epitope, can be used as a mucosal adjuvant to improve protection against respiratory

infection with murine cytomegalovirus (MCMV) [74] and also that encapsulation of EP67-conjugated CTL epitope in biodegradable nano- and micro- particles improves protection against MCMV when compared to free vaccine [153]. However, the effect of conjugating EP67 onto the surface of biodegradable NPs on the immune responses generated against encapsulated antigen is not known.

Recombinant *Listeria monocytogenes* expressing ovalbumin (LM-OVA) has been routinely utilized as a model pathogen to study the efficacy of novel immunization strategies using ovalbumin as a model antigen [154, 155]. Although the natural route of infection for *Listeria monocytogenes* is the oral route, mice were demonstrated to be susceptible to intranasal challenge with peak bacterial titers in lungs, liver and spleen on day 3[156]. Thus, the respiratory challenge with LM-OVA can be used as a model for testing efficacy of mucosal immune responses generated by immunization.

To determine if surface modification of PLGA NPs with EP67 affects the efficacy of immune response against the encapsulated antigen, we administered 6-week old female C57Bl/6 mice with vehicle alone (PBS), encapsulated OVA NPs (NP(OVA)) and encapsulated OVA NPs surface-modified with EP67 (EP67-NP(OVA)) in a volume (50 µL) which is expected to reach the total respiratory tract, including the lungs [141] (Fig. 5A). We then infected the mice 14 days' post-immunization with sub-lethal dose[156] (2×10^7 CFU/ 50µL) of *Listeria monocytogenes* expressing OVA (LM-OVA) and compared peak bacterial titers in lungs, liver and spleen three days post-infection[156] (Fig. 4.8A).

LM-OVA titers in mice immunized with EP67-NP(OVA) [2.751×10^8 CFU/g] in lungs (Fig. 4.8B) were significantly lower when compared to NP(OVA)- [1.275×10^9 CFU/g; P = 0.0122] and PBS- [1.528×10^9 CFU/g; P = 0.0026] treated mice. In liver (Fig. 4.8C) the titers in EP67-NP(OVA)- [2.724×10^6 CFU/g] treated mice were significantly lower than in NP(OVA)- [1.430×10^8 CFU/g; P = 0.0099] treated mice and no statistical difference when compared to PBS- [1.015×10^8 CFU/g; P = 0.0573] treated mice. Similarly, in spleen (Fig. 4.8D), titers in EP67-NP(OVA) [2.529×10^7 CFU/g] treated mice were significantly lower than in NP(OVA) [1.738×10^8 CFU/g; P = 0.0028] treated mice; whereas there was no statistical difference when compared to PBS- [9.242×10^7 CFU/g; P = 0.1356] treated mice. Thus, immunization with EP67 surface-modified nanoparticles increased the efficacy of immune response against the encapsulated antigen.

4.4 Respiratory immunization with EP67 surface-modified OVA encapsulated PLGA NPs increases the magnitude of mucosal and systemic antigen-specific T-cells.

Effective protection against viral and intracellular bacterial infection requires potent pathogen-specific CTL responses[14, 157]. Resistance to intravenous challenge with LM-OVA, an intracellular pathogen, has been demonstrated to be primarily due to OVA-specific CD8+ T-cells but not antigen-specific humoral responses. Given that respiratory immunization with EP67-NP(OVA) increased protection against respiratory challenge with LM-OVA, it is expected that respiratory

immunization with EP67-NP(OVA) would increase the proportions of OVA-specific systemic and mucosal T-cells.

To determine if respiratory immunization with EP67-NP(OVA) increases the magnitude of OVA-specific T cell responses, we again administered mice with vehicle alone (PBS), encapsulated OVA NPs (NP(OVA)) and encapsulated OVA NPs surface-modified with EP67 (EP67-NP(OVA)) under the same dosage regimen (Fig. 5A). We then compared the proportions of OVA-specific activated T cells, on the day of infection (14 days post immunization), in lungs and spleen by staining with OVA-specific tetramers for CD4 (I-A^b / HAAHAEINEA) and CD8 (H-$2K^b$ / SIINFEKL) and analyzing by flow cytometry. Immunization with EP67-NP(OVA) generated a higher proportion of CD4+$CD44^{hi}$tet+ cells in lungs (Fig. 4.9A) and spleen (Fig. 4.9B) by about 0.5% and 2% over NP(OVA)- or PBS-treated animals. Furthermore, EP67-NP(OVA) generated a higher proportion of CD8+$CD44^{hi}$tet+ cells than NP(OVA) or PBS in lungs (Fig. 4.9C) by 0.2% and in spleen (Fig. 4.9D) by 0.8%. Thus, respiratory immunization with EP67-NP(OVA) increases the proportion of systemic and mucosal antigen-specific T-cells.

4.5 Respiratory immunization with EP67 surface-modified OVA-encapsulated NPs affects CD127/KLRG1 memory subsets of systemic antigen-specific CD8+ and CD4+ T-cells.

Effective immunization against a pathogen requires the generation of a stable pool of long-lived antigen-specific memory T-cells which can respond quickly to protect against infection [158]. It is possible to identify memory precursor cells with the

potential to become long-lived memory cells during the peak of T-cell response generated post-immunization by analyzing the cell surface expression of IL-7Rα (CD127) and KLRG1 [159, 160]. Based on CD127 and KLRG1 expression, T-cells can be classified into early effector cells (EEC - $CD127^{-}KLRG1^{-}$), memory precursor effector cells (MPEC - $CD127^{+}KLRG1^{-}$), short-lived effector cells (SLEC - $CD127^{-}KLRG1^{+}$) and, double positive effector cells (DPEC - $CD127^{+}KLRG1^{+}$)[160, 161]. Although studies have shown that all the subsets have similar potential to clear pathogens, only EECs and MPECs have the potential to become long-lived memory cells[161].

To determine if respiratory immunization with EP67-NP(OVA) affects CD127/KLRG1 memory subsets of systemic antigen-specific CD4+ and CD8+ T-cells, we compared the proportions of CD127/KLRG1 subsets within the CD4+CD44+tet+ and CD8+CD44+tet+ cells generated in the spleens using flow cytometry. EP67-NP(OVA) generated higher proportions of SLEC (CD4 - ~16% / CD8 - ~13%), DPEC (CD4 - ~3% / CD8 - ~0.8%) and MPEC (CD4 - 5% / CD8 - ~38%) compared to NP(OVA) (Fig. 4.10). On the other hand, the proportion of EEC was found to be higher in NP(OVA) by ~23% in CD4+ cells and ~42% in CD8+ cells. Thus, surface modification of PLGA NPs with EP67 affects the CD127/KLRG1 memory subsets of systemic CD4+ and CD8+ T-cells.

4.6 Respiratory immunization with EP67 surface-modified OVA-encapsulated NPs affects CD127/KLRG1/CD62L memory subsets of systemic antigen-specific CD8+ and CD4+ T-cells.

Activated antigen-specific CD44+ T-cells can also be classified into functionally heterogeneous memory subsets based on the cell surface expression of CD127, KLRG1, and lymph node homing receptor CD62L [162]. SLECs (SLEC– $CD127^{-}$ $KLRG1^{-}CD62L^{-}$) have high cytolytic potential *in vitro*, but lack long-term survival. Effector memory precursor cells (T_{EM} MPEC- $CD127^{+}KLRG1^{-}CD62L^{-}$) demonstrate both cytolytic activity and long-term survival, whereas central memory precursor cells (T_{CM} MPEC- $CD127^{+}KLRG1^{-}CD62L^{+}$) have low cytolytic activity but have long-term survival and high homeostatic proliferation.

To determine if respiratory immunization with EP67-NP(OVA) affects CD127/KLRG1/CD62L memory subsets of systemic antigen-specific CD4+ and CD8+ T-cells, we compared the proportions of CD127/KLRG1/CD62L subsets within the CD4+CD44+tet+ and CD8+CD44+tet+ cells generated in the spleens using flow cytometry. EP67-NP(OVA) generated higher proportions of T_{EM} MPEC (CD4 - ~9% / CD8 - ~28%), and T_{CM} MPEC (CD4 – 0.3% / CD8 - ~2%) compared to NP(OVA) (Fig. 4.11). In contrast, the proportion of SLEC was found to be higher in NP(OVA) by ~8% in CD4+ cells and ~2% in CD8+ cells. Thus, surface modification of PLGA NPs with EP67 affects the CD127/CD62L memory subsets of systemic CD4+ and CD8+ T-cells.

4.7 Respiratory immunization with EP67 surface-modified OVA encapsulated NPs affects cytokine secretion profile of epitope responsive systemic T-cells.

To determine the effect of respiratory immunization with EP67-NP(OVA) on the cytokine secretion profile of epitope responsive T-cells, we *ex vivo* restimulated the splenocytes harvested 14 days post-immunization with the immunodominant OVA CD4 epitope (ISQAVHAAHAEINEAGR) for 48 h and analyzed the supernatants for Th1 (IL-2, IL-6) (Fig. 4.12A), Th17 (IL-17A, IL-22) (Fig. 4.12B) and Th2 (IL4, IL-5) (Fig. 4.12C) cytokines. Splenocytes isolated from EP67-NP(OVA) treated mice produced significantly higher quantities of Th1 (~100%) and Th17 (~300%) cytokines when compared to both PBS- and NP(OVA)-treated mice, whereas no difference was found in the production of Th2 type cytokines. Thus, respiratory immunization with EP67-NP(OVA) affects the cytokine secretion profile of CD4 epitope responsive T-cells and shifts it towards Th1 and Th17 type responses.

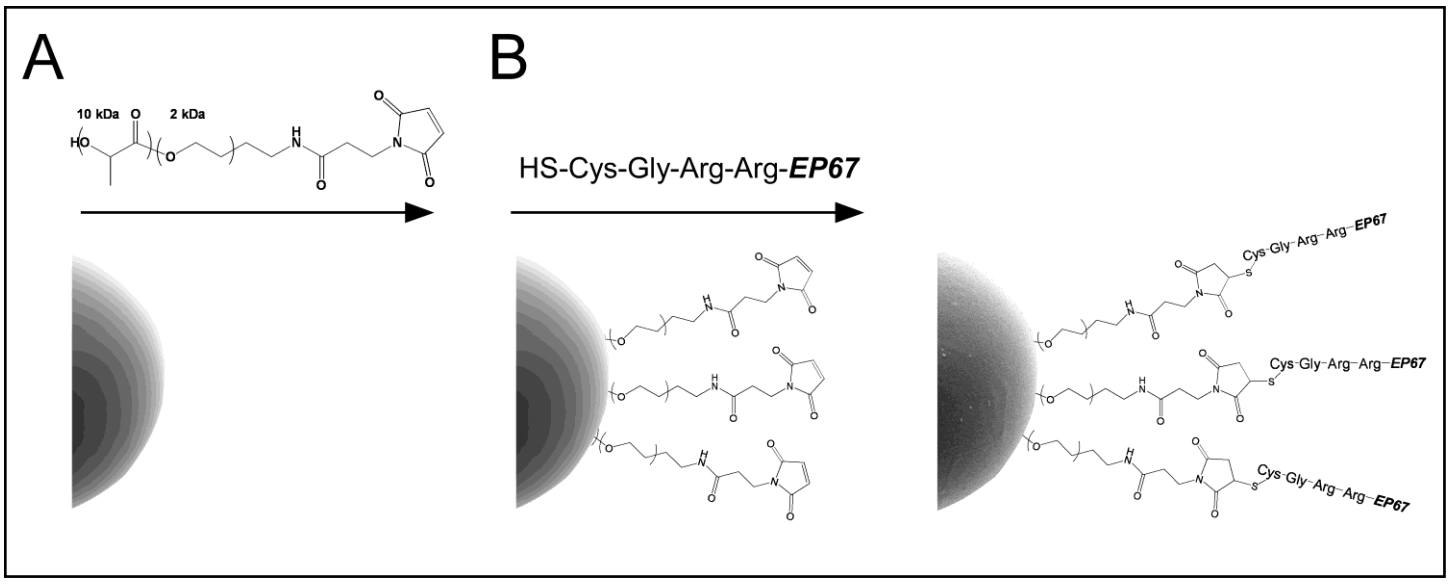

Figure 4.12 Synthetic strategy for modifying the surface of PLGA 50:50 nanoparticles with EP67

Endotoxin-free ovalbumin (OVA) was encapsulated in PLGA 50:50 nanoparticles by the W/O/W emulsification / solvent extraction method. (A) Maleimide-activated 2 kDa PEG linkers were added to the nanosphere surfaces during OVA encapsulation as part of PLLA(10 kDa)-b-PEG(2 kDa)-maleimide diblock copolymers that were physically partitioned into nascent PLGA 50:50 nanospheres before complete solvent extraction and subsequent lyophilization. The extent that the surfaces of lyophilized particles were functionalized with maleimide was determined by 1H-NMR (Fig.4.2). (B) EP67 was activated with sulfhydryl groups by the addition of N-terminal Cys through a Gly-Arg-Arg-linker and reacted with lyophilized maleimide-activated nanospheres resuspended in PBS [pH 7.4]. The extent that the surfaces of lyophilized particles were modified with EP67 was determined by amino-acid analysis (Fig.4.3).

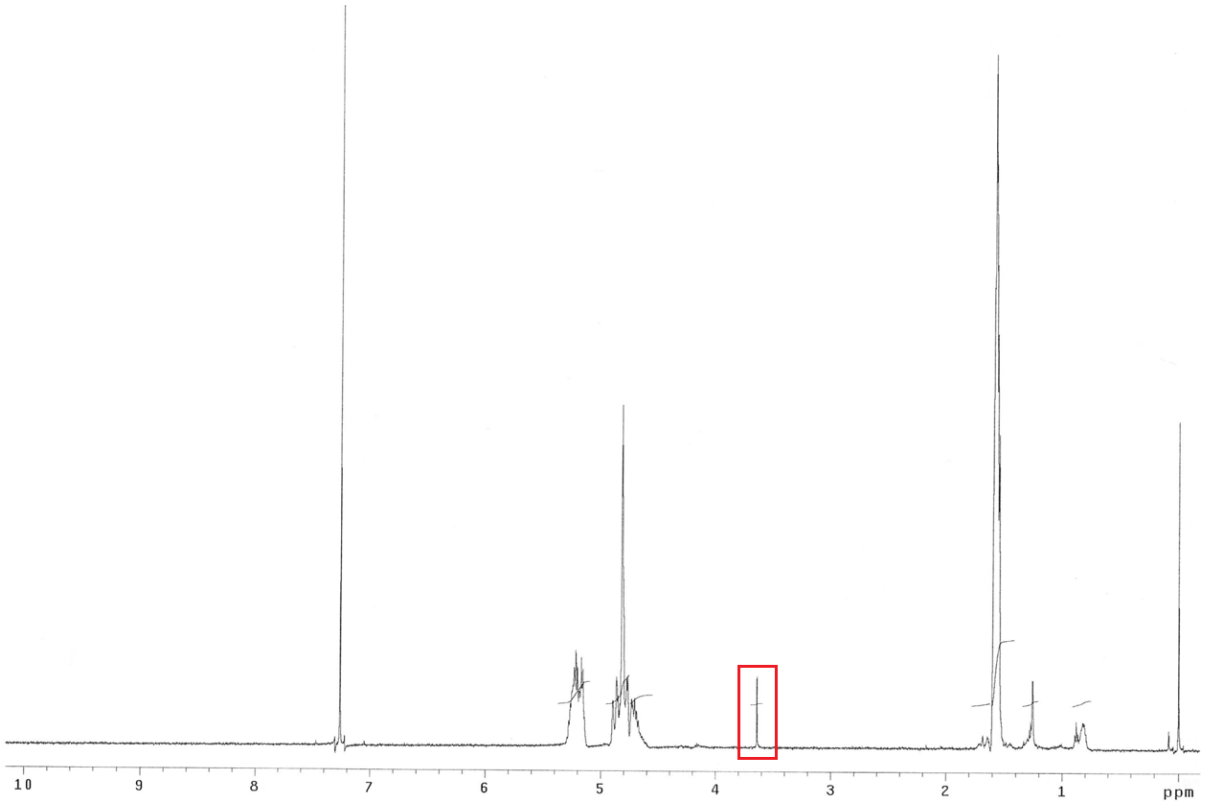

Figure 4.2 Surface functionalization of PLGA NPs with PLLA-PEG-MAL was confirmed by the presence of PEG peak in the 1H-NMR spectra of the NPs

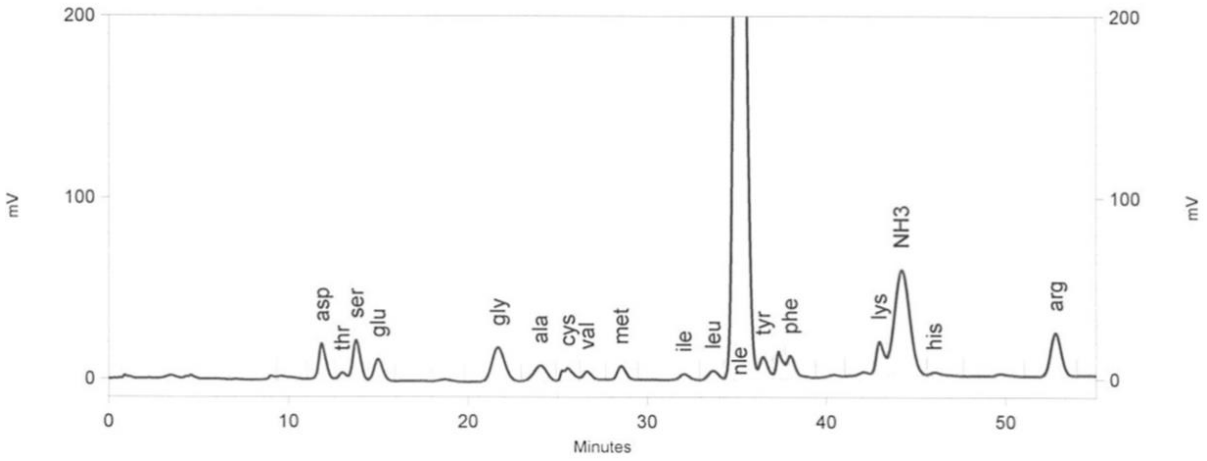

Figure 4.3 Coating of maleimide functionalized NPs with EP67 was confirmed by amino acid analysis of blank PLGA-EP67 NPs.

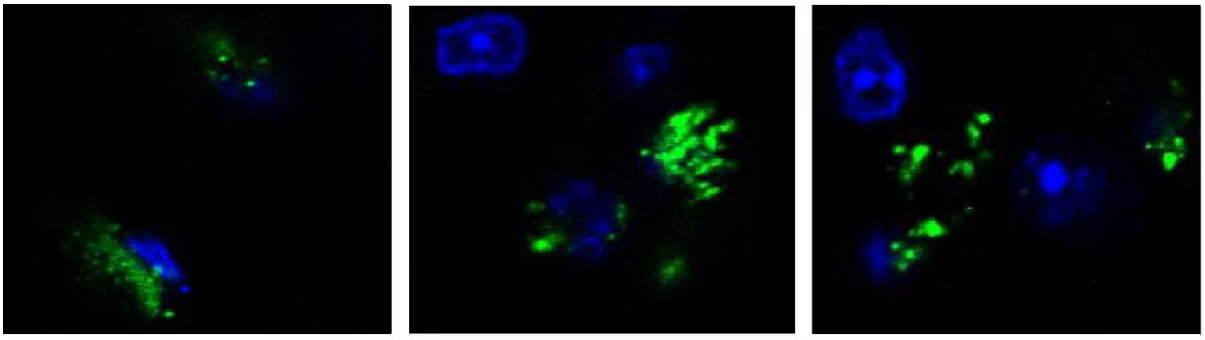

Figure 4.4 Internalization of FITC-OVA loaded NPs by BMDCs.

NP(FITC-OVA), scEP67-NP(FITC-OVA) and EP67-NP(FITC-OVA) were incubated with BMDCs on a cover slip placed inside a 24 well plate for 1h, washed 3X with PBS, nuclei stained with DAPI and analyzed by confocal microscopy.

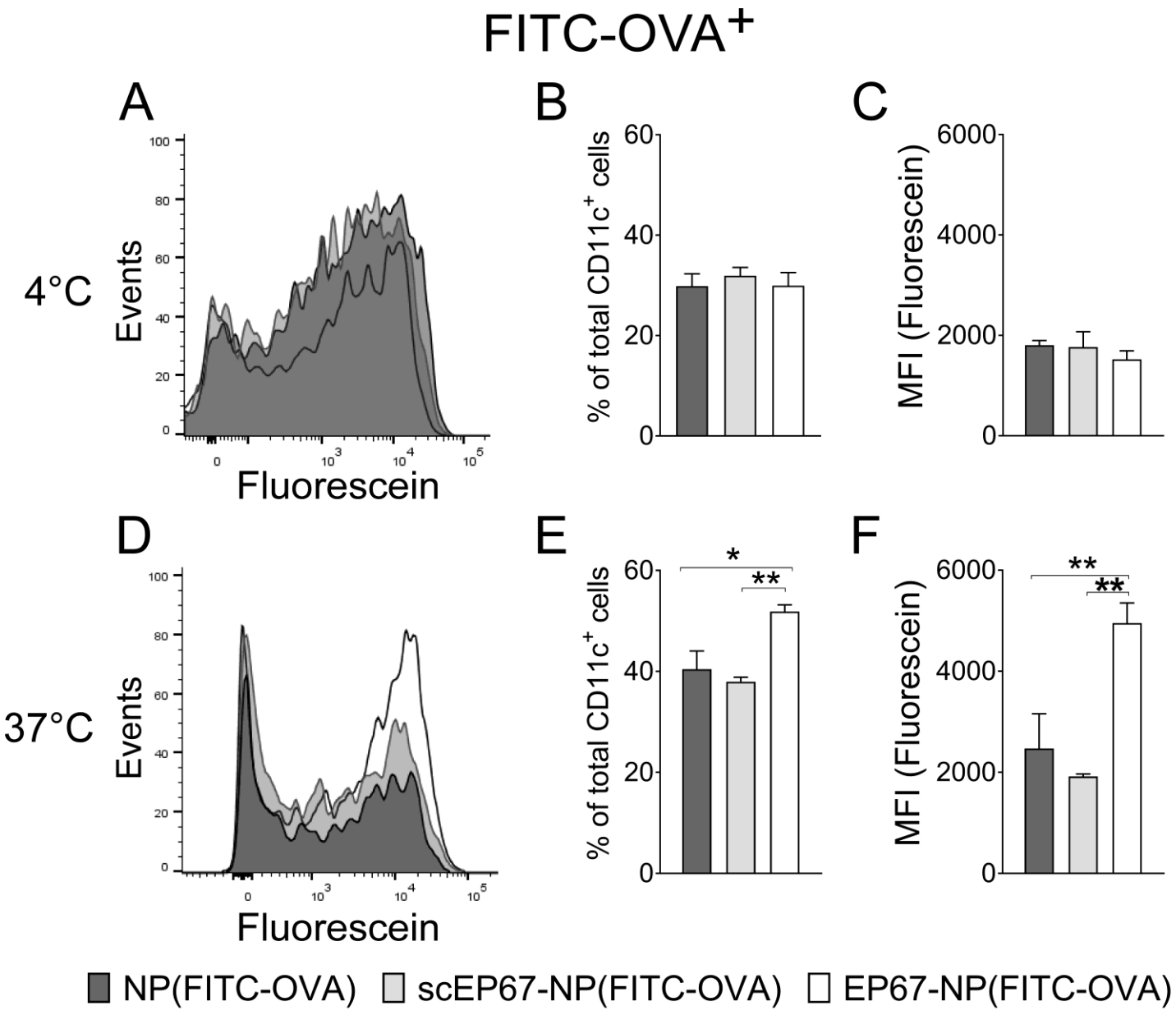

Figure 4.13. Modifying the surface of PLGA 50:50 nanoparticles with EP67 increases internalization by immature murine bone marrow-derived DCs.

Immature BMDCs (male C57BL/6) were incubated at (A, B, C) 4˚C or (D, E, F) 37°C for 2 h with ~1.5 µg of endotoxin-free fluorescein-modified OVA (FITC-OVA) loaded in ~20 µg of unmodified PLGA 50:50 nanoparticles [NP(FITC-OVA), dark grey bars], PLGA 50:50 nanoparticles surface-modified with inactive scEP67 [scEP67-NP(FITC-OVA), grey bars], or PLGA 50:50 nanoparticles surface-modified with EP67 [white

bars, EP67-NP(FITC-OVA)] as described in Fig 4.1. Cells were rinsed with PBS, stained with viability dye and PE-Cy5 anti-CD11c antibodies, then analyzed by flow cytometry. Representative FACS data of median fluorescein staining EVENTS from viable $CD11c^+$ BMDCs incubated with the indicated nanoparticles for 2 h at (A) 4°C or (D) 37°C. The average percent of total viable $CD11c^+$ BMDCs at (B) 4°C or (E) 37°C that were FITC-OVA^+ ±SD (n=2 wells) and the average median fluorescence intensities (MFI) of fluorescein staining of live $CD11c^+$FITC-OVA^+ BMDCs at (C) 4°C or (F) 37°C ±SD (n=2) 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 three independent experiments.

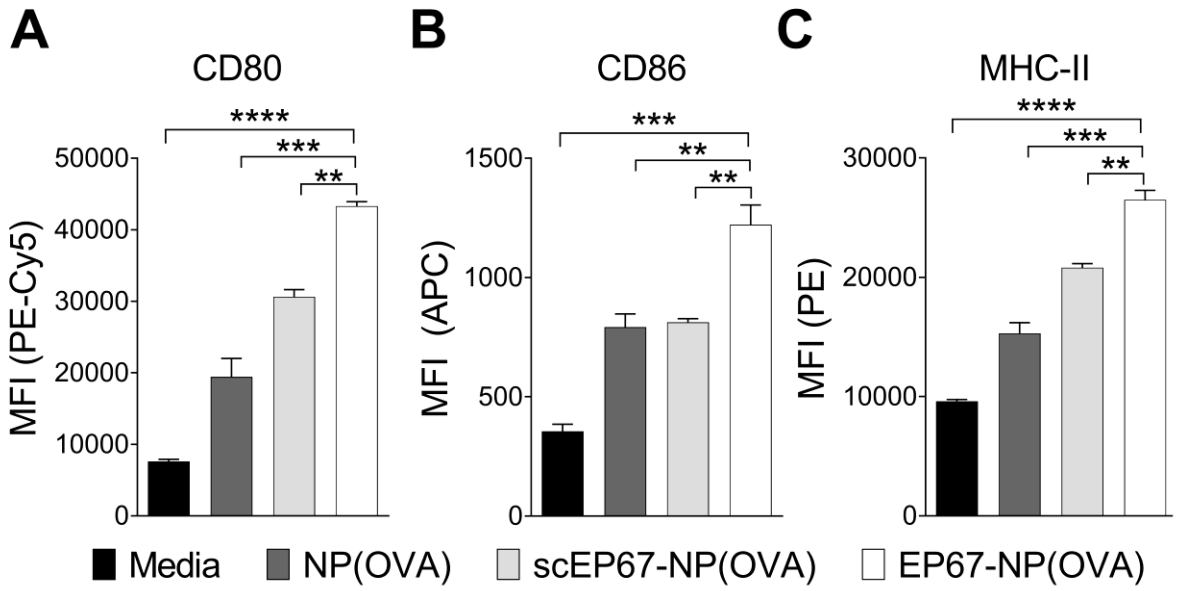

Figure 4.6 Modifying the surface of PLGA 50:50 nanoparticles with EP67 increases the expression of activation markers on the surface of immature murine BMDCs

Immature BMDCs (male C57BL/6) were incubated at 37°C for 24 h with media alone [black bars, Media] or media containing ~7.9 µg endotoxin-free OVA loaded in ~100 µg of uncoated 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)]. BMDCs were then stained with viability dye, anti-CD11c, anti-CD80, anti-MHC-II and anti-CD86 antibodies and analyzed by flow cytometry. Average median fluorescence intensity ±SD (n=2) of staining CD11c+ cells with (A) PE-Cy5 anti-CD80, (B) anti-CD86, or (C) anti-MHC II between the indicated treatment groups were compared using one-way ANOVA with Dunnett's post-test where **p≤0.01, ***p≤0.001, and ****p≤0.0001. Data are representative of at least three independent experiments.

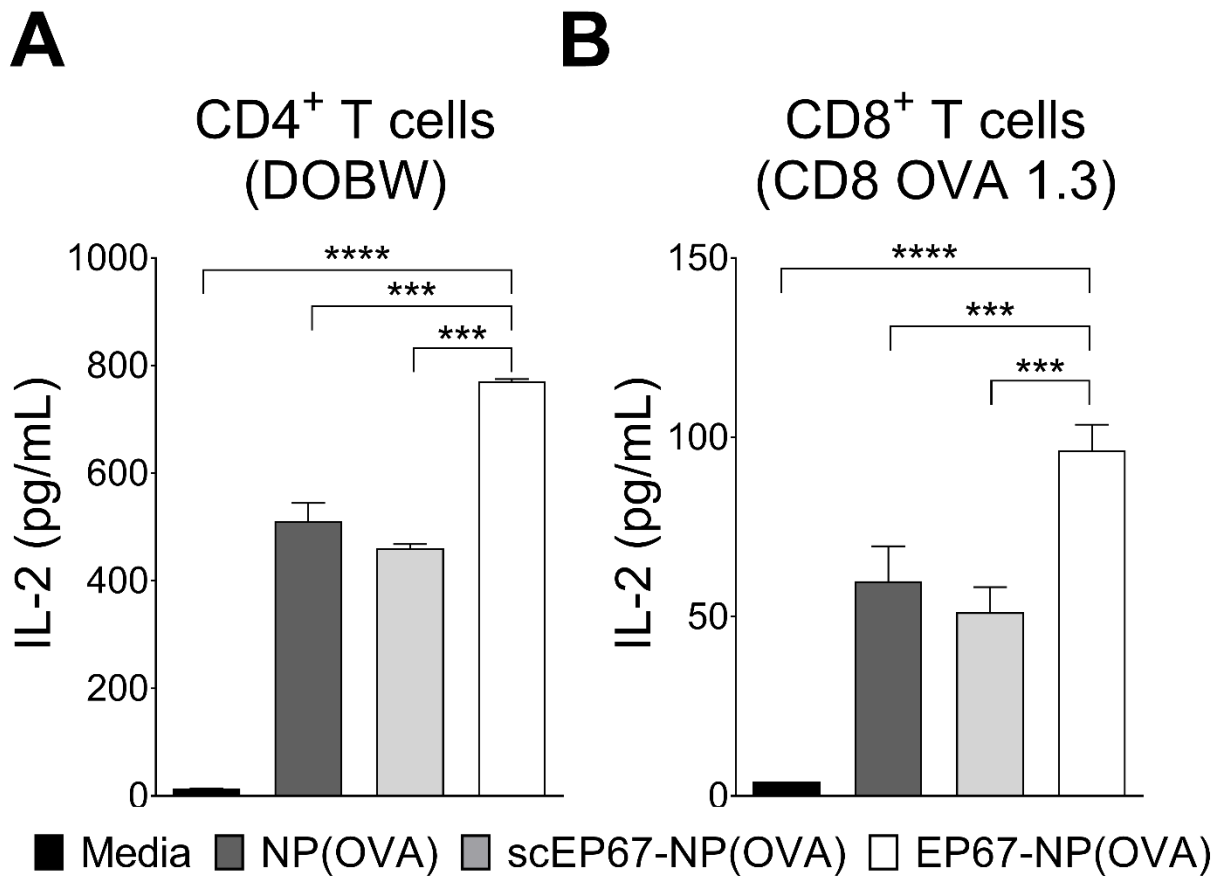

Figure 4.14. Modifying the surface of PLGA 50:50 nanoparticles with EP67 increases murine BMDC activation of model naïve T cells against encapsulated protein in vitro.

Immature BMDCs (C57BL/6) were incubated at 37°C for 2 h with media alone [black bars, Media] or media containing ~7.9 µg endotoxin-free OVA-loaded in ~ x mg unmodified nanoparticles (~400 nm diam.) [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% EP67). BMDCs were then washed and incubated with OVA-specific $CD4^+$ (DOBW) or $CD8^+$ (CD8 OVA 1.3) T cell hybridomas for 18 h at 37°C. Average concentrations of IL-2 ± SD (n=3) released into the media by (A) $CD4^+$ (DOBW) or (B)

$CD8^+$ (CD8 OVA 1.3) T cells were determined by ELISA and compared between treatment groups using one-way ANOVA with Dunnett's post-test where **p≤0.01, ***p≤0.001, ****p≤0.0001.

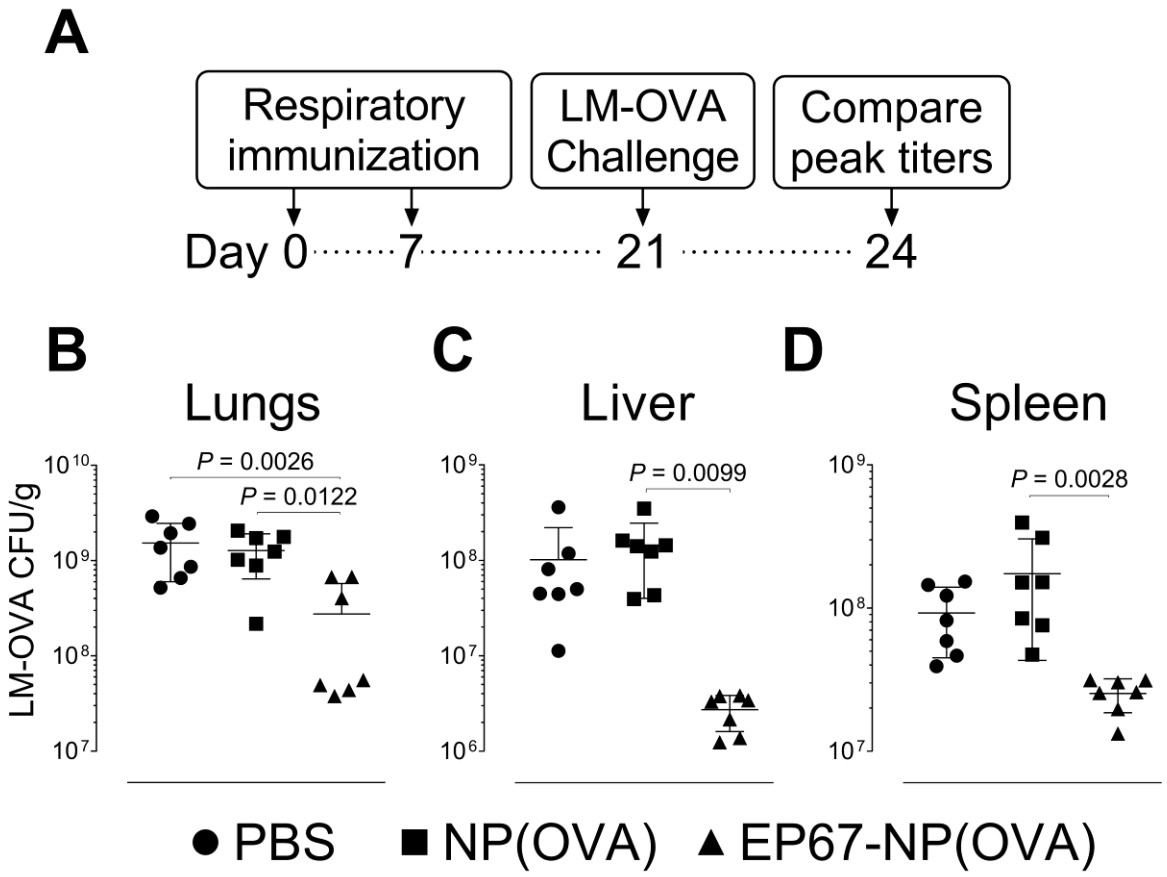

Figure 4.8 Modifying the surface of PLGA 50:50 nanoparticles with EP67 increases the efficacy of respiratory immunization

Modifying the surface of PLGA 50:50 nanoparticles with EP67 increases the efficacy of respiratory immunization with encapsulated protein against primary respiratory infection with L. monocytogenes that ectopically expresses OVA. (A) Vehicle alone (PBS), or vehicle containing ~25 µg LPS-free OVA encapsulated in unmodified PLGA 50:50 nanoparticles [NP(OVA)] or PLGA 50:50 nanoparticles surface-modified with EP67 [EP67-NP(OVA)] was administered to naïve female C57BL/6 mice (~6 wk old) on Day 0 and Day 7 by IN administration (50 µL). Fourteen days after the final

immunization (Day 21), L. monocytogenes ectopically expressing OVA (LM-OVA) was administered IN (2 x 107 CFU in 50 µL) and peak titers of LM-OVA (Day 3 post-infection; Day 24) were determined by CFU assay. Average peak LM-OVA colony forming units (CFU) per gram of tissue ± SD (n = 7 mice) in the (B) lungs, (C) liver, and (D) spleen were compared using one-way ANOVA with uncorrected Fisher's LSD test. Data are representative of two independent experiments.

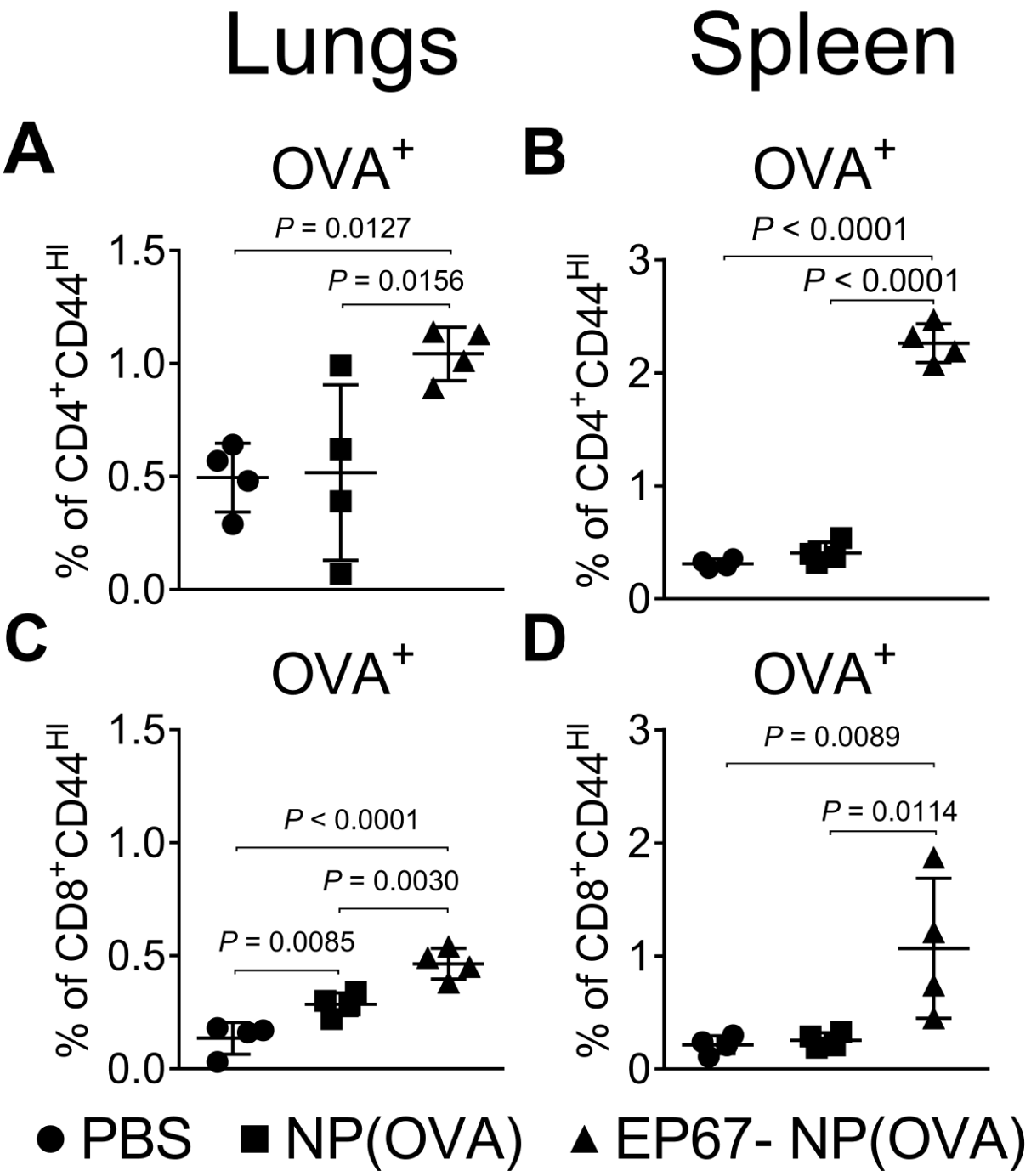

Figure 4.15 Modifying the surface of PLGA nanoparticles with EP67 increases proportions of antigen-specific mucosal and systemic T cells after respiratory immunization.

Modifying the surface of PLGA 50:50 nanoparticles with EP67 increases proportions of encapsulated protein-specific mucosal and systemic T cells after respiratory

immunization. Naïve female C57BL/6 mice (~6 wk old) were immunized as described in Fig.4.8. (Days 0 and 7) and sacrificed on the day of challenge (Day 21). Proportions of (A, B) OVA-specific (tet+) $CD4^{+}CD44^{HI}$ or (C, D) OVA-specific (tet+) $CD8a^{+}CD44^{hi}$ cells in the lungs and spleen were determined by flow cytometry. Average percentages of $CD4^{+}CD44^{HI}tet^{+}$ cells and $CD8+CD44^{HI}tet^{+}$ cells ±SD (n=4 mice) between treatment groups were compared in the respective organs using one-way ANOVA with uncorrected Fisher's LSD test.

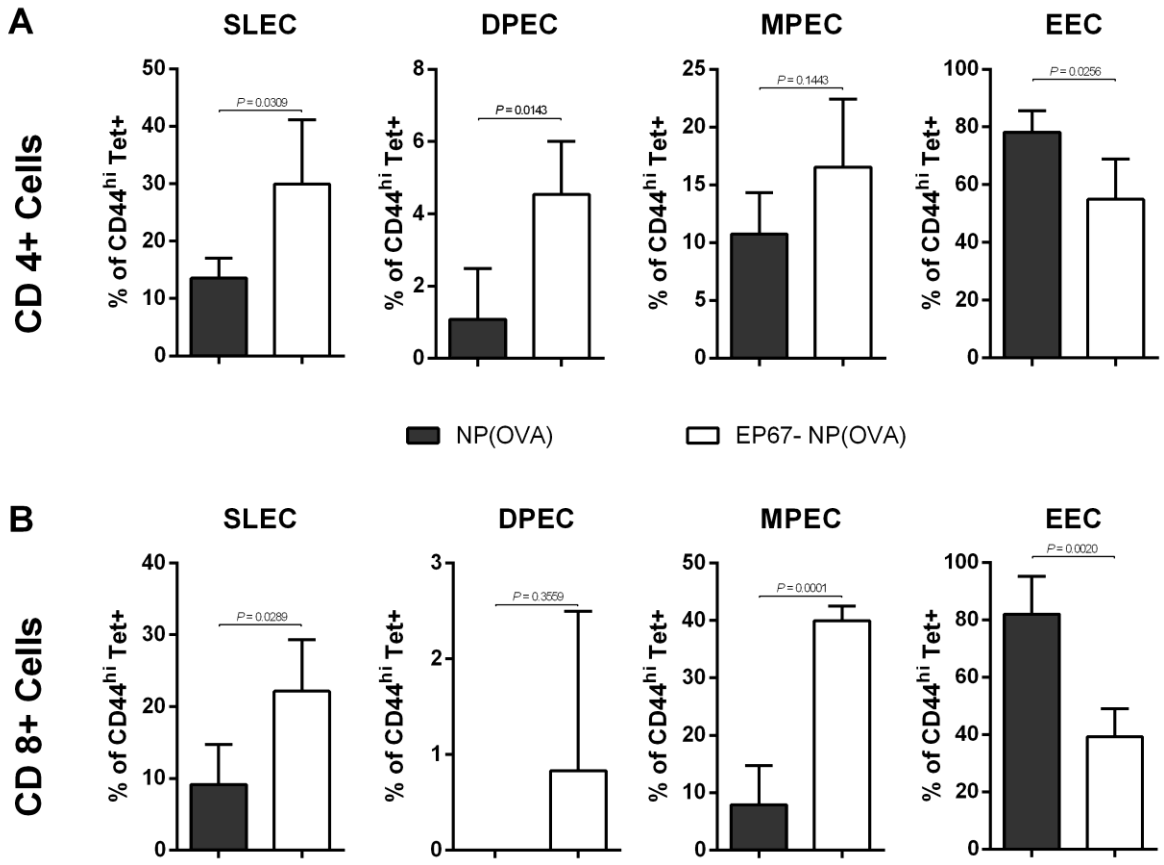

Figure 4.10 Modifying the surface of PLGA nanoparticles with EP67 affects CD127/KLRG1 memory subsets of mucosal T-cells.

Modifying the surface of PLGA nanoparticles with EP67 affects CD127/KLRG1 memory subsets of mucosal CD4+ and CD8+ T-cells generated against encapsulated protein by respiratory immunization. Naïve female C57BL/6 mice (~6 wk old) were immunized as in Fig. 5 and sacrificed on the day of challenge (Day 21). Average percent of (A) CD4+CD44HITet+ or (B) CD8a+CD44HITet+ cells in the lungs that were SLEC (CD127-KLRG1+), DPEC (CD127+KLRG1+), MPEC (CD127+KLRG1-), or EEC (CD127-KLRG1-) ± SD (n=4 mice) were determined by flow cytometry and compared using two-tailed unpaired t-test.

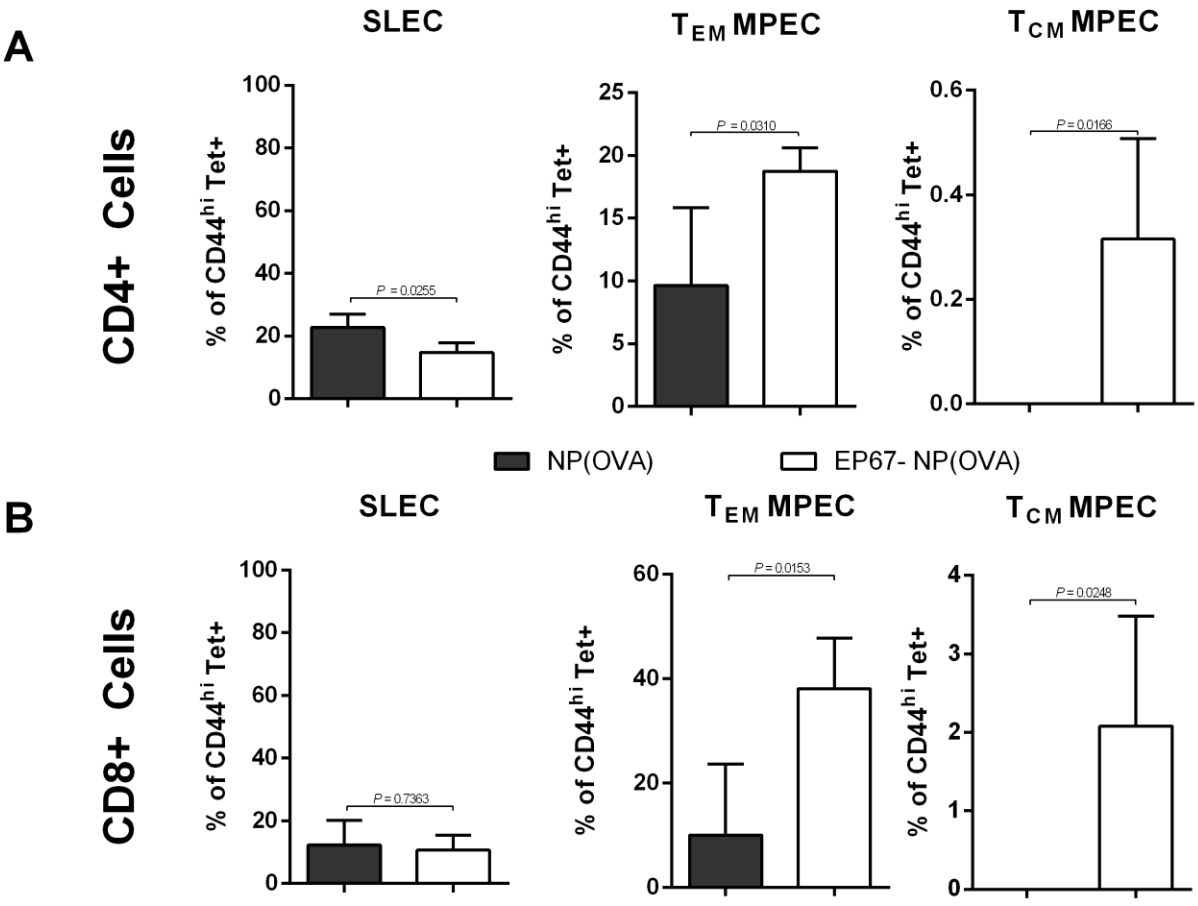

Figure 4. 16 Modifying the surface of PLGA nanoparticles with EP67 affects CD127/KLRG1/CD62L memory subsets of T cells.

Modifying the surface of PLGA nanoparticles with EP67 affects CD127/KLRG1/CD62L memory subsets of systemic $CD4^+$ and $CD8^+$ T cells generated against encapsulated protein after respiratory immunization. Naïve female C57BL/6 mice (~6 wk old) were immunized as in Fig. 4.8 and sacrificed on the day of challenge (Day 21). Average proportions of (A) $CD4^+CD44^{HI}Tet^+$ or (B) $CD8a^+CD44^{HI}Tet^+$ cells in the spleen that were SLEC ($CD127^-KLRG1^-CD62L^-$), TEM MPEC ($CD127^+KLRG1^-CD62L^-$), or TCM MPEC ($CD127^+KLRG1^-CD62L^+$) ± SD (n=4 mice) were determined by flow cytometry and compared using two-tailed unpaired t-test.

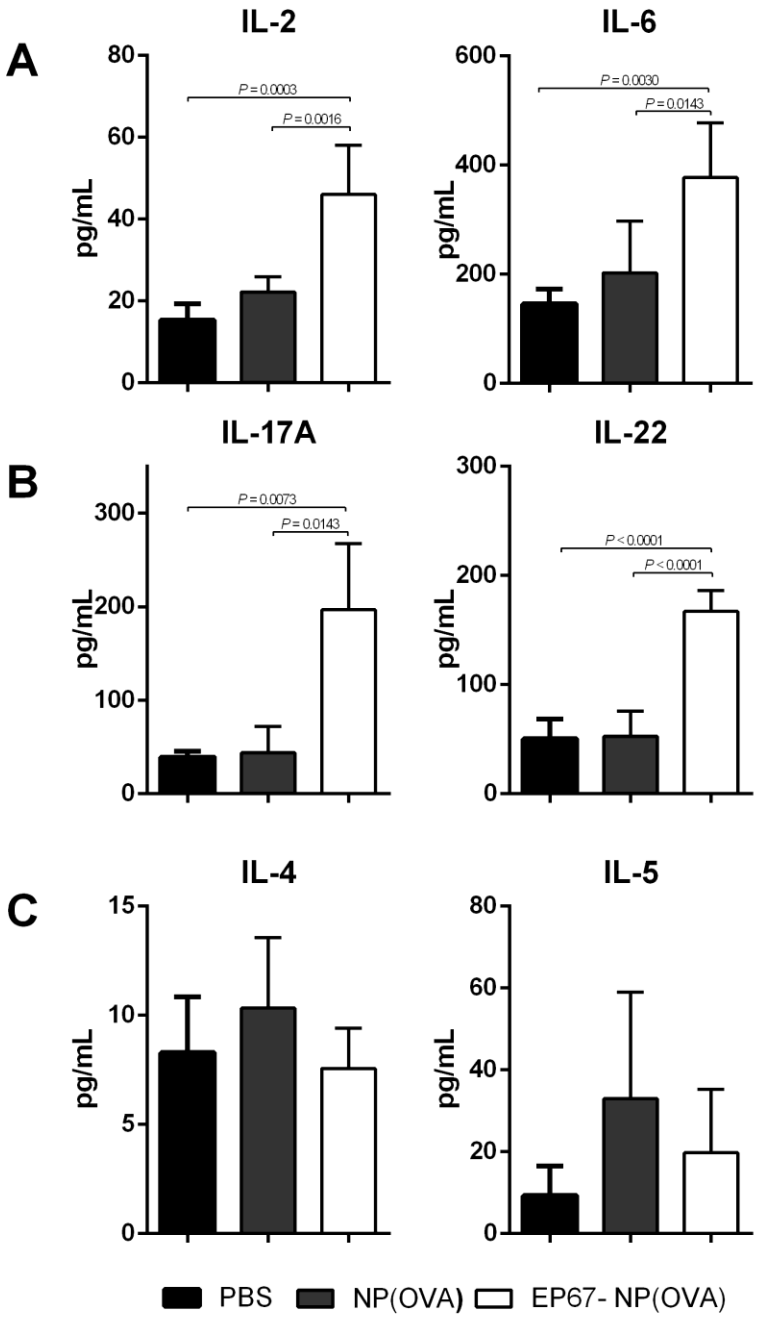

Figure 4.17 Modifying the surface of PLGA nanoparticles with EP67 selectively affects the cytokine secretion pattern of CD4+ epitope-responsive splenocytes. Naïve female C57BL/6 mice (~6 wk old) were immunized as in Fig. 4.8. Splenocytes were harvested on the day of infection (Day 21) and incubated with OVA CD4+ epitope

for 48h. Average concentrations ±SD (n=4) of (A) Th1, (B) Th17, or (C) Th2 cytokines in the media were determined by multiplex assay and compared using one-way ANOVA with uncorrected Fisher's LSD test. Results are representative of at least two independent experiments.

Table 4.1 Characteristics of OVA-encapsulated PLGA nanoparticles

Formulation	Loading (µg/mg ± SD)	Diameter (nm ± SD)	Polydispersity Index (PDI ± SD)	Zeta Potential (mV ± SD)	Peptide Content (µg/mg)
NP(OVA)	78 ± 3	332 ± 4	0.20 ± 0.02	-16.8 ± 0.5	-
scEP67-NP(OVA)	79 ± 2	432.8 ± 0.8	0.41 ± 0.03	-12.5 ± 0.6	1.75 ± 0.2
EP67-NP (OVA)	78 ± 4	382 ± 7	0.171 ± 0.002	-13.3 ± 0.7	0.99 ± 0.2

CHAPTER 5

DISCUSSION

This study provides evidence that surface modification of OVA encapsulated nanoparticles with EP67 increases the efficacy of immune responses against OVA in female C57BL/6 mice after respiratory immunization. We found that respiratory immunization with EP67 surface modified OVA encapsulated PLGA nanoparticles (i) increased protection against respiratory infection with OVA-expressing *Listeria monocytogenes* by significantly reducing bacterial burden at mucosal (lungs) and systemic (liver and spleen) sites (Fig 4.8), (ii) increased magnitudes of OVA-specific CD4+/CD8+ T-cells in lungs and spleen (Fig 4.9), (iii) increased proportions of short-lived effector cells (SLECs), double positive effector cells (DPECs), memory precursor effector cells (MPECs) and decreased early effector cells (EECs) in lungs (Fig 4.10), (iv) increased effector memory MPECs and central memory MPECs without affecting SLECs in spleen (Fig 4.11) and (v) affected the cytokine secretion profile of splenocytes responsive to MHC II epitope of OVA (Fig 4.12).

Our study also provides evidence that surface modification of OVA-encapsulated NPs with EP67 (i) increases internalization of nanoparticles by immature BMDCs when compared to uncoated or inactive scEP67 coated nanoparticles (Fig. 4.5), (ii) increases activation of BMDCs, as measured by upregulation of activation markers MHC-II, CD80 and CD86, when compared to uncoated or inactive scEP67

coated nanoparticles (Fig. 4.6) and, (iii) increases antigen-presenting potential of BMDCs, determined by activation of OVA-specific T-cell hybridomas CD8OVA1.3 (CD8+) and DOBW (CD4+) by BMDCs treated with to uncoated, inactive scEP67 or EP67 coated nanoparticles (Fig. 4.7).

Dendritic cells are the most potent antigen-presenting cells that play a major role in generating adaptive immune responses against invading pathogens. Several studies have demonstrated that directing antigen to DCs by targeting pattern recognition receptors (PRRs), using antibodies or PRR ligands, can enhance antigen presentation and thus improve the efficacy of immunization. In our earlier studies, we have shown that EP54-, a sister analog of EP67, containing vaccine constructs are rapidly internalized via the C5aR present on human monocyte-derived DCs and presented in the context of HLA I/II. Given that EP67 has a 1000-fold higher affinity to C5aR than EP54, it is reasonable to expect that DCs will similarly internalize and present EP67-containing vaccines. EP67 is also expected to improve antigen processing and presentation directly by activating DCs and, indirectly by inducing DCs to produce various cytokines and chemokines that favor a pro-inflammatory immune response, as evidenced by the upregulation of maturation markers CD80, CD40, and CD54, inducing secretion of chemokines (CCL2, CCL3, CCL4, CXCL8, and CXCL10)

and cytokines (IL-6, IL-1β, TNF-α, and IL-10) by human monocyte-derived DCs (unpublished data). These studies together can be used to explain the results of our current study. EP67 on the surface of NPs upon interaction with C5aR on antigen presenting cells (i) induces receptor-mediated internalization of NPs, (ii) followed by activation of APCs resulting in increased surface expression of antigen-presenting molecules and, consequently, (iii) better activation of CD4+ and CD8+ T-cells resulting in enhanced immune responses against the encapsulated antigen.

The effective immune response against an invading pathogen involves the generation of a large pool of pathogen-specific effector T-cells (T_{EFF}), some of which, after the infection is resolved, transition into effector memory (T_{EM}) and central memory cells (T_{CM}) with high proliferative and survival potential. Although, the rate of transition from T_{EFF} to T_{EM} and T_{CM} depends on the nature of immunization and presence of antigen, the long-term fate of T_{EFF} cells is decided during the initial phase of T-cell activation. The long-term fate of T-cells depends on the duration of initial DC-T-cell interactions which is affected by the dose of antigen-experienced by DCs. T-cells that interact with DCs exposed to high antigen dose are expected to differentiate into memory T-cells.

Keeping the above information in mind, the increased memory precursor effector cells observed after respiratory immunization with EP67 surface-modified NPs when compared to unmodified NPs may be due to faster translocation of EP67 surface modified NPs across the respiratory epithelium, through M-cells which may express C5aR alike intestinal M-cells, into mucosal-associated lymphoid tissue (MALT) which is rich in the lymphoid cells required to generate an immune response. The higher proportions of MPECs can also be explained by increased uptake of EP67 surface-modified NPs by mucosal APCs through C5aR, and this is supported in part, by the increased internalization of EP67 surface-modified NPs by BMDCs *in vitro* observed in this study. However, the effect of particle size on internalization by APCs and trafficking across epithelium cannot be overlooked. As such, both unmodified and EP67 surface modified NPs are in the optimal size range required for APC phagocytosis and translocation across the epithelium (although not receptor mediated in the case of unmodified NP), therefore it is possible that EP67 surface modification is only accelerating the transition of effector cells into memory precursors, as supported by the higher proportion of early effector cells in the unmodified NP-treated animals (Fig. 4.9).

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Summary and Conclusions

Vaccination is one of the most effective ways to prevent infectious diseases, however, there are several diseases against which conventional vaccination strategies have failed to generate effective immune responses or have a low safety profile. Furthermore, conventional vaccines consisting of live attenuated or inactivated pathogens are difficult to develop and have the potential to revert to pathogenicity. Subunit vaccines composed of antigenic proteins or peptides can potentially overcome the limitations of conventional vaccines, but have limited efficacy because of low immunogenicity, inability to generate T-cell immunity, rapid clearance from administration site and insufficient delivery to antigen presenting cells. Thus, there is an urgent need to develop novel strategies that can overcome the disadvantages of subunit vaccines.

In this study, we report the development of C5aR-targeted biodegradable PLGA nanoparticles that improve the efficacy of respiratory immunization by enhancing the immune responses generated against the encapsulated model antigen ovalbumin. We have shown that surface modification of PLGA nanoparticles with C5aR ligand EP67 will increase the internalization of nanoparticles by BMDCs and simultaneously activate BMDCs as shown by the upregulation of MHC II and costimulatory molecules. The activated BMDCs also appear to have higher antigen-presenting potential as shown by the increased secretion of IL-2 by OVA-specific T-cell hybridomas DOBW and CD8 OVA1.3.

Furthermore, we have demonstrated respiratory immunization with EP67 surface-modified OVA-encapsulated PLGA nanoparticles (i) increased protection

against respiratory infection with LM-OVA by significantly reducing bacterial numbers (ii) increased magnitudes of OVA-specific CD4+/CD8+ T-cells in lungs and spleen, (iii) increased proportions of short-lived effector cells (SLECs), double positive effector cells (DPECs), memory precursor effector cells (MPECs) in lungs, (iv) increased effector memory MPECs and central memory MPECs without affecting SLECs in spleen and (v) affected the cytokine secretion profile of splenocytes responsive to MHC-II epitope of OVA.

In summary, our findings suggest that surface modification of biodegradable nanoparticles increases the efficacy of respiratory immunization against the encapsulated antigen by increasing magnitude and proportions of memory precursor effector cells, which have the highest potential to become long-lived memory cells, and provide long-term immunity. Thus, surface modification of biodegradable nanoparticles encapsulating the desired antigen with EP67 may be an effective approach to increase the efficacy of mucosal vaccines,

6.2 Future Directions

Although the work presented here demonstrates the proof of concept that surface modification of PLGA nanoparticles can increase the efficacy of respiratory immunization against the encapsulated antigen, the formulation by itself is not optimized. It is important to understand the effect of different formulation parameters

like the size of the particle, release rate of antigen, the length of PEG cross-linker and the extend of surface modification with EP67 will have on the immune responses. Therefore, the next step would be to modify the above-mentioned parameters and optimize the vaccine formulation. After an optimized formulation is achieved, the next goal is to test the efficacy of the formulation in protecting mice from respiratory infection with murine cytomegalovirus, using protective antigens from MCMV as the encapsulated antigen, when compared to immunization with co-administration of inactivated virus with EP67.

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