Immunization with Synthetic Nanoparticles to Generate Mucosal CD8 T Cell Responses

by

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ABSTRACT

Vaccines have benefited global health by controlling or eradicating life threatening diseases. With better understanding of infectious diseases and immunity, more interest has been placed on stimulating mucosal immune responses with vaccines as mucosal surfaces function as a first line of defense against infections. Progress made in nanoparticle research, in particular the successful use of liposomes for drug delivery, has made liposomes an attractive candidate for vaccine delivery. Here, we investigate the efficacy of using a novel nanoparticle system, Interbilayer Crosslinked Multilamellar Vesicles (ICMVs), as a mucosal vaccine to stimulate mucosal and systemic CD8 immunity.

We first assessed the ability of ICMVs to elicit mucosal CD8 response, against the model antigen ovalbumin (OVA), by administration of the nanoparticles through the lungs. We explored the use of 2 different Toll-like receptor agonists (TLRa), monophosphoryl lipid A (MPLA) and Polyinosinic:polycytidylic acid (poly (I:C) or pIC) added to ICMVs as adjuvants. Pulmonary administration of ICMV with both adjuvants was found to give the most potent CD8 T cell response in both systemic and mucosal compartments. We looked further into the quality of the immune response and detected the presence of antigen-specific memory CD8 T cells in the system at ~2.5 months after immunization. The majority of these cells were found to be effector memory cells ($CD44^{hi}CD62L^{lo}$) and expressed markers for long term survival ($CD127^{hi}KLRG1^{lo}$), suggesting that long term protection against infection can be induced by pulmonary delivery of ICMVs. We also explored using this system to deliver a model HIV peptide epitope, AL11, and ICMV successfully induced CD8 response against this epitope. Animals immunized against AL11 were challenged with a live virus expressing the same epitope and protection was seen only in the pulmonary ICMV treatment group. Virus was delivered via the lungs and viral titre was decreased in both the lungs and ovaries. Neither the soluble form of the vaccine or ICMV delivered via parenteral injection conferred protection. Safety of the ICMV system was also assessed and no significant negative effects were observed in body weight and histological analysis on lungs. Finally, mechanism of using nanoparticles as pulmonary vaccines was investigated to gain better understanding in how particulate vaccine and route of immunization improved the efficacy of a vaccine.

Overall, this thesis describes a comprehensive study of systemic and mucosal CD8 responses generated by pulmonary delivery of a novel nanoparticle system. This data provides evidence that mucosal delivery of ICMVs can safely and effectively stimulate disseminated mucosal $CD8^+$ T cells at sites relevant for protection against mucosal infection. A better understanding of nanoparticles for pulmonary immunization was also gained.

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1. Background

1.1. The need for vaccines

Vaccines are one of medicine's most important accomplishments and essential to global public health. Diseases such as measles, mumps, rubella, diphtheria, tetanus, pertussis, polio and yellow fever are now under control because of vaccination. Smallpox has been completely eradicated and polio is on the verge of elimination.[1] However, we are still threatened by emerging and re-emerging infectious diseases such as HIV, avian flu and SARS. The discovery of the link between cancer and infectious agents such as HPV and *Helicobacter pylori* accelerates the need for vaccine development.

1.2. Importance of mucosal vaccines

Mucosal surfaces are a portal of entry for the majority of pathogens. This includes respiratory and gastrointestinal disease that kill approximately 5 million children in developing countries each year and sexually transmitted mucosal pathogens (including HIV) that affects millions of adults.[2] Therefore, triggering immunity at mucosal surfaces is essential to protect against infectious disease as a first line of defense.[3,4,5] However, The majority of vaccines in use today are administered by parenteral injections. Parenteral vaccines protect individuals by triggering systemic immunity and often fail to elicit protective mucosal immunity, while immunization via mucosal routes is more effective at inducing immunity against pathogens at their sites of entry in addition to systemic immunity. [3,5] Hence, mucosal vaccines can fight against pathogens at the site of entry and prevent systemic spread if the first line of defense has been breached.

In addition to conferring immune protection at mucosal surfaces, needle-free administration of mucosal vaccines offers additional advantages. Needle and syringe vaccination is associated with unwanted infection in both patients and healthcare workers through inappropriate re-use of needles or syringes, discomfort, and the fear of needles affects compliance rates, particularly in children.[6]

Needle-free vaccination includes all methods for delivering vaccines that do not require a needle and syringe for administration. All mucosal surfaces, including oral, nasal, rectal, conjunctival, and vaginal mucosa have been considered as potential route of vaccination sites. Due to practical reasons and expected lack of cultural acceptance of certain of these sites, most research in this area has focused on oral and nasal administration.[7]

1.3. Common mucosal immunity

The concept of the "common mucosal immunological system" was proposed nearly 40 years ago, suggesting that mucosal sites function together as one system-wide organ.[8] The idea rose when John Beinenstock and his group observed that bronchus-associated lymphoid tissues were found to be similar to those in the gastrointestinal tract. Since then, it has become increasingly evident that immunization at one mucosal surface triggers immune response at other mucosal sites (and often in systemic responses).[4,8] Of note, the strength of the response at distal mucosal sites is dependent on the site of application (see Figure 1-1), further work is to be done to gain better understanding of crosstalk between different mucosal compartments within the common mucosal immune system.[9]

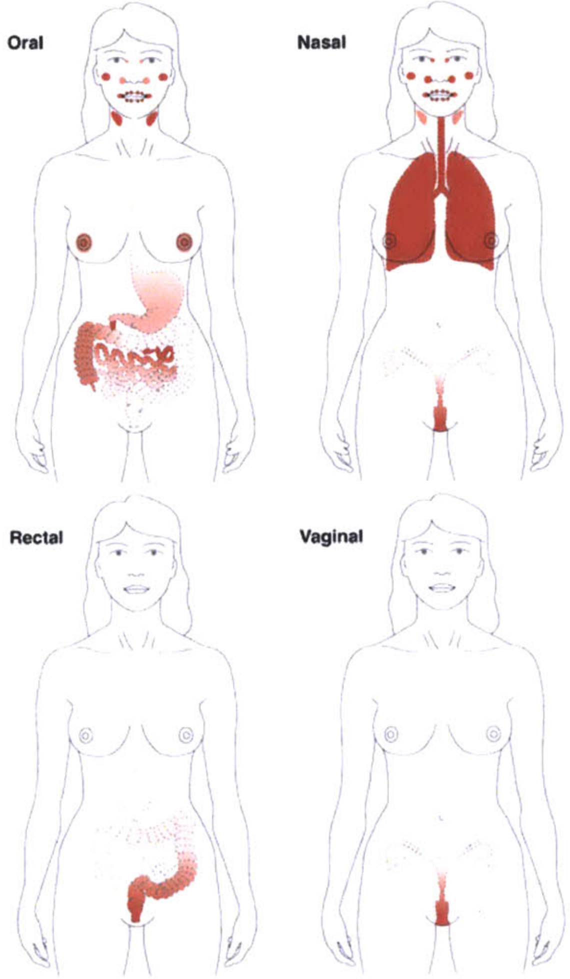


Figure 1-1. Common mucosal immunity and mucosal IgA response after different routes of immunization.
Evidence of common mucosal system from detection of IgA at sites distal to vaccination site, however, different mucosal routes result in varying levels of response at different sites depending on route of immunization. Shading indicates strength of response. (adapted from Holmgren *et al.* [10])

Current understanding of common mucosal response is as follows: Both B and T cells, leave the site of initial encounter with antigen (*e.g.*, a Peyer's patch), transit through the lymph, enter the circulation and then seed selected mucosal sites, mainly back to the mucosa of origin. The anatomic affinity of such cells seems to be largely determined by site-specific integrins and chemokine receptors ('homing receptors') on their surface and complementary mucosal tissue–specific receptors ('addressins') on vascular endothelial cells.[10] These mucosal homing receptors are imprinted onto lymphocytes by mucosal dendritic cells (DCs). Recent studies indicate that mouse DCs isolated from mesenteric lymph nodes and Peyer's Patches, but not from spleen and peripheral lymph nodes, increase the expression of the mucosal homing receptor α4β7[11, 12] and CCR9[11, 12], the receptor for the gut-associated chemokine TECK/CCL25 on memory T cells, and license effector/memory $CD8^+$ T cells to home preferentially to the intestinal epithelium.[10] Therefore, mucosal DCs influence both expression of homing and chemokine receptors on T cells and affect homing to mucosal sites. Because chemokines, integrins and cytokines are differentially expressed among mucosal tissues, there is a significant degree of compartmentalization linking specific mucosal inductive sites with particular effector sites.

1.4. Synthetic particles as vaccine vectors

Currently, more than 30 injectable vaccines have been licensed for human use while only a handful of mucosal vaccines are on the market. Most of these mucosal vaccines are for oral use against enteric infections with the exception of two nasal attenuated influenza vaccines (see Table 1-1).[2]

Oral polio virus vaccines (OPV)
Oral live-attenuated typhoid vaccine (Vivotif™)
Oral inactivated B subunit-whole cell cholera vaccine (Dukoral™)
Oral live-attenuated rotavirus vaccines (RotaTeq™ and ROTARIX™)
Nasal cold-adapted live-attenuated influenza vaccine (FluMist™)

Table 1-1. Internationally licensed mucosal vaccines currently used in humans. Table from Czerkinsky *et al.*[2]

As seen above, all of the licensed mucosal vaccines are live or inactivated whole-pathogen vaccines. Live vaccines, such as smallpox, measles, mumps, rubella, varicella, adenovirus (and others) and oral polio vaccine mentioned above, have the advantage of producing both humoral and cellular immunity and often require only one boost. However, live vaccines include a serious risk of reverting back to their virulent form and intrinsic instability, making them untenable for a number of diseases.[1] In fact, the oral polio vaccine is no longer recommended due to rare cases of vaccine-associated paralytic poliomyelitis. The rotavirus vaccine, Rotashield was also withdrawn from the market when post-licensure surveillance detected a rare association between the vaccine and intussusception.[7] Live vaccines also induce anti-vector immune responses, thus, the same vector often cannot be used to boost a response. Inactivated vaccines are safer but because they cannot replicate, they tend to provide shorter length of protection and are more likely to require boosters to create long-term immunity. Given these issues, increasing efforts have been focused on developing DNA and subunit vaccines. These vaccines are attractive because of their increased safety since they cannot revert to a virulent form and their lack of contaminants remaining from the original pathogenic organism. Additionally, the ability to consistently produce large, well defined quantities of antigen from recombinant methods is highly desirable.[1] The development of new delivery methods/vehicles have accompanied the rise of new subunit vaccines as in many cases, the antigen itself is easily degradable and weakly immunogenic.

Needleless methods to deliver vaccine are actively under development. These include fluid/solid jet injectors,electroporation, microneedle/patches and various particulate carriers for different routes of mucosal immunization.[7] Currently, the most common particulate carriers used for mucosal delivery are listed in Table 1-2.

Carrier	Comments
Liposomes	Liposomes vaccine may enhance uptake and processing by enclosing the antigen in the lipid vesicles. Liposomes offer advantages such as easy surface modification and a wide range of antigen encapsulation. However, antigen loading and stability are low.
Polymer nanoparticles and microparticles	Nanoparticles/microparticles have an advantage over live systems, in which immune response to the live vector can dominate. The synthesis process usually involves the use of organic solvents that may cause degradation of antigen during encapsulation.
ISCOM	ISCOMs are cage-like structures into which antigen can be incorporated, resulting in enhanced immune response after their administration. ISCOMs are resistant to solubilization by the bile salts deoxycholate, cholate and taurocholate.
Virosomes	Virosomes can be regarded as a special category of liposome vaccine delivery systems whereby viral membrane proteins are integrated into unilamellar vesicles composed of viral and other natural or synthetic lipids. Viral surface glycoproteins possess high affinity for receptors on mucosal surfaces, thus providing a mechanism for efficient attachment of antigen to mucosal surfaces.
Virus-like particles	VLPs are formed from the self-assembly of one or more viral capsid or envelope proteins that are expressed recombinantly in mammalian or insect cells. They are highly immunogenic and stimulate a high rate of uptake while lacking viral genes. However, they are formulated by recombinant technology.

Table 1-2. Particulate carriers commonly employed to deliver vaccine antigen to mucosal sites.
Information from *Woodrow et al*[5] and *Vyas et al*[13].

Synthetic particles are widely explored for vaccine design as the entrapment of antigen in particles clearly enhances its acquisition and processing by antigen presenting cells and ensuing adaptive immunity. The particle itself, exhibit adjuvant properties on a number of different levels: (1) uptake of antigen by antigen-presenting cells (APC) is favored in particulate form rather than soluble; (2) an antigen-loaded degradable particle slowly releases antigen in either an intra or extracellular manner to prolong antigen availability, acting as an antigen depot to extend antigen release which has shown to enhance immunogenicity; (3) depending on the route of antigen trafficking within the APC which is dictated by the particle size and composition; delivery of particulate antigen to the cytoplasm versus an endosomal compartment can direct a different pattern of MHC presentation and acquired immunity; (4) when given alone, particles directly stimulate a pronounced innate response in dendritic cells (DCs) and in animal models;[14] The adjuvant activity of particles has also recently been described at the molecular level as engaging the Nalp3 inflammasome and complementing the activity of toll-like receptor ligands.[14]

Inflammasomes are large multiprotein complexes which plays a key role in innate immunity by participating in the production of the pro-inflammatory cytokines, leading to cell recruitment at injection site followed by the activation of antigen presenting cells.[15, 16] The best characterized inflammasome is the NLRP3 (also known as NALP3 and cryopyrin) inflammasome. It comprises the NLR protein NLRP3, the adapter ASC and pro-caspase-1 but the mechanisms underlying activation of the NLRP3 inflammasome have only been partly resolved.[17]

Particulate compounds that have been shown to activate inflammasomes include silica crystal and asbestos. Endocytosis of these particles by pulmonary macrophages results in NLRP3 inflammasome activation involving ROS and lysosome destabilization, leading in turn to silicosis and asbestosis, respectively.[18, 19] Calcium phosphate crystals were also recently shown to activate NLRP3. Hydroxyapatite crystals, a component of bone, are frequently found in osteoarthritis synovial fluid, activate IL-1β production by means of the NLRP3 inflammasome, and mediate inflammation and joint disease.[20] The commonly used vaccine adjuvant, alum, a crystalline compound of an aluminium salt has also been found to cause inflammation via the activation of the NLRP3 inflammasome [19, 21] Two other particulate adjuvants, chitosan and Quil-A, can also induce IL-1β secretion *in vitro* by a NLRP3-dependent mechanism.[21] Another study has shown that poly(lactide-*co*-glycolide) and polystyrene microparticles activate NLRP3 *in vitro* in a process dependent on lysosomal acidification and on the cysteine protease Cathepsin B.[22] These reports collectively demonstrate that uptake of microparticulate by DCs activates the NALP3 inflammasome for proinflammatory cytokine production (including IL-1β, IL-18), thus, enhancing effects on innate and antigen-specific cellular immunity.

In addition to their adjuvant properties, synthetic particles are attractive for vaccine delivery because the can be mass produced with consistent quality at low cost and transported without being refrigerated. This is an important consideration in vaccine development as the pressing need for vaccines in developing countries has called for research in affordable vaccines.[23]

1.5. T cell vaccines

Most vaccines confer protection by eliciting a protective humoral response (see Table 1-3). Long-lived plasma cells produce antibodies that limit disease by neutralizing a toxin or blocking the spread of the infectious agent.[24] With the threat of more emerging/re-emerging diseases, researchers have begun to realize that these 'B cell vaccines' that confer protection via antibodies alone are not adequate to prevent diseases caused by viral or intracellular pathogens. The discovery of HIV/AIDS further highlights that B cell vaccines may not be enough when confronted by an agent that is not easily blocked by antibody. Researchers have turned to the elicitation of cellular immunity, or 'T cell vaccines,' which recognize and kill infected cells.[25] Cellular immunity is useful not only for intracellular pathogens, but also for treating cancer (therapeutic cancer vaccines).[26] Ideally, a vaccine that triggers both humoral and cellular response is likely to be most effective to fight against a pathogen.[27]

Vaccine	Serum IgG	Mucosal IgG	Mucosal IgA	T Cells
Diphtheria toxoid	++	(+)		
Hepatitis A	++			
Hepatitis B (HbsAg)	++			
HiB PS	++	(+)		
Hib glycoconjugates	++	++		
Influenza	++	(+)		
Influenza intranasal	++	+	+	+(CD8$^+$)
Measles	++			+(CD8$^+$)
Meningococcal PS	++	(+)		
Meninggococcal conjugates	++	++		
Mumps	++			
Papilloma virus	++	++		
Pertussis, whole cell	++			
Pertussis, acellular	++			+?(CD4$^+$)
Pneumococcal PS	++	(+)		
Pneumococcal conjugates	++	++		
Polio Sabin	++	++	++	
Polio Salk	++	+		
Rabies	++			
Rotavirus			++	
Rubella				
Tetanus toxoid	++			
Tuberculosis(BCG)	++			++(CD4$^+$)
Typhoid PS	+	(+)		
Varicella	++			+?(CD4$^+$)
Yellow Fever	++			

Table 1-3. Correlates of vaccine induced immunity.
Adapted from Siegrist, C-A.[28] PS : polysaccharide. Note: this table may not be exhaustive and only includes currently licensed vaccines.

The importance of CD8 T cells came to attention particularly in the case of HIV infection, for example: (i) Cytotoxic T-lymphocyte (CTL) escape is a major force driving HIV evolution[29] (ii) Highly functional CD8$^+$ T-cell responses are correlated with slow AIDS disease progression[30] (iii) evidence of HLA class I mediated responses is associated with good outcomes in HIV-infected people.[31] In addition, nonhuman primates have demonstrated the value of a vaccine-induced T-cell response in conferring protection against the clinical progression of disease after virus infection.[32] The ability of $CD8^+$ T cell populations to proliferate upon antigen encounter has also been associated with control of HIV replication in humans [33] Furthermore, depletion of peripheral $CD8^+$ cells in SIV-infected macaques significantly increased virus loads.[32] Detailed flow cytometry analyses of multiple effector functions found the association of polyfunctional $CD8^+$ T cells and their *in vivo* efficacy.[34]

To stimulate CD8 T cell response using subunit vaccines, enhancing crosspresentation of antigens onto class I MHC is of great interest. Crosspresentation is the process by which professional APCs are able to load peptides from a processed extracellular protein antigen onto MHC class I molecules, triggering a CTL response.

Efficient MHC presentation of vaccine proteins by antigen presenting cells (APC) is a prerequisite for the induction of a protective immune response. Purified proteins, which are the antigen component in most new generation vaccines, are usually internalized, processed and presented by DC mainly on class II MHC. Class I presentation of extracellular antigens is generally not very efficient. This results in poor CD8 T cell priming. Recent reports have elegantly demonstrated that the pathway for crosspresentation resides in the early endocytic compartment of DC and is physically separated from both the class II presentation pathway of exogenous antigen and the standard class I presentation of intracellular proteins.[35, 36] Adjuvants that specifically activate this pathway in the APCs are expected to improve the efficacy of vaccines for which a CTL response is of paramount importance.[15] A study by Shen *et al.* showed that in primary mouse bone marrow-derived dendritic cells (BMDCs), the MHC class I presentation of PLGA-encapsulated ovalbumin (OVA) stimulated T cell interleukin-2 secretion at 1000-fold lower concentration than soluble antigen.[37] This was found to be due to increased protein escape from endosomes into the cytoplasm via PLGA particles, thereby increasing the access of exogenous antigen to the classic MHC class I loading pathway. In the same study, PLGA particles with OVA encapsulated was also found to serve as an intracellular antigen reservoir as MHC class I presentation of OVA was sustained for 72 hr, decreasing by only 20% after 96 hr, a time at which the presentation of soluble and latex bead-associated antigens was undetectable.[37] Hence, encapsulation of antigens into particles can prolong presence of antigen and promote crosspresentation for improved CTL induction.

1.6. Toll-like receptors (TLRs) and mucosal vaccine adjuvants

The most potent mucosal adjuvants which are available for mucosal immunization are heat labile enterotoxin from *Escherichia coli* (LT) and cholera toxin (CT) from *Vibrio cholerae*. These molecules and their sub-units have shown to successfully induce antibodies and CTL response.[38, 39] Protection against challenge with *B. pertussis*[40], *S. pneumoniae*[41] and herpes simplex virus[42] following intranasal immunization are also documented in mice. However, since the native toxins CT and LT are the causative agents for cholera and traveler's diarrhea, they are considered to be too toxic for use in humans. Several groups have focused on the development of detoxified mutants of LT and CT by mutating enzymatic activity in ADP-ribosylation (which causes abnormal intracellular accumulation of cAMP and excess fluid secretion from intestinal epithelial cells). Toxicity was significantly reduced, but detectable.[39, 43] Therefore, different kinds of adjuvants are being explored for mucosal vaccination.

Toll-like receptors (TLRs) have been recently recognized to play a major role in pathogen recognition and innate immunity. Agonists for Toll-like receptors (TLRs) have been investigated for use as mucosal vaccines. These receptors recognize pathogen-associated molecular patterns (PAMPs) such as bacterial cell wall components (e.g., peptidoglycan, lipoteichoic acid) and uncommon forms of nucleic acids (e.g., double-stranded RNA, CpG) and trigger immune responses to activate innate immune response. This, in turn, orchestrates the adaptive immune response through the activation of antigen-presenting cells (APCs) or induction of increased M cell activity.[5]

Synthetic polymer particles can be engineered to activate innate immune signalling pathways by incorporating structures that mimic natural PAMPs. Hence, they have been used in conjunction with TLR agonists (TLRa) frequently as an adjuvant for particle vaccines.[44] A study by Blander *et al* demonstrated that the delivery of antigen and adjuvant within the same phagocytosed cargo can improve antigen presentation efficiency, thus, stimulating stronger immune response.[45] Although the intended targets of adjuvant innate immune triggers are APCs, additional cells including airway epithelial cells also express TLRs and are also triggered to produce inflammatory cytokines, chemokines and antimicrobial peptides.[46-49] In addition, delivery of TLRa in synthetic particles can limit the potential for adverse events by restricting their systemic distribution to the injection site.[50]

Among various TLRs, we focus on Monophosphoryl Lipid A (MPLA) and Polyinosine-polycytidylic acid (poly (I:C) or pIC) as many studies have shown them to be effective adjuvants for eliciting CD8 immune cells. MPLA is a TLR4 ligand component of LPS (purified from the cell wall of *Salmonella minnesota* R595 and detoxified by mild hydrolytic treatment) is considerably less toxic yet maintains immunostimulatory activity.[44] Many studies have used MPLA as a mucosal vaccine adjuvant mainly for intranasal[51-53] and oral vaccines[52, 54]. MPLA is approved for clinical use and is used as a vaccine adjuvant for in the human papillomavirus and hepatitis B vaccine. Poly (I:C) is a synthetic analog of dsRNA recognized by TLR3. Since poly (I:C) interacts with additional receptors (including retinoic acid-inducible gene I, melanoma differentiation-associated gene 5 and double-stranded RNA-dependent protein kinase), it's adjuvanticity cannot be uniquely ascribed to TLR3 activation.[44] Poly (I:C) has also been applied as a mucosal adjuvant mainly to elicit CD8 T cell response.[55-61] So far, poly (I:C) had limited applications in primates (including human) because higher doses caused severe safety problems. Derivative of poly (I:C) with lower toxicity are being researched and clinical trials have been initiated. No published data on their activity and safety is currently available.[44]

1.7. Scope and outline of thesis

This thesis explores the use of nanoparticles as vaccine delivery agents to elicit mucosal CD8 T cell immunity. The nanoparticle system we used is a novel multilamellar liposome system, interbilayer-crosslinked multilamellar vesicles (ICMVs), recently developed in the Irvine laboratory. Compared to traditional liposomes, this new liposomal vesicle has enhanced stability in serum making it capable of eliciting potent CD8 response when administered parenterally.[62] This motivated the exploration of whether the enhanced stability could also be used to penetrate mucosal barriers without disruption to stimulate mucosal immune response. Among various mucosal routes for administration, we chose to employ pulmonary administration of ICMVs as it is one of the more easily accessible mucosal routes and previous studies have shown it is a promising route to elicit strong local protective immunity in the airways. In addition to a local mucosal immune response, we focused on investigating whether disseminated CD8 responses could be detected systemically and at distant mucosal sites. The goal was to determine if a totally synthetic, well-defined system can easily deliver subunit antigens and elicit a broad spectrum (over whole organism) CD8 response. Such a response would indicate that synthetic particles can be vaccine delivery vectors that are as effective as live-attenuated vaccines and at the same time offer advantages of safety and ease of manufacturing over vaccines currently in use (live attenuated vaccines).

Interbilayer-crosslinked multilamellar vesicles (ICMVs) is a system composed of phospholipid capsules with covalent bonds crosslinking between multiple lipid bilayers. The simple composition makes them easy to synthesize with no organic solvents required, therefore, it is ideal for incorporating fragile antigens into the particle. Covalent bonds introduced between the lipid bilayers allow the particles to encapsulate high amounts of antigen with improved stability *in vivo*, hence, high amounts of antigen will be delivered in each 'package' into antigen presenting cells (APCs) and stay intact for a longer time, improving immune response. Lyophilized ICMVs have been tested and showed similar efficacy *in vivo* compared to fresh particles, pointing to the possibility of eliminating liquid/cold-chain storage of these vaccines.

Chapter 2 describes the synthesis of ICMVs and analysis of their efficacy in stimulating CD8 T cells after pulmonary administration. Optimization of TLR agonists to be used as adjuvants was done to ensure that a robust CD8 T cell response was elicited. The potency of ICMVs administered through the lungs and parenterally was compared to demonstrate that mucosal immunization can elicit a better response than systemic administration. A significant finding was that pulmonary administration of ICMVs elicited strong CD8 T cell responses that can disseminate to systemic and distal mucosal effector sites. To our knowledge, this is the first demonstration of disseminated mucosal immunity elicited using a totally synthetic nanoparticle delivery system.

Chapter 3 examines the quality of the CD8 response generated by pulmonary vaccination with ICMVs. Both humoral and cellular responses elicited were measured at ~2.5 months after priming. A focus is placed on the cellular response and characterization of CD8 memory T cells induced by the vaccine.

Chapter 4 evaluates the safety and efficacy of ICMVs administered into the airway. To move towards translating our system into clinical application, the safety of system has to be ensured. Clinical signs of distress in immunized animals were evaluated and toxicity was evaluated in histological sections from lungs. Evaluation of efficacy was done by challenging immunized animals with tumor cells and infectious agents. Mice immunized with OVA encapsulated in ICMVs (OVA-ICMV) showed protection against tumors indicating this system can be employed as a therapeutic cancer vaccine. We then immunized mice with ICMVs with a different antigen (AL11, a peptide derived from SIV *gag*) encapsulated. Mice were then challenged with *gag*-expressing vaccinia virus and ICMVs successfully prevented/controlled infection of the virus. This shows that the system is effective in conferring protection and versatile as different antigens can be used with the particles.

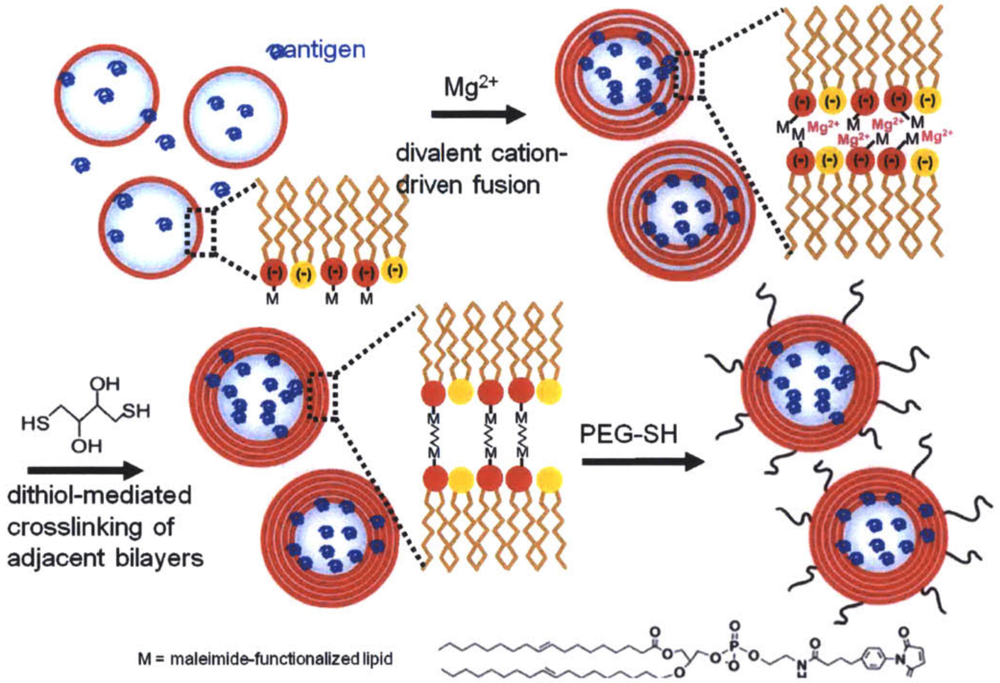
Chapter 5 documents our findings on the mechanisms of eliciting strong CD8 response when ICMVs were delivered via the pulmonary route. We compared the amount of antigen-presenting cells (APCs) present in the draining lymph nodes (LNs) of the pulmonary administration site and the parenteral (tailbase) injection site, and gained an understanding of why mucosal immunization can generate a stronger CD8 response than conventional subcutaneous injection. We also gained an understanding of why antigen encapsulated in particles (antigen in particulate form) improved CD8 responses compared to a soluble version of the antigen in terms of amount of antigen uptake/delivery, speed of uptake and draining, stimulated cells' antigen presentation ability and the mechanism behind dissemination of CD8 cells after immunization.

2. Interbilayer-crosslinked multilamellar vesicles (ICMVs) for pulmonary immunization

2.1. Introduction

Use of nanoparticles has attracted a lot of interest for vaccine delivery. Among the numerous particulate systems developed for vaccine delivery, liposomes are one of the most popular systems as these vesicles can indeed deliver a wide range of molecules. They have been shown to enhance considerably the immunogenicity of weak protein antigens or synthetic peptides. In fact, there are commercially available liposome formulations for drug delivery applications, and two virosomal vaccines (based on hybrid liposome-viral protein compositions) are licensed for human use in Europe.[63] Liposomes are made of materials that are all biocompatible and ease of manufacturing makes them attractive as vaccine delivery vehicles. However, liposomes suffer from low encapsulation efficiency and low stability.[5]

The Irvine laboratory recently developed a novel lipid-based system, interbilayer-crosslinked multilamellar vesicles (ICMVs), where lipid bilayers are covalently crosslinked together, stabilizing the structure (see Figure 2-1, Figure 2-2)[62]. These particles are ~250 nm in diameter, have high encapsulation efficiency and an organic solvent-free synthesis process, hence, allowing a high loading of conformationally-intact antigens. The crosslinked lipid layers prolong antigen release compared to regular liposomes (see Figure 2-2), leaving more antigen to be delivered into antigen presenting cells (APCs) once the antigen-particle complex is phagocytosed, leading to better antigen presentation to CD8 cells. With the addition of the TLR4 agonist, monophosphoryl lipid A (MPLA), ICMVs have been shown to effectively elicit CD8 T cell responses in blood after subcutaneous vaccination in mice (see Figure 2-3).



Nature Materials 10, 243–251 (2011)

Figure 2-1. Schematic for synthesis of ICMV
(Schematic: courtesy of James Moon)
(1)Anionic, maleimide-functionalized liposomes are prepared from dried lipid films. (2) divalent cations are added to induce fusion of liposomes and the formation of multilamellar liposomes. (3) Membrane-permeable dithiols are then added, which crosslink maleimide lipids on apposed lipid bilayers in the vesicle walls, and (4) the resulting lipid particles are PEGylated with thiol-terminated PEG.[62]

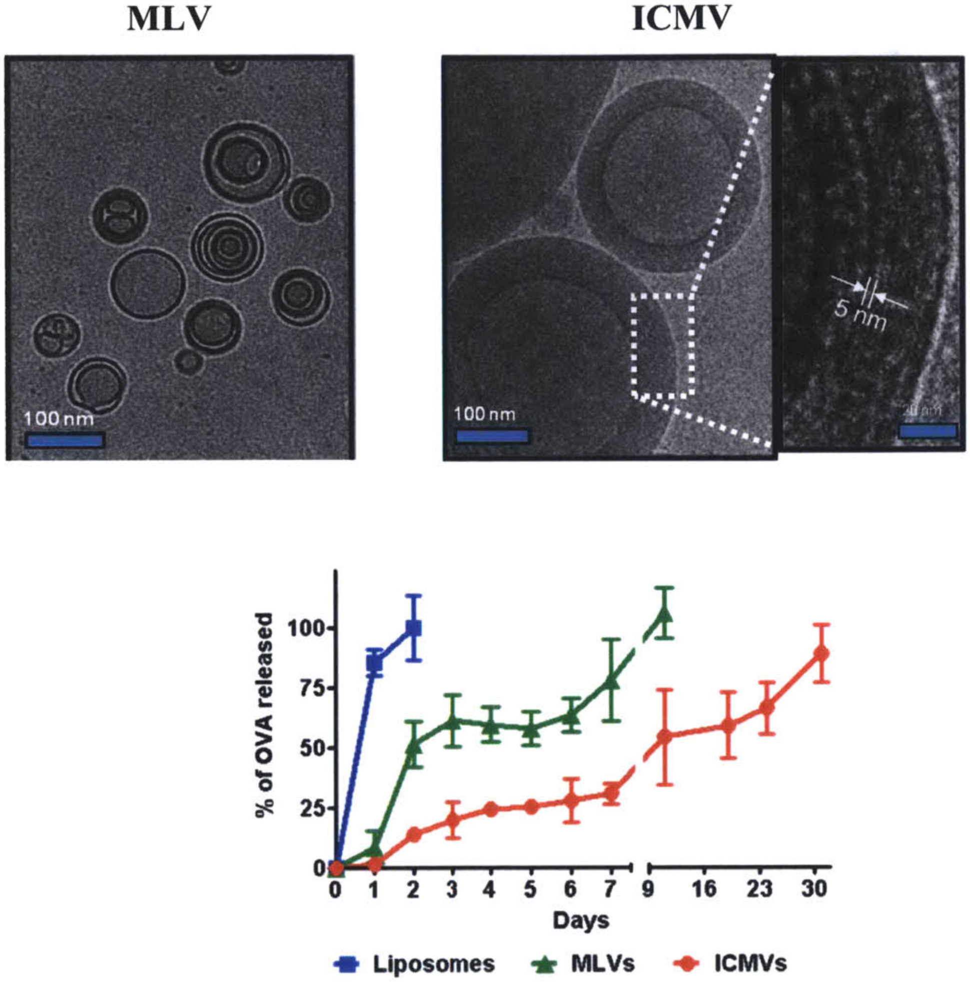


Figure 2-2. Interbilayer-crosslinked multilamellar vesicles (ICMVs).
(Top) Image of mutlilamellar liposomes (MLVs) and ICMVs. Phospholipid bilayers are crosslinked by covalent bonds in ICMVs. (Bottom) In vitro release kinetics of OVA entrapped in ICMVs compared to regular unilamellar liposomes or mutlilamellar liposomes (MLVs). (from Moon *et al.* [62])

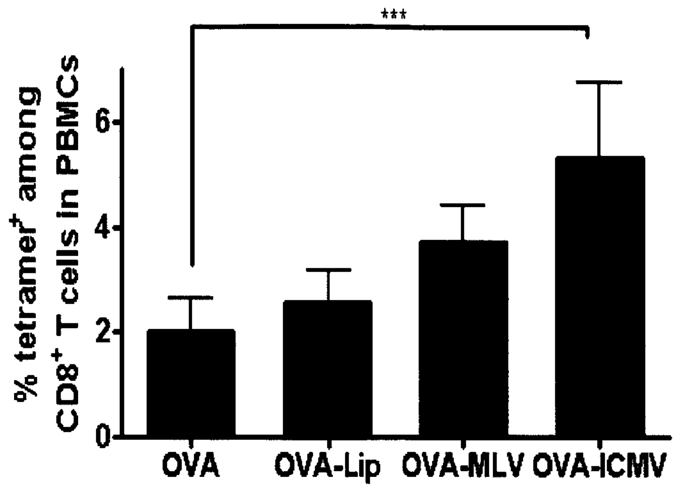


Figure 2-3. Immune response elicited by subcutaneous injection of ICMVs.
(figure: courtesy of James J. Moon)
Tetramer staining on cells from blood 7 days after immunization with 10 μg OVA and 0.3 μg MPLA in soluble form or entrapped in liposomes, MLVs, or ICMVs.

With a growing interest in mucosal vaccines, nanoparticles have also been used to deliver mucosal vaccines. In the past decade, the use of the nasal cavity as a route for drug delivery has been an area of considerable interest. Liposomes have been used to deliver nasal vaccines and have shown to effectively elicit humoral and cellular immune responses.[52, 58, 64-90]

In this chapter, we report on the successful stimulation of mucosal and systemic CD8 T cell responses using ICMVs as a delivery vehicle via the pulmonary route. We first optimized the choice of molecular adjuvant to use with ICMV. In previous studies, we only used MPLA as an adjuvant.[62] To determine if we could further improve CD8 responses at mucosal sites, we examined if poly (I:C), a TLR3 agonist shown to promote CD8 T cell responses and confer T-cell-mediated protection[58, 59, 91-93], can enhance the efficacy of our vaccine. Our results confirmed that poly (I:C) can improve antigen specific CD8 frequency and the combination of both MPLA and poly (I:C) gave the best responses, comparable to live viral vaccines[94,95-97]. We further explored if pulmonary delivery is a better route for vaccine administration compared to parenteral injections. Since we envision that ICMVs can be delivered via a nasal spray/inhaler, this needle-free approach can provide practical benefits if it can achieve immune stimulation similar to delivery via injection. Our results show that in fact, pulmonary administration with MPLA + poly(I:C) provides superior CD8 stimulation; we saw higher frequency of CD8 T cells in systemic compartments and dissemination of antigen specific CD8 T cells into distal mucosal compartments, providing evidence that with the correct delivery system and adjuvants, synthetic particles have the potential to perform as well as a live-attenuated for mucosal vaccination.

2.2. Materials and Methods

2.2.1. Materials

Interbilayer-crosslinked multilamellar vesicles (ICMV) were composed DOPC (1,2-Dioleoyl-sn-Glycero-3-Phosphocholine) and MPB (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl) butyramide). All lipids for interbilayer-crosslinked multilamellar vesicles (ICMV) synthesis were purchased from Avanti Polar Lipids (Alabaster, AL). MPLA from *Salmonella* Minnesota was purchased from sigma (cat#L6895) and poly (I:C) (MW = 0.2-1kb) was purchased from Invivogen (cat #tlrl-picw). Ovalbumin is from Worthington, Lakewood, NJ. PEG-thiol (2kDa) was purchased from Laysan Bio (Arab, AL). LavaPep™ Peptide Quantification Kit was from Fluorotechnics (cat# LP-022010). All reagents were used as received unless otherwise noted.

Wild type C57BL/6 mice (stock #: 000664) were purchased from Jackson Labs. Avertin to anesthetize mice for intratracheal administration was made by dissolving 2-2-2 Tribromoethanol (T48402) into Tert amyl alcohol (240486) both purchased from sigma. For administration of vaccines into lungs, Exel Safelet IV catheters (22 gauge, 1 inch, Fisher, cat. no. 14-841-20), Intubation platform (Steve Boukedes, labinventions@gmail.com) and Fiber-Lite Illuminator (Dolan-Jenner Industries, Inc., Model 3100-1) and Flat forceps (Roboz, cat. no. RS-8260) were used.

Evaluation of antigen specific CD8 T cells were done by staining with SIINFEKL/H-2Kb peptide–MHC tetramers (Becton Dickinson T03000), anti-CD8a antibody (BD Biosciences) and 4,6-diamidino-2-phenylindole (DAPI) and collagenase D (cat# 11088882001) are from Roche. Fc block from BD Pharmingen (Cat# 553142) was used to prevent non-specific binding.

Intracellular cytokine staining required SIINFEKL peptide, MW 963 (Anaspec 60193), Brefeldin A (E-biosciences 00-4506-51), phorbol myristate acetate (PMA) and ionomycin from sigma.Fixation and permeabilization kit (BD #554714) from BD was used and staining was done with anti-CD8, anti IFNγ and anti-TNFα purchased from BD Bioscience.

2.2.2. Synthesis of ICMVs

ICMVs were synthesized as previously described with slight modifications[62]. (see Figure 2-1 for illustration of ICMV synthesis). Briefly, dried lipid films consisted of 1.26 μmol of lipids in chloroform (typical lipid composition: DOPC (1,2-Dioleoyl-sn-Glycero-3-Phosphocholine): MPB (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl) butyramide) = 1:1 molar ratio, all lipids from Avanti Polar Lipids, Alabaster, AL) were prepared. For samples with MPLA embedded, 2.9 mg MPLA was added to the lipid film. The lipid films were then rehydrated in 20 mM bis-tris propane at pH 7.0 with cargo proteins/peptides, including ovalbumin, at 1.625 mg/ml. After vigorous vortexing every 10 min for 1 hr, the liposomal suspension was then sonicated in alternating power cycles of 6 watts and 3 watts in 30s intervals for 5 min on ice (Misonix Microson XL probe tip sonicator, Farmingdale, NY). DTT and $CaCl_2$ were added together at a final concentration of 3 mM and 40 mM, respectively and incubated for 1 hr at 37°C. After the particles were washed twice in deionized water by centrifugation at 14,000 x *g* for 4 mins, 10 mg/ml of 2kDa PEG-thiol was then added and incubated for 30 mins at 37°C. The final product was washed twice before resuspension in PBS and stored at 4°C. The particles were used within 24 hours of synthesis. For samples with poly (I:C) added as an adjuvant, poly (I:C) was mixed into the particle suspension just before immunization to give a final concentration of 0.13 mg/mL. The amount of protein/peptide encapsulated in ICMVs was determined by digesting the particles in 0.2% Triton X-100, and measuring the protein/peptide amount with LavaPep™ Peptide Quantification Kit (Fluorotechnics, LP-022010).

2.2.3. Intratracheal administration of particles

Intratracheal administration was done following the procedure described in Dupage et al.[98] A detailed protocol is provided in "Appendix C: Protocol for intratracheal instillation". Briefly, mice were anaesthetized by i.p. injection of avertin. Then the animal was placed on a custom-made platform so that it is hung from its top front teeth on a horizontal bar. The mouth of the mouse was opened and the tongue was gently pulled out with a flat forceps. An illuminator directed at the mouse chest aided in identifying the trachea in the mouth. After locating the trachea, a catheter was inserted into it. The needle in the catheter was then removed. The vaccine solution was then pipetted directly into the opening of the catheter until the entire volume (75 μL) was inhaled.

2.2.4. In vivo immunization studies

6-10 week old female C57Bl/6 mice (Jackson Laboratories) were used for immunization studies. Vaccines were first administered on D0 then again as a boost at 4-6 weeks after the priming dose. Tissues were harvested at indicated timepoints and homogenized through a cell strainer or between the frosted ends of 2 glass slides then filtered, except for intraepithelial lymphocytes (IEL) from the small intestine. A detailed protocol for IEL extraction is provided in

Appendix B: Protocol for intestinal intraepithelial cell isolation. Vaginal tissue was first digested in collagenase for 30mins at 37C before meshing through a cell strainer. Blood cells were collected into tubes spray-coated with EDTA as an anticoagulant and isolated by performing lysis of red blood cells with ACK lysis buffer. Cell suspensions were then assessed by various assays.

2.2.5. Peptide-MHC tetramer staining

Cells were resuspended in 1% BSA/PBS and Fc block was first added. SIINFEKL/H-2K^b peptide–MHC tetramer was added to the cell solution and incubated at RT for 30mins. Anti-CD8 antibody was added and incubated for an additional 20 min at RT. Cell suspensions were then washed and DAPI was added to discriminate live/dead cells. Sample was then analyzed with a FACSCantoII flow cytometer.

2.2.6. Intracellular cytokine staining

Cells were resuspended in RPMI supplemented with 10% fetal bovine serum (FBS), Beta Mercaptoethanol (bME), Penicillin and Streptomycin (P/S), Sodium pyruvate, Glutamine, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), non-essential amino acids (NAAs). SIINFEKL peptides were added to media and incubated for 2 hours at 37°C. For positive controls, 50 ng/mL phorbol myristate acetate (PMA) and 1 μM ionomycin were added instead. After 2 hours of incubation, 1x brefeldin A was added and incubated for an additional 3-4 hours. Stimulated cells were then washed with 1%BSA/PBS, Fc blocked and stained for cell membrane proteins (20mins 4°C) then for intracellular cytokines (30mins 4°C). After washing, samples were analyzed by a FACSCantoII (Becton Dickinson) flow cytometer.

2.2.7. Statistical analysis

All data was analysed by two-way analysis of variance followed by Bonferroni post-test. Data represent the mean±s.e.m. with $n \geq 3$. *, $p<0.05$; **, $p<0.01$, *** $p<0.01$

2.3. Results and Discussion

2.3.1. Dual adjuvant gives potent CD8 response

We first focused on the TLR4 agonist MPLA, which primed strong CTL responses in combination with ICMVs following parenteral vaccination[62] and the TLR3 agonist poly(I:C) (pIC), which can both stimulate airway epithelial cells[49, 99] and promote cross-presentation of protein antigens by dendritic cells.[100] Groups of C57Bl/6 mice were immunized by intratracheal (*i.t.*) administration of particles with or without addition of

MPLA or poly(I:C) on days 0 and 35 or 42, and OVA-specific T-cell responses were analyzed by peptide-MHC tetramer staining. The amount of MPLA and pIC added was determined following preliminary *in vivo* dose titration experiments. No significant enhancement of CD8 T cell frequency was observed beyond 10 μg pIC and we found that increasing amounts of MPLA decreased the CD8 T cell response (see Figure 2-4). A low dose of 0.3 μg MPLA embedded into lipid bilayers that had given potent responses in our previous *in vivo* studies was used for the vaccine.[62] We further optimized whether pIC should be added externally or entrapped within ICMVs together with antigen and found that external pIC gave a better response (Figure 2-5). We also compared administering a boost on D28 or D42 and found that boosting on D28 gave similar results to D42 (Figure 2-6).

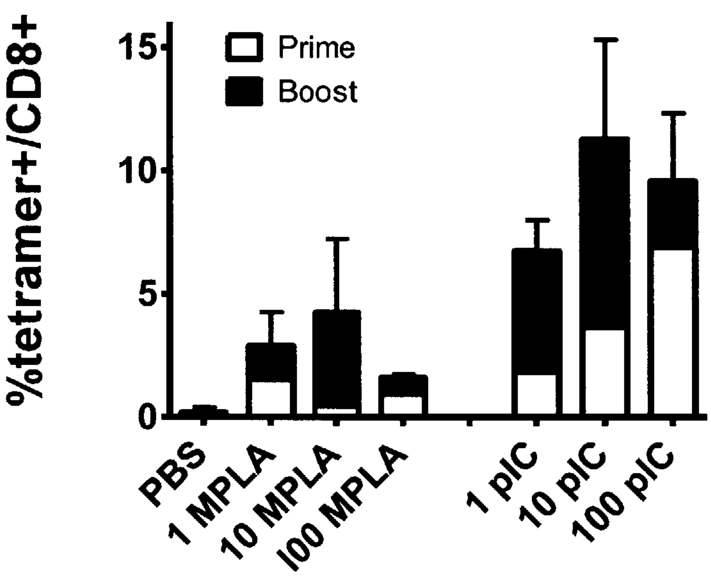


Figure 2-4. Dose titration of MPLA and poly (I:C).
Antigen specific CD8 T cells in mice 7 days after prime/boost in blood. MPLA (1ug, 10ug or 100ug) or poly (I:C) was added to ICMV with 10ug of ovalbumin encapsulated (OVA-ICMVs).

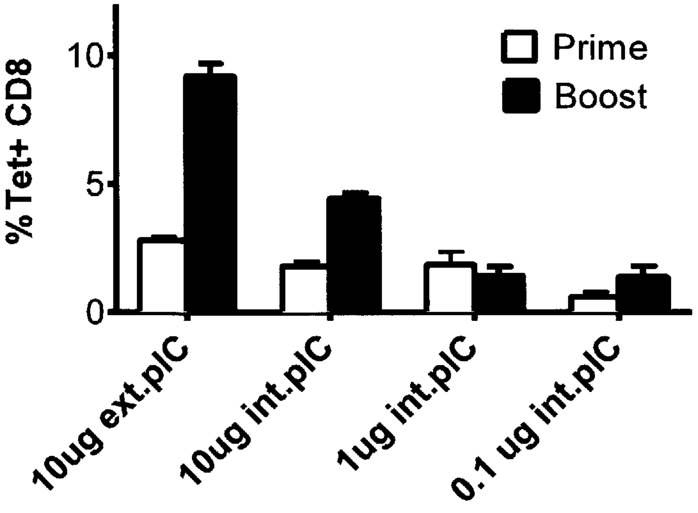


Figure 2-5. Internal vs external poly (I:C) as adjuvants.
Antigen specific CD8 T cells in mice 7 days after prime/boost in blood. Poly (I:C) (10ug, 1ug or 0.1ug) was added externally added (ext) or encapsulated internally (int) into OVA-ICMVs (ICMV with 10 ug ovalbumin encapsulated). Poly (I:C) added externally induced better CD8 response.

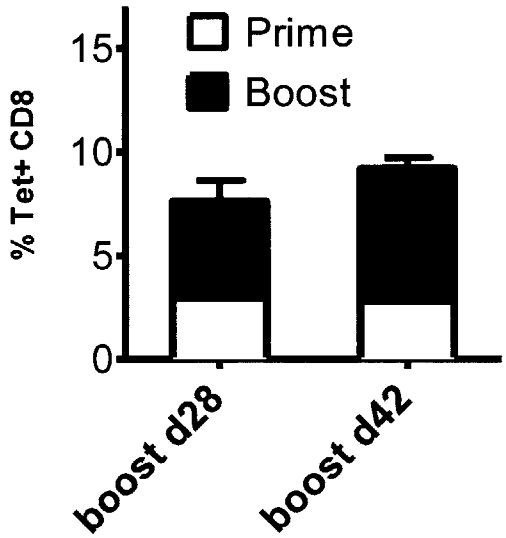


Figure 2-6. Determining when a boost should be administered.
Antigen specific CD8 T cells in mice 7 days after prime and boost (D28 or D42) in blood. Poly(I:C) (10ug) was added externally into OVA-ICMVs (ICMV with 10 ug ovalbumin encapsulated). Boosting on D28 gave similar results to boosting on D42.

ICMV lipid nanoparticles encapsulating the model antigen ovalbumin (OVA) were prepared with or without MPLA embedded in the capsule walls as previously described.[62] Because combinations of TLR agonists (TLRa) can act in a synergistic manner to promote B- and T-cell responses[101, 102], we also assessed the relative of potency of MPLA and pIC co-administered with ICMVs in pulmonary vaccination. As shown in Figure 2-7, ICMVs adjuvanted by MPLA or poly (I:C) both elicited easily detectable OVA-specific CD8+ T-cell responses in the blood, spleen, and lungs, which were further expanded by boosting with the same formulations. Poly(I:C) was more potent than MPLA, but the combination of these two TLRa provided the strongest response, with the dual TLRa vaccine eliciting 15% tetramer+ CD8+ T-cells in the blood and 65% tetramer+ CD8 cells in the lungs at 7 days post-boost (Figure 2-7). ICMVs administered with poly(I:C) also elicited greater frequencies of cytokine-producing CD8+ T-cells both systemically in the spleen and in the lungs when assessed by ICS 7 days post boost (Figure 2-8). Notably, these large frequencies of antigen-specific CD8+ T-cells expanded in both the blood and local mucosal compartments compare favorably to OVA-specific immune responses elicited by live vectors[94,95-97], demonstrating that mucosal nanoparticle vaccination in concert with TLR agonists can prime robust T-cell responses to protein antigens.

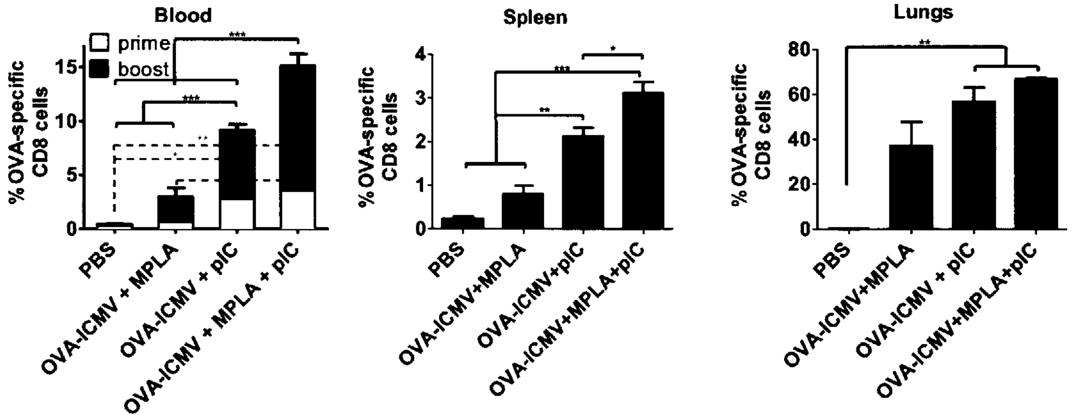


Figure 2-7. Effect of dual TLR agonists on antigen-specific CD8+ T cell response. The effect of dual TLR agonists on CD8+ T cell responses were measured *in vivo*; we immunized C57Bl/6 mice with 10 μg of OVA in ICMVs formulated with 0.3 μg MPLA, 10 μg pIC, or the combinations of the two via intratracheal administration (*i.t.*) on d 0 and 42. Frequency of OVA-specific CD8 T cells was analyzed 7 days after prime (blood) and 7 days after boost (blood, spleen and lungs) by SIINFEKL-MHC I tetramer staining.

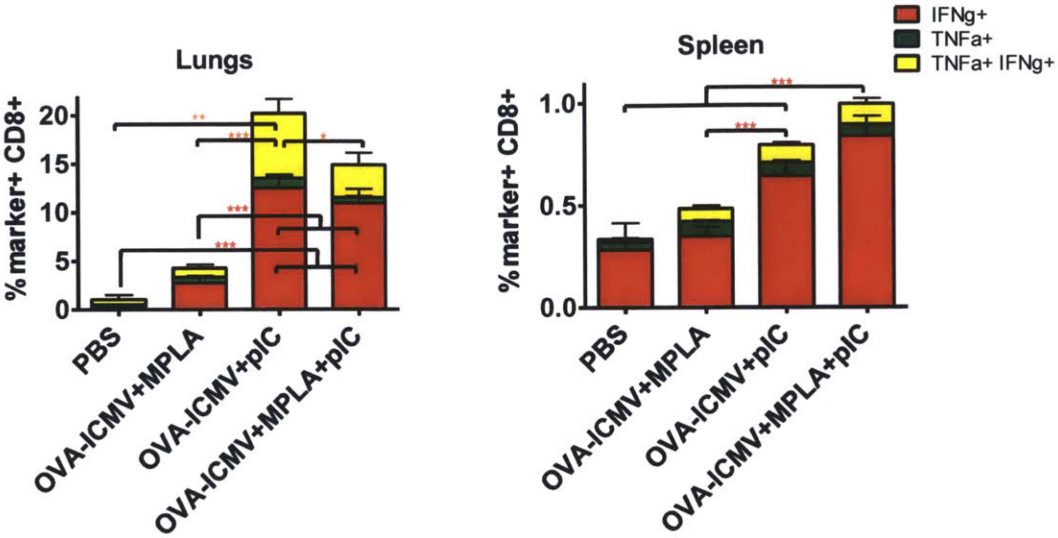


Figure 2-8. Functionality of OVA-specific CD8 T cells with different adjuvants. Functionality of OVA-specific Cd8 T cells was assayed 7 days after boost by restimulation *ex vivo* with SIINFEKL peptide. Presence of intracellular IFN-γ and/or TNF-α was determined by intracellular cytokine staining. 10 µg of OVA in ICMVs formulated with 0.3 µg MPLA and/or 10 µg pIC was administered.

2.3.2. Pulmonary vaccination stimulate stronger response than parenteral injections

After determining that the vaccination regimen and adjuvant formulation giving the most potent response, we continued all our experiments with the same formulation, employing MPLA encapsulated in the ICMVs together with antigen, and poly (I:C) mixed externally with the particles just prior to vaccination. Currently, most vaccines available are delivered by a needle injection, hence, we compared the efficacy of ICMVs given via pulmonary administration against conventional parenteral subcutaneous injection. Mice were immunized on D0 and D28 by either intratracheal instillation or subcutaneous tailbase injection. Seven days after boost (D35) tetramer staining was done on cells isolated from the blood, spleen and lungs (Figure 2-9). Figure 2-9 showed that pulmonary administration of either ICMV or soluble antigen elicited a greater antigen specific CD8 T cells response than a subcutaneous injection in all compartments analyzed. The effect of having antigen encapsulated in ICMV rather than administration of antigen in free soluble form is evident in the results from the pulmonary administration, as antigen encapsulated in ICMVs gave a significantly higher antigen-specific CD8 cell frequency than the soluble antigen; a ~3-4-fold increase in blood and spleen and ~1.5-fold increase in the lungs. Of note, antigen-specific frequency among CD8 T cells in the lungs reached as high as 40-80%, indicating the lungs are a site that can effectively stimulate a strong local CD8 T cell response.

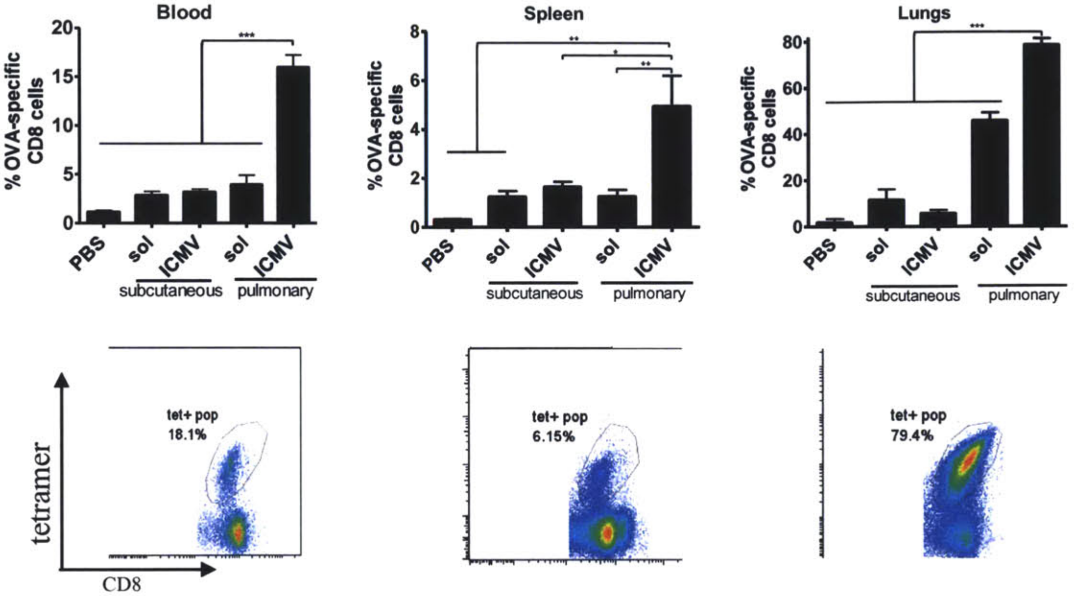


Figure 2-9. Antigen-specific CD8 response to vaccines given through the airway vs parenteral injection.
Frequency of OVA-specific CD8 T cells was analyzed on 7 days after boost in blood, spleen and lungs by SIINFEKL-MHC I tetramer staining. Formulation determined previously: 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and 10 μg pIC, was administered. (ICMV=OVA-ICMV+MPLA + pIC. Sol = soluble OVA+MPLA+pIC). Representative scatter plots are shown.

2.3.3. Pulmonary vaccination stimulates potent disseminated CD8 response

Recent advances suggest that mucosal sites in the body can function together as a system-wide organ.[103] Various studies have found that administration of a vaccine at one mucosal surface can elicit both local and distal mucosal immune responses.[103,104,10] However, the emphasis has been placed on antibody responses and few studies have performed a thorough analysis of the mucosal CD8 T cell response at distal sites. To this end, cells from the vaginal tract and intestinal intraepithelial cells were also isolated after pulmonary immunization in the experiments shown above, and the amount of antigen-specific CD8 T cells were assessed (Figure 2-10). Pulmonary immunization with antigen entrapped in ICMV nanoparticles programmed greater accumulation of memory $CD8^+$ T-cells in the reproductive tract and the gut compared to equivalent soluble antigen/TLRa vaccines. Assessed one week post boost, nanoparticle immunization elicited a 1.7-fold higher frequency of OVA-specific T-cells in the vaginal tract and at least 1.5-fold higher frequency of antigen-specific cells among intraepithelial lymphocyte in the small intestine (depending on the adjuvant used), relative to soluble OVA vaccines.

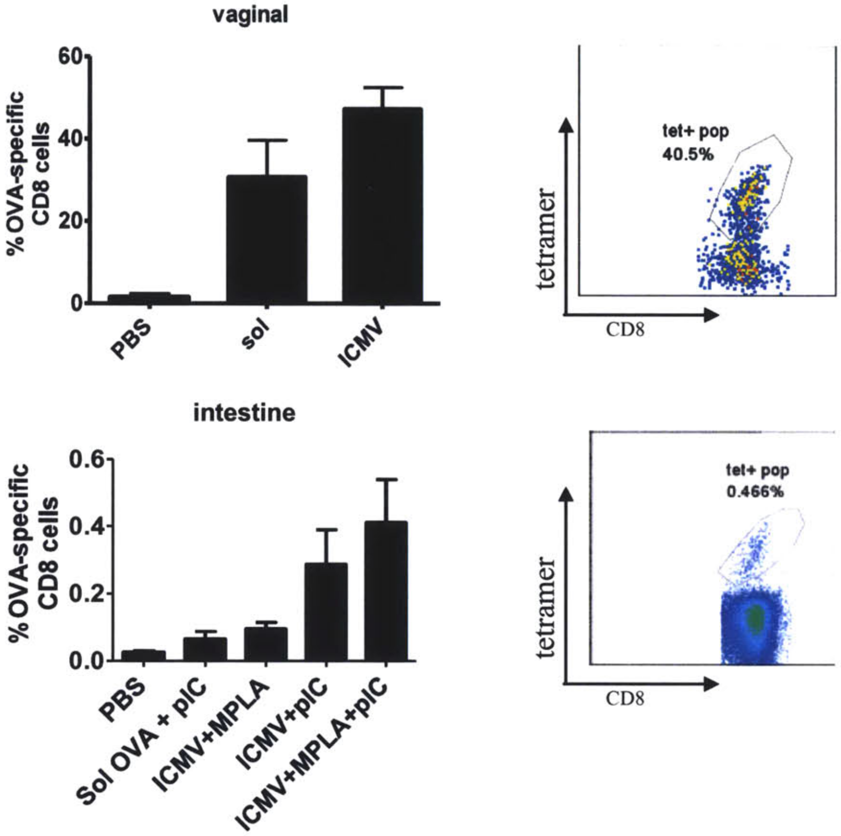


Figure 2-10. Pulmonary immunization elicits disseminated CD8 T cells response at distal mucosal sites.
Frequency of OVA-specific CD8 T cells was analyzed in on 7 days after boost (d35 after prime) in vaginal tissue and small intestine by SIINFEKL-MHC I tetramer staining. No significant difference was found between ICMV and sol group. Formulation determined previously: 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and/or 10 μg pIC, was administered. (ICMV=OVA-ICMV+MPLA + pIC. Sol = soluble OVA+MPLA+pIC).

2.4. Conclusions

In this chapter, we demonstrated that antigen-carrying ICMVs adjuvanted with MPLA and poly (I:C) are highly immunogenic following pulmonary vaccination and promote local and distal mucosal immunity as well as systemic immunity. In addition, pulmonary administration elicited a disseminated CD8 T cell response to the intestine and the vaginal tract. We focused further studies on this TLRa combination and continued to characterize immune response elicited by this system. In chapter 3 we will determine if memory CD8 T cells are generated with ICMVs as long-term protection is an important practical aspect of vaccine development.

3. Pulmonary immunization primes a long lasting disseminated effector memory CD8 T cell response

3.1. Introduction

In Chapter 2, we showed that ICMVs with dual adjuvants can be a potent mucosal vaccine for the CD8 T cell response. When delivered via the pulmonary route, antigen-specific T cells were detected in the systemic and mucosal compartments. In this chapter, we examined the long term response after vaccination.

The goal of T cell vaccines is to generate long-lived memory CD8 cells capable of recognizing and rapidly expanding when re-encountering a pathogen. Reports of disseminated CD8 response after mucosal administration (usually with a live attenuated vaccine)[91, 105-108] are found but few investigate memory cells at disseminated sites. Here, we report a more comprehensive investigation of memory cells not only at the local site but also distal sites with the ICMV synthetic particle vaccine.

We first examined humoral responses generated by pulmonary delivery of ICMVs. Systemic and mucosal IgG was measured and found to be present. We then focused our efforts on memory CD8 T cell characterization.The frequency of antigen specific CD8 T cells at 77 days after immunization was determined for both pulmonary and subcutaneous vaccinations. We then examined the phenotype and quality of antigen-specific memory cells.

The majority (~90%) of $CD8^+$ effector T cells will die after immunization, while the remaining subset survives to become long-lived memory cells, protecting the host from re-infection.[109, 110] Once the long-term memory T cell population is established, these cells can persist for many months or years, undergoing slow basal homeostasis while at the same time maintaining the ability to proliferate extensively should their cognate antigen be re-encountered.[110] Memory T cells are classified into two major subsets: (1) $CD44^{hi}CD62L^{lo}$ T effector-memory (T_{EM}) populations are believed to be responsible for tissue surveillance and able to mount rapid response to antigen challenge. They reside primarily in peripheral tissues and provide a first line of defense against re-infection. (2) The $CD44^{hi}CD62L^{hi}$ central memory T-cell (T_{CM}) subset largely recirculates between the secondary lymphoid organs. They reside primarily in secondary lymphoid organs have a greater capacity for in vivo expansion and require effector function after re-exposure to Ag.[111] The differentiation of effector cells to memory cells involves the progressive acquisition of memory traits over time, generating heterogeneous phenotypic subsets. Recently, it has been shown that the molecules KLRG1 and IL-7R (CD127) can be used to differentiate between two types or subsets of differentiating cells: $KLRG1^{hi}CD127^{lo}$ effector T cells which are rapidly produced during infection and can transiently occupy the memory compartment, and $KLRG1^{lo}CD127^{hi}$ memory T cells which emerge later during infection and generate longer-lived memory cells.[110] For characterization of memory cells, we performed CD44/CD62L and CD127/KLRG1 staining staining to gauge the phenotype and longevity of these cells, respectively.

Our data shows that pulmonary administration of vaccines stimulated generation of memory CD8 cells as antigen-specific CD8 T cells persist for over 2 months after immunization. The majority of memory cells were effector memory ($CD44^{hi}CD62L^{lo}$) cells located at mucosal sites, and they display a $CD127^{hi}KLRG1^{lo}$ phenotype suggesting they are longer-lived memory cells. These observations were more pronounced in the animals receiving ICMV for vaccination. In contrast, subcutaneous injection of vaccines led to a smaller expansion of antigen-specific CD8 cells leading to less effector memory cells. These cells also do not display a tendency to reside in mucosal tissues.

3.2. Materials and methods

3.2.1. Materials

Materials used to make our vaccine and to assess CD8 T cell response in mice were described in Chapter 2. In addition, anti-CD44 (BD), anti-CD62L(ebioscience), anti-CD127(ebioscience), anti-KLRG1 (ebioscience) and anti-granzyme B (ebioscience) were used. Counting beads from invitrogen (cat# PCB100) were used to enumerate cell numbers with flow cytometry. To measure antibody concentrations, HRP conjugated anti-mouse IgG (H+L; Zymed catalog # 81-6720), TMB (3,3′,5,5′-tetramentylbenzidine) (Thermo 34028) and 2N sulphuric acid was used for ELISA. OVA (worthington) was used to coat plates to capture antigen specific antibodies.

3.2.2. In vivo immunization studies

6-10 week old female C57Bl/6 mice were used for immunization studies. Vaccines were administered on D0 and D28 by intratracheal instillation (as described in Chapter 2) or subcutaneous injection at the tail base (*s.c.*). For CD8 T cell analysis, tissues were harvested at indicated timepoints, homogenized and then assayed by tetramer and antibodies staining (all antibodies incubated with cells for 20 mins at RT, after incubation with tetramer) or intracellular cytokine staining as described in Chapter 2. To enumerate cell numbers, counting beads were added before flow cytometry analysis.

3.2.3. Enumerating cell number with counting beads

To enumerate cell numbers, counting beads (invitrogen cat# PCB100) were added after staining, before flow cytometry analysis. The number of cells of interest was calculated as follow:

$$\#\ \text{cells of interest in well} = \#\ \text{counting beads in well} \times \frac{\#\ \text{events within gate of interest}}{\#\ \text{events within gate for counting beads}}$$

total number of cells of interest in whole sample = # cells of interest in well × dilution factor

3.2.4. Characterization of humoral response by ELISA

Serum, vaginal washes and fecal pellets were collected at 10-11 weeks after immunization. Blood was collected by retro-orbital bleed and serum was separated by centrifugation and stored at -80°C. Vaginal washes were collected by washing the vaginal cavity with 10 mL of PBS x 4 times (40 mL total) and stored at -80 °C. Fecal samples were prepared according to protocol detailed in "Appendix A: Protocol for processing fecal samples for antibody measurement by ELISA". Samples were assayed for OVA-specific IgG using ELISA Anti-OVA IgG concentration was determined by including a monoclonal mouse anti-ova IgG1 (clone OVA-14, Sigma-Aldrich, St. Louis, Missouri) as a standard reference in each assay.

3.2.5. Statistical Analysis

Data were pooled from multiple repeated experiments with $n \geq 3$. All data was analyzed by two-way analysis of variance followed by Bonferroni post-test. Data represent the mean±s.e.m. *, $p<0.05$; **, $p<0.01$, *** $p<0.01$

For vaginal tissue in Figure 3-9, data was pooled from multiple repeated experiments with $n \geq 3$. Data was analyzed by two-tail t-test. Data represent the mean±s.e.m. *, $p<0.05$; **, $p<0.01$, *** $p<0.01$

3.3. Results and Discussion

3.3.1. Pulmonary immunization elicits systemic and mucosal antigen specific humoral responses

In addition to the CD8 T cell response, we also confirmed that pulmonary vaccination with OVA-ICMVs can elicit local and distal mucosal antibody responses. Both soluble OVA and OVA-ICMV vaccines elicited OVA-specific IgG in serum and vaginal washes (
Figure 3-1), although no significant difference was detected between the 2 groups.

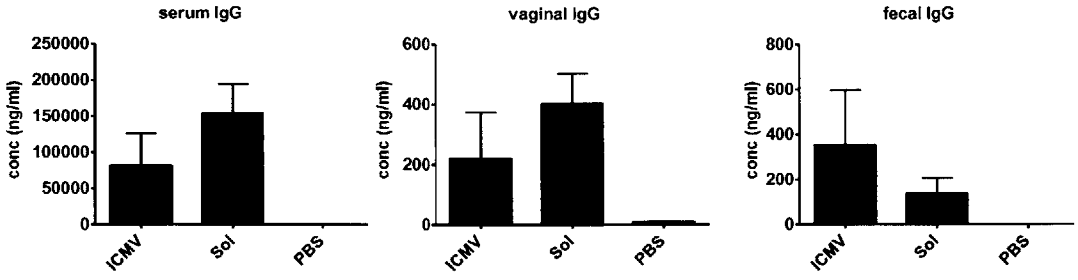


Figure 3-1 Characterization of humoral response elicited by pulmonary immunization.

OVA-specific IgG in serum, vaginal washes and fecal samples on D77 after prime measure by ELISA. Formulation as determined previously: 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and 10 μg pIC was administered on D0 and D28. (ICMV=OVA-ICMV+MPLA + pIC. Sol = soluble OVA+MPLA+pIC).

3.3.2. Persistence of antigen-specific memory T cells after pulmonary immunization

We compared the persistence of antigen specific T cells following pulmonary immunization with ICMVs or vaccines comprised of the same antigen and adjuvant doses administered in soluble form. C57Bl/6 mice were immunized with soluble OVA+MPLA+pIC or OVA-ICMV+MPLA+pIC vaccines *i.t.* on days 0 and 28, and the frequency and absolute number of tetramer+ $CD8^+$ T-cells in lymphoid organs, blood, and peripheral tissue sites were evaluated over time. As shown in Figure 3-2, pulmonary nanoparticle vaccination triggered a remarkable expansion of OVA-specific CD8+ cells compared to the soluble OVA with adjuvants, with 5.3-fold more OVA-specific CD8+ T-cells in the lung tissue, 5.3-fold more in the mediastinal lymph nodes (mLN) and 5.4-fold more in the spleen. These responses represent an approximate 100-1000 fold expansion of the naive OVA-specific T-cell population in these mice (previously estimated at between 70 and 600 OVA-specific CD8+ T-cells per mouse.[112] Antigen-specific T-cells primed by the nanoparticle vaccine established a substantially greater memory population in both the local lung tissue and the systemic spleen compartment, with 6.7-fold and 3.5-fold more OVA-specific $CD8^+$ cells in these tissues at 11 weeks post-prime, respectively, compared to the same doses of antigen and adjuvant given in soluble form. We also assessed dissemination of OVA-specific T-cells to distal mucosal sites, and found that pulmonary immunization with antigen entrapped in ICMV nanoparticles programmed substantially greater accumulation of memory $CD8^+$ T-cells in the reproductive tract compared to equivalent soluble OVA+MPLA+pIC vaccine. Assessed 11 weeks post-prime, nanoparticle immunization elicited a 3.5-fold higher frequency of OVA-specific

T-cells in the vaginal tract ($p < 0.001$, Figure 3-3). T-cells primed by mucosal nanoparticle vaccination also exhibited greater functionality compared to soluble protein vaccines: At day 7 post boost, ~15% of $CD8^+$ T-cells in the lungs produced effector cytokines on *ex vivo* restimulation with OVA peptides, compared to ~4% cytokine-producing cells following soluble OVA vaccination, and 6-fold more T-cells produced multiple cytokines following nanoparticle vaccination ($p < 0.05$, Figure 3-4). In addition, expression of granzyme B was elevated 4.9-fold in tetramer^{++} T-cells following nanoparticle vaccination compared to soluble protein immunization ($p < .01$, Figure 3-5). Thus, ICMVs can stimulate durable antigen-specific CD8 T cell response at various sites; systemic / mucosal immune tissue, lymphoid / effector sites, through pulmonary administration. These cells display functional characteristics and are capable of initiate a response when reencountering their specific antigen.

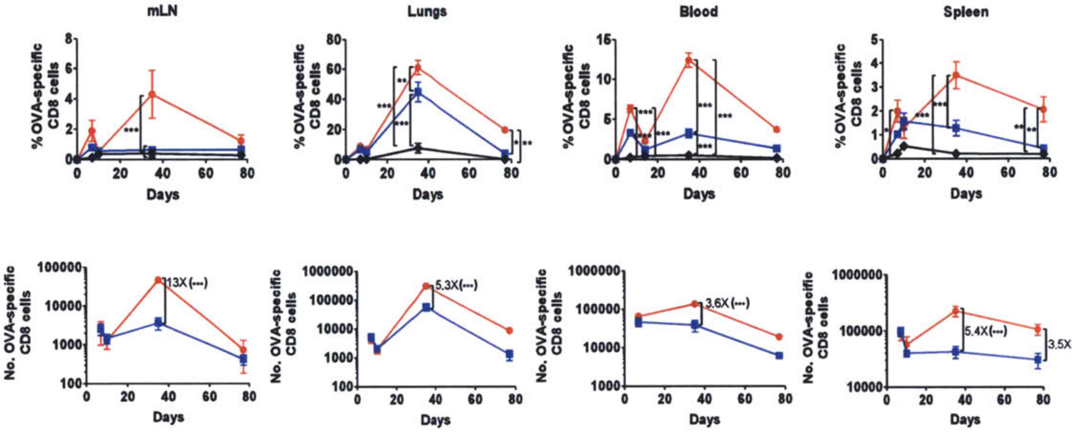


Figure 3-2. Expansion and persistence of antigen-specific CD8 T cells over time. Frequency and absolute number of antigen-specific CD8 T cell in mediastinal LN, Lungs, blood and spleen after pulmonary immunization of OVA encapsulated in ICMV or in soluble form up to D77 after prime. Formulation determined previously: 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and 10 μg pIC was administered on D0 and D28. (Red = OVA-ICMV+MPLA+pIC, Blue=soluble OVA+MPLA+pIC)

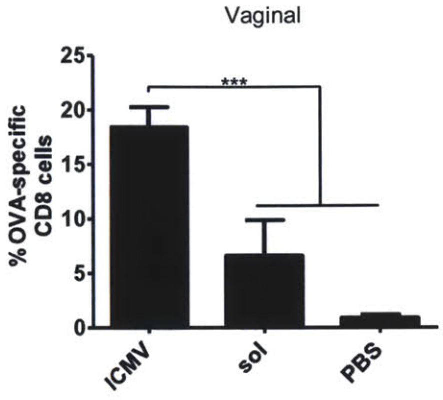


Figure 3-3. Persistence of antigen-specific tissue in vaginal tissue.
Frequency and absolute number of antigen-specific CD8 T cell in vaginal tract after pulmonary immunization of OVA encapsulated in ICMV or in soluble form up on D77 after prime. Formulation determined previously: 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and 10 μg pIC was administered on D0 and D28. (ICMV = OVA-ICMV+MPLA+pIC, Sol=soluble OVA+MPLA+pIC)

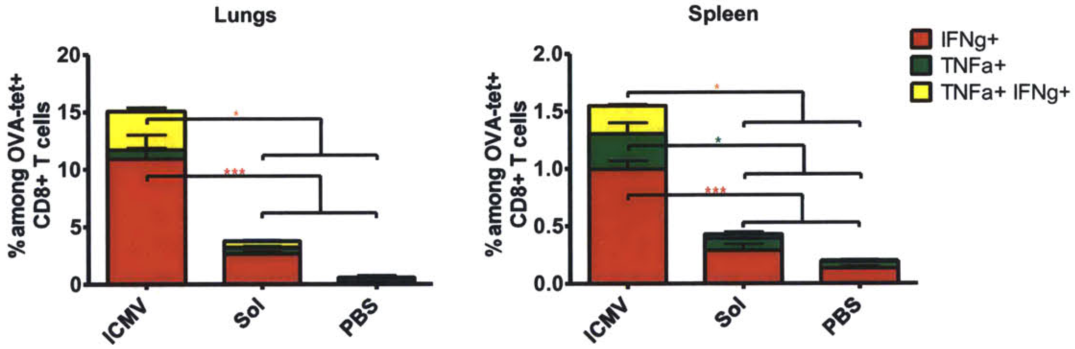


Figure 3-4. Functionality of OVA-specific CD8 T cells after pulmonary immunization with OVA-ICMV or soluble OVA.
Functionality of OVA-specific CD8 T cells was assayed 7 days after boost by restimulation *ex vivo* with SIINFEKL. Presence of intracellular IFN-γ and/or TNF-α was determined by intracellular cytokine staining. Formulation determined previously: 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and 10 μg pIC was administered on D0 and D28. (ICMV = OVA-ICMV+MPLA+pIC, Sol=soluble OVA+MPLA+pIC)

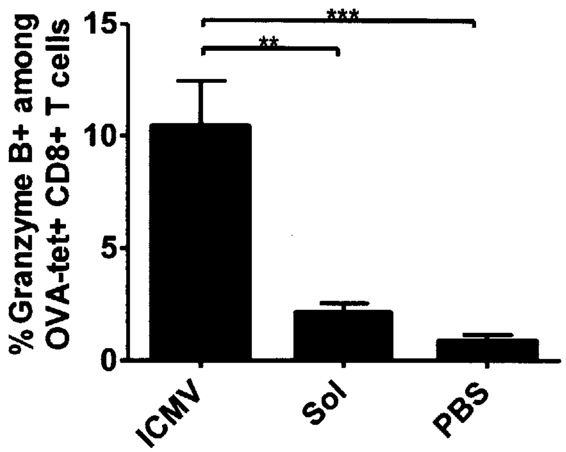


Figure 3-5. Secretion of granzyme B in antigen-specific cells.
Presence of intracellular granzyme B was determined by intracellular cytokine staining after ex vivo restimulation of cells from lungs on D7 after boost with SIINFEKL. Formulation determined previously: 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and 10 μg pIC was administered on D0 and D28. (ICMV = OVA-ICMV+MPLA+pIC, Sol=soluble OVA+MPLA+pIC)

3.3.3. ICMV promotes robust effector memory T cell response

The priming of persistent CD8+ T-cell populations in both systemic and mucosal tissue compartments led us to examine the phenotype of memory cells induced by nanoparticles in mucosal vs. *s.c.* vaccination. C57Bl/6 mice were vaccinated *i.t.* or *s.c.* with soluble OVA+MPLA+pIC or OVA-ICMV+MPLA+pIC, boosted on day 28, and cells were isolated from the lungs, mediastinal LNs (mLN), blood, spleen, vaginal tract (VAG), and inguinal LNs (ING), for analysis of memory markers by flow cytometry. Pulmonary vaccination with soluble or ICMV vaccines elicited antigen-specific T-cells biased to an effector memory ($CD44^{hi}CD62L^{lo}$) phenotype (Figure 3-6), but this was particularly pronounced for the nanoparticle vaccine, where more than 90% of the total antigen-specific cells were $T_{EM.}$ (Figure 3-7) The greater proportion of T_{EM} cells elicited by mucosal nanoparticle vaccination was accompanied by ~5-7-fold greater absolute numbers of T_{EM} cells in both systemic and mucosal tissues compared to soluble vaccines (Figure 3-6). In addition, the ratio of mucosal:systemic memory CD8 T cells resulting from each vaccine showed that mucosal nanoparticle vaccines primed a larger fraction of the total elicited memory T cell pool to home into the mucosal compartment (Figure 3-8).

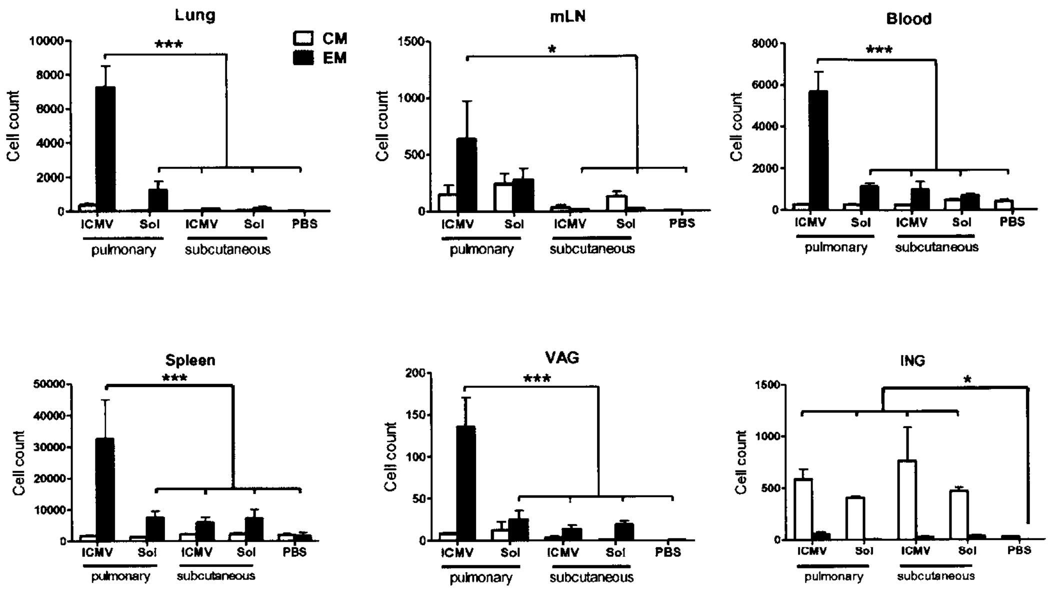


Figure 3-6. Analysis of central and effector memory cells in various tissues.
The absolute number of central (CD44^{hi}CD62L^{hi}) and effector (CD44^{hi}CD62L^{lo}) antigen –specific CD8 cells in different compartments (Lungs, mediastinal LN, blood, spleen vaginal tract and inguinal LNs) were determined on D77 after prime by CD44/CD62L staining on tetramer+ cells. Formulation determined previously: 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and 10 μg pIC was administered on D0 and D28. (ICMV = OVA-ICMV+MPLA+pIC, Sol=soluble OVA+MPLA+pIC)

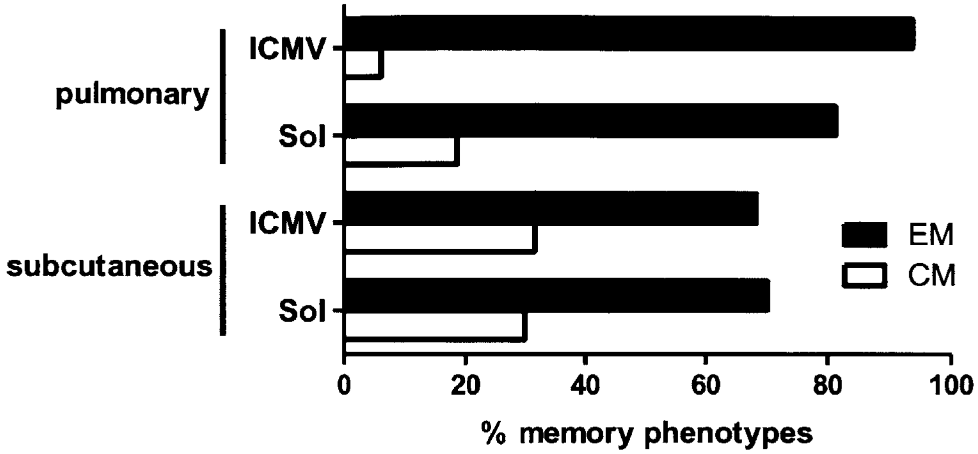


Figure 3-7. Overall frequency of effector and central memory cells in immunized mice.

Effector and central memory cell numbers from tissue analyzed in Figure 3-6 were summed together for a systemic view of memory cells present in a whole mouse after vaccination. Effector memory and central memory antigen-specific CD8 T cells in the whole mouse were determined by summing the mean number of effector cells and central memory cells in all tissues (Lungs, mLN, blood, spleen, vaginal tissue (VAG), inguinal LN(ING)) analyzed in each group [i.e. sum of mean number of effector or central memory tetramer+CD8 T / sum of mean number of tetramer+ CD8 T)].

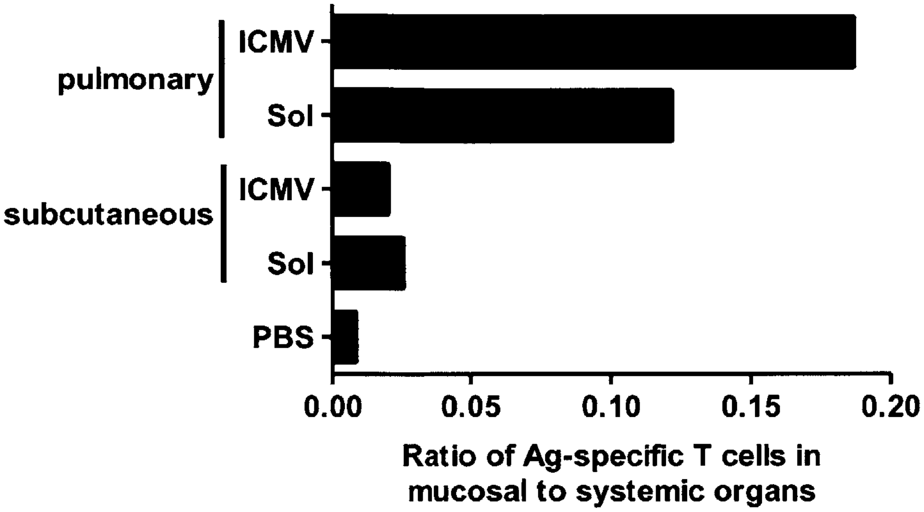


Figure 3-8. Ratio of antigen-specific CD8 T cells in mucosal to systemic organs. The ratio of mucosal:systemic memory CD8+ T-cells resulting from each vaccine group was calculated as follow: (sum of mean number of effector memory CD8 T in lungs and vaginal tract) / (sum of mean number of effector memory CD8 T in spleen, blood, mediastinal LNs).

3.3.4. ICMV promotes long lasting effector memory T cell response

Finally, we assessed whether effector memory cells observed in systemic and mucosal compartments exhibited markers of long-lived cells to provide durable protection against pathogens. This was done by analyzing the frequency of antigen-specific T-cells expressing markers of long-lived memory cells. Recently, it has been shown that the coordinate expression of CD127 and killer cell lectin-like receptor G1 (KLRG1) can distinguish short-lived effector T-cells ($CD127^{lo}KLRG1^{hi}$) from those that will become long-lived memory cells ($CD127^{hi}KLRG1^{lo}$).[113, 114] Wild type C57Bl/6 mice were immunized with soluble or ICMV vaccines *i.t.*, and we compared the frequency of antigen-specific memory cell precursors present 7 days post-prime and 7 days post-boost in the lungs, mediastinal LNs, spleen, and blood. At 7 days following prime, the frequency of $tetramer^{+}CD127^{hi}KLRG1^{lo}$ cells was at a very low level for both OVA-ICMV and soluble OVA vaccines. However, 7 days post boost, 20-30% of antigen-specific T-cells in the blood and spleen in both groups expressed memory cell markers (Figure 3-9A). However, the frequency of $CD127^{hi}KLRG1^{lo}$ memory precursors among OVA-specific T-cells was increased ~5-fold in the lungs and mLNs compared to soluble vaccines (Figure 3-9A). This difference in the frequency of memory cell precursors combined with the quantitatively much greater expansion of T-cells gave a much larger pool of T-cells entering the memory pool post ICMV-vaccination, compared to the soluble vaccine (Figure 3-9B), is consistent with the increased number of memory cells found in the blood and mucosal compartments at late times post vaccination.

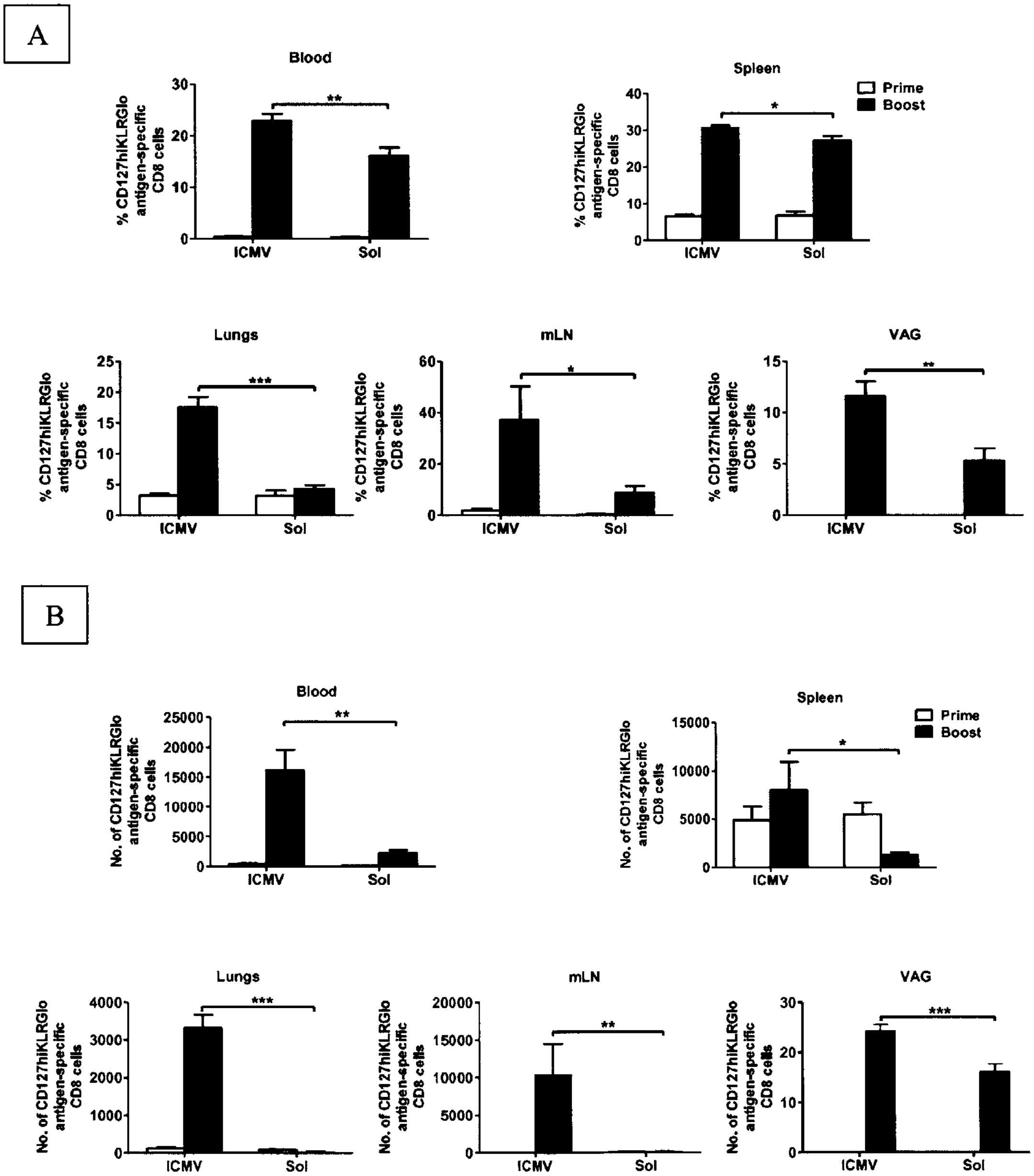


Figure 3-9. Nanoparticle vaccination increases generation of long lasting memory cells.

C57Bl/6 mice were immunized with OVA-ICMV with dual adjuvants as above on D0 and D28. Number (A) and frequency (B) of long lived effector memory CD8 T cells

($CD127^{hi}KLRG1^{lo}$) in different compartments (Lungs, mediastinal LN, blood, spleen) were determined on 7 days after prime and boost by CD127/KLRG1 staining on tetramer+ cells. For vaginal tissue, only data on D7 after boost was collected.

3.4. Conclusions

In this chapter, we confirmed that pulmonary vaccination of ICMVs can stimulate potent antigen-specific memory CD8 T cell response. Antigen specific memory cells were detected at disseminated effector sites and were mainly effector memory cells. These effector memory cells have the ability to reside at effector tissues and launch an immune response immediately upon reencountering the specific antigen. Results from staining markers of memory cells suggest that nanoparticle vaccination drives a more efficient induction of memory cell precursors compared to soluble antigen/adjuvant vaccines. Comparison to conventional subcutaneous injection of the vaccine suggests that the pulmonary route of vaccination can provide more potent and broader protection in animals and confirms that mucosal immunization triggers a stronger mucosal immune response than a parenteral injection through the common mucosal immune network.

4. Efficacy and safety of pulmonary immunization with ICMV nanoparticles

4.1. Introduction

In previous chapters, we established that pulmonary delivery of ICMVs is a potent inducer of CD8 T-cell responses. In this chapter, we investigate key preclinical development issues of ICMV nanoparticles and focus on efficacy and safety of this system.

An important hallmark of a successful vaccine is demonstrating ability to protect from a live challenge. Currently, there are many studies exploring the use of synthetic nanoparticles for mucosal vaccines but only a few studies have demonstrated mucosal protection.[38, 73, 78, 81, 83, 100, 115-120] When investigating mucosal immune response triggered by the pulmonary administration of liposome vaccines, all studies evidenced a local production of specific IgA in bronchoalveolar lavages or nasal secretions, whatever the nature of the transported molecule (DNA, peptide or protein) or the targeted pathogen[121] and local cellular response have also been reported[78].Few have investigated distal cellular immune response[76, 87, 122, 123] with the majority demonstrating cellular response in splenocytes only. Efforts towards showing distal mucosal cellular response[122] is particularly of interest as the possibility of inducing a genital/rectal immune response is attractive for vaccines targeting pathogens that disseminate during sexual contact, such as HIV.[121]

To ensure that ICMVs effective as a pulmonary vaccine, we first challenged mice with OVA-expressing tumors cells and confirmed that CD8 T cell responses induced with ICMV particles are capable of killing antigen-expressing tumor cells in a therapeutic setting. We then tested the versatility of this system and its potential to deliver different antigens. We chose AL11, an immunodominant pepide present on SIV *gag* protein as a new antigen. This SIV *gag* target is a model antigen for HIV vaccines. At this point, we also compared immunization using intratrachael delivery vs. intranasal delivery as intranasal immunization is a potentially simpler method to deliver vaccine into the lungs. Finally, an AL11-expressing vaccinia virus that infects mice was used to challenged immnuized mice to confirm efficacy of ICMVs *in vivo*.

Besides efficacy, safety is a major concern for any pharmaceutical product, especially vaccines that will be administered to healthy individuals. We evaluated inflammation and possible toxicity of ICMVs following pulmonary vaccination, and observed no significant difference compared negative control groups in terms of clinical signs of distress or damage in the lungs.

4.2. Materials and methods

4.2.1. Materials

Materials used to make our vaccines and to assess the CD8 T cell response in mice were as described in Chapter 2. In addition, AL11 tetramer was used to identify antigen-specific CD8 T cells. PADRE (AKFVAAWTLKAAA) and AL11 (AAVKNWMTQTL) peptides were synthesized at the Koch Institute and Tufts University, respectively. Counting beads from invitrogen (cat# PCB100) were used to enumerate cell numbers with flow cytometry. A bead-based multiplex assay from BD biosciences (Cytometric Bead Array, mouse Th1/Th2/Th17 kit, cat# 560485) was used to analyze cytokines.

B16-OVA cells were purchased from ATCC. Vaccinia virus expressing AL11 peptide was kindly provided by the laboratory of Prof Dan Barouch (Harvard Medical School). CV-1 cells (cat# CCL70) and Eagle's Minimum Essential Medium (EMEM) (cat# 30-2003) were purchased from ATCC. Crystal violet (sigma cat# C0775-25G) was dissolved in 20% ethanol for staining cells. Medroxyprogesterone was purchased from sigma. 10% Neutral buffered formalin (cat# 3800598) from Leica was used for fixing tissue for histology.

4.2.2. Immunization and AL11 tetramer staining

Animals were cared for following federal, state and local guidelines. Groups of 6- to 10-week old female C57Bl/6 mice (Jackson Laboratories) were immunized via intra-tracheal administration (*i.t.*), intra-nasal administration (i.n.) (see Appendix D: Intranasal immunization with ICMVs for detailed protocol) or subcutaneous injection at the tail base (*s.c.*) with antigens (AL11 peptide, PADRE), each encapsulated in separate ICMVs, with optimized doses (10 μg AL11, and 3.3 μg PADRE) with MPLA and poly (I:C) at the same doses as before on days 0 and 28. Control groups included immunization with the equivalent dose of soluble antigen and TLR agonists or PBS. Mice were sacrificed on D77 after prime and cells were isolated from tissue as mentioned in chapter 2. Evaluation of antigen specific CD8 T cells was performed by staining with AL11/H-2K^b peptide–MHC tetramers (Becton Dickinson) following the same protocol as staining with SIINFEKL tetramer mentioned in chapter 2.

4.2.3. Tumor challenge

Mice were inoculated with 50,000 B16F10-OVA cells *s.c.* in the flank, and on days 3 and 10, the mice were administered with OVA-ICMV+MPLA+pIC or soluble OVA+MPLA +pIC intratracheally.

4.2.4. Vaccinia challenge

Mice were immunized with AL11 and PADRE in separate ICMVs with MPLA and pIC on D0 and D28. Two weeks after boost, a single dose of AL11-expressing vaccinia (~4 x 10^6 PFU) was administered intratracheally or intravaginally. Intra-tracheal administration of virus follows the same procedure as administration of ICMVs described above. For intravaginal infections, immunized mice were injected subcutaneously with 2 mg of medroxyprogesterone to synchronize their estrus cycles 5 days before challenge. On the day of challenge, mice were anaesthetized with avertin and ~4×10^6 PFU of vaccinia virus (20uL) was administered into the vagina with a pipette. Weights of mice were monitored every day after challenge. On D5 after challenge, mice were euthanized and tissue (lungs and ovaries) were collected to determine viral titers by plaque assay.

4.2.5. Plaque assay

Ovaries were harvested in 1 ml PBS, homogenized through a 40 µm cell strainer and snap frozen in liquid nitrogen. Samples were then thawed, vortexed, and snap frozen again for 4 times. Samples were then placed in a sonication bath for 1 min before serially diluting stepwise 1:10 (10^2-10^7). One ml of each dilution was placed on a confluent layer of CV-1 cells in 6 well plates and incubated at 37°C for two hours, prior to aspiration and addition of 2 ml EMEM + 10%FCS to each well. Plates were incubated for an additional 48 hours at 37°C prior to aspiration and staining and fixing with ~500 µl of 0.1% crystal violet in 20% ethanol for 5-10 min. After removing the staining solution, the plates were air-dried and then counted to determine viral titers.

4.2.6. Histology

Mice were immunized on D0 and D28. Lungs were removed on D1, D7, D29 and D35 and placed into 10% neutral buffered formalin immediately for fixation. After 24 hrs, tissues were transferred to 70% ethanol for dehydration and storage. Samples were then embedded in paraffin and sectioned. H&E staining were performed and inflammation was scored by a pathologist. To determine clinical signs of distress after *i.t.* immunizations, pathological assessment of lungs were performed. Mice were immunized on days 0 and 28 as denoted above, lungs were collected on days 1, 7, 29, and 35, fixed in 10% neutral buffered formalin, processed for sectioning and H&E staining, then scored by a pathologist.

4.2.7. Cytokine analysis

Mice were immunized and lungs, serum and bronchoalveolar lavage were collected. Cytokine analysis (IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17A, IFNγ, TNFα) was done according to manufacturer's protocol of the "mouse Th1/Th2/Th17 kit" (BD cat# 560485).

4.2.8. Statistical analysis

Experiments were done with $n \geq 3$ for tetramer staining, histology and cytokine studies, and $n \geq 5$ per group for challenge studies. Comparison of survival curve for tumor challenge was performed using the log rank test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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4.3. Results and Discussion

4.3.1. Pulmonary ICMV vaccination confers protection in a therapeutic model of cancer therapy

To determine whether the strong T_{EM} responses elicited by pulmonary vaccination with ICMV lipid nanoparticles enhanced the protection elicited by subunit vaccines, we tested the efficacy of these vaccines in both therapeutic tumor and prophylactic viral challenge models. To first test the efficacy of immunization against the model antigen, OVA, we inoculated C57Bl/6 mice *s.c.* with $5x10^5$ OVA-expressing B16F10 melanoma tumor cells and gave therapeutic pulmonary vaccinations on d3 and 10 with soluble OVA+MPLA+pIC or OVA-ICMV+MPLA+pIC. As shown in Figure 4-1, mucosal vaccination with soluble OVA protein delayed tumor growth but did not improve the ultimate survival of animals compared to untreated controls. In contrast, ICMV vaccination led to 100% rejection of tumors and long-term survival of all mice.

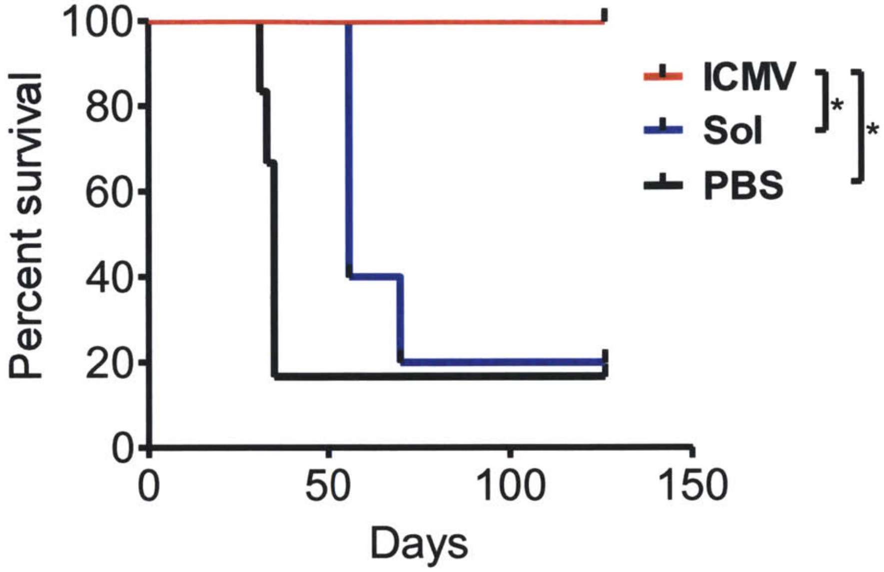


Figure 4-1. Survival of mice challenged with B16-OVA tumor cells.
Mice inoculated with 5×10^4 B16-OVA tumor cells in the flank were immunized *i.t.* with OVA-ICMV nanoparticles or soluble OVA on D3 and D10. Survival of mice was monitored after administration of the therapeutic vaccine. Formulation determined previously: 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and 10 μg pIC was administered. (ICMV = OVA-ICMV+MPLA+pIC, Sol=soluble OVA+MPLA+pIC)

4.3.2. ICMV nanoparticles carrying a peptide vaccine mount strong CD8 T-cell responses against a model HIV antigen

We then proceeded to test the ability of ICMVs to enhance the efficacy of a mucosal peptide vaccine. We chose to immunize against AL11, an immunodominant CTL epitope (in C57Bl/6 mice) derived from SIV-*gag*, AAVKNWMTQTL.[124] Since this antigen is a peptide, a helper epitope was added to induce CD4 T cells that could promote the antigen-specific CD8 immune response. Hence, the universal $CD4^+$ T-cell helper epitope PADRE (AKFVAAWTLKAAA)[125, 126] was included in the vaccine. Figure 4-2 show that addition of PADRE significantly enhanced the frequency of AL11-specific CD8 cells elicited by vaccination with the peptide vaccine in both soluble or particulate form. The adjuvants MPLA and pIC were added to all groups as before. Note that data shown in Figure 4-2 was obtained from mice immunized with an sub-optimized dose. Additional titration studies were done (data not shown) to optimize both the AL11 and PADRE dose for vaccination. All subsequent immunizations were later done at the optimized dose (10 μg AL11 and 3.3 μg PADRE) with MPLA and pIC.

We administered vaccines comprised of 10ug AL11 (AAVKNWMTQTL)[124] and 3.3ug PADRE (AKFVAAWTLKAAA)[125, 126] each encapsulated in separate ICMVs (AL11-ICMV and PADRE-ICMV, respectively) or administered in soluble form *i.t.* together with MPLA and poly (I:C) on days 0 and 28. Control mice received equivalent vaccines administered subcutaneously. Enumeration of AL11-specific $CD8^+$ T-cells in the blood and lungs at 11 weeks via tetramer staining revealed increased numbers of antigen-specific cells in both the systemic circulation and local tissue elicited by the mucosal nanoparticle vaccine, similar to our findings with OVA protein (Figure 4-3).

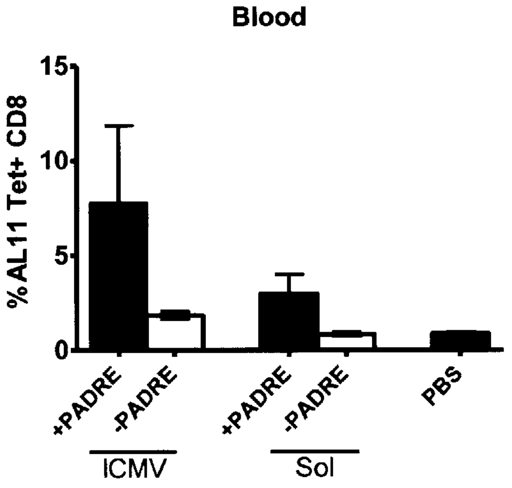


Figure 4-2. PADRE is needed to enhance AL11-specific CD8 T cell response. Preliminary data comparing AL11 administered to mice in ICMV or soluble form with or without PADRE (75 μg AL11, 13 μg PADRE). Mice were immunized *i.t.* on D0 and D28. Blood is collected on D35 and AL11 tetramer staining is performed. MPLA and pIC were added to each group as described before. Note that AL11 and PADRE given were not at the optimized dose in this experiment. (ICMV = AL11-ICMV+PADRE-ICMV+MPLA+pIC, Sol=soluble AL11+soluble PADRE+MPLA+pIC)

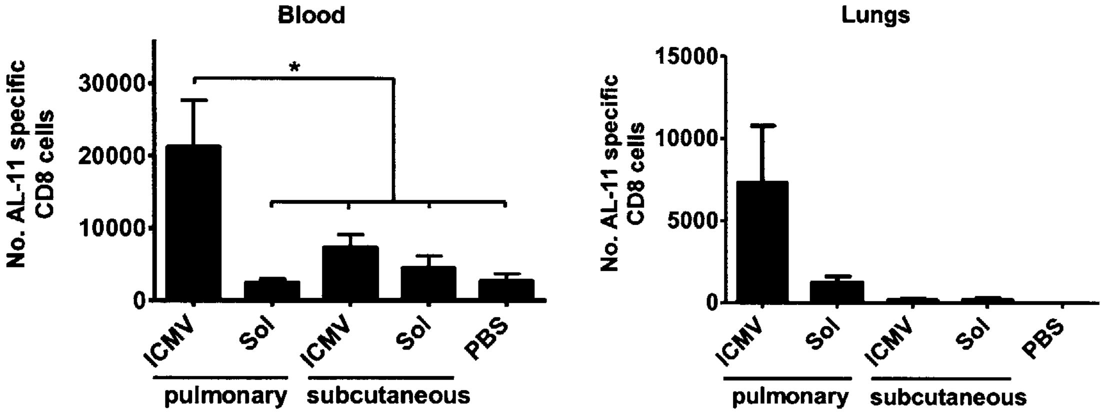


Figure 4-3. Pulmonary immunization with ICMVs generates potent AL11 specific CD8 T cells response.

C57Bl/6 mice were immunized with AL11 peptide (10 μg) and PADRE helper peptide (3.3 μg) in solution or encapsulated in ICMVs formulated with MPLA (0.3ug) and pIC (10ug) on d0 and d28. Frequency of AL11-specific CD8 T cells on D77 in blood and lungs were determined using AL11-MHC I tetramer staining and flow cytometry. (ICMV = AL11-ICMV+PADRE-ICMV+MPLA+pIC, Sol=soluble AL11+soluble PADRE+MPLA+pIC)

4.3.3. Intranasal administration of ICMVs

So far, we chose to deliver ICMVs intratracheally into the lungs since a defined and consistent dose of vaccine can be delivered, making our comparison between different treatments groups more accurate. This approach models aerosol-based pulmonary vaccines in various stages of development.[127-129] To evaluate the possibility of translating this vaccine system towards a simpler clinical route of administration (e.g. vaccination with a nasal spray), we administered vaccines intranasally and compared the response to the response delivered intratracheally and found that intranasal delivered ICMVs elicited similar levels of AL11 specific CD8 T cell frequencies to that obtained by *i.t.* administration, while the soluble antigen remained significantly less effective (Figure 4-4).

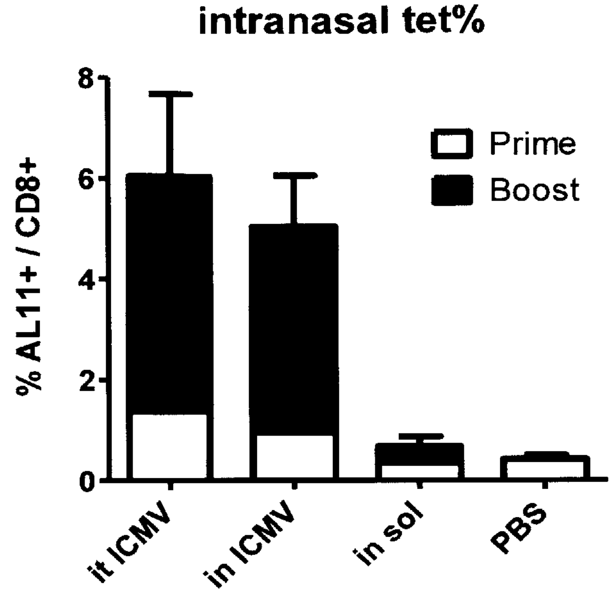


Figure 4-4. Intranasal vs intratracheal administration of vaccines.
Mice were administered the same AL11 and PADRE vaccines encapsulated in ICMVs or soluble form and delivered into the airway via 2 different methods on D0 and D28 (10ug g AL11, 3.3ug PADRE). Blood was taken 7 days after prime and after boost to determine frequency of AL11 specific CD8 T cells by tetramer staining.(it = intratracheal, in = intranasal, ICMV = AL11-ICMV+PADRE-ICMV+MPLA+pIC, Sol=soluble AL11+soluble PADRE+MPLA+pIC)

4.3.4. Pulmonary ICMV nanoparticle vaccines confer protection against vaccinia virus challenge

Vaccinia virus was used for our live pathogen challenge studies. Vaccinia is the virus used for immunization against smallpox and a well-studied laboratory model particularly for poxvirus biology and immunity. The virus has broad cellular tropism and can infect almost any cell line in culture.[130] *In vivo* studies have shown that vaccinia virus exhibits a strong tropism for ovarian tissue and can cause ovary pathology and sterility.[131, 132]

A SIV-*gag*-expressing vaccinia[133] was used as the challenge virus to assess protection afforded by prophylactic ICMV vaccination. Mice were immunized with AL11 and PADRE in soluble or particulate form via the pulmonary or subcutaneous routes on D0 and D28. Vaccinia-SIV-*gag* (~4 x 10^6) was administered intratracheally on D42. As shown in Figure 4-5, mice receiving *s.c.* or soluble peptide pulmonary vaccines showed steady weight loss not statistically different from naive animals, leading to 100% mortality by day 5 post challenge, while animals receiving the mucosal ICMV vaccine showed only minor weight loss that was recovered by day 5, and no mortality. Plaque assays on the lungs and ovaries of animals at Day 5 showed that *s.c.* vaccines or soluble vaccine given *i.t.* had a minor impact on viral titers in the lungs (Figure 4-6). Pulmonary soluble vaccine and *s.c.* ICMV particles protected a fraction of mice from viral dissemination to the ovaries (3/6 and 4/6 respectively, though this did not lead to protection from mortality). In contrast, pulmonary ICMV vaccination elicited a 2-log reduction in vaccinia PFU in the lungs (Figure 4-6A) and completely blocked dissemination of the virus to the ovaries (Figure 4-6B). Thus, the enhanced numerical expansion of CD8+ T-cells and their higher level of effector functions elicited by mucosal nanoparticle vaccination provided substantially enhanced protection from mucosal virus challenge.

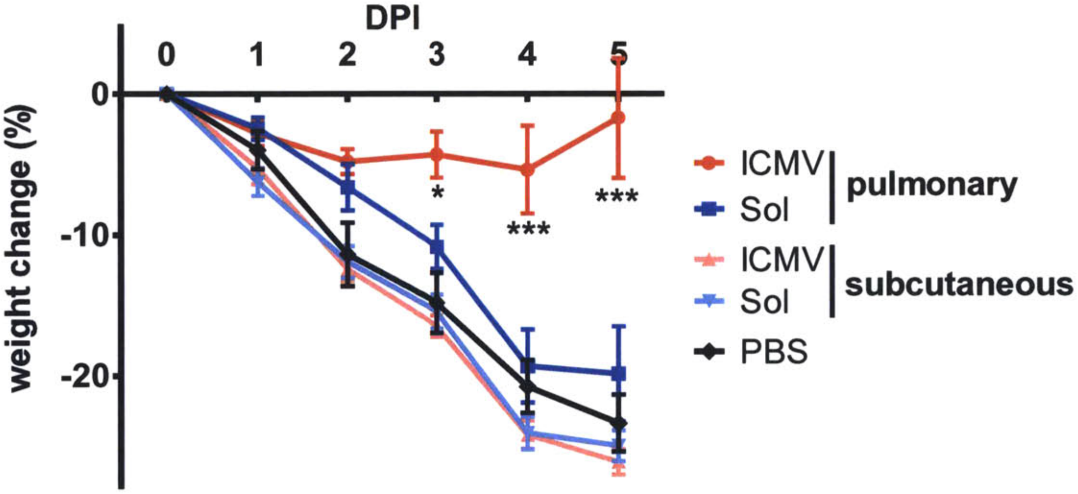


Figure 4-5. Change in body weight of mice infected with vaccinia virus.
Mice were administered the same AL11 and PADRE vaccines encapsulated in ICMVs or soluble form and delivered *i.t.* or *s.c.* on D0 and D28 (10ug g AL11, 3.3ug PADRE).Immunized mice were challenged with a dose of vaccinia (~4x10^6 PFU) administered via the lungs 14 days after boosting. Weights of mice were tracked to monitor clinical signs of distress. Only pulmonary ICMV group conferred protection against the vaccinia; there was little weight loss and signs of recovery on D5. Asterisks represents statistically significant differences between it ICMV and PBS control group. (ICMV = AL11-ICMV+PADRE-ICMV+MPLA+pIC, Sol=soluble AL11+soluble PADRE+MPLA+pIC)

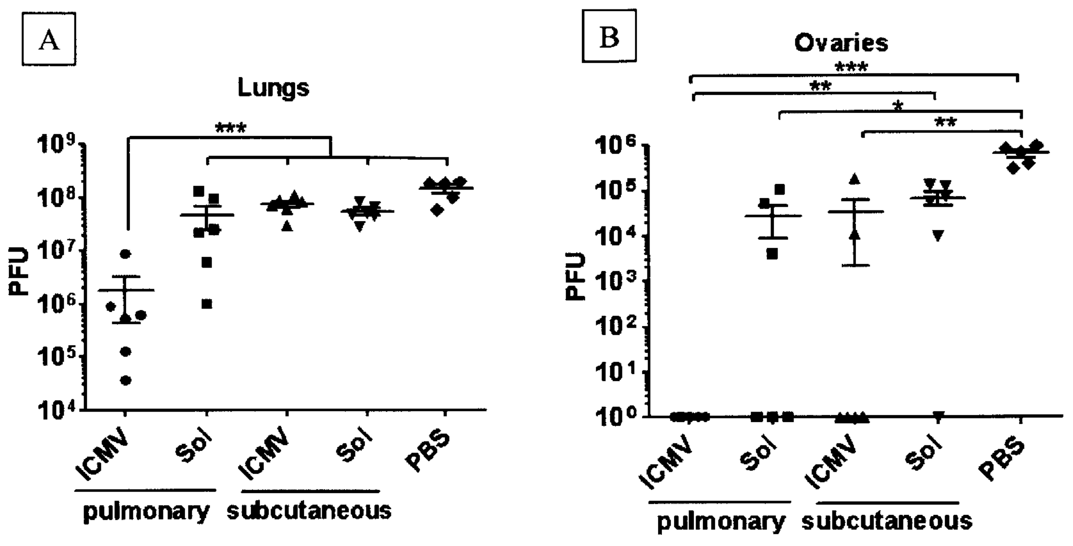


Figure 4-6. Viral titers in tissue after vaccinia challenge.
Mice were administered the same AL11 and PADRE vaccines encapsulated in ICMVs or soluble form and delivered *i.t.* or *s.c.* on D0 and D28 (10ug g AL11, 3.3ug PADRE).Immunized mice were challenged with a dose of vaccinia ($\sim 4\text{x}10^6$ PFU) administered via the lungs 14 days after boosting. Only pulmonary ICMV group showed significant reduction in viral titers in lungs (A) and ovaries (B) determined by a plaque assay on harvested tissues. ICMV = AL11-ICMV+PADRE-ICMV+MPLA+pIC, Sol=soluble AL11+soluble PADRE+MPLA+pIC)

Finally, we attempted to challenge mice intravaginally with vaccinia virus to test for disseminated protection in pulmonary-immunized mice. Mice were challenged on D42 as before. However, infection of vaccinia virus through the vaginal tract was inconsistent. No weight loss was detected in PBS group and no infection was seen in a portion of the animals in the PBS groups. No conclusion can be drawn regarding protection from pathogens entering through the vaginal mucosa.

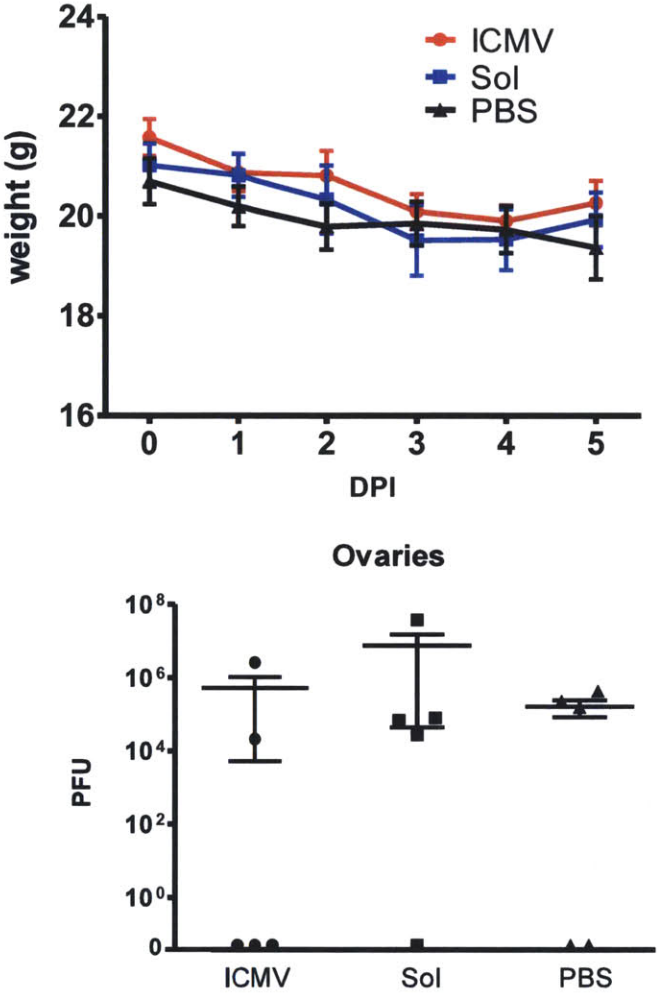


Figure 4-7. Intravaginal challenge with vaccinia virus in pulmonary immunized mice.

Mice were administered the same AL11 and PADRE vaccines encapsulated in ICMVs or soluble form and delivered *i.t.* on D0 and D28 (10ug g AL11, 3.3ug PADRE).Immunized mice were challenged with a dose of vaccinia (~4×10^6 PFU) administered intravaginally 14 days after boosting. (Top) Weights of mice after intravaginal challenge with vaccinia virus. No significant weight loss was observed in all groups. (Bottom) Ovaries were harvested on D5 after intravaginal challenge. Infection via the vaginal tract was determined to be inconsistent as virus failed to infect unimmunized mice. ICMV = AL11-ICMV+PADRE-ICMV+MPLA+pIC, Sol=soluble AL11+soluble PADRE+MPLA+pIC)

4.3.5. Safety of ICMV for pulmonary immunization

A chief concern for the development of pulmonary vaccines is the potential for particles/strong adjuvants to induce airway damage. Thus, we assessed potential systemic and local side effects following *i.t.* immunization with ICMV/MPLA/poly (I:C) vaccines. Mice mucosally vaccinated with ICMVs and TLR agonists (TLRa) showed no significant weight loss following priming or boosting (Figure 4-8), the only difference detected between ICMV and PBS groups was detected 2 days post boost.

To further evaluate if inflammation or necrosis was induced in the local tissues, lung tissues were harvested for histological analysis at various time points (Figure 4-9). Histopathology scores provided by a blinded pathologist showed that TLRa administration with or without ICMVs induced mild inflammatory responses in the lungs with increased macrophages and lymphocytes in the local tissues. However, no tissue damage was seen in all samples and lungs were not scored as diseased. (Figure 4-9).

Multiplex ELISA measurement of cytokines produced in the BAL fluid, lung tissue and serum following priming or boosting immunization showed the transient presence of IL-6 and very low levels of TNFα and IL-2 in the BAL and lungs that resolved within 24 hrs. We also detected IFN-γ that appeared by 24 hr only after boost. Cytokines were confined locally to the site of administration and not detected in serum, except for a low level of IL-6 present only after boost. IL-4, IL-10, IL-17 and type I IFN were also measured but found to remain at basal levels in the lung, BAL fluid and serum (data not shown). These results indicate that *i.t.* immunization with ICMVs induces only mild and transient inflammatory responses in the lung tissues and draining LNs with minimal toxicity or systemic side effects.

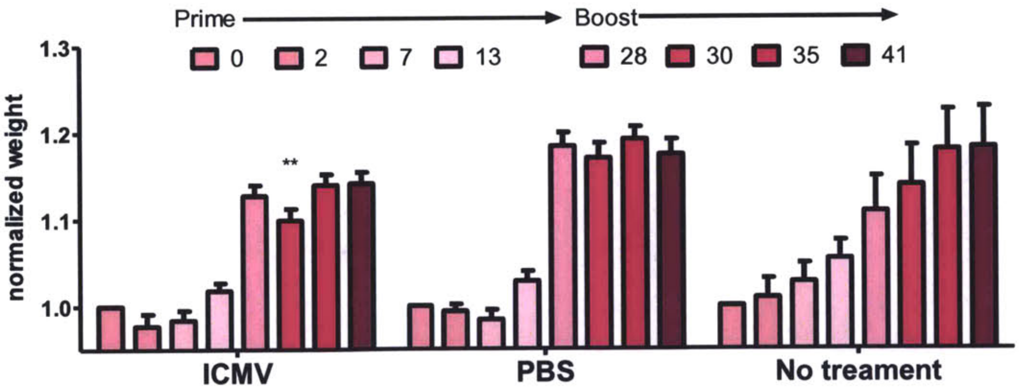


Figure 4-8. Weight change in mice after immunization.
Weight of mice is normalized to weight on D0. Mice were untreated or immunized intratracheally on D0 and D28 with OVA-ICMV+MPLA+pIC or PBS. D0, 2, 7, and 13 after prime and boost are shown above.

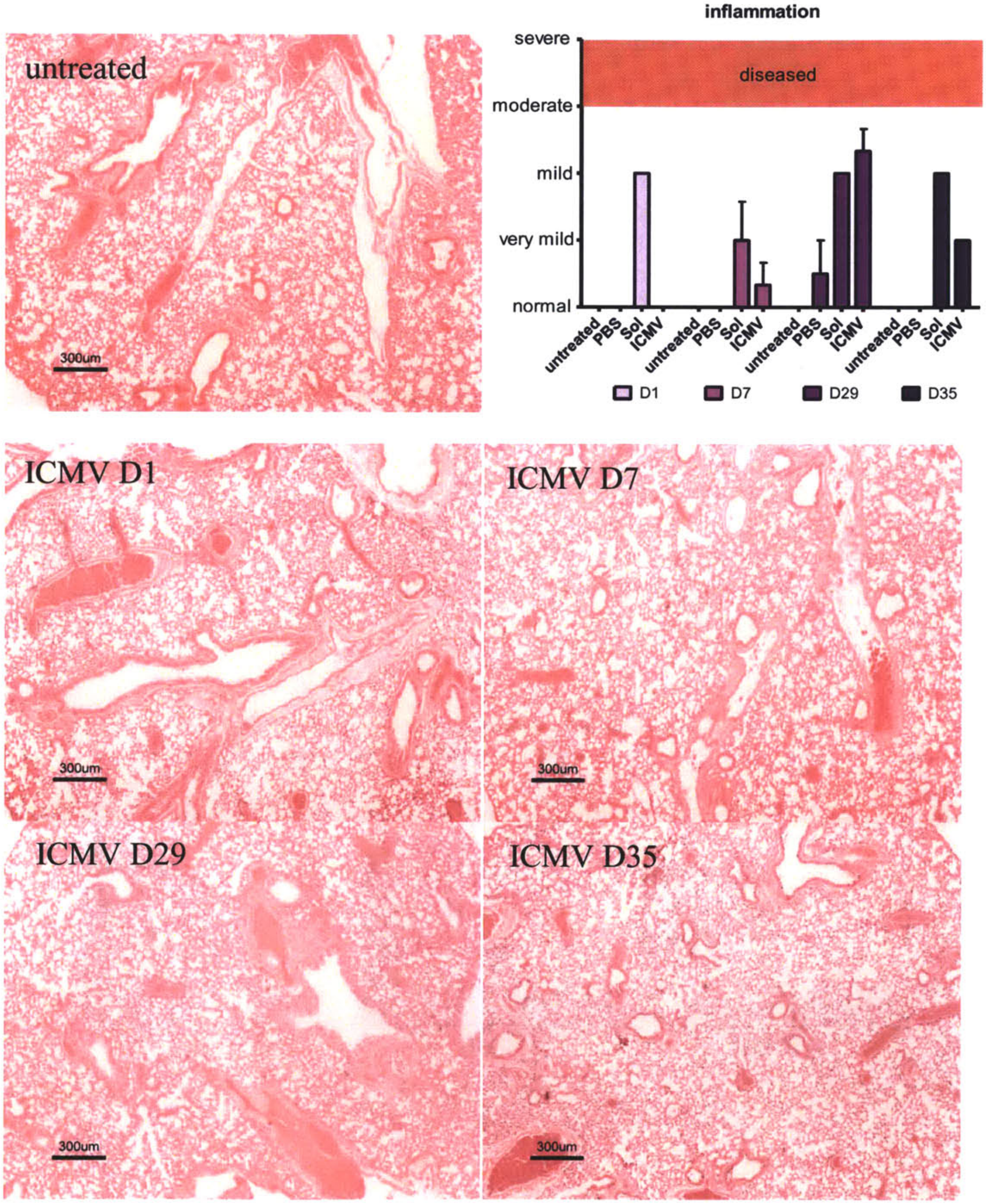


Figure 4-9. Histological analysis of pulmonary administration of ICMVs
Mice were untreated or immunized intratracheally on D0 and D28 with OVA-ICMV+MPLA+pIC. H&E staining on lungs sections on D1 and D7 after prime and boost.(Scale bar, 300 µm.) Representative image from each group with $n \geq 2$ are shown. Inflammation scores given by pathologist on are shown. Results are presented as mean ± SD.

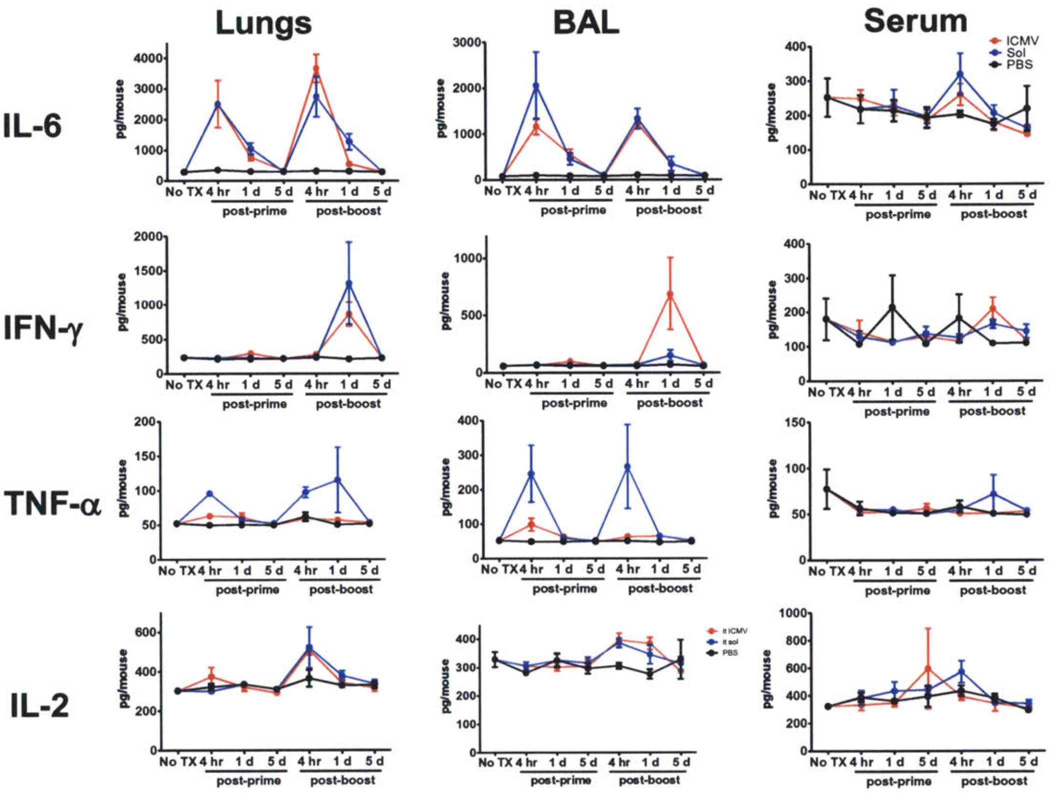


Figure 4-10. Cytokine analysis after pulmonary administration of vaccine.
Mice were immunized intratracheally on D0 with OVA-ICMV+MPLA+pIC. Serum, supernatants from lungs cell homogenates and broncholveoloar lavage (BAL) were collected at difference timepoints after immunization and analyzed for presence of cytokines with a multiplexed bead-based array for cytokine quantification. Data represent the mean±s.e.m. with $n \geq 3$. *, $p<0.05$; **, $p<0.01$, *** $p<0.01$ analysed by two-way analysis of variance followed by Bonferroni post-test.

4.4. Conclusions

The above results demonstrated that antigen-carrying ICMV particles adjuvanted with MPLA and poly (I:C) are safe and capable of stimulating immune responses against various antigens (proteins or peptides). The induced immune response successfully confers protection against tumors and infectious disease, indicating that this system can be useful for both therapeutic and prophylactic vaccination. Of note, protection was not only seen at sites local to vaccination, establishment of tumors at the flank and dissemination of infection from lungs to the ovaries was also prevented after pulmonary immunization, indicating broad immune protection at various sites in the body. We will continue to investigate if pulmonary vaccination can prevent invasion of pathogens through distal mucosal sites. If proven, this can be a versatile platform to vaccinate against various diseases. In addition, with confirmation that intranasal administration is as effective as intratracheal instillation, we envisage that ICMVs can be administered through an aerosol spray and be transformed into a non-invasive needle-free, universal vaccine platform.

5. Understanding the mechanism of potent immune response elicited by pulmonary immunization with nanoparticles

5.1. Introduction

Various vaccine systems has reported successful stimulation of mucosal CD8 T cells and conferred protection. However, the development of effective T cell vaccines remains elusive. This is due, in part, to the lack characterization of the determinants of successful T cell immunity. In this chapter, we attempt to understand data we gathered from the previous studies. We will first investigate why pulmonary administration induced a stronger immune response compared to parenteral injections, a phenomenon that has also been observed in other studies.[105, 134-136] Then, we will present data to explain why antigen in particulate form can stimulate better CD8 responses than antigen in free soluble form. Finally, we try to gain a better understanding of the mechanism of disseminated cellular immunity induced by pulmonary immunization.

Pulmonary immunization has shown to be very effective since the lungs are a highly responsive immune system. It is sensitive to mount an immune response as it is one of the easiest surfaces for pathogens to invade a host. Pulmonary macrophages and dendritic cells (DCs) are the main cell types playing a role in both innate and adaptive immunity. Alveolar macrophages are very abundant, with over a billion in the periphery and interstitium of the lungs[137]. DCs are found in epithelial linings of the conducting airways, submucosa below the airway epithelium, within alveolar septal walls and on the alveolar surfaces.[138] The specific roles of macrophages and DCs are still being investigated. The two cell populations are both professional antigen-presenting cells[137, 139] and different subtypes of macrophages and DCs are now being investigated. For example, one study reported that among various DC substypes in the lungs, $CD11c^{+}CD11b^{lo}CD103^{+}$ DC exclusively promote the proliferation of naïve $CD8^{+}$ T cells, whereas $CD11c^{+}CD11b^{hi}CD103^{-}$ DC preferentially seem to induce proliferation of $CD4^{+}$ T cells.[140] Another study reports that alveolar macrophages are not particularly efficient stimulators of immune responses when compared to other macrophages.[139] While the role of macrophages and DC are not well defined, it is consistently shown that APCs migrate to the airway draining LNs and prime T cells at the LNs,[141] although a few studies have reported T cell activation occurring at lymphoid structures call bronchus-associated lymphoid tissue (BALT) occasionally found in the lungs.[137, 142]

Dissemination of CD8 cells after mucosal immunization is also not completely understood. It can be due to a dissemination of antigen-loaded DCs towards non-draining lymph nodes and subsequent proliferation of resident T cells, or to a redistribution of T cells primed in the lymphoid compartment draining the immunization site. Many studies done in gut tissue agree that at the priming site, DC presents antigen and plays a role in imprinting gut homing markers (e.g. $\alpha4\beta7$) onto T cells to selectively home to gut tissue.[11, 12, 143, 144] Using an adoptive transfer model, Ciabattini *et al* showed that intranasal immunization with ovalbumin and *Streptococcus gordonii* increased number of antigen-specific T cells in genital and intestinal draining lymph nodes. Upregulation of α4β7 on T cells was also recorded.[145, 146] Intranasal immunization with fluorescent OVA indicated Ag-loaded APCs are only localized in mediastinal lymph nodes that drain the respiratory tract, and did not disseminate towards distal lymphoid sites.[146]

We found that the airway can prime better T cell response since it has a high number of APC, together with particles increasing and prolonging antigen delivery to the priming site, potent CD8 T cells response was induced by pulmonary administration of ICMVs. We also traced trafficking of antigen-specific CD8 cells after immunization and found that ICMV stimulates strong trafficking of CD8 all over the animal. Dissemination of primed CD8 T cells is facilitated by imprinting of mucosal homing markers onto T cells at the draining LNs of the airway.

5.2. Materials and methods

5.2.1. Materials

Double transgenic OT-1 mice expressing luciferase (OT-1/Luc) were bred in house. CD8 T cell enrichment kits were purchased from Stemcell Technologies (Cat#19753). Luciferin was purchased from Caliper Life Sciences (cat# 122796) and bioluminescent signal in mice were detected with an IVIS® Spectrum (Caliper Life Sciences). 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) was purchase from sigma (cat#21888). Materials used to make our vaccines and to assess CD8 T cell responses in mice were as described in Chapter 2. In addition, anti-α4β7 (ebioscience) was used as a mucosal homing marker. Counting beads from invitrogen (cat# PCB100) were used to enumerate cell numbers with flow cytometry.

5.2.2. In vivo imaging of CD8 proliferation

CD8+ T cells were isolated from 6- to 10-week old double-transgenic OT-1 mice expressing luciferase (OT-1/Luc) using a CD8+ T cell negative selection kit (Stemcell Technologies), and 0.75×10^6 OT-1/Luc CD8+ T cells were then adoptively transferred into 6- to 10-week old female C57Bl/6-albino mice (Jackson Laboratories) by retro-orbital injection. Twenty-four hours after adoptive transfer, mice were immunized as described above. On days 3 and 5 after immunization, the mice were injected with D-luciferin (150 mg/kg, Xenogen, Alameda, CA) i.p., and 10 min later, bioluminescence signals from OT-1/Luc CD8+ T cells *in vivo* were acquired with a Xenogen IVIS Spectrum Imaging System (Xenogen) before and after necropsies. Proliferation of CD8 T cells was determined by using the Living Image software to calculate signal flux within a region of interest (ROI). Results were also confirmed by harvesting cells and performing tetramer and antibodies staining and analysed by flow cytometry.

5.2.3. Isolating cells from Peyer's Patches

Peyer's Patches (PP) along the small intestine were identified, cut off with scissors and placed in cold RPMI. Fecal matter was then cleaned off of each PP on moist paper towels. After removal of fecal matter, PPs were placed in a petri dish and needles were used to shred each PP open to release lymphocytes within each PP. All cells were then collected in 3-4mls of 1%BSA/PBS and passed through a filter (80um pore size) into a tube and collected by centrifugation. Cells were then ready for antibody staining as described in chapter 2 followed by flow cytometry analysis. When staining for α4β7, anti-α4β7 (ebioscience) was incubated with cells for 20 mins at RT before incubation with tetramer.

5.2.4. In vitro CFSE dilution assay

Lungs, mediastinal LN and spleens were harvested from mice 3 days after immunization. Whole tissue homogenates were co-incubated with 50,000 CD8+ T cells isolated from OT-1 mice expressing Thy1.1+ and labelled with 1 μM 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE). After 3 days, dilution of CFSE was analyzed by staining the culture with DAPI, anti-CD8a (Becton Dickinson) anti-α4β7 integrin (ebioscience), anti-CCR9 (Becton Dickinson), anti-Thy1.1 (Becton Dickinson) followed by flow cytometry analysis.

5.2.5. In vivo CFSE dilution assay

Cells from OT-1 mice were isolated and labeled with CFSE. CFSE^{+}OT-1/Luc CD8+ T cells (0.75x10^{6}) were then adoptively transferred into 6- to 10-week old female C57Bl/6-albino mice (Jackson Laboratories) by retro-orbital injection. Twenty-four hours after adoptive transfer, mice were immunized as described above. Three days after immunization, tissues were harvested and homogenized into a single cell suspension. Cells were then stained with DAPI, anti-CD8a (Becton Dickinson), anti-α4β7 integrin (ebioscience), anti-CCR9 (Becton Dickinson), anti-Thy1.1 (Becton Dickinson) following antibody staining protocol in chapter 2 and analysed with a FACSCantoII.

5.2.6. In vivo antigen uptake assays

Fluorophore-tagged OVA was synthesized by reacting OVA with Alexa Fluor 647-succinimidyl ester (Invitrogen, Carlbad, CA). Mice were immunized by intra-tracheal administration with 10 μg of fluorophore-tagged OVA in either soluble or ICMV formulations with or without 0.3 μg MPLA and 10 μg poly (I:C) at various time points. Lungs, mediastinal LNs and bronchoalveolar lavage samples were collected to assess the amount of OVA in each compartment. The amount of OVA present in each tissue was measured with a fluorescent microplatereader. Images of lungs and mediastinal LNs cryosections were also taken with a Zeiss LSM 510 confocal microscope for histological analysis. Cell types responsible for antigen was determined by staining cells obtained from lungs, mediastinal lymph nodes and bronchoalveolar lavage with anti-CD11c, -CD11b, -MHC II, -F4/80, -B220, -CD205, -IA8 and analyzing with flow cytometry.

5.2.7. Statistical analysis

Experiments conducted with n = 3 in each group. Results are presented as mean ± SEM. n.d. = not detectable.

5.3. Results and Discussion

5.3.1. Antigen presenting cells (APCs) efficiently capture ICMV particles in the lungs

As a first step in dissecting the differences between subcutaneous and pulmonary immunization, we asked whether the significantly greater CD8 T cell response seen in pulmonary immunization compared to parenteral immunization might be attributed to the presence of more antigen-presenting cells at the site of administration. We immunized mice with fluorescently-tagged OVA in ICMV or soluble form. Three days after administration, cells from the mediastinal LNs, inguinal LNs, and lungs were extracted to identify OVA+ CD11c+ cells with flow cytometry. At least 4-fold more antigen-presenting cells (APC) captured the antigen at the draining lymph nodes when vaccine was administered via the lungs compared to a subcutaneous injection (Figure 5-1). Furthermore, ~1000x more APC cells captured OVA in the lungs than in the inguinal LNs. These cells may traffic to mediastinal LN and have the ability to prime more CD8 T cells in the mediastinal LN.

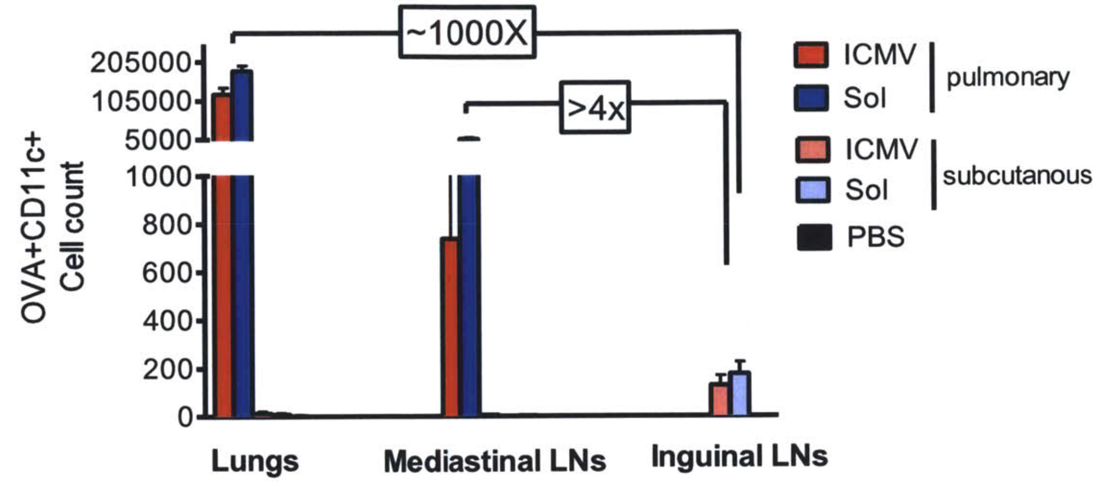


Figure 5-1. Number of antigen positive APC in draining LNs after pulmonary and subcutaneous administration.

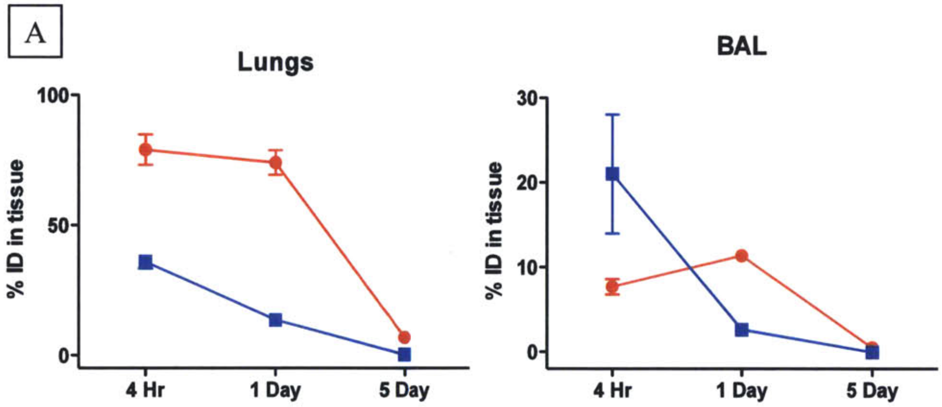
More antigen-presenting cells take up antigen with pulmonary immunization. Fluorescent OVA was administered to mice via the pulmonary or subcutaneous route. Number of OVA+ cells was counted using a flow cytometer and a significantly higher number of OVA+ CD11c+ cells (>4 fold) were found in the draining lymph node for pulmonary administration. Mice were immunized intratracheally on D0 with 10 µg of OVA in ICMVs or in soluble form with 0.3 µg MPLA and 10 µg pIC was administered. (ICMV = OVA-ICMV+MPLA+pIC, Sol=soluble OVA+MPLA+pIC)

5.3.2. ICMV promote uptake and draining of antigen to site of priming

In chapter 3, we compared mucosal vaccination of soluble mixtures of protein or peptide antigen combined with TLR agonists (MPLA+pIC) to mucosal vaccination with ICMV nanoparticles. We saw that nanoparticles promoted expansion of a much larger and more durable population of antigen-specific CD8$^+$ T-cells of an effector memory phenotype, disseminated to multiple mucosal surfaces, and exhibiting enhanced effector functions.

To understand how nanoparticle vaccination was promoting this enhanced response over vaccines comprised of the same doses of antigen and adjuvant administered in soluble form, we analyzed the kinetics and magnitude of antigen uptake and antigen presentation in the lungs. We first administered fluorescent OVA+MPLA+pIC or OVA-ICMVs+MPLA+pIC *i.t.* and quantified the amount of antigen retained in the lung tissue or collected in the bronchoalveolar lavage (BAL) by spectrofluorimetry. Soluble antigen was rapidly cleared from both the lavage fluid and lung tissue within 24 hrs, while nanoparticle uptake into the tissue was more rapid and sustained, with ~65% of the injected antigen dose still in the lung tissue after 1 day (Figure 5-2A). By day 5, fluorescent antigen was cleared from the tissue in both groups. Histological analysis of the lungs and draining mediastinal LNs were consistent with these results– uptake of substantial quantities of punctate packets of OVA by cells in the lung were seen in ICMV-immunized mice, while very low levels of soluble OVA were seen taken up in the lung tissue (Figure 5-2B). Four days post-administration, no OVA was detectable in draining LNs in soluble OVA-treated mice, but OVA was still readily detected in the nodes of ICMV-treated groups (Figure 5-2C).

Analysis of the cell types acquiring antigen by flow cytometry showed that macrophages accumulated the majority of antigen in both OVA and OVA-ICMV groups, and macrophages captured ~10-fold more antigen per cell in the lungs (Figure 5-3). Surprisingly, equivalent numbers of CD11c$^+$ dendritic cells in the lungs acquired antigen following either treatment after 1 day. However, after 5 days there were 10-fold more OVA$^+$ DCs still present in the lungs of mice administered OVA-ICMVs. Similarly, after 1 day similar numbers of OVA$^+$ DCs with identical levels of antigen were observed in mediastinal LNs, but 5 days post-immunization, antigen$^+$ DCs were still readily detectable in the LNs of OVA-ICMV-treated mice, while no antigen-bearing DCs remained in animals treated with soluble OVA.



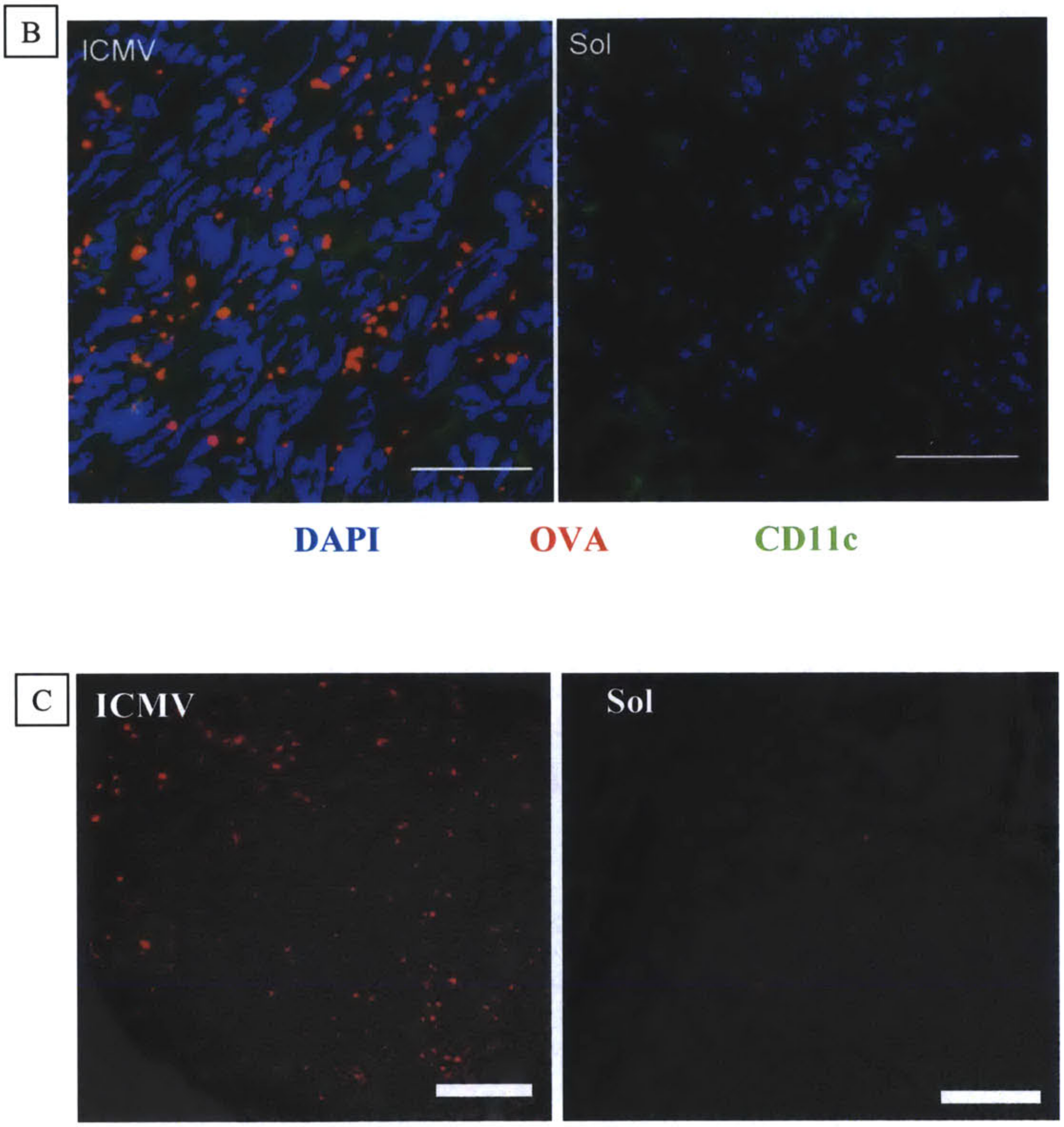


Figure 5-2. ICMVs delivers antigen more efficiently to prime an immune response. Tissue extracted after pulmonary administration of fluorescently-tagged antigen was analyzed. Mice were immunized intratracheally on D0 with 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and 10 μg pIC was administered. (A) Lungs and bronchoalveolar lavage were collected at indicated timepoints. Tissue samples and vaccines from day of administration were measured with a fluorescent microplate reader to determine % dose administered are found in the tissue. (red = OVA-ICMV+MPLA+pIC, blue=soluble OVA+MPLA+pIC).

(B and C) Cryosections from lungs (B) and mediastinal LNs (C) were taken on D1 or D4 after immunization, respectively. Immunohistochemical analysis was performed: sections were stained with anti-CD11c (green), DAPI (blue) and antigen (shown in red = OVA-Alexa Fluor 555). Representative confocal sections of tissue from two independent experiments conducted with n = 2–3 are shown. Scale bars: panel B=50 μm, panel C= 200μm.

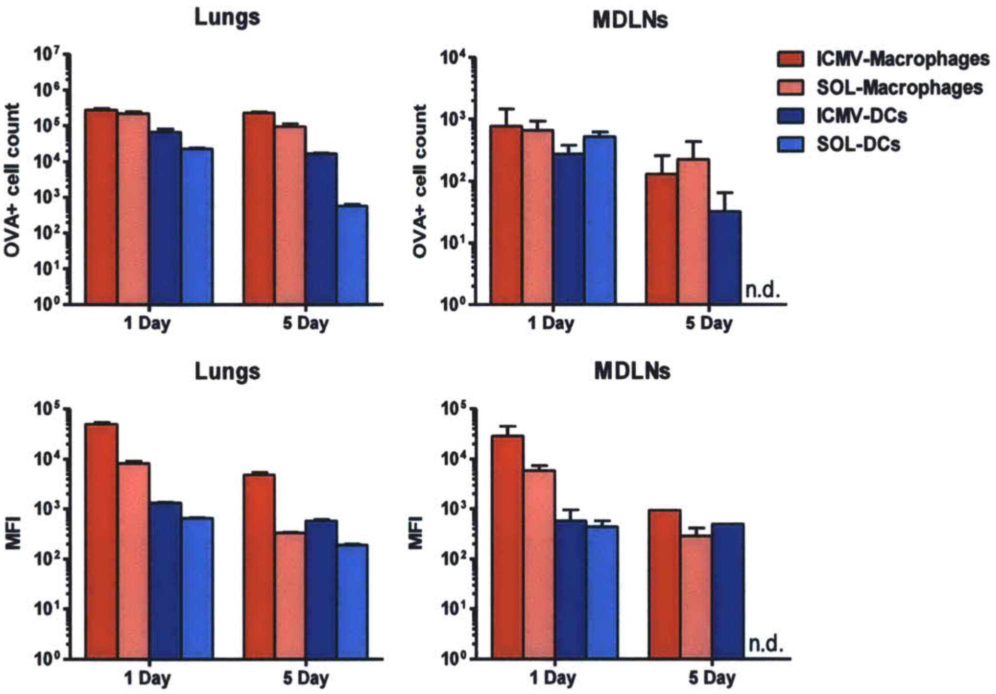


Figure 5-3. Macrophages and dendritic cells take up antigens in lungs.
Mice were immunized intratracheally on D0 with 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and 10 μg pIC was administered Uptake by macrophages and DCs in lungs and mediastinal LNs was examined on D1 and D5 after delivery by flow cytometry. (ICMV = OVA-ICMV+MPLA+pIC, Sol=soluble OVA+MPLA+pIC).

5.3.3. ICMV enhances antigen presentation to CD8 T cells

Prolonged detection of antigen in dendritic cells does not necessarily imply strong or durable antigen presentation, since DCs must process and cross-present captured exogenous protein antigen to prime T-cells. To assess the strength and duration of antigen presentation following pulmonary vaccination, we assessed the capacity of APCs from the lymphoid organs and lungs of immunized mice to prime naive OVA-specific T-cells *in vitro*. Groups of mice were immunized *i.t.* with soluble or ICMV vaccines and 3 days after immunization, leukocytes from the lungs, mediastinal LNs, and spleens were isolated and co-cultured with CFSE-labelled naive OT-I CD8$^+$ T-cells cells. Among the tissues tested, only APCs from the mediastinal LNs stimulated proliferation of naive T-cells, confirming that T-cell priming is initiated in the mLNs (Figure 5-4). Notably, lymph node cells from nanoparticle-immunized mice elicited a substantially greater accumulation of highly-divided T-cells. This suggested that the increased antigen delivery and controlled release of the antigen from the nanoparticles enhanced antigen presentation and CD8 T cell priming. In addition, effective cross presentation of induced by encapsulating antigens in particles is also a cause to stronger CD8 cell proliferation.

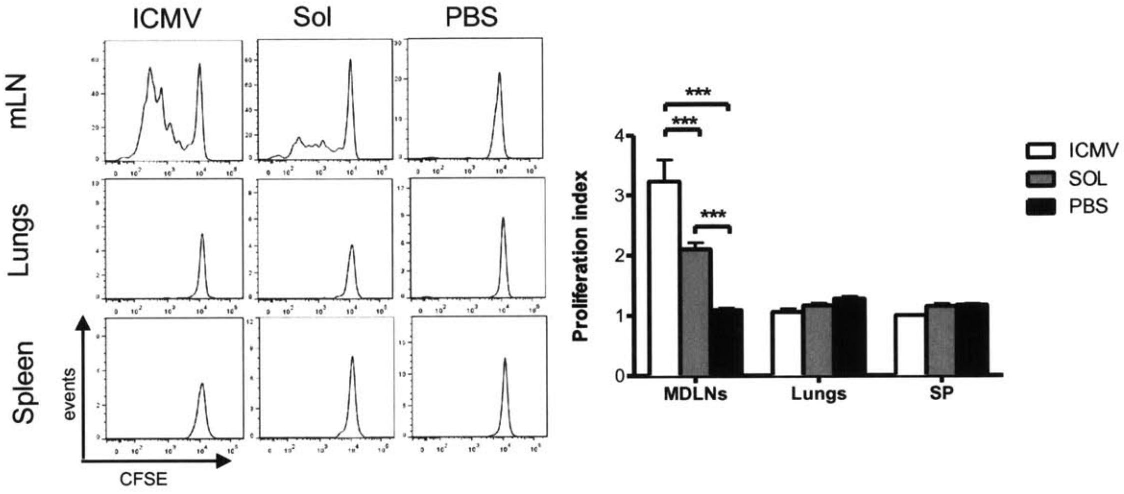


Figure 5-4. ICMV nanoparticles promote uptake and sustained presence of antigen in dendritic cells and macrophages, enhances antigen presentation and imprinting of mucosal homing receptors.
Mice were immunized intratracheally on D0 with 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and 10 μg pIC was administered. Tissues harvested 3 days after *i.t.* immunization was homogenized and cocultured with CFSE-labelled OT-1 cells. Proliferation of OT-1 cells was determined by flow cytometry after 3 days of co-culture.(Left) Representative plots of flow cytometry histograms showing CFSE dilution of OT-1 cells. (Right) Graph of proliferation index calculated from histograms shown on left. Proliferation index = the total number of divisions divided by the number of cells that went into division (calculated by Flowjo flow cytometry analysis software). (ICMV = OVA-ICMV+MPLA+pIC, Sol=soluble OVA+MPLA+pIC).

5.3.4. Pulmonary nanoparticle immunization enhances imprinting of mucosal homing receptors on CD8 T cells

DCs in the mLNs are capable of imprinting expression of mucosa-homing receptors on T-cells[143, 144]and when expression of the mucosal homing integrin $\alpha_4\beta_7$ was assessed on *in vitro*-primed OT-I cells, APCs from ICMV-treated mice primed ~3-fold more OT-I cells to upregulate $\alpha_4\beta_7$ expression compared to the soluble vaccine (Figure 5-5). This result provides a basis for understanding the enhanced mucosal memory cell population observed following nanoparticle immunization.

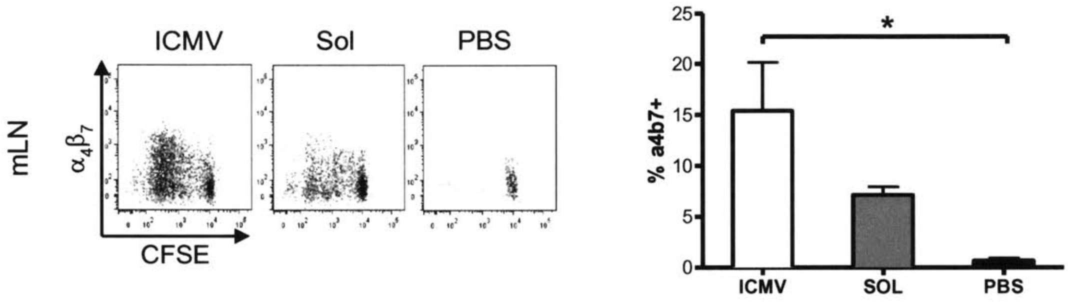
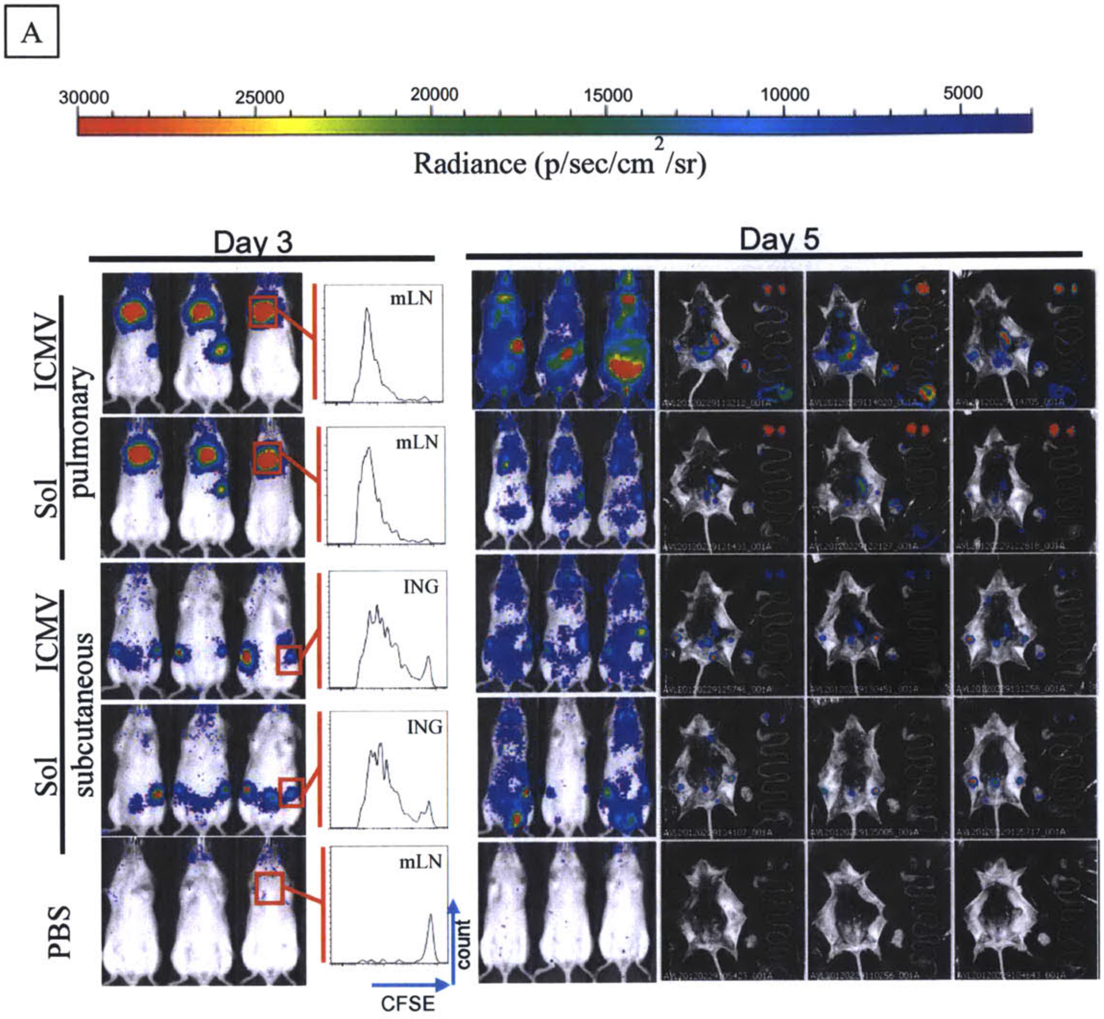


Figure 5-5. $\alpha_4\beta_7$ integrin expression on CD8 cells primed in mediastinal LNs. Mice were immunized intratracheally on D0 with 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and 10 μg pIC was administered. Tissues harvested 3 days after *i.t.* immunization was homogenized and cocultured with CFSE-labelled OT-1 cells. $\alpha_4\beta_7$ expression on OT-1 cells was determined by flow cytometry 3 days after co-culture. Representative flow cytometry dot plots gated on OT-1 cells are shown on the left. (ICMV = OVA-ICMV+MPLA+pIC, Sol=soluble OVA+MPLA+pIC).

5.3.5. CD8 T cells disseminate from priming site and continue to expand

Sustained strong antigen presentation in mLNs could explain the greater T-cell expansion and bias toward an effector memory phenotype observed following nanoparticle vaccination, but only if T-cells remain confined in the mLNs over several days to be exposed for a prolonged duration to high antigen levels on DCs in this lung-draining site. To determine how long T-cells remain localized in the priming mLNs, we used an adoptive transfer model employing luciferase-expressing OVA-specific OT-I TCR-transgenic CD8+ T-cells (OT-I-luc) to trace the proliferation and trafficking of antigen-specific cells following pulmonary immunization. Naive OT-I-luc T-cells were transferred into recipient mice, which were immunized 24 hr later with soluble OVA+MPLA+pIC or OVA-ICMV+MPLA+pIC, via *i.t.* or *s.c.* routes. Bioluminescence imaging and flow cytometry analysis showed that by day 3 post-vaccination, ICMVs or soluble antigen administered *i.t.* were priming OT-1-luc expansion in the mLNs, while as expected[62], *s.c.* vaccinations showed T-cell expansion in the draining inguinal LNs (Figure 5-6A). By day 5, the mean total bioluminescence signal from mice immunized *s.c.* or given soluble vaccine *i.t.* still remained lower than that of the mucosal ICMV vaccination on day 3, though imaging revealed a dissemination of primed T-cells to the spleen, iliac, and mesenteric lymph nodes (Figure 5-6 A, B). In contrast, pulmonary vaccination with ICMVs led to a further near doubling in OT-I-luciferase signal from day 3 to day 5, giving a mean total T-cell signal 3-6-fold greater than each of the other vaccine groups. Further, OT-I signal was detected not only in lymph nodes and spleen but also across the gut of mice and in the reproductive tracts (Figure 5-6A). Quantitative differences in the degree of T-cell expansion from the imaging data (Figure 5-6B) were corroborated by tetramer staining analysis of T-cells in the blood (Figure 5-7), which showed several-fold greater expansion of OT-I-luc cells in this compartment by pulmonary ICMV vaccination compared to *s.c.* ICMVs or *i.t.* soluble vaccine. Differences in the T-cell homing pattern elicited by *i.t.* ICMV vaccination were further illuminated by bioluminescence imaging of freshly-dissected organs on day 5: Subcutaneously-administered vaccines elicited little OT-I T-cell trafficking into the lungs and none to the gastrointestinal tract. In contrast, both soluble antigen and ICMV vaccines administered *i.t* primed T-cell homing into the lungs by day 5, but ICMV nanoparticles uniquely also elicited OT-I-luc cells homing into the cecum and Peyer's patches along the small intestine as assessed by whole-tissue imaging (Figure 5-6A, Figure 5-9) and tetramer staining of lymphoid cells from the Peyer's patches (Figure 5-9). Consistent with our *in vitro* OT-I priming studies, only pulmonary nanoparticle vaccination induced significant expression of the mucosal tissue-homing integrin a_4b_7 in tetramer$^+$ peripheral blood OT-I cells (Figure 5-8). Thus, naive T-cells remain localized in lung-draining lymph nodes for several days following pulmonary immunization; nanoparticle immunization equips DCs in the mLN to provide strong antigen presentation to T-cells throughout this duration and more strongly imprints mucosal homing receptors. By day 5, T-cells disseminate while continuing to expand, with nanoparticle immunization eliciting robust infiltration of antigen-specific cells into distal mucosal sites.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30000
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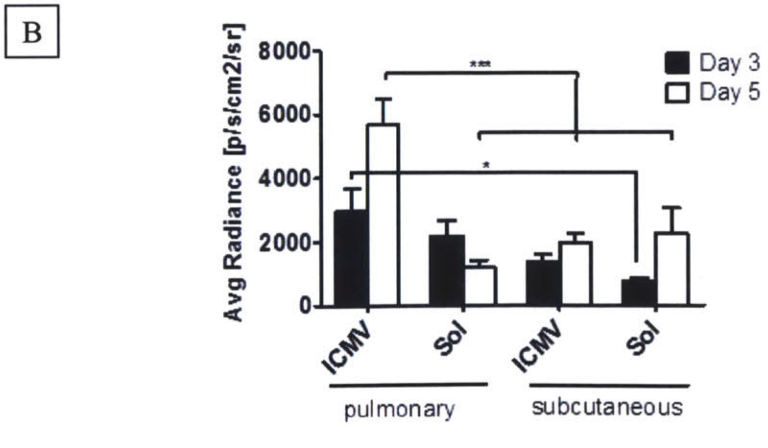
B
Avg Radiance [p/s/cm2/sr]
8000
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2000
0
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*
Day 3
Day 5
ICMV
Sol
ICMV
Sol
pulmonary
subcutaneous

Figure 5-6. Trafficking and proliferation of CD8 T cells after immunization.
OT-1$^+$Luciferase$^+$ cells were adoptively transferred into C57Bl/6 mice one day before immunization via *i.t.* or *s.c.* routes. Mice were immunized *i.t.* or *s.c.* on D0 with 10 μg of OVA in ICMVs or in soluble form with 0.3 μg MPLA and 10 μg pIC was administered. (A) Trafficking and proliferation of OT-1 Luciferase$^+$ cells was monitored in live mice by *in vivo* imaging on D3 and D5 after immunization. Lungs and gastrointestinal tracts were dissected for imagining from mice on D5. In a separate experiment, CFSE-stained OT-1 cells were adoptively transferred before immunization and proliferation of OT-1 cells at draining LNs (mLN = mediastinal LN, ING = inguinal LN) were confirmed by flow cytometry analysis on D3. (B) Quantification of bioluminescent signal of whole mouse from images of live mice on D3 and D5. (ICMV = OVA-ICMV+MPLA+pIC, Sol=soluble OVA+MPLA+pIC).

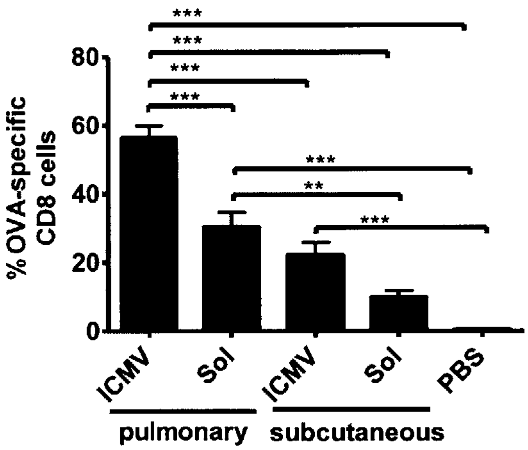

Figure 5-7. Frequency of CD8 T cells in blood.
Frequency of OVA-specific CD8 in blood on D5 after immunization in OT-1 adoptive transfer model determined by tetramer staining and flow cytometry. (ICMV = OVA-ICMV+MPLA+pIC, Sol=soluble OVA+MPLA+pIC).

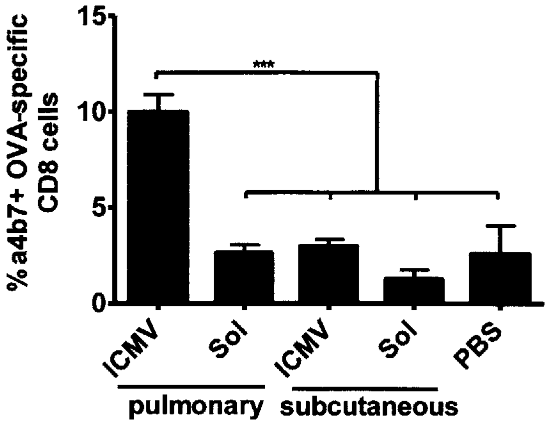

Figure 5-8. Pulmonary nanoparticle immunization efficiently induces mucosal homing markers on CD8 T cells.

Frequency of integrin $\alpha_4\beta_7^+$ cells among tetramer+ blood cells assessed by flow cytometry. (ICMV = OVA-ICMV+MPLA+pIC, Sol=soluble OVA+MPLA+pIC).

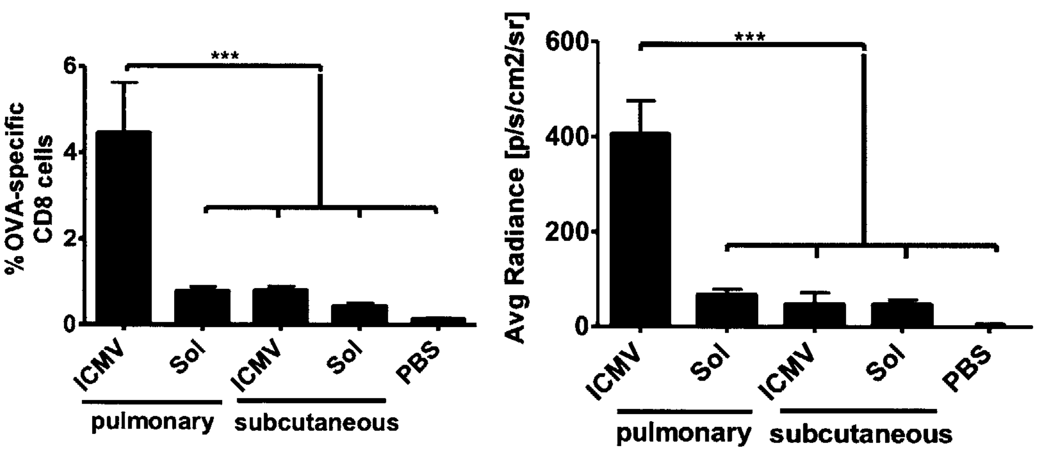

Figure 5-9. Pulmonary immunization with ICMV leads to antigen-specific CD8 T cells homing to the gut.

(Left) Frequency of OVA-specific CD8 T-cells in Peyer's patches on D5 after immunization in OT-1 adoptive transfer model determined by flow cytometry. (Right) Quantification of bioluminescent signal of small intestines from images on D5. (ICMV = OVA-ICMV+MPLA+pIC, Sol=soluble OVA+MPLA+pIC).

5.4. Conclusions

Altogether, we found that the significantly higher number of APCs in the airway and draining LNs in combination with enhanced and prolonged delivery of antigen in ICMVs leads to the potent antigen-specific CD8 T cells stimulation when delivering protein vaccines via ICMVs in the airway. CD8 T cells primed in the draining LNs of the pulmonary system are also imprinted with mucosal homing markers causing robust dissemination of primed antigen-specific CD8 T cells to traffic to other effector (mucosal/systemic) sites.

6. Conclusions and future work

6.1. ICMVs as a safe and versatile platform for delivering vaccines

We have developed a synthetic nanoparticle system for delivery of mucosal vaccines. We were able to demonstrate that mucosal delivery of this vaccine can stimulate potent CD8 T cell responses that confer protection against tumors (in a therapeutic setting) or a live pathogen challenge (prophylactically). Although other studies have explored nanoparticles as carriers for mucosal vaccination[52, 64-70, 72-75, 77-86, 88, 147], the cellular immune responses reported here (for the model antigen OVA, which is often used as a benchmark for vaccine studies) are much stronger than previous reports and few studies have demonstrated disseminated CD8 mucosal responses at a distal mucosal site. To our knowledge, this is the first study to demonstrate the above using lipid-based particles as a vaccine delivery vector.

The ICMV delivery system has previously shown to be a potent inducer of systemic CD8 responses when administered parenterally.[62] Knowing that triggering mucosal immunity is of great benefit to control the spread of viruses/intracellular pathogens, especially for HIV vaccine development, we were motivated to evaluate if the ICMV system can be applied as a mucosal vaccine. Although ICMVs with MPLA incorporated were shown to be effective in inducing CD8 responses, we explored the possibility of further enhancing its efficacy by adding a second adjuvant since synergy exist between TLR agonists.[100, 102] We chose to add the TLR3 agonist, poly (I:C), as it has been proven to be a strong CD8 T cell adjuvant.[56, 59, 60, 91-93, 148] Additionally, since poly (I:C) is also a ligand of RIG-I-like receptors (RLRs), it may be capable of stimulating DCs to promote mucosal homing of CD8 T cells.[149, 150] Initial experiments comparing single and dual TLR agonist added together with ICMVs indicated that dual adjuvants gave us the best CD8 response in blood and lungs. In fact, at 7 days after pulmonary immunization, we saw that nearly 80% of the CD8 T cells in the lungs were antigen-specific cells, hence, we determined that this dual adjuvant approach is optimal for generating strong CD8 responses.

We then focused on examining if the potent CD8 response disseminates to distal mucosal sites, since mucosal surfaces are connected together to form the "common mucosal system". In parallel, we also immunized mice with the same vaccine formulations parenterally to compare the efficacy of the two different immunization routes. Consistent with data previously reported [105], pulmonary immunization elicited a stronger mucosal response than parenteral immunization. The systemic CD8 response was also higher in pulmonary-immunized animals, suggesting pulmonary immunization has the potential to generate stronger, broader protection overall.

We continued to evaluate the quality of the CD8 response generated and ensured that the memory response can be generated to provide durable immunity using this mucosal vaccine system. Surprisingly, we found that pulmonary administration of ICMV nanoparticles can induce a significantly higher number/frequency of long lasting ($CD127^{hi}KLRG1^{lo}$) effector memory ($CD44^{hi}CD62L^{lo}$) T cells compared to soluble antigen or ICMVs delivered parenterally, indicating that both the route of immunization and the delivery vehicle contributed to significant enhancement in CD8 T cell induction.

This led us to look into understanding the mechanism behind the strong response generated. We performed analysis of cells that have taken up the antigen after immunization via the lungs or subcutaneous injection and found that pulmonary administration induces stronger CD8 response because the draining mediastinal lymph nodes contain significantly more antigen$^+$ APCs (at least 4-fold) to stimulate T cells. This is in line with the fact that mucosal tissues are in constant contact with the environment, and hence, requires abundant APC to survey for the presence of foreign antigen in the host. We also compared delivery of antigen in particulate or soluble form and found that the nanoparticle formulation slowed down the clearance of antigen, hence, delivering more antigen to each APC. Particles also carried more antigen to the priming site (found to be mediastinal LNs not lungs) for a longer period of time (shown in confocal images) leading to prolonged CD8 activation which was not investigated in this thesis.

The ability of ICMV to change the kinetics of APC exposure to antigen may be an explanation for the significantly higher number of effector memory cells induced. It is suggested that the decision of a cell to become effector or central memory cells depends on antigen exposure and the type of cytokine present.[109, 151] The initial 'burst size' of the CD8+ effector T cell response also correlates with the magnitude of the long-term memory response.[152] Therefore, particles delivering a higher amount of antigen per APC or the prolonged exposure of APC to antigen when encapsulated may be a reason for the large amount of effector memory cells generated. However, it may also be linked to the type of adjuvant we used as TLR agonist may have direct influence on CD62L expression on lymphocytes. [59, 153]

To gain insight into CD8 dissemination, we used *in vivo* imaging to track proliferation of antigen-specific CD8 T cells and found that after pulmonary immunization, CD8 cells are activated at the site local to injection, which then disseminates throughout the body. This was corroborated by detection of upregulation of mucosal homing integrins ($\alpha_4\beta_7$) in the *i.t.* ICMV group.

To conclude, we have demonstrated that the novel nanoparticle system, ICMV, can be an effective and safe mucosal vaccine, acting through multiple mechanisms to enhance vaccination through the airways. It is a versatile system that can be easily adapted to deliver protein/peptide/DNA vaccine and confer protection against infectious agents or treat cancer.

6.2. Discussion

Numerous pathogens, including influenza, HIV, and HSV, initiate infection at mucosal surface, therefore, vaccines that can induce long-term protection at multiple mucosal surfaces would be ideal. In this work, we tested the efficacy of needle-free delivery of a nanoparticle vaccine system to elicit mucosal immunity. Intratracheal or intranasal administration of ICMV with TLR adjuvant vaccines induced dramatic expansion of

CD8+ T-cell frequency at the site of vaccine administration, followed by dissemination and long-term maintenance of CTLs in distant mucosal tissues. Pulmonary administration of ICMVs delivering whole protein OVA and TLR agonists can elicit up to 65% tetramer+ antigen-specific CD8+ T cells in the lungs and ~15% tetramer+ CD8+ T cells in systemic circulation, representing more than 4-fold increase in CTL frequency compared to soluble protein vaccination after 7 days post-boost (Figure 2-7, Figure 3-2). ICMV nanoparticles also substantially enhanced the frequency and number of CTLs accumulated at distant mucosal tissues at long-term: we observed 3.5-fold increase in the frequency of OVA-specific T cells in the vaginal tract, compared to soluble protein vaccine after 11 wks post-prime (Figure 3-3).

The initial dramatic expansion of CTLs in the local respiratory tract shortly after pulmonary vaccination with ICMVs can be attributed to the enhanced antigen delivery to APCs by the vaccine particles in the local tissues and draining LNs. Compared to soluble antigen that was rapidly cleared from the lungs within 1 d, up to 65% of antigen was still present in the lung tissues after 1 d of priming (Figure 5-2). ICMV vaccination also substantially enhanced draining and prolonged antigen delivery to mediastinal LNs (mLNs) compared to soluble protein vaccination. In particular, after 5 days of priming, a significant number of OVA+ APCs were still detected in the lungs and mLNs in mice immunized with ICMVs, whereas there was a dramatic reduction in OVA+ APC counts in the group with soluble protein vaccination (Figure 5-2). Consequently, the increased antigen delivery to mLNs allowed dramatic expansion of CTLs in the local tissues. As shown in Figure 5-4, cells isolated from mLNs on d 3 post-priming with ICMVs, but not soluble protein vaccine, were capable of cross-priming OVA-specific CD8+ T cells ex vivo, indicating that enhanced antigen uptake and transport to APCs by ICMV vaccines translated into dramatic expansion of CTLs in mLNs. Cells from the lungs or spleen were not able to stimulate OVA-specific CD8+ T cells. Nor did we detect any antigen or particles in organs other than lungs of mdLNs up to 5 d post-prime, suggesting that particles did not enter the circulation to reach distant organs. This is in contrast to previous observations with lipid complexes, where it has been speculated that pulmonary vaccination might promote disseminated T-cell and Ab responses because of antigen delivery to multiple sites such as gut and spleen due to highly vascularized nature of the lung tissues.[154, 155] Instead, we observed significant upregulation of α4β7, integrin receptor for gut-homing phenotype[156, 157] among OVA+ CD8+ T cells primed with mLN cells ex vivo (Figure 5-5), and also in the blood after in vivo priming (Figure 5-8). This line of evidence suggests that intratracheal instillation of vaccine particles delivers a large amount of antigen to the lung and mLNs, leading to sustained high concentration of antigen restricted to the local tissues. Antigen-specific CD8+ T cells are primarily primed by APCs in mLNs, and a large frequency of newly primed CD8+ T cells are imprinted with phenotypes directing their trafficking to mucosal tissues.

Preclinical studies with viral vector vaccinations have demonstrated successful induction of CD8 T cellular responses in mucosal tissues. Intranasal immunization in mice with recombinant adenovirus vectors expressing HSV epitopes generated CTL responses that were compartmentalized in mucosal tissues for more than 1.5 yr following immunization.[105] Nasal administration of vaccinia virus Ankara combined with SIV DNA vaccine stimulated significant SIV-specific mucosal and systemic CTL responses in rhesus monkeys, and vaccinated animals challenged with intravaginal SIVmac251 had a 3-log viremia reduction compared to non-treated animals.[108] These viral vector can generate potent CD8 responses, however, pre-existing anti-vector immunity and manufacturing challenges complicate vector-based vaccine design. Therefore, considerable research effort has been directed at the development of DNA or subunit vaccines.

Pulmonary delivery of plasmid DNA formulated with polyethyleneimine[158], lipid complexes[155], or liposomes[87] have shown to elicit disseminated mucosal immune responses, characterized by mucosal IgA and CTL responses in genital, rectal, and gut-associated tissues. Synthetic subunit vaccines employing HIV peptides formulated with strong experimental adjuvants have also been developed to elicit HIV-specific cytotoxic T cells resident in mucosal tissues. Mice immunized with HIV peptides and cholera toxin via the intrarectal route induced long-lasting HIV-specific CTL memory in gut-associated tissues, such as Peyer's patches and lamina propria, and vaccinated mice were protected against infection with a recombinant virus vaccinia expressing HIV-1 IIIB gp160.[38] Similar approaches have been explored with other peptide vaccines administered via intranasal[159] and transcutaneous routes[160]. These studies have collectively shown success towards mucosal vaccine development. However, plasmid DNA vaccines are not immunogenic enough to be used in humans yet, and peptide vaccines raise the issue of covering HLA of humans broadly. Hence, vaccine systems that can elicit potent immune response with whole proteins as antigens are being investigated.

Effective cross-presentation of epitopes is crucial to subunit vaccines development. The uses of synthetic particles as vaccine delivery vectors have shown to significantly improve induction of CTL response by enhancing cross-presentation of antigens. Peptide antigens derived from influenza virus[119] or lymphocytic choriomeningitis virus (LCMV)[85] were conjugated on liposomes to enhance antigen transport and uptake by DCs, and intranasal vaccination with these peptide-liposome complexes protected mice against intranasal viral challenge. Similar approach was taken to co-deliver influenza A peptides encapsulated in liposomes and anti-CD40 antibody, resulting in effective reduction of influenza viral titers in the lung by CD8- and CD4-T cell mediated cellular immunity.[78] Hubbell *et al.* have demonstrated that nanoparticle-mediated delivery of whole protein antigen and CpG via intranasal route enhanced antigen uptake and transport to draining lymph nodes.[161] Such approaches led to three- and ten-fold increases in CTL numbers in spleen and lungs, respectively, compared to soluble controls and protected mice against intranasal influenza challenge. Taken together, these studies collectively demonstrate that particles or vesicles delivering peptide or protein antigen via mucosal route of administration can elicit compartmentalized CTL responses in local sites of mucosal vaccination. In our study, we further prove that mucosal administration of subunit antigen encapsulated in particles can elicit long lasting broad disseminated mucosal CD8 response.

Previous studies have highlighted the importance of effector memory cells at mucosal surfaces. A study by Li *et al* combined in situ tetramer (IST) staining and in situ hybridization (ISH) to locate and enumerate virus-specific tetramer+ T cells in macaques infected with SIV and showed that timing, ratio, and spatial colocalization of virus-specific CTLs to infected cells determined the outcome of infection.[162] They found that it is crucial to have enough effectors cells at the portal of entry before infection to prevent mucosal transmission,[162] highlighting the importance of generating T_{EM} at mucosal surfaces. Live vectors have successfully shown to generate mucosal effector memory cells. Non-replicating recombinant adenovirus (rAd) vectors[163] as well as rhesus cytomegalovirus (RhCMV)[164, 165] were able to induce durable SIV-specific mucosal T cell response in rhesus monkeys. The latter provided evidence suggesting that T_{EM} at mucosal surfaces can prevent establishment of systemic infection after a mucosal viral infection without the involvement of central memory cells and antibodies. Hence, mucosal effector memory cells acting as a first line of defense is critical to preventing establishment of HIV and central memory cells at systemic sites may only be needed as a second line of defense.

Generation of effector memory cells are not well defined but it is believed to be determined at the early stages of vaccination[166, 167] Initial signal strength, concentration of Ag, stimulation duration and co-stimulatory molecule expression, can determine effector/central memory differentiation.[168] Strong antigen stimulation promotes cell survival and responsiveness to IL-7 and IL-15, which is closely associated with expansion of T_{EM}.[168, 169] In vivo studies done by altering DC:T cell ratio by adoptive transfer of OT-1 or artificially increase of DC population by Flt3L before infection[170, 171] have shown that DC:T cell ratio has plays a part in memory cell differentiation; low DC:T-cell ratio preferentially generates T_{CM}, while higher ratios tend to generate T_{EM}.

In the present study, we introduce a synthetic subunit vaccine system that can also achieve a strong T_{EM} biased immune response at mucosal surfaces. In addition to finding that pulmonary delivery of ICMVs induced potent long lasting CD8 T cell response that preferentially resides at mucosal effector sites (Figure 3-8), further analysis on antigen-specific memory T cells revealed that pulmonary delivery of ICMVs generated a strong effector memory-biased phenotype (Figure 3-6, Figure 3-7). Data from both systemic and mucosal compartments showed that the absolute number of effector memory cells was 2-10-fold higher than vaccine delivered in the soluble form or subcutaneously at more than 2 months post-vaccination (Figure 3-6). Effector cells reside in mucosal sites are especially important as they immediately respond to pathogens at the site of entry. CD44/CD62L staining at >2 months days after immunization revealed that this system can induce 15-fold more total number of effector memory cells than central memory cells, while soluble antigen or subcutaneous delivery of the vaccine only results in a 2-5-fold increase (Figure 3-7). We speculate the increased and prolonged antigen delivery to the priming site (Figure 5-2) by ICMVs, in combination with the high number of antigen-presenting cells present in the respiratory tissue (Figure 5-1), allow for an environment with increased antigen concentration, prolonged antigen stimulation and high DC:T cell ratio, resulting in the significant effector memory generation. These results are further confirmed by higher expression of $CD127^{hi}KLRG^{lo}$, an indicator of longer-lived effector cells (Figure 3-9). These results indicate that our nanoparticle vaccines can elicit effector memory cells residing in mucosal sites, a crucial feature for a successful vaccine as T_{EM} in mucosal tissues immediately respond to pathogens at the site of entry.[162]

In addition to efficacy, vaccine safety is a major concern. We have found no alarming evidence of toxicity in lung pathology (Figure 4-9) and systemic inflammation as assessed with serum cytokine levels (Figure 4-10). Clinical signs of distress were also not found (Figure 4-8). Recent reports of intranasal vaccine as a cause for neural damage and facial paralysis[172] have proven to be due to monosialoganglioside (GM1) binding adjuvants such as cholera toxin.[173, 174] Here, we have shown that dual TLR agonists, MPLA and poly I:C, are safe alternative adjuvants. MPLA is a FDA-approved adjuvant for human papillomavirus and hepatitis B vaccines.[44] Although poly (I:C) had so far limited usage in the clinic due to severe side effects at high doses, we have not observed as harmful toxicity in our animal studies, and alternatively, derivatives of poly (I:C) with lower toxicity in various clinical trials may be used with ICMV vaccines in future studies.[175, 176]

6.3. Future work

Based on our studies so far, ICMV vaccines can trigger disseminated CD8 T cell responses as pulmonary administration can protect against tumor establishment at the flank and prevent dissemination of viral infection from the lungs to the ovaries. However, blocking pathogens at the site of entry can prevent entry of pathogens into the host. Hence, experiments to test if an intravaginal / gut infection can be prevented will be of great interest for mucosal vaccine development

An alternative approach would be to investigate if heterologous prime-boost regimens can stimulate still stronger responses at distal mucosal surfaces. After priming via pulmonary administration, one could administer a boost at the mucosal surface of interest to stimulate stronger proliferation of antigen-specific CD8 T cell that have been seeded at the site by the priming dose.

Alternative adjuvants can also be explored to improve/target homing of T cells to mucosal tissues. For example, including retinoic acid, which has been shown to induce mucosal homing receptors CCR9 and $\alpha 4\beta 7$ on both mouse and humans[177, 178] may enhance spreading of CD8 T cells to different mucosal compartments.

Further understanding of induction of effector vs central memory cells will also be helpful for mucosal vaccine development, especially for pulmonary vaccines which has gained a lot of attention. Most mucosal vaccines are live attenuated vaccines, therefore, it is difficult to isolate different components that influence the immune response. Using a synthetic particle system, different aspects can be changed, e.g. immunization regimen, particle size, release rate, various adjuvants, etc can be isolated and explored to gain better understanding and rationally design better mucosal vaccines.

7. Appendix A: Protocol for processing fecal samples for antibody measurement by ELISA

1. Lyophilize fecal pellets (collected from mice, placed in pre-weighed screw cap tubes and snap frozen in freezer)
2. Get dry weight of feces after lyophilisation
3. Make cocktail (**Reference:** Journal of Immunological Methods, 67 (1984) 101-108) [179]
    a. 1%BSA/PBS+0.1% tween 20
    b. 1:10 dilution of P2714 protease inhibitor
    c. 50mM EDTA
    d. 1mM PMSF (dissolve PMSF in 100% EtOH first make 100mM stock, make fresh everytime and discard leftovers)
4. Add cocktail to feces, 10uL/mg—keep on ice and let samples sit to soften pellets (~15mins)
5. Use toothpicks to homogenize sample in cocktail
6. Sonicate for 5 mins in water bath
7. Vortex 30mins at RT
8. Spin max speed 10-15mins and collect supernatant
9. Spin supernatant at max speed 10-15mins
10. Collect supernatant
11. Freeze down supernatant (spin again before doing ELISA to pellet down any remaining debris)
12. For OVA-specific ELISA:
    a. Coat plates with 100uL of 1mg/ml OVA solution
    b. Start with 1:10 dilution

8. Appendix B: Protocol for intestinal intraepithelial cell isolation

As mentioned in chapter 2, intestinal intraepithelial cells were isolated to determine amount of antigen-specific CD8 T cells were present in the small intestine. Simple meshing does not allow one to extract lymphocytes as various cell types and bowel contents needs to be separated out.

*For IEL medium and HBSS+Hepes, see recipes at the end.

1) Euthanize the mouse.
2) Remove the small intestine: Cut the small intestine at the junction with the pyloric valve and slowly draw it out of the peritoneal cavity. Then cut the small intestine at the junction with the cecum. **While drawing it out, remove the fat with your fingers**/tweezers. Place the intestine in a Petri Dish containing ice cold HBSS or PBS (should be on ice, too). This step is critical to obtaining a large and vibrant population of cells from this tissue!

3) Place the SI in a 10 mm Petri dish with cold HBSS. Cut the intestine longitudinally. Wash the open small intestine several times in cold HBSS and remove as much bowel contents as possible.
4) Remove the fat as much as possible using forceps.
5) Carefully remove Peyer's patches (cut them beyond their border to make sure that no remaining lymphoid tissue is left).
6) Cut the SI into 0.5-1.0 cm pieces.
7) Place the SI pieces in a 50 ml tube containing **20 ml of serum free media w/ 5mM EDTA and 0.145mg/ml of DTT per intestine.** Incubate with shaking (150/min) for 30 min at 37oC.
8) strain the content of the tube through a 100μm cell strainer. Transfer the pieces of small intestine on the strainer to a 50 ml conical tube containing **10 ml of serum free media w/ 2mM EDTA per intestine** (NO SERUM!). Vortex the tube with tissue for 30 seconds and then strain the content of the tube through the same strainer into the same beaker.
9) Repeat the shaking/straining 2-3 more times. *During all these procedures the small intestine pieces will start to turn pink.*
10) Filter the cell suspension through 70 μm cell strainers atop 50 ml Falcon tube on ices.
11) Centrifuge the filtered suspension 5 min at 1800 rpm, 4°C

12) Discard supernatant (SN) and resuspend the pellets in 8 ml 44% Percoll. Vortex briefly and put the cells in a 15 ml tube.

13) Very carefully, underlaid 5 ml 67% Percoll (using a 2 ml pipet). Two distinct phases should be clearly visible and delimited.

14) Important: In order to obtain a better separation of the cells, use the Percoll solutions at RT (20°C).

15) Centrifuge at 1800 rpm for 20 min, 20°C with smooth acceleration and NO brake.

16) If the gradient was successful, the lymphoid fractions should be visible as a turbid ring in the 44%-67% Percoll interphase. The epithelial cells (and other low-density cells) will float on top of the 44% layer and erythrocytes, dead cells, and debris should be in the pellet.

17) Carefully remove the epithelial and other low-density stuff with a Pasteur pipet and gentle aspiration. Collect the lymphoid fraction from the 44%-67% interphase using a 2 ml pipet and put it in a new 15 ml tube (about 1-2 ml).

18) Add IEL medium up to 12 ml. Centrifuge

19) Resuspend the IEL in FACS buffer for staining.

RECIPES:

HBSSS:
500 ml Hanks balanced salt solution without Ca^{++}/Mg^{++} (1x)
+ 10 ml 1M Hepes buffer
+ 5 ml 100x penicillin/streptomycin
+ 0.25 ml gentamicin (40 mg/ml)

IEL medium:
1000 ml RPMI
+ 20 ml FBS
+ 20 ml 1M Hepes buffer
+ 10 ml 100x penicillin/streptomycin
+ 0.50 ml gentamicin (40 mg/ml)

100% Percoll (Final pH should be approx. 7.2. Stable up to 1 month at 4°C)
For 50 ml add:
45 ml stock Percoll
+ 4.48 ml HBSS w/o Ca^{++}/Mg^{++} (10x)
+ 0.50 ml 1 M Hepes buffer
+ 0.23 ml 1 N HCl (Sigma, already prepared)

Percoll quantities required for 1 mouse (if isolating LP and IEL from SB and colon), 2 mice (if only isolating IEL from LP and colon), or 4 mice (if only isolating IEL from SB):

67% Percoll (72% if isolating neutrophils)
6.6 ml 100% Percoll
3.4 ml HBSS

44% Percoll
8.80 ml 100% Percoll
11.2 ml HBSS

*67% and 44% Percoll should be freshly prepared every time and used at RT (20 °C).

9. Appendix C: Protocol for intratracheal instillation

To deliver vaccines directly into the lungs, we followed an intratracheal instillation protocol published by the Jacks Lab in MIT[98]:

1) Anesthetize mice by intra-peritoneal injection of room temperature 20 mg/mL avertin (use 0.4 mg/g body weight for females and 0.45 mg/g body weight for males). Confirm the mice are fully anesthetized by ensuring that they lack a toe reflex.

2) Place the mouse on an intubation platform (purchased from Steve Boukedes, labinventions@gmail.com) so that it is hanging from its top front teeth on the bar (Figure 9-1a–c).

3) Push the mouse towards the bar so that the chest is vertical underneath the bar (perpendicular to the platform) (Figure 9-1b).

4) Direct the Fiber-Lite Illuminator (Model 3100-1, Dolan-Jenner, 660000051001), a fiber optic light source, to shine on the mouse's chest, in between the front legs (Figure 9-1b,c).

5) Prepare the Exel Safelet IV catheter (22 gauge, 1 inch, Fisher, cat. no. 14-841-20) for the instillation procedure. To ensure that the needle does not become exposed and impale the mouse, hold the square part of the needle with the thumb and the index finger, and using the middle finger, push the catheter over the end of the needle completely and continue to hold the catheter in place during the administration protocol (Figure 9-2a,b).

6) Using the Exel Safelet IV catheter, open the mouth and gently pull out the tongue with the flat forceps (Figure 9-1d).

7) Locate the opening of the trachea by peering into the mouth and looking for the white light emitted from the trachea (Figure 9-1e).

8) While holding the Exel Safelet IV catheter vertically, position the catheter over the white light emitted from the opening of the trachea, and allow the catheter to slide into the trachea until the top of the catheter reaches the mouse's front teeth (Figure 9-1f). There should be no resistance while inserting the catheter into the trachea.

9) While stabilizing the Exel Safelet IV catheter with one hand, remove the needle from the mouth (Figure 9-1g).

10) The proper placement of the catheter in the trachea can be confirmed by visualizing the white light shining through the opening of the catheter in the mouth (Figure 9-1h).

11) Pipette the formulation directly into the opening of the catheter to ensure the entire volume is inhaled (Figure 9-1i).

12) If the catheter is correctly inserted into the trachea, the mouse will begin inhaling the formulation immediately. Once the formulation is no longer visible in the opening of the catheter, wait a few seconds for the entire volume to travel down the catheter before removing the catheter from the trachea and disposing of it.

13) Place the mouse under a heat lamp (Figure 9-3a) or on a latex glove filled with warm water (Figure 9-3b) or heat pad to recover from anaesthesia.

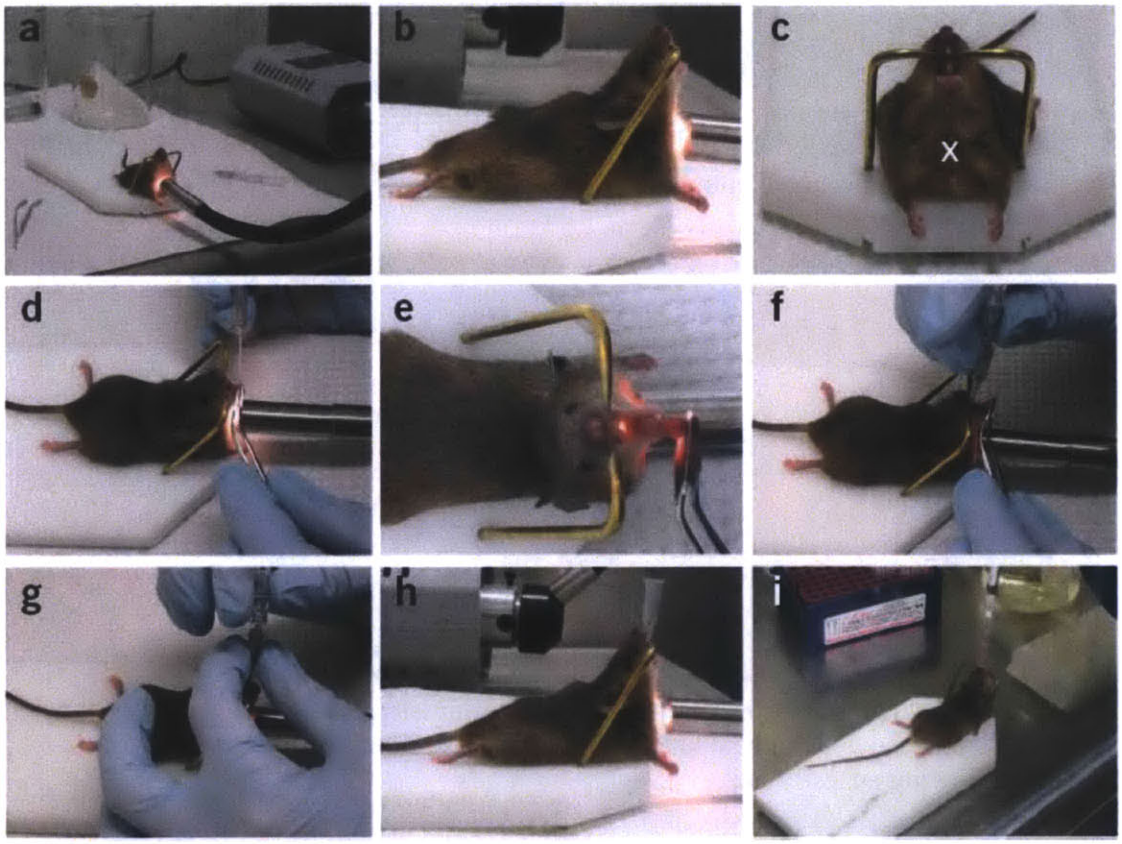

Figure 9-1. Intratracheal instillation procedure.
Anesthetized mice are placed on the platform by their front teeth so that their chest hangs vertically beneath them (**a,b**). The light is directed on the mouse's upper chest (**b,c**), on the spot marked by the 'X' (**c**). The mouth is opened using the Exel Safelet IV catheter (**d**), and the tongue is gently pulled out using the flat forceps. After locating the white light emitted from the trachea (**e**), the Exel Safelet IV catheter is slid into the trachea (**f**), and the needle is removed (**g**). The mouse with the inserted catheter (**h**) on the platform is moved into a biosafety hood, where the virus is dispensed into the opening of the catheter (**i**).

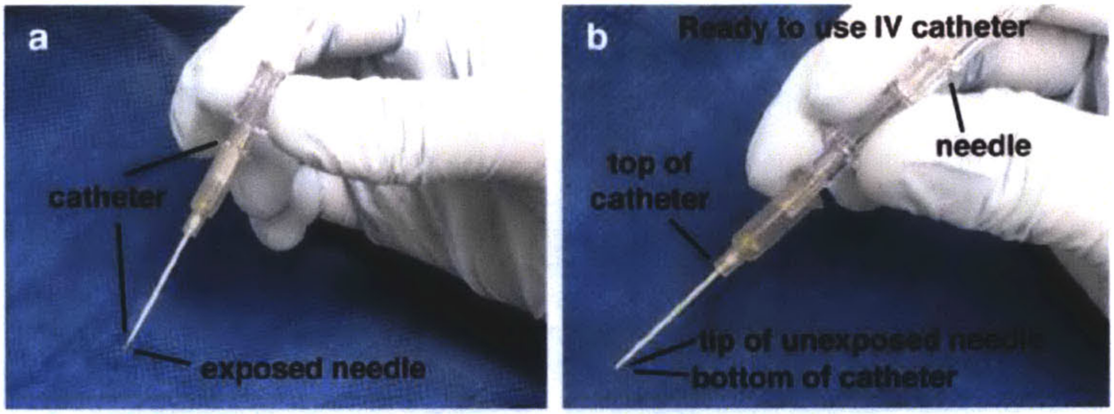

Figure 9-2. Preparation of the Exel Safelet IV catheter for intratracheal instillation.
Upon opening the Exel Safelet IV catheter, the needle is exposed (**a**). Slide the catheter over the end of the needle to completely cover the tip (**b**) and the Exel Safelet IV catheter is now ready to use.

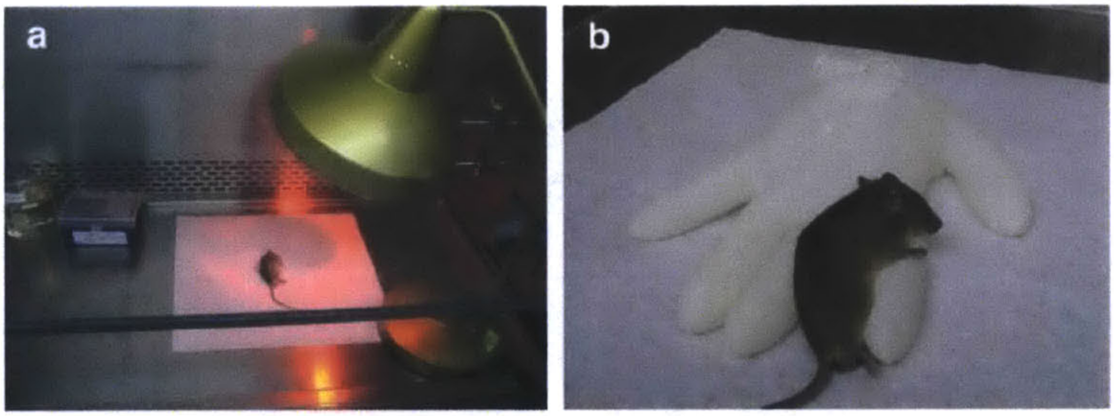

Figure 9-3. Recovery following intratracheal administration.
Mice can be placed under a heat lamp (**a**) or on a glove filled with warm water (**b**) to recover following anaesthesia

Text and figures adapted from:
Michel DuPage, Alison L Dooley and Tyler Jacks; Nature Protocols **4**, p.1064 - 1072 (2009).

Notes:

Inadequate anesthesia increases the probability that the mouse will swallow the formulation, therefore the amount of avertin administered to the mice is crucial to the success of the procedure. Mice administered with too much avertin are more likely to stop breathing during the procedure and recover poorly from the anesthesia. Conversely, mice administered with too little avertin may struggle to inhale the formulation and should be given more avertin before continuing. Therefore, it is recommended to start with the smallest volume of avertin needed to keep mice anesthetized during the procedure and if necessary, administer more avertin, 50 –100 μL at a time. If mouse is over-anesthetized and breathing very slowly, wait until breathing becomes more regular but ensure the mouse still lacks a reflex response before attempting administration. Following the procedure, mice will recover better if they are kept warm to maintain their normal body temperature after anesthesia.

To locate the white light marking the opening of the trachea, it is recommended to look at the ventral surface of the throat. This can be done by leaning further over the mouth and pushing the tongue with the catheter towards the ventral surface of the throat. Sometimes, saliva may be covering the opening of the trachea. It is recommended that one gently probe at the back of the throat with the Exel Safelet IV catheter to expose the trachea. If the catheter is correctly inserted into the trachea, the mouse will begin inhaling the formulation immediately. If it does not inhale the formulation (formulation stays in the catheter), it is likely that the catheter is inserted into the esophagus. In this case, one should pipette the formulation out of the catheter for reuse and begin the catheter insertion procedure again.

10. Appendix D: Intranasal immunization with ICMVs

Intratracheal instillation can allows one to deliver more defined and consistent doses to the lungs, therefore, pulmonary immunization was delivered intratracheally in this project. However, as mentioned in chapter 4, intranasal delivery is a less invasive, more clinically relevant method to deliver solutions into the lungs. The protocol we used for intranasal administration is as follow (modified from DuPage et al [98]):

1) Administer avertin by i.p. injection as described in Appendix C: Protocol for intratracheal instillation to anesthetize mouse.
2) Hold mouse in hand. Hold the mouse up so that the head is positioned above its feet
3) Use fingers to gently hold jaw shut to prevent solution from draining down esophagus during inhalation
4) Administer the solution dropwise (~10ul each drop) onto the nostrils with a pipette.
5) Solution will be inhaled immediately, keep fingers on jaw until drop is all inhaled.
6) Wait until breathing becomes regular
7) Administer another drop of solution and repeat procedure until all solution is inhaled.
8) Lay mouse down with ventral side faces up for recovery.

Notes: Do not grasp the mouse tightly, as this will inhibit the mouse's breathing. Do not attempt to insert the pipette tip into the nostril.

Figure 10-1. Intranasal administration
The anesthetized mouse lies gently in the hand of the investigator (a), and solution is administered dropwise (b) into nostril until the virus is completely inhaled (b, c). In the modified protocol, use fingers to gently hold jaw shut to prevent solution from draining down esophagus during inhalation.(from DuPage *et al* [98])

11. Appendix E: Protocol for Ki67 staining

Ki-67 protein is strictly associated with cell proliferation. The protein is present during all active phases of the cell cycle but absent in resting cells, making it an excellent marker for proliferation[180]. For this project, Ki67 staining was performed to identify sites of active antigen-specific CD8 T cells proliferation.

1) Prepare cell suspension in 1%BSA/PBS
2) Add Fc block to prevent non-specific binding
3) Incubate cells with tetramer of interest for 30mins, 4C
4) Stain with antibodies against cell surface markers of interest (e.g. anti-CD8, antiCD127). Incubate 30mins, 4C
5) Use BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD cat# 554714) and follow manufacturer's protocol to fix and permeabilize cells.
6) Stain with anti-Ki67 (Abcam cat# ab27619) in 1x perm/wash buffer for 30mins, 4C
7) Wash with 1%BSA/PBS twice
8) Store in 1%BSA/PBS. Add counting beads (Invitrogen cat#PCB100) if necessary.

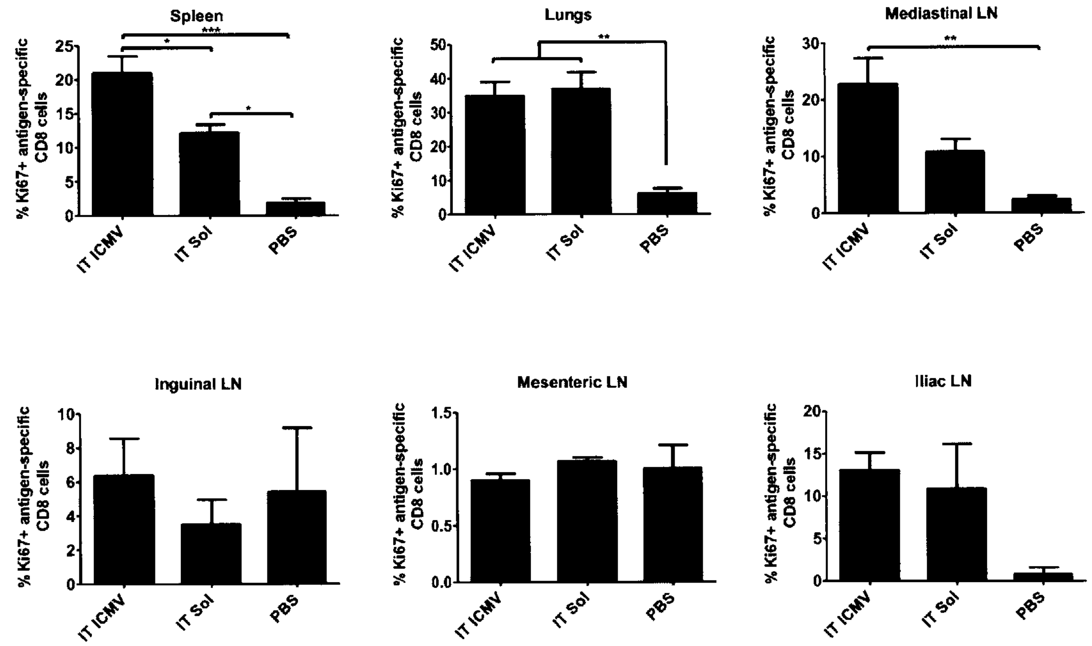

Figure 11-1. Proliferating (Ki67$^+$) antigen-specific CD8 T cells 10 days after pulmonary immunization.

Preliminary data we gathered suggest that pulmonary immunization with ICMV continues to prime more antigen-specific CD8 T cells at sites local to vaccine administration (Lungs, mediastinal LN). Cells disseminated to distal sites (spleen, inguinal LN, mesenteric and iliac LN) also continues to proliferate.

12. References

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