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PHARMACEUTICALLY ENGINEERED NANOPARTICLES FOR ENHANCING IMMUNE RESPONSES TO HIV-1 TAT AND GAG p24 PROTEINS

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ABSTRACT OF DISSERTATION

Jigna D. Patel

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Pharmacy
at the University of Kentucky

By
Jigna D. Patel

Lexington, Kentucky

Director: Dr. Russell J. Mumper, Associate Professor of Pharmaceutical Sciences

Lexington, Kentucky

2006

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These studies were aimed at investigating the potential application of nanoparticles engineered from oil-in-water microemulsion precursors for enhancing immune responses to HIV-1 Tat and Gag p24 proteins. Both of the HIV-1 proteins have been reported to be critical in the virus life cycle and are being evaluated in clinical trials as vaccine candidates.

Anionic nanoparticles were prepared using emulsifying wax as the oil phase and Brij 78 and sodium dodecyl sulfate as the surfactants. The resulting nanoparticles were coated with Tat and were demonstrated to produce superior immune responses after administration to BALB/c mice compared to Tat adjuvanted with Alum. Similarly, cationic nanoparticles were prepared using emulsifying wax and Brij 78 and cetyl trimethyl ammonium bromide as the surfactants. The cationic nanoparticles were investigated for delivery of immunostimulatory adjuvants, namely three Toll-like receptor ligands, for obtaining synergistic enhancements in immune responses to a model antigen, Ovalbumin (OVA).

In vitro and *in vivo* studies were carried out to elucidate possible mechanisms by which nanoparticles may result in enhancements in immune responses. *In vitro* studies were carried out to evaluate the uptake of nanoparticles into dendritic cells and to assess the release of pro-inflammatory cytokines from dendritic cells in the presence of nanoparicles. *In vivo* studies were carried out using a MHC class I restricted transgenic mouse model to investigate the potential for nanoparticles coated with OVA to enhance presentation of the protein to $CD8^+$ T cells compared to OVA alone.

Finally, the preparation of nanoparticles with a low amount of surface chelated nickel for high affinity binding to histidine-tagged (his-tag) proteins was investigated. It was hypothesized that this strengthened interaction of his-tag protein to the nickel chelated nanoparticles (Ni-NPs) would result in a greater uptake of antigen *in vivo*; therefore, enhanced immune responses compared to protein bound to anionic nanoparticles. *In vivo* evaluation of his-tag HIV-1 Gag p24 bound to Ni-NPs resulted in enhanced immune responses compared to protein either adjuvanted with Alum or coated on the surface of nanoparticles.

Jigna D. Patel

July 24, 2006

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By

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DISSERTATION

Jigna D. Patel

The Graduate School

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To my sister, Deena and

in memory of my mother, Dhanu

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Chapter 1

Introduction and statement of problem

Adjuvants can be defined as any material included as a component in a vaccine to aid in producing more robust cellular and/or humoral immune responses to the antigen of interest. Adjuvants can be broadly categorized as immunostimulatory or particulate, including particulate delivery systems. Many of the early developments in vaccines, including the use of adjuvants, have been empirically derived on a trial and error basis without in depth knowledge of the exact mechanisms involved in generating effective immune responses. As we gain a better understanding of the immune system and its components, we can design more effective vaccines. An important strategy in designing more effective vaccines would be to explore the use of novel delivery systems for effectively eliciting the immune responses necessary for protection from viral and bacterial pathogens. The need for safe and effective delivery systems is even more evident with new generation vaccines where peptides, protein subunits, or DNA are the antigens. Unlike traditional vaccines such as live attenuated or whole killed pathogens, new generation vaccines are considered to be much safer; however, new generation vaccines produce poor immune responses when administered alone. Therefore, in many cases, adjuvants are typically used in conjunction with the antigen to enhance the immune response [1,2].

Currently, aluminum-based mineral salts (Alum) are the most widely used adjuvants for human vaccination. Alum has proven to be safe and effective for antibody

production (humoral response); however, it is not very effective for generating strong cellular responses with recombinant proteins [3], which are considered to be important in providing protection from many viruses such as the human immunodeficiency virus (HIV) [4]. While many new adjuvants have entered clinical trials, most have been proven too toxic to be used routinely in humans [1]. Ideal adjuvants for routine human vaccine applications should be safe, cost-effective, relatively simple to manufacture and scale-up, versatile, and should produce balanced (humoral and cellular) immune responses.

Particulate delivery systems such as microparticles and liposomes have been extensively investigated for enhancing immune responses to protein-based vaccines [5-9]. These systems offer several advantages over other adjuvants for enhancing immune responses since: 1) they are naturally targeted for uptake by APCs due to their similar size to pathogens; 2) targeting ligands for APCs can be incorporated on the surface of the particles; and 3) immunostimulatory molecules can be incorporated with the particles for synergistic enhancements in immune responses. Moreover, a number of studies suggest that smaller particles (<1 micron) are more effective at generating immune responses compared to larger particles (>10 microns) [10-13].

Nanoparticles prepared from oil-in-water microemulsion precursors have been shown to be effective at enhancing immune responses to plasmid DNA [14,15] and a model protein, β-galactosidase [16]. The present research was focused on further investigating the utility of these nanopariticles for enhancing immune responses to two HIV proteins, Tat and Gag p24. Both proteins are of significant interest for HIV vaccine development since they have been reported to be relatively conserved and critical in the

HIV life cycle. In addition, both proteins are currently being evaluated in clinical trials [17-19]. *In vitro* and *in vivo* studies evaluating parameters such as uptake and cell activation by nanoparticles were carried out to gain a better understanding of the mechanism by which nanoparticles may be enhancing immune responses. These types of studies are considered to be important as they would have implications on improvements that could be made to develop of more effective vaccine delivery systems.

Chapter 2

Plan of research

The overall goal of this research was to investigate the application of pharmaceutically engineered nanoparticles for HIV-1 Tat and Gag p24 proteins to elicit enhanced as well as balanced immune responses compared to protein adjuvanted with Alum. This research was guided by three main hypotheses:

Hypothesis 1. Mice dosed with anionic nanoparticles coated with HIV-1 proteins will result in enhanced humoral and cellular immune responses compared to those dosed with protein adjuvanted with Alum.

Hypothesis 2. Increasing the affinity of the protein antigen for the nanoparticles will result in a more stable attachment and lead to a corresponding enhancement in the immune responses *in vivo* compared to antigens coated on anionic nanoparticles by charge interaction.

Hypothesis 3. Mice dosed with nanoparticles co-formulated with protein antigen and an immunostimulatory molecule will produce enhanced immune responses compared to those dosed with protein antigen with nanoparticles or immunostimulatory molecule alone.

To evaluate these hypotheses, the research plan described in sections 2.1 to 2.4 was carried out.

2.1   Formulation and *in vivo* assessment of anionic nanoparticles

The main objectives of this section were: 1) to demonstrate that stable anionic nanoparticles could be prepared; 2) to formulate Tat with anionic nanoparticles; and 3) to evaluate the *in vivo* responses of Tat-coated nanoparticles compared to Tat adjuvanted with Alum. Anionic nanoparticles in this study were prepared from oil-in-water microemulsion precursors using emulsifying wax as the oil phase and Brij 78 and sodium dodecyl sulfate (SDS) as the surfactants. The nanoparticles were characterized by particle size, charge, and transmission electron microscopy. Tat was formulated with anionic nanoparticles and further characterized by measuring the resulting size and charge of the particles. The *in vivo* immune responses to Tat were evaluated by dosing BALB/c mice with 0.2 to 5 μg Tat either coated on anionic nanoparticles or adjuvanted with Alum. The Tat-specific immune responses were evaluated by measuring the serum IgG levels, the ability of the anti-sera to neutralize extracellular Tat, and mapping the antibody epitopes generated to Tat.

2.2   Formulation and *in vivo* assessment of immunostimulatory molecules and Ovalbumin (OVA) coated on cationic nanoparticles

The immunostimulatory molecules investigated for use with nanoparticles are classified as Toll-like receptor (TLR) ligands. The aims of this section were as follows: 1) demonstrate the preparation of cationic nanoparticles; 2) demonstrate that OVA could be coated on cationic nanoparticles; 3) demonstrate that TLR ligands and OVA could be coated on cationic nanoparticles; 4) evaluate the TLR ligands and OVA formulations in BALB/c mice to identify the optimal TLR ligand formulation for future studies with

nanoparticles. Cationic nanoparticles were prepared from oil-in-water microemulsion precursors using emulsifying wax and Brij 78 and cetyl trimethyl ammonium bromide (CTAB) as the surfactants. The TLR ligands evaluated in this section were lipoteichoic acid (LTA), a synthetic double stranded RNA analog (Poly I:C), and a 20-mer synthetic oligonucleotide containing CpG motifs (CpG). The nanoparticles were characterized before and after coating with OVA or the TLR ligands by measuring the size and charge of the particles. Initially, a short-term *in vivo* study was carried out in mice to identify the best TLR ligand for further evaluation. For this study, mice were dosed once with 50 μg of the various TLR ligands and 5 μg OVA coated on nanoparticles. The immune responses were evaluated on day 8 by measuring T cell proliferation in the lymph nodes. Based on the results of this study, the TLR ligand CpG was chosen for further evaluation and a follow up *in vivo* study was carried out to evaluate the immune responses to CpG (10 μg) and OVA (5 μg) coated on nanoparticles compared to CpG or nanoparticles alone formulated with OVA. The immune responses in this follow up study were evaluated by measuring OVA-specific serum IgG titers and OVA-specific splenocyte proliferation.

2.3 **_In vitro_ and _in vivo_ assessment of mechanism(s) of immune response enhancement by nanoparticles**

This main objective of this section was to elucidate possible mechanism(s) by which nanoparticles may be enhancing immune responses to antigens *in vivo*. These studies were separated into two parts. In the first part, *in vitro* studies were carried to evaluate: 1) the uptake of various types of nanoparticles (neutral, anionic, and cationic) by murine bone-marrow derived dendritic cells (BMDDCs); 2) the release of pro-

inflammatory cytokines in human DCs and BMDDCs; and 3) the release of IL-12 from BMDDCs using CpG-coated NPs compared to CpG alone. The second part of this study involved the use of a MHC class I restricted OVA transgenic mouse model (OT-1) for evaluating the uptake and presentation to T cells *in vivo* of OVA-coated nanoparticles compared to OVA alone. For these studies, T lymphocytes from the spleens of OT-1 mice were labeled with a green-fluorescent marker and transferred by tail-vein into C57BL/6 mice. The OVA containing formulations were injected 24 hours later and T cell proliferation in the lymph nodes was measured after 3 days.

2.4   Formulation and *in vivo* evaluation of nickel-coated nanoparticles

The goal of this section was to strengthen the interaction of antigens with the nanoparticles for obtaining greater enhancements in immune responses compared to simple anionic nanoparticles. The use of chelated nickel for purification of proteins containing a hexa-histidine tag (his-tag) has been used extensively and this interaction has been reported to be extremely strong, in the order of magnitude of biotin-avidin interactions. This approach of incorporating nickel on the nanoparticles for strengthening the interaction with proteins was investigated. More specifically, the aim of these studies was to: 1) demonstrate that nanoparticles containing a small amount of chelated nickel could be prepared; 2) demonstrate that the nickel coated nanoparticles could bind to his-tag proteins; and 3) evaluate the immune response to his-tag HIV-1 Gag p24 bound on nickel nanoparticles compared to protein adsorbed on anionic nanoparticles or adjuvanted with Alum. Nanoparticles having a small amount of surface-chelated nickel were prepared using emulsifying wax, Brij 78, and a nickel chelating lipid 1,2-dioleoyl-*sn*-

glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl]. The resulting nickel nanoparticles (Ni-NPs) were characterized by particle size and the amount of nickel entrapped in the particles was determined by atomic emission spectroscopy. Initial studies to demonstrate binding of his-tagged proteins to Ni-NPs were performed using his-tag green fluorescent protein (GFP). The optimal binding and stability of his-tag GFP bound to Ni-NPs was determined. Based on the GFP binding results, the optimal binding ratios for his-tag HIV-1 Gag p24 were determined and further *in vivo* immune responses to the protein formulated with Ni-NPs, Alum, and anionic nanoparticles were evaluated.

Chapter 3

Background and literature review

3.1   Vaccines

3.1.1   A brief history

Vaccination can be defined as "an overt attempt to use part or all of a microbial pathogen to protect against that microbe" [20] with the ultimate goal being the induction of appropriate immune responses to provide protection against a pathogen without causing serious disease. Vaccines have proven to be highly effective at controlling many infectious diseases and reducing disease related mortality over the last two centuries. In fact, vaccines have been regarded as one of the greatest achievements in the reduction of deaths due to infectious diseases, with the exception of safer water [21].

The introduction of vaccination is accredited to Edward Jenner, an English physician, for his effort in deliberately inoculating a young boy with cowpox and demonstrating this as an effective strategy for controlling smallpox infection when it was introduced to the boy six weeks later [21]. Jenner referred to the inoculum as *vaccine* (derived from the Latin word for cow, *vacca*) and termed this procedure *vaccination* [22], entitling Jenner as the father of modern vaccinology in historical records. However, the idea of protection from infectious diseases was long before recognized in China and India in the 16th century where a process termed variolation was practiced to reduce severity of smallpox. This process involved the introduction of dried pus from smallpox pustules into healthy individuals through the nose or the skin [21]. The process of variolation via

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the skin (cutaneous variolation) eventually spread to through the Middle East, Africa, Turkey, and finally introduced to Great Britain by Lady Mary Wortley Montagu in 1721 [20,21,23].

The next major advance in vaccination came about in 1879 with Louis Pasteur's work on the attenuation of chicken cholera bacterium. Pasteur noted that a culture of chicken cholera left exposed to air over a long period provided protection against challenge with non-attenuated cholera in immunized chickens. Based on these initial observations with the chicken cholera cultures, Pasteur believed that pathogens could be attenuated by exposure to various environmental factors such as elevated temperatures, oxygen, and chemicals. Further experimentation during this period by Pasteur and colleagues confirmed his hypothesis and led to the development of both a rabies vaccine and an anthrax vaccine in the 1880's [21].

These early pioneering efforts by Jenner and Pasteur paved the foundation for future developments in vaccines. A brief historical outline of the major developments in vaccines is presented in Table 3.1. As evident from this Table, since the initial discovery of an effective smallpox vaccination strategy, numerous other vaccines have come into development. Moreover, vaccination has been effective in controlling many diseases including: smallpox, diphtheria, tetanus, yellow fever, pertussis, *Haemophilus influenza* type b disease, poliomyelitis, measles, mumps, and rubella [21]. However, the ultimate triumph in vaccinology to date has been the global eradication of smallpox, declared in 1980 by the World Health Organization (WHO) [22].

3.1.2 Types of vaccines

Vaccines can be classified into three main categories: live attenuated, whole-killed, or genetically engineered vaccines. The use of live attenuated or whole killed organisms as vaccine components have been recognized since the 18th century, as described above. These approaches are typically referred to as traditional vaccines. Recently, a better understanding of molecular mechanisms and genetic techniques has led to the development of a new class of vaccines that are comprised of DNA, peptides or proteins, more commonly referred to as new generation vaccines.

3.1.2.a Traditional vaccines

The first successful vaccines demonstrated by Jenner and Pasteur were based on live attenuated organisms. Live attenuated vaccines are composed of pathogens weakened by passage or culture conditions causing the organism to be non-virulent but still immunogenic. Examples of the earliest live attenuated vaccines used in humans include the smallpox and rabies virus vaccines. It took another 40 years after the introduction of rabies vaccine by Pasteur for the next major live attenuated vaccine to be developed, which occurred in 1920's with the introduction of the Bacille Calmette-Guérin (BCG) vaccine for *tuberculosis* (TB) by Camille Calmette and Alphonse Guérin. The first BCG vaccine was developed by repeated passage of the mycobacterium in bovine bile and after 13 years and approximately 230 passages, the attenuated BCG strain was obtained and evaluated orally in clinical trials with children in 1921, with the widespread use in humans initiated in 1927 [21]. The value of BCG vaccines has been questioned because of the varying effectiveness observed in controlled trials with the

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vaccine where some trials demonstrate benefits while others show no benefit in providing protection from the bacteria by vaccination. Despite this ongoing controversy over the use of BCG vaccines, they are still in use throughout the world with the exception of a few industrialized countries such as United States and Netherlands [24]. Moreover, renewed interests in these vaccines have been stimulated with the emergence of tuberculosis associated in human immunodeficiency virus (HIV) infected patients. Perhaps the most significant achievement in the use of live attenuated vaccines is the introduction of an oral poliovirus vaccine (OPV) in 1962, resulting in a dramatic decrease in poliomyelitis – one of the diseases targeted of eradication by WHO [25]. Additional examples of live attenuated vaccines that have made significant contributions in reducing disease rates include the mumps, rubella, and measles vaccines, which have become the foundation of routine pediatric immunization and were licensed as combination vaccines (MMR) in 1971 [26,27] . Although live attenuated vaccines have been used widely and are generally more effective than inactivated vaccines, a major disadvantage of these types of vaccines is that they tend to pose numerous safety concerns as the organisms could revert back to a more virulent strain and cause disease.

The use of whole-killed vaccines began soon after Pasteur's initial success in generating attenuated strains of chicken cholera. Daniel Salmon and Theobald Smith are credited for demonstrating that whole-killed pathogens retained their immunogenicity [28]. In 1896, the work by Wilhelm Kolle demonstrated that agar-grown, heat inactivated *Vibrio cholerae* could be used as a human vaccine for cholera. Around the same time Richard Pfeiffer and Almroth Wright demonstrated that cultures of *Salmonella typhi* could be inactivated with heat and preserved in phenol. This demonstration led to

the development of a typhoid fever vaccine in 1896 that eventually (1915) became widely used in the military in Europe [29]. Some examples of vaccines to infectious agents that have been developed in this category include the inactivated poliovirus (IPV) vaccine, Hepatitis A vaccine, and the influenza vaccine. The use of IPV vaccine has replaced the more effective OPV vaccine in the U.S. and many European countries due to some safety concerns raised by some reported cases of paralysis due to the live attenuated vaccine and more importantly, because the disease is very rare in these countries [30].

3.1.2.b New generation vaccines

The majority of early vaccines developed were based on live attenuated or whole killed organisms. However, the growing knowledge of infectious diseases during late 19th and early 20th century paved the pathway for the identification of pathogen components and exploring the use of these individual components for developing vaccines. In the late 1880s, Roux, Yersin, Behring, and Kitasato realized that diphtheria bacillus produced an extracellular toxin and that sera from infected animals contained an antitoxin able to neutralize the diphtheria toxin in culture. These discoveries paved the foundation for future developments of inactivated toxins, referred to as toxoids, as vaccine components. In fact, as early as 1923 it was realized that the diphtheria toxin could be inactivated by formalin and enabled Gaston Ramon to develop the earliest subunit vaccines, namely diphtheria and tetanus vaccines [21]. With additional technological advancements, the ability to separate and extract bacterial components led to the development of polysaccharides, components of bacterial cell walls, as viable

vaccine strategies in cases such as the early pneumococcal and meningococcal vaccines [31].

From these early discoveries, it is apparent that advancements in technology as well as an understanding of both pathogens and immune functions have contributed immensely to the development of safer, more effective vaccines throughout their history. This is even more apparent in the recent years where genetic engineering techniques have contributed immensely in developing safer vaccines. For example, the use of recombinant DNA has enabled production of large quantities of well-defined, purified proteins that have been used for a variety of applications including the development of safer protein-based vaccines. In fact, the earliest success in using genetic engineering for vaccine development came about in the early 1980's with the introduction of a yeast derived Hepatitis B subunit vaccine, which had previously been obtained from purification of infected individual's plasma [32,33]. Genetic engineering techniques have also opened up avenues in the rational design of proteins using site-directed mutagenesis to produce proteins with altered properties that are not naturally occurring in the pathogen and thus, may be less detrimental or reactogenic in the host, i.e. toxoids. One example of this application is in the improvement of an existing pertussis vaccine, where pertussis toxin was detoxified by a double mutations to the protein which rendered it safer while still retaining its antigenic conformation and immunogenicity *in vivo* [34,35]. This protein-based pertussis vaccine is approved for human use and has replaced the whole-cell pertussis vaccine in many countries [36].

Another advancement in vaccines permitted by genetic engineering technology occurred in 1992 when Johnston's group reported that plasmid DNA (pDNA) was

effective for immunization, referred to as genetic immunization [37]. Genetic vaccines take advantage of the use of plasmid DNA for expression of a gene encoding for a specific antigen(s) under the control of a eukaryotic or viral promoter (such as a CMV promoter) [38]. DNA vaccines, once administered, allow for the expression of protein inside the cells leading to processing and presentation by the major histocompatability (MHC) class I pathway and generating strong cellular responses. Indeed, strong cytotoxic T lymphocyte (CTL) responses have been reported with genetic vaccines in several different animal models [39-41]. Genetic vaccines enable the delivery and co-expression of multiple antigens or epitopes of a pathogen on one plasmid [42]. Moreover, the delivery of antigen-cytokine fusions encoded on the same plasmid has also been investigated as a method for obtaining robust enhancements in immune responses [43-45]. The most common route of administration for delivery of genetic vaccines has been via intramuscular injection; although, many new devices and alternative approaches, such as topical application and use of gene gun delivery devices, have been investigated more recently in effort to enhance immune responses by targeting dendritic cells (DCs) [14,42,46-48]. Unlike many of the approaches to vaccines discussed so far, genetic immunization has proven to be successful in mice; however, it has demonstrated limited effectiveness in non-human primates and humans after conventional intramuscular administration, requiring extremely high doses of pDNA and in some cases failing to induce antibody responses [38]. More recently, genetic immunization using low doses of DNA in humans has been reported with the use of gene gun devices as an alternative to intramuscular injection by PowderMed, Inc. (http://www.powdermed.com). This success has been brought about by the PMED™ technology, which involves the delivery of DNA

coated onto gold particles (1-3 micron in size) into the epidermis using a high pressure helium gene gun. Phase I clinical trials with PMED™ for influenza vaccine resulted in 100% seroconversion with as little as 4 μg dose of DNA. This approach is currently being explored for cancer, genital herpes, Hepatitis B, and HIV vaccines in phase I clinical trials. In addition, genetic immunization show promising applications in prime-boost vaccine strategies, where the pDNA vaccine may be used for initial immunization followed by boosting with another type of vaccine, i.e. viral vectors or subunit-based vaccines [49]. Thus, genetic immunization may prove successful with the development of more effective delivery devices in combination with heterologous prime-boost immunization strategies. A major safety concern raised with the use of pDNA based vaccines is the potential of the plasmid to integrate into the host genome [42].

The candidate subunit or pDNA based vaccines deem themselves much safer than traditional approaches as they are purified and well-defined entities; however, they are less immunogenic than their counterparts due to removal of various components of the whole pathogen which can function as recognition elements in the body and trigger the innate immune system – naturally mediating enhanced immune responses. Therefore, one downfall of new generation vaccines is that they are not very immunogenic when administered by themselves and many of these approaches require the use of adjuvants to generate strong immune responses.

3.1.3   Trends and future developments in vaccines

Although significant developments have been made in vaccines, a number of challenges still lie ahead for the prevention of numerous diseases. The growing

knowledge and technological advancements in various areas of vaccinology have made this an exciting and prevailing field. It has become evident from past experiences that a greater understanding of immunological functions and viral pathogenesis for many organisms will be essential in developing effective vaccines for chronic infections such as HIV, human cytomegalovirus, herpes simplex virus (HSV) and hepatitis C virus (HCV). Moreover, vaccines have been traditionally used for preventing infectious diseases; however, current trends demonstrate a shift in their application as there is a great deal effort being focused on designing vaccines for non-infectious diseases such as Alzheimer's, cancer, autoimmune diseases, and for therapeutic applications for controlling progression on to disease as in the case of HIV, HSV, and human papillomavirus (HPV) [28].

Whereas the historical basis for vaccines was largely empirical, technological advancements, in particular genetic engineering techniques, can potentially lead to a more rational approach in design of vaccines. Several examples of the application of genetic engineering techniques in generating safer, more effective vaccines have already been presented in the previous section in the discussion of new generation vaccines. Additional examples of the benefits of these techniques include, 1) attenuation of live vaccines via gene deletions or mutations, 2) engineering bacterial or viral vectors to express foreign proteins of interest, 3) generating reassortant viruses (i.e. influenza vaccines) by combination of genes from two or more different viral strains, and 4) generating T cell epitope based vaccines [50]. In spite of the successes of generating more stable, less virulent live attenuated vaccines with these techniques, there is still a significant concern in using this approach for many diseases. Moreover, the growing

requirement for safer and molecularly well-defined entities imposed by regulatory agencies lends future developments in this area towards the use of subunit based vaccines. Therefore, a great deal of focus in this area has been placed on identifying the key antigens in preventing or controlling infection as well as on finding newer, more effective adjuvants due to their inherent need for enhancing immune responses with these purified antigens [51]. Other key considerations for developing new vaccines include: storage, stability, ease of manufacture and scale up, ease of administration or needle free administration, and feasibility of single dose. The use of needle-free devices and single-dose vaccination are particularly important for increased patient compliance.

3.2   Vaccinology and key mediators of immunity

Despite the successes of some of the earliest vaccines such as smallpox and polio, many of these developments have been made empirically without much knowledge on the functions of immune system and its components in regard to generating an effective immune response. The most successful vaccines developed to date have been made for pathogens that cause acute infections and that could be potentially cleared from the body by the host's immune system. Moreover, the effectiveness of many of these vaccines has largely relied on the generation of high levels of neutralizing antibodies. An understanding of the immune system and its components has greatly advanced with emerging chronic infections such as HCV, HIV etc. that cause persistent infections that cannot be cleared by the body's immune system even in the presence of strong immune responses. Many, if not most, of these infections are intracellular and offer additional challenges due to mutations in the virus, giving rise to varying strains. Thus, with the

aim of developing effective vaccines for these more challenging infections, a greater understanding of the immunological functions has been and continues to be gained. The contributions of immunology to this area will be necessary for developing safer and more effective vaccines, adjuvants, and delivery systems.

The immune systems and its functions can be divided into two categories: the innate and adaptive immune system [52,53]. Both are essential in providing protection from organisms and have specialized components that are involved in generating an immune response. Although both systems are generally thought of as distinct, there is considerable interplay in the two systems in fighting infections and they in fact share some of components (i.e. antigen presenting cells). Key features of each system are presented in Table 3.2.

3.2.1 Innate immune system

The innate immune system is often referred to the non-specific component of the immune system [53]. This system is the first line of defense against pathogens and is activated through recognition of non-specific, conserved molecular structures commonly present on pathogens or groups of pathogens, but not found in the host, called pathogen-associated molecular patterns (PAMPs) [54,55]. Some examples of PAMPs include the lipopolysaccharides (LPS) found in gram-negative bacteria and double-stranded RNA produced by many viruses. These PAMPs are recognized by a limited number of pathogen recognition receptors (PRR), which are germline-encoded receptors present in cells comprising the innate immune system [55,56]. Activation of the innate immune system occurs within hours of encountering an organism and results in the production of

pro-inflammatory cytokines such as interferons (IFN-α, β, and γ), interleukins (ILs), and activation of complement pathway [52,55]. The immune response generated mediates the clearance of the pathogen from the host; however, the immune response to the pathogen is constant – no heightened immune response is generated with successive exposures to the pathogen [52,53].

The activation of innate immune system is mediated by the recognition of pathogens by PRR including Toll-like receptors (TLRs), scavenger receptors, and Fc receptors. A detailed review of the recognition, binding, and signaling processes involved in the innate immune response is presented elsewhere (see references [55,57,58]).

3.2.2 Adaptive immune system

Hallmarks of the adaptive immune system include high specificity and memory of the specific immune responses generated [52]. Unlike the innate immune system, the adaptive immune system is capable of generating stronger and higher immune responses with successive exposures to the pathogen. Thus, it is possible to prevent or reduce the course of infection upon re-exposure to the same pathogen – via the generation of a memory response. This branch of the immune system is composed of three main cells: antigen presenting cells (APCs), B lymphocytes (B cells) and T lymphocytes (T cells). The APCs provide a key bridge between the innate and adaptive immune systems, whereas, the lymphocytes are the mediators of the high specificity and memory components unique to the adaptive immune system. Both lymphocytes involved in the adaptive immune response are derived from a common lymphoid progenitor cell in the

bone marrow; however, their maturation at different sites in the body raises these two cell types [52]. Moreover, both naïve lymphocytes are located in specialized areas in secondary lymphoid organs (i.e. the mucosal lymphoid tissues, draining lymph nodes and spleen) where antigen recognition by these cells occurs, generating effector cells that can participate in the antigen-specific response. Therefore, unlike innate immune system which initiates responses at the site of infection, the adaptive immune responses are initiated in the secondary lymphoid organs. In addition, memory cells that are able to respond more robustly to subsequent exposures to the same pathogen are generated during adaptive immune responses. One major difference between the two lymphocytes for antigen recognition is that B cells can directly recognize the antigen or pathogen through receptors present on the cell surface, whereas T cells do not have the ability to directly recognize the antigen and one of the functions of APCs is to process the antigen and present peptide fragments bound to cell surface molecules for recognition by T cells. The main interactions between the cells of the adaptive immune system leading to the production of antigen specific immune responses are summarized in Figure 3.1. A brief description of the various functions of the cells in generating an adaptive immune response is given in the sections below (see references [52,53] for a comprehensive review).

3.2.2.a Antigen presenting cells

Pathogens taken up and processed by APCs initiate a sequence of events that ultimately leads to the activation of APCs and naïve T cells. APCs are found on most tissues (i.e. skin and mucosa) and circulating throughout the body, constantly surveying

the environment for invading organisms. The ingested pathogens are then processed into peptide fragments (peptides of 9-15 amino acids), are conjugated to molecules encoded by genes of the major histocompatability complex (MHC) and are presented on the surface of APCs for T cell recognition. This process is referred to as antigen presentation. Two types of MHC molecules are present: MHC class I, which are present on all cell types, and MHC class II, which are specifically expressed on APCs. MHC class I molecules bind peptides of 9-10 amino acids, whereas MHC class II molecules bind to longer peptide fragments of 10-15 amino acids in length. The processing pathway for the peptide fragments binding to these two MHC molecules is also distinct. MHC class I molecules are generally thought to bind to processed fragments of a protein synthesized in the cytoplasm, such as those resulting from infection from a virus. On the other hand, MHC class II molecules primarily bind to peptide fragments of exogenously derived proteins, which have been processed in the lysosomes. Naïve T cells possessing receptors of the same specificity as the epitopes presented on these peptide-MHC complexes can then bind to the cell surface; however, this interaction alone will not result in activation of naïve T cells. Recognition of other cell surface molecules on the APCs commonly referred to as co-stimulatory molecules by the T cell receptors is also necessary to result in activation of the naïve T cells. While all APCs have the potential of stimulating naïve T cells by up-regulating expression of MHC class II and co-stimulatory molecules during infection, the most efficient APC and the primary APC responsible for activation of naïve T cells are the dendritic cells (DCs) (discussed in detail in section 3.4).

3.2.2.b T lymphocytes

Immature lymphocytes that leave the bone marrow and mature in the thymus give rise to mature T lymphocytes or T cells. These cells can be further subdivided into two categories depending on the expression on cell surface markers as CD4 and CD8 positive T lymphocytes. Both T cells also express the T cell receptor (TCR), which has specificity for certain peptides and is involved in recognition of the MHC-peptide complex presented on APCs. Immune responses mediated by T cells are generally referred to as cell-mediated immune (CMI) responses.

The CD8 positive (CD8$^+$) T cells also called cytotoxic T lymphocytes (CTLs) are activated by the recognition of peptides presented on MHC class I molecules. Activation of CTLs results in lysis of infected cells by recognition of the MHC class I-peptides on these cells. The CD4 positive (CD4$^+$) T cells are referred to as the T helper cells (Th) and these cells can be further subdivided into Th1 and Th2 cells. T helper cells are activated by the recognition of peptides presented on MHC class II molecules and the type of Th cell formed is influenced by various factors (i.e. cytokines) during an infection. For example, the release of pro-inflammatory cytokines such as IL-12 by APCs during the innate immune response causes differentiation of naïve Th cells into Th1 cells. The two Th cell types also result in production for distinctly different cytokine profiles which are associated with mediating either cellular or humoral based immune responses. Th1 cells release IL-2, IL-3, and IFN-γ and are associated with enhancing cellular responses, which are necessary for combating intracellular pathogens. On the other hand, Th2 cells mediate humoral responses by releasing the cytokines IL-4, IL-5,

IL-6 and IL-10. Th2 responses are most effective at eliminating extracellular pathogens such as bacteria.

3.2.2.c B lymphocytes

B lymphocytes, more commonly referred to as B cells, mature in the bone marrow and are primarily responsible for producing antibodies (immunoglobulin, Ig) referred to as the humoral immune response. Mature B cells express two types of Ig receptors on their cell surface: IgM and IgD, which can directly recognize and bind to antigens. The binding of an antigen to the B cell receptor results in endocytosis of the antigen-receptor complex and processing of the antigen. This process also ultimately leads to the activation and differentiation of B cells into plasma cells, which are able to secrete antigen specific antibodies that can circulate in the blood. The initial antibodies secreted in an immune response are IgM and upon interaction with cytokines released from activated Th cells, the isotypes of antibody produced by plasma cells can be varied to IgG, IgE or IgA (a process referred to as isotype switching).

The uptake and processed antigen can also be presented on the surface of MHC class II molecules on B cells. Although they are not very efficient stimulators of naïve T cells, B cells that have been induced to express the necessary co-stimulatory molecules can function as APCs, stimulating naïve T cells.

3.3   Adjuvants and mechanism of action

Adjuvants, derived from the Latin word *adjuvare* which means to help, have been described as any material used with antigens in immunization that aid in enhancing the

cellular and/or humoral immune responses [2,59]. The use of adjuvants was first introduced by Ramon in 1925, who demonstrated that immune responses to diphtheria and tetanus toxoid could be enhanced by injection with other compounds such as agar, tapioca, lecithin, starch oil, saponin, and breadcrumbs [60]. In the recent years, a great deal of attention has focused on the development and application of new adjuvants. This trend has been introduced partially by the interest in using new generation vaccines, which are purified and lack many of the components of pathogens recognized by the innate immune system and therefore, tend to produce poor immune responses when given alone. Adjuvants can also be used to modulate the type of immune response generated to the antigen. Appropriate selection of the adjuvant with the antigen can result in primarily cellular (CTL and Th1) or humoral based immune responses. For example, aluminum salts (commonly referred to as Alum) tend to produce predominantly humoral based immune responses [61-63] where as bacterial derived components such as Lipid A and Saponins (QS21) are associated with producing cellular type responses [1,64]. Thus, the selection of the appropriate adjuvant has to be made based on the type of immune response desired or that is necessary for providing protection. However, the selection is often empirical in many cases because the exact immune responses necessary to provide protection from pathogens in many diseases are not known.

Alum was first introduced as an adjuvant in 1926 and continues to be the only FDA approved adjuvant for routine human vaccination in the U.S. [65]. It has been used widely and proven effective for enhancing humoral immune responses. Although Alum has been used extensively in vaccines for over 70 years, the exact mechanisms by which they enhance immune responses have not been fully elucidated. Proposed mechanisms

include: 1) enhancing the uptake of associated antigen into APCs, 2) forming a depot in macrophages present in muscle, and 3) causing a local inflammatory response due to necrosis at the injection site possibly resulting in the release of inflammatory cytokines and activation of APCs [66-69]   Alum is a weak adjuvant for mediating cellular immunity and is associated with generating the production of immunoglobulin E associated with allergic reactions [3,62,70]. Thus, there is a great need for more effective adjuvants to enhance cell-mediated responses with protein-based vaccines, as these are regarded to be important for many chronic infections such as HIV. Many new adjuvants have been evaluated in clinical trials; however, most have proven too toxic for routine human vaccination. In addition to demonstrated safety and biocompatibility, other ideal adjuvant properties for consideration include ease of manufacture, wide applicability, cost effective, and stability.

Although the exact mechanisms by which adjuvants enhance immune responses are not completely understood, the three general mechanisms proposed by which adjuvants enhance immune response [2,65,71] are listed below.

1. Adjuvants may be used to form a depot of the antigen at the site of injection, avoiding rapid clearance of the antigen from the body. The antigen can then be slowly released over a period of time. Some examples of adjuvants that are thought to enhance immune responses by depot formation include water-in-oil emulsions (CFA and IFA), Alum, and PLGA microparticles (>10 microns in size).

2. Adjuvants can promote uptake of the antigen by APCs leading to higher antigen-specific immune responses. Adjuvants of particulate nature, as in the case of pathogens, are naturally targeted for uptake by APCs.

3. Adjuvants can induce release of pro-inflammatory cytokines and induce expression of cell-surface molecules on APCs, causing them to become more efficient at stimulating naïve T cells. Some examples of adjuvants exerting their effects via this mechanism include: PAMPs or cytokines such as IL-2 or IL-12, which is induced during inflammation.

A general list of adjuvants used for enhancing immune responses is presented in Table 3.3. These adjuvants can be more generally classified as immunostimulatory or particulate.

3.3.1 Immunostimulatory adjuvants

Immunostimulatory adjuvants enhance immune responses by initiating intracellular signaling pathways that lead a number of events including: maturation of APCs, the release of pro-inflammatory cytokines or upregulation of cell surface molecules such as MHC class II and co-stimulatory molecules on APCs.

3.3.1.a Cytokines

Cytokines are small, secreted proteins that are naturally found in the body and play a critical role in antigen-presentation, activation and proliferation of antigen-specific lymphocytes during an immune response. In addition, many cytokines are involved in biasing an immune response to Th1 or Th2 and can be used to selectively modulate the

immune response generated. For example, IL-12 released by APCs during an innate immune response is associated with biasing the development of naïve CD4$^+$ T cells into Th1 cells. Moreover, the release of IFN-γ by Th1 cells mediate cellular responses by causing activation and proliferation of CD8$^+$ T cells [72,73]. On the other hand, the presence of IL-4 or IL-10 during an immune responses directs development of Th2 cells [74]. Thus, the use of cytokines has been attractive for manipulating or directing the types of immune responses obtained. In addition, cytokines may also enhance immune responses via recruitment of APCs at the site of injection for antigen uptake and influencing expression of MHC II and co-stimulatory molecules on these cells. The most extensively evaluated cytokines include: IFN-γ and IFN-α, IL-2, IL-4, IL-7, IL-12, IL-15, IL-18, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [75]. The main disadvantages in using cytokines are that the proteins can be degraded rapidly *in vivo* and/or dose-related toxicity is associated with cytokine use [76,77]. To overcome these issues, the use of particulate delivery systems for slow release at the site of injection as a means to minimize toxicity has been investigated [78-82]. In addition, cytokines encoded on pDNA alone or encoded along with an antigen have been investigated for enhancing immune responses [43,44,77].

3.3.1.b Bacterial derived adjuvants: PAMPs and TLRs

PAMPs are naturally recognized by cells of the innate immune system and upon binding to the PRRs initiate a signaling pathway resulting in the production of cytokines or upregulation in the expression of costimulatory molecules. This activation of the innate immune system subsequently triggers adaptive immune responses. TLR ligands

have been evaluated as adjuvants for enhancing immune responses [56]. Currently, there are 10 identified TLRs and they are located either intracellularly (TLR3, 7, 8, 9) or expressed on the cell surface (1, 2, 6, 4, 5, 11). Ligands for many of the TLRs have been identified [57]. The binding of ligands to TLRs on DCs modulates the expression of chemokine receptors and causes immature DCs to undergo maturation as well as migration from peripheral tissues into the draining lymph nodes where they can stimulate naïve T cells and initiate the adaptive immune responses. Many of the TLR ligands are associated with generating strong cellular type immune responses [56,83-85]. Two bacterial derived immunostimulatory adjuvants that have been extensively investigated will be discussed. Both mediate their effects via binding to TLRs, initiating the innate immune response.

Lipopolysaccharide derivatives

LPS is derived from the cell wall of gram negative bacteria and is composed of two domains: a hydrophilic polysaccharide portion which extends out from the cell surface and a hydrophobic domain known as Lipid A embedded in the cell wall [86]. LPS binds to TLR 4 and has shown to be extremely strong adjuvant for generating cellular responses [56,87,88]. However, LPS can cause septic shock and this severe toxicity limits it's use as an adjuvant for humans [56,88]. The adjuvant activity of LPS is contributed to Lipid A and a chemically modified derivative of LPS called monophophosphoryl Lipid A (MPL) has demonstrated to retain the adjuvant properties with dramatically decreased toxicity [89]. MPL has been shown to generate strong Th1 type responses [90] and has been extensively investigated in clinical trials [91-93]. MPL

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is currently under investigation for use with Leishmania, malaria, TB and cancer antigens and has been evaluated in more than 30,000 humans. Moreover, MPL is approved for use in Canada for a melanoma vaccine called Melacine®. Synthetic MPL derivatives (RC-529) have also been evaluated in phase II clinical trial for Hepatitis B vaccine. Additionally, the Lipid A derivative OM-174 has been evaluated in cancer patients and demonstrated acceptable safety profile [77,94].

CpG

Bacterial DNA has demonstrated potent immunostimulatory activity due to the presence of unmethylated CpG (cytosine phosphate guanosine) dinucleotides in a particular sequence context, referred to as CpG motifs. Unlike bacterial DNA, CpG motifs are not as prevalent or usually methylated in mammalian DNA, making them unable to stimulate the immune system [95]. Bacterial DNA containing CpG motifs are recognized by TLR9, which is expressed in the endoplasmic reticulum. Recognition by TLR9 results in signal transduction once the receptor translocates to the lysosomal compartment, where it can bind to bacterial DNA taken up by cells [96]. The binding of CpG motifs to TLR9 triggers the production of various pro-inflammatory cytokines such as IL-6, TNF-α as well as the Th1 promoting cytokine, IL-12, and induces the expression of co-stimulatory molecules on APCs, enabling them to mature into professional APCs and become more efficient at antigen presentation [97]. More importantly, the identification of synthetic oligodeoxynucleotides (ODN) containing CpG motifs (referred to as CpG ODNs) that mimic the potent immunostimulatory properties of bacterial DNA has generated considerable interest to investigate them as potential adjuvants. Extensive

in vivo experiments in mice have shown that CpG ODNs provide significant enhancements in immune responses to various antigens using various routes of administration [98-104] . In addition, studies with CpG ODNs demonstrate the ability to generate strong Th1 and cellular immune responses [105-107]. As with most immunostimulatory adjuvants, adverse side effects such as enlarged spleen [108] and lymph nodes [109] have been reported in mice; however, these were reported to be sequence- and dose-dependent [108]. Moreover, some concern has been raised that CpG ODN use may generate autoimmune diseases due to over stimulation of the innate immune system; however, repeated doses administered *in vivo* in mice and non-human primates to date have shown no toxicity [110]. Therefore, the concerns raised with the use of CpG ODNs may be prevented by judicious selection of the appropriate CpG sequence and dose. It is important to note that the strength of the immune response generated by each CpG ODN can vary and the optimal sequences differ from species to species. However, optimal CpG ODN have been identified for a number of different species including mice, rabbit, sheep, goat, cattle, swine, horse, rhesus monkey, chimpanzee, and humans [111]. Clinical trials evaluating the use of CpG ODNs for adjuvants in hundreds of patients have reported no adverse reactions due to CpG [110]. In fact, Phase I clinical trials have demonstrated that hepatitis B surface antigen (HBsAg) adjuvanted with CpG ODN resulted in stronger, more robust immune responses compared to the HBsAg alone [94,112].

3.3.1.c Saponins

Saponins, isolated from the tree *Quillaja saponaria* Molina, have demonstrated to possess potent immunostimulatory activity. Saponins are amphipathic molecules possessing a hydrophilic carbohydrate and hydrophobic steroid or triterpene moiety [113]. A major disadvantage of saponins is their surface-active character which causes hemolysis of red blood cells *in vitro* [114]. Quil A, the active component of saponins, has been widely investigated as adjuvants in veterinary vaccines for years; however, its hemolytic activity has limited its use in human vaccines [2,115]. HPLC analysis of saponins led to the identification of a heterogeneous group of triterpene glycosides with varying adjuvant activity and toxicity [113]. Among these highly purified saponins, QS-21 has been the most extensively evaluated and has demonstrated to possess reduced toxicity (considerably reduced hemolytic activity) while retaining the potent adjuvant properties [113,116-120]. QS-21 has been demonstrated as a strong adjuvant for both cellular, CTL and Th1, and humoral immune responses including enhancements in IgG2a isotype with subunit-based vaccines [114,121-123]. The application of QS-21 with HIV-1 DNA for systemic and mucosal immune response enhancements has also been demonstrated [124].

The adjuvant activity of QS-21 has not been fully elucidated. Saponins can interact with cholesterol on cell membranes resulting in pore formation, which may be one way antigens could gain access to the cytoplasm for antigen presentation via the MHC class I pathway to generate CTL responses [125]. *In vitro* studies have demonstrated that murine macrophages stimulated with QS-21 result in cytokine production [77]. Moreover, *in vivo* evaluation in BALB/c mice has demonstrated the

ability of QS-21 to initiate innate immune responses by enhancing the activity of natural killer cells in a dose-dependent manner [77]. QS-21 has been evaluated in numerous clinical trials as an adjuvant for cancer [126-129], malaria [130], HIV-1 [131], and pneumococcal [77] vaccines. QS-21 has now been administered to over 3500 patients in over 90 clinical trials, including some children [77,94] and the most common side effect is reported to pain or tenderness at the site of injection [131,132]. Doses of QS-21 equivalent to or greater than 200 μg have been reported with significant local reactions raising concerns of adverse effects with this adjuvant [77,133]. Hence, a fine balance between the QS-21 dose and the adverse effects must be determined for each antigen and taken into consideration for each vaccine application.

3.3.2 Particulate delivery systems

Particulate delivery systems have been investigated extensively for enhancing immune responses with new generation vaccines. This class of adjuvants exerts their effect mainly through formation of an antigen depot at the site of injection or by enhancing the uptake of the associated antigen by APCs. Particulate delivery systems are passively targeted for uptake by APCs since they have dimensions that are comparable to naturally occurring pathogens (Table 3.4) [125]. The use of emulsions, immune stimulating complexes, liposomes, microparticles, and nanoparticles as adjuvants will be reviewed.

3.3.2.a Emulsions

Emulsions are one of the oldest adjuvants investigated for enhancing immune responses, with the first reported use in 1916 [134]. Emulsions are generated by mixing antigen solubilized in the aqueous phase with an oil phase in the presence of surfactant. Emulsions can be classified into two types depending on the continuous phase: oil-in-water emulsions are oil droplets dispersed in the water phase and water-in-oil emulsions are water droplets dispersed in the oil phase. Interest in using emulsions as adjuvants was stimulated by the initial demonstration of Freund in 1937 that a water-in-oil emulsion composed of mineral oil mixed with killed mycobacteria could serve as a potent adjuvant. This adjuvant is more commonly referred to as complete Freund's adjuvant (CFA) and it has been used extensively in laboratory animal research for generating strong cellular and humoral immune responses. However, the use of CFA is associated severe side effects [135-138] and is too toxic to be used as an adjuvant in humans. An alternative adjuvant preparation without killed mycobacteria, incomplete Freund's adjuvant (IFA), has been used in a number of veterinary vaccines and has been evaluated in humans with influenza and killed poliomyelitis vaccines [134,139]. However, localized toxicity such as formation of abscesses and granulomas at the site of injection prevented further evaluation of IFA for human vaccines. It is thought that many of these localized reactions were due to impurities in the antigen or the formulation components, i.e. the oil or emulsifier, and that the use of highly purified mineral oils and surfactants available may reduce the side effects of IFA [139].

In attempt to replace Freund's adjuvant, the use of an oil-in-water emulsion based on the biodegradable oil squalene was reported in the 1980s [1]. One adjuvant

formulation based on this system, syntax adjuvant formulation (SAF), induced strong cellular responses [140-142]; however, it was too toxic for human vaccination due to the inclusion of an immunostimulatory muramyl dipeptide (MDP) derivative [1]. These studies led to the development of MF59, a squalene-in-water emulsion without MDP, as an adjuvant (see refs [143,144] for detailed review). MF59 has been investigated with numerous antigens including influenza [145-148], hepatitis B [149,150], HIV-1 [151], HSV [152] and HPV [153]. *In vivo* studies in mice suggest that MF59 is taken up by macrophages and DCs at the site of injection and in the draining lymph nodes [154]; however, a second study evaluating the distribution of the co-administered antigen demonstrated that clearance of the antigen from the injection site was independent of MF59 [155]. MF59 was shown to be safe and well tolerated with several vaccines in clinical trials involving over 32,000 patients, including elderly patients, toddlers, and infants [143]. MF59 is currently approved as an adjuvant (in the influenza vaccine FLUAD[®]) in many European countries – the only other adjuvant besides Alum to be approved for use in routine human vaccination [156].

3.3.2.b Liposomes

Dispersion of phospholipids and other polar ampiphiles in aqueous buffers result in the formation of closed, concentric bilayer vesicles called liposomes. These systems contain an aqueous core and a lipid bilayer, thus allowing for delivery of both hydrophilic and hydrophobic molecules. Liposomes can vary in size from 20 nm to several microns and can be classified into two groups depending the number of concentric lipid bilayers: small or large unilamellar vesicles (SUV or LUV, respectively) consist of one lipid

bilayer and large multilamellar vesicles (MLV) consist of multiple lipid bilayers [7]

Liposomes have been investigated for numerous drug delivery applications, are marketed

for delivery of cancer drugs [157] and have demonstrated to be relatively safe with no

reports of granulomas after parenteral administration [7,134]. The application of

liposomes as adjuvants was first reported with diphtheria toxoid in 1974 [158] and have

since been shown to be effective for enhancing immune responses to numerous other

antigens [6,159-161] . Moreover, liposomes have been reported to generate good CTL

responses with various antigens in animal models [162-166]. Studies to elucidate the

mechanism of immune response enhancement using liposomes suggest that liposomes

taken up into endosomes release part of the encapsulated antigen directly in the

cytoplasm, allowing the antigen to gain access to the MHC class I pathway and leading to

stronger CTL responses [167].

The stability of liposomes *in vivo* is dependent on the fluidity of the lipid bilayer,

which is determined by the type of phospholipids used in preparation. Many preparations

include the use of cholesterol to improve stability of the liposome formulations.

However, the use of phospholipids also poses limitations because they can be rapidly

degraded in the host by phospholipases [2]. In addition, the composition and physical

characteristics of liposomes are critical parameters influencing particle uptake by

macrophages [168] and dendritic cells [169] and could potentially affect the immune

responses obtained *in vivo* [170]. The use of stable polymerized liposomes have been

investigated for enhancing immune responses to encapsulated antigens via the oral route

[171]. More recently, different types of liposomes have been prepared using alternative

amphipathic molecules to phospholipids and are classified based on their composition as

[172]: Virosomes (fusogenic liposomes), Transferosomes, Archaeosomes, Niosomes, Chochleates, and Proteosomes. These systems have been evaluated in animal models and demonstrate to be as effective or superior to conventional liposomes at enhancing immune responses [160,173-179]. In some cases, these alternative liposomes show enhanced stability compared to the conventional liposome formulations [179,180]. Of these alternative liposomes, virosomes, which resemble SUVs and contain influenza hemagglutinin (HA), have demonstrated greatest potential for use in human vaccines [51,164,176].

3.3.2.c Immune stimulating complexes (ISCOMS)

Immune stimulating complexes, ISCOMS, were introduced by Morein *et al*. in 1984 as an antigen delivery system with immunostimulatory properties [181]. ISCOMS form spontaneously upon mixing appropriate ratios of phospholipids, cholesterol, Quil A, and an amphipathic antigen (i.e. viral membrane proteins). Electron micrographs of ISCOMs demonstrate characteristic, rigid cage-like structures of about 40 nm size [172]. This adjuvant has been shown to be effective at enhancing immune responses by both the parenteral and mucosal routes [182,183]. The use of ISCOMs has been investigated in small animals with antigens for measles [184-186], influenza [187,188], and HIV [189,190] vaccines and both humoral and cell mediated immunity were induced. More importantly, these strong, protective immune responses were maintained when tested in larger animal models including non-human primates [182] and ISCOMs has been licensed for use in an anti-influenza vaccine for horses [172]. The hemolytic activity and toxicity normally associated with Quil A is greatly reduced in this system due to the

hydrophobic interactions with cholesterol, preventing significant interactions with the cell [191]. Recent studies suggest that IL-12 plays a vital role in the strong cell mediated responses observed *in vivo* with ISCOMs and found that the main APCs involved in priming CD4^{+} and CD8^{+} T cells are DCs [192,193]. ISCOMs are currently being evaluated in phase I clinical trials with influenza, HPV, and human cytomegalovirus [94,172]. The major limitation of this approach is its applicability to different antigens. ISCOMs typically involve the use of membrane proteins; however, inclusion of antigens that do not possess this hydrophobic character may prove difficult and require extensive modification for efficient incorporation into ISCOMS.

3.3.2.d Nanoparticles and microparticles

Solid colloidal particles can be classified depending on their size as nanoparticles for particles in the 10-1000 nm in size range or microparticles for those particles greater than 1 μm in size. The preparation of nanoparticles and microparticles has been reported using a wide range of materials [194]. Moreover, numerous methods have been reported for preparation of nanoparticles and microparticles; however, the most commonly practiced techniques include emulsion and interfacial polymerization, high-pressure homogenization, solvent extraction/evaporation (including the double-emulsion-solvent evaporation technique used for entrapping proteins) and microemulsion precursor technology. In addition to parameters such as cost-effectiveness, applicability, ease of scale up, the method used for preparing the particulate delivery system is extremely critical and must be carefully selected as this can potentially influence the stability of the antigen and ultimately affect the quality of immune response obtained.

The interest in using solid colloidal particles for enhancement in immune responses was stimulated by studies reported by Kreuter and Speiser on the adjuvant properties of polymeric nanoparticles [195-197]. These studies reported the use of poly(methyl methacrylate) (PMMA) nanoparticles for enhancing immune responses to both adsorbed and entrapped vaccines. Although the use of PMMA nanoparticles was shown to enhance immune responses to a number of different antigens by Kreuter [198], the utility of these particles is limited by their accumulation in the body due to the extremely slow rate of degradation of the polymer [199]. The use of biodegradable poly(alkylcyanoacrylates) (PACA) for preparing nanoparticles was introduced by Couvreur *et al.* [200] and their use has been investigated for delivery of various hydrophilic and lipophilic drugs; however, the use of these nanoparticles has not been reported in vaccines and controversy over the toxicity due to alkylcyanoacrylate monomers exists.

The idea of using solid colloidal particles was further advanced by O'Hagan *et al.* [201,202] and Eldridge *et al.* [11,203] by demonstrating that antigens entrapped in poly(lactide-co-glycolide) (PLGA) microparticles resulted in similar enhancements in immune responses as the antigen emulsified with Freund's adjuvant. Although other polymeric materials such as chitosan [48,204,205], poloxamers [206], polyphosphazenes [207], and polyanhydrides [208,209] have been investigated for enhancing immune responses, the use of PLGA polymers has been most preferred due to the extensive history of use and biocompatibility in humans. PLGA polymers have been found numerous applications in humans for medical purposes such as surgical sutures, have demonstrated excellent biocompatibility and safety, and have been approved for use as

drug delivery devices for a number of therapeutic products [210]. A detailed review of the various methods used for preparation of PLGA microparticles and nanoparticles is presented elsewhere [211]. PLGA microparticles have been effective at entrapping a range of different antigens and *in vivo* evaluation in animals have demonstrated them to be effective at enhancing both cellular (Th1/CTL) and humoral immune responses using various routes of administration [8,212-217]. More recently, the utility of PLGA microparticles has been attractive for enhancing immune responses to pathogens that may be used as potential biowarfare agents [218]. In addition, PLGA microparticles have also been investigated for enhancing mucosal immune responses, which are regarded to be important for many diseases as the nasal, rectal, and vaginal sites are the routes of entry for most pathogens [171,219,220]. The potential of PLGA microparticles for oral delivery of entrapped antigens has also been evaluated and shown to induce both systemic and mucosal immune responses [213,217,221-225]. This enhanced immune response via the oral route is speculated to be due to uptake of the microparticles by mucosal associated lymphoid tissue (MALT) [226]. Although microparticles have demonstrated good enhancements in mucosal immune responses in small animal models, generating these responses non-human primates and humans has been challenging [227].

The use of PLGA polymer for preparation of controlled release drug delivery systems has been of great interest and currently there are a number of products on the market using this technology. The release rate of the entrapped molecule from the microparticle can be modified by altering the co-polymer composition, molecular weight, crystallinity of the polymer, and the size of the microparticles [228,229]. This property has made PLGA microparticles extremely attractive for development of single-dose

vaccines [230-234]. The concept of oral delivery and single-dose vaccines using microparticles rely on the antigen being entrapped within the microparticle. However, it must be realized that a great deal of work is necessary to ensure the stability of the entrapped antigen as this can influence the *in vivo* results [235]. The entrapment of the protein in microparticles is associated with a number of issues such as protein stability during encapsulation, storage and hydration, as well as the environment experienced *in vivo* during antigen release [229].

As an alternative to antigen entrapment, the surface characteristics of PLGA microparticles have been modified by incorporation anionic or cationic surfactant during the microparticle preparation for adsorption of the antigen on the surface of the particle via electrostatic and van der waals interactions [9,212,236]. Antigens adsorbed to the surface of microparticles have shown enhanced immune responses *in vivo* in mice [237,238] and these systems are undergoing evaluation in phase I clinical trials (http://www.iavireport.org/trialsdb/vaccinedetail1.asp?i=82).

As noted before, one of the main mechanisms by which particulate delivery systems are thought to enhance immune response is by enhanced uptake into APCs. *In vitro* studies demonstrate both nanoparticles [239,240] and microparticles [241,242] are taken up by APCs. In addition, the efficiency of antigen presentation was found to be enhanced by 10-100 fold using PLGA microparticles [241]. The uptake of particles by APCs is influenced by several factors including charge, hydrophobicity/hydrophilicity, and size [168,242]. The increase in hydrophobicity of particles was reported to have greater adjuvant activity *in vivo* in one study [243]. Interestingly, *in vivo* studies have demonstrated that smaller particles are taken up into lymphatics better than larger

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particles [11,12,244]; thus, having greater chances of being taken up by residing APCs. Combined, these two factors, size and drainage into the lymphatics, correlate with enhanced immunogenicity of smaller microparticles compared to larger microparticles [11-13]. The inverse correlation of particle size and immune response has also been reported by Kreuter with nanoparticles in the 62-306 nm range [10].

The superior immune responses obtained *in vivo* using smaller particles have stimulated a great deal of interest in further investigating nanoparticles for subunit-based vaccines. To this end, PLGA nanoparticles have been shown to enhance immune responses in mice [245-247]. The potential of cationic nanoparticles adsorbed with hepatitis B surface antigen and β-galactosidase to induce antigen-specific systemic (IgG, Th1/Th2) and mucosal (IgA) responses was demonstrated after intranasal administration [248]. More recently, a novel method for preparation of nanoparticles from oil-in-water microemulsion precursors was demonstrated by Mumper *et al*. [14-16,249,250]. The microemulsion precursors are prepared by mixing appropriate ratios of oil, water, and pharmaceutically acceptable surfactant(s) at an elevated temperature and by simply cooling these microemulsions to room temperature nanoparticles approximately 100 nm in size are obtained. These nanoparticles have the advantage of being prepared in a single step, one vessel process without the use of organic solvents and with relative ease in modifying the physical characteristics of the particles by choosing the appropriate surfactants. In addition, the nanoparticles are potentially biocompatible as the oil phase used for preparation of these nanoparticles is comprised of cetyl alcohol and polysorbate 60, both found in many pharmaceutical products. More importantly, these nanoparticles are hemocompatible [251] and shown to be degraded via the alcohol dehydrogenase

system [252]. *In vivo* evaluation in mice have demonstrated enhanced Th1 and humoral responses to the model antigen β-galactosidase [16] and HIV-1 Tat protein [253] coated on the surface of anionic nanoparticles after subcutaneous administration.

3.3.3 Combined adjuvant formulations

With the exception of ISCOMs and CFA, the adjuvants described up to this point involve the use of individual systems, i.e. particulate or immunostimulatory, for enhancing immune responses. Many researchers have investigated the combination of various adjuvant systems as a means for obtaining synergistic enhancements in immune responses. For example, the use HIV-1 gp120 and Gag p24 entrapped in microparticles that were dispersed in MF59 demonstrated significantly stronger antigen-specific humoral and Gag p24-specific CTL responses compared to either adjuvant alone [254]. Alving *et al.* reported the use of lipid A entrapped liposomes adsorbed to Alum as a safe and effective method for enhancing humoral and antibody responses to surface bound or co-entrapped antigen in human clinical trials [166]. The use of QS-21 with low doses of IL-12 was effective for synergistic enhancement in immune responses to respiratory syncytial virus, demonstrating the combined adjuvant formulation was effective while reducing the dose-related systemic toxicity of IL-12 [82]. Moreover, the addition of MPL to QS-21 adjuvant was effective at directing immune responses to HIV-1 gp120 from a Th2 to a Th1 type response [255]. The adsorption of IL-12 on Alum has also been investigated to enhance Th1 type immune responses to several antigens [78,81,256].

In the quest for developing safer, more potent adjuvants, a great deal of interest has turned towards the investigation of various delivery systems for co-delivery of the

antigen and immunostimulatory adjuvants [8,79,246,257-260]. The use of delivery systems entrapping an immunostimulatory adjuvant may provide an avenue for reducing the dose of antigen or the dose of immunostimulatory adjuvant by targeting uptake by APCs, providing a controlled release environment, and most importantly, decreasing toxic side effects, as in the case of ISCOMs. The entrapment of the immunostimulatory adjuvant MDP in microparticles was shown to reduce the toxicity [261] while still providing potent immune responses to entrapped antigen [262]. Furthermore, CpG entrapped in PLGA nanoparticles was reported to generate potent cellular responses using a ten-fold lower dose compared to unentrappped CpG [263]. A particularly attractive approach for many investigators involves the use of immunostimulatory adjuvants with particulate systems for modulating the immune responses to obtain enhanced cellular based responses. Table 3.5 lists several particulate systems that have been investigated with immunostimulatory adjuvants for improving immune responses to various antigens.

3.4 Dendritic cells and targeting

The body's defense mechanism relies on a coordinated set of actions involving the components of the innate and adaptive immune system to generate strong, long-lasting immune responses to pathogens. As described in section 3.2, one of the key cells involved in this process are the APCs such as DCs. DCs are regarded as professional APCs as they are the only cells capable of inducing primary immune responses [264]. Although macrophages can function as APCs, their primary function involves phagocytosis and clearance of the pathogens from the body. On the other hand, DCs

primarily function to sense, ingest, and process invading pathogens (innate immune response) and present them to naïve T cells present in the secondary lymphoid organs (adaptive immune response). DCs are also considered to play a vital role in naïve CD4$^+$ T cell differentiation into Th1 or Th2 type and therefore, controlling both the quality of the immune response as well as the strength of the immune response [72,265]. Moreover, studies have demonstrated that DCs have the ability to present exogenous antigens (i.e., from non-replicating pathogens and apoptotic or necrotic cells) on MHC class I molecules resulting in stimulation of CD8$^+$ T cells. This process, referred to as cross-priming, demonstrates a non-classical or alternative pathway present in DCs for processing and complexing exogenously derived peptides to MHC class I molecules and for inducing CTL responses [266]. In addition to the antigen presenting function, DCs play an important role in the viability, growth, and differentiation of activated B cells [267]. Consequently, the key link DCs provide between the innate and adaptive immune responses combined with their ability to cross-prime exogenous antigens have made these cells particularly attractive targets for manipulating immune responses and for targeting vaccines.

3.4.1 Types of dendritic cells

DCs are derived from hematopoietic progenitor cells in the bone marrow that give rise to DC precursors, which circulate the blood and lymphatics. These DC precursors further develop into a heterogeneous population of DCs that can be classified into different subsets based on origin, phenotype, localization, and function. In mice and humans, DC precursors can be derived from two different types of hematopoietic

progenitor cells classified as the myeloid and lymphoid progenitors [268]. Furthermore, the DC precursors generated from these two progenitor cells give rise to phenotypically distinct subsets of DCs that have been identified in both mice and humans (described in detail in [264,268,269]). For example, three phenotypically distinct DC subtypes have been identified in humans: 1) Langerine^{+} DCs, more commonly referred to as Langerhans cells (LCs), 2) myeloid DCs, and 3) plasmacytoid DCs. Cell culture studies with human CD34^{+} hematopoetic progenitor cells suggest that LCs and myeloid DCs are derived from a common myeloid progenitor cell, whereas plasmacytoid DCs are thought to be derived from a lymphoid progenitor cell [268]. The complex nature of the DC subtypes is even more evident in mice, where DCs can be classified into six subtypes based on the expression of different cell surface markers [269]. Regardless of the species, the different DC subtypes contain both immature and mature DCs and they differentially express PRRs, which affects their ability to recognize and respond to pathogens and ultimately influence the quality of immune response generated. For example, plasmacytoid DCs express TLR7 and 9 only, while both the LCs and myeloid DCs express TLR1, 2, 3, 4, 5, 8 but not 7 and 9. Moreover, further differences have been reported between LCs and myeloid DCs. Myeloid DCs have about a 10-fold higher efficiency of antigen capture and are more potent stimulators of CD4^{+} T cells compared to LCs [264]. Taken together, these differences in the DC subsets enable them to greatly influence the adaptive immune responses generated to various pathogens.

3.4.2 Maturation of DCs into professional APCs

DC precursors in the blood can migrate to various tissues and in the presence of certain cytokines, such as GM-CSF and IL-4, they differentiate into immature DCs, which reside in the peripheral tissues surveying for invading pathogens. Immature DCs are extremely efficient at capturing antigens and can do this by the following pathways: macropinocytosis, receptor-mediated endocytosis, and phagocytocysis [266]. The uptake of the antigen causes immature DCs to undergo a maturation process which involves phenotypic and morphological changes, migratory capability from the tissue into draining lymph nodes, upregulation of co-stimulatory molecules, and enhancement MHC class II molecule synthesis as well as transportation to cell surface. This entire process ultimately leads to the immature DCs being converted into mature DCs that possess antigen presentation capability and are referred to as professional APCs. Morphological changes that are associated with mature dendritic cells include the formation of fine, long veils (>10 μm) extending from the cell body [267]. Functionally, the mature DCs become less phagocytic, migrate to T cell areas of in the secondary lymphoid organs, and are highly efficient at stimulating the antigen-specific naïve T cells. The main features of immature and mature DCs are summarized in Figure 3.2. Several factors can trigger immature DCs to undergo maturation including binding of bacterial or viral components to TLRs (LPS or dsRNA), inflammatory cytokines (TNF-α, IL-1, IL-6), T cell derived signals (CD40), binding of immune complexes to Fc receptors, and other endogenous factors or cell death. The presence of IL-10 during an immune response inhibits the maturation of DCs; thus, they become less efficient stimulators of naïve T cells and could lead to antigen-specific tolerance [270].

3.4.3 Receptor-mediated targeting of DCs

The unique properties of DCs (discussed in the previous sections) have made them attractive and widely investigated for targeting antigens to obtain enhanced immune responses. In addition to TLRs, DCs express several endocytic receptors, such as the C-lectin and Fc receptors [74] that could be utilized for enhancing immune responses to protein-based vaccines. The main challenge in targeting DCs is that many of these receptors have only been recently identified and therefore, have not been extensively studied. In addition, the ligands that could be utilized for targeting some of these receptors are yet to be determined.

3.4.3.a C-lectin receptors

The various DC subsets express a family of C-lectin type receptors that recognize their targets via single or multiple carbohydrate recognition domains (CRDs). C-lectin receptors bind to their ligands in a calcium-dependent manner [271]. There are two types of C-lectin receptors: Type I consisting of the mannose and DEC-205 receptors; Type II consisting of Langerin, DC-SIGN and Dectin-1 receptors [272].

<u>Mannose receptor</u>

The mannose receptor (MR) has been most investigated for targeting antigens to DCs and macrophages for obtaining enhanced immune responses [273-276]. The MR contains 8 carbohydrate recognition domains (CRD), of which CRD 4-8 are involved in binding and recognition of terminal mannose containing ligands [277,278]. The captured antigen is transported to early endosomes and the MR is recycled back to the cell surface, allowing large amounts of antigen to be captured via the MR [279].

Mannosylation of proteins resulted in a 200-10,000 fold increase in simulation of CD4$^+$ T cells compared to non-mannosylated proteins *in vitro* [280]. Many groups reported the use of hydrophobized mannan for enhancing cellular and humoral immune responses to antigens when formulated with particulate delivery systems such as liposomes and nanoparticles [15,163,281-283]. Moreover, mannan conjugated to the cancer antigen HER2 was reported to induce strong enough CTL responses to reject HER2+ tumors *in vivo* [284]. One limitation reported to using mannan is its *in vivo* toxicity (in mice by iv) and high immunogenicity [283]. More recently, Mizuochi *et al*. have investigated various alternative oligosaccharide ligands and demonstrated that mannopentaose [has five mannose (Man) residues linked in the following order: Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man] attached to the surface of liposomes generated enhanced cellular responses compared uncoated liposomes [283,285,286]. Therefore, alternative mannose type ligands may have potential applications for targeting antigen uptake into DCs and for enhancing cellular immune responses with particulate delivery systems.

DEC-205

DEC-205, composed of 10 CRDs, is significantly homologous to the MR [287,288]; however, no specific ligands have been defined for DEC-205 [287]. Targeting to DEC-205, similar to MR, results in the antigen-receptor complex being endocytosed and recycled back to the cell surface; however, endocytosis via DEC-205 targets the antigen to the late endosomal compartment [279]. *In vitro* and *in vivo* targeting to DEC-205 using an anti-DEC 205 antibody-antigen complex was reported to enhance the antigen presentation on MHC class I and II molecules (by 100 to 400 fold) compared to

non-targeted antigens [279,289]. Moreover, targeted liposomes coated with an anti-DEC 205 antibody were reported to induce strong CTL responses to an anti-tumor antigen when co-delivered with immunostimulatory adjuvants [290]. Furthermore, a more recent study reported that DEC-205-targeted HIV-1 Gag p24 was effective at inducing potent $CD4^+$ T cell responses and long-lived memory T cells in mice compared to untargeted protein or adenovirus expressing Gag p24 [291].

3.4.3.b Fc receptors

DCs express surface immunoglobulin receptors called Fc receptors (FcR) that recognize the constant portion (Fc) of IgG. One type of FcR, FcγR, has been investigated for targeting antigens to DCs. Unlike MR and DEC-205 receptor, FcγR is not transported back to the cell-surface after endocytosis and thus, have a lower uptake capacity compared to MR and DEC-205 [275]. *In vitro* studies demonstrating that antigen presentation can be enhanced by 100-1000 fold by targeting them to the FcR have been reported [292,293]. In addition, antigen entrapped in FcγR-targeted liposomes was reported to be 1000 to 100,000 fold more efficient at stimulating antigen-specific T cells *in vitro* compared to free antigen or untargeted liposomes [294,295]. *In vivo* studies targeting FcγR using a antibody-antigen conjugate demonstrate enhanced cellular and humoral immune responses compared to antigen alone [296,297]. Moreover, targeting FcγR has been demonstrated to result in efficient cross-priming of the antigen [298,299] and thus, have the potential to enhance CTL responses to antigens. Targeting antigens to FcγR could be limited by potential competition with other IgG and B cells present *in vivo*.

3.4.4 Targeting DCs by route of immunization

Traditionally, vaccines have been administered by the parenteral route, with intramuscular (i.m.) and subcutaneous (s.c.) routes being most utilized. The choice in immunization route is of important consideration as it can significantly affect the type of immune responses generated and side effects, such as local reactions [124,300-304]. The immunization route may influence the uptake of the antigen by APCs, affecting the strength and type of responses obtained. For example, i.m. injection is considered to enhance immune responses by offering a depot site for the antigen as few APCs are present at the muscle sites [301]. The use of s.c. route has been investigated for targeting colloids to draining lymph nodes, with the size of the colloid being a major factor in determining drainage [244]. Therefore, antigens formulated with nanoparticles, as described previously in section 3.3.2.d, are potentially targeted for passive uptake into APCs residing in the lymphatic system after s.c. injection.

The use of mucosal routes for immunization has been of particular interest as it can generate both systemic and mucosal immune responses (secretory IgA), providing an additional barrier of protection at the site of infection for pathogens that are transmitted via mucosal routes. Traditionally, the oral and nasal routes have been the most investigated for mucosal immunization [124,220,305]. Non-toxic derivatives of cholera toxin (CT) and heat labile enterotoxin (LT) adjuvants have been often investigated for enhancing immune responses to antigens via mucosal immunization [306]. Moreover, the use of a genetically modified LT, LTK63, is being evaluated for intranasal delivery of an influenza vaccine human clinical tirals [94]. Although the oral route has been investigated extensively with antigens entrapped in particulate delivery systems, the nasal

route is more attractive since it avoids many challenges associated with oral delivery, such as the harsh acidic environment of the GI tract experienced by antigens [125,227]. More recently, there has been a great deal of interest in using the skin as an immunization site, referred to as transcutaneous immunization (TCI) [307-310]. As described in the earlier section, LCs reside in the viable epidermis and although they compose only 1-4% of the cells in the viable epidermis, they cover over 20% of the surface area of the skin due to their characteristic DC-like veils that extend from the cell body [308]. Therefore, immunization via the skin can be used to passively target the uptake of antigens by LCs, potentially leading to strong systemic and mucosal immune responses. The main challenge in targeting the LCs in the viable epidermis is penetration through the stratum corneum layer [309]. One system reported the use of a Macroflux® skin, which consists of titanium microprojections (~330 μm in length), for delivery of protein-based vaccines through the skin [311]. Macroflux® skin patch coated with OVA demonstrated 50-fold higher IgG titers compared to i.m. or s.c. routes and comparable to the intradermal route (i.d.) at the lowest dose investigated in hairless guinea pigs. The ability of the Macroflux® patch to co-deliver an immunostimulatory adjuvant, resulting in augmented OVA-specific antibodies, was also reported [311]. In other animal studies the use of detoxified LT and CT mutants is most often investigated to obtain enhanced systemic and mucosal immune responses to an antigen after direct application to shaven skin [310,312-314]. Moreover, TCI using a patch was demonstrated to be safe in phase I clinical trials for immunizing humans with LT, generating robust systemic and mucosal antibodies for protection against travelers diarrhea [315]. This technology has been further extended for evaluation in numerous clinical trials including anthrax, hepatitis B, and influenza

vaccines using a patch infused with the adjuvant LT [94]. This technology may be a viable strategy for advancing the idea of needle-free immunization; however, the variability in immune responses generated among individuals due to differences in penetration across the stratum corneum layer in larger populations needs to be evaluated.

Another delivery technology which bypasses the conventional needle-based immunization approach involves the use of a high powered helium device (PowderJect$^{®}$) to deliver the antigen of interest into the epidermis, where an abundant population of LCs are present [316]. This system has been reported to significantly enhance immune responses to hepatitis B surface antigen [317,318], influenza [317], and HIV-1 gp 120 [319]. Moreover, the hepatitis B surface antigen could be co-formulated with CpG ODN and delivered to the epidermis for further enhancements in immune responses, including Th1 type responses [318]. The use of this technology has also been extended for delivery of DNA coated on gold particles to LCs in the epidermis [316]. Unlike traditional approaches for genetic immunization which rely on transfecting muscle cells after intramuscular administration, this system allows for direct delivery of the DNA into the LCs responsible for generating the immune response. This technology termed, PMED$^{™}$, has demonstrated success in recent phase I clinical trials with influenza vaccine and is currently in phase I clinical trials for a number of additional vaccines (www.powdermed.com).

3.5   HIV vaccine development

Although the cause of acquired immunodeficiency syndrome (AIDS), HIV [320], was identified and fully sequenced in the early 1980's [321], there is still no effective

HIV vaccine and HIV continues to infect millions of people. The worldwide prevalence of AIDS has focused attention on the urgent need to develop a safe and effective vaccine. The difficulties in developing an effective HIV vaccine are mainly due to: 1) rapid mutations; 2) genetic variability; 3) formation of latent proviral DNA and 4) lack of knowledge on the immune correlates of protection [19,322,323].

3.5.1   HIV infection

HIV is classified as a retrovirus and more specifically grouped with lentiviruses that are characteristic of long incubation periods following infection which eventually lead to immunosupression and diseased states [52]. It is thought to have evolved from the less pathogenic simian immunodeficiency virus (SIV) [324] and studies of SIV in simian species have been useful in developing an understanding of the pathogenesis of HIV [325,326]. There are two types of HIV viruses identified: HIV-1, the more common and main cause of AIDS worldwide, and HIV-2, the less pathogenic strain of HIV and more closely related strain to SIV [324]. Rapid mutations in the virus are caused by the error prone nature of the reverse transcriptase giving rise to genetically varying strains of HIV-1 throughout the world [327]. Currently, there are three main categories of HIV-1: group M, N, and O. Group M is prevalent worldwide and consists of various HIV-1 strains classified as subtypes or clades denoted A to J. The presence of groups N and O are less prevalent and are predominantly identified in Africa and eastern Europe [324].

HIV can be transmitted by sexual intercourse, exposure to contaminated blood, or maternal transmission (mother to child). The hallmark of HIV infection is depletion of $CD4^+$ T cells, which are critical in mediating cellular immunity. There are several stages

in HIV infection correlated with the presence of specific events during infection demonstrated in Figure 3.3 [52,327]. In the early or acute phase of infection (2-8 weeks), a burst in viral replication is observed, correlated with a dramatic decrease in the number of $CD4^+$ T cells. During this phase, the first immune responses to emerge are HIV-specific CTL responses, which eliminate HIV-infected cells and help decrease the viral load. CTL responses are followed by an increase in circulating levels of anti-HIV antibodies and the patient is said to have undergone seroconversion at this point. Following this phase, an asymptomatic phase is observed during which viral replication persists and a gradual decrease in $CD4^+$ T cells occurs. When the $CD4^+$ T cells drop below a certain level (< 200-400 cells/μL) necessary to generate effective immune responses to other infections, the onset of AIDS begins [328] and the patient becomes susceptible to numerous opportunistic infections – infections that normal, healthy individuals can fight but prove fatal in AIDS patients. These opportunistic infections are the ultimate cause of death in AIDS patients.

The asymptomatic phase in HIV-infected patients is highly variable, lasting from a few months to over 16 years. Individuals that are able to control the infection (>12-16 years) and progression to disease are referred to as long-term non-progressors (LTNP) [327]. Studies of these patients have been of great interest to many scientists in this area to gain a better understanding of the immune correlates necessary to prevent onset of AIDS. To this end, many studies with LTNP and HIV-infected patients report an inverse correlation between disease progression and the presence of strong CTL responses to multiple HIV antigens, suggesting the importance of CTL responses in controlling viral replication [329-331].

3.5.2 HIV life cycle

The HIV viral genome consists of nine genes flanked by long-terminal repeats (LTRs) that are composed of the transcription regulatory elements (Figure 3.4A). These genes translate for the HIV structural, regulatory, and accessory proteins that have various functions in the viral life cycle (Table 3.6) [52,327]. A schematic representation of the mature HIV virus is presented in Figure 3.4B. The various stages in the HIV life cycle have been described in detail elsewhere [332,333] and are highlighted in Figure 3.5.

HIV infection of CD4$^+$ T cells leads to a productive infection of the cell and subsequently their demise. Two receptors, CD4 and chemokine, are necessary for viral infection. The envelope protein first binds to CD4 receptors found on T cells, macrophages, and DCs causing conformational changes that allow it to bind to chemokine co-receptors (CCR5 or CXCR4) also present on the cells. The majority of HIV infections occur through the use of CCR5 co-receptor; however, viral strains utilizing CXCR4 are more pathogenic and the use of this receptor has been observed after long periods of infection [324,334]. The differential use of the two receptors during HIV infection is likely due to the variability in the envelope proteins, which mediate binding to these receptors. It is important to note that the spread of the virus is also mediated by infecting DCs and macrophages, which posses CD4 receptors. Infection of DCs does not result in viral replication. Instead, they harbor the virus allowing for spread to CD4$^+$ T cells by cell to cell contact upon migration into LNs [333]. Moreover, DCs abundantly express the C-lectin receptor called DC-SIGN which binds strongly to the envelope protein, enabling the virus to enter the cell [335]. Thus, LNs can harbor the virus and act as reservoirs for the virus [333].

3.5.3 Vaccine status

Initially, it was believed that neutralizing antibodies to HIV would provide protection from the virus (sterile immunity) [3]. Therefore, early HIV vaccines were based on the env gene encoding for the envelope proteins, gp 160 and gp 120, which harbor the neutralizing epitopes. Envelope based vaccines seem to provide neutralizing antibodies strain specific, and thus, broad, cross-reactive neutralizing antibodies are not generated [19,322,336-338]. The ineffectiveness of neutralizing antibodies in providing protection from infection was even more evident after the reported failures of bivalent envelope-based vaccines in two different phase III clinical trials [339]. This is due to the high variability in the envelope proteins, which gives rise to the various groups and subtypes of HIV mentioned in section 3.5.1. The envelope proteins can vary by more than 30% in their amino acids among the various subtypes [340], with differences greater than 50% reported between group M and O [341]. Also, few or no CTL responses are observed with the envelope based vaccines [19,322,338].

Difficulties thus far in achieving sterile immunity based on the envelope vaccines have prompted an alternative avenue for developing an HIV vaccine − disease prevention. Studies of LTNP and HIV-infected patients suggest that CTL responses are important in preventing disease. The presence of strong CTL responses correlates with low viral loads. Although it is realized that CTL responses alone will not prevent virus entry or replication, many studies suggest that they will aid in maintaining low viral loads and controlling viral replication. Thus, CTL responses may delay or prevent the onset of the disease [19,322,337,338,342]. In order to develop a vaccine for controlling infection and blocking the onset of disease against various HIV subtypes, the candidate antigen

should be immunogenic, conserved (to produce cross-reactive responses), and critical in the virus life cycle. Currently, numerous candidate HIV vaccines are being evaluated in human clinical trials [343]. The vaccine components, unlike early approaches, are targeted towards multiple HIV antigens. Two HIV proteins that have received considerable interest for inclusion in potential HIV vaccines are Gag [323,343] and Tat [18,344-346].

3.5.4 HIV-1 Gag

The gag gene encodes for the positively charged Gag poly-protein precursor, p55. Non-replicating, non-infectious virus like particles are formed by p55 in the absence of viral proteins or RNA. Gag p55 is composed of four domains, individually known as the mature Gag proteins: p24 capsid (CA), p17 matrix (MA), p7 nucleocapsid (NC), p6 and p1. After virus budding from the host cell, p55 is cleaved by the HIV protease into the mature Gag proteins. The Gag precursor is involved in virus assembly and membrane targeting, whereas the mature Gag proteins are involved in virus uncoating and disassembly. In the mature virus, MA is associated with the inner viral protein coat, while NC complexes with the viral RNA in the core and CA forms a shell around the RNA and core-associated proteins [17]. The Gag proteins are well conserved among diverse HIV strains and subtypes. Furthermore, strong Gag-specific CTL responses have been found to correlate with low viral loads in LTNP and HIV-infected patients [337,347-349]. In fact, a recent study using peripheral blood mononuclear cells from chronically infected patients found that 96% of the patients responded to challenge by Gag peptides, found to be the highest response out of all proteins used (Env, Pol, and

Nef). In addition, the magnitude of the T cell responses to Gag was inversely correlated with the viral load in plasma and directly with the CD4$^+$ T cell counts in the patients [350]. Numerous studies performed by O'Hagan have demonstrated the ability of recombinant Gag p55 or pDNA adsorbed to poly[d,l-lactide-co-glycolide] (PLGA) microparticles to induce potent CTL and antibody responses in animals [13,238,351] and these microparticles coated with Gag p55 pDNA are being further evaluated in phase I clinical trials (http://www.iavireport.org/trialsdb/vaccinedetail1.asp?i=82).

One of the most conserved domains of Gag is found in the CA protein, referred to as Gag p24 [352-354]. In fact, this Gag p24 contains a region (the major homology region) that is conserved among different genera of retroviruses [17]. Cytotoxic T, B, and T helper cell eptiopes have been identified in the Gag p24 protein by Ikuta *et al*. [355]. Moreover, other groups have demonstrated Gag p24 to be the target of Gag-specific CTL responses [238,354,356]. The importance of Gag p24-specific CTL responses in controlling viral replication and CD4$^+$ cell counts was further demonstrated in a recent study of HIV-infected patients [357]. Combined these features of Gag proteins have made them extremely attractive for including them in HIV vaccines. Indeed, numerous clinical trials are investigating Gag as vaccine component including a phase I clinical trial that is exploring the use of microparticles with Gag (pDNA) [343].

3.5.5 HIV-1 Tat

The two exons of the tat gene of HIV encode for a small regulatory protein of 86-102 aa. The first exon is comprised of the first 72 aa necessary for the HIV transactivating activity [358,359] and is composed of four domains: the amino-terminal

(aa 1-21); the cysteine-rich domain (aa 22-37) representing the transactivation domain; the core (aa 38-48); and the basic domain (aa 49-72) containing nuclear localization signals and regions mediating cellular uptake of Tat [360]. In addition, the second exon contains an RGD sequence required for the interaction with cell surface intergin receptors [361] and mediates cellular uptake of extracellular Tat [362]. HIV-1 Tat is expressed in the early stages of infection [363] and reported to be necessary for production of an infectious virus, with only minute amounts of the structural proteins (Env, Gag, and Pol) expressed in the absence of Tat [364,365]. Moreover, Tat released from infected cells is found in the extracellular milieu and shown to be taken up by uninfected cells, where it can have numerous effects in addition to enhancing virus gene expression. Biologically active Tat was reported to be taken up by APC's, particularly DCs, more efficiently than other cells and promote Th1 type responses [366]. In addition, conjugation of proteins to Tat has been shown to be an effective method in presenting exogenous proteins in context of MHC class I, and generating antigen-specific CTL responses [367]. Tat induces the expression of the chemokine receptors CCR5 and CXCR4, which facilitate the transmission of macrophage-tropic and T cell-tropic strains of HIV-1, respectively [368]. In addition, Tat binds to CXCR4 receptor and prevents viral infection of cells expressing this receptor, which may potentially cause the virus to adapt to using the CCR5 receptors in the early phase of HIV infection [369]. Tat also plays an important role in development of diseases such as AIDS-related Kaposi sarcoma [370], AIDS-related vasculopathy [371] and HIV-dementia [372] in infected patients. Tat has been shown to be both immunogenic as well as well conserved among different HIV subtypes [23,26]. Both antibody [373-376] and CTL responses [377,378] to Tat have been inversely

correlated with disease progression. In fact, a high frequency of CTL responses to Tat is observed in 80% of HIV-infected patients that were able to effectively control the viral replication (< 1000 RNA copies/mL) [378]. Extensive work by Ensoli's group has demonstrated Tat to be immunogenic, safe, and effective in inducing humoral and cellular responses to Tat [18]. In addition, they reported that 9 out of 12 cynomolgus monkeys vaccinated with Tat (protein or DNA) were protected upon challenge with a highly pathogenic strain (SHIV89.6P) and the protection correlated with the presence of anti-Tat CTLs [379,380]. Studies done by other groups have also found Tat to be safe and immunogenic [381-383]; however, two groups have reported failure in controlling viral replication upon challenge with SHIV89.6P [382,383]. The authors caution that the conflicting results obtained could be due to differences in the study designs including different monkey species, dose, immunization schedules, adjuvants, and dose and route of challenge with viruses [382-384]. Despite the conflicting data reported in non-human primate studies, many researchers agree on the potential of Tat-based vaccines [368,385-387] and results from phase I clinical trials evaluating Tat for preventative and therapeutic vaccines are being compiled [388].

3.5.6 Therapeutic vaccines for HIV-infected patients

It is estimated that 14,000 new HIV infections occur every day and even more astounding is that greater than 95% of these new infections are in underdeveloped nations [389]. Although the use of highly active anti-retroviral therapy (HAART) has been greatly effective in controlling viral replication and disease progression, it has not been effective at eliminating the virus completely and there are numerous challenges faced

with this therapy regimen. The regimen itself is complex, often required multiple drugs to be taken every day. Moreover, drug-resistant viruses have been found to develop during HAART that require alternative drug combinations for effectively controlling HIV, which may ultimately limit the effectiveness of the therapy. The regimens are also often accompanied by complicated side effects such as increased plasma lipids, hyperglycemia, and metabolic abnormalities [324]. More importantly, the high cost and limited availability of HAART to individuals in underdeveloped countries, where the majority of new emerging infections are occurring, is extremely concerning.

As an alternative to HAART, a great deal of interest has been generated in the concept of using therapeutic vaccines for controlling disease progression in HIV-infected patients (reviewed in reference [390]). The goal of therapeutic vaccines would be to stimulate appropriate cellular and humoral immune responses in the infected patient capable of controlling the viral replication and therefore, eliminating or decreasing the need for HAART. This approach is especially attractive for regions where accessibility to HAART is costly and limited because a single dose of the vaccine could be administered at appropriate intervals, aiding to reduce viral loads and possibly decrease HIV transmission and thus, the number of new infections. Many therapeutic vaccines have demonstrated to be effective in animal models and have entered human clinical trials; however, so far the results from these trials have failed to demonstrate significant benefits in using this approach. Some of the challenges that are faced in developing and evaluating therapeutic vaccines include: 1) little is known on the immune correlates necessary for viral control, 2) the appropriate time to administer the vaccine and to initial or cease HAART if used in combination with the vaccine, and 3) limitations in the

current assays available to evaluate responses from patients after vaccination. Nonetheless, current work continues to evaluate therapeutic vaccines as a viable alternative to HAART due to the potential benefits that could be reaped from this approach. In this respect, the important roles Gag and Tat play in the viral life cycle have targeted these antigens as components in therapeutic vaccines [384,390-392].

Table 3.1. Historical outline for the major developments in vaccines. (Adapted from Plotkin and Plotkin [21] and Bramwell [23])

Vaccine Introduced	Type of Vaccine	Major Achievements
18th Century		
Smallpox	Live attenuated	The idea of vaccination introduced using a closely related animal virus (cowpox)
19th Century		
Rabies Anthrax	Live attenuated	First demonstration of chemical attenuation of pathogens for live vaccines
Typhoid Cholera Plague	Whole-killed	Demonstration that inactivated organisms could retain their immunogenicity
20th Century		
(1920s-1940s)		
Whole-cell Pertussis	Whole-killed	-
BCG	Live-attenuated	First demonstration of *in vitro* passage for attenuation using artificial media (bile)
Yellow-Fever	Live-attenuated	*In vitro* passage using mouse brain and chick embryo
Influenza	Whole-killed	Production in chick embryo
Tetanus Diphtheria	Protein	First use of inactivated protein vaccines – Toxoids
(1950s-1970s)		
Injected polio (IPV)	Whole-killed	Cell culture introduced for producing vaccines
Oral Polio (OPV) Measles Rubella	Live-attenuated	
Influenza	Whole-Killed	Introduction of reassortants – RNA segments from attenuated strain combined with RNA from circulating strain.
Pneumococcal Meningococcal	Purified Polysaccharides	Polysaccharide use for vaccination
(1980s to date)		
H. Influenzae type b	Protein	Conjugation of protein (i.e. bacterial toxins) to polysaccharides for enhancing immune response
Hepatitis B	Protein	First protein-based vaccine produced by genetic engineering technology
Acellular Pertussis	Protein	Genetic engineering for protein modification and avoiding side effects associated with bacterial cell walls

Table 3.2. Features of innate and adaptive immune systems. (Adapted from Janeway and Medzhitov [55]).

Feature	Innate immune system	Adaptive immune system
Action Time	Rapid (hours)	Slow (days-weeks)
Immune response	Invariant	Progressive
Cells	Macrophages Dendritic cells Mast cells Neutrophils Eosinophils NK Cells	Antigen presenting cells B lymphocytes T lymphocytes
Receptor variability	Germ-line encoded and not variable	Encoded in gene segments and variable by rearrangement
Recognition	Conserved molecular patterns present on pathogens recognized by PRR	Details of molecular structure and sequences recognized by highly specific receptors
Outcome of Immune Response	1) Pathogen is cleared 2) Adaptive immune system is initiated	1) Antigen-specific effector and memory cells generated 2) Antigen-specific antibodies generated 3) Pathogen is eliminated or infection is controlled

Table 3.3. Examples of commonly investigated adjuvants for vaccines.

Class of Adjuvant	Examples
Immunostimulatory	Cytokines: *IL-2, IL-12, IFN-γ, GM-CSF* QS21 Bacterial DNA (CpG) Lipid A Bacterial Toxins: *cholera toxin,* *heat labile enterotoxin*
Particulate	Emulsions: *CFA, MF59* ISCOMs Liposomes Microparticles Nanoparticles Virus-like particles (VLPs) Mineral Salts: *Aluminum hydroxide, Aluminum* *phosphate and Calcium phosphate*

Table 3.4. Comparison of relative sizes of pathogens with commonly investigated particulate delivery systems for vaccines. (Adapted from O'Hagan *et al*. [125])

Pathogens		Particulate delivery systems	
Bacteria	~0.5-3 µm	Microparticles	1-10 µm
Herpes virus	~250 nm	Nanoparticles	10-1000 nm
HIV;	~100 nm	Liposomes	50 nm – 10 µm
Influenza virus			
Poliovirus	~20-30 nm	MF59	~200 nm
		ISCOMs	~40 nm
		VLPs	~20-50 nm

Table 3.5.   Examples of combined adjuvant formulations investigated for enhancement in immune responses.

Particulate System	Immunostimulatory Adjuvant	Antigen	References
Alum	IL-12	Malaria antigen	[256]
		HIV-1 gp 120	[81]
		Respiratory syncytial virus	[78]
	CpG	Hepatitis B surface antigen	[107]
	Lipid A derivative	Herpes simplex virus	[91]
Liposomes	CpG	Ovalbumin	[393,394]
		Influenza Hepatitis B surface antigen	[258]
		Hepatitis C antigen (NS3)	[159,162]
		HIV-1 gp 140	[99]
	Lipid A derivative	HIV-1 gp 140	[99]
		Influenza	[395]
	IL-6	HIV-1 gp 120	[79]
	IL-2	Influenza	[80,396,397]
	IFN-γ	Influenza hemagglutinin: T and B cell epitopes	[45,79]
Nanoparticles and Microparticles	CpG	HIV-1 gp120	[398]
		HIV-1 Gag p55	[398]
		Neisseria meningitides type B	[237]
		Anthrax	[260]
		Tetanus toxoid	[111,263]
	Lipid A derivative	Neisseria meningitides serotype B (Men B)	[257]
		HIV-1 gp 120	[257]
		Hepatitis B core antigen	[246]

Table 3.6. HIV proteins and their main functions in viral life cycle.

Gene		Protein encoded	Major Function(s)
Structural	gag	Gag p55 precursor; capsid (p24); matrix (p17); nucleocapsid (p7); p6; p1	Virus assembly and structure
	pol	Reverse transcriptase (p66/51); Integrase (p32); Protease (p11)	Viral genome transcription, integration into host genome, and cleavage of precursor proteins in immature virus
	env	gp 160 Envelope precursor; gp 120; gp 41	Glycoproteins (gp) required for binding and internalization of virus
Regulatory	tat	Transactivator (Tat)	Promotes infection of cells and LTR-mediated viral gene transcription
	rev	Regulator of viral expression (Rev)	Binds to Rev responsive element on HIV mRNA, allowing transport of unspliced RNA from nucleus to cytoplasm and production of structural proteins
Accessory	nef	Negative-regulation factor (Nef)	Down regulates expression of cell surface molecules (CD4 and MHC I) in infected cells
	vif	Viral infectivity (Vif)	Involved in viral replication by stabilizing DNA intermediate and
	vpr	Viral protein R (Vpr)	Plays a role in transporting HIV-preintegration complex to nucleus
	vpu	Viral protein U (Vpu)	Down regulates CD4 expression and forms ion channels in cell membranes to allow release of virons from infected cells

Figure 3.1
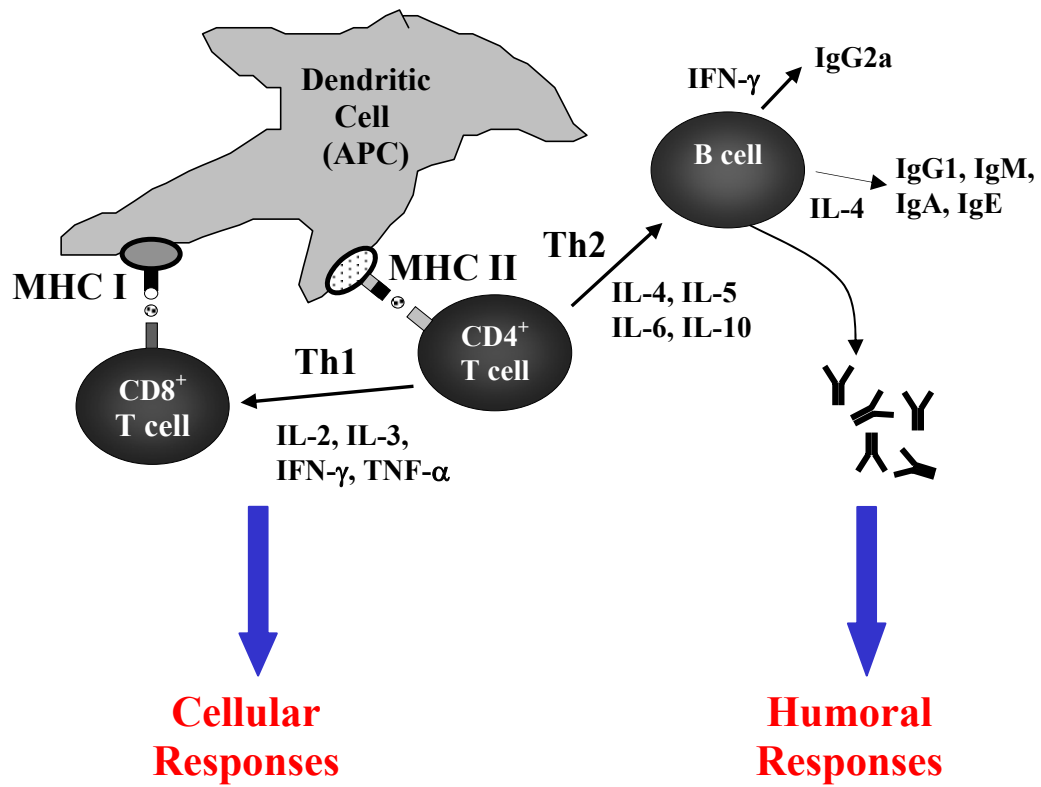
Figure 3.1. Adaptive immune response. The key cellular interactions involved in generating antigen-specific immune responses are depicted.   Cellular responses are mediated by CD8$^+$ and CD4$^+$ T cells and humoral responses are mediated by B cells with additional help provided by T helper 2 (Th2) cells. The cytokines released by the CD4$^+$ T cell influence the isotype of antibodies produced by B cells (Adapted from Singh and O'Hagan [1]).

Figure 3.2
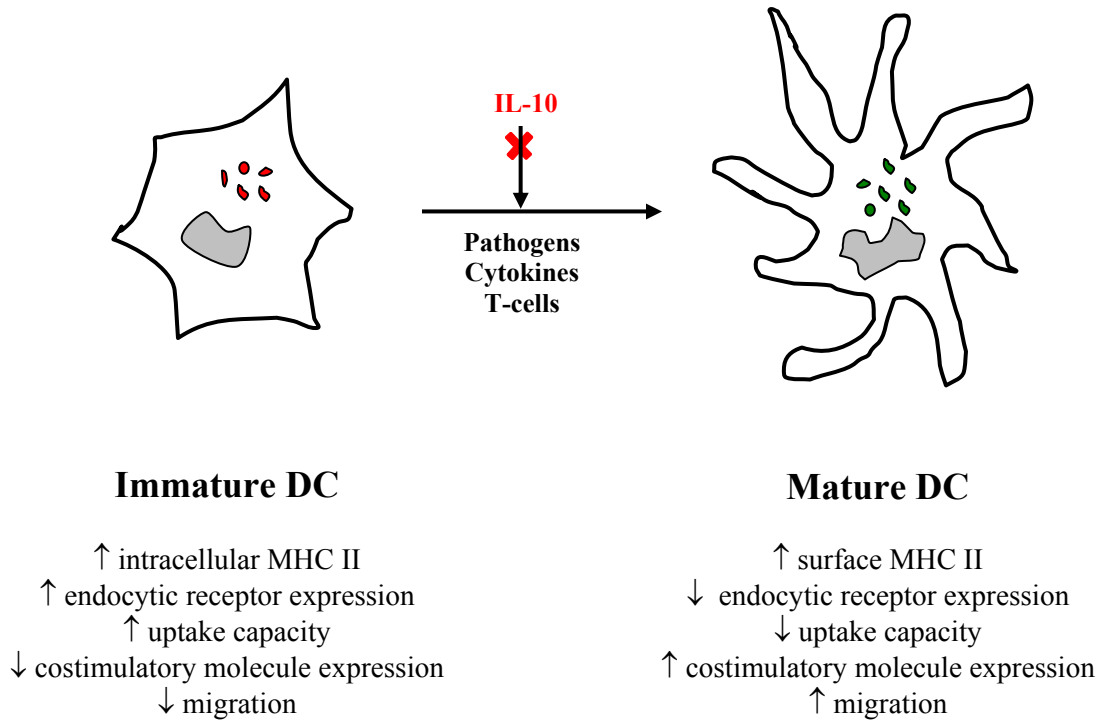
Figure 3.2. Features of dendritic cells.   Immature DCs are triggered to mature by environmental factors such as encountering a pathogen or release of inflammatory cytokines.   Immature and mature DCs possess different structural, phenotypical and functional features. (Adapted from Banchereau and Steinman [267]).

Figure 3.3
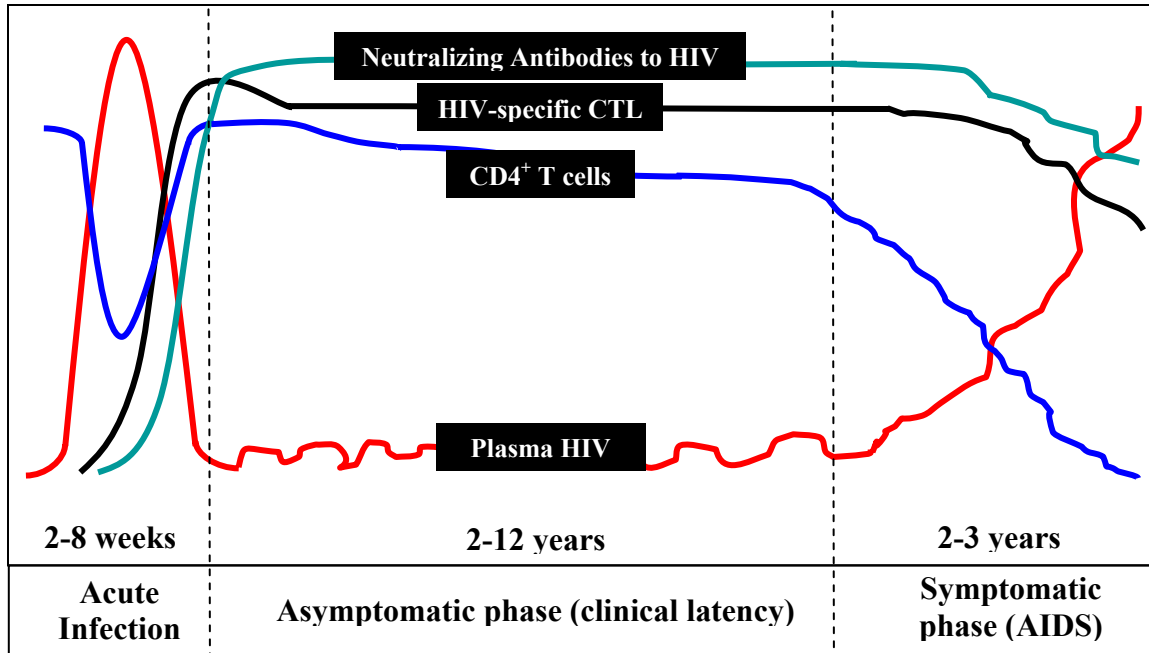
Figure 3.3. Adaptive immune responses during HIV infection. An increase in circulating virus correlates with decrease in the CD4$^+$ T cells in the initial phase of infection. The induction of HIV-specific CTL responses helps to maintain CD4$^+$ T cells in the asymptomatic phase of infection. However, the immune system is unable to replace the dying CD4$^+$ cells and their subsequent depletion leads to AIDS. (Adapted from Girard and Excler [327] and Parham [52]).

Figure 3.4

A)

B)
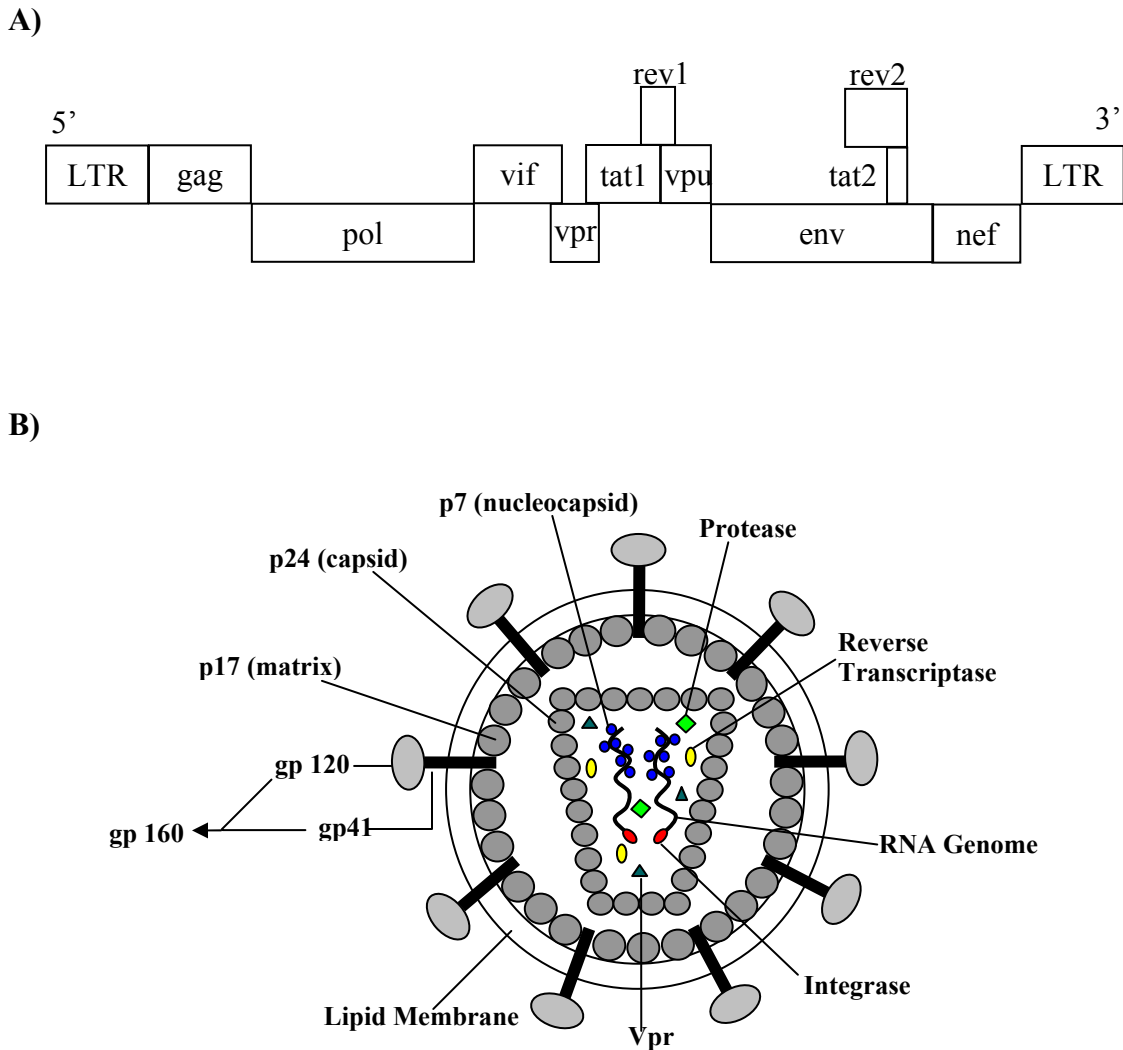
Figure 3.4. HIV genome and schematic representation of the HIV viron. A) The viral genome consists of nine genes flanked by LTR regions. B) Schematic representation of HIV viron, showing the location of the various proteins in the mature virus. (Adapted from Girard and Excler [327] and http://hivinsite.ucsf.edu/ InSite?page=kb-02-01-01#S2X).
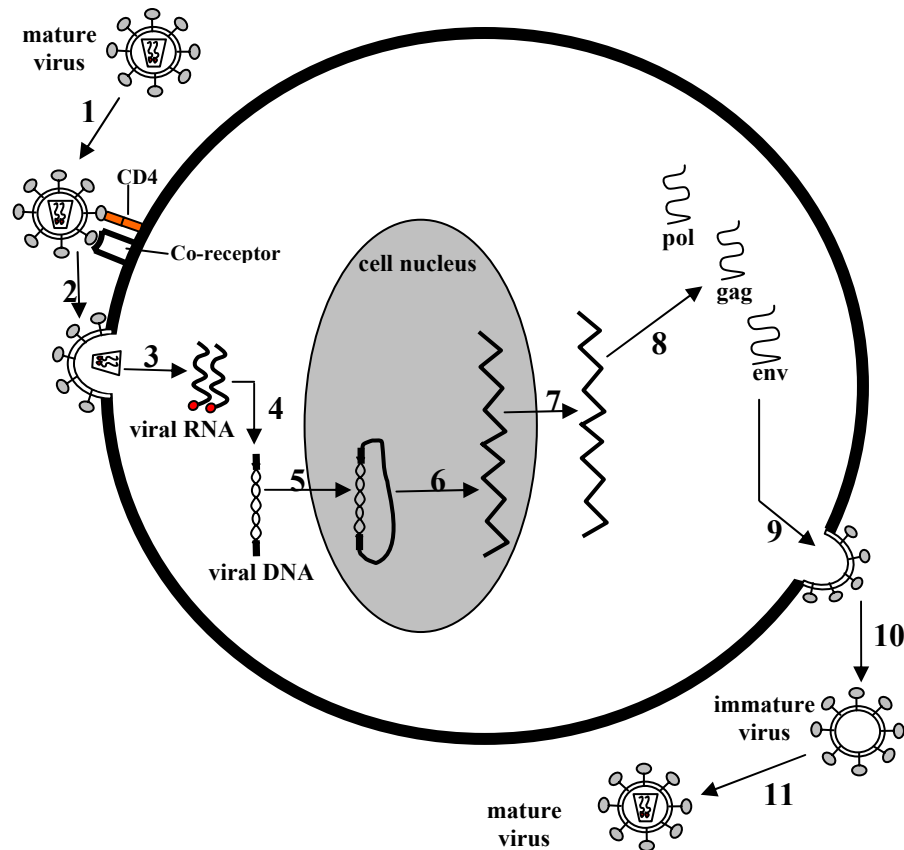
Figure 3.5

Figure 3.5. HIV life cycle. 1) gp120 interacts with CD4 and chemokine co-receptors present on the cell. 2) The virus membrane fuses with cell membrane. 3) Viral contents are emptied into cell cytoplasm. 4) The viral RNA is transcribed into double stranded DNA by the reverse transcriptase. 5) Viral DNA is transported into the nucleus and integrated into host genome by integrase. 6) The DNA is transcribed into RNA. 7) RNA is transported to the cell cytoplasm. 8) The viral proteins are synthesized in the cytoplasm. 9) The structural proteins are transported to the cell membrane and virus assembly begins. 10) The immature virus buds from the cell membrane. 11) The protease enzyme cleaves the polyproteins to give the mature virus. (Adapted from Freed [17]).

Chapter 4

HIV-1 Tat-coated nanoparticles result in enhanced humoral immune responses and neutralizing antibodies compared to Alum adjuvant

4.1   Summary

HIV-1 Tat has been identified as an attractive target for vaccine development and is currently under investigation in clinical trials as both a therapeutic and preventative vaccine for HIV-1. It is well known that protein based vaccines produce poor immune responses by themselves and therefore require adjuvants to enhance immune responses. We have previously reported on the use of anionic nanoparticles for enhancing cellular and humoral immune responses to Tat (1-72). The purpose of this study was to further evaluate the immune response of HIV-1 Tat (1-72) coated on anionic nanoparticles (NPs) compared to Alum using various doses of Tat (1-72). Nanoparticles were effective at generating comparable antibody titers at both 1 μg and 5 μg doses of Tat (1-72), whereas the antibody titers significantly decreased at the lower dose of Tat (1-72) using Alum. Anti-sera from Tat (1-72) immunized mice reacted greatest to the N-terminal and basic regions of Tat, with the NP groups showing stronger reactivity to these regions compared to Alum. Moreover, the anti-sera from all Tat (1-72) immunized groups contained Tat-neutralizing antibodies and were able to significantly inhibit Tat-mediated long terminal repeat (LTR) transactivation.

4.2   Introduction

Ideally, an HIV vaccine would be effective at blocking viral entry and thus, provide sterilizing immunity. However, recent failures with the envelope (Env) based HIV vaccine have demonstrated that this may be a difficult goal to attain [339]. These experiences have prompted investigators to look at alternative strategies in developing an HIV vaccine; for example, to limit viral replication and thereby block or delay progression onto AIDS. In pursuit of this secondary goal, it is well recognized that the candidate antigen must be conserved among different HIV subtypes, as this is a major drawback for Env based vaccines. In addition, the antigen should be immunogenic and play a critical role in the virus life cycle. To this end, the HIV-1 regulatory protein Tat has received considerable interest as a potential HIV vaccine or as a component in a HIV vaccine [384,385,399,400].

Tat, a small regulatory HIV protein, is encoded by two exons, with the size of the full length protein varying from 86 to 102 amino acids (aa) depending on the viral strain. The first exon of Tat encodes the first 72 aa of the protein, which includes: the amino-terminal (aa 1-21); the cysteine-rich domain (aa 22-37) representing the transactivation domain; the core (aa 38-48); and the basic domain (aa 49-72) containing nuclear localization signals and regions mediating cellular uptake of Tat [360]. This region of Tat has been shown to be necessary for Tat-mediated transactivation of HIV-1 gene expression [358,359] and is highly conserved among different viral subtypes [401,402], with the cysteine-rich domain (aa 25-38) being conserved among human, bovine and simian species [403]. While the second exon, encoding the C-terminal domain of Tat is not required for the transactivation, it contains an arginine-glycine-aspartic acid (RGD)

sequence that mediates the binding of extracellular Tat to integrin receptors [361]. Additionally, the C-terminal domain of Tat is of significant importance in the cellular uptake of extracellular Tat [362].

Tat plays a vital role in the viral life cycle and mediates many processes allowing for spread of the virus throughout the body and potentiating disease. Of these, possibly the most significant role of Tat in the virus life cycle is the enhancement of HIV gene expression. Tat is expressed in the very early stages of infection before the expression of the structural components (Env, Gag, Pol) [363]. In fact, Tat is necessary for efficient viral gene expression and in the absence of Tat, no or only minute amounts of the structural proteins are expressed, preventing the production of an infectious virus [364,365]. In addition to promoting viral gene expression in HIV-infected cells, extracellular Tat released from infected T lymphocytes has numerous effects on uninfected cells that aid in the progression of disease. Tat induces the expression of the chemokine receptors CCR5 and CXCR4, which function as co-receptors for HIV-1 and facilitate the transmission of macrophage and T cell tropic HIV-1 strains [368]. Some *in vitro* studies have demonstrated that Tat induces the production of Interferon-α, inhibiting T cell proliferation, [400] and also promotes apoptosis of T cells by increasing the expression of CD95L/Fas ligand on macrophages [381]. Fanales-Belasio *et al.* have demonstrated that biologically active Tat at high concentrations is taken up by antigen presenting cells (APCs), particularly monocyte-derived dendritic cells, inducing expression of MHC II, co-stimulatory molecules, and causing production of Th1 type cytokines such as IL-12 [366]. On the contrary, Izmailova *et al.* did not observe DC activation or maturation in the presence of HIV infection or Tat expression; however,

they reported that Tat was involved in up-regulation of chemoattractant proteins in immature DC which are involved in recruiting of T cells and macrophages thus, aiding in the spread of infection [404]. The ability of Tat to be efficiently taken up by cells has become attractive for the delivery of proteins into cells [405] and conjugation of proteins to Tat has been shown to be an effective method in presenting exogenous proteins in context of MHC class I, and generating antigen-specific CTL responses [367].

The importance of Tat in the progression to diseased states has been demonstrated in numerous reports [373-378,406-408]. In fact, both strong antibody [373,374,376] and CTL responses [377,378] to Tat have been inversely correlated with viral loads and disease progression. Moreover, Tat is involved in the progression of AIDS-related Kaposi sarcoma and high serum levels anti-Tat antibodies have been correlated with reduced Kaposi sarcoma in HIV-infected patients [387]. Extensive studies carried out in mice and non-human primates have demonstrated that immunization with Tat is safe and effective at generating humoral and cellular immune responses [18]. However, conflicting data exists on the effectiveness of a Tat-based vaccine in controlling infection upon challenge with a highly pathogenic strain (SHIV-89.6P) in non-human primates [379,381,383,409,410]. In spite of these data, many researchers agree that the potential of Tat based vaccines warrant further investigation and Phase I clinical trials evaluating Tat for preventative and therapeutic vaccines are currently ongoing in Italy [18,407]. Moreover, a Tat toxoid (chemically modified Tat) vaccine has already been evaluated in both HIV negative and positive patients and was shown to be safe and effective [387]. Futhermore, other ongoing clinical trials evaluating preventative HIV-1 vaccines include

Tat as component of the vaccine in addition to other HIV-1 antigens (IAVI Report, February 2005).

We have previously reported on the preparation and purification of the first exon of Tat protein (referred to as Tat (1-72)) [362]. We have demonstrated that Tat (1-72) is immunogenic and that Tat (1-72) coated anionic nanoparticles were effective at generating humoral and cellular immune responses to Tat [253]. In the present study, the use of nanoparticles for generating immune responses to various doses of Tat (1-72) was further investigated. More specifically, we sought to determine the lowest effective dose of Tat (1-72) that could produce immune responses when administered with anionic nanoparticles. In addition, the antibody epitopes generated in mice immunized with Tat (1-72) were mapped and the Tat-neutralizing activity in the sera from immunized mice was evaluated.

4.3 Materials and methods

Materials

Emulsifying wax, comprised of cetyl alcohol and polysorbate 60 (molar ratio of 20:1), was purchased from Spectrum (New Brunswick, NJ). Sodium dodecyl sulfate (SDS), PBS/Tween 20 buffer, bovine serum albumin (BSA), triethanolamine, and mannitol were purchased from Sigma Chemical Co. (St. Louis, MO). Brij 78 was purchased from Uniqema (New Castle, DE). Sheep anti-mouse IgG, peroxidase-linked species specific F(ab')$_2$ fragment was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Goat anti-mouse IgG2a and IgG1 horseradish peroxidase (HRP) conjugates were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Tetramethylbenzidine (TMB) substrate kit was purchased from Pierce (Rockford, IL). Incomplete Freund's adjuvant and mycobacterium tuberculosis were purchased from Fisher Scientific (Hampton, NH). Lipid A from *Salmonella Minnesota* R595 (Re) was purchased from List Biological Laboratories (Campbell, CA). HIV-1 Clade B consensus Tat peptides (15 aa) were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MA). Recombinant HIV-1 Tat (1-72 aa) was prepared as previously described [362].

Preparation of anionic NPs

Nanoparticles from oil-in-water microemulsion precursors were prepared as previously described previously [253] with slight modification. Briefly, 2 mg of emulsifying wax and 3.5 mg of Brij 78 was melted and mixed at ~60-65°C. Deionized

and filtered (0.2 μm) water (980 μL) was added to the melted wax and surfactant while stirring to form an opaque suspension. Finally, 20 μL of sodium dodecyl sulfate (50 mM) was added to form clear microemulsions at 60-65°C. The microemulsions were cooled to room temperature, while stirring, to obtain NPs (2 mg/mL). The final concentration of components in the NP suspension was emulsifying wax (2 mg/mL), Brij 78 (3 mM), and SDS (1 mM). The NP sizes were measured using a Coulter N4 Plus Sub-Micron Particle Sizer (Coulter Corporation, Miami, FL) at 90°. The overall charge of the NPs was measured using Malvern Zeta Sizer 2000 (Malvern Instruments, Southborough, MA).

Coating of the anionic NPs with Tat

Varying amounts of Tat were added to NPs (1000 μg/mL) in 5% (v/v) mannitol. The suspension was vortexed gently and placed on a horizontal shaker at room temperature for a minimum of 30 min to allow for coating. The coated NPs were diluted appropriately in de-ionized water for measuring the size and charge of the particles.

Mouse immunization study

Two animal studies were carried out to determine the immune response to different doses of Tat. A summary of the experimental design is presented in Table 4.1. For both studies, female BALB/c mice (8-10 weeks old) obtained from Harlan Sprague-Dawley Laboratories (Indianapolis, Indiana) were immunized subcutaneously with 100 μL of the formulations. In the initial mouse study (Study 1), mice (n=5-6/group) were dosed on day 0, 21 and 28 with 1 μg or 5 μg of Tat-coated NPs or 1 μg or 5 μg of Tat

adjuvanted with Alum. The dose of NPs and Alum administered in both cases was 100 µg. As a positive control, mice were immunized on day 0 with 5 µg of Tat adjuvanted with complete Freund's adjuvant (CFA) followed by boost with 5 µg Tat adjuvanted with incomplete Freund's adjuvant (IFA). On day 35, mice were bled by cardiac puncture and sera were separated. All sera collected were stored at -20°C.

In the follow up study (Study 2), mice (n=6-8/group) were dosed on day 0, 14 and 28 with 0.2 µg or 1 µg of Tat-coated NPs or 0.2 µg or 1 µg of Tat adjuvanted with Alum. Again, the dose of NPs and Alum given to animals was 100 µg. Mice were immunized with 1 µg of Tat adjuvanted with Lipid A (50 µg) as a positive control. To assess the kinetics of Tat specific antibodies generated using the different treatments, mice were bled on day 13 and 34 by tail vein and sera were collected. On day 42, all mice were bled by cardiac puncture and sera were collected. All sera were stored at -20°C.

Determination of antibody titers

Tat-specific serum IgG, IgG1 and IgG2a antibody titer were determined using an ELISA. Briefly, 96-well plates (Costar) were coated with 50 µL of Tat (5-8 µg/mL in 0.01 M phosphate buffered saline, pH 7.4) overnight at 4°C. The plates were blocked for 1 hr at 37°C with 200 µL of 4% BSA prepared in PBS/Tween 20. The plates were then incubated with 50 µL per well of mouse serum diluted appropriately in 4% BSA/PBS/Tween 20 for 2 hr at 37°C. The plates were washed with PBS/Tween 20 and incubated with 50 µL/well anti-mouse IgG HRP F(ab')$_2$ fragment from sheep (1:3000 in 1% BSA/PBS/Tween 20) for 1 hr at 37°C. For IgG1 and IgG2a determination, the plates were similarly incubated with goat anti-mouse IgG1-HRP or goat anti-mouse IgG2a-HRP

diluted 1:8000 (1% BSA/PBS/Tween 20). After washing the plates with PBS/Tween 20, the plate was developed by adding 100 μL of TMB substrate and incubating for 30 min at RT. The color development was stopped by addition of 100 μL of 2 M H_2SO_4 and the OD at 450 nm was read using a Universal Microplate Reader (Bio-Tek Instruments, Inc.). The OD at 450 nm versus log serum dilution for each animal was plotted and fit to a four parameter logistic equation using GraphPad Prism software. The titer was defined as $0.5*OD_{max}$.

B cell epitope mapping

Tat anti-sera were tested by ELISA to determine reactivity to various regions of Tat. Tat peptides (50 μL of 1 μg/mL Tat peptide in 0.05 M carbonate buffer, pH 9.6) were coated onto 96-well Costar plates by incubating overnight at 4°C. The plates were blocked for 1 hr at 37°C with 200 μL of 4% BSA prepared in PBS/Tween 20. Tat anti-sera diluted at 1:100 in 4% BSA/PBS/Tween 20 were added to the wells and incubated for 2 hr at 37°C. The wells were washed, reacted with anti-mouse IgG HRP F(ab')$_2$ fragment from sheep, and developed as described for total IgG titer.

Tat-mediated LTR-transactivation assay

SVGA LTR-chloramphenicol acetyltransferase (CAT) was produced by stable transfection of the astrocytic cell line SVGA with pHIV-CAT [411]. SVGA LTR-CAT cells were seeded into 6-well plates a minimum of 24 hr prior to use in Dulbecco's Modified Eagle Medium (DMEM; GibcoBRL) with 10% heat-inactivated fetal bovine serum (FBS; Sigma) and 1% antibiotic-antimycotic solution (penicillin G sodium,

streptomycin sulfate, and amphotericin B in 0.85% saline; GibcoBRL) (DMEM+10% FBS+Ab). Ten (10) µL of sera was mixed with 2 µL (1000 µg) recombinant Tat (1-72) derived from the first exon of the HIV-1 tat gene and incubated for 30 min at 37°C in a water bath. During the incubation, medium was replaced on the SVGA LTR-CAT cells with 2 mL fresh DMEM+10% FBS+Ab. The sera/Tat mixtures were then added to the cells. Cells were then lysed at 24 hr post-treatment and CAT levels were quantitated by ELISA according to the manufacturer's directions (Roche).

Statistical analysis

Statistical analysis was performed using one-way analysis of variances (ANOVA) followed by pair-wise comparisons using Newman-Keuls multiple comparison test using GraphPad Prism software.

4.4   Results and discussion

Many researchers have reported on the potential of HIV-1 Tat-based vaccine or supported the idea including Tat as a component in a cocktail for vaccine development [384,385,399,400]. As new targets for HIV vaccine development are identified, it is also important to investigate novel delivery systems for effectively enhancing immune responses to the target antigen(s). Our laboratory has reported on the use of anionic and cationic NPs for effectively enhancing immune responses to plasmid DNA and protein based vaccines [14-16]. These NPs are prepared from oil-in-water microemulsion precursors and form solid stable particles. The emulsifying wax oil phase used to prepare the NPs is comprised of cetyl alcohol and polysorbate 60 (20:1 molar ratio) which are both employed as components in parenteral products and are potentially non-toxic materials. We have recently shown that these nanoparticles are hemocompatible and metabolized via endogenous alcohol dehydrogenase enzyme systems [251]. In addition, these NPs have the advantage of being prepared in a single step, one vessel process with relative ease in modifying the physical characteristics of the particles by using appropriate surfactants. We recently reported the use of anionic NPs as effective delivery systems for enhancing immune responses to Tat (1-72) [253]. In the present studies, the dose-response to Tat (1-72) using NPs compared to standard Alum adjuvanted protein was further evaluated. In addition, the humoral immune responses to Tat (1-72) were characterized for reactivity to various regions of Tat and for activity in a LTR-transactivation assay.

Adsorption of Tat (1-72) on anionic NPs

The NPs prepared in these present studies are stabilized by the inclusion of Brij 78 and made to be negatively-charged by the use of the anionic surfactant SDS. The net charge of the NPs prior to coating with Tat is approximately -56 mV (Table 4.2). Tat, a cationic protein, is expected to be coated on the surface of the NPs via ionic interactions. It is thought that the negatively charged amino acids of Tat are exposed on the surface of NPs while the positively charged amino acids interact with the negatively charged NPs. As demonstrated in Table 4.2, there is a slight increase in the zeta potential with increasing amounts of Tat coated on NPs; however, the Tat-coated NPs continue to have a net negative charge possibly due to the exposed negatively charged amino acids. The coating of Tat on NPs at a 1:100 w/w ratio was found to be approximately 85% by SDS-PAGE/densitometry (data not shown).

Dose response to Tat in immunized mice

We previously reported that Tat (5 μg) coated on anionic NPs result in similar Tat-specific IgG levels as Alum and Lipid A adjuvanted with Tat [253]. Thus, a dose response of Tat was evaluated in these present studies to determine the minimum Tat dose that could be administered while maintaining the Tat-specific IgG response. In the first study, use of 1 μg and 5 μg of Tat coated on NPs or adjuvanted with Alum was investigated. Tat (5 μg) with CFA adjuvant was used as a positive control. The results shown in Figure 4.1 demonstrate that Tat-specific IgG antibody titers are maintained at both the 1 μg and 5 μg of Tat coated on NPs, while Tat (1 μg) adjuvanted with Alum produced significantly lower titers compared to Tat (5 μg) adjuvanted with Alum.

Moreover, both the 1 μg and 5 μg Tat-coated NPs produced comparable Tat-specific IgG titers compared to CFA. Only the higher dose of Tat adjuvanted with Alum produced comparable Tat-specific IgG levels to CFA adjuvant. These results suggested that a lower dose of Tat using NPs compared Alum was still effective at eliciting a strong Tat-specific antibody response.

In the follow up study, the effectiveness of lower doses of Tat coated on NPs and adjuvanted with Alum in generating Tat specific antibodies was evaluated. Lipid A with 1 μg of Tat was used as a positive control. The results (Figure 4.2) indicated that Tat (1 μg) coated NPs were able to generate significantly higher IgG levels at all time points tested compared to both Alum groups and the NP group receiving the lower dose of Tat. These data combined with IgG results from the first study suggest that the total serum anti-Tat IgG levels are similar with immunization doses of 1 μg or 5 μg of Tat coated on NPs, however, doses of less than 1 μg of Tat, cause a significant decrease in the anti-Tat IgG levels. More importantly, the data taken together demonstrate that the Tat-coated NPs were capable of generating stronger and more robust humoral immune responses at lower doses of antigen compared to Tat adjuvanted with Alum.

To evaluate the type of response (Th1- or Th2-type) that was generated using NPs compared to the other adjuvants, both Tat-specific IgG2a and IgG1 titers were determined using Tat anti-sera from the first study evaluating 1 μg and 5 μg Tat. The Tat-specific IgG2a and IgG1 titers along with the mean IgG2a/IgG1 ratio are presented in Figure 4.3. Significantly lower Tat-specific IgG2a titers were produced using 1 μg Tat adjuvanted with Alum compared to Tat (1 μg) coated NPs and the mean IgG2a/IgG1 ratios were lower for both Alum groups compared to the NP groups. Interestingly, CFA,

a strong adjuvant for Th1 responses in mice, produced similar Tat-specific IgG2a levels as Tat coated NPs or Tat (5 μg) adjuvanted with Alum. This high level of IgG2a seen with all groups may be attributed to Tat, which has been demonstrated to enter the MHC class I pathway and enhance Th1 responses [366,367]. These results are consistent with a greater stimulation of Th1 responses by NPs compared to Alum.

Tat-specific antibody epitope mapping

The Tat used in this study only corresponds to the first 72 aa acids of Tat, which is encoded by the first exon of the tat gene. Tat anti-sera collected from both studies were analyzed to determine reactivity to overlapping 15-mer peptides spanning aa 1-83 of the Tat sequence. Table 4.3 presents the reactivity pattern for anti-sera collected from each animal for each Tat immunized group in Study 1. Sera from all animals recognized aa 1-15 or 5-19, corresponding to the N-terminal region of Tat. In addition, the sera from Tat immunized mice also recognized aa 45-59 and 49-63, corresponding to the basic region of Tat; however, this was to a lesser degree than recognition of the N-terminal and was only observed in some of the animals. Interestingly, Tat-coated NPs which demonstrated the highest total serum Tat-specific IgG titers also demonstrated the strongest reactivity in both the N-terminal and basic regions. Moreover, the strong reactivity to both the N-terminal and basic regions of Tat is maintained using 1 μg of Tat-coated NPs compared to the Alum groups. In fact, Tat (1 μg) coated on NPs showed stronger reactivity in the basic region of Tat compared to Tat (5 μg) adjuvanted with Alum. Similarly, evaluation of the Tat anti-sera from Study 2 demonstrated strong reactivity of 1 μg Tat-coated NPs compared to Alum at both the N-terminus and basic

region (Table 4.4). Although the sera from both positive controls in the two studies, CFA and Lipid A, demonstrated reactivity across the entire Tat sequence analyzed, the strongest recognition was in the N-terminal and basic regions of the protein.

These data are in agreement with the antibody epitopes reported for Tat (aa 1-86) in different species [387,412-414]. Sera from healthy and HIV-infected volunteers immunized with a Tat-toxoid vaccine reacted primarily with the N-terminus (aa 1-24) and the basic domain (aa 46-60) of Tat [387]. Moreover, sera from mice, rabbits, macaques and humans immunized with recombinant Tat, synthetic Tat, Tat toxoid or Tat peptides demonstrated reactivity to N-terminus and basic domains of Tat [413]. While both the N-terminal and basic regions of Tat are highly conserved among different HIV-1 subtypes [414], the recognition of the basic region may be of particular importance since it mediates the cellular uptake and nuclear localization of Tat [360]. Thus, generating antibodies that strongly recognize this region is advantageous for inhibiting Tat-mediated HIV-gene expression in infected cells. It is important to note that that the strongest reactivity in the basic domain in the Tat immunized animals was produced by the NP groups, suggesting that NPs may be effective at enhancing immune responses to this region.

Inhibition of Tat-mediated LTR-transactivation

Extracellular Tat has been demonstrated to enter HIV-infected cells and promote gene expression [358,359]. Tat mediates transactivation of HIV-1 via the long-terminal repeat promoter by migrating from the cell cytoplasm into the cell nucleus and binding to the Tat responsive element, TAR region [18]. Thus, neutralization of extracellular Tat may be crucial in preventing spread of HIV infection and slowing the progression on to

disease. The anti-sera from Tat immunized mice were evaluated for extracellular Tat neutralization activity using SVGA cells (an astrocytic cell line) transfected with pHIV-CAT plasmid. Sera from the naïve group were used as controls and the percent inhibition in CAT expression for each group compared to the naïve group is presented in Figure 4.4. All groups demonstrated significantly higher Tat neutralizing antibody compared to the naïve sera. As shown, the anti-sera from Tat (5 μg) coated NPs group produced significantly higher inhibition in CAT expression compared to all groups. Nonetheless, the other groups also demonstrated ability of the anti-sera to neutralize extracellular Tat. These data suggest that neutralizing antibodies to Tat (1-72) can be generated with the various adjuvants employed. Interestingly, Moreau *et al.* have reported that only anti-sera reacting with the N-terminal and basic regions of Tat were able to block Tat-mediated LTR-transactivation [413]. In the present studies, a correlation between reactivity in these regions of Tat and ability to block extracellular Tat entry into cells was not observed. All the anti-sera were able to neutralize extracellular Tat to some degree regardless of the Tat epitopes recognized in the peptide ELISA. However, Tat (5 μg) coated on NPs which demonstrated the strongest reactivity to the basic region in the peptide ELISA also demonstrated the highest neutralization activity in the LTR-transactivation assay. Moreover, this group also demonstrated the strongest reactivity in the basic region of Tat, which may be of significant importance in preventing entry of Tat and thus, Tat-mediated LTR-transactivation. Differences observed between the results obtained in this study compared to those observed by Moreau *et al.* could be due to a number of factors. First, different cell types were used in the two studies which may affect the sensitivity of the assay. Second, the present studies used the first exon of Tat

90

(aa 1-72) for the LTR-transactivation assay whereas Moreau *et al.* used full length Tat (aa 1-86), which has been shown to enter cells more efficiently than Tat (1-72). Third, the Tat concentrations employed in the present LTR-transactivation studies are approximately 80-fold greater than those reported by Moreau *et al.* Fourth, Tosi *et al.* have shown that only the monomeric form of exogenous Tat is able induce LTR-transactivation and took significant precautions using high concentrations of DTT to inhibit oligomer formation [415]. Thus, taken together these differences may offer some explanation as to why a correlation was not observed between the reactivities of the anti-sera in the N-terminal and basic regions of Tat and the neutralization activity in the LTR-transactivation assay. Further work to optimize the LTR-transactivation assay using a HeLa cell line and a difference source of extracellular Tat are on going in our laboratory. Preliminary results, presented in Chapter 8, demonstrate that improved sensitivity in the assay can be obtained with these modifications.

It is well recognized that there is an urgent need for a safe and effective vaccine against HIV. Numerous failures to neutralize the virus using envelope-based vaccines have caused researchers to look at alternative avenues to provide some protection or to slow down the progression of the disease in HIV-infected patients by the development of therapeutic vaccines. One of the requirements for an effective HIV vaccine will be that the antigen is conserved among the different viral subtypes. Thus, many researchers are investigating the potential of HIV regulatory proteins Nef, Rev, and Tat, which are expressed early in the life cycle and are also fairly well conserved among the different HIV subtypes, as alternative targets for vaccine development [406,416,417]. Several studies have demonstrated the importance of Tat in the virus life cycle and demonstrated

that anti-Tat antibodies [373,374,376] and CTL responses [377,378] correlate inversely with disease progression, making Tat an attractive candidate for HIV vaccine development.

In conclusion, we previously reported that anionic NPs were effective for enhancing immune humoral and cellular immune responses to HIV-1 Tat [253]. The present studies further demonstrated the advantages of using NPs over Alum as an adjuvant for protein based vaccine in that NPs allowed for lower doses of Tat to be administered. Moreover, Tat-coated NPs were able to generate antibodies that strongly recognized both the N-terminal and basic regions of the protein. Tat-mediated LTR-transactivation studies also revealed that the antibodies generated with all Tat groups were able to block Tat entry into cells, with Tat coated NPs showing superior Tat neutralization activity over other forms of delivery. Together the data presented here demonstrate the potential of these novel anionic NPs as effective vaccine delivery systems for enhancing immune responses to HIV-1 Tat-based vaccines and possibly other HIV protein-based vaccines.

Table 4.1. Experimental design for mouse immunization study.

Study	Treatment	Tat Dose	Immunization Schedule	Sera Collected
Study 1	Naïve	-	-	Day 35
	NPs + Tat	1 and 5 µg	Day 0, 21, 28	
	Alum + Tat	1 and 5 µg		
	CFA + Tat	5 µg		
Study 2	Naïve	-	-	Day 13, 34, 42
	NPs + Tat	0.2 and 1 µg	Day 0, 14, 28	
	Alum + Tat	0.2 and 1 µg		
	Lipid A + Tat	0.2 and 1 µg		

Table 4.2. Physical properties of anionic NPs coated with HIV-1 Tat (1-72).

Sample (n=3)	Mean Size (nm)	Mean Charge (mV)
Anionic NPs	102.5 ± 7.6	-55.7 ± 0.7
Tat-coated NPs (1:500 w/w)	130.6 ± 3.3	-48.6 ± 3.7
Tat-coated NPs (1:100 w/w)	110.9 ± 3.3	-43.9 ± 1.9
Tat-coated NPs (1:20 w/w)	111.6 ± 5.1	-43.6 ± 1.7

Table 4.3. Tat anti-sera reactivity to 15-mer Tat peptides in Study 1. All sera were diluted 1:100. The reactivity of anti-sera from each animal is presented. The value in parenthesis represents the dose of Tat in μg. *Cutoff = (AVG Naïve response) + (3*SD). (-) indicates no response – ELISA OD values equal to cutoff/background.

| Group | Tat Peptide (aa) | | | | | | | | | | | | | | | | | |
	1-15	5-19	9-23	13-27	17-31	21-35	25-39	29-43	33-47	37-51	41-55	45-59	49-63	53-67	57-71	61-75	65-79	69-83
NPs 1 (5)	3.444	2.983	0.273	-	-	-	-	-	-	-	-	3.470	0.992	-	-	-	-	-
NPs 2 (5)	3.123	2.588	-	-	-	-	-	0.240	0.361	-	-	-	-	-	-	-	-	-
NPs 3 (5)	2.878	3.207	-	-	-	-	-	-	-	-	-	-	1.006	-	-	-	-	-
NPs 4 (5)	3.440	2.859	0.821	-	-	-	-	-	-	-	-	-	0.275	-	-	-	-	-
NPs 5 (5)	1.909	3.301	0.712	-	-	-	-	-	-	-	-	-	1.069	-	-	-	-	-
NPs 6 (5)	3.360	2.943	-	0.242	-	-	-	0.249	-	0.31	-	0.487	0.321	-	-	-	-	-
Alum 1 (5)	0.277	0.337	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 2 (5)	0.383	1.624	-	-	-	-	-	-	-	-	-	0.573	-	-	-	-	-	-
Alum 3 (5)	2.929	2.759	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 4 (5)	-	0.508	0.337	-	-	-	-	-	-	-	-	-	0.265	-	-	-	-	-
Alum 5 (5)	-	0.578	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 6 (5)	0.998	1.794	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CFA 1 (5)	3.289	3.416	3.032	0.46	0.386	0.35	0.396	0.473	0.444	0.538	0.614	0.688	0.510	0.799	0.416	0.385	0.385	0.303
CFA 2 (5)	1.834	3.061	-	0.247	-	-	0.285	0.256	0.351	-	-	-	-	-	-	-	-	-
CFA 3 (5)	1.639	3.108	0.426	0.378	0.355	0.369	0.423	0.451	0.591	0.515	0.573	0.685	0.430	0.35	0.485	0.413	0.445	0.371
CFA 4 (5)	2.593	-	-	-	-	-	-	-	-	-	-	-	0.236	-	-	-	-	-
CFA 5 (5)	2.329	0.724	-	-	-	-	-	-	-	-	-	-	0.234	1.87	-	-	-	-
NPs 1 (1)	3.266	2.979	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NPs 2 (1)	3.004	2.668	-	-	-	-	-	-	0.292	-	-	-	0.754	-	-	-	-	-
NPs 3 (1)	3.114	3.065	-	-	-	-	-	-	-	-	-	0.648	1.277	-	-	-	-	-
NPs 4 (1)	3.037	3.279	0.49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NPs 5 (1)	0.625	0.765	2.139	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 1 (1)	0.277	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 2 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 3 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 4 (1)	2.719	3.202	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 5 (1)	0.392	0.936	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 6 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cutoff*	0.26	0.26	0.24	0.24	0.22	0.22	0.25	0.23	0.28	0.28	0.29	0.45	0.22	0.25	0.25	0.27	0.27	0.25

Table 4.4. Tat anti-sera reactivity to 15-mer Tat peptides in Study 2. All sera from mice immunized with 1 µg of Tat were diluted 1:100. The reactivity of anti-sera from each animal is presented.   *Cutoff = (AVG Naïve response) + (3*SD). (-) indicates no response – ELISA OD values equal to cutoff/background.

Group	Tat Peptide (aa)																	
	1-15	5-19	9-23	13-27	17-31	21-35	25-39	29-43	33-47	37-51	41-55	45-59	49-63	53-67	57-71	61-75	65-79	69-83
NPs 1	1.395	2.121	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NPs 2	0.828	3.262	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NPs 3	3.071	3.008	-	-	-	-	-	-	-	-	-	-	0.555	0.242	-	-	0.165	.172
NPs 4	2.194	3.337	2.828	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NPs 5	2.537	2.872	3.044	-	-	-	-	-	-	-	-	0.621	-	-	-	0.181	-	-
NPs 6	2.946	2.423	-	-	-	-	-	0.173	-	0.294	0.373	-	-	-	-	-	-	-
NPs 7	2.998	2.413	-	-	-	-	-	-	-	-	-	-	1.422	-	-	-	-	-
NPs 8	3.232	3.086	0.693	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 1	0.253	3.210		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 2	0.562	1.471	0.205	-	-	-	-	-	-	0.233	-	-	-	-	-	-	-	-
Alum 3	1.207	2.377	-	-	-	0.164	-	0.273	-	0.772	0.746	-	0.778	-	-	-	-	0.214
Alum 4	3.353	3.191	2.983	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 5	-	0.452	0.803	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 6	0.357	0.354	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alum 7	0.805	2.479	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lipid A 1	3.542	2.839	0.458	0.290	0.296	0.291	0.304	0.364	0.441	0.638	0.817	0.646	0.953	0.378	0.390	0.345	0.483	0.407
Lipid A 2	2.919	3.282	0.512	0.348	0.348	0.357	0.375	0.440	0.498	0.719	0.989	1.967	1.423	0.397	0.464	0.438	0.569	0.487
Lipid A 3	3.347	2.996	0.831	0.495	0.531	0.557	0.566	0.585	0.722	1.169	1.707	1.736	2.019	0.712	0.771	0.644	0.912	0.555
Lipid A 4	3.506	3.045	0.383	0.228	0.220	0.222	0.262	0.320	0.429	0.473	0.696	0.603	1.042	0.370	0.348	0.310	0.365	0.269
Lipid A 5	2.556	2.378	1.337	0.364	0.435	0.345	0.338	0.378	0.494	0.880	0.907	0.882	2.487	0.463	0.491	0.441	0.597	0.422
Lipid A 6	2.912	2.941	2.369	0.349	0.401	0.347	0.336	0.377	0.404	0.680	0.884	0.810	0.802	0.397	0.432	0.392	0.521	0.428
Lipid A 7	2.627	2.496	2.562	0.274	0.286	0.258	0.280	0.304	0.369	0.572	0.579	0.522	0.831	0.287	0.325	0.305	0.346	0.322
Lipid A 8	2.590	2.597	0.310	0.233	0.212	0.192	0.215	0.240	0.306	0.473	0.670	0.584	0.561	0.251	0.271	0.239	0.271	0.239
Cutoff*	0.24	0.21	0.16	0.19	0.18	0.15	0.20	0.15	0.22	0.23	0.33	0.61	0.34	0.14	0.15	0.15	0.15	0.12

Figure 4.1
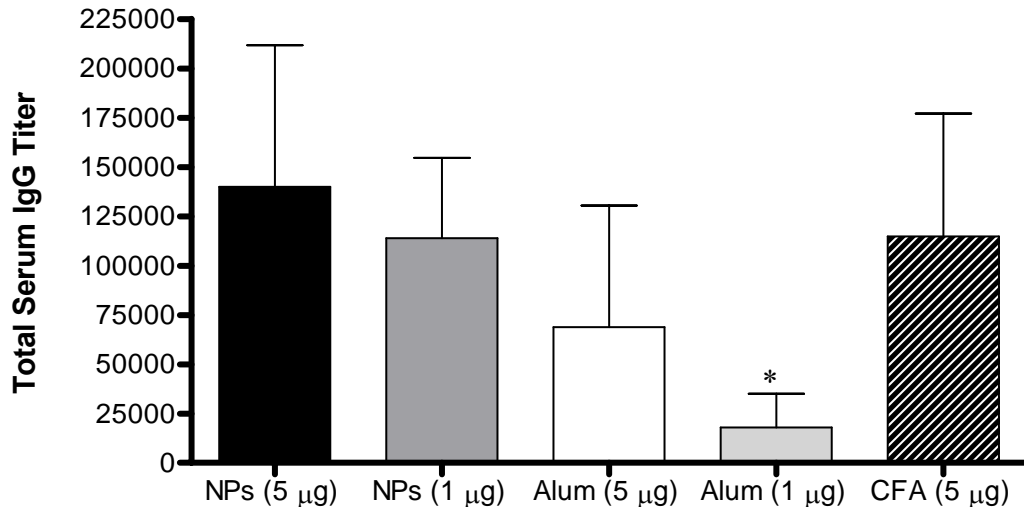
Figure 4.1. Study 1: Tat-specific total serum IgG titers. The numbers in parentheses refer to the dose of Tat. BALB/c mice were immunized on day 0, 21, and 28 with 100 μL of each formulation. Tat-specific total serum IgG Titers were evaluated on day 35 by ELISA. Data represent the mean ± SD. *$p<0.05$ compared to all groups.

Figure 4.2
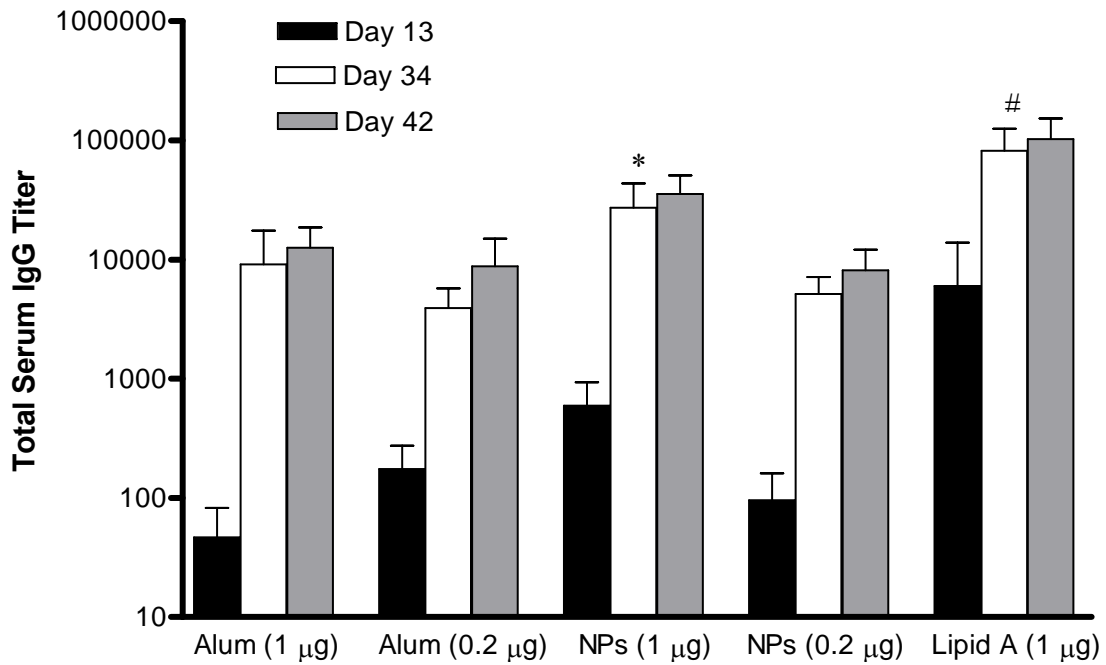
Figure 4.2. Study 2: Tat-specific total serum IgG titers. The numbers in parentheses refer to the dose of Tat. BALB/c mice were immunized on day 0, 14, and 28 with 100 µL of each formulation. Tat-specific total serum IgG Titers were evaluated on day 13, 34, and 42 by ELISA. Data represent the mean ± SD. *p<0.05 compared to NPs (0.2 µg) and both Alum groups at all time points; #p<0.05 compared to all groups at all time points.

Figure 4.3
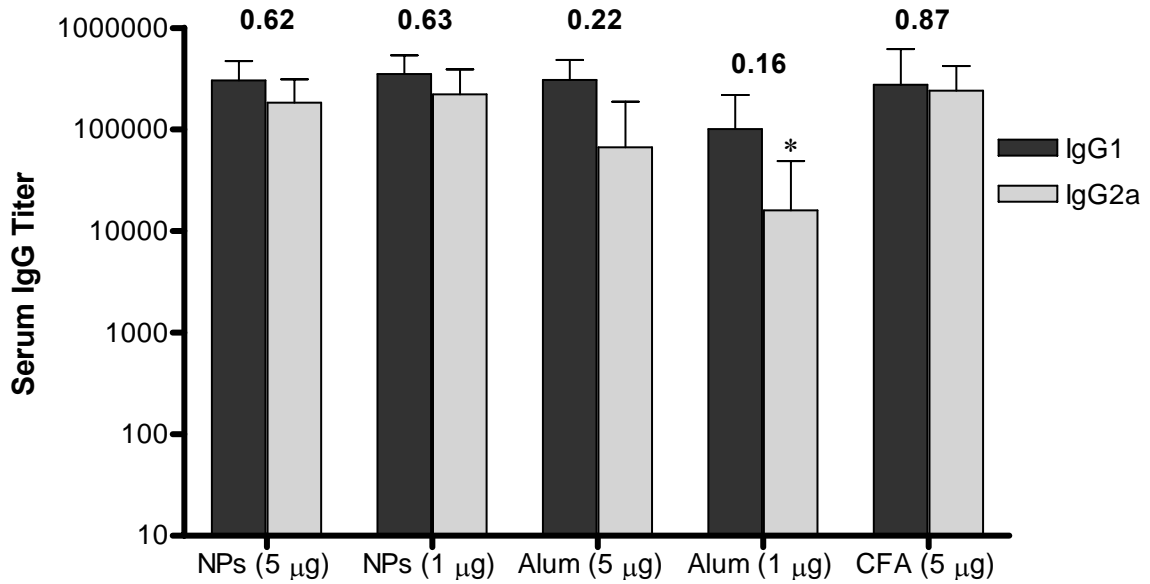
Figure 4.3. Tat-specific IgG2a and IgG1 titers. BALB/c mice were immunized on day 0, 21, and 28 with 100 μL of each formulation. The numbers in parentheses refer to the dose of Tat. Tat-specific serum IgG2a and IgG1 Titers were evaluated on day 35 by ELISA. The mean IgG2a/IgG1 ratio is indicated on top of the graphed titers for each group. Data represent the mean ± SD. *p<0.05 compared to NPs (1 μg) group.

Figure 4.4
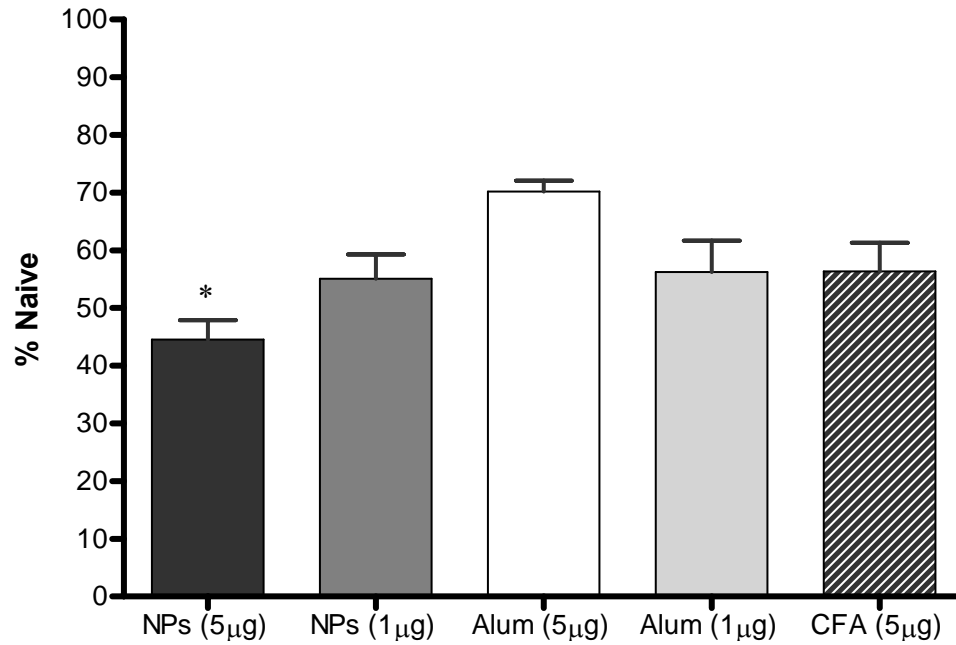
Figure 4.4. Inhibition of Tat-mediated LTR-transactivation. BALB/c mice were immunized on day 0, 21, and 28 with 100 µL of each formulation. The numbers in parentheses refer to the dose of Tat. Serum from each mouse was evaluated for Tat neutralizing antibodies using a LTR-transactivation assay. The percent inhibition in CAT expression for each group is expressed in comparison to naïve sera. Data represent the mean ± SD. *p<0.05 compared to all groups.

Chapter 5

Formulation of Toll-like receptor ligands with cationic nanoparticles and *in vivo* evaluation using Ovalbumin as a model antigen

5.1 Summary

The use of cationic nanoparticles formulated with immunostimulatory adjuvants was investigated for obtaining enhancements in immune responses to ovalbumin (OVA) compared to either nanoparticles or adjuvant alone. More specifically, three Toll-like receptor ligands were investigated: lipoteichoic acid (LTA), a synthetic CpG oligonucleotide (CpG), and a synthetic double-stranded RNA analog (Poly I:C). Initial studies revealed the strong potency of CpG formulated with nanoparticles for stimulating cell responses in the lymph nodes 8 days after initial immunization. Based on these data, further work was carried out to evaluate the humoral immune responses in BALB/c mice using nanoparticles coated with OVA and CpG. The data demonstrated that more robust OVA-specific immune responses could be obtained with CpG coated on nanoparticles compared to either CpG or nanoparticles alone.

5.2 Introduction

Unlike traditional vaccines such as live attenuated or whole killed pathogens, new generation vaccines, composed of peptides, proteins or DNA, are considered to be potentially much safer; however, they produce poor immune responses when administered alone. Thus, the use of immunological adjuvants is crucial with new generation vaccines to elicit stronger, more robust immune responses. Of particular importance is the choice of adjuvant used with the antigen, as the adjuvant can greatly influence the type of immune response generated, biasing towards either a Th1/cellular or Th2/humoral type response. For example, aluminum-based mineral salts such as Alum produce strong humoral responses but Alum is considered a weak adjuvant for mediating cellular responses [134]. In contrast, bacterial derived products such as Lipid A typically bias the immune response towards a cellular or Th1 type response [64]. Therefore, the key to eliciting optimal immune responses with new generation vaccines may lie in the selection of an appropriate adjuvant or combination of adjuvants.

While many new adjuvants have been explored and evaluated in clinical trials, the majority of adjuvants have been proven to be too toxic to be used in routine human vaccination. Alum continues to be the only approved adjuvant for use in human vaccines in the United States [2]. It is well recognized that in order to conquer emerging chronic infections, such as HIV, safer and more effective adjuvants that produce cellular in addition to humoral immune responses will be necessary [77].

Adjuvants can be broadly classified as immunostimulatory or particulate, including particulate delivery systems. Immunostimulatory adjuvants function mainly at the cytokine level, enhancing the immune responses via activation and up regulation of

co-stimulatory molecules on antigen presenting cells (APCs). Immunostimulatory adjuvants derived from bacterial components, such as lipopolysaccharides and lipoproteins, are recognized by Toll-like receptors (TLRs) expressed on antigen presenting cells and mediate enhancement in immune responses through activating the innate immune system [418]. Presently, ten TLRs have been identified, denoted TLR1 to TLR10, and ligands that bind to most of these receptors have been identified [57]. For example, the well-investigated immunostimulant lipopolysaccharide is reported to bind to TLR4, whereas lipoproteins bind to TLR2. Moreover, the cooperation of different TLRs in recognition of microbes has been reported. One example reported includes the cooperation of TLR2 with TLR1 [419] and TLR6 [420] in recognition and innate immune responses to pathogens. An additional feature of TLRs is their differential expression on different dendritic cell subtypes [269]. Furthermore, TLRs are expressed either on the cell surface (TLR1, 2, 4-5, 11) or intracellularly (TLR3, 7-9) [83].

In contrast to immunostimulatory adjuvants, particulate delivery systems are thought to mediate their effects through enhanced delivery and uptake by APCs [1]. Particulate systems are naturally targeted for uptake by APCs due to the similarity in sizes compared to pathogens [125]. The use of immunostimulatory adjuvants in combination with particulate delivery systems has been of great interest in obtaining more robust enhancements in immune responses to antigens [4,92,257,421,422]. Particulate delivery systems offer numerous advantages for delivery of immunostimulatory adjuvants including: 1) reducing the toxicity or side effects of the immunostimulant by providing controlled delivery or release [255], 2) allowing for lower

doses of immunostimulant to be used by enhancing uptake into APCs [263], and 3) biasing immune responses toward cellular/Th1 type with particulate systems [99,394].

To this end, our laboratory has reported on the preparation of nanoparticles (NPs) from oil-in-water microemulsion precursors and reported on initial studies demonstrating the application of these NPs for enhancing immune responses to plasmid DNA [14,15], cationized β-galactosidase protein [16], and the HIV-1 Tat protein [253]. These NPs are approximately 100 nm in size and have the advantage of being prepared in a single step, one vessel process with relative ease in modifying the physical characteristics of the particles by using appropriate surfactants. More importantly, the oil phase used for preparation of these NPs is comprised of cetyl alcohol and polysorbate 60, both which are found in many pharmaceutical products and are biocompatible [251]. The present studies investigated the potential application of these NPs formulated with three different TLR ligands for enhancing immune responses to a model antigen, ovalbumin (OVA). To formulate with cationic NPs, three negatively charged TLR ligands were used: Poly I:C, a synthetic analog of double-stranded RNA (dsRNA) that binds to TLR3; lipoteichoic acid (LTA), a cell wall component from gram positive bacteria that binds to TLR2; and CpG oligonucleotide (CpG), a synthetic 20-mer oligonucleotide containing unmethylated CpG motifs typically present in bacterial DNA that bind to TLR9.

5.3   Materials and methods

Materials

Emulsifying wax, comprised of cetyl alcohol and polysorbate 60 (molar ratio of 20:1), was purchased from Spectrum (New Brunswick, NJ). Cetyl trimethyl ammonium bromide (CTAB), PBS/Tween 20 buffer, bovine serum albumin (BSA), ovalbumin grade VI (OVA), and mannitol were purchased from Sigma Chemical Co. (St. Louis, MO). Brij 78 was purchased from Uniqema (New Castle, DE). Sheep anti-mouse IgG, peroxidase-linked species specific F(ab')$_2$ fragment was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Goat anti-mouse IgG2a and IgG1 horseradish peroxidase (HRP) conjugates were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Tetramethylbenzidine (TMB) substrate kit was purchased from Pierce (Rockford, IL). RPMI 1640, 10% heat-inactivated fetal calf serum, Hanks Balanced Salt Solution (HBSS), HEPES, L-glutamine, penicillin, and streptomycin were from GIBCO (Carlsbad, CA). The TLR ligands: murine CpG oligodeoxynucleotide (ODN 1826: 5'-tcc atg a<u>cg</u> ttc ctg a<u>cg</u> tt-3'), synthetic dsRNA (Poly I:C), and Lipoteichoic acid (LTA) were purchased from Invivogen (San Diego, CA). Incomplete Freund's adjuvant, mycobacterium tuberculosis and 2-mercaptoethanol were purchased from Fisher Scientific (Hampton, NH). For *in vivo* studies, Female BALB/c mice (8-10 weeks old) obtained from Harlan Sprague-Dawley Laboratories (Indianapolis, Indiana) were used. Unless stated otherwise, all water used in experiments was filtered (0.2 μm) deionized water.

Preparation of cationic NPs

Nanoparticles from oil-in-water microemulsion precursors were prepared as described previously with slight modification [14,15]. Briefly, 2 mg of emulsifying wax and 3.5 mg of Brij 78 was melted and mixed at ~60-65°C. Water (980 μL) was added to the melted wax and surfactant while stirring to form an opaque emulsion. Finally, 20 μL of CTAB (50 mM) was added to form clear microemulsions at 60-65°C. The microemulsions were cooled to room temperature while stirring to obtain solid NPs (2 mg/mL). The final concentration of components in the NP suspension was emulsifying wax (2 mg/mL), Brij 78 (3 mM), and CTAB (1 mM). The NP sizes were measured using a Coulter N4 Plus Sub-Micron Particle Sizer (Coulter Corporation, Miami, FL) at 90°. The overall charge of the NPs was measured using Malvern Zeta Sizer 2000 (Malvern Instruments, Southborough, MA).

Coating of the cationic NPs with OVA and TLR ligands

Varying amounts of OVA were added to NPs (1000 μg/mL) in 5% (v/v) mannitol. The suspension was vortexed gently and placed on a horizontal shaker at room temperature for a minimum of 30 min to allow for coating. A similar procedure was followed to coat the TLR ligands on cationic NPs. For formulations where TLR ligands and OVA were coated on NPs, the required amount of OVA was first coated on the NPs followed by coating with the ligand as described above. The coated NPs were diluted appropriately in water for measuring the size and charge of the particles.

Mouse immunization study evaluating cationic NPs coated with TLR ligands and OVA

Mice (n=5/group) were immunized (s.c.) once, on day 0, with 100 μL of NPs, OVA-coated NPs, OVA- and LTA-coated NPs, OVA- and Poly I:C-coated NPs, OVA- and CpG-coated NPs, and OVA adjuvanted with complete Freund's adjuvant (CFA). The doses were as follows: 5 μg of OVA; 50 μg of TLR ligand; and 100 μg of cationic NPs. The immune responses in draining lymph nodes (brachial, axillary and inguinal) were assessed on day 8.

Lymphocyte proliferation assay for day 8 harvested lymph nodes

The draining lymph nodes collected for each mouse were prepared individually and stimulated in triplicate with media, Con A (2 μg/mL), or OVA (50 μg/mL). Briefly, single cell suspensions were prepared by teasing the lymph nodes apart in 1X Hanks Balanced Salt Solution (HBSS). Single cell suspensions were transferred into 15 mL of 1X HBSS in a centrifuge tube and spun down at 1,500 rpm for 10 min at $4^{o}C$. The cells were resuspended in RPMI 1640 (supplemented with 10% heat-inactivated fetal calf serum, 1 mM HEPES, 2 μM L-glutamine, 10 U/mL penicillin, 100 U/mL streptomycin, 50 μM 2–mercaptoethanol). The cells (5×10^5 cells/well) were incubated with media, Con A, or OVA at $37^{o}C$, 7% CO_2 for 4 days and then pulsed with 1 μCi of ^{3}H-thymidine and incubated for an additional 24 hr at $37^{o}C$, 7% CO_2. The cells were harvested and counted on day 5 to measure T cell proliferation.

Mouse immunization study with cationic NPs coated with CpG and OVA

Mice (n=4-5/group) were immunized (s.c.) on day 0 and day 14 with either CpG-coated NPs, OVA adjuvanted with CpG, OVA-coated NPs or CpG- and OVA-coated NPs. CpG was coated on NPs at a 1:10 w/w and the dose of CpG or OVA given to animals was 10 μg and 5 μg, respectively. As a positive control, mice were immunized with 50 μg of OVA adjuvanted with CFA on day 0. On day 14, mice were bled via tail vein prior to boosting and blood was collected into Microtainer tubes (BD) and sera were separated according to manufacturer's instructions. On day 28, mice were bled by cardiac puncture and sera were separated. All collected sera were stored at -20°C. Spleens were harvested and used for splenocyte proliferation assay.

Determination of antibody titers

OVA-specific serum IgG, IgG1 and IgG2a antibody titer were determined using an ELISA. Briefly, 96-well plates (Costar) were coated with 100 μL of OVA (10 μg/mL in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6) overnight at 4°C. The plates were blocked for 1 hr at 37°C with 200 μL of 4% BSA prepared in PBS/Tween 20. The plates were then incubated with 50 μL per well of mouse serum diluted appropriately in 4% BSA/PBS/Tween 20 for 2 hr at 37°C. The plates were washed with PBS/Tween 20 and incubated with 50 μL/well anti-mouse IgG HRP F(ab')$_2$ fragment from sheep (1:3000 in 1% BSA/PBS/Tween 20) for 1 hr at 37°C. For IgG1 and IgG2a determination, the plates were similarly incubated with goat anti-mouse IgG1-HRP or goat anti-mouse IgG2a-HRP diluted 1:8000 (1% BSA/PBS/Tween 20). After washing the plates with PBS/Tween 20, the plates were developed by adding 100 μL of TMB substrate and

incubating for 30 min at RT. The color development was stopped by the addition of 100 μL of 2 M H_2SO_4 and the OD at 450 nm was read using a Universal Microplate Reader (Bio-Tek Instruments, Inc.). The OD at 450 nm versus log serum dilution for each animal was plotted and fit to a four parameter logistic equation using GraphPad Prism software. The titer was defined as the anti-sera dilution giving $0.5*OD_{max}$.

Splenocyte proliferation assay

The spleens were crushed in 1X HBSS using a stomacher homogenizer for 60s at normal speed to obtain single cell suspensions and the suspensions were then transferred into centrifuge tubes. Red blood cells were lysed adding 1X ACK buffer (156 mM NH_4Cl, 10 mM $KHCO_3$ and 100 μM EDTA) and incubating for 1-2 min at RT. The cells were spun down at 1500 rpm, 4°C for 10 min. Supernatants were decanted and the cells were washed 2 more times with 1X HBSS. The cells were resuspended in RPMI 1640 (supplemented with 10% heat-inactivated fetal calf serum, 1 mM HEPES, 2 μM L-glutamine, 10 U/mL penicillin, 100 U/mL streptomycin, 50 μM 2–mercaptoethanol). For splenocyte proliferation assay, cells ($5x10^5$ cells/well) were added to a 96-well plate and incubated in triplicate with media, Con A (2 μg/mL), or OVA (50 μg/mL) at 37°C, 7% CO_2 for 4 days. The cells were pulsed with 1 μCi of ^{3}H-thymidine on day 4 and incubated for an additional 24 hr at 37°C, 7% CO_2.

Statistical analysis

Statistical analysis was performed using one-way analysis of variances (ANOVA) followed by pair-wise comparisons using Tukey's multiple comparison test using GraphPad Prism software.

5.4   Results and discussion

Preparation and characterization of cationic NPs coated with OVA and TLR ligands

The preparation of NPs from warm oil-in-water microemulsion precursors has previously been reported by our laboratory [15,16]. These NPs are approximately 100 nm in size and have the advantage of being prepared in a single step, one vessel process with relative ease in modifying the physical characteristics of the particles by using appropriate surfactants. More importantly, the oil phase used for preparation of these NPs is comprised of cetyl alcohol and polysorbate 60, both which are found in many pharmaceutical products and are biocompatible [251].

The NPs used in these present studies were stabilized by the inclusion of Brij 78 and made to be positively-charged by the use of the cationic surfactant CTAB. The net charge of the NPs prior to coating with OVA or TLR ligands was approximately +50 mV (Figure 5.1). OVA, a negatively charged protein, was expected to be coated on the surface of the NPs via ionic interactions. As demonstrated in Figure 5.1, there was a net decrease in the overall charge with increasing concentrations of OVA adsorbed to the surface of the NPs. At a protein concentration of 50 μg/mL, approximately 95% of OVA was estimated to be coated on the NPs using SDS-PAGE densitometry (data not shown). At this concentration of OVA, it was expected that there was an excess of NPs as the net charge of the OVA-coated NPs was still positive and thus, allowed for the adsorption of TLR ligands via the negative charges of the molecules. In the present studies, the adsorption of TLR ligands on NPs led to a further decrease in the charge, resulting in an

overall negative charge for the particles (Table 5.1). The coating of CpG did not significantly affect the nanoparticle particle size; however, the coating with LTA and Poly I:C resulted in an increase in the mean particle sizes to around 300 and 200 nm, respectively.

Lymphoproliferative responses to OVA using TLR ligand-coated NPs

Initial *in vivo* studies were carried out to evaluate the ability of cationic NPs coated with three different negatively charged TLR ligands in providing more robust immune responses to OVA compared to NPs alone. The immune responses were evaluated on day 8 by measuring T cell proliferation in the draining lymph nodes. Lymph node cell counts on day 8 demonstrated the highest activity with CpG-coated NPs (Figure 5.2), suggesting that this TLR ligand had extremely potent immunostimulatory activity compared to the Poly I:C and LTA. Interestingly, this response was even stronger than the positive control CFA. As expected, the OVA-specific proliferative responses demonstrated the strongest responses with the CFA positive control (Figure 5.3). All NP groups with OVA demonstrated positive OVA-specific responses, with the OVA-coated NPs showing the strongest response. However, the responses with the NP groups were modest compared to the CFA. It is possible that day 8 may not be optimal for evaluating the T cell responses. Nonetheless, these data demonstrate the potential application of NPs with or without TLR ligands for enhancing immune responses to OVA. The results suggested that CpG was a potent immunostimulatory adjuvant when coated on cationic NPs compared to Poly I:C and LTA, and was chosen for further work with NPs. Moreover, the use of CpG has been of great interest to numerous researchers.

The potent activity of CpG does pose potential side effects such as enlarged spleen and lymph nodes, which have been reported in literature to be dose-dependent [108,109]. Thus, the use of cationic NPs coated with CpG may have the advantage in minimizing these side effects by enhancing the adjuvant activity of CpG and allowing for lower doses of CpG to be used. In fact, recent reports demonstrated that PLGA nanoparticles were able to effectively reduce the doses of CpG required for obtaining more robust immune responses to tetanus toxoid compared to CpG in saline [263].

Immune response to OVA

In vivo studies in BALB/c mice were carried out to investigate the use of cationic NPs as potential delivery systems to improve the adjuvant effect of CpG and thus, obtain significant enhancements in the immune responses to OVA. Mice were immunized with either OVA-coated NPs (NPs+OVA), OVA mixed with CpG (CpG+OVA), or OVA- and CpG-coated NPs (NPs+CpG+OVA). As a positive control, mice were immunized with OVA adjuvanted with CFA. Sera collected from mice were analyzed for total OVA-specific IgG at 2 weeks post initial immunization and 2 weeks post second immunization. As expected, animals immunized with OVA (50 μg) adjuvanted with CFA (a 10-fold higher dose than all other groups) resulted in the highest total IgG titers at both time points (Figure 5.4). More importantly, 3-4-fold higher IgG titers were observed at both 2 and 4 weeks using NPs coated with both CpG and OVA compared to CpG with OVA or NPs coated with OVA alone. There were no significant differences at either time point in the IgG titers of the CpG with OVA group compared to OVA-coated NP group. Also, the CpG-coated NP control group did not result in significant OVA-specific IgG

compared to naïve animals (data not shown). The data suggest that NPs coated with CpG and OVA can result in robust enhancements in the humoral immune response. These results are consistent with reports from Singh *et al.* who demonstrated that the immune responses to HIV antigens using microparticles coated with CpG are more effective at enhancing immune responses than either adjuvant alone [398]. Surprisingly, this study demonstrated greatest OVA-specific proliferative responses with the NP group (Figure 5.5). This is in line with the initial study; however, it is unclear why the positive control group CFA did not produce a positive response in the assay.

As mentioned earlier, there have been several reports on the use of CpG in shifting the immune response to antigens from a Th2 to a Th1 type response [105,107,159]. The presence of Th1/cellular response can be inferred by measuring the production of different IgG isotypes, as this is directly under the influence of cytokines secreted by T helper cells. Th1 responses are characterized by production of IFN-γ, which promotes production of IgG2a isotype, whereas Th2 responses produce IL-4 which promotes the presence of the IgG1 isotype [423]. Although no significant differences existed among the groups for the OVA-specific IgG2a titers in this study, there is a shift in the type of immune response generated using NPs coated with CpG compared to NPs alone (Figure 5.6). It is likely that the route of immunization and dose of CpG and/or the antigen may influence these types of responses. Interestingly, Weeratna *et al.* have reported that the intramuscular route (i.m.) is superior to the subcutaneous route (s.c.) in obtaining optimal adjuvant activity with CpG [424]. Nonetheless, Samuel *et al.* have reported significant reduction in the CpG dose can be obtained using nanoparticles after subcutaneous injection [111,263]. However, the CpG was entrapped inside the

nanoparticles with the antigen, which may allow for slow release of the antigen at the injection site. Moreover, the antigen in this case is tetanus toxoid and the dose and effectiveness of adjuvant, i.e. CpG, may be dependent on the antigen. Samuel *et al*. also used C57BL/6 mice in their studies, which have been reported to express higher levels of TLR9 compared to BALB/c mice, hence affecting the IL-12 production and Th1 responses in the two strains of mice in response to CpG adjuvant [425]. In fact, this difference in the two strains of mice was suggested to be the underlying cause of the higher susceptibility of BALB/c mice to *Listeria monocytogenes* infection compared to C57BL/6 mice [425]. Therefore, the data with the current strategy in this study does suggest that NPs can be used for enhancing the adjuvant effect with CpG; however, further studies evaluating the route of immunization, CpG and antigen doses, as well as different antigens may be necessary to truly reveal the range of enhancements in immune responses that can be achieved with these NPs.

As the field moves toward an era where well-defined antigenic components are becoming more attractive candidates for vaccines, there is an urgent need for more effective and safer adjuvants to aid in generating cellular and humoral immune responses to these antigens. Toxicity is one of the greatest barriers in the development of new adjuvants for use in routine human vaccination. These studies highlight the potential of NPs for enhancing immune responses to protein-based vaccines. In addition, the use of an optimal murine CpG coated on NPs was shown to generate more robust immune responses than either adjuvant alone, further illustrating the potential of these NPs as adjuvants. Although some concern has been raised that CpG use may generate autoimmune diseases due to over stimulation of the innate immune system, repeated

doses administered *in vivo* in mice and non-human primates to date have shown no toxicity. More importantly, the use of CpG have been evaluated in hundreds of human subjects in Phase I clinical trials and no adverse reactions due to CpG have been reported [110]. In fact, Phase I clinical trials have demonstrated that hepatitis B surface antigen (HBsAg) adjuvanted with CpG resulted in stronger, more robust immune responses compared to the HBsAg alone [94,112]. The strength of the immune response generated by each CpG oligonucleotide can vary and the optimal sequences differ from species to species. However, optimal CpG oligonucleotides have been identified for a number of different species including mice, rabbit, sheep, goat, cattle, swine, horse, rhesus monkey, chimpanzee, and humans [111].

Acknowledgements

Table 5.1. Physical properties of cationic NPs coated with TLR ligands and OVA.

Sample (n=3)	Mean Size (nm)	Mean Charge (mV)
NPs	111.9 ± 5.4	48.1 ± 1.6
NPs + OVA	116.7 ± 5.9	41.8 ± 1.9
NPs + OVA + LTA	299.1 ± 3.4	-35 ± 6
NPs + OVA + Poly I:C	220 ± 1.8	-30 ± 10
NPs + OVA + CpG	107.1 ± 1.1	-26.0 ± 3

All OVA formulations were prepared at a concentration of 50 µg/mL OVA and 1000 µg/mL cationic NPs. All TLR ligands were present at a concentration of 500 µg/mL.

Figure 5.1
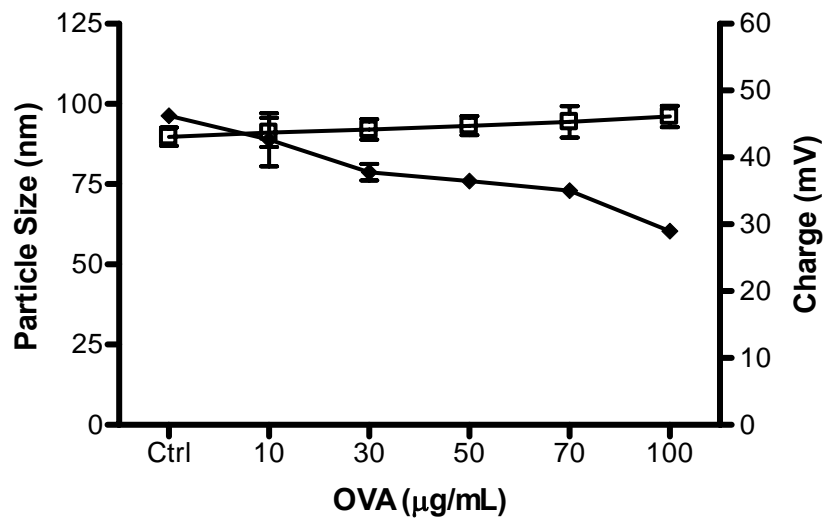
Figure 5.1. **Physical characterization of cationic NPs coated with increasing concentrations of OVA**. The particle size (□) and charge (♦) of cationic NPs (Ctrl) and OVA-coated on cationic NPs are shown. Data reported are the mean ± S.D. (n=3).

Figure 5.2
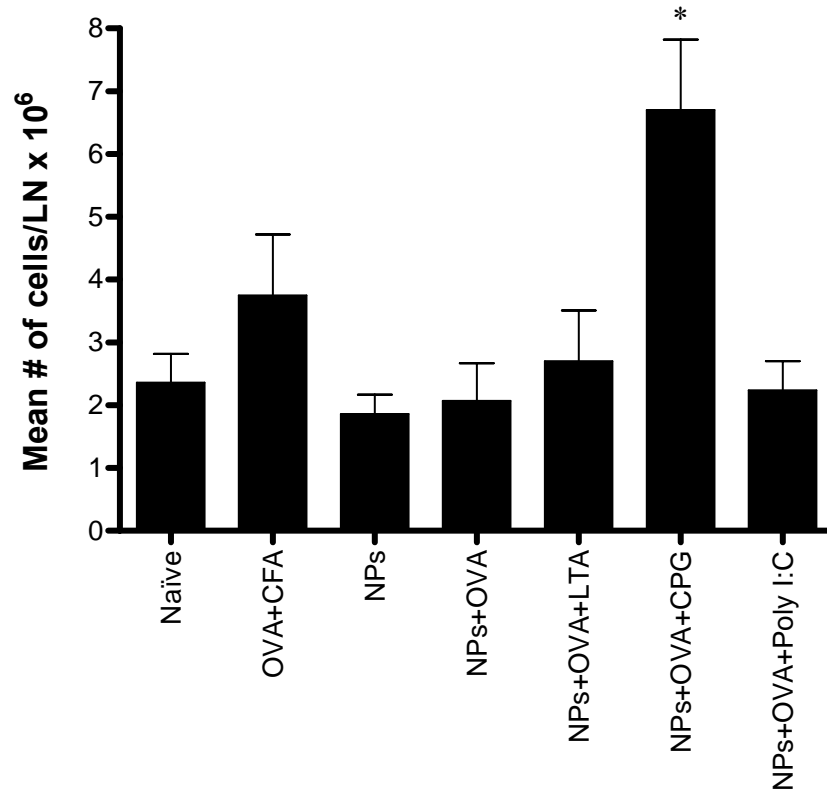
Figure 5.2. Mean number of cells recovered from the draining lymph nodes. Brachial, axillary and inguinal lymph nodes were collected from mice 8 days after immunization with the appropriate formulations. The data represent the mean number of cells $\pm$ S.D. (for n=5/group). *p<0.05 compared to all groups.

Figure 5.3
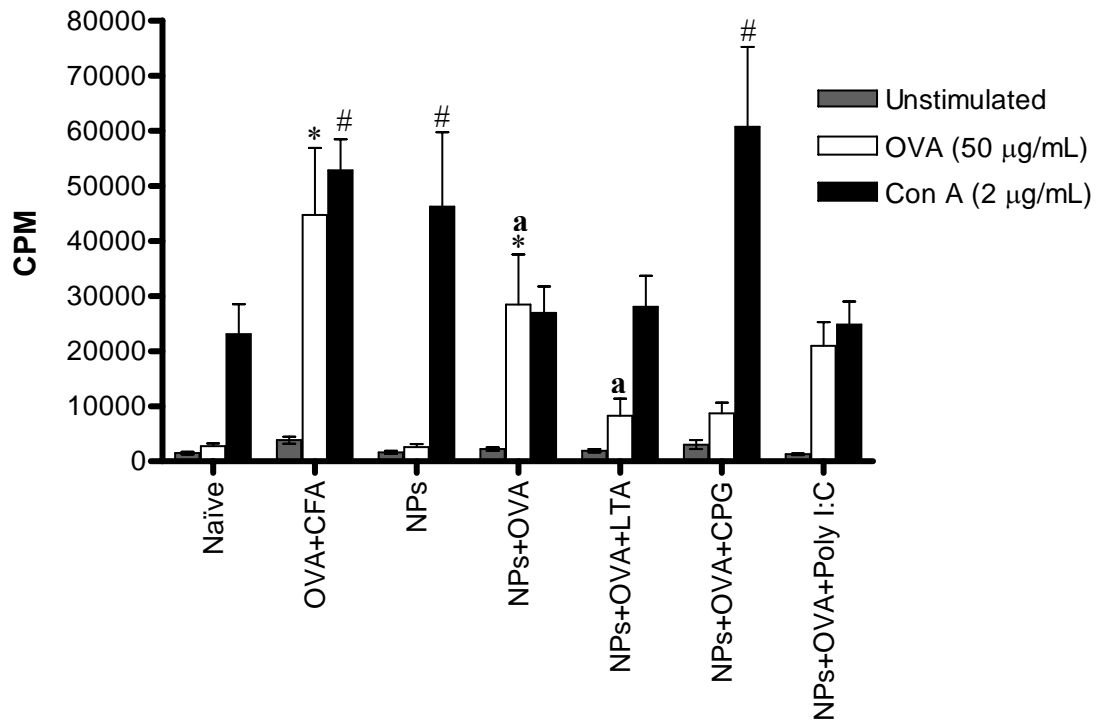
Figure 5.3.   T cell proliferation in draining lymph nodes on day 8.   Single cell suspensions of the draining lymph nodes were stimulated for 4 days with Con A (2 μg/mL) or OVA (50 μg/mL). The cells were evaluated for ^{3}H-thymidine incorporation on day 5. The data represent the mean ± S.D. (for n=5/group). *p<0.05 compared to naïve group for OVA-stimulated cells; $^#$p<0.05 compared to naïve group for Con A stimulated group; ^{a}p<0.05 for NPs+OVA group compared to NPs+OVA+LTA.

Figure 5.4
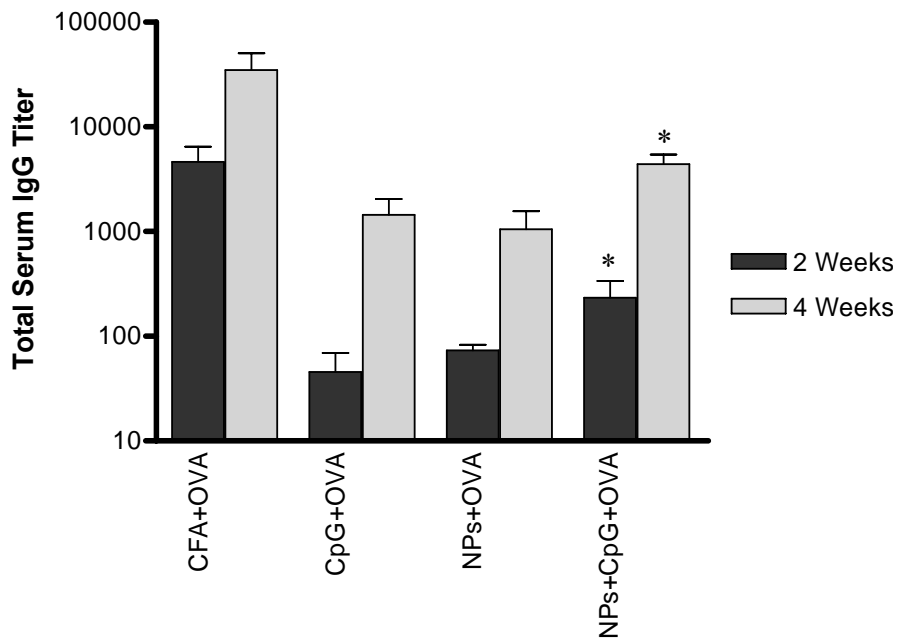
Figure 5.4. **OVA-specific serum IgG titers at 2 weeks and 4 weeks post initial immunization.** Mice were immunized on day 0 and 14 with OVA (5 μg) adjuvanted with CpG, OVA (5 μg) coated on NPs (100 μg) or OVA (5 μg) and CpG (10 μg) coated on NPs (100 μg). As a positive control, OVA (50 μg) adjuvanted with CFA was injected on day 0 only. Titers reported are the mean ± S.D. (n=4-5). * indicates that the titers are significantly higher compared to CpG group and NPs group.

Figure 5.5
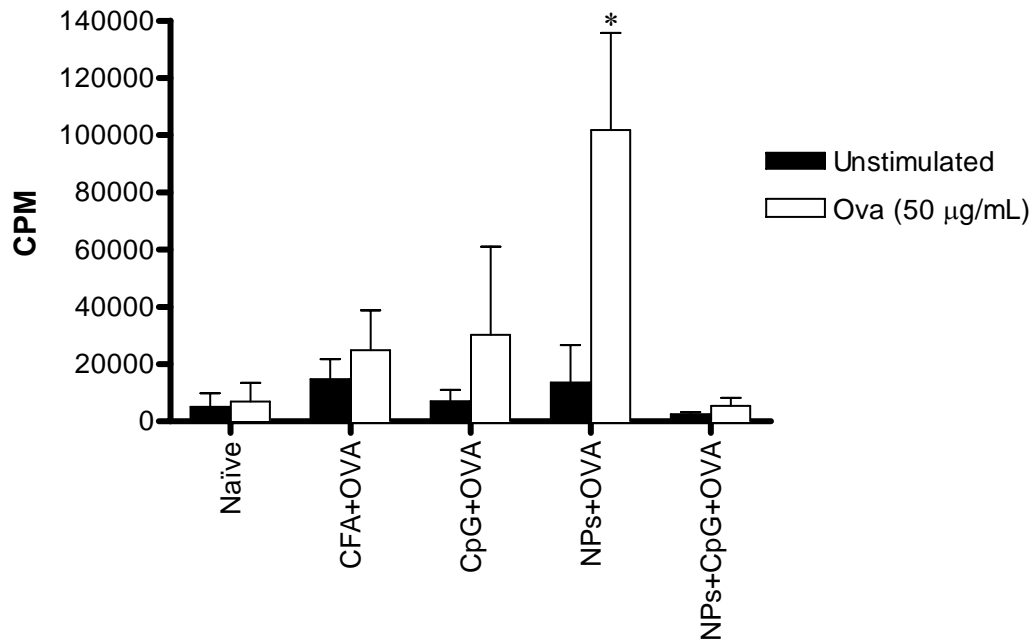
Figure 5.5. OVA-specific proliferative responses in spleen on day 5. Spleens were harvested four weeks post initial immunization and single cell suspensions of the spleen were stimulated with OVA for 4 days. The incorporation of ^{3}H-thymidine was evaluated on day 5. The data represent the mean $\pm$ S.D. (for n=4-5/group). *p<0.05 compared to all groups.

Figure 5.6
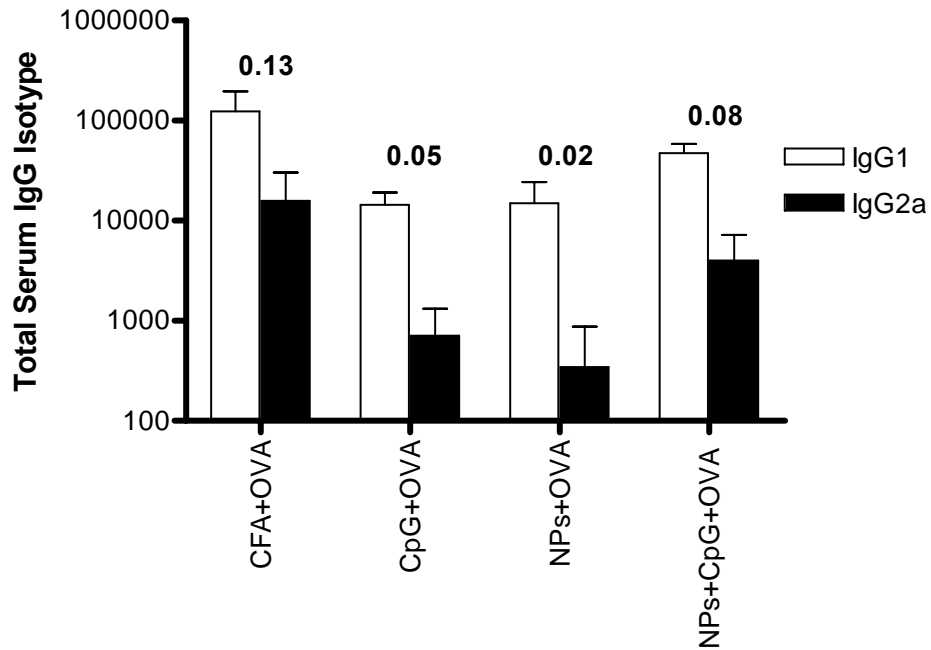
Figure 5.6. OVA-specific serum IgG1 and IgG2a titers. The IgG1 and IgG2a titers in sera were analyzed at four weeks post initial immunization. The mean IgG2a to IgG1 ratio for each group is indicated on top of the graphed titers. Data for each group represents the mean ± S.D. (n=4-5).

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Chapter 6

Mechanistic investigation of immune response enhancement using nanoparticles

6.1 Summary

Nanoparticles prepared from oil-in-water microemulsion precursors have been shown to be effective for enhancing immune responses to pDNA and protein-based vaccines in mice. The *in vitro* and *in vivo* studies in this chapter were carried out to assess the mechanism(s) by which nanoparticles may be enhancing immune responses. *In vitro* studies demonstrated that nanoparticles were taken up efficiently by murine bone-marrow derived dendritic cells (BMDDCs) and the presence of nanoparticles intracellulary was confirmed by confocal microscopy. Furthermore, the release of pro-inflammatory cytokines from human dendritic cells and BMDDCs was not observed after incubation with nanoparticles for 24 hr; however, nanoparticles coated with the immunostimulatory adjuvant, CpG, resulted in the release of IL-12, which was higher than the cytokine levels with CpG alone. Adoptive transfer experiments using cells from OT-1 mice, MHC class I restricted Ovalbumin (OVA) transgenic mouse model, demonstrated higher proliferation of the OT-1 cells using OVA-coated nanoparticles compared to OVA, especially at lower doses of OVA. Taken together, these data suggest that nanoparticles may be enhancing immune responses by promoting uptake of antigen by antigen presenting cells and by facilitating the delivery of antigen into MHC class I restricted antigen presentation pathway.

6.2   Introduction

The need for safer, more effective adjuvants is clearly recognized for development of vaccines comprised of proteins, peptides and pDNA [4,5,218,426]. One of the major limitations in development of vaccines is their toxicity or adverse side effects [134]. Many of the adjuvants evaluated have been empirically chosen for use and the mechanisms by which these adjuvants enhance immune responses are not fully understood and continue to be evaluated. For example, although Alum has been used in routine human vaccination for over 70 years, the exact mechanism of immune response enhancement has not been fully elucidated [67]. There are several proposed mechanisms for the immune response enhancement using Alum and it is possible that each contributes, at least in part, to stimulating stronger immune responses to the antigen *in vivo*. The following have been proposed for immune response enhancement with Alum: enhancement of uptake of associated antigen into antigen presenting cells (APCs), formation of depot at the site of injection and in macrophages present in muscle, local inflammatory response due to necrosis at injection site possibly resulting in activation of APCs and stimulating release of cytokines [66-69].

Studies over the last several years with various adjuvants have allowed for broad classification of adjuvants depending on their mode of action [1,64]. The first class of adjuvants, immunostimulatory adjuvants, functions mainly at the cytokine level enhancing immune responses via activation and up regulation of co-stimulatory molecules on APCs. Many of the adjuvants belonging to this class are derived from bacterial components. On the contrary, the second class of adjuvants is mainly of synthetic/chemical nature consisting of particulate systems. This class includes

particulate delivery systems, such as microparticles, nanoparticles, and liposomes, and they are thought to exert their activities by acting as delivery vehicles for the antigens and promoting the uptake of the antigen by: 1) directly enhancing uptake into APC via passive and active targeting; and/or 2) forming a depot of the antigen at the site of injection, providing a slow release and uptake by APCs. Studies with many particulate delivery systems such as nanoparticles and microparticles have demonstrated that they are taken up by APCs [239-242,427] and these systems are believed to enhance immune responses *in vivo* by promoting the uptake of the associated antigen [4,13]. Moreover, in one study a modest upregulation in co-stimulatory and MHC class II molecules was also reported [239], suggesting a possible role of these delivery systems in the maturation of APCs or enhancement in antigen presentation.

To this end, nanoparticles (NPs) prepared from oil-in-water microemulsion precursors have been reported to enhance immune responses to pDNA [14,15], cationized β-galactosidase [16], and HIV-1 Tat [253]. In addition, further enhancements in immune responses with these NPs were observed by incorporating mannan to target mannose receptors present on macrophages and dendritic cells (DCs) [14,15]. It was also demonstrated that the uptake of nanoparticles in macrophages could be enhanced with the mannan ligand [428]. One of the most potent APCs are DCs and the targeting and presentation of antigens by DCs is of great interest. The present studies were aimed at further understanding the mechanism(s) by which NPs enhance immune responses. *In vitro* studies were carried out to evaluate the uptake and release of various pro-inflammatory cytokines from DCs. In addition, an *in vivo* study using a MHC class I-

restricted OVA transgenic mouse model was carried out to evaluate the utility of these

NPs in enhancing presentation of exogenous protein via this pathway.

6.3   Materials and methods

Materials

Emulsifying wax, comprised of cetyl alcohol and polysorbate 60 (molar ratio of 20:1), Alum and sodium dodecyl sulfate (SDS) were from Spectrum (New Brunswick, NJ). Cetyl trimethyl ammonium bromide (CTAB), PBS/Tween 20 buffer, bovine serum albumin (BSA), ovalbumin grade VI (OVA) and Sepharose CL4B were purchased from Sigma Chemical Co. (St. Louis, MO). Brij 78 was purchased from Uniqema (New Castle, DE). TNF-α, IL-1β, and IL-12 ELISA kits were purchased from Pierce (Rockford, IL). Murine CpG oligodeoxynucleotide (ODN 1826) was purchased from Invivogen (San Diego, CA). ScintiVerse liquid scintillation cocktail and 2-mercaptoethanol was purchased from Fisher Scientific (Hampton, NH). Prolong® Antifade kit, Carboxy-fluorescein diacetate, succinimidyl ester (CFSE), and 3,3'-dioctadecyloxacarbocyanine perchlorate (DiOC$_{18}$) were purchased from Molecular Probes (Eugene, OR). Biotinylated anti-mouse CD11c primary antibody, and the monoclonal antibodies, anti-Valpha2 and anti-Vbeta5 were purchased from BD Bioscience (San Jose, CA). RPMI 1640, 10% heat-inactivated fetal calf serum, Hanks Balanced Salt Solution (HBSS), HEPES, L-glutamine, penicillin, and streptomycin were from GIBCO (Carlsbad, CA). ^{3}H-cetyl alcohol was purchased from Moravek Biochemicals (Brea, CA). Lysis buffer (5X) was purchased from Promega (Madison, WI). Lipid A from *Salmonella Minnesota* R595 (Re) was purchased from List Biological Laboratories (Campbell, CA). APC-conjugated Streptavidin was purchased from eBioscience (San Diego, CA). Unless stated otherwise, all water used in experiments was filtered (0.2 μm) deionized water.

Preparation of cationic NPs

Nanoparticles from oil-in-water microemulsion precursors were prepared as described previously with slight modification [14,15]. Briefly, 2 mg of emulsifying wax and 3.5 mg of Brij 78 was melted and mixed at ~60-65°C. Water (980 μL) was added to the melted wax and surfactant while stirring to form an opaque emulsion. Finally, 20 μL of CTAB (50 mM) or SDS (50 mM) was added to form clear microemulsions at 60-65°C. The microemulsions were cooled to room temperature, while stirring, to obtain solid NPs (2 mg/mL). The final concentration of components in the NP suspension was emulsifying wax (2 mg/mL), Brij 78 (3 mM), and CTAB or SDS (1 mM). To prepare neutral NPs, the amount of Brij 78 was increased to 4.6 mg or 4 mM final concentration, with no additional co-surfactants. The NP sizes were measured using a Coulter N4 Plus Sub-Micron Particle Sizer (Coulter Corporation, Miami, FL) at 90°. The overall charge of the NPs was measured using Malvern Zeta Sizer 2000 (Malvern Instruments, Southborough, MA).

Coating of the cationic NPs with OVA and CpG

Varying amounts of OVA were added to NPs (1000 μg/mL) in 5% (v/v) mannitol. The suspension was vortexed gently and placed on a horizontal shaker at room temperature for a minimum of 30 min to allow for coating. A similar procedure was followed to coat CpG on cationic NPs at a 1:3 w/w ratio. The coated NPs were diluted appropriately in water for measuring the size and charge of the particles.

In vitro generation of murine BMDDCs

Bone marrow cells were obtained by flushing the femurs of BALB/c mice with 1X HBSS. Cells were cultured in 100 mm bacteriological petri dishes at 2 x 10^5 cells/mL in 10 mL of complete RPMI 1640 medium (supplemented with 10% heat-inactivated fetal calf serum, 1 mM HEPES, 2 μM L-glutamine, 10 U/mL penicillin, 100 U/mL streptomycin, 50 μM 2–mercaptoethanol) containing 20-25 ng/mL GM-CSF at 37^oC, 7% CO_2. The cells were supplemented with an additional 10 mL of complete RPMI 1640 with 20-25 ng/mL GM-CSF on day 3. On day 6, 10 mL of supernatant was removed from each plate and spun down. The cells were resuspended in fresh 10 mL of complete RPMI 1640 with 20-25 ng/mL GM-CSF and added back to the Petri dishes. Non-adherent to lightly adherent cells were harvested on day 7 and used for *in vitro* studies.

In vitro uptake of NPs using murine BMDDCs

Cationic (CTAB), anionic (SDS), and neutral (Brij 78) NPs radiolabeled with ^{3}H-cetyl alcohol (50 μCi/mL) were prepared for quantitating uptake of NPs by BMDDCs. The preparation, entrapment and stability of the radiolabeled NPs has been described previously [429]. Day 7 harvested BMDDCs were plated in 200 μL of complete RPMI 1640 at 2 x 10^5 cells/well in 48-well tissue culture plates. After incubating the cells overnight at 37^oC, 7% CO_2, the wells were washed once with 500 μL of cold 1X HBSS and 100 μL of complete RPMI 1640 was added to each well. The radiolabeled NPs were diluted appropriately in complete RPMI 1640 and 100 μL (equivalent of 1 μg of NPs) was added to each well and plates were incubated at 37^oC, 7% CO_2 for 1, 2, 4, 6 and 12 hr. As a control, cells were incubated at 4^oC for 1, 2 and 4 hr. At each time point, the

media containing radiolabeled NPs was removed and the cells were washed three times with 400 μL of 1X cold PBS. The cells were lysed with 100 μL of 1X lysis buffer with one freeze thaw cycle at -20°C. The lysed cells were collected and counted in ScintiVerse liquid scintillation cocktail using a Beckman LS 6500 scintillation counter (Fullerton, CA).

Confocal microscopy of BMDDCs incubated with fluorescent NPs

Fluorescent NPs were prepared by using 2% w/w DiOC$_{18}$. Briefly, 40 μl of DiOC$_{18}$ (1 mg/ml stock in chloroform) was added to a vial containing 2 mg of emulsifying wax and 3.5 mg of Brij 78. The vial was mixed at ~60-65°C allowing for all solvent to be evaporated. After complete evaporation of the solvent, 1 ml of water was added to the vial at 60-65°C and NPs were formulated as described above. Unentrapped DiOC$_{18}$ was separated from the NPs by gel permeation chromatography using a Sepharose CL4B packed column (15 x 70 mm) with 0.01 M PBS, pH 7.4 as the mobile phase. Purified NPs were used further for *in vitro* studies with BMDDCs. Immunofluorescence staining was used to visualize the uptake of NPs by DCs. BMDDCs were seeded into sterile Petri dish at concentrations 4 x 10^5 cells/mL and incubated with 2 or 10 μg/mL of fluorescent NPs at 37°C, 7% CO$_2$ in the dark. After incubating for 24 hr, the cells were washed with 1X PBS to remove excess NPs. DCs were stained by labeling the cells with biotinylated anti-mouse CD11c primary antibody followed by labeling with APC-conjugated Streptavidin. Cells were washed with 1X PBS after staining, fixed on a glass slide using 2% formaldehyde for 15 min at room temperature in the dark, and mounted using Prolong$^{\circledR}$ Antifade kit. Cells were visualized

using Leica Laser scanning confocal microscope equipped with Argon laser (488 nm green), Krypton laser (568 nm red), and HeNe laser (633 far red) using Leica confocal software.

In vitro stimulation of with monocyte derived dendritic cells (MDDCs) with NPs

Immature human MDDCs ($5x10^5$ cells/well) were plated in triplicate in 48-well plates and incubated for 24 hrs at 37^oC, 5% CO_2 with media alone (X-Vivo 15 supplemented with 10% FBS, 1% penicillin-streptomycin, 20 ng/mL GM-CSF, and 10 ng/mL IL-4) or in the presence of Alum, anionic NPs, or Lipid A at concentrations of 10, 50, and 100 μg/mL. The supernatants collected at 24 hr and assessed for TNF-α, IL-1β, and total IL-12 release by ELISA.

In vitro stimulation of murine BMDDCs

BMDDCs ($2x10^5$ cells/well) were plated in triplicate in a 96-well plate and incubated overnight at 37^oC, 5% CO_2. The cells were further incubated with varying concentrations of cationic NPs, Lipid A, CpG, or CpG coated on cationic NPs (1:3 w/w) for 24 hr and supernatants were collected. The size and charge of CpG-coated NPs was 130 ± 15.2 nm and -32 ± 2.2, mV, respectively. The total IL-12 release in the supernatants was quantified using ELISA kits.

Adoptive transfer experiments using OT-1 cells

OT-1 T cell receptor transgenic mice expressing a T cell receptor specific for an ovalbumin peptide presented by the H-2K^b molecule were used in adoptive transfer

experiments as previously described [430]. Briefly, spleen and lymph node cells from OT-1 mice were labeled with CFSE and transferred into C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) such that each mouse received 2.5 x 10^6 OVA-specific T cells. After 24 hr, mice (n=3/group) were immunized subcutaneously on the back and flanks with various concentrations of OVA alone or OVA-coated NPs. Three days following immunization, cells were isolated from draining lymph nodes and stained with monoclonal antibodies to the transgenic T cell receptor (anti-Valpha 2 and anti-Vbeta 5) and analyzed by three-color flow cytometry.

6.4   Results and discussion

In vitro uptake

Particulate delivery systems are thought to enhance immune responses mainly by promoting uptake or delivery of the associated antigen into APCs, such as DCs. To this end, Singh *et al*. speculated that the enhanced immune responses obtained with the immunostimulatory adjuvant CpG coated on cationic microparticles may be due to enhanced delivery and uptake of both coated CpG and antigen into APCs [398]. *In vitro* studies with PLGA microparticles [241] and nanoparticles [239,240,431] demonstrated that the particles are taken up by DCs. Consequently, the uptake also resulted a modest increase in the expression of MHC class II and co-stimulatory molecules with the nanoparticles [239]; however, this was not observed with microparticles.   It was suggested that particle size may have an effect on inducing the expression of these molecules [239].

In the present studies, the uptake of NPs prepared from microemulsion precursors were evaluated using BMDDCs. To determine the extent of uptake, radiolabelled NPs with varying charges were incubated with BMDDCs and the associated radioactivity was measured over time.   Figure 6.1 demonstrates that significantly more NPs were associated with BMDDCs at 37°C than at 4°C, suggesting active internalization. There is some association of the particles to the cells at 4°C probably due to adsorption of the NPs to the cell surface. In addition, Figure 6.2 confirms that all NPs, regardless of charge, were taken up by BMDDCs, with approximately 50-70% of the NPs taken up over 12 hr. The uptake of cationic NPs was found to be significantly higher than anionic and neutral

NPs between 4 and 12 hr. Interestingly, there was a linear increase in the number of particles taken up over 6 hr, after which there appeared to be a reduction in the uptake as indicated by the plateau around the 12 hr time point. This highly efficient uptake of NPs is clearly evident in Figure 6.3, where BMDDCs incubated with fluorescent labeled NPs demonstrate that the NPs, indicated by green fluorescence, are taken up and located intracellulary in DCs, outlined by red fluorescence.

***In vitro* evaluation of anionic NPs using human MDDCs**

In vitro data indicate that the NPs are taken up into DCs. To further evaluate if NPs are immunostimulatory after taken up by DCs, anionic NPs were incubated with human DCs for 24 hr and the supernatants were analyzed for three pro-inflammatory cytokines, IL-12, TNF-α, and IL-1β. Lipid A, an immunostimulatory adjuvant, was used a positive control in these studies and Alum, which is a humoral immune response mediator was also included for comparison. As shown in Figure 6.4 at the 10 μg/mL concentration of adjuvant, the positive control Lipid A, but not Alum or anionic NPs, caused significant release of IL-12 (a Th1 promoting cytokine), TNF-α and IL-1β from human DCs. Furthermore, Lipid A caused a dose-dependent release of the pro-inflammatory cytokines at the higher concentrations used; however, the anionic NPs and Alum treated cells did not cause release of pro-inflammatory cytokines at the higher concentrations (data not shown). These data suggest that NPs are not immunostimulatory and do not enhance immune responses via the release of pro-inflammatory cytokines.

***In vitro* stimulation using murine BMDDCs**

CpG has been shown to induce maturation and activation BMDDCs resulting in a release of several pro-inflammatory cytokines including IL-12 [97]. It has been reported that CpG treated DCs had an enhanced ability to activate T cells, which was significantly diminished in DCs derived from IL-12 knockout mice. This suggested that CpG-mediated IL-12 release from DCs plays a significant role in enhancement of Th1/cellular immune responses [432]. Since the receptor for CpG, TLR9, is located intracellularly, it has to be first taken up into BMDDCs where CpG can then bind to the TLR9 initiating the signaling cascade to cause IL-12 secretion. Thus, it was expected that there would be significantly higher IL-12 release by BMDDCs if the uptake of CpG was enhanced using cationic NPs. As shown in Figure 6.5, cationic NPs did not stimulate significant release of IL-12 from BMDDCs confirming that NPs by themselves have no significant immunostimulatory activity. However, cationic NPs coated with CpG resulted in IL-12 in a dose-dependent manner that was similar to Lipid A (positive control). There was a significant amount of IL-12 release compared to unstimulated cells at a CpG or Lipid A dose of greater than 50 ng/mL. NPs coated with CpG resulted in higher IL-12 release compared to CpG alone and this was statistically significant at a dose of 100 ng/mL of CpG. These data in combination with the uptake data suggest that NPs functioned to enhance delivery, causing enhanced uptake of CpG by BMDDCs and therefore, allowing for more of the oligodeoxynucleotide to be available intracellulary to bind to TLR9 and causing enhanced IL-12 release. Interestingly, Kwon *et al*. reported over a 10-fold increase in IL-12 release from BMDDCs after incubating with CpG coated on acid degradable nanoparticles [431] and in comparison, the enhancements in IL-12 release in

the present studies were only modest. Several factors may influence these discrepancies.

First, the BMDDCs used by Kwon *et al*. were derived from C57BL/6 mice and reports

suggest that these mice express TLR9 to a greater extent compared to BALB/c mice

[425]. More importantly, BMDDCs are derived from primary cultures of cells from

femurs of mice and several factors including the age of mice, the method used for

generation of DCs, and the composition of the cultures at time of use may have an effect

on the results. Taken together, these factors may allow for more efficient stimulation of

BMDDCs at lower concentrations of CpG than were obtained with our experiments. An

additional concern during these *in vitro* experiments is the presence of FBS in the media,

which may disrupt electrostatic interactions between the cationic particle and CpG,

possibly causing at least some of the CpG to dissociate from the NPs.   This would be

less of a concern in the *in vivo* situation when the formulation is injected into the

subcutaneous space.   Other factors that may also be of critical consideration is in

identifying greater differences in IL-12 release such as the time of incubation/stimulation

and the source of CpG.

OT-1 cell proliferation in adoptive transfer experiments

One of the major goals in vaccine development is to stimulate CD8$^+$ T cell

responses by presentation of antigen on MHC class I molecules. To determine the extent

to which antigens coupled to NPs stimulate CD8$^+$ T cells *in vivo*, we utilized an adoptive

transfer system with transgenic T cells from the OT-1 mouse, which express a class I

restricted receptor specific for OVA presented by the H-2K^b class I molecule. OT-1 cells

were labeled with CFSE and transferred into normal C57BL/6 mice. After 24 hr, the

mice were immunized with OVA alone (no adjuvant) or OVA-coated NPs. Flow cytometry was performed on day 3 to assess the extent of cell division by measuring CFSE dye dilution in the draining lymph nodes, which is a direct measure of the degree of antigen recognition. All of the OT-1 cells in the control, unimmunized mice, remained CFSE bright indicating no cell division. In contrast, OT-1 T cells from mice immunized with OVA alone or OVA-coated NPs showed extensive CFSE dye dilution indicating strong cell division in the draining lymph nodes. These responses were at a plateau between 10 and 20 μg of OVA coated on NPs, as there were no significant differences in the T cell proliferation with a decrease in the antigen dose by one-half. However at the lowest dose evaluated, mice immunized with OVA-coated NPs showed significantly more cell division than OVA alone, indicating that OVA coated on NPs is superior to an equivalent concentration of soluble OVA in terms of stimulation of $CD8^+$ T cells (Figure 6.6 and 6.7). These results demonstrate that NPs can facilitate the entry of coated proteins into the MHC class I processing pathway resulting in enhanced presentation to $CD8^+$ T cells *in vivo*. This enhanced presentation via the MHC class I pathway is thought to be due to enhanced delivery into DCs, which are able to cross-present exogenous antigens and are the primary cells involved in stimulation of T cells [74]. Moreover, these results are in agreement with the *in vitro* results using CpG coated NPs demonstrating that NPs were effective at enhancing delivery of the coated molecule. Studies evaluating CpG-coated NPs, lower doses of OVA with and without NPs, and time points of immunization in the adoptive transfer experiments will be helpful in further elucidating mechanism(s) of immune response enhancement.

Taken together, these studies demonstrate that NPs prepared from oil-in-water microemulsions are effective systems for enhancing delivery of the associated antigen or immunostimulatory adjuvant into DCs. It is hypothesized that this enhanced delivery causes the significant enhancements observed with antigen coated on NPs *in vivo*. However, the uptake and fate of NPs *in vivo* need to be further evaluated to further characterize their mechanism(s). *In vitro* studies suggest that NPs do not enhance immune responses by release of cytokines; however, *in vivo* cell death by necrosis or apoptosis after injection may contribute to the immune response generation and will need to be further investigated.

Acknowledgements
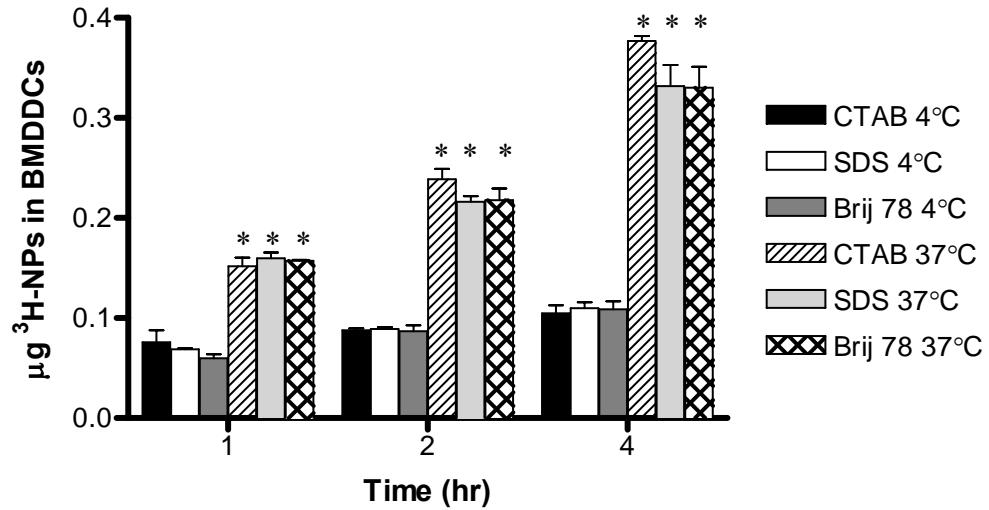
Figure 6.1

Figure 6.1. Uptake of ³H-NPs by BMDDCs at 37°C versus 4°C. BMDDCs $(1\times10^6/\text{mL})$ were incubated for various times with 1 μg of ³H-NPs prepared using various surfactants, CTAB, SDS, and Brij 78, to obtain cationic, anionic, and neutral NPs, respectively. NPs were incubated with BMDDCs at 4°C as a control to distinguish association with the cells versus uptake at 37°C. *indicates $p<0.05$ at 37°C compared to 4°C at 1-4 hr by Students t-test.

Figure 6.2
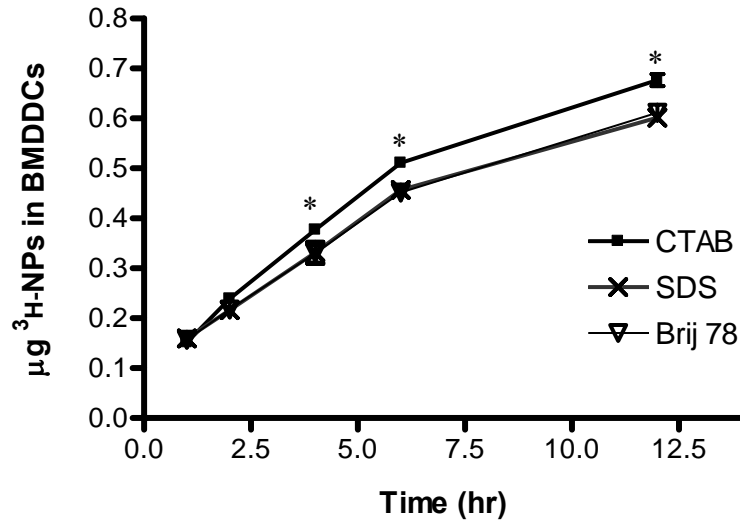
Figure 6.2. Uptake of ³H-NPs by BMDDCs at 37°C. BMDDCs (1x10⁶/mL) were incubated for various times with 1 μg of ³H-NPs prepared using various surfactants, CTAB, SDS, and Brij 78, to obtain cationic, anionic, and neutral NPs, respectively. Data reported are the mean ± S.D. (n=3). *p<0.05 for CTAB NPs compared to SDS and Brij 78 NPs.

Figure 6.3
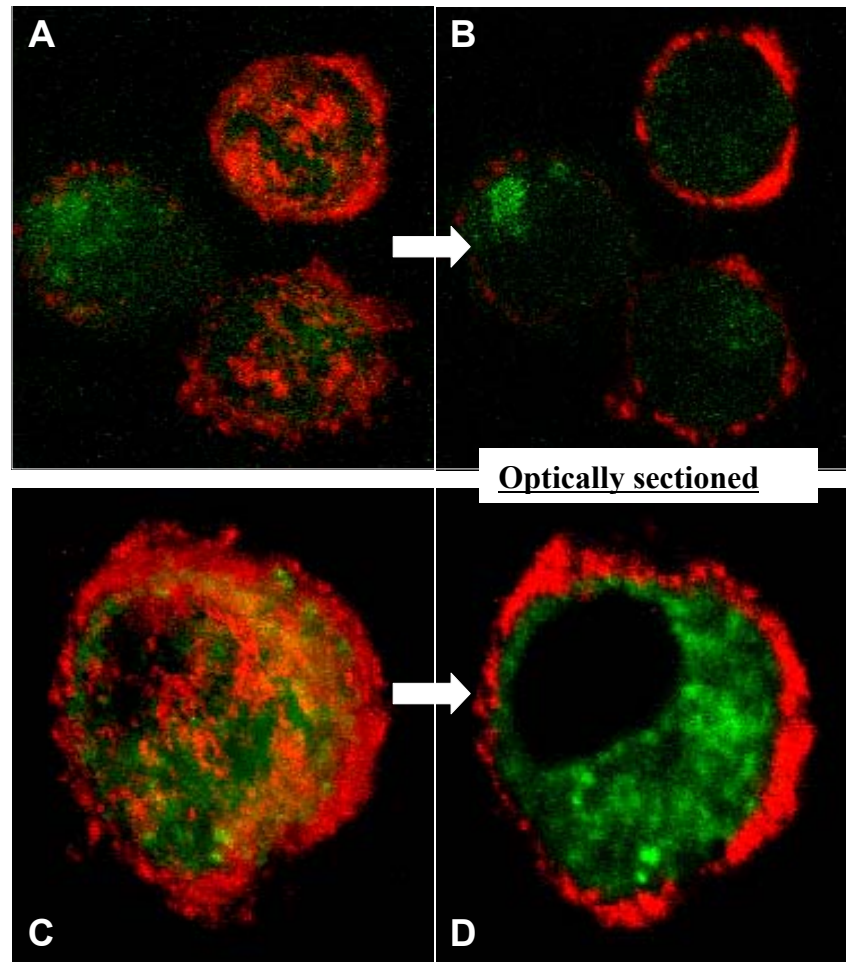
Figure 6.3. Laser-scanning confocal microscopy images of fluorescent NPs present intracellularly in DCs. DCs were visualized by staining with biotinylated anti-mouse CD11c primary antibody and APC-conjugated Streptavidin secondary antibody (red). NPs were labeled with $DiOC_{18}$ (green). All images were obtained at 100X magnification. A) Whole DC confocal image of BMDDCs incubated with 2 μg/ml fluorescent NPs. B) Optically sectioned DC confocal image of BMDDCs incubated with 2 μg/ml fluorescent NPs. C) Whole DC confocal image of BMDDCs incubated with 10 μg/ml fluorescent NPs. D) Optically sectioned DC confocal image of BMDDCs incubated with 10 μg/ml fluorescent NPs.

Figure 6.4
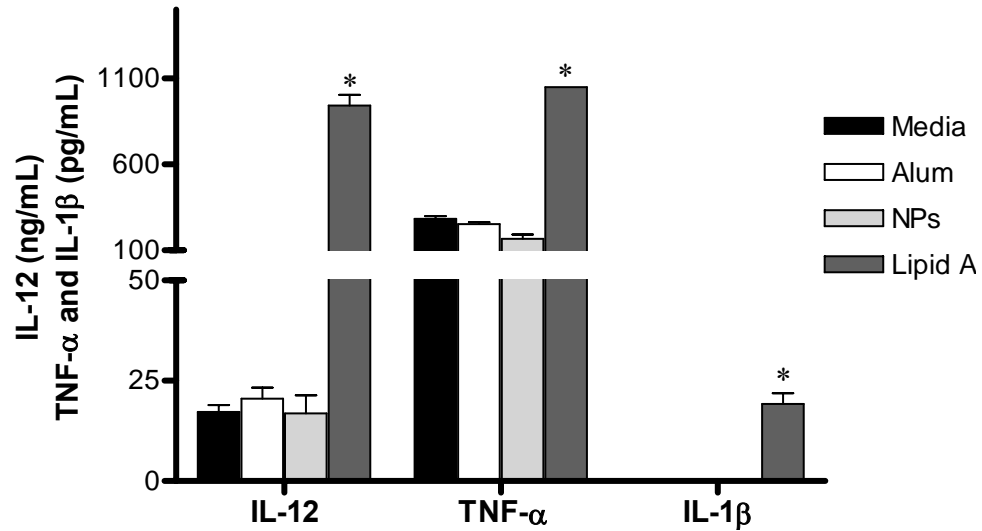
Figure 6.4. Pro-inflammatory cytokine release from human MDDCs. Day 7 MDDCs (1×10^6/mL) were cultured with media or 10 μg/mL Alum, anionic NPs, or Lipid A for 24 hr at 37°C, 5% CO_2. Pro-inflammatory cytokines were assessed in 24 hr supernatants by ELISA. TNF-α levels for Lipid A were >1050 pg/mL. Data reported are the mean ± S.D. (n=3). *p<0.05 compared to all other groups.

Figure 6.5
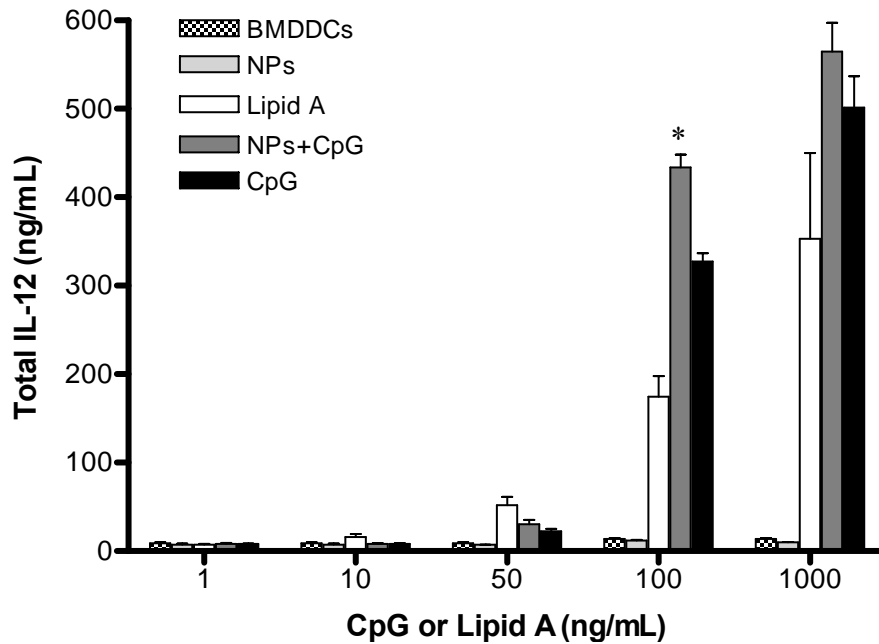
Figure 6.5. IL-12 release from BMDDCs after *in vitro* stimulation. Day 7 BMDDCs $(1 \times 10^6/\text{mL})$ were cultured in complete RPMI 1640 alone (BMDCCs) or with cationic NPs, CpG, CpG coated on NPs (NPs+CpG) or Lipid A (positive control). Total IL-12 release from cells was measured in 24 hr supernatants by ELISA. NP concentrations used were 3.35 times that indicated for CpG or Lipid A. Data represents the mean ± S.D. (n=3). Experiment was performed in duplicate with similar trends and data shown here is representative of one experiment. *p<0.05 for CpG-coated NPs compared to CpG alone by Students t-test.

Figure 6.6
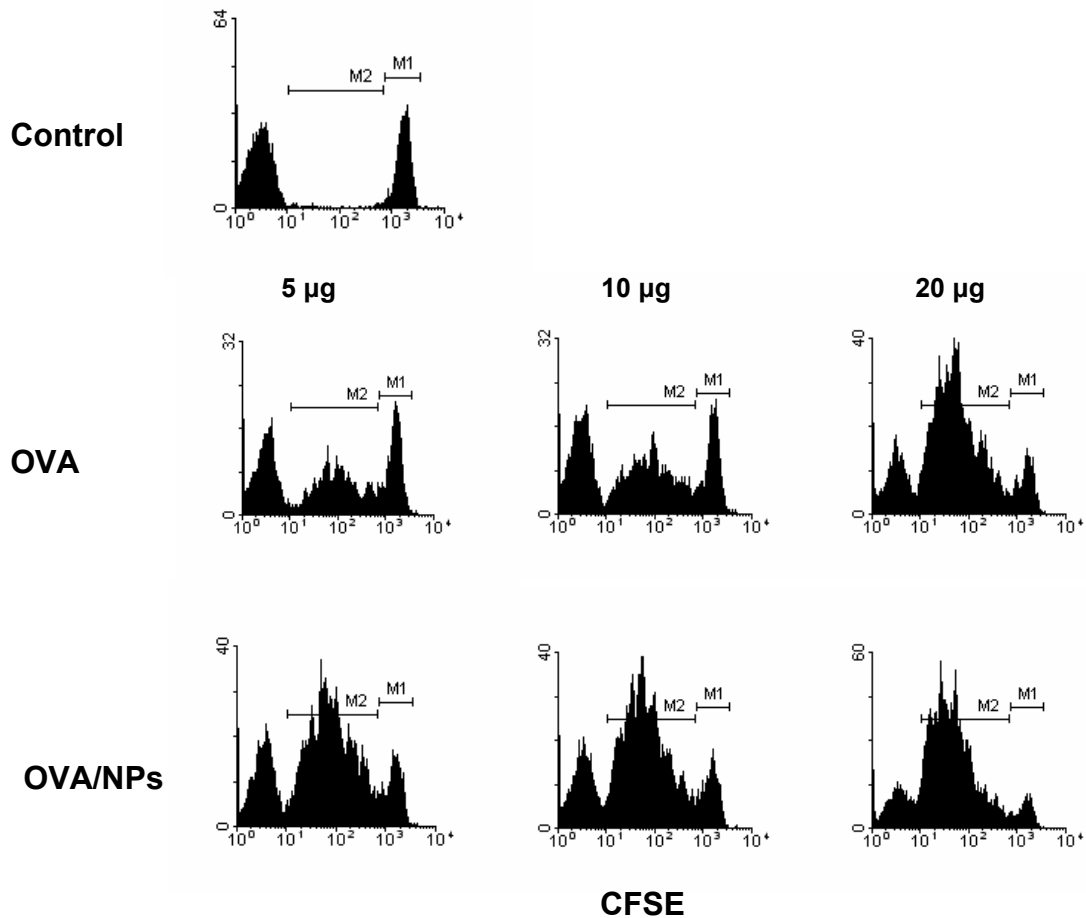
Figure 6.6. Flow cytometry histograms comparing OVA-coated NPs to soluble OVA for stimulating a CD8$^+$ T cell clonal expansion *in vivo*. The flow cytometry histograms from a representative mouse of the CFSE fluorescence of cells expressing the Valpha 2, Vbeta 5 T-cell receptor. The left-most peak represents endogenous Valpha 2, Vbeta 5 positive T-cells in the B6 mice and thus are CFSE negative. The cells in peak M1 represent OT-1 cells that have not divided while cells in peak M2 represent OT-1 cells that have undergone various numbers of cell divisions.

Figure 6.7
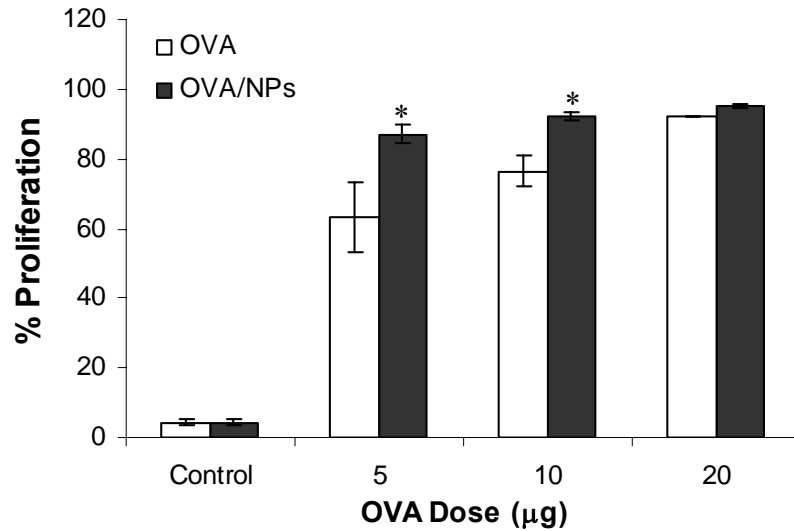
Figure 6.7.   OVA-coated NPs are superior to soluble OVA at stimulating a CD8$^+$ T cell clonal expansion *in vivo*.   The percent cell division (mean +/- S.D.) of the OT-1 cells was determined by calculating the number of cells under marker M2 as a percent of the total cells under marker M1 + M2.   *$p < 0.05$ compared to OVA by Student's t-test.

Chapter 7

Preparation and characterization of nickel nanoparticles for enhanced immune responses to his-tag HIV-1 Gag p24

7.1   Summary

Particulate delivery systems have been widely investigated for obtaining enhanced immune responses to protein-based vaccines. Previous reports from our laboratory have demonstrated that anionic or cationic nanoparticles prepared from oil-in-water microemulsion precursors can be used to enhance immune responses to antigens coated on the surface of the particle by charge interactions. A stronger interaction of the antigen to the surface of nanoparticles may provide greater association of antigen with the particles *in vivo* and prove beneficial in further enhancing the immune responses. The purpose of these studies was to prepare nanoparticles with a small amount of surface-chelated nickel to enable stronger interactions with histidine-tagged (his-tag) proteins. The surface-chelated Ni nanoparticles were shown to bind to his-tag green fluorescent protein and his-tag HIV-1 Gag p24. Furthermore, his-tag Gag p24 bound to the nickel nanoparticles resulted in significant enhancements in humoral responses, including IgG2a responses, compared to the protein adjuvanted with Alum or coated on the surface of negatively charged nanoparticles.

7.2   Introduction

The need for improved adjuvants for enhancing immune responses to protein-based vaccines is widely recognized [2,7,59,60,94,433-435]. Presently, Alum continues to be the only approved adjuvant for routine human vaccination in the U.S [3]. However, there has been considerable interest in the development of particulate delivery systems for enhancing immune responses with protein-based vaccines over the past few years [4,6,8,172,198,263,436]. In fact, both PLGA microparticles and liposomes are currently under clinical evaluation with potential HIV and hepatitis vaccines, respectively [77,94]. Particulate delivery systems are attractive as they offer numerous advantages such as the ability to: control the release of the antigen [202,230,232], target the delivery of antigen to antigen presenting cells (APCs) [169,283,296], and incorporate immunostimulatory adjuvants for synergistic enhancements in immune responses [79,257,260]. Moreover, particulate delivery systems are of similar sizes as naturally occurring pathogens and considered to be rapidly taken up by APCs, leading to increased accumulation of the associated protein inside the cell [125].

Particulate delivery systems for protein-based vaccine applications have most often utilized entrapment of the antigen within the particle for obtaining enhanced immune responses [8,213,214,216,217,437]. Although effective, concerns associated with this approach include protein instability and entrapment efficiency. For example, the protein stability is of significant concern with the most often investigated PLGA microparticles [235]. The protein degradation can occur during the entrapment by conditions such as the presence of organic solvents, during freeze drying, and also by the acidic environment created by polymer degradation *in vivo* [229]. Moreover, low

entrapment of the protein and instability of the delivery system are challenges faced with using liposomal systems. Alternatively, the use of charged particles has been investigated for coating antigens to the particle surface by ionic interactions and thus, enhancing immune responses to the coated antigen *in vivo* [9,235,237,248,398]. Negatively charged PLGA microparticles have been shown to enhance immune responses to HIV-1 Gag p55 [238]. To this end, reports from our laboratory have demonstrated the potential of charged nanoparticles (NPs) prepared from oil-in-water microemulsion precursors for enhancing immune responses with cationic proteins such as β-galactosidase [16] and HIV-1 Tat protein [253,438], and with the anionic model antigen ovalbumin (OVA) as shown in Chapter 5.

Several studies suggest that the higher uptake of antigens into APCs using particulate delivery systems may result in enhanced immune response to associated antigens *in vivo* [239-242]. Studies in our laboratory suggest that NPs are taken up effectively *in vitro* by DCs and that the enhanced delivery of associated antigen or molecules, at least in part, contributes to the enhanced immune responses observed *in vivo* [439]. It is important to note that these are simply antigens coated on charged particles and some dissociation of the coated protein from the particle may occur *in vivo* due to other charged molecules present, resulting in a decreased accumulation in APCs. It is hypothesized that increasing the affinity of the antigen for the particles could allow even more of the antigen accumulate in the APCs compared to antigen coated on the surface of the NPs and thus, enabling greater enhancements in immune responses *in vivo*.

The attachment of proteins and antibodies to particulate delivery systems has been investigated extensively by covalent linkages that involve the use of sulfhydryl-, amine-

and carboxyl-reactive moieties on the protein or the particle [440-443]. However, these approaches are often cumbersome and require the use of activating or reducing agents. In addition, these methods have the potential for causing protein degradation during attachment, random or multiple point attachment of the protein, and quite often, low coupling efficiencies are obtained. An alternative approach that takes advantage of affinity interactions has been extensively used for purification of recombinant proteins [444-447]. This approach exploits the interaction between chelated divalent metal ions such as nickel, copper, or cobalt and a short sequence of histidine residues (4 to 10 repeating units) added to the N- or C-terminus of the protein, referred to as histidine-tags (his-tag). These purification methods generally involve immobilizing the metal ion onto the column packing material using a chelating agent such as nitrilotriacetic acid (NTA) [444]. In the case of Cu^{2+} and Ni^{2+} which have six coordination sites, NTA forms a strong complex with four of the metal sites, leaving two additional sites for interaction with the his-tag present on the protein [445]. Moreover, the interaction of his-tags with NTA-Ni has been reported to be equivalent or stronger than that of antibody interactions (10^{-6} to 10^{-9}), with a dissociation constants (K_d) in the range of 10^{-6} to 10^{-13} M at pH 7-8 depending on the protein and location of the his-tag on the protein [448,449]. The binding is reversible by competing off with excess imidazole (> 100 mM) or by lowering the pH which results in release of the his-tag protein due to protonation of electron donating histidine groups (pKa = 6.0). In addition to its extensive use in protein purification, the use of NTA-Ni has been also reported for immobilization of his-tag proteins onto surfaces for structural and functional studies [450] and for studying protein interactions by flow cytometry [449]. More recently, hydrophobized NTA-Ni ligand

integrated in the liposomal lipid bilayer was reported for attaching his-tag peptides and proteins on the surface [451] and for targeting the uptake of entrapped antigen to DCs via surface immobilization of his-tag antibodies for DC-specific receptors [290].

The interaction of chelated Ni with his-tag proteins for enhancing immune responses to antigens with particulate delivery systems would be advantageous since it is simple, applicable to a wide range of proteins, and offers stronger interactions between the particle and antigen compared to conventional charged particles, consequently allowing for higher accumulation of the antigen inside the cell. Once the antigen is taken up into the cells, it can also be released because the interactions weaken in the acidic environment of the lysosomes. Therefore, the present studies were aimed at investigating the preparation of NPs with a small amount of surface-chelated nickel for binding to his-tag proteins. In addition, the utility of these NPs for enhancing the immune responses to protein-based vaccines was evaluated *in vivo* using his-tag HIV-1 Gag p24 protein (his-tag p24).

7.3   Materials and methods

Materials

Emulsifying wax, comprised of cetyl alcohol and polysorbate 60 (molar ratio of 20:1) and Alum were purchased from Spectrum (New Brunswick, NJ). Phosphate buffered saline, pH 7.4 (PBS), PBS, pH 7.4 with 0.05% Tween 20 (PBS/Tween 20), bovine serum albumin (BSA), and Sepharose CL4B were from Sigma Chemical Co. (St. Louis, MO). Brij 78 was purchased from Uniqema (New Castle, DE). Sheep anti-mouse IgG, peroxidase-linked species specific F(ab')$_2$ fragment was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).   IFN-γ ELISA kit, streptavidin-horseradish peroxidase (Sv-HRP) and biotinylated rat anti-mouse IgG1 and IgG2a monoclonal antibodies were from BD Biosciences Pharmingen (San Diego, CA). Tetramethylbenzidine (TMB) substrate kit and HisGrab™ nickel-coated plates were purchased from Pierce (Rockford, IL). Microcon® YM-100, CentriPlus® YM-100, certified nickel standard (Claritas® certified reference material), nitric acid (trace metal grade), and 2-mercaptoethanol were purchased from Fisher Scientific (Hampton, NH). RPMI 1640, 10% heat-inactivated fetal calf serum, Hanks Balanced Salt Solution (HBSS), HEPES, L-glutamine, penicillin, and streptomycin were from GIBCO (Carlsbad, CA). 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] nickel and ammonium salt, DOGS-NTA-Ni and DOGS-NTA, respectively, were purchased from Avanti Polar Lipids (Alabastar, AL).   PVDF membranes, 15% Tris-HCl SDS-PAGE gels, and Immun-star HRP substrate kit were from Bio-Rad (Hercules, CA). Histidine-tag HIV-1 Gag p24 (his-tag p24) was obtained through the Centralised Facility for AIDS Reagents supported by EU Programme

EVA/MRC and the UK Medical Research Council (donated by Dr. I. Jane). For *in vivo* studies, female BALB/c mice (6-8 weeks old) were obtained from Harlan Sprague-Dawley Laboratories (Indianapolis, IN).

Preparation of NPs with surface-chelated Ni

Nanoparticles were prepared from oil-in-water microemulsion precursors using emulsifying wax as the oil phase and Brij 78 as the surfactant. In a 7 mL glass vial, 2 mg of emulsifying wax and 3.5 mg (3 mM) of Brij 78 was added. To this vial, 10.6 μL (0.1 mM) of DOGS-NTA-Ni (10 mg/mL stock in chloroform) was added and the chloroform was evaporated on a hot plate (~60-65°C) while stirring. Water (1000 μL) was added to the vial at 60-65°C and the contents of the vial were mixed on the hot plate to form clear microemulsions. NPs were obtained by cooling the vials to room temperature while stirring. NPs of similar composition but without Ni were prepared in the same manner using 0.1 mM of DOGS-NTA lipid instead, referred to as NTA-NPs. The NPs were characterized by measuring their size using a Coulter N4 Plus Sub-Micron particle sizer (Coulter Corporation, Miami, FL) at 90° and charge, using a Malvern Zeta Sizer 2000 (Malvern Instruments, Southborough, MA).

Binding of his-tag p24 and initial *in vivo* studies

For the initial studies, his-tag p24 was bound to the surface-chelated Ni nanoparticles (Ni-NPs) at a 1:10 w/w ratio in PBS, pH 7.4 at 4°C overnight. The effectiveness of these formulations was evaluated *in vivo* using BALB/c mice (n=5-6 per group). The animals were dosed on day 0 and day 14 with his-tag p24 bound to Ni-NPs,

adjuvanted with Alum, or as a control coated on NTA-NPs. All mice were given 100 μL

s.c. injections on the back containing 2.5 μg of his-tag p24 and 25 μg of the NPs or

Alum. The mice were bled by cardiac puncture on day 28 and the sera were separated

and stored at –20°C for antigen-specific IgG analysis.

Determination of his-tag p24-specifc total IgG levels

His-tag p24-specific serum IgG levels were determined using an ELISA. The

plates (96-well Costar plates) were coated with 50 μL of his-tag p24 (5 μg/mL in PBS,

pH 7.4) overnight at 4°C. The plates were blocked for 1 hr at 37°C with 200 μL of 4%

BSA prepared in PBS/Tween 20. The plates were then incubated with 50 μL per well of

mouse serum diluted at 1:100 and 1:1000 in 4% BSA/PBS/Tween 20 for 2 hr at 37°C.

The plates were washed with PBS/Tween 20 and incubated with 50 μL/well anti-mouse

IgG HRP F(ab')$_2$ fragment from sheep (1:3000 in 1% BSA/PBS/Tween 20) for 1 hr at

37°C. After washing the plates with PBS/Tween 20, the plates were developed by adding

100 μL of TMB substrate and incubating for 30 min at RT. The color development was

stopped by the addition of 100 μL of 2 M H$_2$SO$_4$ and the OD at 450 nm was read using a

Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT).

Optimization of Ni-NP formulation for binding to his-tag proteins using his-tag GFP

To further optimize the binding of Ni-NPs to his-tag p24, studies evaluating the

entrapment and binding ratios to a model protein, his-tag green fluorescent protein (GFP)

were carried out. Excess DOGS-NTA-Ni was separated from the Ni-NPs using a gravity

packed Sepharose CL4B gel permeation chromatography (GPC) column (15 x 70 mm).

Briefly, 200 µL of the Ni-NPs was passed down the GPC column using PBS, pH 7.4 as the mobile phase. Fractions (1 mL) were collected and the fractions containing the NPs were used for binding to his-tag GFP. To determine the optimal binding ratios, his-tag GFP was mixed with the GPC-purified Ni-NPs at a 1:16.9 and 1:33.7 w/w ratios in PBS, pH 7.4 at 4°C overnight. Free protein was separated from bound protein by passing through the Sepharose CL4B column using PBS, pH 7.4 as the mobile phase. Fractions collected (1 mL) were analyzed by fluorescence to determine the percent of his-tag GFP bound to Ni-NPs. The stability of the binding at 1:33.7 w/w ratio at 37°C in PBS, pH 7.4 was evaluated by removing aliquots at over 4 hr and passing through the GPC column. The fluorescence associated in fraction 1-12 was measured and particle sizes were measured using fraction 4. The fluorescence was measured using a Hitachi F-2000 fluorescence spectrophotometer (Fairfield, OH) with the excitation and emission wavelengths set at 395 nm and 508 nm, respectively.

Optimization of his-tag p24 binding to Ni-NPs

The Ni-NPs were purified by GPC as described and further reacted with the his-tag p24 at 1:8.85, 1:17.7, 1:35.4, and 1:70.8 w/w ratios to determine the optimal binding conditions. Unlike his-tag GFP, the binding of his-tag p24 to the Ni-NPs cannot be assessed directly. Therefore, after GPC purification, fractions 7-13 were evaluated for the presence of free protein by ELISA. The NP containing fractions, fractions 3 to 5, were combined, concentrated using Microcon® YM-100 ultracentrifuge devices and analyzed by western blot.

ELISA for analysis of free his-tag p24

To detect the free his-tag p24 eluting from GPC column, a qualitative ELISA method was developed using HisGrab™ nickel-coated plates. Samples (100 μL) were added to the plate and incubated for 1 hr at room temperature (RT) while shaking. The wells were washed 3 times with PBS/Tween 20 and blocked with 200 μL of 1% fetal bovine serum (heat inactivated) in PBS/Tween 20 for 1 hr at RT. The wells were washed 3 times with PBS/Tween 20 and incubated with for 1 hr at RT with 100 μL of His-tag p24 anti-sera (from initial studies) at 1:1000 in the blocking solution. The wells were washed again and incubated for 1 hr with 100 μL of anti-mouse IgG HRP F(ab')$_2$ fragment from sheep diluted at 1:3000 in the blocking solution. After washing the wells with PBS/Tween 20, they were developed by incubating with 100 μL of TMB substrate for 30 min at RT and the color development was stopped by the addition of 100 μL of 2 M H$_2$SO$_4$. The OD at 450 nm was measured using a plate reader.

Western blot analysis for his-tag p24 bound to Ni-NPs

The concentrated NP fractions after GPC purification along with various amounts of his-tag p24 as controls were loaded on 15% Tris-HCl SDS-PAGE gels using Bio-rad power supply (200 V constant for 45 min). A semi-dry transfer of the proteins from the SDS-PAGE gel onto a PVDF membrane was performed using Trans-Blot semi dry transfer cell (Bio-rad) using the Bio-rad power supply (15 V, 120 mA, 400 W for 24 min). The membrane was blocked for 1 hr with 4% BSA prepared in PBS/Tween 20 and then incubated with a 1:1000 dilution of his-tag p24 anti-sera from the initial experiment for 2 hr. Finally, the membrane was incubated with a 1:5000 anti-mouse IgG HRP

F(ab')$_2$ fragment from sheep for 1 hr. All antibodies were diluted in 4% BSA in PBS/Tween 20. All steps were performed with shaking at RT and three to five washings for 5 min using PBS/Tween 20 were included in between each step. The protein on the membrane was detected using the Immun-star HRP substrate kit and the membrane was exposed using a Kodak Image Station 2000 mm (New Haven, CT) and analyzed using Kodak 1D software.

Atomic emission spectroscopy for quantitating the surface-chelated Ni on NPs

Atomic emission spectroscopy (AES) using inductively coupled plasma as the excitation source was used to quantify Ni present on the Ni-NPs before and after GPC purification. The method parameters set on the Varian Vista-PRO CCD simultaneous ICP-OES instrument (Palo Alto, CA) were as follows: plasma flow at 15.0 L/min; auxillary flow at 1.50 L/min; nebulizer flow at 0.90 mL/min; sample uptake 30 sec; rinse time 10 sec; and pump rate 15 rpm. Yttrium was used as an internal standard for correction as needed. The Ni was detected at 216.55 and 231.604 nm and the final results were calculated based on an average of both wavelengths. The data was collected and analyzed by Vista-PRO ICP software v.4.1.0. All samples and standards were prepared using 5% nitric acid. A standard curve for Ni was prepared from 10 to 200 ppb Ni. For quality control purposes, independent Ni standards at 20 and 100 ppb were prepared and analyzed prior to sample analysis. The acceptance criteria for the quality control standards were based on greater 90% of theoretical Ni concentration. To determine the recovery of Ni from the NP matrix, 0.4 and 2.0 mg of NTA-NPs were spiked with 10 and 50 ppb of Ni and analyzed for Ni content. For quantitation of Ni on the Ni-NPs, the NPs

were purified by GPC to obtain a total of 2.0 mg of purified NPs and fractions 3-6 were collected. The combined fractions were further desalted and concentrated using CentriPlus® YM-100 ultracentrifuge devices to a final volume of 1 mL and diluted in nitric acid for analysis.

In vivo assessment of optimized his-tag p24 bound to Ni-NPs

BALB/c mice (n = 6-8 per group) were immunized (s.c.) on day 0 and day 14 with 100 µL of his-tag p24 bound to GPC purified Ni-NPs, coated on NTA-NPs, or adjuvanted with Alum. As an additional control, his-tag p24 bound to unpurified Ni-NPs were also assessed. The dose of his-tag p24 was 2.5 µg and of the NPs or Alum was 88.5 µg. On day 28, mice were bled by cardiac puncture; the sera were collected and stored at $-20^{o}C$ for IgG analysis. The spleens for were collected and pooled for each group for splenocyte proliferation and IFN-γ release assays.

His-tag p24-specific antibody isotype analysis

His-tag p24 specific IgG1 and IgG2a levels were determined using an ELISA procedure similar to that described for total IgG levels. Briefly, the plates were coated with 50 µL of his-tag p24 (1 µg/mL in PBS, pH 7.4) overnight at $4^{o}C$. The plates were blocked with 4% BSA in PBS/Tween 20 for 1 hr at $37^{o}C$. The sera (50 µL) diluted at 1:1000 in the blocking solution were added to the wells and incubated for 1 hr at RT. The plates were incubated with 50 µL of IgG1 or IgG2a diluted at 1:5000 in blocking solution for 1 hr at RT and finally with Sv-HRP diluted at 1:4000 in blocking solution for

30 min at RT. The plates were washed 3-5 times with PBS/Tween 20 in between each step. The plates were developed and read as described for the total IgG levels.

Splenocyte proliferation and IFN-γ release assay

The spleens were crushed in 1X Hanks Balanced Salt Solution (HBSS) using a stomacher homogenizer for 60 s at normal speed to obtain single cell suspensions and the suspensions were then transferred into centrifuge tubes. Red blood cells were lysed adding 1X ACK buffer (156 mM NH_4Cl, 10 mM $KHCO_3$ and 100 μM EDTA) and incubating for 1-2 min at RT. The cells were spun down at 1500 rpm, 4^oC for 10 min. Supernatants were decanted and the cells were washed 2 more times with 1X HBSS. The cells were resuspended in RPMI 1640 (supplemented with 10% heat-inactivated fetal calf serum, 1 mM HEPES, 2 μM L-glutamine, 10 U/mL penicillin, 100 U/mL streptomycin, 50 μM 2–mercaptoethanol). For splenocyte proliferation assay, cells ($5x10^5$ cells/well) were added to a 96-well plate and incubated with media, Con A (2 μg/mL), or his-tag p24 (1 μg/mL) at 37^oC, 7% CO_2 for 4 days. The cells were pulsed with 1 μCi of ^3H-thymidine on day 4 and incubated for an additional 24 hr at 37^oC, 7% CO_2. The cells were harvested on filters and counted on day 5 to measure T cell proliferation. To measure IFN-γ release from stimulated splenocytes, parallel 48-well plates were set up using $1x10^6$ cells/well in 400 μL of media and stimulated as described for the proliferation assay. The supernatants were collected at 72 hr and stored at -80^oC for IFN-γ analysis by ELISA.

Statistical analysis

Statistical analysis was performed using one-way analysis of variances (ANOVA) followed by pair-wise comparisons using Tukey's multiple comparison test using GraphPad Prism software.

7.4 Results and discussion

Preparation of Ni-NPs and initial *in vivo* study with Ni-NPs

NPs prepared from oil-in-water microemulsion precursors have been reported from our laboratory [15,16,249]. These NPs offer great versatility in entrapment of ligands and molecules and can be easily engineered to be neutral, anionic, or cationic based on the appropriate choice of surfactant(s). In these studies, neutral NPs were prepared using non-ionic emulsifying wax as the oil phase and the neutral surfactant Brij 78. To further incorporate a small amount of surface-chelated nickel, the use of the lipid DOGS-NTA-Ni (Figure 7.1) was explored. The hydrophobic portion of this molecule is thought to be entrapped within the oil phase, exposing the NTA-Ni portion on the surface of the NPs for interaction with the histidine-tag on protein. The Ni-NPs prepared were approximately 150 nm in size with a slightly negative charge (-30 to -20 mV). Based on theoretical calculations, these Ni-NPs were initially bound to his-tag p24 at a 1:10 w/w ratio for initial *in vivo* evaluation to determine the applicability of this technology for further development. The binding of his-tag p24 to the Ni-NPs was confirmed by SDS-PAGE (data not shown). The use of DOGS-NTA entrapped in NPs was also investigated to control for non-specific adsorption of the protein on the surface of the NPs. The carboxylic groups of the NTA are thought to give the NPs a net negative charge and could allow his-tag p24, a cationic protein, to be coated on the surface of the particles. As shown in Figure 7.2, the Ni-NPs resulted in a significant enhancement in the antibody responses compared to both Alum and NTA-NPs. These initial studies were very

encouraging in that they demonstrated that superior humoral responses could be obtained with these Ni-NPs compared to the conventional coated NPs and Alum.

Ni-NP formulations optimized and characterized with his-tag GFP

Based on the promising results obtained with the initial *in vivo* studies, further work to optimize and characterize the Ni-NPs using his-tag GFP was performed. The use of this protein offered numerous advantages in optimizing the formulations such as ease of detection by fluorescence, direct quantitation of the protein bound to the NPs and released from the NPs. In addition, GFP is similar in molecular weight, 28 kDa for GFP versus 24 kDa for Gag p24, to the Gag p24 used in the *in vivo* studies. Separation of his-tag GFP bound to Ni-NPs from unbound his-tag GFP was achieved using a gravity column packed with Sepharose CL4B resins. The eluent from the GPC purification can be fractionated (1 mL) and based on the particle size intensity and fluorescence intensity measurements, NPs and protein associated with NPs were found to elute in fractions 3 to 6, where as free protein eluted in later fractions, 8 to 12 (Figure 7.3). Moreover, during these binding studies, it was discovered that the Ni-NP formulation contained some unentrapped DOGS-NTA-Ni, which could also be separated from the NPs by GPC since it elutes mostly in Fractions 7-9 (Figure 7.4). Thus, GPC purification with a Sepharose CL4B column allowed efficient separation of the unentrapped lipid from Ni-NPs and for separating unbound his-tag protein from the Ni-NP bound protein. As shown in Figure 7.4, the GPC purification of excess lipid from Ni-NPs resulted in a higher amount of his-tag protein being bound to the surface of the NPs and subsequently, GPC purification of NPs was performed prior to reacting with his-tag proteins.

The binding of his-tag GFP with Ni-NPs was evaluated at two ratios and it was found that at a 1:33.7 w/w ratio of his-tag GFP to Ni-NPs, greater than 80% of the protein was bound to the surface of the NPs (Figure 7.5). Furthermore, to determine the specificity of the binding, GPC purified NTA-NPs were mixed with his-tag GFP at a 1:33.7 w/w ratio and the binding was evaluated. As shown in Figure 7.6, only 7% of the protein was associated with the NTA-NPs and the majority of the protein eluted in later fractions, where free protein is expected to elute, suggesting that the binding to the Ni-NPs was stronger and more specific than simple adsorption on the surface of the particles. The binding of his-tag GFP to Ni-NPs was found to be stable as determined by particle size and binding efficiency over 4 hr in PBS, pH 7.4 (Figure 7.7). Furthermore, his-tag GFP bound to Ni-NPs at 1:33.7 w/w ratio was stable at 4°C, with comparable binding efficiency on day 7 as the initial day of preparation (data not shown).

Entrapment efficiency of DOGS-NTA-Ni in NPs based on Ni

The amount of DOGS-NTA-Ni entrapped in NPs was calculated indirectly by quantitating the amount of Ni associated with the NPs before and after GPC purification using AES. The molar ratio of Ni chelated with DOGS-NTA is 1:1, therefore the entrapment efficiency of the lipid can be calculated based on the Ni. As controls, the amount of Ni present in NTA-NPs was evaluated and the recovery of Ni from this matrix was also determined using NTA-NPs. As expected, no Ni was detected in the control NTA-NP preparations. In addition, the spike-recovery studies suggested that the Ni could be recovered from the NP matrix, with greater than 80% recovery at the highest amount of NPs evaluated (Table 7.1). Based on the amount of Ni associated with NPs

before and after GPC purification in four independent Ni-NP preparations, it was calculated that approximately 5% of the lipid initially used in the formulation was entrapped within the NPs (Table 7.2). Furthermore, using these results, it was calculated that there was about a 3-fold excess of Ni present on the NPs at the 1:33.7 w/w ratio of his-tag GFP to NPs. Interestingly, increasing the protein to NP ratio from 1:16.9 to 1:33.7 w/w only resulted in a slight increase in binding efficiency (Figure 7.5) from about 70% to 80%. This combined with the fact that there is still a 3-fold excess of Ni on the NPs at the higher binding ratio suggests that there may have been some steric hindrance preventing accessibility to at least some of the Ni present on NPs for binding to the protein.

Optimized Ni-NP formulations with his-tag p24

Based on the binding studies for his-tag GFP with Ni-NPs, the optimal binding ratios of his-tag p24 to Ni-NPs was evaluated for further use in *in vivo* studies. Evaluating the binding of his-tag p24 was more challenging because, unlike GFP, it could not be assessed directly on Ni-NPs. To determine the optimal binding ratios, the relative amounts of unbound his-tag p24 were detected using ELISA (Figure 7.8) and the fractions containing Ni-NPs were analyzed for presence of bound his-tag p24 by western blot (Figure 7.9). The western blot was further analyzed by densitometry and the results suggested that approximately 80% of the protein was associated with the Ni-NPs at ratios greater that 1:35.4 w/w. Thus, for further *in vivo* evaluation the 1:35.4 w/w ratio was used to prepare the formulation with Ni-NPs and with control NTA-NPs.

***In vivo* results using optimized Ni-NP formulations with his-tag p24**

Initial *in vivo* studies demonstrated the potential of Ni-NPs for enhancing humoral immune responses to his-tag p24. However, these formulations were not optimized and some of the protein could have been associated with excess, unentrapped lipid instead of NPs, leading to suboptimal *in vivo* responses. The goal of this follow up *in vivo* study was to confirm that humoral immune responses could be obtained with the optimized formulations of Ni-NPs and to further evaluate the cellular immune responses. Mice were immunized with his-tag p24 bound to Ni-NPs, coated on NTA-NPs or adjuvanted with Alum. In addition, the use of unpurified Ni-NPs was also investigated to control for the immune responses that may have been enhanced by unentrapped lipid reacted with his-tag p24. At the 1:1000 serum dilution, significantly higher his-tag p24- specific IgG levels were detected using the Ni-NPs compared to all groups (Figure 7.10). Moreover, NTA-NPs, Alum, and unpurified Ni-NP groups were statistically insignificant compared to the naïve group.   Interestingly, the unpurified Ni-NPs demonstrated similar potency in generating antibodies as the NTA-NPs and Alum, but significantly less compared to the purified Ni-NPs. It is hypothesized that his-tag p24 would interact with unentrapped lipid, which exists freely in solution, in micelles, or loosely adsorbed on the NP surface, to a greater extent because the Ni would be more accessible for interactions compared to the Ni-chelated to lipid which is entrapped in the NPs. Thus, while there may be some enhancement in antigen uptake and subsequently antibody production with the unpurified Ni-NP formulation, the results demonstrate that they are less effective compared to the responses that can be generated with the antigen being bound to the Ni on the NPs.

Moreover, these data support that protein bound to Ni-NPs was superior to Alum and NTA-NPs in enhancing humoral immune responses to his-tag p24.

The cellular immune responses in these studies were evaluated by splenocyte proliferation and IFN-γ release assays. Splenocytes from immunized mice that were stimulated *in vitro* with his-tag p24 demonstrated significantly higher proliferation with all groups compared to naïve group, with the exception of the unpurified Ni-NPs group (Figure 7.11). In addition, the IFN-γ released from the stimulated splenocytes showed a similar trend in that all groups, except the unpurified Ni-NPs, produced significantly higher IFN-γ compared to the naïve group (Figure 7.12). However, the IFN-γ release was only modest with both the Ni-NP and Alum groups (cytokine levels in the picogram per mL range). It is interesting to note that although weak antibody responses could be generated with the unpurified Ni-NPs, this group did not induce strong cellular responses as demonstrated by both the splenocyte proliferation and IFN-γ release assays. This could be due to very little antigen actually being bound to the Ni on the NPs because of binding with the more accessible unentrapped lipid, as discussed above. Alternatively, the dose of DOGS-NTA-Ni given with these NPs is higher than that of the purified Ni-NPs and could have an affect on the immune responses. Therefore, further assessment various doses of Ni-NPs would be beneficial in elucidating the effect of Ni on the immune responses and the optimal doses for enhancing both cellular and humoral immune responses. It is important to note that based on the AES characterization of Ni-NPs, the dose of Ni administered to mice in each 100 μL injection in the purified Ni-NPs was 20 ng. This amount of Ni is significantly lower than the levels of Ni that have shown adverse effects in mice [452-455]. To provide additional perspective, the average human

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diet is estimated to contain 0.15 mg Ni per day and drinking water alone contains 0.001 to 0.01 mg Ni per Liter [456]. Thus, the dose of Ni used in these studies was considered to be within the tolerable range.

An additional indicator of the type of immune response generated (i.e. Th1 or Th2) are the serum isotype levels, IgG1 versus IgG2a. During an immune response, the release of Th1 or Th2 cytokines will affect the production of these antibodies. BALB/c mice normally produce antibodies of IgG1 isotype; however, in the presence of Th1 type immune responses, the cells produce IFN-γ which causes a switch in the isotype produced to IgG2a. The isotype analysis in these studies revealed that the Ni-NPs resulted in the highest levels of IgG2a compared to all groups, while the IgG1 levels were comparable to the other three immunized groups (Figure 7.13).

In conclusion, the preparation of novel NPs containing a small amount of surface-chelated nickel was shown to be effective for enhancing the interaction with his-tag proteins compared to simple charged particles. This interaction of the antigen to the Ni-NPs resulted in superior humoral immune responses *in vivo* compared to protein adjuvanted with Alum or coated on charged NPs. Moreover, the Ni-NPs are also promising for generating Th1 type immune responses. It is hypothesized that the Ni-NPs enhance immune responses by increasing the interaction of the antigen with the NPs and allowing for greater amount of the antigen to be taken up into APCs. Although further studies are necessary to elucidate the exact mechanism(s) of immune response enhancement, preliminary *in vitro* data suggests that Ni-NPs do not enhance immune responses by causing the release of IL-12, a Th1 driving cytokine, from DCs (data not shown). Taken together, these data demonstrate the potential applications of Ni-NP for

vaccine delivery and warrant further investigation of these systems for enhancing cellular and humoral immune responses to protein-based vaccines.

Acknowledgements

Table 7.1. Ni spike and recovery from NTA-NPs. Spike and recovery studies with Ni were performed at 10 and 50 ppb using 0.4 and 2.0 mg of NTA-NPs. Results shown are average of n=3.

NTA-NPs	Ni spike	Average Ni Recovery (%)	Standard Deviation
0.4 mg	10 ppb	102.1	19.0
0.4 mg	50 ppb	104.8	1.8
2.0 mg	10 ppb	87.8	9.6
2.0 mg	50 ppb	89.6	5.1

Table 7.2. Quantitation of Ni on NP surface before and after GPC purification by AES. *Values reflect amount of Ni per 2 mg of NPs**.

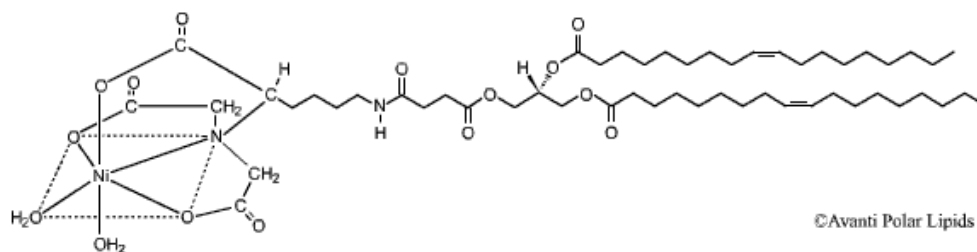| Ni-NP Preparation	μg Ni before GPC*	μg Ni after GPC*	Molecules Ni per particle	Molecules of GFP per particle	Molar ratio of GFP to Ni
1	8.19	0.53			
2	7.87	0.38	3557	1039	1 to 3
3	7.82	0.27			
4	7.85	0.49			

Figure 7.1

Figure 7.1. Structure of DOGS-NTA-Ni. NTA occupies four of the Ni coordination sites, leaving two unoccupied sites (shown coordinating with water in figure) for interaction with the histidine residues. (Structure was taken from Avanti Polar Lipids website: http://www.avantilipids.com/SyntheticNickel-ChelatingLipids.asp).
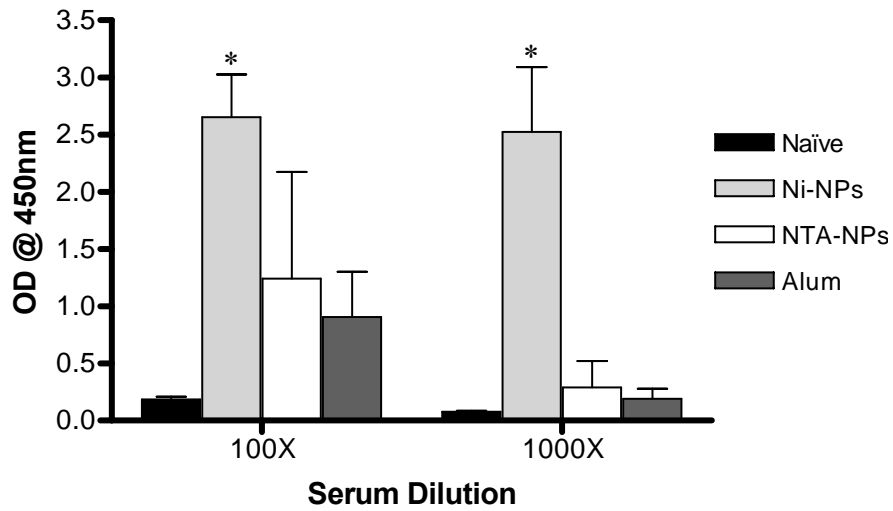
Figure 7.2

Figure 7.2. **Gag p24-specific IgG levels in serum at 4 weeks post initial immunization**.   Mice were immunized with 2.5 μg of his-tag p24 bound to Ni-NPs (25 μg), coated on NTA-NPs (25 μg), or adjuvanted with Alum (25 μg) on day 0 and day 14. Data for each group represents the mean ± S.D. (n=5-6).   *p<0.01 compared to all groups.

Figure 7.3
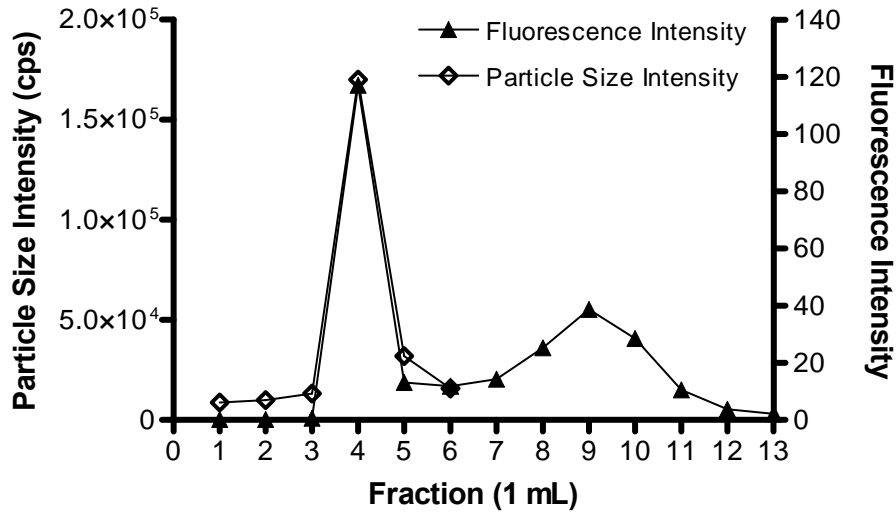
Figure 7.3. Elution profile of Ni-NPs, his-tag GFP bound to Ni-NPs, and unbound his-tag GFP on Sepharose CL4B GPC column. Ni-NPs eluted in fraction 3-6 as determined by particles size intensity. The extent of his-tag GFP bound to Ni-NPs was determined by separating protein bound to Ni-NPs from free protein, which elutes in faction 8-12 as determined by fluorescence intensity measurements. Fluorescence of the protein when bound to Ni-NPs overlaps with correlating particle size intensities in fraction 3-6.

Figure 7.4
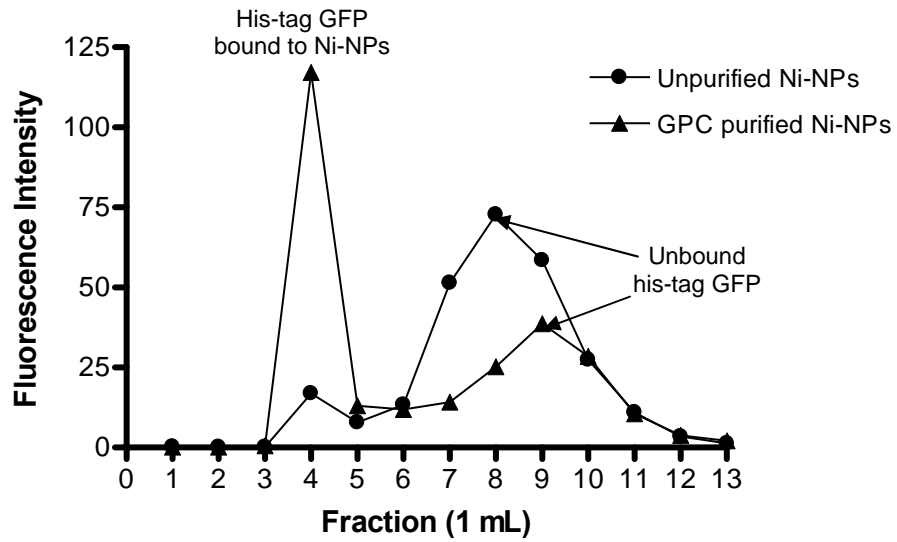
Figure 7.4. Separation profiles for his-tag GFP bound to GPC purified Ni-NPs and unpurified Ni-NPs. Free protein elutes in fraction 8-12, whereas the peak for the protein bound to unentrapped lipid in unpurified Ni-NPs was shifted to the left to fractions 7-12. The binding efficiency of his-tag GFP to Ni-NPs was increased by first separating the unentrapped lipid from the NPs by GPC.

Figure 7.5
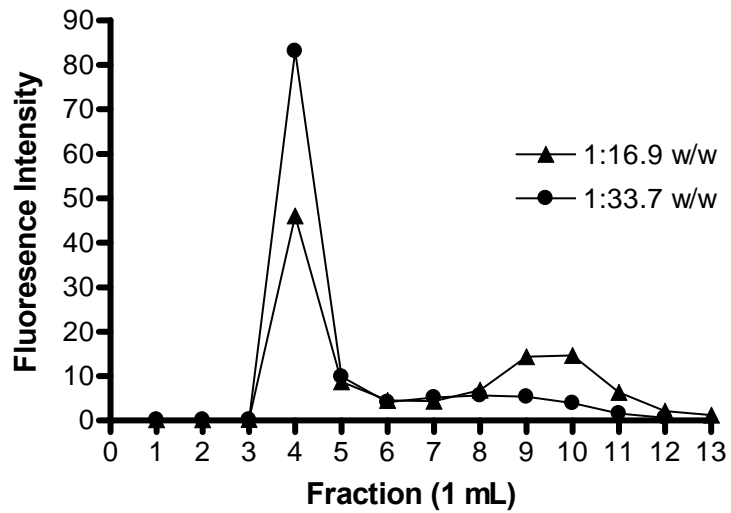
Figure 7.5. GPC purification profiles for his-tag GFP bound to Ni-NPs at 1:16.9 and 1:33.7 w/w ratios of protein to Ni-NPs. The binding of his-tag GFP to Ni-NPs was evaluated at the ratios indicated by separating free protein from protein bound to Ni-NPs using GPC. At the 1:33.7 w/w ratio, greater than 80% of the protein was associated with the Ni-NPs.

Figure 7.6
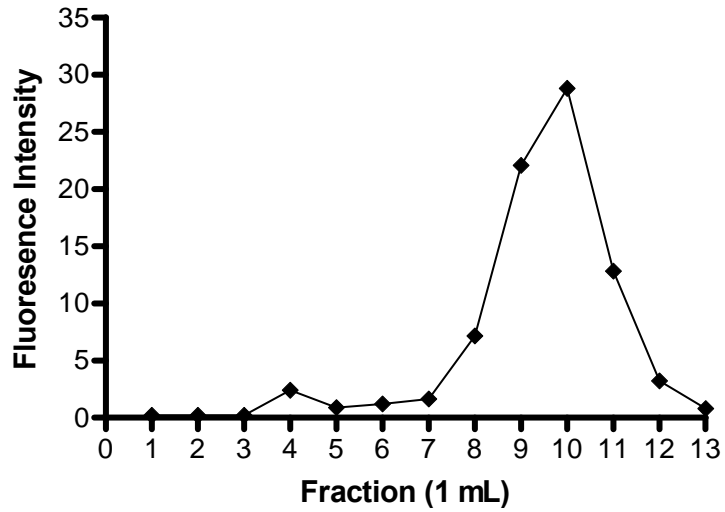
Figure 7.6. GPC profile for his-tag GFP mixed with NTA-NPs. His-tag GFP was incubated with NTA-NPs at 1:33.7 w/w ratio overnight at 4°C in PBS, pH 7.4. The interaction of the protein with the NPs was evaluated to determine specificity of the binding with NTA-NPs compared to Ni-NPs. Only 7% of the protein was associated with the NTA-NPs, based on fluorescence intensity measurements associated with fractions 3-6. The majority of the protein was detected in later fractions, 8-12, where free his-tag GFP was expected to elute.

Figure 7.7
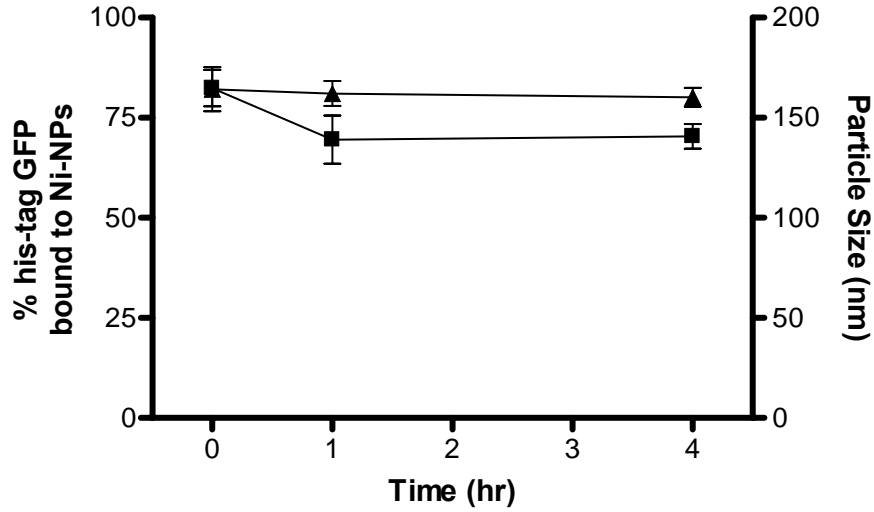
Figure 7.7. Stability of his-tag GFP bound to Ni-NPs at 37°C in PBS, pH 7.4. His-tag GFP was bound to Ni-NPs (1:33.7 w/w) by overnight incubation at 4°C in PBS, pH 7.4. The formulation was then incubated at 37°C to evaluate stability of the interaction with the Ni-NPs. Aliquots of the formulation were taken at various time points and passed through a Sepharose CL4B GPC column to evaluate the percent of his-tag GFP remaining associated with the Ni-NPs. The particle sizes were measured using fraction 4 from the GPC purification.

Figure 7.8
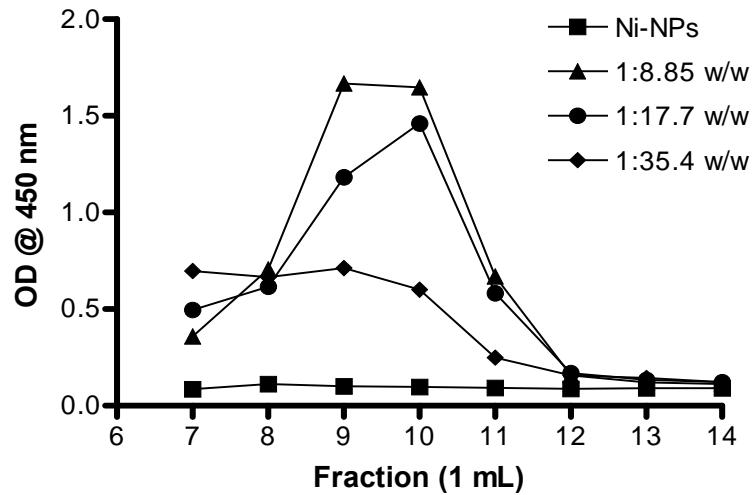
Figure 7.8. Free his-tag p24 eluting from GPC column. His-tag p24 was bound to Ni-NPs at various ratios and the relative amounts of free protein eluting from the Sepharose CL4B GPC column were traced in the fractions were free protein was expected to elute using ELISA. Ni-NPs with no protein were run as control with the ELISA to account for any interference.

Figure 7.9
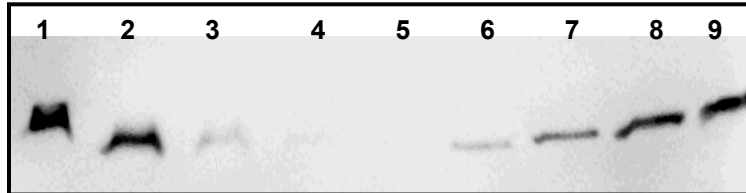
Figure 7.9. Western blot of his-tag p24 bound to Ni-NPs. His-tag p24 was incubated to Ni-NPs at various ratios and the protein bound to Ni-NPs was separated from free protein by passing through a Sepharose CL4B GPC column. Fractions 3-5 from the GPC purification were collected, combined, and analyzed by western blot to determine the relative amounts of his-tag p24 bound to the Ni-NPs. The lanes correspond to the following samples: 1) 1:70.8 w/w; 2) 1:35.4 w/w; 3) 1:17.7 w/w; 4) 1:8.85 w/w; 5) Ni-NPs as control; and lanes 6-9 are 50, 100, 200, and 250 ng of his-tag p24 standards loaded as controls, respectively. Greater than 80% of the his-tag p24 was bound to the Ni-NPs at ratios greater than 1:35.4 w/w.

Figure 7.10
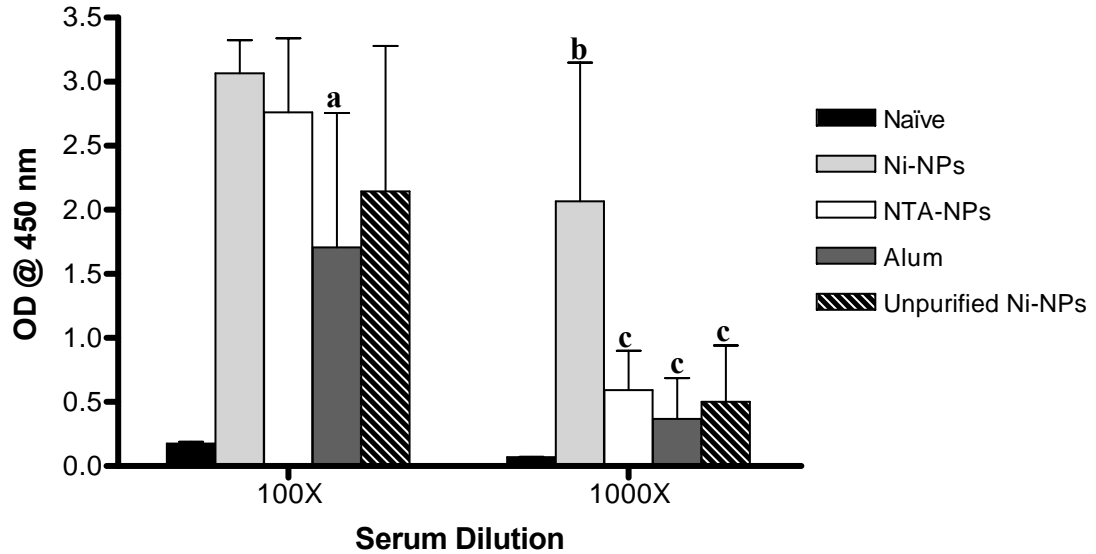
Figure 7.10. **Total serum IgG levels for his-tag p24 immunization with optimized formulations**. Mice were immunized with 2.5 μg of his-tag p24 bound to Ni-NPs (88.5 μg), coated on NTA-NPs (88.5 μg), adjuvanted with Alum (88.5 μg) or mixed with unpurified Ni-NPs (88.5 μg) on day 0 and day 14. Serum IgG levels were measured on day 28. Data for each group represents the mean ± S.D. (n=6-8). [a]p<0.01 compared to Ni-NP group; [b]p< 0.001 compared to all groups; [c]p>0.05 compared to naïve group.

Figure 7.11
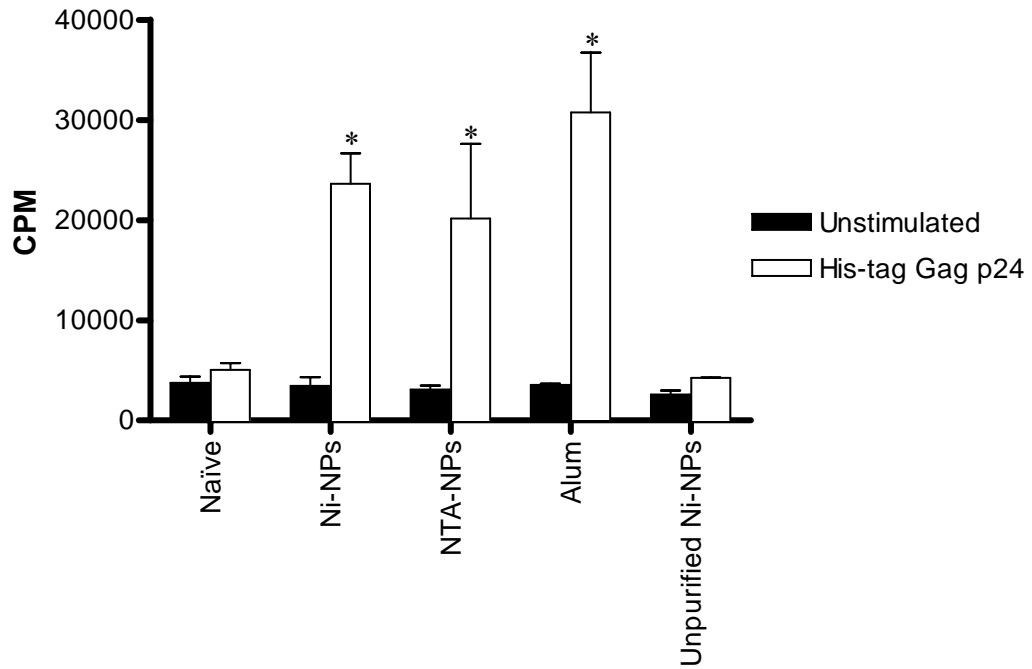
Figure 7.11. Splenocyte proliferative responses to his-tag p24 on day 5. Mice were immunized with 2.5 µg of his-tag p24 on day 0 and day 14. Spleens were harvested and pooled for each group on day 28. Cells (5×10^5/well) were stimulated with 1 µg/mL his-tag p24 and the incorporation of ^{3}H-thymidine in cells was evaluated on day 5. The data represents the mean ± S.D. (n=3). *p<0.05 compared to unstimulated cells.

Figure 7.12
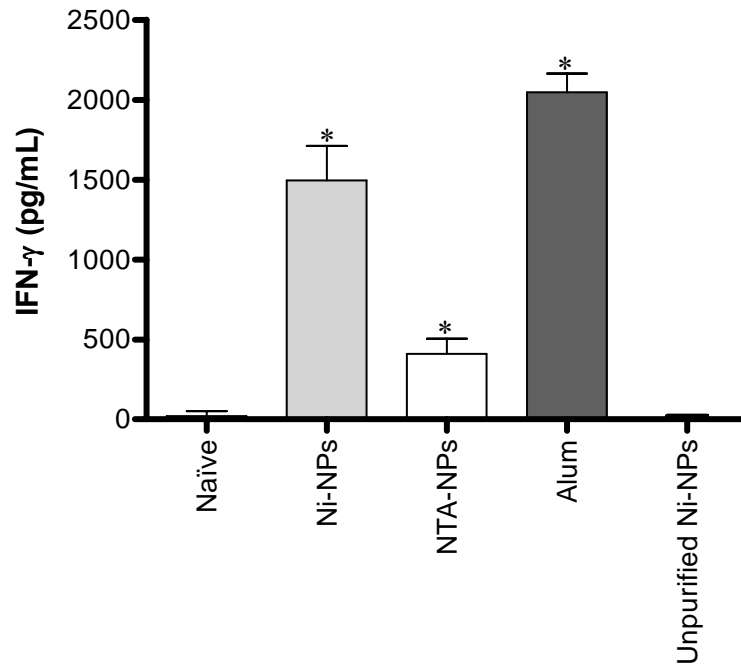
Figure 7.12. 72 hr IFN-γ release from stimulated splenocytes. Mice were immunized with 2.5 μg of his-tag p24 on day 0 and day 14. The spleens were harvested and pooled for each group on day 28. Cells ($1x10^6$) were stimulated with 1 μg/mL his-tag p24 and the supernatants were evaluated for IFN-γ release at 72 hr by ELISA. The data represents the mean ± S.D. (n=3). *p<0.05 compared to unstimulated cells.

Figure 7.13
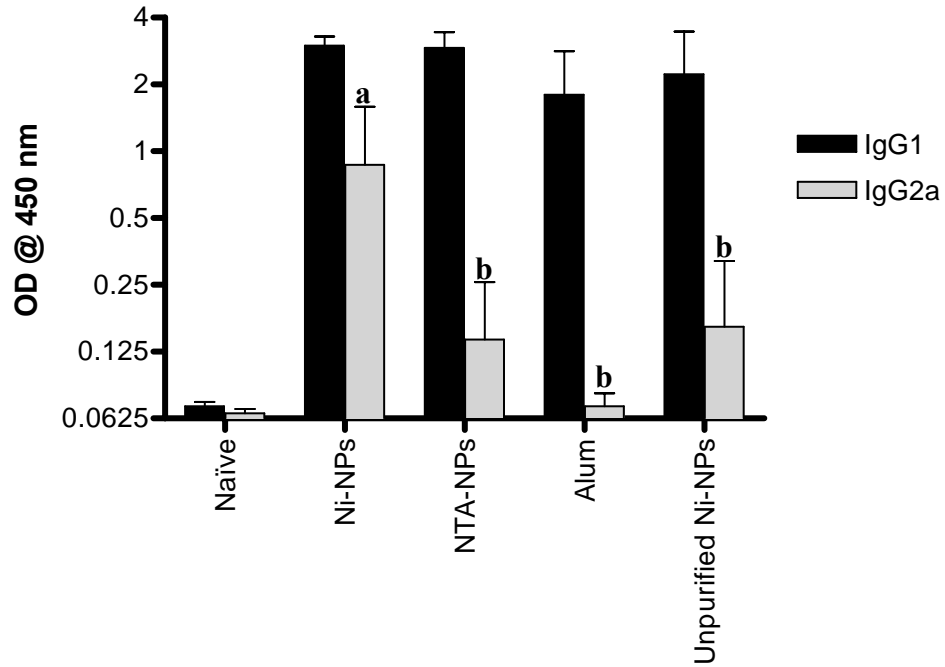
Figure 7.13. His-tag p24-specific serum IgG1 and IgG2a levels. Serum IgG isotype levels were measured on day 28 by ELISA using a 1:1000 serum dilution. Data for each group represents the mean ± S.D. (n=6-8). [a]p<0.001 compared to all groups; [b]p>0.05 compared to naïve group.

Chapter 8

In vivo immune responses to Tat coated on different anionic nanoparticle formulations

8.1   Summary

Anionic nanoparticles can be prepared from oil-in-water microemulsion precursors using emulsifying wax as the oil phase and sodium dodecyl sulfate (SDS) as the surfactant. The stability of these anionic nanoparticles in buffered solutions was improved by the inclusion of the co-surfactant Brij 78. The studies in this chapter were carried out to evaluate the immune responses to nanoparticles prepared using different surfactant compositions. Three anionic NP formulations were prepared using the following composition of surfactants: 15 mM SDS, 15 mM SDS/1 mM Brij 78 and 1 mM SDS/3 mM Brij 78. These nanoparticle formulations were coated with the cationic HIV-1 Tat protein and evaluated *in vivo*. No significant differences in the humoral immune responses were generated among all the Tat-coated nanoparticle formulations evaluated. Moreover, an improved LTR-transactivation assay demonstrated that significant Tat-neutralizing antibodies were generated with all formulations evaluated. For the cellular responses, some differences were observed in an initial study using a 1 µg Tat dose; however, no significant differences among the groups were detected in a follow up study using a 5 µg Tat dose. Taken together, these data suggest that the composition of surfactants used to prepare the anionic nanoparticles do not have a significant influence on the immune responses generated to HIV-1 Tat protein.

8.2   Introduction

Nanoparticles prepared from oil-in-water microemulsions precursors have been reported to enhance cellular and humoral immune responses to pDNA- and protein-based vaccines [14-16,46,253]. These nanoparticles (NPs) are prepared using emulsifying wax as the oil phase and appropriate surfactants to obtain particles with the desired surface properties. For example, cationic surfactants such as cetyl trimethyl ammonium bromide (CTAB) can be used to obtain net positively charged particles and surfactants with a negative charge such as sodium dodecyl sulfate (SDS) can be used to obtain anionic particles. These particles with different surface properties can be further used to coat anionic or cationic molecules and/or proteins on the NP surface for enhanced immune responses compared to the protein alone or adjuvanted with Alum.

Previous studies in our laboratory have shown that anionic NPs prepared using 15 mM SDS as the surfactant generate superior cellular and humoral immune responses to β-galactosidase [16] and HIV-1 Tat protein [253]. However, these NPs were also shown to contain an excess amount of the SDS surfactant, which could be removed by purification using gel permeation chromatography (GPC) [16]. In a more recent study, the use of sterically stabilized NPs prepared using 1 mM SDS/3 mM B78 was investigated with Tat as it allowed for preparation of net negatively charged NPs with a minimal amount of excess SDS present [438]. In our studies, this is advantageous because it avoids the need for GPC purification and estimating the dose of NPs used for *in vivo* studies. Moreover, these NPs coated with Tat demonstrated superior humoral immune responses compared to Alum.

To evaluate if anionic NPs prepared from different surfactant compositions could influence the type of immune responses generated to the coated antigen *in vivo*, anionic NPs having different surfactant compositions were investigated. Anionic NPs were prepared using emulsifying wax as the oil phase and the following surfactant combinations: 1) 15 mM SDS/1 mM Brij 78; 2) 1 mM SDS/3 mM Brij 78; and 3) 15 mM SDS. All anionic NP formulations were coated with HIV-1 Tat protein and the Tat-specific cellular and humoral immune responses were evaluated to identify differences in the NP formulations.

8.3   Materials and methods

Materials

Emulsifying wax, comprised of cetyl alcohol and polysorbate 60 (molar ratio of 20:1) and sodium dodecyl sulfate (SDS), were purchased from Spectrum (New Brunswick, NJ). PBS/Tween 20 buffer, bovine serum albumin (BSA), Sephadex G75 and mannitol were purchased from Sigma Chemical Co. (St. Louis, MO). Brij 78 was purchased from Uniqema (New Castle, DE). Sheep anti-mouse IgG, peroxidase-linked species specific F(ab')$_2$ fragment was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Goat anti-mouse IgG2a and IgG1 horseradish peroxidase (HRP) conjugates were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). IFN-γ ELISA kit was from BD Biosciences Pharmingen (San Diego, CA). Tetramethylbenzidine (TMB) substrate kit was purchased from Pierce (Rockford, IL). Centricon$^®$ YM-50 ultracentrifuge devices were from Fisher Scientific (Hampton, NH). HIV-1 Clade B consensus Tat peptides (15 aa) were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MA). Recombinant HIV-1 Tat (1-72 aa) was prepared as previously described [362].

Preparation of anionic NPs

NPs from oil-in-water microemulsion precursors were prepared as previously described with slight modification [16,253]. The composition of the various formulations is described in Table 8.1. For formulations incorporating Brij 78 (B78), 4 mg of emulsifying wax and appropriate amount of Brij 78 was melted and mixed at ~60-65°C.

Deionized and filtered (0.2 μm) water was added to the melted wax and surfactant while stirring to form an opaque suspension. Finally, an appropriate volume of sodium dodecyl sulfate (50 mM) was added to form clear microemulsions at 60-65°C. The microemulsions were cooled to room temperature while stirring to obtain NPs (2 mg/mL). The NP sizes were measured using a Coulter N4 Plus Sub-Micron Particle Sizer (Coulter Corporation, Miami, FL) at 90°. The overall charge of the NPs was measured using Malvern Zeta Sizer 2000 (Malvern Instruments, Southborough, IL).

GPC to remove excess surfactant in anionic NP formulations

Previously, it was demonstrated that there was an excess of surfactant present when formulating NPs with 15 mM SDS [16]. This excess surfactant was shown to be separated from the NPs by passing through a GPC column packed with Sephadex G75 (15 x 75 mm). Therefore, to remove excess surfactant from the NPs prepared in this study, 300 μL of the NP formulation was passed through a Sephadex G75 column and the fractions containing the NPs (fraction 3-5) were collected for further use in coating with Tat. The GPC purification was also performed on the 1 mM SDS/3 mM B78 NPs to serve as an additional control in the *in vivo* experiments. All purifications were performed three times to obtain a total of 1.8 mg of purified NPs and the fractions collected from the GPC purifications were further concentrated using Centricon$^{®}$ YM-50 ultracentrifuge devices to obtain NPs at a final concentration of 2 mg/mL.

Coating of the anionic NPs with Tat

To prepare formulations for *in vivo* analysis, Tat, at a final concentration of 10 or 50 μg/mL, was added to the appropriate NP formulations (1000 μg/mL) in 5% (v/v) mannitol. The suspensions were vortexed gently and placed on a horizontal shaker at room temperature for a minimum of 30 min to allow for coating. The coated NPs were diluted appropriately in de-ionized water for measuring the size and charge of the particles.

Mouse immunization study

Two animal studies were carried out to determine the immune response to Tat coated on various anionic NP formulations. A summary of the experimental design is presented in Table 8.2. For both studies, female BALB/c mice (8-10 weeks old) obtained from Harlan Sprague-Dawley Laboratories (Indianapolis, Indiana) were immunized subcutaneously with 100 μL of the formulations. In the first study (study 1), mice were dosed three times at 2 week intervals with 1 μg of Tat coated on anionic NPs that had been purified by GPC or with 1 μg Tat coated on unpurified NPs (100 μg) prepared using 1 mM SDS/3 mM B78. The dose of anionic NPs for the GPC purified formulations was also estimated to be about 100 μg. The IgG responses were monitored by collecting sera via tail-vein bleed prior to boosting with Tat-coated NPs. On day 42, mice were bled by cardiac puncture and sera were separated. All sera collected were stored at -20°C. In addition, spleens were harvested from all mice and pooled for each group for splenocyte proliferation and IFN-γ release assays. In the second study (study 2), a similar protocol was followed except a 5 μg dose of Tat was evaluated with the anionic NP formulations

and the spleens in the study 2 were collected on day 42 and prepared individually for splenocyte proliferation and IFN-γ release assays.

Determination of antibody titers

Tat-specific serum IgG, IgG1 and IgG2a antibody titers were determined using an ELISA. This procedure is described in detail in Chapter 4 of the dissertation.

Tat anti-sera recognition of N-terminal and basic regions of Tat protein

Tat anti-sera in the first study were tested by ELISA to determine reactivity to the N-terminal and basic regions of Tat. The ELISA procedure is described in detail in Chapter 4.

LTR-transactivation assay

The LTR-transactivation assay was performed essentially as described in Chapter 4 with the following modifications. First, HeLa cell lines transfected with pHIV-CAT were used for the assay. Secondly, supernatant containing Tat released from HeLa cell lines (transfected with a Tat plasmid) was used as the source for extracellular Tat. The Tat-contatining supernatants were diluted at 1:40 and incubated with the sera for the assay. Sera were diluted at 1:10, 1:50, 1:250, and 1:1250 to evaluate for Tat-neutralizing antibodies.

Splenocyte proliferation and IFN-γ release assays

The spleens were crushed in 1X Hanks Balanced Salt Solution (HBSS) using a stomacher homogenizer for 60 s at normal speed to obtain single cell suspensions and the suspensions were then transferred into centrifuge tubes. Red blood cells were lysed adding 1X ACK buffer (156 mM NH_4Cl, 10 mM $KHCO_3$ and 100 μM EDTA) and incubating for 1-2 min at RT. The cells were spun down at 1500 rpm, 4^oC for 10 min. Supernatants were decanted and the cells were washed 2 more times with 1X HBSS. The cells were resuspended in RPMI 1640 (supplemented with 10% heat-inactivated fetal calf serum, 1 mM HEPES, 2 μM L-glutamine, 10 U/mL penicillin, 100 U/mL streptomycin, 50 μM 2–mercaptoethanol). In Study 1, for the splenocyte proliferation assay, cells ($5x10^5$ cells/well) were added to a 96-well plate and incubated with media, Con A (2 μg/mL), Tat (0.1 and 1 μg/mL) or pooled 15-mer Tat peptides (10 μg/mL each peptide) at 37^oC, 7% CO_2 for 4 days. The cells were pulsed with 1 μCi of ^{3}H-thymidine on day 4 and incubated for an additional 24 hr at 37^oC, 7% CO_2. The 15-mer Tat peptides were pooled as follows: Set 1 = aa 1-15, 5-19, 9-23, 13-27; Set 2 = aa 17-31, 21-35, 25-39: Set 3 = 29-43, 33-47, 37-51; Set 4 = aa 41-55, 45-59, 49-63, 53-67; Set 5 = 57-71, 61-75, 65-79, 69-83. To measure IFN-γ release from stimulated splenocytes, parallel 48-well plates were set up using $1x10^6$ cells/well in 400 μL of media and stimulated as described for the proliferation assay. The supernatants were collected at 72 hr and stored at -80^oC for IFN-γ analysis by ELISA.

For study 2, the splenocyte proliferation and IFN-γ release assays were carried out as described as above. The IFN-γ assay was set up in parallel to splenocyte proliferation assay using 96-well plates using $5 x 10^5$ cells/well.

Statistical analysis

Statistical analysis was performed using one-way analysis of variances (ANOVA) followed by pairwise comparisons with Tukey's multiple comparison test, when appropriate, using GraphPad Prism software.

8.4　Results and discussion

Preparation and characterization of Tat coated anionic NPs

Previously our laboratory reported on the preparation of anionic NPs from microemulsions precursors using 15 mM SDS as the anionic surfactant. These NPs were demonstrated to effective enhance immune responses to a model protein, β-galactosidase [16], and HIV-1 Tat [253]. Further work with this system revealed that upon removal of excess surfactant from this formulation, the NPs were unstable in buffered solutions. To further stabilize these systems, the used of B78 as a co-surfactant was investigated and it was found that at least 1 mM of B78 was required to provide stable NPs in buffered solutions (data presented in Appendix B). In addition, it was found that NPs prepared using various compositions of surfactant (Table 8.1) resulted in sizes of around 100-150 nm with a net charge of approximately -60 mV (Table 8.3). Upon GPC purification to remove excess surfactant all formulations retained a net negative charge, with the 1 mM SDS/3 mM B78 having a slightly lower zeta potential. Coating the NPs with an increasing concentration of Tat resulted in a decrease in the overall charge of the particles; however, the Tat-coated NPs continued to have a net negative charge possibly due to the exposed negatively charged amino acids of the protein or due to an excess amount of anionic NPs present.

Immune responses to Tat coated on anionic NPs:　Study 1

In vivo responses to the different anionic NPs were evaluated in BALB/c mice to identify if using different surfactant compositions would affect the cellular and/or

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humoral immune responses generated to Tat. As shown in Figure 8.1, there were no significant differences in the Tat-specific antibody titers produced at all time points assessed. Moreover, the evaluation of IgG1 and IgG2a antibody titers did not demonstrate any significant differences in the formulations (Figure 8.2). These data combined suggest that the different formulations were equally effective at generating humoral immune responses and Th1 type immune responses, as indicated by the IgG2a production with the various formulations.

Previous results with Tat coated on anionic NPs (1 mM SDS/3 mM Brij 78) demonstrated that the anti-sera from Tat immunized animals produced antibodies with specificity in the N-terminal and basic regions of the protein [438]. To further assess the different anionic formulations in their ability to produce antibodies to Tat specific for these regions, the anti-sera collected in Study 1 was evaluated for binding to 15-mer Tat peptides covering the N-terminal and basic regions of Tat. As shown in Table 8.4, all groups contained antibodies that recognized these regions of Tat and no obvious differences due to the different formulations of NPs were noted.

The Tat-neutralizing antibodies generated using different anionic NP formulations were assessed using an LTR-transactivation assay. In the previous study presented in Chapter 4, modest Tat-neutralizing activity was demonstrated with all Tat anti-sera compared to literature reports. This was speculated to be due to differences in assay conditions and thus, further work to optimize the LTR-transactivation assay was carried out. In attempt to improve the assay, two modifications were made to the existing LTR-transactivation assay: 1) HeLa cells transfected with pHIV-CAT were used instead of the SVGA cells, and 2) Tat-containing supernatants replaced the use of recombinant Tat as

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the source for extracellular protein in the assay. Using this modified assay, the sera from all Tat-immunized groups demonstrated the ability to neutralize extracellular Tat as shown in Figure 8.3. More importantly, the Tat-neutralizing activity was retained at higher serum dilutions, which was not possible in the assay described in Chapter 4. Therefore, the altered conditions improved the sensitivity of the assay and studies to further optimize the assay are ongoing.

To evaluate cellular immune responses, the spleens were pooled and stimulated with 0.1 and 1.0 μg/mL Tat or with pooled 15-mer Tat peptides. No significant Tat-specific splenocyte proliferative responses compared to the naïve group were detected at a concentration of 0.1 μg/mL Tat (Figure 8.4). However, at Tat concentration of 1.0 μg/mL, significantly higher Tat-specific proliferation was seen with all NP groups compared to the naïve group. In addition, the Tat-specific splenocyte proliferative response with the 15 mM SDS group was significantly higher than both of the 1 mM SDS/3 mM B78 groups ($p < 0.05$). Tat has been observed to induce non-specific proliferation of naïve cells as seen in Figure 8.4. This has also been reported by other groups [412]. Thus, the use of pooled 15-mer Tat peptides was also evaluated in effort to identify the appropriate sequence(s) for future assays. As shown in Figure 8.5, the strongest response compared to the naïve group was observed with peptide set 5, which included the C-terminus peptides for Tat; however, no significant differences were observed among the test groups.

The IFN-γ release from stimulated splenocytes was evaluated in 72 hr supernatants by ELISA. Once again, Tat-induced non-specific IFN-γ release in naïve cells, proving it difficult to assess cellular responses with the test groups (Figure 8.6).

The strongest IFN-γ release was observed with the 1 mM SDS/3 mM B78 control (Ctrl) and 15 mM SDS NP groups using the 1.0 μg/mL Tat concentration. Moreover, IFN-γ release from splenocytes stimulated with the pooled 15-mer Tat peptides demonstrated strongest responses with the 15 mM SDS NP group with set 4 and 5 as shown in Figure 8.7; however, it should be noted that the IFN-γ release was quite modest compared to that obtained with Tat protein. In addition, non-specific IFN-γ release from naïve cells stimulated with peptide set 5 was also observed. Taken together, these data suggested that while no differences existed in the humoral immune responses to the various formulations, potential differences in the cellular responses (IFN-γ release) to Tat using the different formulations may exist and were further evaluated in a second study.

Immune responses to Tat coated on anionic NPs: Study 2

Based on the immune responses results from Study 1, a second study to evaluate the immune responses to Tat was carried out with these three formulations: 15 mM SDS/1 mM B78; 1 mM SDS/3 mM B78 (unpurified control); and 15 mM SDS. In this study, purification of 1 mM SDS/3 mM B78 was not included because of the lack of significant differences between this group and the control group in the first study. In the second immunization study, a higher dose of Tat, 5 μg, was used for evaluating Tat-specific cellular responses to enable easier identification of differences among the NP groups. As observed in Study 1, no significant differences were observed in the Tat-specific IgG titers on day 42 (Figure 8.8). Furthermore, the splenocyte proliferation assay demonstrated Tat-specific proliferation with all groups including the naïve group making it difficult to point out any differences among the groups (Figure 8.9).

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Surprisingly, no proliferation to any of the pooled Tat peptide sets was observed with the groups and in the IFN-γ release assay only one animal from the 1 mM SDS/3 mM B78 group demonstrated a strong response after stimulation with all of the pooled Tat peptide sets (data not shown). The IFN-γ release data also did not show any significant differences among the groups, and non-specific IFN-γ release after stimulation with the Tat protein in the naïve group was observed (Figure 8.10).

In summary, previous reports from our laboratory have demonstrated that anionic NPs were effective at enhancing the immune responses to protein-based vaccines. In the present studies, further work was carried out to evaluate if any differences in the cellular and/or humoral immune responses could be identified using anionic NPs of varying surfactant composition. Based on the data from two *in vivo* studies using 1 and 5 μg of Tat coated on these various anionic NP formulations, no significant differences in the humoral responses were observed. Likewise, all NP formulations used were effective at generating neutralizing antibodies to Tat as demonstrated by the improved LTR-transactivation assay. Overall, the cellular responses to the anionic NPs also did not show strong differences. However, there were numerous challenges faced with assessing cellular immune responses to the Tat protein. For example, Tat demonstrates mitogenic activity in the cellular assays. The use of Tat peptides was also investigated but it was difficult to identify the optimal regions to include in future studies and further studies optimizing the cellular assays to evaluate Tat-specific responses are necessary.

Acknowledgements

I would like to thank Dr. Avindra Nath's laboratory at John's Hopkins University for performing the LTR-transactivation assay. I would also like to thank Martin Ward in Dr. Woodward's laboratory for his help with the *in vivo* studies.

Table 8.1. Composition of anionic NP formulations.

Formulation	Brij 78 (mg)	Volume of 50 mM SDS (μL)	DI Water (mL)
15 mM SDS	0	600	1.4
15 mM SDS/1 mM Brij 78	2.3	600	1.4
1 mM SDS/3 mM Brij 78	6.9	40	1.96

Table 8.2. Experimental conditions for mouse immunization studies.

Study	Formulation	Tat Dose	Immunization Schedule	Sera Collected
Study 1	Naïve	-	-	Day 13, 35, 42
	15 mM SDS	1 μg	Day 0, 14, 28	
	15 mM SDS/1 mM B78	1 μg		
	1 mM SDS/3 mM B78	1 μg		
	1 mM SDS/3 mM B78 (unpurified Control)	1 μg		
Study 2	Naïve	-	-	Day 42
	15 mM SDS	5 μg	Day 0, 14, 28	
	15 mM SDS/1 mM B78	5 μg		
	1 mM SDS/3 mM B78 (unpurified Control)	5 μg		

Table 8.3. Physical characteristic of anionic NP formulations.

Formulation	Before GPC		After GPC		Tat Coated (10 µg/mL)		Tat Coated (50 µg/mL)	
	Mean size (nm)	Mean Charge (mV)	Mean size (nm)	Mean Charge (mV)	Mean size (nm)	Mean Charge (mV)	Mean size (nm)	Mean Charge (mV)
15 mM SDS	113.3 ± 13.3	-64.6 ± 1.8	150.6 ± 5.4	-63.2 ± 2.0	119.7 ± 9.8	-58.9 ± 6.2	114.8 ± 19.4	-51.1 ± 2.3
15 mM SDS/1 mM B78	121.4 ± 10.1	-61.3 ± 1.9	145.9 ± 11.4	-63.9 ± 3.6	130.7 ± 10.5	-62.0 ± 4.3	122.2 ± 0.3	-51.7 ± 2.6
1 mM SDS/3 mM B78	147.9 ± 10.9	-61.2 ± 3.6	172.4 ± 9.9*	-43.3 ± 5.5*	146.7 ± 13.3	-44.4 ± 5.8	-	-
1 mM SDS/3 mM B78 (unpurified Control)	147.9 ± 10.9	-61.2 ± 3.6	-	-	151.27 ± 20.8	-53.8 ± 3.3	153.4 ± 14.9	-46.5 ± 1.4

The mean sizes and charges ± S.D. (n=3) are shown.
*represents mean ± S.D. (n=2).

Table 8.4. Tat anti-sera reactivity to N-terminal and basic regions of Tat. All sera from Study 1 were diluted 1:100. The reactivity of anti-sera from each animal is presented. *Cutoff = (AVG Naïve response) + (3*SD). (-) indicates no response – ELISA OD values equal to cutoff/background.

Tat Peptide	aa 1-15	aa 5-19	aa 9-23	aa 45-59	aa 49-63
15 mM SDS/1 mM B78					
1	1.880	2.887	-	-	-
2	1.998	2.831	0.509	0.358	0.423
3	2.743	2.862	-	-	-
4	-	-	-	0.286	0.314
5	1.073	1.355	0.184	-	-
6	0.884	2.515	0.187	-	-
7	2.691	2.656	0.168	-	0.227
1 mM SDS/3 mM B78					
1	0.847	0.749	0.183	-	0.235
2	1.118	2.042	2.837	-	-
3	2.183	1.010	0.181	-	-
4	2.526	2.515	-	0.472	0.388
5	0.397	1.515	0.231	0.356	0.305
6	1.363	1.695	-	-	0.32
15 mM SDS					
1	1.407	2.267	0.234	0.593	0.341
2	2.443	2.630	0.447	-	-
3	2.984	2.708	0.192	-	0.551
4	2.423	2.875	0.167	-	0.274
5	2.457	2.465	-	-	-
6	2.715	2.565	1.734	-	-
7	0.680	1.392	0.194	-	0.254
1 mM SDS/3 mM B78 Ctrl					
1	2.520	2.999	1.356	-	-
2	1.726	2.89	0.685	-	0.351
3	1.609	2.523	0.215	-	-
4	-	0.790	0.200	-	0.228
5	0.368	1.440	-	-	-
6	1.110	1.468	-	-	-
7	2.984	3.047	-	-	-
8	2.934	2.531	0.172	0.275	0.228
Cutoff*	0.31	0.18	0.16	0.27	0.20

Figure 8.1

Figure 8.1. Tat-specific serum IgG titers. BALB/c mice were immunized (s.c.) on day 0, 14, and 28 with 1 μg of Tat coated on the anionic NPs (100 μg). Sera were collected and analyzed by ELISA at various time points. Data represent the mean ± S.D. (n=6-8).
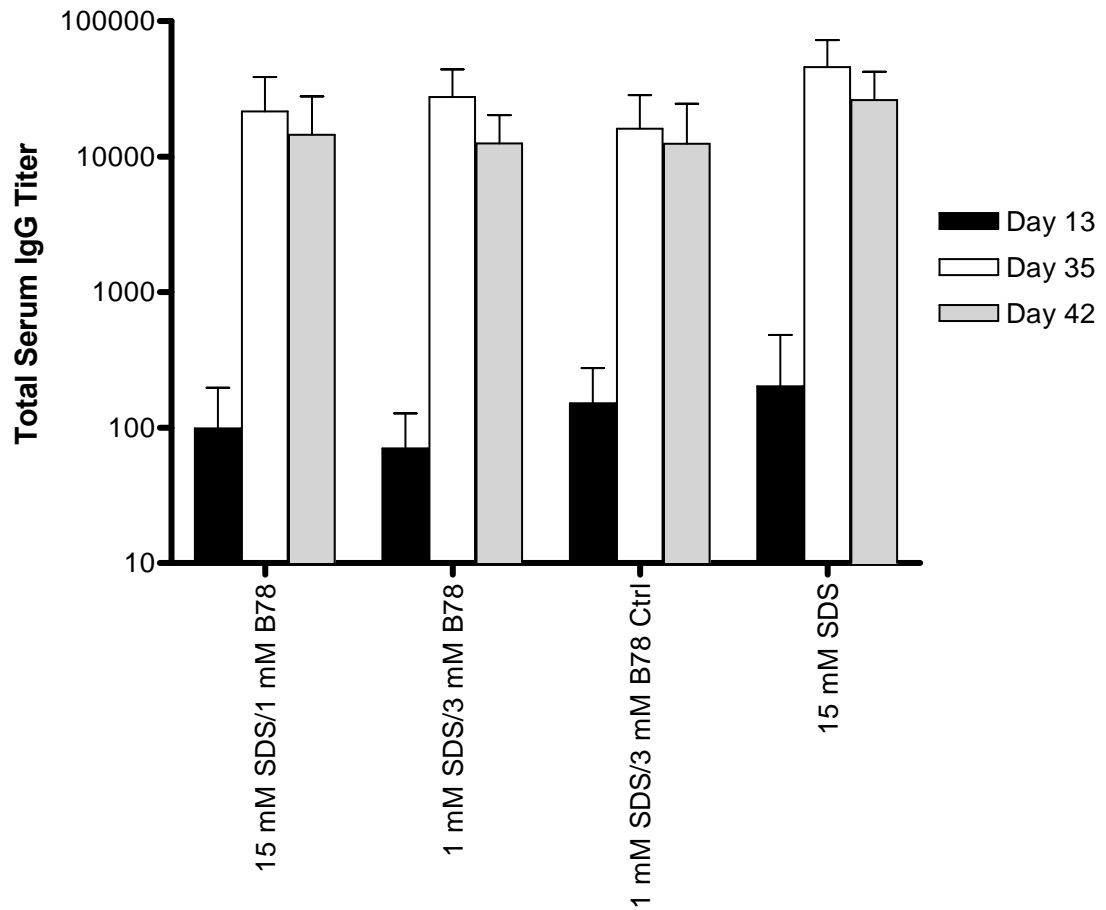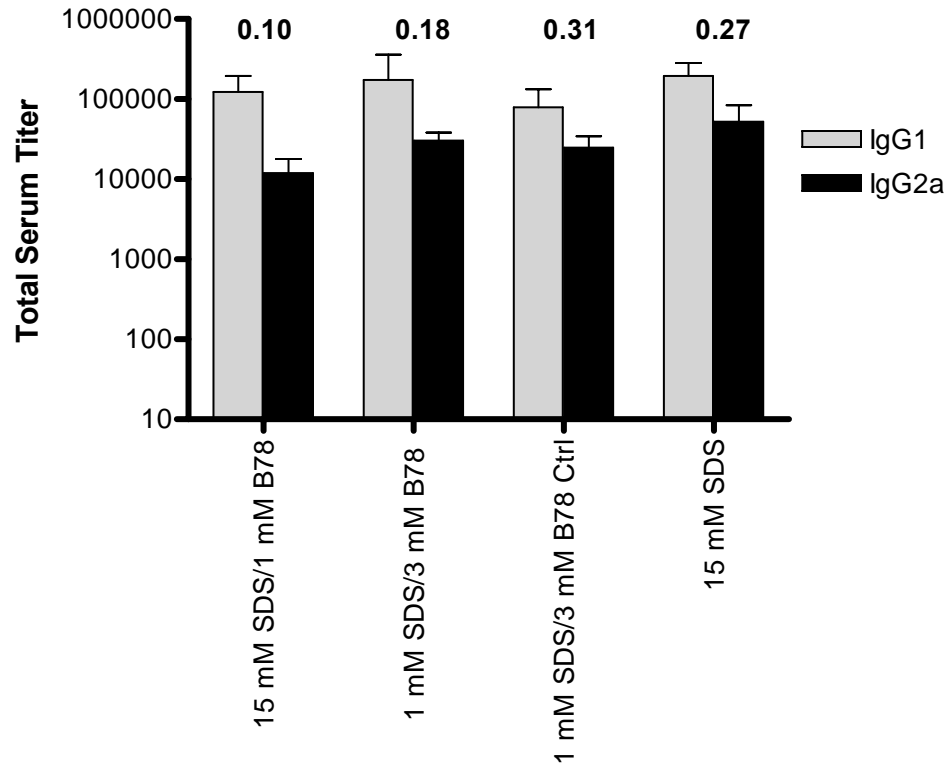
Figure 8.2

Figure 8.2. Tat-specific IgG1 and IgG2a titers. BALB/c mice were immunized (s.c.) on day 0, 14, and 28 with 1 μg of Tat coated on the anionic NPs (100 μg). Tat-specific serum IgG1 and IgG2a titers were evaluated on day 42 by ELISA. The mean IgG2a/IgG1 ratio is indicated on top of the graphed titers for each group. Data represent the mean ± S.D. (n=6-8).

Figure 8.3
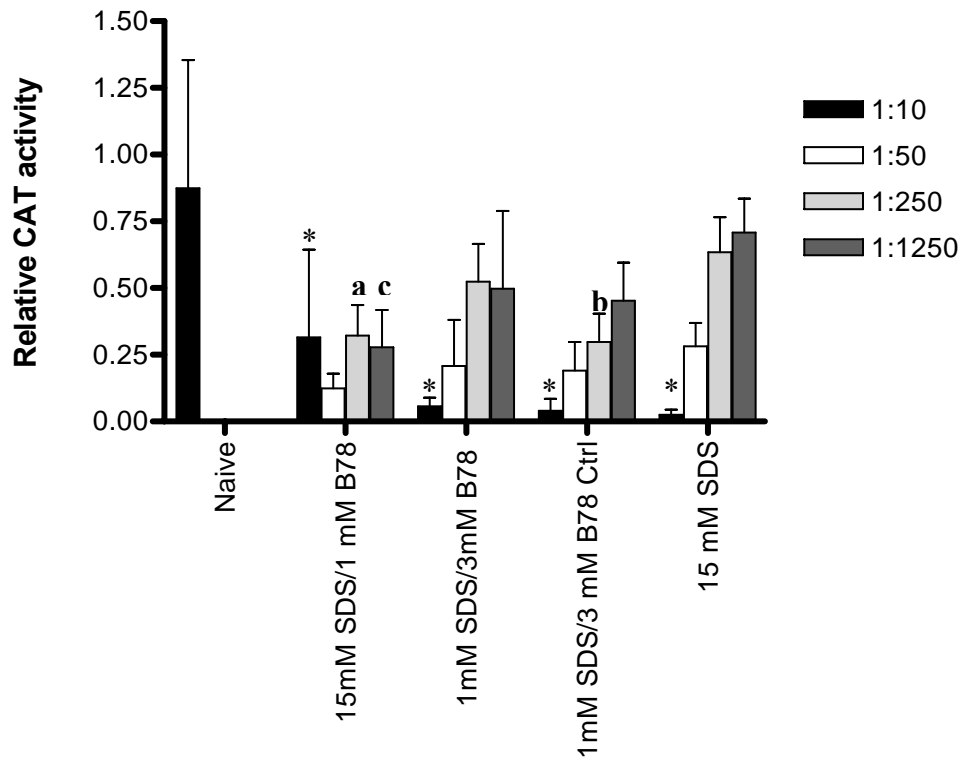
Figure 8.3. Neutralizing activity of Tat anti-sera using an improved LTR-transactivation assay. BALB/c mice were immunized on day 0, 14, and 28 with 1 µg of Tat-coated NPs and sera were collected on day 42. Serum from each mouse was diluted as indicated in the figure legend and evaluated for Tat neutralizing antibodies using an optimized LTR-transactivation assay. The relative amounts of CAT expression determined using an ELISA. Data represents mean ± SD (n = 6-8). *p<0.05 compared to naïve group; [a]p<0.05 compared to 1mM SDS/3mM B78 and 15mM SDS group; [b]p<0.05 compared to 1mM SDS/3mM B78 and 15mM SDS group; [c]p<0.05 compared to 15 mM SDS group.

Figure 8.4

Figure 8.4. Splenocyte proliferation on day 5. BALB/c mice (n=6-8 per group) were immunized (s.c.) on day 0, 14, and 28 with 1 μg of Tat coated on the anionic NPs (100 μg) and spleens were harvested and pooled for each group on day 42. Single cell suspensions of the spleen were stimulated with Con A or Tat for 4 days and the incorporation of ^{3}H-thymidine was evaluated on day 5. The data represent the mean ± S.D. (n=3). *p<0.01 compared to naïve group; #p<0.05 compared to the Ctrl and 1 mM SDS/3 mM B78 group.

Figure 8.5
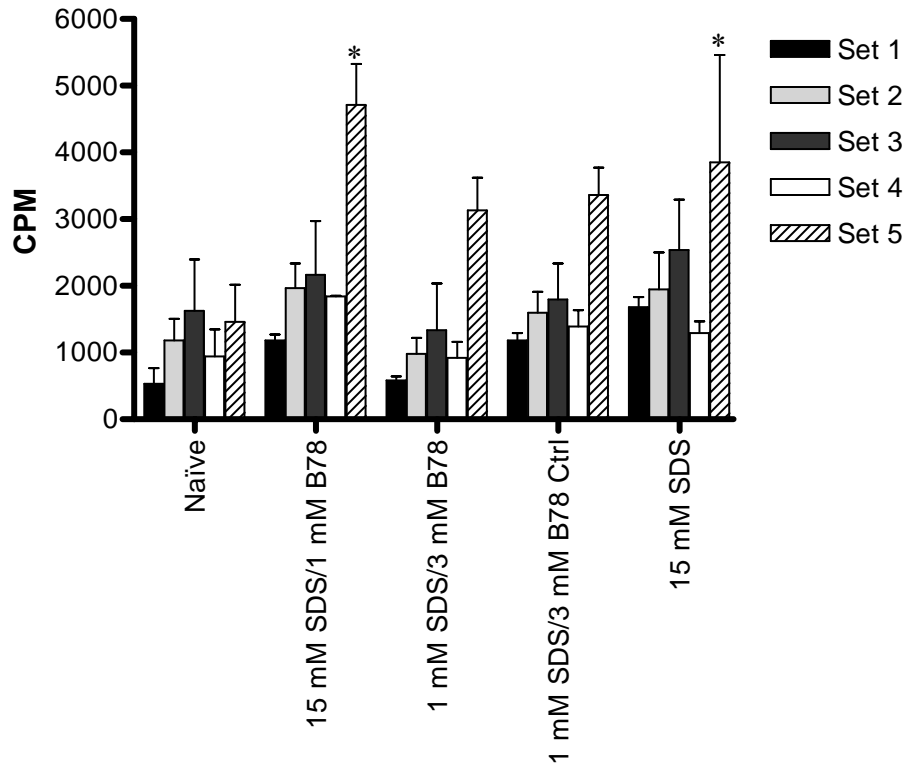
Figure 8.5. Splenocyte proliferative responses to 15-mer Tat peptides. BALB/c mice (n=6-8 per group) were immunized (s.c.) on day 0, 14, and 28 with 1 μg of Tat coated on the anionic NPs (100 μg) and spleens were harvested and pooled for each group on day 42. Single cell suspensions of the spleen were stimulated with pooled 15-mer Tat peptides for 4 days. The incorporation of ^{3}H-thymidine was evaluated on day 5. Set 1 = aa 1-15, 5-19, 9-23, 13-27; Set 2 = aa 17-31, 21-35, 25-39; Set 3 = 29-43, 33-47, 37-51; Set 4 = aa 41-55, 45-59, 49-63, 53-67; Set 5 = 57-71, 61-75, 65-79, 69-83. The data represent the mean ± S.D. (n=3). *$p<0.05$ compared to naïve group.

Figure 8.6
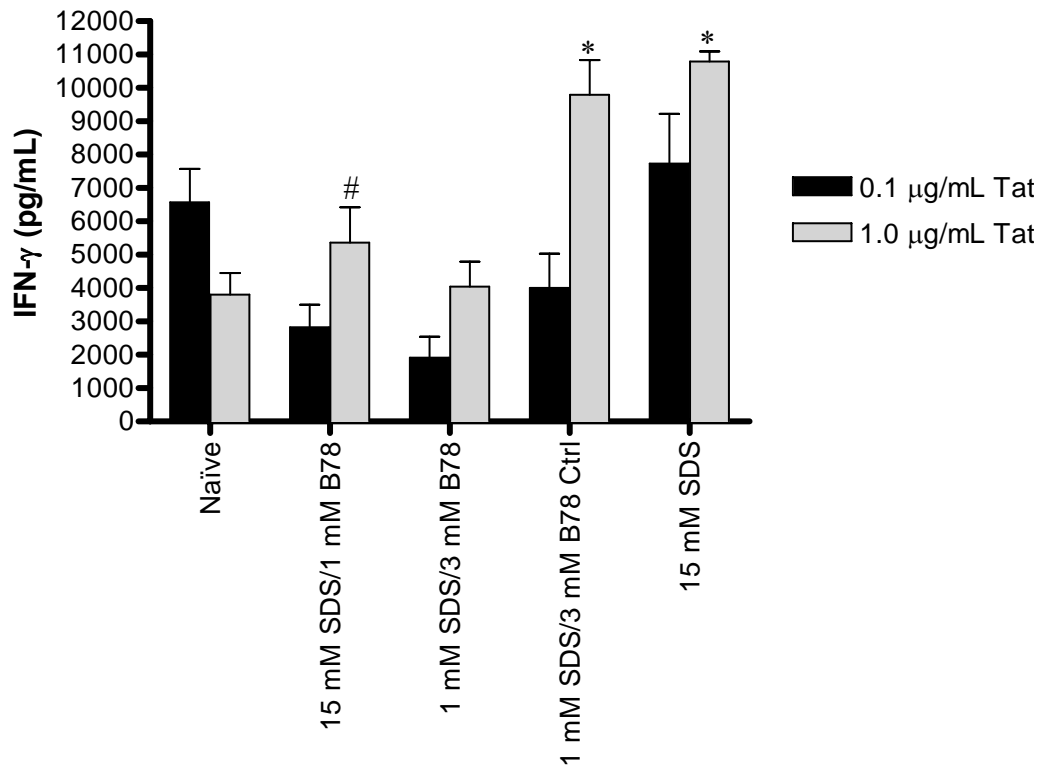
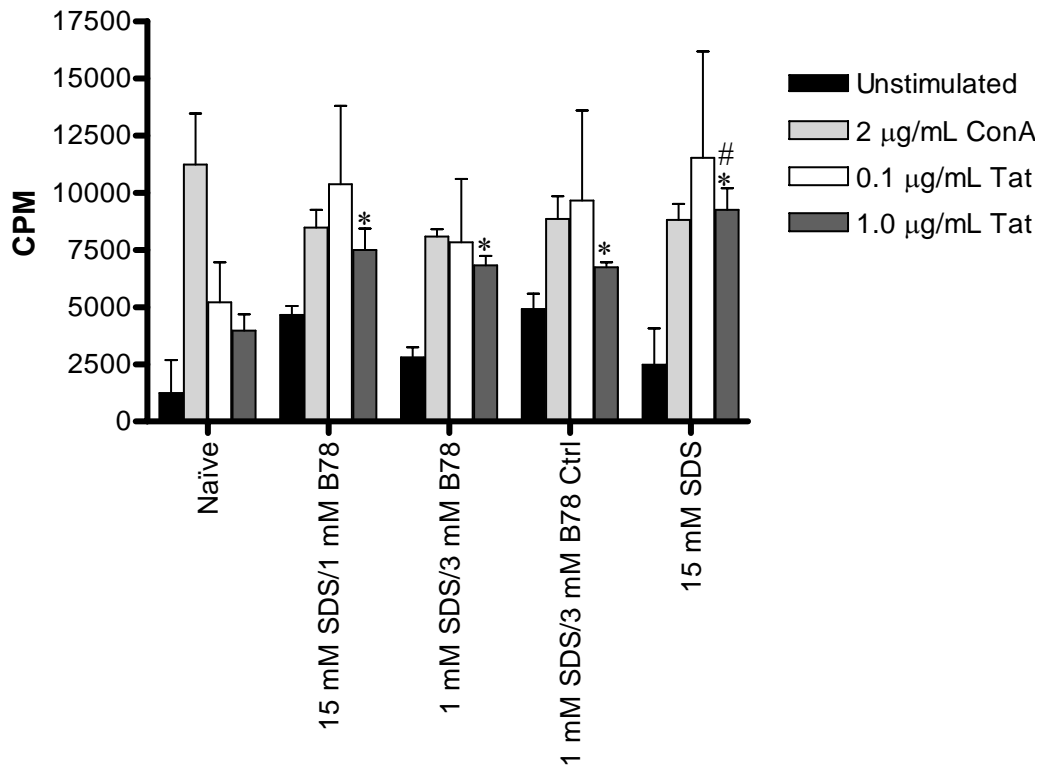**Figure 8.6. INF-γ release from Tat stimulated splenocytes.** BALB/c mice (n=6-8 per group) were immunized (s.c.) on day 0, 14, and 28 with 1 µg of Tat coated on the anionic NPs (100 µg) and spleens were harvested and pooled for each group on day 42. Single cell suspensions of the spleen were stimulated with Tat for 72 hr and the IFN-γ release in the supernatants was quantitated by ELISA. The data represent the mean ± S.D. (n=3). *p<0.001 compared to naïve group; [#]p<0.05 compared to naïve group.

Figure 8.7
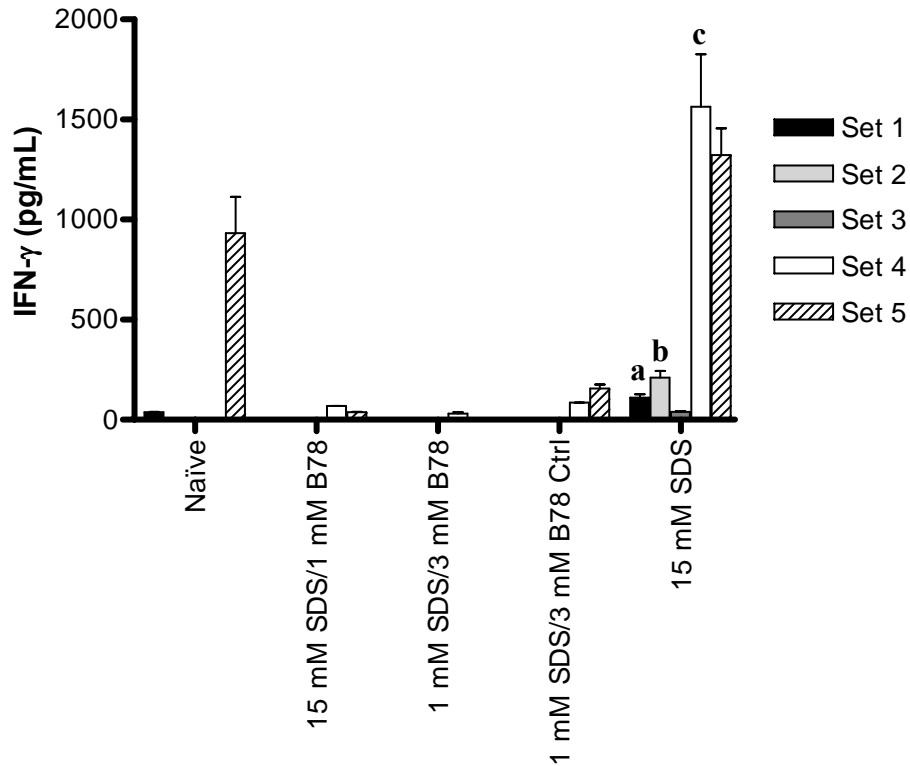
Figure 8.7. INF-γ release from splenocytes stimulated with 15-mer Tat peptides.

BALB/c mice (n=6-8 per group) were immunized (s.c.) on day 0, 14, and 28 with 1 μg of Tat coated on the anionic NPs (100 μg) and spleens were harvested and pooled for each group on day 42. Single cell suspensions of the spleen were stimulated in triplicate with pooled 15-mer Tat peptides and the supernatants were pooled at 72 hr from triplicate wells and the assayed for IFN-γ by ELISA. Set 1 = aa 1-15, 5-19, 9-23, 13-27; Set 2 = aa 17-31, 21-35, 25-39; Set 3 = 29-43, 33-47, 37-51; Set 4 = aa 41-55, 45-59, 49-63, 53-67; Set 5 = 57-71, 61-75, 65-79, 69-83. The data represent the mean ± S.D. (n=2). [a]p<0.01 compared to all groups; [b]p<0.001 compared to all groups; [c]p<0.001 compared to all groups.

Figure 8.8
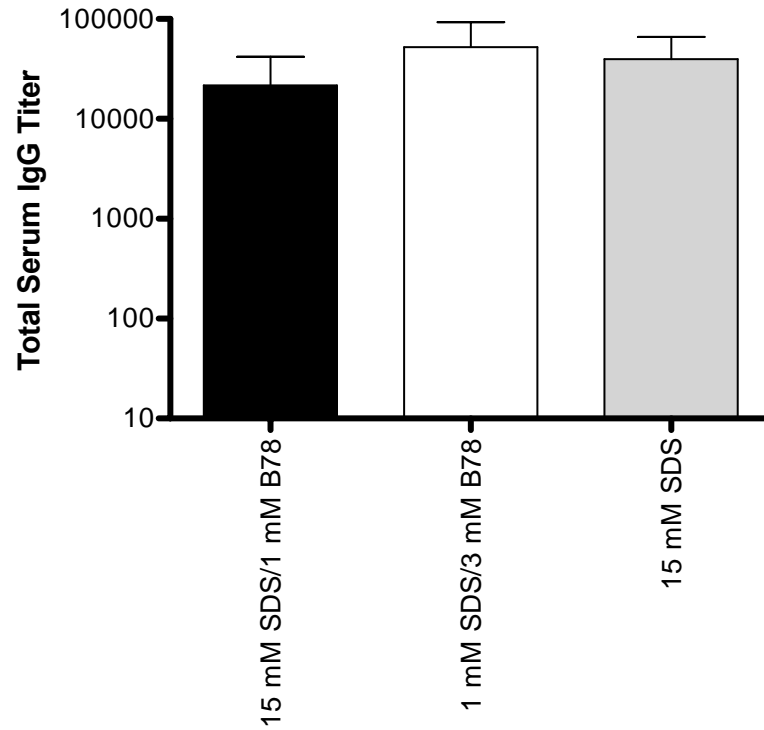
Figure 8.8. Tat-specific serum IgG titers. BALB/c mice were immunized (s.c.) on day 0, 14, and 28 with 5 μg of Tat coated on the anionic NPs (100 μg). Sera were collected and analyzed by ELISA at various time points. Data represent the mean ± S.D. (n=6-8).

Figure 8.9
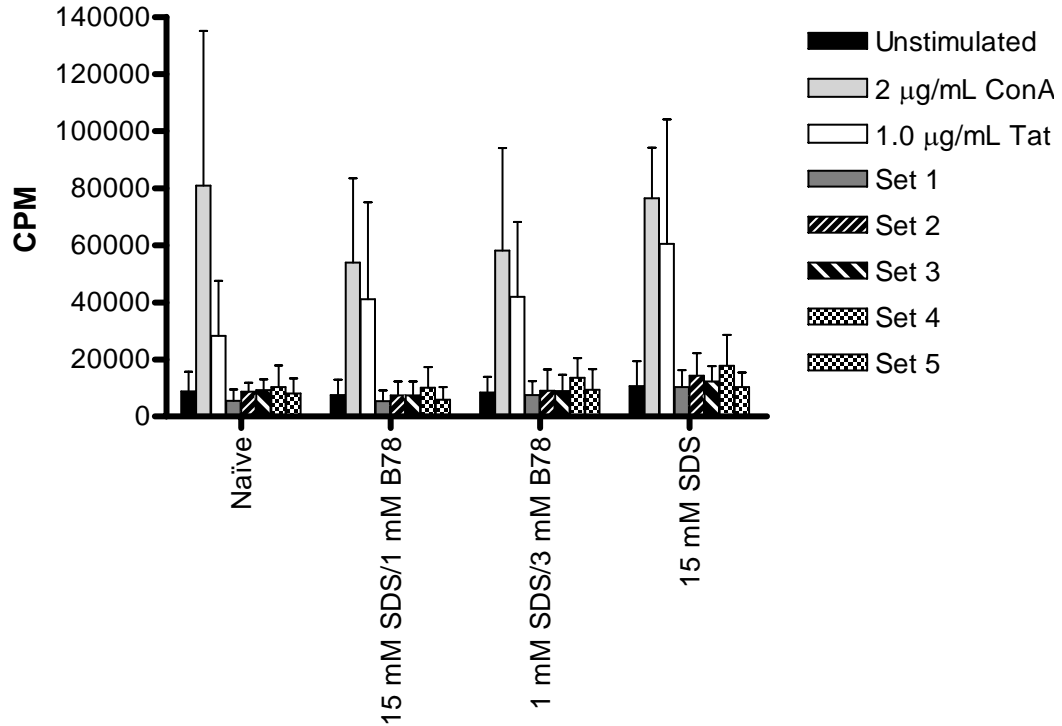
Figure 8.9. Splenocyte proliferation on day 5. BALB/c mice were immunized (s.c.) on day 0, 14, and 28 with 5 μg of Tat coated on the anionic NPs (100 μg) and spleens were harvested on day 42. Single cell suspensions of the spleen were stimulated in triplicate for 4 days. The incorporation of ^{3}H-thymidine was evaluated on day 5. Set 1 = aa 1-15, 5-19, 9-23, 13-27; Set 2 = aa 17-31, 21-35, 25-39; Set 3 = 29-43, 33-47, 37-51; Set 4 = aa 41-55, 45-59, 49-63, 53-67; Set 5 = 57-71, 61-75, 65-79, 69-83. The data represent the mean ± propagated error. (n=6-8).

Figure 8.10
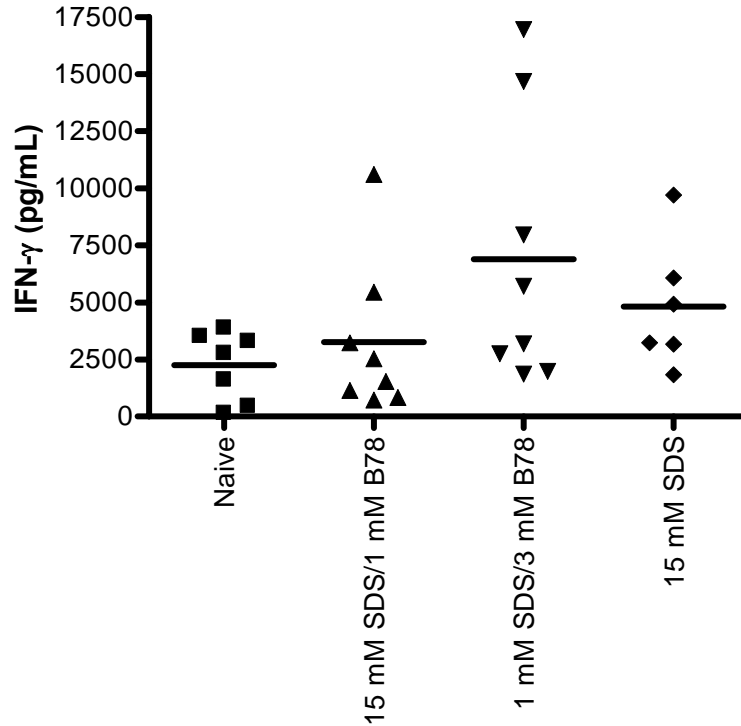
Figure 8.10. IFN-γ release from Tat stimulated splenocytes. BALB/c mice (n=6-8) were immunized (s.c.) on day 0, 14, and 28 with 5 μg of Tat coated on the anionic NPs (100 μg) and spleens were harvested on day 42. Single cell suspensions of the spleen were stimulated with Tat (1.0 μg/mL) for 72 hr and the IFN-γ release in the supernatants was quantitated by ELISA. The graph show IFN-γ release from stimulated splenocytes for each mouse in the group and the horizontal line represents the mean for each group.

Chapter 9

Summary and conclusions

The purpose of the studies presented herein was to investigate the potential of nanoparticles for enhancing immune responses to the HIV-1 proteins, Tat and Gag p24. The hypotheses driving this research were that: 1) mice dosed with anionic nanoparticles coated with HIV-1 proteins would result in enhanced humoral and cellular immune responses compared to those dosed with protein adjuvanted with Alum, 2) increasing the affinity of the protein antigen for the nanoparticles would result in a more stable attachment and lead to a corresponding enhancement in the immune responses *in vivo* compared to antigens coated on anionic nanoparticles by charge interaction, and 3) mice dosed with nanoparticles co-formulated with protein antigen and an immunostimulatory molecule would produce enhanced immune responses compared those dosed with protein antigen with either nanoparticles or immunostimulatory molecule alone.

In these studies, anionic NPs were prepared from oil-in-water microemulsions using emulsifying wax as the oil phase and Brij 78 and SDS as the surfactants. The overall composition of surfactants used to prepare the anionic NPs did not significantly affect the immune responses generated to Tat. Anionic NPs coated with HIV-1 Tat were demonstrated to generate superior immune responses compared to Tat adjuvanted with Alum. A dose-response study with Tat demonstrated that NPs were able to generate comparable levels of Tat-specific IgG titers using a 1 and 5 μg Tat dose, whereas Alum resulted in significantly lower IgG titers at the lower dose of Tat. To determine the

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antibody epitopes generated, the Tat anti-sera was evaluated for recognition to 15-mer Tat peptides by ELISA. It was found that the Tat anti-sera from immunized animals reacted greatest with the N-terminal and basic regions of the protein, which is consistent with literature reports [387,412-414]. The NP groups demonstrated higher levels of antibodies recognizing the basic region compared to Alum. Moreover, the anti-sera from Tat-immunized mice were capable of blocking extracellular Tat in a LTR-transactivation assay.

To investigate the potential of these NPs for synergistic enhancements in immune responses with immunostimulatory molecules, cationic NPs were prepared and formulated with three different immunostimulatory molecules. These molecules are TLR ligands and were expected to enhance immune response via stimulation of the innate immune system. The cationic NPs were coated with the immunostimulatory molecules, LTA, CpG, and Poly I:C, and evaluated for immune responses generated to OVA. From these studies, CpG demonstrated the greatest immunostimulatory activity and further *in vivo* evaluation demonstrated that CpG coated NPs resulted in significant enhancements in the OVA-specific antibody titers compared to either adjuvant alone.

To gain an understanding of the mechanism(s) by which nanoparticles may be enhancing immune responses to the associated antigens, two studies were carried out. In the *in vitro* studies, the uptake of NPs into DCs and consequently, the release of pro-inflammatory cytokines were evaluated. These studies demonstrated that NPs were taken by into DCs and accumulated intracellularly in the cytoplasm. Analysis of pro-inflammatory cytokines after 24 hr incubation of NPs with DCs demonstrated that the NPs did not cause release of any significant levels of TNF-α, IL-1β or IL-12.

Furthermore, significant enhancements in IL-12 release were observed after incubating the CpG coated NPs with DCs compared to CpG alone. These *in vitro* studies suggested that NPs were enhancing the uptake of the associated molecule into DCs and this mechanism, at least in part, contributes to the enhancements in immune responses observed *in vivo*.

In the second study, the extent to which antigens coupled to NPs stimulated $CD8^+$ T cells *in vivo* were evaluated by performing an adoptive transfer of transgenic T cells from the OT-1 mouse into C57BL/6 mice. The OT-1 transgenic mouse model expresses a class I restricted receptor specific for OVA presented by the $H-2K^b$ class I molecule. These studies demonstrated that NPs significantly enhanced the uptake of the associated protein into the MHC class I processing pathway and thus, resulted in enhanced presentation to CD8+ T cells *in vivo* compared to soluble OVA. This enhanced presentation via the MHC class I pathway was thought to be due to enhanced delivery of coated OVA into DCs compared to soluble OVA.

These *in vitro* and *in vivo* data together suggest that NPs enhance immune responses primarily through enabling greater accumulation of the associated antigen into DCs compared to soluble antigen. The NPs investigated so far have taken advantage of coating the antigen on the surface through simple ionic interactions. It was hypothesized that by increasing the affinity of the antigen for the NPs, a greater accumulation of the antigen into DCs can be achieved and this would result in further enhancements in the immune responses compared to the surface coated antigen on anionic NPs. For this, NPs containing a small amount of surface-chelated Ni were prepared and shown to bind to his-tag proteins with more strongly compared to NPs prepared without the Ni.

Furthermore, *in vivo* evaluation of his-tag Gag p24 bound to Ni-NPs generated superior IgG responses, including IgG2a, compared to protein adjuvanted with Alum or coated on the surface of NPs.

In conclusion, these studies highlight the potential applications of NPs prepared from oil-in-water microemulsion precursors for enhancing immune responses to both HIV-1 Tat and Gag p24. Of significant importance is the demonstration that the 72 aa Tat protein was effective at generating antibodies that recognized the same regions of the protein when compared to the full-length Tat protein (1-86 or 1-101) [387,412-414]. Many researchers believe that a Tat-based vaccine may play a vital role in controlling the disease progression [346,368,400]. To this end, the use of the full-length Tat protein as a potential HIV vaccine has been investigated [384]. However, some reports suggest that the full-length Tat is immunosuppressive. As an alternative, one group has reported on the use of Tat-toxoid as a potential Tat vaccine [457]. A previous study in our laboratory demonstrated that Tat (1-72) is not immunosuppressive [253] and the studies presented here further confirm the ability to generate antibodies to both the N-terminal and basic regions, which is consistent with literature reports using the full-length Tat protein. In addition, the antibodies to Tat (1-72) are able to neutralize extracellular Tat as demonstrated by the LTR- transactivation assays. Therefore, the use of Tat (1-72) coated on NPs in a potential HIV vaccine may be a good alternative to the current approaches with the full-length Tat protein or Tat-toxoid.

A significant contribution in the advancement of delivery systems for protein-based vaccines was further demonstrated through the development of a simple and rapid approach for attaching protein antigens to the surface of NPs via surface-chelated nickel.

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Several studies, including those presented herein, suggest that particulate delivery systems may result in enhanced immune responses *in vivo* due to the higher uptake of antigen associated with the particles [239,427]. However, the conventional approach of coating the protein antigen on a charged particle, while effective at enhancing immune responses, may be inefficient or sub-optimal due to possible dissociation of some of the antigen from the particle after *in vivo* administration. Typical approaches utilized for attachment of antibodies or proteins to particles often require activating agents or multiple steps and pose challenges in obtaining high conjugation yields. As an alternative, the studies presented in this dissertation demonstrated that NPs containing a small amount of surface-chelated nickel could be prepared. The Ni-NPs provide a relatively simple approach for attaching protein antigens containing his-tag on the surface of the NPs. More importantly, this technology may be widely applicable to protein-based vaccines because the interaction uses a simple histidine tag that could be incorporated in the expression vector during the protein production process.

Although the studies presented here illustrate the potential applications of NPs for HIV-1 protein-based vaccines, more experiments to further characterize and optimize these systems will be necessary. The studies presented in this dissertation were based on using the *in vivo* immune responses as the end point; however, minimal work was carried out to correlate the effect of formulation parameters on the *in vivo* immune responses. It must be recognized that several steps are involved between the protein formulation with the NPs and the *in vivo* responses that may prove to be critical in the generating the immune responses but are missing in the work presented here. For example, the protein stability and release, as well as the uptake of the associated antigen by DCs using both

the charged NPs and Ni-NPs are critical parameters to evaluate. These types of *in vitro* studies may provide clues for further improvements that could be made in the formulations, which may lead to greater enhancements in the cellular and humoral immune responses. Moreover, additional *in vitro* studies investigating the expression of co-stimulatory molecules on DCs and the processing and presentation of antigens on DCs may provide insight into the possible mechanisms by which NPs are enhancing the immune responses *in vivo*.

While *in vitro* studies may provide a basic understanding of mechanistic processes, ultimately *in vivo* studies will be necessary to providing a greater understanding of the possible functions of NPs. Along these lines, the fate of the protein associated with the NPs after *in vivo* administration would provide a greater understanding of their role in stimulating immune responses. Additional studies evaluating the responses occurring at the site of injection, i.e. inflammation, may reveal other contributing mechanisms through which NPs enhance the immune responses to antigens. Furthermore, if the uptake of the NPs is found to be critical in enhancing the immune responses, exploring the use of a DC-targeting ligand may also provide additional benefits in improving the immune responses to the antigen. Combined these additional studies may reveal further improvements or alternative formulation approaches that could be investigated for building better nanoparticle-based delivery systems for HIV-1 protein-based vaccines.

Appendices

This section contains the following information and additional experiments:

- **Appendix A**: Structures and physical properties for various materials used in the dissertation

- **Appendix B**: Preparation and characterization of sterically stabilized anionic nanoparticles for delivery of HIV-1 Tat and Gag proteins

- **Appendix C**: Synthesis of mannopentaose targeting ligand and *in vitro* evaluation

Figure A1

HO(CH$_2$CH$_2$O)$_W$ (OCH$_2$CH$_2$)$_X$OH

CH(OCH$_2$CH$_2$)$_Y$OH

O

CH$_2$(OCH$_2$CH$_2$)$_Z$ -O-C-CH$_2$ (CH$_2$)$_{15}$CH$_3$

Polysorbate 60 (Polyoxyethylene 20 sorbitan monostearate);
W+X+Y+Z = 20; Mw 1312; HLB 14.9

CH$_3$ (CH$_2$)$_{14}$CH$_2$OH

Cetyl Alcohol; Mw 242; m.p. 49°C
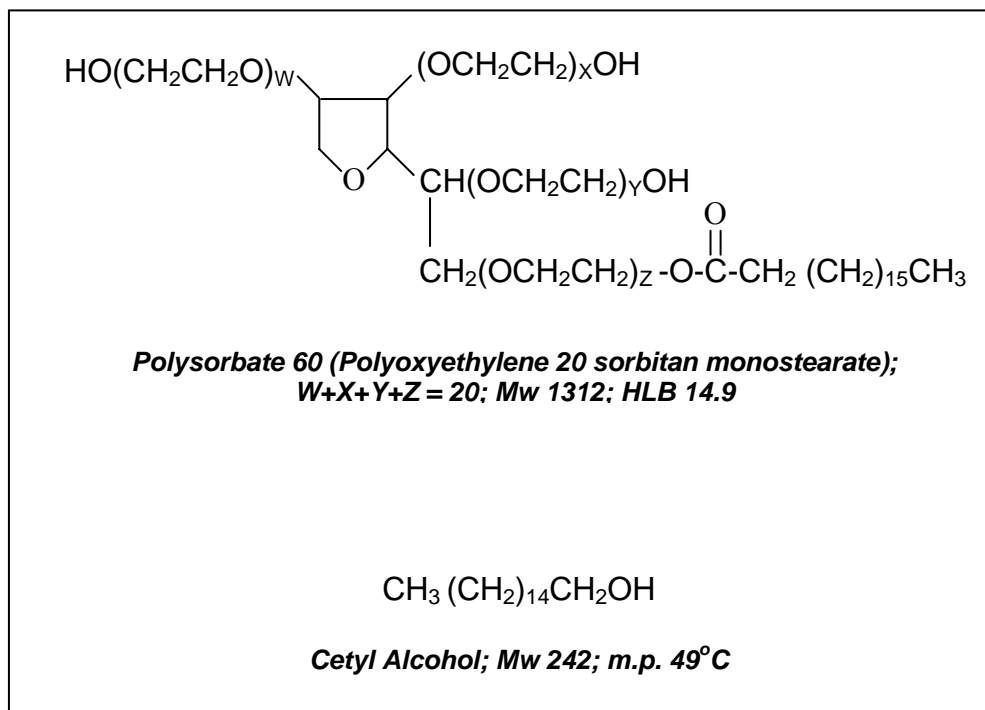
Figure A1. Structure and properties of emulsifying wax. Emulsifying wax is comprised of cetyl alcohol and polysorbate 60 in a 20:1 molar ratio.

$$CH_3 (CH_2)_{17} (OCH_2CH_2)_{20}OH$$

Polyoxyethylene 20 Stearyl Ether (Brij® 78); Mw 1150;
m.p. 38°C; HLB 15.3

$$CH_3(CH_2)_{11} SO_4Na$$

Sodium Dodecyl Sulfate (SDS); Mw 288; HLB 40

$$CH_3(CH_2)_{15}N(CH_3)_3Br$$

Cetyl Trimethyl Ammonium Bromide (CTAB); Mw 364.5; HLB 10
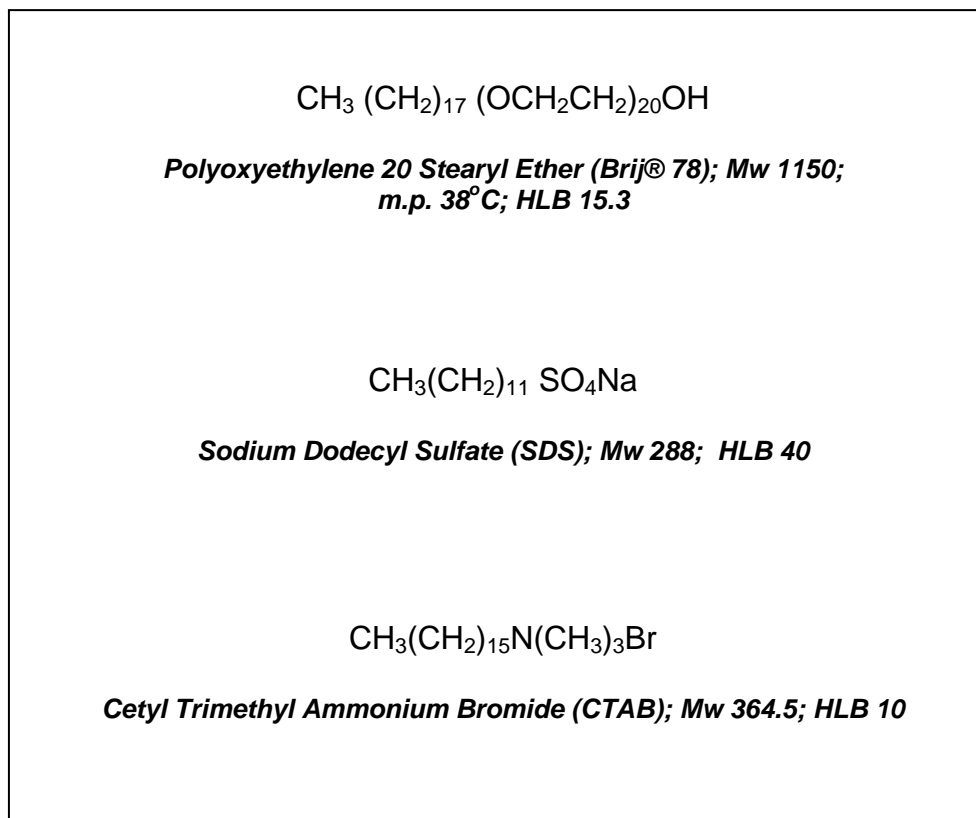
Figure A2. Structure and physical properties of surfactants used for preparing nanoparticles. Neutral, anionic, and cationic nanoparticles were prepared using Brij 78, SDS, and CTAB, respectively.

Figure A3

3,3'-dioctadecyloxacarbocyanine perchlorate (DiOC$_{18}$); Mw 881.72
Ex: 484 nm and Em: 501nm
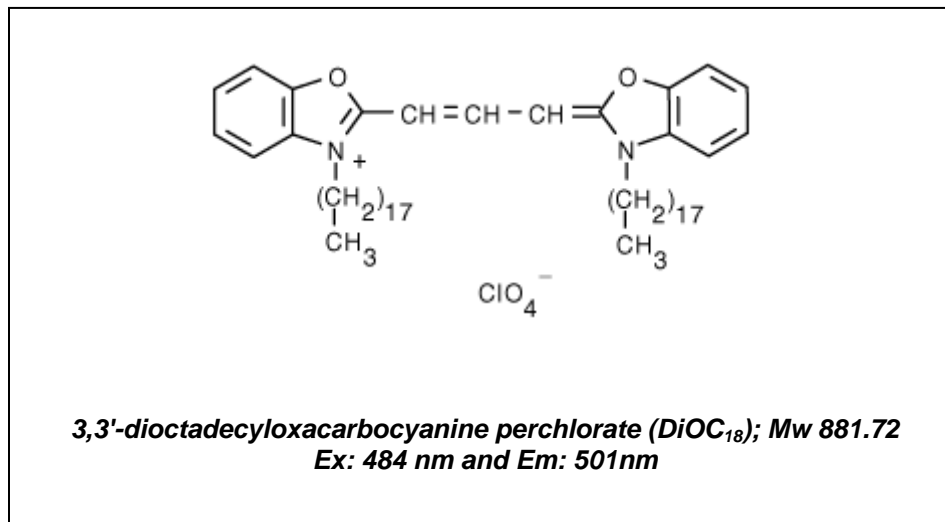
Figure A3.   Chemical structure and physical properties of DiOC$_{18}$.   This green fluorescent marker was used for labeling nanoparticles for confocal microscopy studies looking at nanoparticle uptake into BMDDCs. (Structure obtained from http://probes.invitrogen.com/servlets/structure?item=275)

Appendix B

Preparation and characterization of sterically stabilized anionic nanoparticles for delivery of HIV-1 Tat and Gag Proteins

B.1   Preparation of sterically stabilized anionic NPs

Anionic NPs have been previously prepared in Dr. Mumper's laboratory from microemulsion precursors [16]. Briefly, the microemulsions were prepared at 50-55°C by forming a homogeneous slurry of emulsifying wax (comprised of 20:1 cetyl alcohol/polysorbate 60) in water followed by the addition of sodium dodecyl sulfate (SDS) at a final concentration of 15 mM to result in the formation of stable, clear systems.   The mircoemulsion systems were cooled to room temperature while stirring to form NPs of approximately 100 nm in size. Excess surfactant was removed by gel permeation chromatography (GPC) using a Sephadex G75 column (15 mm x 70 mm) and the particle size was measured by photon correlation spectroscopy (PCS) using a Coulter N4 Plus Particle Sizer at 90° for 60 s.

Initial stability studies of these anionic NPs prepared from 15 mM SDS revealed that the particles aggregated at 25°C in 150 mM sodium chloride (NaCl) (Figure B1). To sterically stabilize these NPs, the use of Brij 78 as a co-surfactant was investigated. The anionic NPs were prepared as stated above using concentrations of 0.5 to 1 mM Brij 78. NPs were further purified by GPC to remove the excess surfactant and then evaluated in 150 mM NaCl at 25°C to determine the concentration of Brij 78 required for stabilization of the particles.   As shown in Figure B1, a minimum of 0.5 mM Brij 78 was required to

stabilize the NPs under the conditions tested. Anionic NPs prepared using 0.5 and 1.0 mM of Brij 78 were further evaluated at 37°C in simulated biological media. Anionic NPs prepared with 1.0 mM Brij 78 were found to be stable in 150 mM NaCl, 10 mM phosphate buffered saline (PBS) pH 7.4, 10% (v/v) fetal bovine serum (FBS) in 150 mM NaCl and 10% (w/v) lactose at 37°C (Figure B2).   In addition, characterization of these anionic NPs prepared using 15 mM SDS and 1 mM Brij 78 by Transmission Electron Microscopy (TEM) revealed that the particles were approximately 50 to 100 nm in size with spherical morphology (Figure B3). This formulation was chosen for further work for coating with the HIV-1 protein antigens.

Figure B1
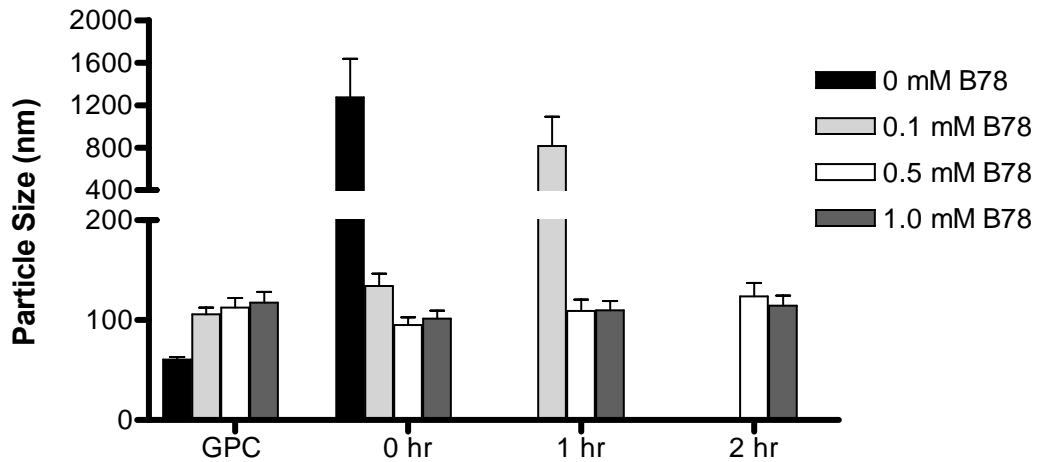
Figure B1. Stability of GPC purified anionic NPs in 150 mM NaCl at 25°C. All NPs were prepared using 15 mM SDS and varying concentrations of Brij 78 (B78) as indicated on the graph. The formulations were passed through a GPC column to remove excess surfactant and the size after purification (GPC) was measured. The stability of the particles was determined over 2 hr in 150 mM NaCl by measuring the size and 0 hr on graph indicates the sized immediately after adding the NaCl. Data represents mean ± S.D. (n=3).
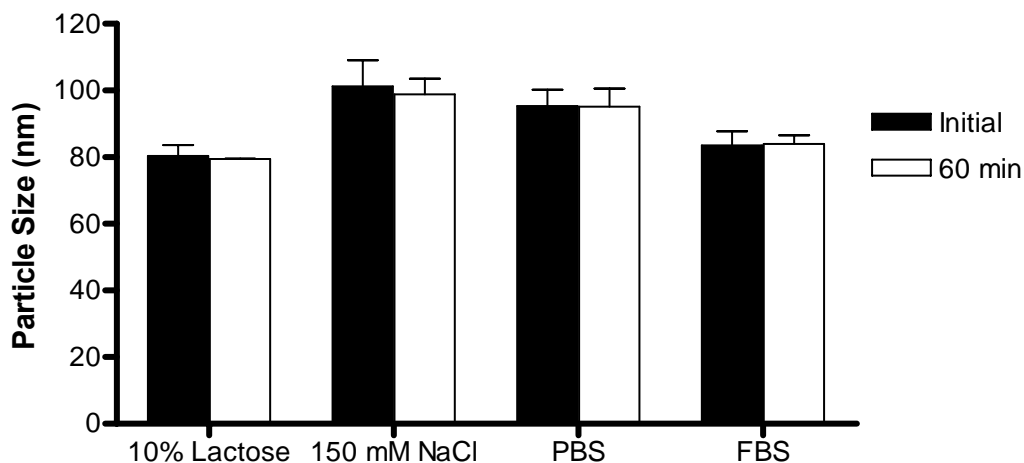
Figure B2. **Stability of anionic NPs in simulated biological media at 37°C.** Negatively charged NPs were prepared using emulsifying wax as the oil phase and 15 mM SDS and 1 mM Brij 78 as the surfactants. NPs were purified by GPC to remove any excess surfactant and stability of the particles was evaluated based on particle size measurements. Data represents mean ± SD (n=3).
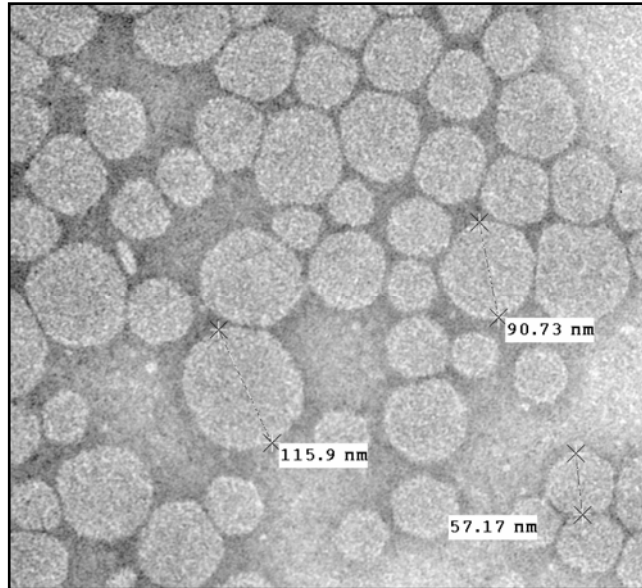
Figure B3. TEM of anionic NPs. Anionic NPs prepared using 15 mM SDS and 1 mM

Brij 78 surfactants were purified by GPC.

B.2   Preparation and characterization of HIV-1 Tat-coated NPs

Anionic NPs were purified by GPC to remove excess surfactant, and filtered through a 0.2 μm filter. A volume of 200 μL of the purified NPs (~40 μg) was gently mixed at varying concentrations of Tat for 1 hr at room temperature. The adsorption of Tat on the surface of NPs was verified by measuring the overall charge (Figure B4) using a Zeta Sizer 2000 from Malvern Instruments, Inc. and TEM analysis of the Tat-coated NPs suggested that the particles retained a spherical shape and ~100 nm size (Figure B5). As expected, an increase in the concentration of Tat adsorbed on the surface of the NPs resulted in an increase in the overall charge of the NPs (Figure B4) up to a final concentration of 25 μg/mL, above which a plateau observed. This may be due to the negative residues in the Tat protein being exposed at the surface of the particle.

Figure B4

Figure B4. Anionic NPs coated with HIV-1 Tat. The size and charge of the particles was characterized before (Ctrl) and after coating with increasing concentrations of Tat. Data represents mean ± SD (n=2).
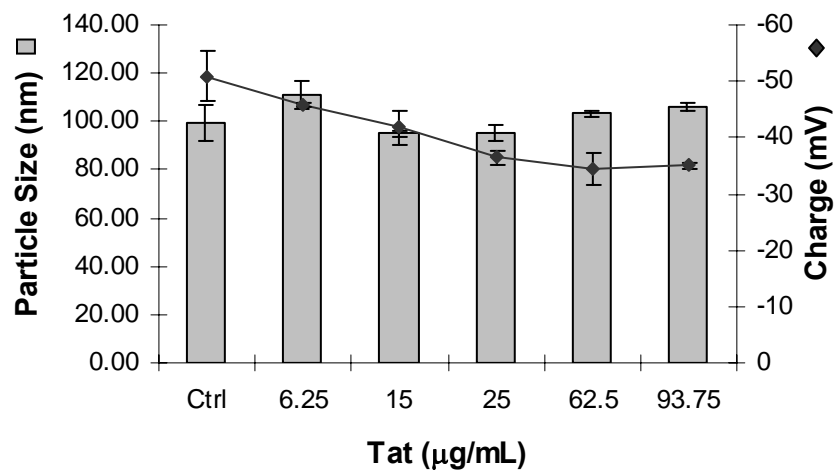
Figure B5

Figure B5. TEM of HIV-1 Tat-coated NPs. Anionic NPs were prepared using 15 mM

SDS and 1 mM Brij 78 and excess surfactant was separated by GPC. The GPC purified

NPs were coated with Tat (25 μg/mL).

B.3   Preparation and characterization of HIV-1 Gag-coated NPs

HIV-1 Gag p55 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV SF2 Gag p55 from Chiron Corporation and the DAIDS) is insoluble in water and most other buffer systems. Therefore, the protein is supplied in 50 mM sodium phosphate (pH 7.9) with 0.4 M NaCl and 6 M urea. To coat anionic NPs with Gag p55, first, excess surfactant was removed by GPC using 6 M urea as the mobile phase. The purified NPs were collected and filtered through a 0.2 μm filter, and 200 μL (40 μg) of NPs were gently mixed with 1, 5, and 10 μg of Gag p55 for 1 hr at room temperature. The coated and uncoated NPs (Ctrl) were centrifuged at 8000 x g at 20°C using Microcon® YM-100 (Millipore, MWCO 100 kDa) ultracentrifuge devices to desalt and remove urea by washing three times with water. The NPs were re-suspended in water (0.2 μm filtered), and characterized by size and charge (Figure B6). The adsorption of Gag p55 on the NPs was also confirmed by TEM (Figure B7), where small, 50 nm size particles formed by Gag p55 were shown to be adsorbed on anionic NPs.

NPs coated with Gag p55 were analyzed using SDS-PAGE and densitometry to quantify the amount of protein adsorbed on the surface. Samples and standards were loaded on pre-cast Novex® 4-20% Tris-Glycine gradient gels and were run under reducing conditions at 125V for 90 min. The gels were developed by silver staining, photographed using GeneSnap, and analyzed by densitometry using GeneTools software. For densitometry, five standards (100, 200, 300, 500, and 700 ng of Gag p55) were run on each gel along with the samples. The amount of protein in all samples was calculated from the standard curve and the coating efficiency for Gag p55 was

determined using these results. The percent of protein adsorbed after ultrafiltration was calculated based on the initial (before ultrafiltration) concentration of Gag p55 for NPs having a final protein concentration of 25 μg/mL and 50 μg/mL. For NPs coated with Gag p55 at a final concentration of 5 μg/mL, the samples were below detection limit; thus, the sample was only analyzed after ultrafiltration and the percent of protein adsorbed was calculated based on the theoretical concentration. As shown in Figure B8, approximately 100% of the protein was adsorbed on the surface of the NPs when the Gag p55 was at a final concentration of 5 μg/mL and 25 μg/mL. In contrast, when the Gag p55 was at a final concentration of 50 μg/mL, only 75% of the protein was adsorbed on the surface of NPs. Gag p55 coated NPs were found to be stable (as determined by retention in particle size) for up to 60 min in 150 mM NaCl, 10 mM PBS, 10% (v/v) FBS, and 10% (w/v) lactose at 37°C (data not shown).

As mentioned above, the use of Gag p55 provides some difficulties in handling the protein since it is insoluble and must be dissolved in 6M urea. As a result, the urea must be removed. This poses potential difficulties in carrying out *in vivo* studies for immunization with protein adjuvanted with Alum. For further studies, the use of Gag p24, which is positively-charged and water-soluble, was investigated. More importantly, it has been previously shown that Gag p24 is highly conserved and leads to cross-reactive CTL responses [353,354,356]. After GPC purification of the NPs, 1, 5, and 10 μg of Gag p24 (Trinity Biotech; Carlsbad, CA) were gently mixed with approximately 40 μg of NPs for 1 hr at room temperature. The size and charge of Gag p24-coated NPs is presented in Figure B9. The overall charge of the NPs becomes more positive with increasing concentrations of Gag p24, confirming adsorption to the surface of NPs. As seen with

Tat, a plateau in the charge of the NPs results above a Gag p24 concentration of 25 μg/mL thought to be possibly due to negatively charged residues of the protein being exposed on the surface of the NPs.

Figure B6. Anionic NPs coated with HIV-1 Gag p55. The size and charge of the particles was characterized before (Ctrl) and after coating with increasing concentrations of Gag p55. Data represents mean ± SD (n=2).
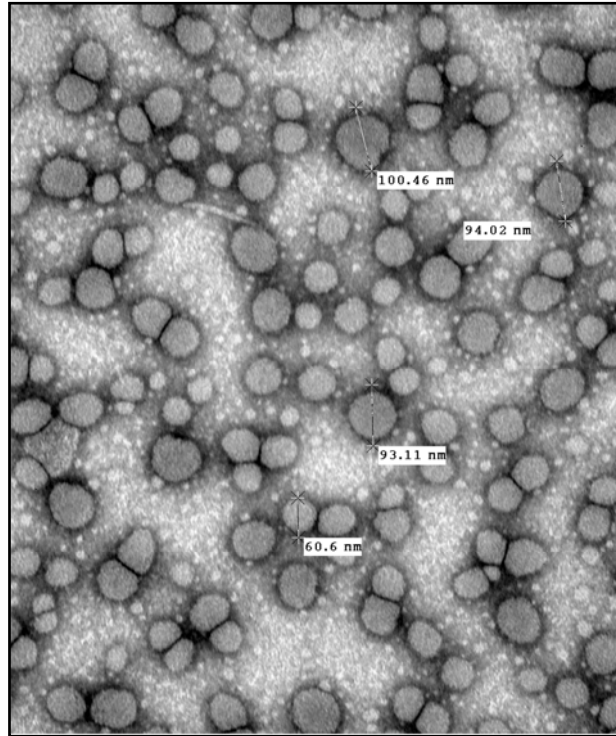
.

Figure B7
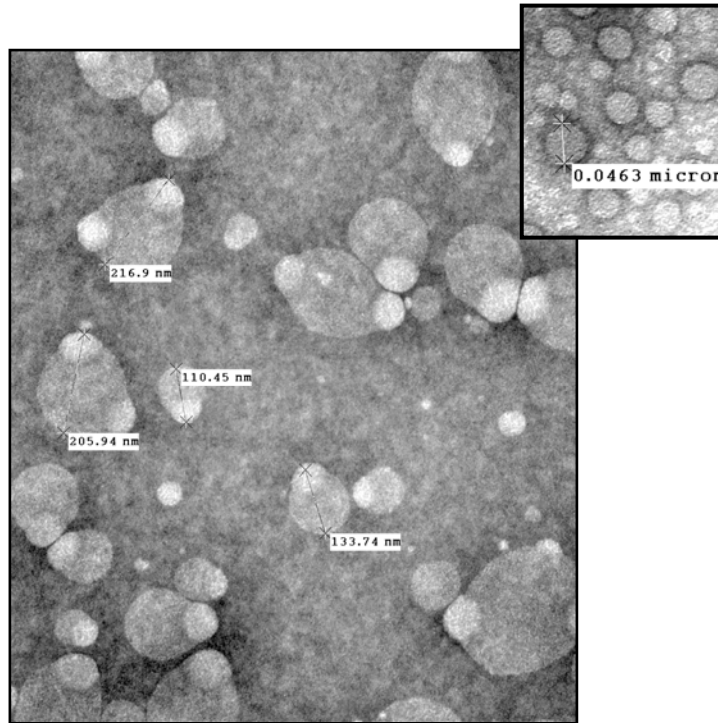
Figure B7. TEM of HIV-1 Gag p55-coated NPs. Anionic NPs were prepared using 15 mM SDS and 1 mM Brij 78 and excess surfactant was separated by GPC. The GPC purified NPs were coated with Gag p55 (25 µg/mL). Gag p55 forms small particles of approximately 50 nm in size as shown on the overlayed TEM. Thus, the smaller particles adsorbed on the larger size particles were confirmed to be Gag p55.
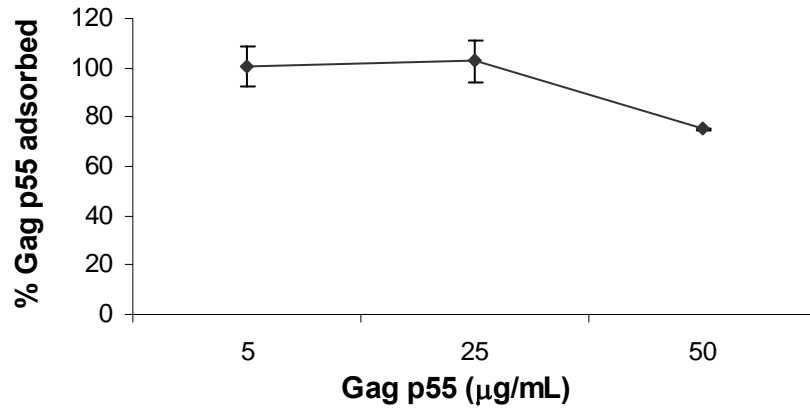
Figure B8. Coating efficiency of HIV-1 Gag p55 on anionic NPs. The amount of Gag p55 associated with the NPs was determined by SDS-PAGE/densitometry. Data represent mean ± S.D. (n=2-6).
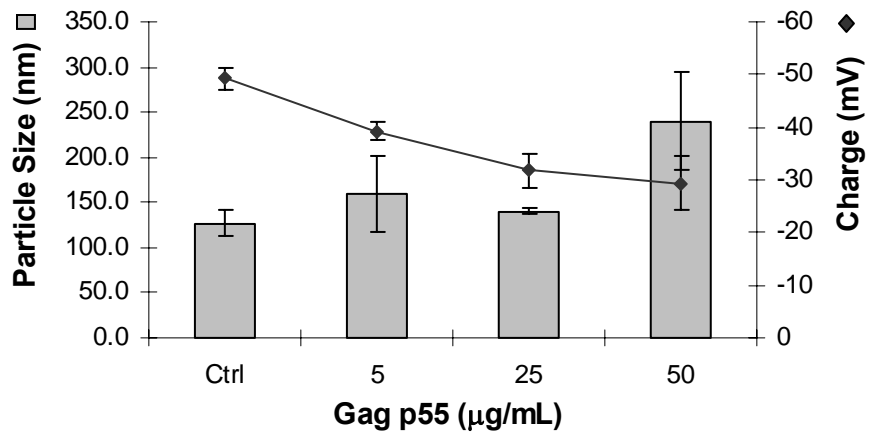
Figure B9
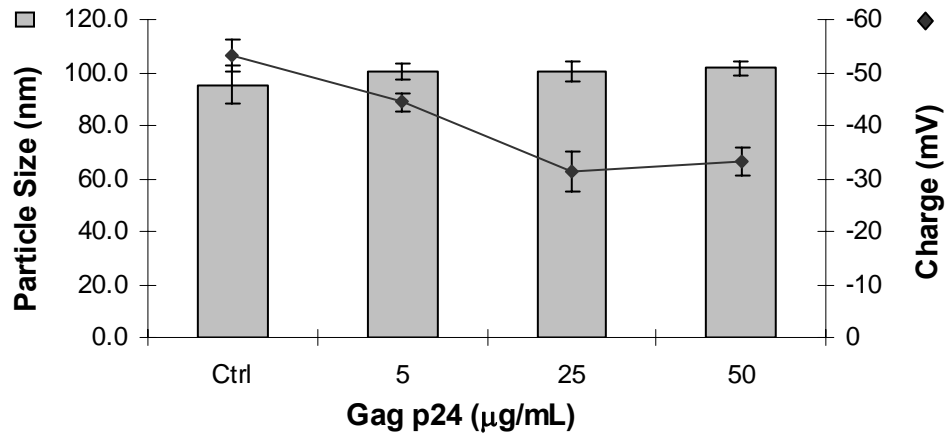
Figure B9. Anionic NPs coated with HIV-1 Gag p24. The size and charge of the particles was characterize before (Ctrl) and after coating with increasing concentration of Gag p24. Data represents mean ± SD (n=2).

B.4　Immune responses to HIV-1 Tat- and Gag p24-coated NPs

BALB/c mice (n=5-6/group) were immunized intramuscularly (i.m., 50 μL per gastrocnemius muscle) on day 0 and day 20 with 2.5 μg Tat or Gag p24 adjuvanted with Alum (Spectrum) or 2.5 μg Tat or Gag p24 coated on NPs. In this study, Alum was used as a positive control for a Th2 type immune response. The sera from all mice were collected on day 36 for further assessment of humoral immune responses generated in the various groups. The Tat- and Gag p24-specific serum IgG levels, determined by ELISA, are shown in Figure B10 and B11, respectively.

The IgG antibody responses from the Tat-immunized mice were stronger than the Gag p24-immunized mice, which may be indicative of the higher immunogenicity of Tat compared to Gag p24. However, in both the Tat- and Gag p24-immunized mice, the antibody levels for mice immunized using NPs versus Alum, a control for Th2 type immune response, were comparable and statistically insignificant. This demonstrates the potential of anionic NPs for producing similar enhancements in immune responses to the approved Alum adjuvant after i.m. administrations. However, previous results using anionic NPs coated with the model antigen β-galactosidase suggests that NPs produced superior immune responses to Alum after subcutaneous (s.c.) immunization. The differences in the responses with NPs obtained in the two studies may be antigen specific or may indicate that the s.c. route may be better compared to i.m. for generating immune responses with the NPs.

Figure B10
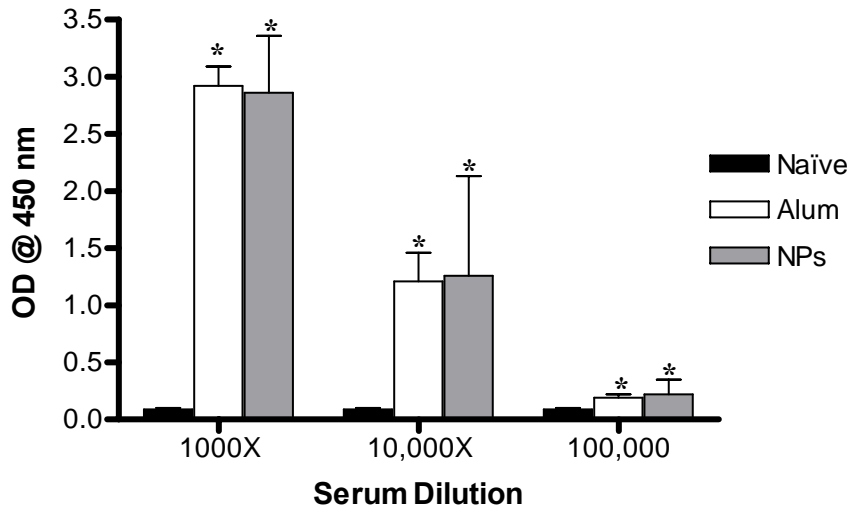
Figure B10. Tat-specific total serum IgG levels on day 36. Mice were immunized with 2.5 μg of Tat coated on anionic NPs or adjuvanted with Alum on day 0 and day 20 by 50 μL injection into each gastrocnemius muscle. Data represent mean ± S.D. (n=5-6). *$p < 0.05$ compared to naïve group by ANOVA.

Figure B11
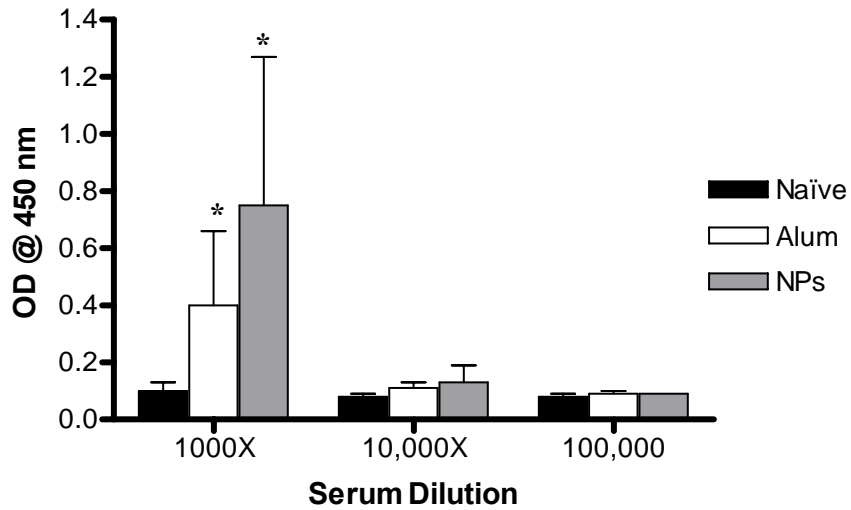
Figure B11. Gag p24-specific total serum IgG levels on day 36. Mice were immunized with 2.5 μg of Gag p24 coated on anionic NPs or adjuvanted with Alum on day 0 and day 20 by 50 μL injection into each gastrocnemius muscle. Data represent mean ± S.D. (n=5-6). *p<0.05 compared to naïve group by ANOVA.

B.5 Conclusions

These studies demonstrated that simple anionic NPs prepared using 15 mM SDS could be stabilized by inclusion of Brij 78. Furthermore, the resulting anionic NPs could be coated with HIV-1 Tat, Gag p55, and Gag p24. The *in vivo* immune responses to Tat- and Gag p24-coated NPs demonstrated that these systems were similar in enhancing immune responses compared to protein adjuvanted with Alum after i.m. immunization.

Appendix C

Synthesis of mannopentaose targeting ligand and *in vitro* evaluation

The mannose receptor (MR) has been utilized for targeting antigens to DCs and thus, obtaining enhanced immune responses [275]. Many groups have investigated the use of hydrophobized mannan for enhancing immune responses to antigens [15,282,283]. Mizuochi *et al*. have investigated various oligosaccharide ligands and demonstrated that hydrophobized mannopentaose (Figure C1) attached to the surface of liposomes generated enhanced cellular responses compared uncoated liposomes [283,285]. Thus, the use of hydrophobized mannopentaose was further investigated as a potential targeting ligand for increasing NPs uptake *in vitro* into DCs.

The conjugation of mannopentaose to the lipid, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) has been previously described and was followed with minor modifications [458]. Briefly, 7.7 mg of DPPE (Avanti Polar Lipids; Alabaster, AL) was reacted with 1.2 mg mannopentaose (Sigma; St. Louis, MO) in 1.5 mL of chloroform/methanol (1:1 v/v) at 60°C for 2 hr in an 8 mL glass vial with a Teflon coated cap. Sodium cyanoborohydride was added after 2 hr and the reaction was allowed to proceed at 60°C. The progress of the reaction was monitored by thin layer chromatography using chloroform/methanol/water 105:100:28 (v/v/v) using orcinol reagent (Fisher; Hampton, NH) to visualize the mannopentaose and the lipid conjugate. The reaction was stopped when the mannopentaose was undetectable by TLC (~5 days). The product was purified by column chromatography using silica gel 60 (EM Science;

Gibbstown, NJ) with the following solvent systems (all indicate volume to volume ratios): 1) 1:1 chloroform/methanol; 2) 11:9:2 chloroform/methanol/water; and 3) 10:10:3 chloroform/methanol/water. The eluent from the column was fractionated, checked for presence of the lipid-mannopentaose conjugate by TLC, and the fractions positive for conjugate were combined and the solvent was evaporated. The product was confirmed to be DPPE-mannopentaose by mass spectrometry with the molecular ion peak present at 1504 m/z (Figure C2).

To determine if the DPPE-mannopentaose could serve as a targeting ligand to enhance the uptake of NPs into BMDDCs, radiolabeled anionic NPs using Brij 78 and SDS (3 mM and 1 mM, respectively) as the surfactant were prepared with 1 to 10% w/w of DPPE-mannopentaose. BMDDCs were confirmed to express the mannose receptor by flow cytometry (Figure C3). The uptake by BMDDCs of the DPPE-mannopentaose NPs was compared to the anionic NPs (B78/SDS) at 37°C. As a control, the various formulations were also incubated with BMDDCs at 4°C to differentiate the binding versus active uptake expected at 37°C and significantly accumulation of all NP formulations was observed at 37°C compared to 4°C (data not shown). As shown in Figure C4, there were no significant differences in the uptake of the particles with inclusion of DPPE-mannopentaose over the 4 hr evaluated. It is important to note that the NPs without the targeting ligand were efficiently taken up by the BMDDCs, with about 40% accumulation into the cells over the 4 hr. This highly efficient uptake of NPs may present difficulty in evaluating differences in uptake due to the targeting ligand *in vitro*. However, this strategy may be viable *in vivo* and may cause enhanced immune responses since there are other cells present and the presence of the ligand may facilitate

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targeting and higher uptake into DCs versus other cell types. Alternatively, it is possible that the ligand on the NPs is not accessible for interaction with the mannose receptor on the BMDDCs. This may be evaluated by inclusion of a hydrophilic spacer such as polyethylene glycol between the lipid and mannopentaose moieties to allow for more flexibility and accessibility for interaction with the receptor.

Acknowledgments

Figure C1

Figure C1. Structure of DPPE lipid conjugated to mannopentaose. The molecular

weight of this ligand was calculated to be 1504.70. (Structure from Shimizu *et al*. [286])
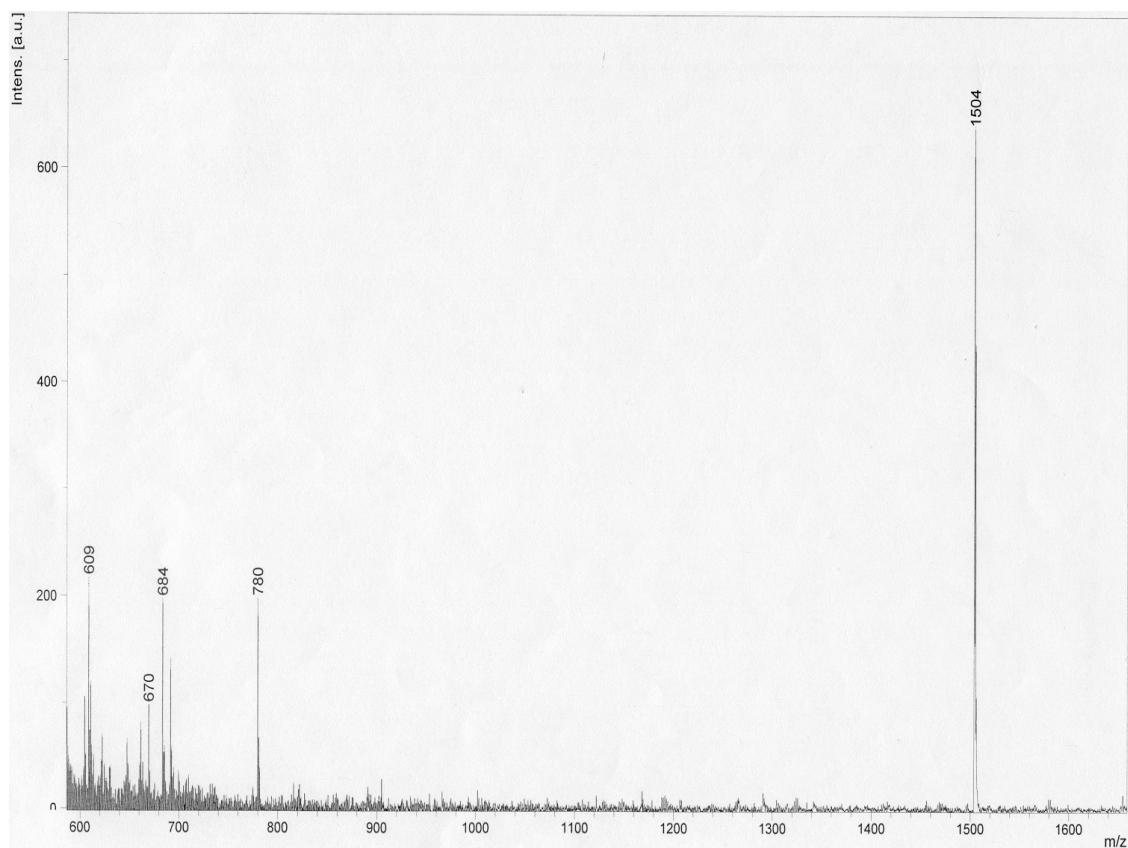
Figure C2. Mass spectrum for purified DPPE-mannopentaose ligand. The mass spectrum was obtained in a negative ion mode by MALDI-TOFMS.

Figure C3
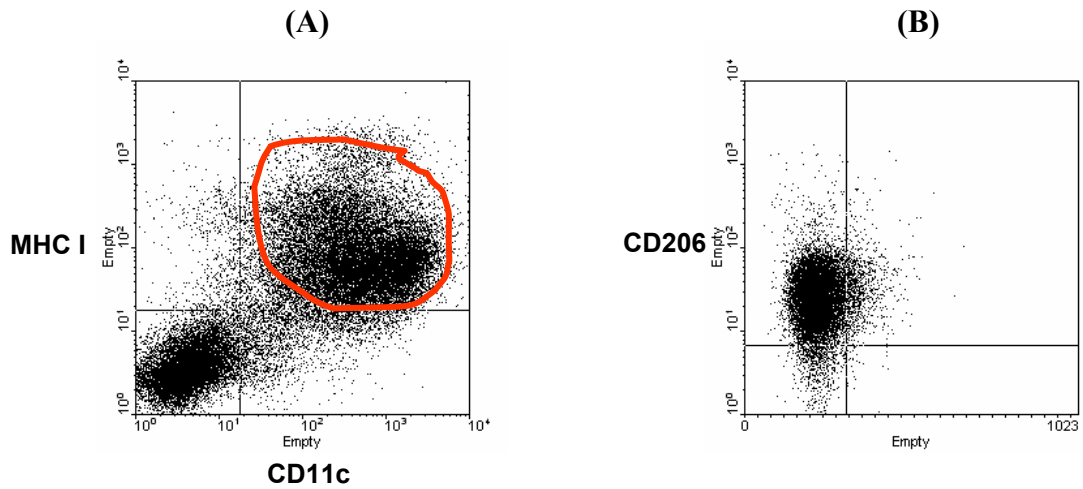
Figure C3. Mannose receptor expression on BMDDCs. A) Cells from primary culture of murine bone-marrow were identified as DCs by staining the cells with CD11c and MHC I antibodies. The double positive cells, circled, were gated to see expression of mannose receptor on the BMDDCs. B) Cells were labeled with a murine mannose receptor antibody (CD206). Double positive cells from (A) that are also positive for mannose receptor are shown.
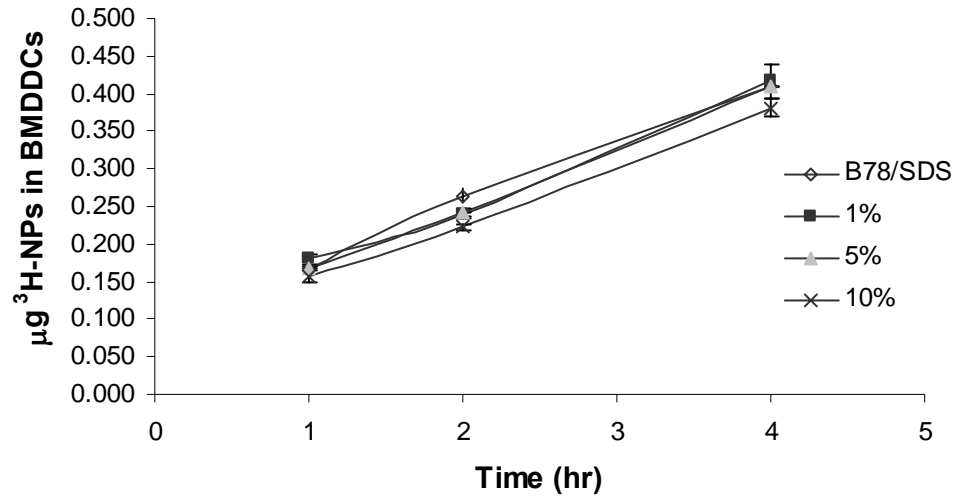
Figure C4. Uptake of radiolabeled NP formulations in BMDDCs. An amount of 1 µg of anionic NP (B78/SDS) or anionic NPs containing 1, 5 and 10% w/w DPPE-mannopentaose were incubated with BMDDCs at 37°C. The radioactivity associated with the cells was measured at the time points shown to evaluate uptake of the various NP formulations.

References

1   Singh, M. & O'Hagan, D. Advances in vaccine adjuvants. *Nat Biotechnol* 1999, **17**(11), 1075-1081.

2   Gupta, R.K. & Siber, G.R. Adjuvants for human vaccines--current status, problems and future prospects. *Vaccine* 1995, **13**(14), 1263-1276.

3   Gupta, R.K. Aluminum compounds as vaccine adjuvants. *Adv Drug Deliv Rev* 1998, **32**(3), 155-172.

4   Singh, M. & Srivastava, I. Advances in vaccine adjuvants for infectious diseases. *Curr HIV Res* 2003, **1**(3), 309-320.

5   Bramwell, V.W. & Perrie, Y. Particulate delivery systems for vaccines. *Crit Rev Ther Drug Carrier Syst* 2005, **22**(2), 151-214.

6   Green, S., Fortier, A., Dijkstra, J. *et al.* Liposomal vaccines. *Adv Exp Med Biol* 1995, **383**, 83-92.

7   Gregoriadis, G. Immunological adjuvants: a role for liposomes. *Immunol Today* 1990, **11**(3), 89-97.

8   O'Hagan, D.T. & Singh, M. Microparticles as vaccine adjuvants and delivery systems. *Expert Rev Vaccines* 2003, **2**(2), 269-283.

9   Singh, M., Kazzaz, J., Ugozzoli, M., Chesko, J. & O'Hagan, D.T. Charged polylactide co-glycolide microparticles as antigen delivery systems. *Expert Opin Biol Ther* 2004, **4**(4), 483-491.

10   Kreuter, J., Berg, U., Liehl, E., Soliva, M. & Speiser, P.P. Influence of the particle size on the adjuvant effect of particulate polymeric adjuvants. *Vaccine* 1986, **4**(2), 125-129.

11   Eldridge, J.H., Staas, J.K., Meulbroek, J.A., Tice, T.R. & Gilley, R.M. Biodegradable and biocompatible poly(DL-lactide-co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralizing antibodies. *Infect Immun* 1991, **59**(9), 2978-2986.

12   Tabata, Y., Inoue, Y. & Ikada, Y. Size effect on systemic and mucosal immune responses induced by oral administration of biodegradable microspheres. *Vaccine* 1996, **14**(17-18), 1677-1685.

13   Singh, M., Briones, M., Ott, G. & O'Hagan, D. Cationic microparticles: A potent delivery system for DNA vaccines. *Proc Natl Acad Sci U S A* 2000, **97**(2), 811-816.

14   Cui, Z. & Mumper, R.J. Topical immunization using nanoengineered genetic vaccines. *J Control Release* 2002, **81**(1-2), 173-184.

15   Cui, Z. & Mumper, R.J. Genetic immunization using nanoparticles engineered from microemulsion precursors. *Pharm Res* 2002, **19**(7), 939-946.

16   Cui, Z. & Mumper, R.J. Coating of cationized protein on engineered nanoparticles results in enhanced immune responses. *Int J Pharm* 2002, **238**(1-2), 229-239.

17   Freed, E.O. HIV-1 gag proteins: diverse functions in the virus life cycle. *Virology* 1998, **251**(1), 1-15.

18   Caputo, A., Gavioli, R. & Ensoli, B. Recent advances in the development of HIV-1 Tat-based vaccines. *Curr HIV Res* 2004, **2**(4), 357-376.

19   Bojak, A., Deml, L. & Wagner, R. The past, present and future of HIV-vaccine development: a critical view. *Drug Discov Today* 2002, **7**(1), 36-46.

20   Plotkin, S.A. Vaccines: past, present and future. *Nat Med* 2005, **11**(4 Suppl), S5-11.

21   Plotkin, S.L. & Plotkin, S.A. A Short History of Vaccination. In *Vaccines* (Eds. Plotkin, S.A. & Orenstein, W.A.) W.B. Saunders Company, Philadelphia, 1999. 1-12.

22   Henderson, D.A. & Moss, B. Smallpox and Vaccinia. In *Vaccines* (Eds. Plotkin, S.A. & Orenstein, W.A.) W.B. Saunders Company, Philadelphia, 1999. 74-97.

23   Bramwell, V.W. & Perrie, Y. The rational design of vaccines. *Drug Discov Today* 2005, **10**(22), 1527-1534.

24   Smith, K.M. & Starke, J.R. Bacille Calmette-Guerin Vaccine. In *Vaccines* (Eds. Plotkin, S.A. & Orenstein, W.A.) W.B. Saunders Company, Philadelphia, 1999. 111-139.

25   Aylward, R.B., Tangermann, R., Sutter, R. & Cochi, S.L. Polio Eradication: Capturing the Full Potential of a Vaccine. In *New Generation Vaccines* (Eds. Levine, M.M., Kaper, J.B., Rappuoli, R., Liu, M. & Good, M.F.) Marcel Dekker, Inc., New York, 2004. 145-157.

26   Decker, M.D. & Edwards, K.M. Combination Vaccines. In *Vaccines* (Eds. Plotkin, S.A. & Orenstein, W.A.) W. B. Saunders Company, Philadelphia, 1999. 508-530.

27   Orenstein, W.A., Hinman, A.R. & Rodewald, L.E. Public Health Considerations - United States. In *Vaccines* (Eds. Plotkin, S.A. & Orenstein, W.A.) W. B. Saunders Company, Philadelphia, 1999. 1006-1031.

28   Plotkin, S.A. Vaccines, vaccination, and vaccinology. *J Infect Dis* 2003, **187**(9), 1349-1359.

29   Levine, M.M. & Lagos, R. Vaccines and vaccination in historical perspective. In *New Generation Vaccines* (Eds. Levine, M.M., Kaper, J.B., Rappuoli, R., Liu, M. & Good, M.F.) Marcel Dekker, Inc., New York, 2004. 1-10.

30   Levine, M.M. & Sztein, M.B. Vaccine development strategies for improving immunization: the role of modern immunology. *Nat Immunol* 2004, **5**(5), 460-464.

31   Payette, P.J. & Davis, H.L. History of vaccines and positioning of current trends. *Curr Drug Targets Infect Disord* 2001, **1**(3), 241-247.

32   McAleer, W.J., Buynak, E.B., Maigetter, R.Z., Wampler, D.E., Miller, W.J. & Hilleman, M.R. Human hepatitis B vaccine from recombinant yeast. *Nature* 1984, **307**(5947), 178-180.

33   Szmuness, W., Stevens, C.E., Zang, E.A., Harley, E.J. & Kellner, A. A controlled clinical trial of the efficacy of the hepatitis B vaccine (Heptavax B): a final report. *Hepatology* 1981, **1**(5), 377-385.

34   Nencioni, L., Pizza, M., Bugnoli, M. *et al.* Characterization of genetically inactivated pertussis toxin mutants: candidates for a new vaccine against whooping cough. *Infect Immun* 1990, **58**(5), 1308-1315.

35   Jadhav, S.S. & Gairola, S. Composition of acellular pertussis and combination vaccines: a general review. *Biologicals* 1999, **27**(2), 105-110.

36   Kaper, J.B. & Rappuoli, R. An Overview of Biotechnology in Vaccine Development. In *New Generation Vaccines* (Eds. Levine, M.M., Kaper, J.B.,

Rappuoli, R., Liu, M.A. & Good, M.F.) Marcel Dekker, Inc., New York, 2004. 11-17.

37 Tang, D.C., DeVit, M. & Johnston, S.A. Genetic immunization is a simple method for eliciting an immune response. *Nature* 1992, **356**(6365), 152-154.

38 Johnston, S.A., Talaat, A.M. & McGuire, M.J. Genetic immunization: what's in a name? *Arch Med Res* 2002, **33**(4), 325-329.

39 Otten, G.R., Doe, B., Schaefer, M. *et al.* Relative potency of cellular and humoral immune responses induced by DNA vaccination. *Intervirology* 2000, **43**(4-6), 227-232.

40 Heppell, J. & Davis, H.L. Application of DNA vaccine technology to aquaculture. *Adv Drug Deliv Rev* 2000, **43**(1), 29-43.

41 van Drunen Littel-van den Hurk, S., Gerdts, V., Loehr, B.I. *et al.* Recent advances in the use of DNA vaccines for the treatment of diseases of farmed animals. *Adv Drug Deliv Rev* 2000, **43**(1), 13-28.

42 Mumper, R.J. & Ledebur, H.C., Jr. Dendritic cell delivery of plasmid DNA. Applications for controlled genetic immunization. *Mol Biotechnol* 2001, **19**(1), 79-95.

43 Nimal, S., McCormick, A.L., Thomas, M.S. & Heath, A.W. An interferon gamma-gp120 fusion delivered as a DNA vaccine induces enhanced priming. *Vaccine* 2005, **23**(30), 3984-3990.

44 Nimal, S., Heath, A.W. & Thomas, M.S. Enhancement of immune responses to an HIV gp120 DNA vaccine by fusion to TNF alpha cDNA. *Vaccine* 2006.

45 Faulkner, L., Buchan, G., Slobbe, L. *et al.* Influenza hemagglutinin peptides fused to interferon gamma and encapsulated in liposomes protects mice against influenza infection. *Vaccine* 2003, **21**(9-10), 932-939.

46 Mumper, R.J. & Cui, Z. Genetic immunization by jet injection of targeted pDNA-coated nanoparticles. *Methods* 2003, **31**(3), 255-262.

47 Cui, Z. & Mumper, R.J. Bilayer films for mucosal (genetic) immunization via the buccal route in rabbits. *Pharm Res* 2002, **19**(7), 947-953.

48 Cui, Z. & Mumper, R.J. Chitosan-based nanoparticles for topical genetic immunization. *J Control Release* 2001, **75**(3), 409-419.

49 Dunachie, S.J. & Hill, A.V. Prime-boost strategies for malaria vaccine development. *J Exp Biol* 2003, **206**(Pt 21), 3771-3779.

50 Plotkin, S.A. Vaccines in the 21st century. *Hybrid Hybridomics* 2002, **21**(2), 135-145.

51 Moingeon, P., de Taisne, C. & Almond, J. Delivery technologies for human vaccines. *Br Med Bull* 2002, **62**, 29-44.

52 Parham, P. *The Immune System*, Garland Publishing/Elsevier Science Ltd, New York, 2000.

53 Ada, G.L. Strategies in Vaccine Design. (Ed. Ada, G.L.) R.F. Landes Company, Austin, 1994. 1-16.

54 Medzhitov, R. & Janeway, C.A., Jr. Decoding the patterns of self and nonself by the innate immune system. *Science* 2002, **296**(5566), 298-300.

55 Janeway, C.A., Jr. & Medzhitov, R. Innate immune recognition. *Annu Rev Immunol* 2002, **20**, 197-216.

56 Kaisho, T. & Akira, S. Toll-like receptors as adjuvant receptors. *Biochim Biophys Acta* 2002, **1589**(1), 1-13.

57 Takeda, K., Kaisho, T. & Akira, S. Toll-like receptors. *Annu Rev Immunol* 2003, **21**, 335-376.

58 Kaisho, T. & Akira, S. Regulation of dendritic cell function through toll-like receptors. *Curr Mol Med* 2003, **3**(8), 759-771.

59 Hunter, R.L. Overview of vaccine adjuvants: present and future. *Vaccine* 2002, **20 Suppl 3**, S7-12.

60 Kenney, R.T. & Edelman, R. Adjuvants for the future. In *New Generation Vaccines* (Eds. Levine, M.M., Kaper, J.B., Rappuoli, R., Liu, M. & Good, M.F.) Marcel Dekker, Inc., New York, 2004. 213-223.

61 Brewer, J.M., Conacher, M., Satoskar, A., Bluethmann, H. & Alexander, J. In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to freund's complete adjuvant, but continues to induce T helper 2 cytokine production. *Eur J Immunol* 1996, **26**(9), 2062-2066.

62 Lindblad, E.B. Aluminium adjuvants--in retrospect and prospect. *Vaccine* 2004, **22**(27-28), 3658-3668.

63 Brewer, J.M., Conacher, M., Hunter, C.A., Mohrs, M., Brombacher, F. & Alexander, J. Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. *J Immunol* 1999, **163**(12), 6448-6454.

64 Cox, J.C. & Coulter, A.R. Adjuvants--a classification and review of their modes of action. *Vaccine* 1997, **15**(3), 248-256.

65 Ada, G.L. The Immunology of Vaccination. In *Vaccines* (Eds. Plotkin, S.A. & Orenstein, W.A.) W.B. Saunders Company, Philadelphia, 1999. 28-39.

66 Ulanova, M., Tarkowski, A., Hahn-Zoric, M. & Hanson, L.A. The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. *Infect Immun* 2001, **69**(2), 1151-1159.

67 Brewer, J.M. (How) do aluminium adjuvants work? *Immunol Lett* 2006, **102**(1), 10-15.

68 HogenEsch, H. Mechanisms of stimulation of the immune response by aluminum adjuvants. *Vaccine* 2002, **20 Suppl 3**, S34-39.

69 Rimaniol, A.C., Gras, G., Verdier, F. *et al.* Aluminum hydroxide adjuvant induces macrophage differentiation towards a specialized antigen-presenting cell type. *Vaccine* 2004, **22**(23-24), 3127-3135.

70 Mark, A., Bjorksten, B. & Granstrom, M. Immunoglobulin E responses to diphtheria and tetanus toxoids after booster with aluminium-adsorbed and fluid DT-vaccines. *Vaccine* 1995, **13**(7), 669-673.

71 Schijns, V.E. Immunological concepts of vaccine adjuvant activity. *Curr Opin Immunol* 2000, **12**(4), 456-463.

72 O'Garra, A. & Arai, N. The molecular basis of T helper 1 and T helper 2 cell differentiation. *Trends Cell Biol* 2000, **10**(12), 542-550.

73 Naylor, P.H. & Hadden, J.W. T cell targeted immune enhancement yields effective T cell adjuvants. *Int Immunopharmacol* 2003, **3**(8), 1205-1215.

74   Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C. & Amigorena, S. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 2002, **20**, 621-667.

75   Ahlers, J.D., Belyakov, I.M. & Berzofsky, J.A. Cytokine, chemokine, and costimulatory molecule modulation to enhance efficacy of HIV vaccines. *Curr Mol Med* 2003, **3**(3), 285-301.

76   Raychaudhuri, S. & Rock, K.L. Fully mobilizing host defense: building better vaccines. *Nat Biotechnol* 1998, **16**(11), 1025-1031.

77   Engers, H., Kieny, M.P., Malhotra, P. & Pink, J.R. Third meeting on Novel Adjuvants Currently in or Close to Clinical Testing World Health Organization--Organisation Mondiale de la Sante, Fondation Merieux, Annecy, France, 7-9 January 2002. *Vaccine* 2003, **21**(25-26), 3503-3524.

78   Hancock, G.E., Smith, J.D. & Heers, K.M. The immunogenicity of subunit vaccines for respiratory syncytial virus after co-formulation with aluminum hydroxide adjuvant and recombinant interleukin-12. *Viral Immunol* 2000, **13**(1), 57-72.

79   Lachman, L.B., Shih, L.C., Rao, X.M. *et al.* Cytokine-containing liposomes as adjuvants for HIV subunit vaccines. *AIDS Res Hum Retroviruses* 1995, **11**(8), 921-932.

80   Wales, J.R., Baird, M.A., Davies, N.M. & Buchan, G.S. Fusing subunit antigens to interleukin-2 and encapsulating them in liposomes improves their antigenicity but not their protective efficacy. *Vaccine* 2005, **23**(17-18), 2339-2341.

81   Jankovic, D., Caspar, P., Zweig, M. *et al.* Adsorption to aluminum hydroxide promotes the activity of IL-12 as an adjuvant for antibody as well as type 1 cytokine responses to HIV-1 gp120. *J Immunol* 1997, **159**(5), 2409-2417.

82   Hancock, G.E., Heers, K.M. & Smith, J.D. QS-21 synergizes with recombinant interleukin-12 to create a potent adjuvant formulation for the fusion protein of respiratory syncytial virus. *Viral Immunol* 2000, **13**(4), 503-509.

83   Pulendran, B. & Ahmed, R. Translating innate immunity into immunological memory: implications for vaccine development. *Cell* 2006, **124**(4), 849-863.

84   Klinman, D.M., Currie, D., Gursel, I. & Verthelyi, D. Use of CpG oligodeoxynucleotides as immune adjuvants. *Immunol Rev* 2004, **199**, 201-216.

85   Datta, S.K., Redecke, V., Prilliman, K.R. *et al.* A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. *J Immunol* 2003, **170**(8), 4102-4110.

86   Schletter, J., Heine, H., Ulmer, A.J. & Rietschel, E.T. Molecular mechanisms of endotoxin activity. *Arch Microbiol* 1995, **164**(6), 383-389.

87   Martin, M., Michalek, S.M. & Katz, J. Role of innate immune factors in the adjuvant activity of monophosphoryl lipid A. *Infect Immun* 2003, **71**(5), 2498-2507.

88   Ismaili, J., Rennesson, J., Aksoy, E. *et al.* Monophosphoryl lipid A activates both human dendritic cells and T cells. *J Immunol* 2002, **168**(2), 926-932.

89   Evans, J.T., Cluff, C.W., Johnson, D.A., Lacy, M.J., Persing, D.H. & Baldridge, J.R. Enhancement of antigen-specific immunity via the TLR4 ligands MPL adjuvant and Ribi.529. *Expert Rev Vaccines* 2003, **2**(2), 219-229.

90   Johnson, A.G. & Tomai, M.A. A study of the cellular and molecular mediators of the adjuvant action of a nontoxic monophosphoryl lipid A. *Adv Exp Med Biol* 1990, **256**, 567-579.

91   Bernstein, D. Glycoprotein D adjuvant herpes simplex virus vaccine. *Expert Rev Vaccines* 2005, **4**(5), 615-627.

92   Alving, C.R. Lipopolysaccharide, lipid A, and liposomes containing lipid A as immunologic adjuvants. *Immunobiology* 1993, **187**(3-5), 430-446.

93   Baldridge, J.R., McGowan, P., Evans, J.T. *et al.* Taking a Toll on human disease: Toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents. *Expert Opin Biol Ther* 2004, **4**(7), 1129-1138.

94   Pink, J.R. & Kieny, M.P. 4th meeting on Novel Adjuvants Currently in/close to Human Clinical Testing World Health Organization -- organisation Mondiale de la Sante Fondation Merieux, Annecy, France, 23-25, June 2003. *Vaccine* 2004, **22**(17-18), 2097-2102.

95   Krieg, A.M., Yi, A.K., Matson, S. *et al.* CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995, **374**(6522), 546-549.

96   Latz, E., Schoenemeyer, A., Visintin, A. *et al.* TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol* 2004, **5**(2), 190-198.

97   Sparwasser, T., Koch, E.S., Vabulas, R.M. *et al.* Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur J Immunol* 1998, **28**(6), 2045-2054.

98   Van der Stede, Y., Verdonck, F., Vancaeneghem, S., Cox, E. & Goddeeris, B.M. CpG-oligodinucleotides as an effective adjuvant in pigs for intramuscular immunizations. *Vet Immunol Immunopathol* 2002, **86**(1-2), 31-41.

99   Rao, M., Matyas, G.R., Vancott, T.C., Birx, D.L. & Alving, C.R. Immunostimulatory CpG motifs induce CTL responses to HIV type I oligomeric gp140 envelope protein. *Immunol Cell Biol* 2004, **82**(5), 523-530.

100   Kwant, A. & Rosenthal, K.L. Intravaginal immunization with viral subunit protein plus CpG oligodeoxynucleotides induces protective immunity against HSV-2. *Vaccine* 2004, **22**(23-24), 3098-3104.

101   McCluskie, M.J., Weeratna, R.D. & Davis, H.L. Intranasal immunization of mice with CpG DNA induces strong systemic and mucosal responses that are influenced by other mucosal adjuvants and antigen distribution. *Mol Med* 2000, **6**(10), 867-877.

102   McCluskie, M.J., Weeratna, R.D., Krieg, A.M. & Davis, H.L. CpG DNA is an effective oral adjuvant to protein antigens in mice. *Vaccine* 2000, **19**(7-8), 950-957.

103   McCluskie, M.J., Weeratna, R.D. & Davis, H.L. The potential of oligodeoxynucleotides as mucosal and parenteral adjuvants. *Vaccine* 2001, **19**(17-19), 2657-2660.

104   Verthelyi, D., Wang, V.W., Lifson, J.D. & Klinman, D.M. CpG oligodeoxynucleotides improve the response to hepatitis B immunization in healthy and SIV-infected rhesus macaques. *Aids* 2004, **18**(7), 1003-1008.

105   Maletto, B., Ropolo, A., Moron, V. & Pistoresi-Palencia, M.C. CpG-DNA stimulates cellular and humoral immunity and promotes Th1 differentiation in aged BALB/c mice. *J Leukoc Biol* 2002, **72**(3), 447-454.

106   Weeratna, R.D., Brazolot Millan, C.L., McCluskie, M.J. & Davis, H.L. CpG ODN can re-direct the Th bias of established Th2 immune responses in adult and young mice. *FEMS Immunol Med Microbiol* 2001, **32**(1), 65-71.

107   Brazolot Millan, C.L., Weeratna, R., Krieg, A.M., Siegrist, C.A. & Davis, H.L. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc Natl Acad Sci U S A* 1998, **95**(26), 15553-15558.

108   Sparwasser, T., Hultner, L., Koch, E.S., Luz, A., Lipford, G.B. & Wagner, H. Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hemopoiesis. *J Immunol* 1999, **162**(4), 2368-2374.

109   Lipford, G.B., Sparwasser, T., Zimmermann, S., Heeg, K. & Wagner, H. CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses. *J Immunol* 2000, **165**(3), 1228-1235.

110   Klinman, D.M. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat Rev Immunol* 2004, **4**(4), 249-258.

111   Diwan, M., Tafaghodi, M. & Samuel, J. Enhancement of immune responses by co-delivery of a CpG oligodeoxynucleotide and tetanus toxoid in biodegradable nanospheres. *J Control Release* 2002, **85**(1-3), 247-262.

112   Halperin, S.A., Van Nest, G., Smith, B., Abtahi, S., Whiley, H. & Eiden, J.J. A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. *Vaccine* 2003, **21**(19-20), 2461-2467.

113   Kensil, C.R., Patel, U., Lennick, M. & Marciani, D. Separation and characterization of saponins with adjuvant activity from Quillaja saponaria Molina cortex. *J Immunol* 1991, **146**(2), 431-437.

114   Kensil, C.R. Saponins as vaccine adjuvants. *Crit Rev Ther Drug Carrier Syst* 1996, **13**(1-2), 1-55.

115   Campbell, J.B. & Peerbaye, Y.A. Saponin. *Res Immunol* 1992, **143**(5), 526-530;discussion 577-528.

116   Soltysik, S., Wu, J.Y., Recchia, J. *et al.* Structure/function studies of QS-21 adjuvant: assessment of triterpene aldehyde and glucuronic acid roles in adjuvant function. *Vaccine* 1995, **13**(15), 1403-1410.

117   Kensil, C.R., Wu, J.Y., Anderson, C.A., Wheeler, D.A. & Amsden, J. QS-21 and QS-7: purified saponin adjuvants. *Dev Biol Stand* 1998, **92**, 41-47.

118   Kensil, C.R., Soltysik, S., Wheeler, D.A. & Wu, J.Y. Structure/function studies on QS-21, a unique immunological adjuvant from Quillaja saponaria. *Adv Exp Med Biol* 1996, **404**, 165-172.

119   Kensil, C.R., Wu, J.Y. & Soltysik, S. Structural and immunological characterization of the vaccine adjuvant QS-21. *Pharm Biotechnol* 1995, **6**, 525-541.

120   Liu, G., Anderson, C., Scaltreto, H., Barbon, J. & Kensil, C.R. QS-21 structure/function studies: effect of acylation on adjuvant activity. *Vaccine* 2002, **20**(21-22), 2808-2815.

121   Cribbs, D.H., Ghochikyan, A., Vasilevko, V. *et al.* Adjuvant-dependent modulation of Th1 and Th2 responses to immunization with beta-amyloid. *Int Immunol* 2003, **15**(4), 505-514.

122   Newman, M.J., Wu, J.Y., Gardner, B.H. *et al.* Induction of cross-reactive cytotoxic T-lymphocyte responses specific for HIV-1 gp120 using saponin adjuvant (QS-21) supplemented subunit vaccine formulations. *Vaccine* 1997, **15**(9), 1001-1007.

123   Britt, W., Fay, J., Seals, J. & Kensil, C. Formulation of an immunogenic human cytomegalovirus vaccine: responses in mice. *J Infect Dis* 1995, **171**(1), 18-25.

124   Sasaki, S., Sumino, K., Hamajima, K. *et al.* Induction of systemic and mucosal immune responses to human immunodeficiency virus type 1 by a DNA vaccine formulated with QS-21 saponin adjuvant via intramuscular and intranasal routes. *J Virol* 1998, **72**(6), 4931-4939.

125   O'Hagan, D.T., MacKichan, M.L. & Singh, M. Recent developments in adjuvants for vaccines against infectious diseases. *Biomol Eng* 2001, **18**(3), 69-85.

126   Livingston, P.O., Adluri, S., Helling, F. *et al.* Phase 1 trial of immunological adjuvant QS-21 with a GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma. *Vaccine* 1994, **12**(14), 1275-1280.

127   Sabbatini, P.J., Kudryashov, V., Ragupathi, G. *et al.* Immunization of ovarian cancer patients with a synthetic Lewis(y)-protein conjugate vaccine: a phase 1 trial. *Int J Cancer* 2000, **87**(1), 79-85.

128   Slingluff, C.L., Jr., Yamshchikov, G., Neese, P. *et al.* Phase I trial of a melanoma vaccine with gp100(280-288) peptide and tetanus helper peptide in adjuvant: immunologic and clinical outcomes. *Clin Cancer Res* 2001, **7**(10), 3012-3024.

129   Bocchia, M., Gentili, S., Abruzzese, E. *et al.* Effect of a p210 multipeptide vaccine associated with imatinib or interferon in patients with chronic myeloid leukaemia and persistent residual disease: a multicentre observational trial. *Lancet* 2005, **365**(9460), 657-662.

130   Kashala, O., Amador, R., Valero, M.V. *et al.* Safety, tolerability and immunogenicity of new formulations of the Plasmodium falciparum malaria peptide vaccine SPf66 combined with the immunological adjuvant QS-21. *Vaccine* 2002, **20**(17-18), 2263-2277.

131   Evans, T.G., McElrath, M.J., Matthews, T. *et al.* QS-21 promotes an adjuvant effect allowing for reduced antigen dose during HIV-1 envelope subunit immunization in humans. *Vaccine* 2001, **19**(15-16), 2080-2091.

132   Waite, D.C., Jacobson, E.W., Ennis, F.A. *et al.* Three double-blind, randomized trials evaluating the safety and tolerance of different formulations of the saponin adjuvant QS-21. *Vaccine* 2001, **19**(28-29), 3957-3967.

133   Kensil, C.R. & Kammer, R. QS-21: a water-soluble triterpene glycoside adjuvant. *Expert Opin Investig Drugs* 1998, **7**(9), 1475-1482.

134   Gupta, R.K., Relyveld, E.H., Lindblad, E.B., Bizzini, B., Ben-Efraim, S. & Gupta, C.K. Adjuvants--a balance between toxicity and adjuvanticity. *Vaccine* 1993, **11**(3), 293-306.

135   Edelman, R. Vaccine adjuvants. *Rev Infect Dis* 1980, **2**(3), 370-383.

136   Steiner, J.W., Langer, B. & Schatz, D.L. The local and systemic effects of Freund's adjuvant and its fractions. *Arch Pathol* 1960, **70**, 424-434.

137   Behar, A.J. & Tal, C. Experimental liver necrosis produced by the injection of homologous whole liver with adjuvant. *J Pathol Bacteriol* 1959, **77**(2), 591-596.

138   Laufer, A., Tal, C. & Behar, A.J. Effect of adjuvant (Freund's type) and its components on the organs of various animal species; a comparative study. *Br J Exp Pathol* 1959, **40**(1), 1-7.

139   Jensen, F.C., Savary, J.R., Diveley, J.P. & Chang, J.C. Adjuvant activity of incomplete Freund's adjuvant. *Adv Drug Deliv Rev* 1998, **32**(3), 173-186.

140   Valensi, J.P., Carlson, J.R. & Van Nest, G.A. Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants. *J Immunol* 1994, **153**(9), 4029-4039.

141   Allison, A.C. & Byars, N.E. Syntex adjuvant formulation. *Res Immunol* 1992, **143**(5), 519-525.

142   Allison, A.C. & Byars, N.E. An adjuvant formulation that selectively elicits the formation of antibodies of protective isotypes and of cell-mediated immunity. *J Immunol Methods* 1986, **95**(2), 157-168.

143   Podda, A. & Del Giudice, G. MF59 Adjuvant Emulsion. In *New Generation Vaccines* (Eds. Levine, M.M., Kaper, J.B., Rappuoli, R., Liu, M.A. & Good, M.F.) Marcel Dekker, Inc., New York, 2004. 225-235.

144   Ott, G., Barchfeld, G.L., Chernoff, D., Radhakrishnan, R., van Hoogevest, P. & Van Nest, G. MF59. Design and evaluation of a safe and potent adjuvant for human vaccines. *Pharm Biotechnol* 1995, **6**, 277-296.

145   Squarcione, S., Sgricia, S., Biasio, L.R. & Perinetti, E. Comparison of the reactogenicity and immunogenicity of a split and a subunit-adjuvanted influenza vaccine in elderly subjects. *Vaccine* 2003, **21**(11-12), 1268-1274.

146   Gasparini, R., Pozzi, T., Montomoli, E. *et al.* Increased immunogenicity of the MF59-adjuvanted influenza vaccine compared to a conventional subunit vaccine in elderly subjects. *Eur J Epidemiol* 2001, **17**(2), 135-140.

147   Iob, A., Brianti, G., Zamparo, E. & Gallo, T. Evidence of increased clinical protection of an MF59-adjuvant influenza vaccine compared to a non-adjuvant vaccine among elderly residents of long-term care facilities in Italy. *Epidemiol Infect* 2005, **133**(4), 687-693.

148   Gabutti, G., Guido, M., Durando, P. *et al.* Safety and immunogenicity of conventional subunit and MF59-adjuvanted influenza vaccines in human immunodeficiency virus-1-seropositive patients. *J Int Med Res* 2005, **33**(4), 406-416.

149   Lewis, D.J., Eiden, J.E., Goilav, C., Langenberg, A.G., Suggett, F. & Griffin, G.E. Rapid and frequent induction of protective immunity exceeding UK recommendations for healthcare settings by MF59-adjuvated hepatitis B vaccine. *Commun Dis Public Health* 2003, **6**(4), 320-324.

150   Heineman, T.C., Clements-Mann, M.L., Poland, G.A. *et al.* A randomized, controlled study in adults of the immunogenicity of a novel hepatitis B vaccine containing MF59 adjuvant. *Vaccine* 1999, **17**(22), 2769-2778.

151   Nitayaphan, S., Khamboonruang, C., Sirisophana, N. *et al.* A phase I/II trial of HIV SF2 gp120/MF59 vaccine in seronegative thais.AFRIMS-RIHES Vaccine Evaluation Group. Armed Forces Research Institute of Medical Sciences and the Research Institute for Health Sciences. *Vaccine* 2000, **18**(15), 1448-1455.

152   Stanberry, L.R. Clinical trials of prophylactic and therapeutic herpes simplex virus vaccines. *Herpes* 2004, **11 Suppl 3**, 161A-169A.

153   Harro, C.D., Pang, Y.Y., Roden, R.B. *et al.* Safety and immunogenicity trial in adult volunteers of a human papillomavirus 16 L1 virus-like particle vaccine. *J Natl Cancer Inst* 2001, **93**(4), 284-292.

154   Dupuis, M., Murphy, T.J., Higgins, D. *et al.* Dendritic cells internalize vaccine adjuvant after intramuscular injection. *Cell Immunol* 1998, **186**(1), 18-27.

155   Dupuis, M., McDonald, D.M. & Ott, G. Distribution of adjuvant MF59 and antigen gD2 after intramuscular injection in mice. *Vaccine* 1999, **18**(5-6), 434-439.

156   Podda, A. & Del Giudice, G. MF59-adjuvanted vaccines: increased immunogenicity with an optimal safety profile. *Expert Rev Vaccines* 2003, **2**(2), 197-203.

157   Gulati, M., Bajad, S., Singh, S., Ferdous, A.J. & Singh, M. Development of liposomal amphotericin B formulation. *J Microencapsul* 1998, **15**(2), 137-151.

158   Allison, A.G. & Gregoriadis, G. Liposomes as immunological adjuvants. *Nature* 1974, **252**(5480), 252.

159   Jiao, X., Wang, R.Y., Qiu, Q., Alter, H.J. & Shih, J.W. Enhanced hepatitis C virus NS3 specific Th1 immune responses induced by co-delivery of protein antigen and CpG with cationic liposomes. *J Gen Virol* 2004, **85**(Pt 6), 1545-1553.

160   Mannino, R.J., Canki, M., Feketeova, E. *et al.* Targeting immune response induction with cochleate and liposome-based vaccines. *Adv Drug Deliv Rev* 1998, **32**(3), 273-287.

161   Mazumdar, T., Anam, K. & Ali, N. A mixed Th1/Th2 response elicited by a liposomal formulation of Leishmania vaccine instructs Th1 responses and resistance to Leishmania donovani in susceptible BALB/c mice. *Vaccine* 2004, **22**(9-10), 1162-1171.

162   Engler, O.B., Schwendener, R.A., Dai, W.J. *et al.* A liposomal peptide vaccine inducing CD8+ T cells in HLA-A2.1 transgenic mice, which recognise human cells encoding hepatitis C virus (HCV) proteins. *Vaccine* 2004, **23**(1), 58-68.

163   Ohishi, K., Kabeya, H., Amanuma, H. & Onuma, M. Induction of bovine leukaemia virus Env-specific Th-1 type immunity in mice by vaccination with short synthesized peptide-liposome. *Vaccine* 1996, **14**(12), 1143-1148.

164   Schumacher, R., Adamina, M., Zurbriggen, R. *et al.* Influenza virosomes enhance class I restricted CTL induction through CD4+ T cell activation. *Vaccine* 2004, **22**(5-6), 714-723.

165   Sugita, T., Yoshikawa, T., Gao, J.Q. *et al.* Fusogenic liposome can be used as an effective vaccine carrier for peptide vaccination to induce cytotoxic T lymphocyte (CTL) response. *Biol Pharm Bull* 2005, **28**(1), 192-193.

166   Alving, C.R., Koulchin, V., Glenn, G.M. & Rao, M. Liposomes as carriers of peptide antigens: induction of antibodies and cytotoxic T lymphocytes to conjugated and unconjugated peptides. *Immunol Rev* 1995, **145**, 5-31.

167   Rao, M. & Alving, C.R. Delivery of lipids and liposomal proteins to the cytoplasm and Golgi of antigen-presenting cells. mangala.rao@na.amedd.army.mil. *Adv Drug Deliv Rev* 2000, **41**(2), 171-188.

168   Ahsan, F., Rivas, I.P., Khan, M.A. & Torres Suarez, A.I. Targeting to macrophages: role of physicochemical properties of particulate carriers--

liposomes and microspheres--on the phagocytosis by macrophages. *J Control Release* 2002, **79**(1-3), 29-40.

169 Foged, C., Arigita, C., Sundblad, A., Jiskoot, W., Storm, G. & Frokjaer, S. Interaction of dendritic cells with antigen-containing liposomes: effect of bilayer composition. *Vaccine* 2004, **22**(15-16), 1903-1913.

170 Mazumdar, T., Anam, K. & Ali, N. Influence of phospholipid composition on the adjuvanticity and protective efficacy of liposome-encapsulated Leishmania donovani antigens. *J Parasitol* 2005, **91**(2), 269-274.

171 Brayden, D.J. & Baird, A.W. Microparticle vaccine approaches to stimulate mucosal immunisation. *Microbes Infect* 2001, **3**(10), 867-876.

172 Kersten, G.F. & Crommelin, D.J. Liposomes and ISCOMs. *Vaccine* 2003, **21**(9-10), 915-920.

173 Ambrosch, F., Wiedermann, G., Jonas, S. *et al.* Immunogenicity and protectivity of a new liposomal hepatitis A vaccine. *Vaccine* 1997, **15**(11), 1209-1213.

174 Bungener, L., Huckriede, A., Wilschut, J. & Daemen, T. Delivery of protein antigens to the immune system by fusion-active virosomes: a comparison with liposomes and ISCOMs. *Biosci Rep* 2002, **22**(2), 323-338.

175 Gupta, P.N., Mishra, V., Rawat, A. *et al.* Non-invasive vaccine delivery in transfersomes, niosomes and liposomes: a comparative study. *Int J Pharm* 2005, **293**(1-2), 73-82.

176 Huckriede, A., Bungener, L., Daemen, T. & Wilschut, J. Influenza virosomes in vaccine development. *Methods Enzymol* 2003, **373**, 74-91.

177 Kunisawa, J., Nakagawa, S. & Mayumi, T. Pharmacotherapy by intracellular delivery of drugs using fusogenic liposomes: application to vaccine development. *Adv Drug Deliv Rev* 2001, **52**(3), 177-186.

178 Patel, G.B., Omri, A., Deschatelets, L. & Sprott, G.D. Safety of archaeosome adjuvants evaluated in a mouse model. *J Liposome Res* 2002, **12**(4), 353-372.

179 Vyas, S.P., Singh, R.P., Jain, S. *et al.* Non-ionic surfactant based vesicles (niosomes) for non-invasive topical genetic immunization against hepatitis B. *Int J Pharm* 2005, **296**(1-2), 80-86.

180 Patel, G.B. & Sprott, G.D. Archaeobacterial ether lipid liposomes (archaeosomes) as novel vaccine and drug delivery systems. *Crit Rev Biotechnol* 1999, **19**(4), 317-357.

181 Morein, B., Sundquist, B., Hoglund, S., Dalsgaard, K. & Osterhaus, A. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. *Nature* 1984, **308**(5958), 457-460.

182 Morein, B., Hu, K.F. & Abusugra, I. Current status and potential application of ISCOMs in veterinary medicine. *Adv Drug Deliv Rev* 2004, **56**(10), 1367-1382.

183 Hu, K.F., Lovgren-Bengtsson, K. & Morein, B. Immunostimulating complexes (ISCOMs) for nasal vaccination. *Adv Drug Deliv Rev* 2001, **51**(1-3), 149-159.

184 Pedersen, I.R., Bog-Hansen, T.C., Dalsgaard, K. & Heegaard, P.M. Iscom immunization with synthetic peptides representing measles virus hemagglutinin. *Virus Res* 1992, **24**(2), 145-159.

185 de Vries, P., van Binnendijk, R.S., van der Marel, P. *et al.* Measles virus fusion protein presented in an immune-stimulating complex (iscom) induces haemolysis-

inhibiting and fusion-inhibiting antibodies, virus-specific T cells and protection in mice. *J Gen Virol* 1988, **69 (Pt 3)**, 549-559.

186   Stittelaar, K.J., Boes, J., Kersten, G.F. *et al.* In vivo antibody response and in vitro CTL activation induced by selected measles vaccine candidates, prepared with purified Quil A components. *Vaccine* 2000, **18**(23), 2482-2493.

187   Sambhara, S., Kurichh, A., Miranda, R. *et al.* Enhanced immune responses and resistance against infection in aged mice conferred by Flu-ISCOMs vaccine correlate with up-regulation of costimulatory molecule CD86. *Vaccine* 1998, **16**(18), 1698-1704.

188   Sambhara, S., Switzer, I., Kurichh, A. *et al.* Enhanced antibody and cytokine responses to influenza viral antigens in perforin-deficient mice. *Cell Immunol* 1998, **187**(1), 13-18.

189   Takahashi, H., Takeshita, T., Morein, B., Putney, S., Germain, R.N. & Berzofsky, J.A. Induction of CD8+ cytotoxic T cells by immunization with purified HIV-1 envelope protein in ISCOMs. *Nature* 1990, **344**(6269), 873-875.

190   Akerblom, L., Nara, P., Dunlop, N., Putney, S. & Morein, B. HIV experimental vaccines based on the iscom technology using envelope and GAG gene products. *Biotechnol Ther* 1993, **4**(3-4), 145-161.

191   Ronnberg, B., Fekadu, M. & Morein, B. Adjuvant activity of non-toxic Quillaja saponaria Molina components for use in ISCOM matrix. *Vaccine* 1995, **13**(14), 1375-1382.

192   Robson, N.C., Beacock-Sharp, H., Donachie, A.M. & Mowat, A.M. Dendritic cell maturation enhances CD8+ T-cell responses to exogenous antigen via a proteasome-independent mechanism of major histocompatibility complex class I loading. *Immunology* 2003, **109**(3), 374-383.

193   Robson, N.C., Beacock-Sharp, H., Donachie, A.M. & Mowat, A.M. The role of antigen-presenting cells and interleukin-12 in the priming of antigen-specific CD4+ T cells by immune stimulating complexes. *Immunology* 2003, **110**(1), 95-104.

194   Kumar, V. & Banker, G. Target-Oriented Drug Delivery Systems. In *Modern Pharmaceutics* (Eds. Banker, G.S. & Rhodes, C.T.) Marcel Dekker, Inc., New York, 1996. 611-680.

195   Kreuter, J. & Speiser, P.P. New adjuvants on a polymethylmethacrylate base. *Infect Immun* 1976, **13**(1), 204-210.

196   Kreuter, J. & Speiser, P.P. In vitro studies of poly(methyl methacrylate) adjuvants. *J Pharm Sci* 1976, **65**(11), 1624-1627.

197   Kreuter, J. & Liehl, E. Long-term studies of microencapsulated and adsorbed influenza vaccine nanoparticles. *J Pharm Sci* 1981, **70**(4), 367-371.

198   Kreuter, J. Nanoparticles as adjuvants for vaccines. *Pharm Biotechnol* 1995, **6**, 463-472.

199   Kreuter, J., Nefzger, M., Liehl, E., Czok, R. & Voges, R. Distribution and elimination of poly(methyl methacrylate) nanoparticles after subcutaneous administration to rats. *J Pharm Sci* 1983, **72**(10), 1146-1149.

200   Couvreur, P., Kante, B., Roland, M., Guiot, P., Bauduin, P. & Speiser, P. Polycyanoacrylate nanocapsules as potential lysosomotropic carriers: preparation, morphological and sorptive properties. *J Pharm Pharmacol* 1979, **31**(5), 331-332.

201   O'Hagan, D.T., Rahman, D., McGee, J.P. *et al.* Biodegradable microparticles as controlled release antigen delivery systems. *Immunology* 1991, **73**(2), 239-242.

202   O'Hagan, D.T., Jeffery, H., Roberts, M.J., McGee, J.P. & Davis, S.S. Controlled release microparticles for vaccine development. *Vaccine* 1991, **9**(10), 768-771.

203   Eldridge, J.H., Staas, J.K., Meulbroek, J.A., McGhee, J.R., Tice, T.R. & Gilley, R.M. Biodegradable microspheres as a vaccine delivery system. *Mol Immunol* 1991, **28**(3), 287-294.

204   van der Lubben, I.M., Verhoef, J.C., Borchard, G. & Junginger, H.E. Chitosan for mucosal vaccination. *Adv Drug Deliv Rev* 2001, **52**(2), 139-144.

205   Illum, L., Jabbal-Gill, I., Hinchcliffe, M., Fisher, A.N. & Davis, S.S. Chitosan as a novel nasal delivery system for vaccines. *Adv Drug Deliv Rev* 2001, **51**(1-3), 81-96.

206   Todd, C.W., Balusubramanian, M. & Newman, M.J. Development of adjuvant-active nonionic block copolymers. *Adv Drug Deliv Rev* 1998, **32**(3), 199-223.

207   Payne, L.G., Jenkins, S.A., Woods, A.L. *et al.* Poly[di(carboxylatophenoxy)phosphazene] (PCPP) is a potent immunoadjuvant for an influenza vaccine. *Vaccine* 1998, **16**(1), 92-98.

208   Sanchez-Brunete, J.A., Dea, M.A., Rama, S. *et al.* Influence of the vehicle on the properties and efficacy of microparticles containing amphotericin B. *J Drug Target* 2005, **13**(4), 225-233.

209   Quick, D.J., Macdonald, K.K. & Anseth, K.S. Delivering DNA from photocrosslinked, surface eroding polyanhydrides. *J Control Release* 2004, **97**(2), 333-343.

210   Shive, M.S. & Anderson, J.M. Biodegradation and biocompatibility of PLA and PLGA microspheres. *Adv Drug Deliv Rev* 1997, **28**(1), 5-24.

211   Jain, R.A. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. *Biomaterials* 2000, **21**(23), 2475-2490.

212   Caputo, A., Brocca-Cofano, E., Castaldello, A. *et al.* Novel biocompatible anionic polymeric microspheres for the delivery of the HIV-1 Tat protein for vaccine application. *Vaccine* 2004, **22**(21-22), 2910-2924.

213   Conway, M.A., Madrigal-Estebas, L., McClean, S., Brayden, D.J. & Mills, K.H. Protection against Bordetella pertussis infection following parenteral or oral immunization with antigens entrapped in biodegradable particles: effect of formulation and route of immunization on induction of Th1 and Th2 cells. *Vaccine* 2001, **19**(15-16), 1940-1950.

214   Moore, A., McGuirk, P., Adams, S. *et al.* Immunization with a soluble recombinant HIV protein entrapped in biodegradable microparticles induces HIV-specific CD8+ cytotoxic T lymphocytes and CD4+ Th1 cells. *Vaccine* 1995, **13**(18), 1741-1749.

215   Partidos, C.D., Vohra, P., Anagnostopoulou, C., Jones, D.H., Farrar, G.H. & Steward, M.W. Biodegradable microparticles as a delivery system for measles virus cytotoxic T cell epitopes. *Mol Immunol* 1996, **33**(6), 485-491.

216   Vordermeier, H.M., Coombes, A.G., Jenkins, P. *et al.* Synthetic delivery system for tuberculosis vaccines: immunological evaluation of the M. tuberculosis 38

kDa protein entrapped in biodegradable PLG microparticles. *Vaccine* 1995, **13**(16), 1576-1582.

217   Le Buanec, H., Vetu, C., Lachgar, A. *et al.* Induction in mice of anti-Tat mucosal immunity by the intranasal and oral routes. *Biomed Pharmacother* 2001, **55**(6), 316-320.

218   Bramwell, V.W., Eyles, J.E. & Oya Alpar, H. Particulate delivery systems for biodefense subunit vaccines. *Adv Drug Deliv Rev* 2005, **57**(9), 1247-1265.

219   Singh, M. & O'Hagan, D. The preparation and characterization of polymeric antigen delivery systems for oral administration. *Adv Drug Deliv Rev* 1998, **34**(2-3), 285-304.

220   O'Hagan, D.T. Microparticles and polymers for the mucosal delivery of vaccines. *Adv Drug Deliv Rev* 1998, **34**(2-3), 305-320.

221   Challacombe, S.J., Rahman, D., Jeffery, H., Davis, S.S. & O'Hagan, D.T. Enhanced secretory IgA and systemic IgG antibody responses after oral immunization with biodegradable microparticles containing antigen. *Immunology* 1992, **76**(1), 164-168.

222   Challacombe, S.J., Rahman, D. & O'Hagan, D.T. Salivary, gut, vaginal and nasal antibody responses after oral immunization with biodegradable microparticles. *Vaccine* 1997, **15**(2), 169-175.

223   Kende, M., Yan, C., Hewetson, J., Frick, M.A., Rill, W.L. & Tammariello, R. Oral immunization of mice with ricin toxoid vaccine encapsulated in polymeric microspheres against aerosol challenge. *Vaccine* 2002, **20**(11-12), 1681-1691.

224   Allaoui-Attarki, K., Pecquet, S., Fattal, E. *et al.* Protective immunity against Salmonella typhimurium elicited in mice by oral vaccination with phosphorylcholine encapsulated in poly(DL-lactide-co-glycolide) microspheres. *Infect Immun* 1997, **65**(3), 853-857.

225   Huyghebaert, N., Vermeire, A., Rottiers, P., Remaut, E. & Remon, J.P. Development of an enteric-coated, layered multi-particulate formulation for ileal delivery of viable recombinant Lactococcus lactis. *Eur J Pharm Biopharm* 2005, **61**(3), 134-141.

226   O'Hagan, D.T. The intestinal uptake of particles and the implications for drug and antigen delivery. *J Anat* 1996, **189 (Pt 3)**, 477-482.

227   Brayden, D.J. Oral vaccination in man using antigens in particles: current status. *Eur J Pharm Sci* 2001, **14**(3), 183-189.

228   Pitt, C.G., Gratzl, M.M., Kimmel, G.L., Surles, J. & Schindler, A. Aliphatic polyesters II. The degradation of poly (DL-lactide), poly (epsilon-caprolactone), and their copolymers in vivo. *Biomaterials* 1981, **2**(4), 215-220.

229   Kissel, T. & Koneberg, R. Injectable biodegradable microspheres for vaccine delivery. In *Microparticulate Systems for the Delivery of Proteins and Vaccines*, Vol. 77 (Eds. Cohen, S. & Bernstein, H.) Marcel Dekker, Inc, New York, 1996. 51-87.

230   Gupta, R.K., Singh, M. & O'Hagan, D.T. Poly(lactide-co-glycolide) microparticles for the development of single-dose controlled-release vaccines. *Adv Drug Deliv Rev* 1998, **32**(3), 225-246.

231 Sah, H., Toddywala, R. & Chien, Y.W. Continuous release of proteins from biodegradable microcapsules and in vivo evaluatjion of their potential as a vaccine adjuvant. *Journal of Controlled Release* 1995, **35**, 137-144.

232 Coombes, A.G., Lavelle, E.C., Jenkins, P.G. & Davis, S.S. Single dose, polymeric, microparticle-based vaccines: the influence of formulation conditions on the magnitude and duration of the immune response to a protein antigen. *Vaccine* 1996, **14**(15), 1429-1438.

233 Cleland, J.L., Lim, A., Daugherty, A. *et al.* Development of a single-shot subunit vaccine for HIV-1. 5. programmable in vivo autoboost and long lasting neutralizing response. *J Pharm Sci* 1998, **87**(12), 1489-1495.

234 Singh, M., Li, X.M., Wang, H. *et al.* Immunogenicity and protection in small-animal models with controlled-release tetanus toxoid microparticles as a single-dose vaccine. *Infect Immun* 1997, **65**(5), 1716-1721.

235 Singh, M., Chesko, J., Kazzaz, J. *et al.* Adsorption of a novel recombinant glycoprotein from HIV (Env gp120dV2 SF162) to anionic PLG microparticles retains the structural integrity of the protein, whereas encapsulation in PLG microparticles does not. *Pharm Res* 2004, **21**(12), 2148-2152.

236 Chesko, J., Kazzaz, J., Ugozzoli, M., O'Hagan D, T. & Singh, M. An investigation of the factors controlling the adsorption of protein antigens to anionic PLG microparticles. *J Pharm Sci* 2005, **94**(11), 2510-2519.

237 Singh, M., Kazzaz, J., Chesko, J. *et al.* Anionic microparticles are a potent delivery system for recombinant antigens from Neisseria meningitidis serotype B. *J Pharm Sci* 2004, **93**(2), 273-282.

238 Kazzaz, J., Neidleman, J., Singh, M., Ott, G. & O'Hagan, D.T. Novel anionic microparticles are a potent adjuvant for the induction of cytotoxic T lymphocytes against recombinant p55 gag from HIV-1. *J Control Release* 2000, **67**(2-3), 347-356.

239 Elamanchili, P., Diwan, M., Cao, M. & Samuel, J. Characterization of poly(D,L-lactic-co-glycolic acid) based nanoparticulate system for enhanced delivery of antigens to dendritic cells. *Vaccine* 2004, **22**(19), 2406-2412.

240 Lutsiak, M.E., Robinson, D.R., Coester, C., Kwon, G.S. & Samuel, J. Analysis of poly(D,L-lactic-co-glycolic acid) nanosphere uptake by human dendritic cells and macrophages in vitro. *Pharm Res* 2002, **19**(10), 1480-1487.

241 Sun, H., Pollock, K.G. & Brewer, J.M. Analysis of the role of vaccine adjuvants in modulating dendritic cell activation and antigen presentation in vitro. *Vaccine* 2003, **21**(9-10), 849-855.

242 Thiele, L., Rothen-Rutishauser, B., Jilek, S., Wunderli-Allenspach, H., Merkle, H.P. & Walter, E. Evaluation of particle uptake in human blood monocyte-derived cells in vitro. Does phagocytosis activity of dendritic cells measure up with macrophages? *Journal of Controlled Release* 2001, **76**, 59-71.

243 Kreuter, J., Liehl, E., Berg, U., Soliva, M. & Speiser, P.P. Influence of hydrophobicity on the adjuvant effect of particulate polymeric adjuvants. *Vaccine* 1988, **6**(3), 253-256.

244 Hawley, A.E., Davis, S.S. & Illum, L. Targeting colloids to lymph nodes: Influence of lymphatic physiology and colloidal characteristics. *Adv Drug Deliv Rev* 1995, **17**, 129-148.

245 Raghuvanshi, R.S., Katare, Y.K., Lalwani, K., Ali, M.M., Singh, O. & Panda, A.K. Improved immune response from biodegradable polymer particles entrapping tetanus toxoid by use of different immunization protocol and adjuvants. *Int J Pharm* 2002, **245**(1-2), 109-121.

246 Chong, C.S., Cao, M., Wong, W.W. *et al.* Enhancement of T helper type 1 immune responses against hepatitis B virus core antigen by PLGA nanoparticle vaccine delivery. *J Control Release* 2005, **102**(1), 85-99.

247 Shephard, M.J., Todd, D., Adair, B.M., Po, A.L., Mackie, D.P. & Scott, E.M. Immunogenicity of bovine parainfluenza type 3 virus proteins encapsulated in nanoparticle vaccines, following intranasal administration to mice. *Res Vet Sci* 2003, **74**(2), 187-190.

248 Debin, A., Kravtzoff, R., Santiago, J.V. *et al.* Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses. *Vaccine* 2002, **20**(21-22), 2752-2763.

249 Oyewumi, M.O. & Mumper, R.J. Gadolinium-loaded nanoparticles engineered from microemulsion templates. *Drug Dev Ind Pharm* 2002, **28**(3), 317-328.

250 Oyewumi, M.O. & Mumper, R.J. Engineering tumor-targeted gadolinium hexanedione nanoparticles for potential application in neutron capture therapy. *Bioconjug Chem* 2002, **13**(6), 1328-1335.

251 Koziara, J.M., Oh, J.J., Akers, W.S., Ferraris, S.P. & Mumper, R.J. Blood compatibility of cetyl alcohol/polysorbate-based nanoparticles. *Pharm Res* 2005, **22**(11), 1821-1828.

252 Dong, X. & Mumper, R.J. The metabolism of fatty alcohols in lipid nanoparticles by alcohol dehydrogenase. *Drug Dev Ind Pharm* In Press 2006.

253 Cui, Z., Patel, J., Tuzova, M. *et al.* Strong T cell type-1 immune responses to HIV-1 Tat (1-72) protein-coated nanoparticles. *Vaccine* 2004, **22**(20), 2631-2640.

254 O'Hagan, D.T., Ugozzoli, M., Barackman, J. *et al.* Microparticles in MF59, a potent adjuvant combination for a recombinant protein vaccine against HIV-1. *Vaccine* 2000, **18**(17), 1793-1801.

255 Moore, A., McCarthy, L. & Mills, K.H. The adjuvant combination monophosphoryl lipid A and QS21 switches T cell responses induced with a soluble recombinant HIV protein from Th2 to Th1. *Vaccine* 1999, **17**(20-21), 2517-2527.

256 Su, Z., Tam, M.F., Jankovic, D. & Stevenson, M.M. Vaccination with novel immunostimulatory adjuvants against blood-stage malaria in mice. *Infect Immun* 2003, **71**(9), 5178-5187.

257 Kazzaz, J., Singh, M., Ugozzoli, M., Chesko, J., Soenawan, E. & O'Hagan D, T. Encapsulation of the immune potentiators MPL and RC529 in PLG microparticles enhances their potency. *J Control Release* 2006, **110**(3), 566-573.

258 Joseph, A., Louria-Hayon, I., Plis-Finarov, A. *et al.* Liposomal immunostimulatory DNA sequence (ISS-ODN): an efficient parenteral and mucosal adjuvant for influenza and hepatitis B vaccines. *Vaccine* 2002, **20**(27-28), 3342-3354.

259 O'Hagan, D.T., Singh, M., Kazzaz, J. *et al.* Synergistic adjuvant activity of immunostimulatory DNA and oil/water emulsions for immunization with HIV p55 gag antigen. *Vaccine* 2002, **20**(27-28), 3389-3398.

260 Xie, H., Gursel, I., Ivins, B.E. *et al.* CpG oligodeoxynucleotides adsorbed onto polylactide-co-glycolide microparticles improve the immunogenicity and protective activity of the licensed anthrax vaccine. *Infect Immun* 2005, **73**(2), 828-833.

261 Tabata, Y. & Ikada, Y. Macrophage activation through phagocytosis of muramyl dipeptide encapsulated in gelatin microspheres. *J Pharm Pharmacol* 1987, **39**(9), 698-704.

262 Puri, N. & Sinko, P.J. Adjuvancy enhancement of muramyl dipeptide by modulating its release from a physicochemically modified matrix of ovalbumin microspheres. II. In vivo investigation. *J Control Release* 2000, **69**(1), 69-80.

263 Diwan, M., Elamanchili, P., Cao, M. & Samuel, J. Dose sparing of CpG oligodeoxynucleotide vaccine adjuvants by nanoparticle delivery. *Curr Drug Deliv* 2004, **1**(4), 405-412.

264 Banchereau, J., Briere, F., Caux, C. *et al.* Immunobiology of dendritic cells. *Annu Rev Immunol* 2000, **18**, 767-811.

265 Moll, H. Antigen delivery by dendritic cells. *Int J Med Microbiol* 2004, **294**(5), 337-344.

266 Steinman, R.M. Dendritic cells and the control of immunity: enhancing the efficiency of antigen presentation. *Mt Sinai J Med* 2001, **68**(3), 160-166.

267 Banchereau, J. & Steinman, R.M. Dendritic cells and the control of immunity. *Nature* 1998, **392**(6673), 245-252.

268 Shortman, K. & Liu, Y.J. Mouse and human dendritic cell subtypes. *Nat Rev Immunol* 2002, **2**(3), 151-161.

269 Pulendran, B. Modulating vaccine responses with dendritic cells and Toll-like receptors. *Immunol Rev* 2004, **199**, 227-250.

270 Corinti, S., Albanesi, C., la Sala, A., Pastore, S. & Girolomoni, G. Regulatory activity of autocrine IL-10 on dendritic cell functions. *J Immunol* 2001, **166**(7), 4312-4318.

271 Lebecque, S. Antigen receptors and dendritic cells. *Vaccine* 2000, **18**(16), 1603-1605.

272 Gogolak, P., Rethi, B., Hajas, G. & Rajnavolgyi, E. Targeting dendritic cells for priming cellular immune responses. *J Mol Recognit* 2003, **16**(5), 299-317.

273 Engel, A., Chatterjee, S.K., Al-arifi, A., Riemann, D., Langner, J. & Nuhn, P. Influence of spacer length on interaction of mannosylated liposomes with human phagocytic cells. *Pharm Res* 2003, **20**(1), 51-57.

274 Arigita, C., Bevaart, L., Everse, L.A. *et al.* Liposomal meningococcal B vaccination: role of dendritic cell targeting in the development of a protective immune response. *Infect Immun* 2003, **71**(9), 5210-5218.

275 Foged, C., Sundblad, A. & Hovgaard, L. Targeting vaccines to dendritic cells. *Pharm Res* 2002, **19**(3), 229-238.

276 Sihorkar, V. & Vyas, S.P. Potential of polysaccharide anchored liposomes in drug delivery, targeting and immunization. *J Pharm Pharm Sci* 2001, **4**(2), 138-158.

277   Martinez-Pomares, L. & Gordon, S. Potential role of the mannose receptor in antigen transport. *Immunol Lett* 1999, **65**(1-2), 9-13.

278   Apostolopoulos, V. & McKenzie, I.F. Role of the mannose receptor in the immune response. *Curr Mol Med* 2001, **1**(4), 469-474.

279   Mahnke, K., Guo, M., Lee, S. *et al.* The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. *J Cell Biol* 2000, **151**(3), 673-684.

280   Tan, M.C., Mommaas, A.M., Drijfhout, J.W. *et al.* Mannose receptor-mediated uptake of antigens strongly enhances HLA class II-restricted antigen presentation by cultured dendritic cells. *Eur J Immunol* 1997, **27**(9), 2426-2435.

281   Sasaki, S., Fukushima, J., Arai, H. *et al.* Human immunodeficiency virus type-1-specific immune responses induced by DNA vaccination are greatly enhanced by mannan-coated diC14-amidine. *Eur J Immunol* 1997, **27**(12), 3121-3129.

282   Toda, S., Ishii, N., Okada, E. *et al.* HIV-1-specific cell-mediated immune responses induced by DNA vaccination were enhanced by mannan-coated liposomes and inhibited by anti-interferon-gamma antibody. *Immunology* 1997, **92**(1), 111-117.

283   Sugimoto, M., Ohishi, K., Fukasawa, M. *et al.* Oligomannose-coated liposomes as an adjuvant for the induction of cell-mediated immunity. *FEBS Lett* 1995, **363**(1-2), 53-56.

284   Shiku, H., Wang, L., Ikuta, Y. *et al.* Development of a cancer vaccine: peptides, proteins, and DNA. *Cancer Chemother Pharmacol* 2000, **46 Suppl**, S77-82.

285   Fukasawa, M., Shimizu, Y., Shikata, K. *et al.* Liposome oligomannose-coated with neoglycolipid, a new candidate for a safe adjuvant for induction of CD8+ cytotoxic T lymphocytes. *FEBS Lett* 1998, **441**(3), 353-356.

286   Shimizu, Y., Yamakami, K., Gomi, T. *et al.* Protection against Leishmania major infection by oligomannose-coated liposomes. *Bioorg Med Chem* 2003, **11**(7), 1191-1195.

287   Jiang, W., Swiggard, W.J., Heufler, C. *et al.* The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature* 1995, **375**(6527), 151-155.

288   Guo, M., Gong, S., Maric, S. *et al.* A monoclonal antibody to the DEC-205 endocytosis receptor on human dendritic cells. *Hum Immunol* 2000, **61**(8), 729-738.

289   Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M.C. & Steinman, R.M. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J Exp Med* 2002, **196**(12), 1627-1638.

290   van Broekhoven, C.L., Parish, C.R., Demangel, C., Britton, W.J. & Altin, J.G. Targeting dendritic cells with antigen-containing liposomes: a highly effective procedure for induction of antitumor immunity and for tumor immunotherapy. *Cancer Res* 2004, **64**(12), 4357-4365.

291 Trumpfheller, C., Finke, J.S., Lopez, C.B. *et al.* Intensified and protective CD4+ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine. *J Exp Med* 2006, **203**(3), 607-617.

292 Gosselin, E.J., Wardwell, K., Gosselin, D.R., Alter, N., Fisher, J.L. & Guyre, P.M. Enhanced antigen presentation using human Fc gamma receptor (monocyte/macrophage)-specific immunogens. *J Immunol* 1992, **149**(11), 3477-3481.

293 Liu, C., Goldstein, J., Graziano, R.F. *et al.* F(c)gammaRI-targeted fusion proteins result in efficient presentation by human monocytes of antigenic and antagonist T cell epitopes. *J Clin Invest* 1996, **98**(9), 2001-2007.

294 Serre, K., Machy, P., Grivel, J.C. *et al.* Efficient presentation of multivalent antigens targeted to various cell surface molecules of dendritic cells and surface Ig of antigen-specific B cells. *J Immunol* 1998, **161**(11), 6059-6067.

295 Machy, P., Serre, K. & Leserman, L. Class I-restricted presentation of exogenous antigen acquired by Fcgamma receptor-mediated endocytosis is regulated in dendritic cells. *Eur J Immunol* 2000, **30**(3), 848-857.

296 Bot, A.I., Smith, D.J., Bot, S. *et al.* Receptor-mediated targeting of spray-dried lipid particles coformulated with immunoglobulin and loaded with a prototype vaccine. *Pharm Res* 2001, **18**(7), 971-979.

297 Rafiq, K., Bergtold, A. & Clynes, R. Immune complex-mediated antigen presentation induces tumor immunity. *J Clin Invest* 2002, **110**(1), 71-79.

298 Regnault, A., Lankar, D., Lacabanne, V. *et al.* Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp Med* 1999, **189**(2), 371-380.

299 Guyre, C.A., Barreda, M.E., Swink, S.L. & Fanger, M.W. Colocalization of Fc gamma RI-targeted antigen with class I MHC: implications for antigen processing. *J Immunol* 2001, **166**(4), 2469-2478.

300 Shroff, K.E., Sengupta, S.R. & Kamat, R.S. Route-related variation in immunogenicity of mycobacteria. *Int J Lepr Other Mycobact Dis* 1990, **58**(1), 44-49.

301 Puri, N., Weyand, E.H., Abdel-Rahman, S.M. & Sinko, P.J. An investigation of the intradermal route as an effective means of immunization for microparticulate vaccine delivery systems. *Vaccine* 2000, **18**(23), 2600-2612.

302 Mark, A., Carlsson, R.M. & Granstrom, M. Subcutaneous versus intramuscular injection for booster DT vaccination of adolescents. *Vaccine* 1999, **17**(15-16), 2067-2072.

303 De Rose, R., Tennent, J., McWaters, P. *et al.* Efficacy of DNA vaccination by different routes of immunisation in sheep. *Vet Immunol Immunopathol* 2002, **90**(1-2), 55-63.

304 Ito, K., Shinohara, N. & Kato, S. DNA immunization via intramuscular and intradermal routes using a gene gun provides different magnitudes and durations on immune response. *Mol Immunol* 2003, **39**(14), 847-854.

305 Sakaue, G., Hiroi, T., Nakagawa, Y. *et al.* HIV mucosal vaccine: nasal immunization with gp160-encapsulated hemagglutinating virus of Japan-liposome

induces antigen-specific CTLs and neutralizing antibody responses. *J Immunol* 2003, **170**(1), 495-502.

306   Pizza, M., Giuliani, M.M., Fontana, M.R. *et al.* Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants. *Vaccine* 2001, **19**(17-19), 2534-2541.

307   Partidos, C.D., Beignon, A.S., Semetey, V., Briand, J.P. & Muller, S. The bare skin and the nose as non-invasive routes for administering peptide vaccines. *Vaccine* 2001, **19**(17-19), 2708-2715.

308   Babiuk, S., Baca-Estrada, M., Babiuk, L.A., Ewen, C. & Foldvari, M. Cutaneous vaccination: the skin as an immunologically active tissue and the challenge of antigen delivery. *J Control Release* 2000, **66**(2-3), 199-214.

309   Hammond, S.A., Tsonis, C., Sellins, K. *et al.* Transcutaneous immunization of domestic animals: opportunities and challenges. *Adv Drug Deliv Rev* 2000, **43**(1), 45-55.

310   Scharton-Kersten, T., Glenn, G.M., Vassell, R., Yu, J., Walwender, D. & Alving, C.R. Principles of transcutaneous immunization using cholera toxin as an adjuvant. *Vaccine* 1999, **17 Suppl 2**, S37-43.

311   Matriano, J.A., Cormier, M., Johnson, J. *et al.* Macroflux microprojection array patch technology: a new and efficient approach for intracutaneous immunization. *Pharm Res* 2002, **19**(1), 63-70.

312   Gockel, C.M., Bao, S. & Beagley, K.W. Transcutaneous immunization induces mucosal and systemic immunity: a potent method for targeting immunity to the female reproductive tract. *Mol Immunol* 2000, **37**(9), 537-544.

313   Beignon, A.S., Briand, J.P., Rappuoli, R., Muller, S. & Partidos, C.D. The LTR72 mutant of heat-labile enterotoxin of Escherichia coli enhances the ability of peptide antigens to elicit CD4(+) T cells and secrete gamma interferon after coapplication onto bare skin. *Infect Immun* 2002, **70**(6), 3012-3019.

314   Glenn, G.M., Rao, M., Matyas, G.R. & Alving, C.R. Skin immunization made possible by cholera toxin. *Nature* 1998, **391**(6670), 851.

315   Glenn, G.M., Taylor, D.N., Li, X., Frankel, S., Montemarano, A. & Alving, C.R. Transcutaneous immunization: a human vaccine delivery strategy using a patch. *Nat Med* 2000, **6**(12), 1403-1406.

316   Dean, H.J., Fuller, D. & Osorio, J.E. Powder and particle-mediated approaches for delivery of DNA and protein vaccines into the epidermis. *Comp Immunol Microbiol Infect Dis* 2003, **26**(5-6), 373-388.

317   Chen, D., Weis, K.F., Chu, Q. *et al.* Epidermal powder immunization induces both cytotoxic T-lymphocyte and antibody responses to protein antigens of influenza and hepatitis B viruses. *J Virol* 2001, **75**(23), 11630-11640.

318   Osorio, J.E., Zuleger, C.L., Burger, M., Chu, Q., Payne, L.G. & Chen, D. Immune responses to hepatitis B surface antigen following epidermal powder immunization. *Immunol Cell Biol* 2003, **81**(1), 52-58.

319   Chen, D., Zuleger, C., Chu, Q., Maa, Y.F., Osorio, J. & Payne, L.G. Epidermal powder immunization with a recombinant HIV gp120 targets Langerhans cells and induces enhanced immune responses. *AIDS Res Hum Retroviruses* 2002, **18**(10), 715-722.

320 Barre-Sinoussi, F., Chermann, J.C., Rey, F. *et al.* Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 1983, **220**(4599), 868-871.

321 Gallo, R.C. & Montagnier, L. The chronology of AIDS research. *Nature* 1987, **326**(6112), 435-436.

322 Klein, M. Current progress in the development of human immunodeficiency virus vaccines: research and clinical trials. *Vaccine* 2001, **19**(17-19), 2210-2215.

323 Robinson, H.L. New hope for an AIDS vaccine. *Nat Rev Immunol* 2002, **2**(4), 239-250.

324 Sleasman, J.W. & Goodenow, M.M. 13. HIV-1 infection. *J Allergy Clin Immunol* 2003, **111**(2 Suppl), S582-592.

325 Ahmed, R.K., Biberfeld, G. & Thorstensson, R. Innate immunity in experimental SIV infection and vaccination. *Mol Immunol* 2005, **42**(2), 251-258.

326 Hulskotte, E.G., Geretti, A.M. & Osterhaus, A.D. Towards an HIV-1 vaccine: lessons from studies in macaque models. *Vaccine* 1998, **16**(9-10), 904-915.

327 Girard, M.P. & Excler, J. Human Immunodeficieny Virus. In *Vaccines* (Eds. Plotkin, S.A. & Orenstein, W.A.) W.B. Saunders Company, Philadelphia, 1999. 928-967.

328 McMichael, A.J. Hiv vaccines. *Annu Rev Immunol* 2006, **24**, 227-255.

329 Borrow, P., Lewicki, H., Hahn, B.H., Shaw, G.M. & Oldstone, M.B. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* 1994, **68**(9), 6103-6110.

330 Harrer, E., Harrer, T., Buchbinder, S. *et al.* HIV-1-specific cytotoxic T lymphocyte response in healthy, long-term nonprogressing seropositive persons. *AIDS Res Hum Retroviruses* 1994, **10 Suppl 2**, S77-78.

331 Novitsky, V., Rybak, N., McLane, M.F. *et al.* Identification of human immunodeficiency virus type 1 subtype C Gag-, Tat-, Rev-, and Nef-specific elispot-based cytotoxic T-lymphocyte responses for AIDS vaccine design. *J Virol* 2001, **75**(19), 9210-9228.

332 Vaishnav, Y.N. & Wong-Staal, F. The biochemistry of AIDS. *Annu Rev Biochem* 1991, **60**, 577-630.

333 Stevenson, M. HIV-1 pathogenesis. *Nat Med* 2003, **9**(7), 853-860.

334 Mondal, D., Williams, C.A., Ali, M., Eilers, M. & Agrawal, K.C. The HIV-1 Tat protein selectively enhances CXCR4 and inhibits CCR5 expression in megakaryocytic K562 cells. *Exp Biol Med (Maywood)* 2005, **230**(9), 631-644.

335 Engering, A., Geijtenbeek, T.B., van Vliet, S.J. *et al.* The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J Immunol* 2002, **168**(5), 2118-2126.

336 Esparza, J. & Bhamarapravati, N. Accelerating the development and future availability of HIV-1 vaccines: why, when, where, and how? *Lancet* 2000, **355**(9220), 2061-2066.

337 Girard, M., Habel, A. & Chanel, C. New prospects for the development of a vaccine against human immunodeficiency virus type 1. An overview. *C R Acad Sci III* 1999, **322**(11), 959-966.

338   Johnston, M.I. & Flores, J. Progress in HIV vaccine development. *Curr Opin Pharmacol* 2001, **1**(5), 504-510.

339   Cohen, J. Public health. AIDS vaccine trial produces disappointment and confusion. *Science* 2003, **299**(5611), 1290-1291.

340   Gaschen, B., Taylor, J., Yusim, K. *et al.* Diversity considerations in HIV-1 vaccine selection. *Science* 2002, **296**(5577), 2354-2360.

341   Vanden Haesevelde, M., Decourt, J.L., De Leys, R.J. *et al.* Genomic cloning and complete sequence analysis of a highly divergent African human immunodeficiency virus isolate. *J Virol* 1994, **68**(3), 1586-1596.

342   McMichael, A.J. & Rowland-Jones, S.L. Cellular immune responses to HIV. *Nature* 2001, **410**(6831), 980-987.

343   Cohen, P. & Kresege, K. 2005: Year in review. in *VAX* Vol. 4 (ed. Noble, S.), 2006.

344   Gilliam, B.L. & Redfield, R.R. Therapeutic HIV vaccines. *Curr Top Med Chem* 2003, **3**(13), 1536-1553.

345   Cho, M.W. Subunit protein vaccines: theoretical and practical considerations for HIV-1. *Curr Mol Med* 2003, **3**(3), 243-263.

346   Lambert, J. Tat toxoid: its potential role as an HIV vaccine. *J Hum Virol* 1998, **1**(4), 249-250.

347   Ferrari, G., Kostyu, D.D., Cox, J. *et al.* Identification of highly conserved and broadly cross-reactive HIV type 1 cytotoxic T lymphocyte epitopes as candidate immunogens for inclusion in Mycobacterium bovis BCG-vectored HIV vaccines. *AIDS Res Hum Retroviruses* 2000, **16**(14), 1433-1443.

348   Buseyne, F., Chaix, M.L., Fleury, B. *et al.* Cross-clade-specific cytotoxic T lymphocytes in HIV-1-infected children. *Virology* 1998, **250**(2), 316-324.

349   McAdam, S., Kaleebu, P., Krausa, P. *et al.* Cross-clade recognition of p55 by cytotoxic T lymphocytes in HIV-1 infection. *Aids* 1998, **12**(6), 571-579.

350   Edwards, B.H., Bansal, A., Sabbaj, S., Bakari, J., Mulligan, M.J. & Goepfert, P.A. Magnitude of functional CD8+ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. *J Virol* 2002, **76**(5), 2298-2305.

351   O'Hagan, D., Singh, M., Ugozzoli, M. *et al.* Induction of potent immune responses by cationic microparticles with adsorbed human immunodeficiency virus DNA vaccines. *J Virol* 2001, **75**(19), 9037-9043.

352   Amara, R.R. & Robinson, H.L. A new generation of HIV vaccines. *Trends Mol Med* 2002, **8**(10), 489-495.

353   Yusim, K., Kesmir, C., Gaschen, B. *et al.* Clustering patterns of cytotoxic T-lymphocyte epitopes in human immunodeficiency virus type 1 (HIV-1) proteins reveal imprints of immune evasion on HIV-1 global variation. *J Virol* 2002, **76**(17), 8757-8768.

354   Buseyne, F., McChesney, M., Porrot, F., Kovarik, S., Guy, B. & Riviere, Y. Gag-specific cytotoxic T lymphocytes from human immunodeficiency virus type 1-infected individuals: Gag epitopes are clustered in three regions of the p24gag protein. *J Virol* 1993, **67**(2), 694-702.

355   Nakamura, Y., Kameoka, M., Tobiume, M. *et al.* A chain section containing epitopes for cytotoxic T, B and helper T cells within a highly conserved region

found in the human immunodeficiency virus type 1 Gag protein. *Vaccine* 1997, **15**(5), 489-496.

356 Novitsky, V., Cao, H., Rybak, N. *et al.* Magnitude and frequency of cytotoxic T-lymphocyte responses: identification of immunodominant regions of human immunodeficiency virus type 1 subtype C. *J Virol* 2002, **76**(20), 10155-10168.

357 Zuniga, R., Lucchetti, A., Galvan, P. *et al.* Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control. *J Virol* 2006, **80**(6), 3122-3125.

358 Frankel, A.D., Biancalana, S. & Hudson, D. Activity of synthetic peptides from the Tat protein of human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* 1989, **86**(19), 7397-7401.

359 Sodroski, J., Patarca, R., Rosen, C., Wong-Staal, F. & Haseltine, W. Location of the trans-activating region on the genome of human T-cell lymphotropic virus type III. *Science* 1985, **229**(4708), 74-77.

360 Caselli, E., Betti, M., Grossi, M.P. *et al.* DNA immunization with HIV-1 tat mutated in the trans activation domain induces humoral and cellular immune responses against wild-type Tat. *J Immunol* 1999, **162**(9), 5631-5638.

361 Chang, H.C., Samaniego, F., Nair, B.C., Buonaguro, L. & Ensoli, B. HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. *Aids* 1997, **11**(12), 1421-1431.

362 Ma, M. & Nath, A. Molecular determinants for cellular uptake of Tat protein of human immunodeficiency virus type 1 in brain cells. *J Virol* 1997, **71**(3), 2495-2499.

363 Fisher, A.G., Feinberg, M.B., Josephs, S.F. *et al.* The trans-activator gene of HTLV-III is essential for virus replication. *Nature* 1986, **320**(6060), 367-371.

364 Popik, W. & Pitha, P.M. Role of tumor necrosis factor alpha in activation and replication of the tat-defective human immunodeficiency virus type 1. *J Virol* 1993, **67**(2), 1094-1099.

365 Feinberg, M.B., Baltimore, D. & Frankel, A.D. The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. *Proc Natl Acad Sci U S A* 1991, **88**(9), 4045-4049.

366 Fanales-Belasio, E., Moretti, S., Nappi, F. *et al.* Native HIV-1 Tat protein targets monocyte-derived dendritic cells and enhances their maturation, function, and antigen-specific T cell responses. *J Immunol* 2002, **168**(1), 197-206.

367 Kim, D.T., Mitchell, D.J., Brockstedt, D.G. *et al.* Introduction of soluble proteins into the MHC class I pathway by conjugation to an HIV tat peptide. *J Immunol* 1997, **159**(4), 1666-1668.

368 Ensoli, B. & Cafaro, A. HIV-1 Tat vaccines. *Virus Res* 2002, **82**(1-2), 91-101.

369 Xiao, H., Neuveut, C., Tiffany, H.L. *et al.* Selective CXCR4 antagonism by Tat: implications for in vivo expansion of coreceptor use by HIV-1. *Proc Natl Acad Sci U S A* 2000, **97**(21), 11466-11471.

370 Buonaguro, L., Buonaguro, F.M., Tornesello, M.L. *et al.* Role of HIV-1 Tat in the pathogenesis of AIDS-associated Kaposi's sarcoma. *Antibiot Chemother* 1994, **46**, 62-72.

371 Matzen, K., Dirkx, A.E., oude Egbrink, M.G. *et al.* HIV-1 Tat increases the adhesion of monocytes and T-cells to the endothelium in vitro and in vivo: implications for AIDS-associated vasculopathy. *Virus Res* 2004, **104**(2), 145-155.

372 Pocernich, C.B., Sultana, R., Mohmmad-Abdul, H., Nath, A. & Butterfield, D.A. HIV-dementia, Tat-induced oxidative stress, and antioxidant therapeutic considerations. *Brain Res Brain Res Rev* 2005, **50**(1), 14-26.

373 Zagury, J.F., Sill, A., Blattner, W. *et al.* Antibodies to the HIV-1 Tat protein correlated with nonprogression to AIDS: a rationale for the use of Tat toxoid as an HIV-1 vaccine. *J Hum Virol* 1998, **1**(4), 282-292.

374 Re, M.C., Gibellini, D., Furlini, G. *et al.* Relationships between the presence of anti-Tat antibody, DNA and RNA viral load. *New Microbiol* 2001, **24**(3), 207-215.

375 Re, M.C., Furlini, G., Vignoli, M. *et al.* Effect of antibody to HIV-1 Tat protein on viral replication in vitro and progression of HIV-1 disease in vivo. *J Acquir Immune Defic Syndr Hum Retrovirol* 1995, **10**(4), 408-416.

376 Re, M.C., Vignoli, M., Furlini, G. *et al.* Antibodies against full-length Tat protein and some low-molecular-weight Tat-peptides correlate with low or undetectable viral load in HIV-1 seropositive patients. *J Clin Virol* 2001, **21**(1), 81-89.

377 van Baalen, C.A., Pontesilli, O., Huisman, R.C. *et al.* Human immunodeficiency virus type 1 Rev- and Tat-specific cytotoxic T lymphocyte frequencies inversely correlate with rapid progression to AIDS. *J Gen Virol* 1997, **78 (Pt 8)**, 1913-1918.

378 Allen, T.M., O'Connor, D.H., Jing, P. *et al.* Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. *Nature* 2000, **407**(6802), 386-390.

379 Cafaro, A., Caputo, A., Fracasso, C. *et al.* Control of SHIV-89.6P-infection of cynomolgus monkeys by HIV-1 Tat protein vaccine. *Nat Med* 1999, **5**(6), 643-650.

380 Cafaro, A., Titti, F., Fracasso, C. *et al.* Vaccination with DNA containing tat coding sequences and unmethylated CpG motifs protects cynomolgus monkeys upon infection with simian/human immunodeficiency virus (SHIV89.6P). *Vaccine* 2001, **19**(20-22), 2862-2877.

381 Pauza, C.D., Trivedi, P., Wallace, M. *et al.* Vaccination with tat toxoid attenuates disease in simian/HIV-challenged macaques. *Proc Natl Acad Sci U S A* 2000, **97**(7), 3515-3519.

382 Allen, T.M., Mortara, L., Mothe, B.R. *et al.* Tat-vaccinated macaques do not control simian immunodeficiency virus SIVmac239 replication. *J Virol* 2002, **76**(8), 4108-4112.

383 Silvera, P., Richardson, M.W., Greenhouse, J. *et al.* Outcome of simian-human immunodeficiency virus strain 89.6p challenge following vaccination of rhesus macaques with human immunodeficiency virus Tat protein. *J Virol* 2002, **76**(8), 3800-3809.

384 Fanales-Belasio, E., Cafaro, A., Cara, A. *et al.* HIV-1 Tat-based vaccines: from basic science to clinical trials. *DNA Cell Biol* 2002, **21**(9), 599-610.

385 Goldstein, G. HIV-1 Tat protein as a potential AIDS vaccine. *Nat Med* 1996, **2**(9), 960-964.

386   Gringeri, A., Santagostino, E., Muca-Perja, M. *et al.* Safety and immunogenicity of HIV-1 Tat toxoid in immunocompromised HIV-1-infected patients. *J Hum Virol* 1998, **1**(4), 293-298.

387   Noonan, D.M., Gringeri, A., Meazza, R. *et al.* Identification of immunodominant epitopes in inactivated Tat-vaccinated healthy and HIV-1-infected volunteers. *J Acquir Immune Defic Syndr* 2003, **33**(1), 47-55.

388   Borsutzky, S., Ebensen, T., Link, C. *et al.* Efficient systemic and mucosal responses against the HIV-1 Tat protein by prime/boost vaccination using the lipopeptide MALP-2 as adjuvant. *Vaccine* 2006, **24**(12), 2049-2056.

389   Girard, M.P., Osmanov, S.K. & Kieny, M.P. A review of vaccine research and development: the human immunodeficiency virus (HIV). *Vaccine* 2006, **24**(19), 4062-4081.

390   Puls, R.L. & Emery, S. Therapeutic vaccination against HIV: current progress and future possibilities. *Clin Sci (Lond)* 2006, **110**(1), 59-71.

391   Lindenburg, C.E., Stolte, I., Langendam, M.W. *et al.* Long-term follow-up: no effect of therapeutic vaccination with HIV-1 p17/p24:Ty virus-like particles on HIV-1 disease progression. *Vaccine* 2002, **20**(17-18), 2343-2347.

392   Moss, R.B., Brandt, C., Giermakowska, W.K. *et al.* HIV-specific immunity during structured antiviral drug treatment interruption. *Vaccine* 2003, **21**(11-12), 1066-1071.

393   Gursel, I., Gursel, M., Ishii, K.J. & Klinman, D.M. Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides. *J Immunol* 2001, **167**(6), 3324-3328.

394   Suzuki, Y., Wakita, D., Chamoto, K. *et al.* Liposome-encapsulated CpG oligodeoxynucleotides as a potent adjuvant for inducing type 1 innate immunity. *Cancer Res* 2004, **64**(23), 8754-8760.

395   Mbawuike, I.N., Acuna, C., Caballero, D. *et al.* Reversal of age-related deficient influenza virus-specific CTL responses and IFN-gamma production by monophosphoryl lipid A. *Cell Immunol* 1996, **173**(1), 64-78.

396   Ben-Yehuda, A., Joseph, A., Barenholz, Y. *et al.* Immunogenicity and safety of a novel IL-2-supplemented liposomal influenza vaccine (INFLUSOME-VAC) in nursing-home residents. *Vaccine* 2003, **21**(23), 3169-3178.

397   Ben-Yehuda, A., Joseph, A., Zeira, E. *et al.* Immunogenicity and safety of a novel liposomal influenza subunit vaccine (INFLUSOME-VAC) in young adults. *J Med Virol* 2003, **69**(4), 560-567.

398   Singh, M., Ott, G., Kazzaz, J. *et al.* Cationic microparticles are an effective delivery system for immune stimulatory cpG DNA. *Pharm Res* 2001, **18**(10), 1476-1479.

399   Agwale, S.M., Shata, M.T., Reitz, M.S., Jr. *et al.* A Tat subunit vaccine confers protective immunity against the immune-modulating activity of the human immunodeficiency virus type-1 Tat protein in mice. *Proc Natl Acad Sci U S A* 2002, **99**(15), 10037-10041.

400   Gallo, R.C., Burny, A. & Zagury, D. Targeting Tat and IFN(alpha) as a therapeutic AIDS vaccine. *DNA Cell Biol* 2002, **21**(9), 611-618.

401   Misumi, S., Takamune, N., Ohtsubo, Y., Waniguchi, K. & Shoji, S. Zn2+ binding to cysteine-rich domain of extracellular human immunodeficiency virus type 1

Tat protein is associated with Tat protein-induced apoptosis. *AIDS Res Hum Retroviruses* 2004, **20**(3), 297-304.

402   Butto, S., Fiorelli, V., Tripiciano, A. *et al.* Sequence conservation and antibody cross-recognition of clade B human immunodeficiency virus (HIV) type 1 Tat protein in HIV-1-infected Italians, Ugandans, and South Africans. *J Infect Dis* 2003, **188**(8), 1171-1180.

403   Huang, H.W. & Wang, K.T. Structural characterization of the metal binding site in the cysteine-rich region of HIV-1 Tat protein. *Biochem Biophys Res Commun* 1996, **227**(2), 615-621.

404   Izmailova, E., Bertley, F.M., Huang, Q. *et al.* HIV-1 Tat reprograms immature dendritic cells to express chemoattractants for activated T cells and macrophages. *Nat Med* 2003, **9**(2), 191-197.

405   Fawell, S., Seery, J., Daikh, Y. *et al.* Tat-mediated delivery of heterologous proteins into cells. *Proc Natl Acad Sci U S A* 1994, **91**(2), 664-668.

406   Addo, M.M., Altfeld, M., Rosenberg, E.S. *et al.* The HIV-1 regulatory proteins Tat and Rev are frequently targeted by cytotoxic T lymphocytes derived from HIV-1-infected individuals. *Proc Natl Acad Sci U S A* 2001, **98**(4), 1781-1786.

407   Rezza, G., Fiorelli, V., Dorrucci, M. *et al.* The presence of anti-Tat antibodies is predictive of long-term nonprogression to AIDS or severe immunodeficiency: findings in a cohort of HIV-1 seroconverters. *J Infect Dis* 2005, **191**(8), 1321-1324.

408   Re, M.C., Gibellini, D., Vitone, F. & La Placa, M. Antibody to HIV-1 Tat protein, a key molecule in HIV-1 pathogenesis. A brief review. *New Microbiol* 2001, **24**(2), 197-205.

409   Maggiorella, M.T., Baroncelli, S., Michelini, Z. *et al.* Long-term protection against SHIV89.6P replication in HIV-1 Tat vaccinated cynomolgus monkeys. *Vaccine* 2004, **22**(25-26), 3258-3269.

410   Belliard, G., Hurtrel, B., Moreau, E. *et al.* Tat-neutralizing versus Tat-protecting antibodies in rhesus macaques vaccinated with Tat peptides. *Vaccine* 2005, **23**(11), 1399-1407.

411   Nabel, G. & Baltimore, D. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* 1987, **326**(6114), 711-713.

412   Marinaro, M., Riccomi, A., Rappuoli, R. *et al.* Mucosal delivery of the human immunodeficiency virus-1 Tat protein in mice elicits systemic neutralizing antibodies, cytotoxic T lymphocytes and mucosal IgA. *Vaccine* 2003, **21**(25-26), 3972-3981.

413   Moreau, E., Belliard, G., Partidos, C.D. *et al.* Important B-cell epitopes for neutralization of human immunodeficiency virus type 1 Tat in serum samples of humans and different animal species immunized with Tat protein or peptides. *J Gen Virol* 2004, **85**(Pt 10), 2893-2901.

414   Goldstein, G., Tribbick, G. & Manson, K. Two B cell epitopes of HIV-1 Tat protein have limited antigenic polymorphism in geographically diverse HIV-1 strains. *Vaccine* 2001, **19**(13-14), 1738-1746.

415   Tosi, G., Meazza, R., De Lerma Barbaro, A. *et al.* Highly stable oligomerization forms of HIV-1 Tat detected by monoclonal antibodies and requirement of

monomeric forms for the transactivating function on the HIV-1 LTR. *Eur J Immunol* 2000, **30**(4), 1120-1126.

416   Addo, M.M., Yu, X.G., Rosenberg, E.S., Walker, B.D. & Altfeld, M. Cytotoxic T-lymphocyte (CTL) responses directed against regulatory and accessory proteins in HIV-1 infection. *DNA Cell Biol* 2002, **21**(9), 671-678.

417   Yamada, T. & Iwamoto, A. Comparison of proviral accessory genes between long-term nonprogressors and progressors of human immunodeficiency virus type 1 infection. *Arch Virol* 2000, **145**(5), 1021-1027.

418   Takeda, K. & Akira, S. Toll-like receptors in innate immunity. *Int Immunol* 2005, **17**(1), 1-14.

419   Takeuchi, O., Sato, S., Horiuchi, T. *et al.* Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol* 2002, **169**(1), 10-14.

420   Ozinsky, A., Underhill, D.M., Fontenot, J.D. *et al.* The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci U S A* 2000, **97**(25), 13766-13771.

421   O'Hagan, D.T. Recent developments in vaccine delivery systems. *Curr Drug Targets Infect Disord* 2001, **1**(3), 273-286.

422   Thoelen, S., Van Damme, P., Mathei, C. *et al.* Safety and immunogenicity of a hepatitis B vaccine formulated with a novel adjuvant system. *Vaccine* 1998, **16**(7), 708-714.

423   Snapper, C.M. & Paul, W.E. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 1987, **236**(4804), 944-947.

424   Weeratna, R.D., Makinen, S.R., McCluskie, M.J. & Davis, H.L. TLR agonists as vaccine adjuvants: comparison of CpG ODN and Resiquimod (R-848). *Vaccine* 2005, **23**(45), 5263-5270.

425   Liu, T., Matsuguchi, T., Tsuboi, N., Yajima, T. & Yoshikai, Y. Differences in expression of toll-like receptors and their reactivities in dendritic cells in BALB/c and C57BL/6 mice. *Infect Immun* 2002, **70**(12), 6638-6645.

426   Clements, C.J. & Wesselingh, S.L. Vaccine presentations and delivery technologies - what does the future hold? *Expert Rev Vaccines* 2005, **4**(3), 281-287.

427   Reddy, S.T., Rehor, A., Schmoekel, H.G., Hubbell, J.A. & Swartz, M.A. In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles. *J Control Release* 2006, **112**(1), 26-34.

428   Cui, Z., Hsu, C.H. & Mumper, R.J. Physical characterization and macrophage cell uptake of mannan-coated nanoparticles. *Drug Dev Ind Pharm* 2003, **29**(6), 689-700.

429   Koziara, J.M., Lockman, P.R., Allen, D.D. & Mumper, R.J. In situ blood-brain barrier transport of nanoparticles. *Pharm Res* 2003, **20**(11), 1772-1778.

430   Kim, S.K., Reed, D.S., Olson, S. *et al.* Generation of mucosal cytotoxic T cells against soluble protein by tissue-specific environmental and costimulatory signals. *Proc Natl Acad Sci U S A* 1998, **95**(18), 10814-10819.

431   Kwon, Y.J., Standley, S.M., Goh, S.L. & Frechet, J.M. Enhanced antigen presentation and immunostimulation of dendritic cells using acid-degradable cationic nanoparticles. *J Control Release* 2005, **105**(3), 199-212.

432   Warren, T.L., Bhatia, S.K., Acosta, A.M. *et al.* APC stimulated by CpG oligodeoxynucleotide enhance activation of MHC class I-restricted T cells. *J Immunol* 2000, **165**(11), 6244-6251.

433   Klinman, D.M. CpG DNA as a vaccine adjuvant. *Expert Rev Vaccines* 2003, **2**(2), 305-315.

434   McCluskie, M.J. & Weeratna, R.D. Novel adjuvant systems. *Curr Drug Targets Infect Disord* 2001, **1**(3), 263-271.

435   Singh, M. & O'Hagan, D.T. Recent advances in vaccine adjuvants. *Pharm Res* 2002, **19**(6), 715-728.

436   Jiang, W., Gupta, R.K., Deshpande, M.C. & Schwendeman, S.P. Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. *Adv Drug Deliv Rev* 2005, **57**(3), 391-410.

437   Carcaboso, A.M., Hernandez, R.M., Igartua, M., Rosas, J.E., Patarroyo, M.E. & Pedraz, J.L. Potent, long lasting systemic antibody levels and mixed Th1/Th2 immune response after nasal immunization with malaria antigen loaded PLGA microparticles. *Vaccine* 2004, **22**(11-12), 1423-1432.

438   Patel, J., Galey, D., Jones, J. *et al.* HIV-1 Tat-coated nanoparticles result in enhanced humoral immune responses and neutralizing antibodies compared to alum adjuvant. *Vaccine* 2006, **24**(17), 3564-3573.

439   Patel, J.D., Gandhapudi, S., Jones, J., Woodward, J.G. & Mumper, R.J. Cationic nanoparticles for delivery of CpG oligodeoxynucleotide and ovalbumin: In vitro and In vivo assesment. *Vaccine*, **Submitted May 2006**.

440   Weissig, V., Lasch, J., Klibanov, A.L. & Torchilin, V.P. A new hydrophobic anchor for the attachment of proteins to liposomal membranes. *FEBS Lett* 1986, **202**(1), 86-90.

441   Slinkin, M.A., Curtet, C., Sai-Maurel, C., Gestin, J.F., Torchilin, V.P. & Chatal, J.F. Site-specific conjugation of chain-terminal chelating polymers to Fab' fragments of anti-CEA mAb: effect of linkage type and polymer size on conjugate biodistribution in nude mice bearing human colorectal carcinoma. *Bioconjug Chem* 1992, **3**(6), 477-483.

442   Torchilin, V.P., Levchenko, T.S., Lukyanov, A.N. *et al.* p-Nitrophenylcarbonyl-PEG-PE-liposomes: fast and simple attachment of specific ligands, including monoclonal antibodies, to distal ends of PEG chains via p-nitrophenylcarbonyl groups. *Biochim Biophys Acta* 2001, **1511**(2), 397-411.

443   Heath, T.D. & Martin, F.J. The development and application of protein-liposome conjugation techniques. *Chem Phys Lipids* 1986, **40**(2-4), 347-358.

444   Crowe, J., Masone, B.S. & Ribbe, J. One-step purification of recombinant proteins with the 6xHis tag and Ni-NTA resin. *Methods Mol Biol* 1996, **58**, 491-510.

445   Hochuli, E., Dobeli, H. & Schacher, A. New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J Chromatogr* 1987, **411**, 177-184.

446   Porath, J., Carlsson, J., Olsson, I. & Belfrage, G. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* 1975, **258**(5536), 598-599.

447 Porath, J. Immobilized metal ion affinity chromatography. *Protein Expr Purif* 1992, **3**(4), 263-281.

448 Schmitt, J., Hess, H. & Stunnenberg, H.G. Affinity purification of histidine-tagged proteins. *Mol Biol Rep* 1993, **18**(3), 223-230.

449 Lauer, S.A. & Nolan, J.P. Development and characterization of Ni-NTA-bearing microspheres. *Cytometry* 2002, **48**(3), 136-145.

450 Celia, H., Wilson-Kubalek, E., Milligan, R.A. & Teyton, L. Structure and function of a membrane-bound murine MHC class I molecule. *Proc Natl Acad Sci U S A* 1999, **96**(10), 5634-5639.

451 Chikh, G.G., Li, W.M., Schutze-Redelmeier, M.P., Meunier, J.C. & Bally, M.B. Attaching histidine-tagged peptides and proteins to lipid-based carriers through use of metal-ion-chelating lipids. *Biochim Biophys Acta* 2002, **1567**(1-2), 204-212.

452 Graham, J.A., Gardner, D.E., Miller, F.J., Daniels, M.J. & Coffin, D.L. Effect of nickel chloride on primary antibody production in the spleen. *Environ Health Perspect* 1975, **12**, 109-113.

453 Graham, J.A., Miller, F.J., Daniels, M.J., Payne, E.A. & Gardner, D.E. Influence of cadmium, nickel, and chromium on primary immunity in mice. *Environ Res* 1978, **16**(1-3), 77-87.

454 Smialowicz, R.J., Rogers, R.R., Riddle, M.M. & Stott, G.A. Immunologic effects of nickel: I. Suppression of cellular and humoral immunity. *Environ Res* 1984, **33**(2), 413-427.

455 Smialowicz, R.J., Rogers, R.R., Riddle, M.M., Rowe, D.G. & Luebke, R.W. Immunological studies in mice following in utero exposure to NiCl2. *Toxicology* 1986, **38**(3), 293-303.

456 Association, N.P.E.R. & Institute, N.D. Safe Use of Nickel in the Workplace. 2nd Edn edn, 1997.

457 Gringeri, A., Santagostino, E., Muca-Perja, M. *et al.* Tat toxoid as a component of a preventive vaccine in seronegative subjects. *J Acquir Immune Defic Syndr Hum Retrovirol* 1999, **20**(4), 371-375.

458 Mizuochi, T., Loveless, R.W., Lawson, A.M. *et al.* A library of oligosaccharide probes (neoglycolipids) from N-glycosylated proteins reveals that conglutinin binds to certain complex-type as well as high mannose-type oligosaccharide chains. *J Biol Chem* 1989, **264**(23), 13834-13839.

Vita

Jigna Patel was born on October 19, 1977 in Livingstone, Zambia. She received her Bachelor of Science degree (1998) and her Master of Science degree (1999) in Chemistry from the University of Kentucky. Jigna worked under the supervision of Dr. Sylvia Daunert in the Department of Chemistry and her thesis title was: *Reversible immobilization of enzymes on membranes based on a calmodulin fusion tail.* In May of 1999, Jigna accepted a position as a senior research analyst in the Center for Pharmaceutical Science and Technology (CPST) in the College of Pharmacy, University of Kentucky. She joined the Department of Pharmaceutical Sciences Graduate Program at the University of Kentucky in the Fall of 2001. Jigna is the recipient of many academic honors including: Dissertation Year Fellowship (2005), AAPS Graduate Student Symposium in Drug Delivery and Pharmaceutical Technology (2005), The Peter Glavinos Graduate Scholarship Endowment (2004), and The American Foundation for Pharmaceutical Education Pre-doctoral Fellowship (2003, 2004). In addition, she was awarded 1st place for a graduate student research poster competition at the 2nd Annual Materials Nanotechnology Workshop (Louisville, KY) in 2003 for her poster titled: *Preparation and characterization of sterically stabilized anionic nanoparticles for delivery of HIV-1 antigens.* Jigna is an author and a co-author on one peer-reviewed publication. She has three additional manuscripts in preparation.

1. **J. D. Patel** and R. J. Mumper. Nanoengineered vaccines: Applications in dendritic cell targeting and HIV vaccine development. *Journal of Nanoscience and Nanotechnology.* Manuscript in preparation.

2. **J. D. Patel**, J. Jones, J. G. Woodward, R. J. Mumper. Preparation and characterization of nickel nanoparticles for enhanced immune responses to his-tag HIV-1 Gag p24. *Pharmaceutical Research*. Manuscript in preparation.

3. **J. D. Patel**, S. Gandhapudi, J. Jones, J. G. Woodward, R. J. Mumper. Cationic nanoparticles for delivery of CpG oligodeoxynucleotide and Ovalbumin: In vitro and in vivo assessment. Manuscript in preparation.

4. **J. D. Patel**, D. Galey, J. Jones, P. Ray, J. G. Woodward, A. Nath, R. J. Mumper. HIV-1 Tat-coated nanoparticles result in enhanced humoral immune responses and neutralizing antibodies compared to Alum adjuvant. *Vaccine*. 24 (2006), 3464-3573.

5. Z. Cui, **J. D. Patel**, M. Tuzova, P. Ray, R. Phillips, J. G. Woodward, A. Nath, R. J. Mumper. Strong T-cell type-1 immune responses to HIV-1 Tat (1-72) protein-coated nanoparticles. *Vaccine*. 22 (2004), 2631-2640.

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