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[Continued on next page]

(54) Title: NANOCARRIER COMPOSITIONS WITH UNCOUPLED ADJUVANT

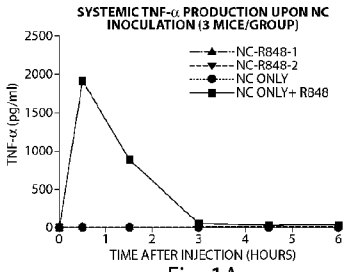

Fig. 1A

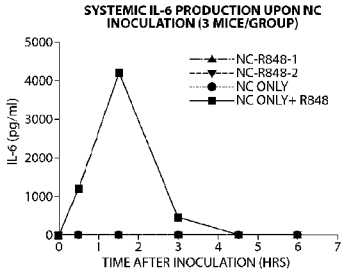

Fig. 1B

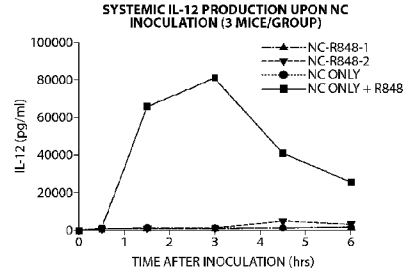

Fig. 1C

(57) Abstract: Disclosed are synthetic nanocarrier compositions with separate adjuvant compositions as well as related methods.

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NANOCARRIER COMPOSITIONS WITH UNCOUPLED ADJUVANT

RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. §119 of United States provisional applications 61/348713, filed May 26, 2010, 61/348717, filed May 26, 2010, 61/348728, filed May 26, 2010, and 61/358635, filed June 25, 2010, the entire contents of each of which are incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to synthetic nanocarrier and separate adjuvant compositions, and related methods, such as for treating diseases in which generating an immune response is desirable.

BACKGROUND OF THE INVENTION

Adjuvants are generally important components for the majority of currently used vaccination regimens. The optimal approach for augmenting the immune response with adjuvant, however, in a number of cases, is not yet known. Therefore, improved compositions and therapeutic methods are needed to provide improved therapies for diseases in which generating an immune response and/or augmenting it is desirable.

SUMMARY OF THE INVENTION

In one aspect, a composition comprising a dosage form that comprises (1) a population of synthetic nanocarriers, (2) a first adjuvant that is not coupled to any synthetic nanocarriers, and (3) a pharmaceutically acceptable excipient is provided. In another aspect, a method comprising administering the composition to a subject is provided.

In one embodiment, the compositions provided herein, including those of the methods provided, comprise a systemic dose of the first adjuvant. In another embodiment, said compositions further comprise a second adjuvant. In still another embodiment, the first adjuvant and second adjuvant are different. In yet another embodiment, the first adjuvant and the second adjuvant are the same. In still another embodiment, the compositions provided herein, including those of the methods provided, comprise a systemic dose of the first adjuvant and/or second adjuvant. In one embodiment, the systemic dose results in the

systemic release of TNF-α, IL-6 and/or IL-12. In another embodiment, the systemic dose results in the systemic release of IFN-γ, IL-12 and/or IL-18.

In one embodiment, the second adjuvant of the compositions provided, including those of the methods provided, is coupled to the synthetic nanocarriers. In another embodiment, the second adjuvant is not coupled to any synthetic nanocarriers. In yet another embodiment, the second adjuvant is coupled to another population of synthetic nanocarriers.

In another embodiment, the compositions provided herein, including those of the methods provided, comprise one or more antigens. In a further embodiment, the one or more antigens are coupled to the synthetic nanocarriers. In yet a further embodiment, the one or more antigens are coupled to another population of synthetic nanocarriers. In another embodiment, the one or more antigens are not coupled to any synthetic nanocarriers.

In one embodiment, the one or more antigens of the compositions provided, including those of the methods provided, comprise a B cell antigen and/or a T cell antigen. In another embodiment, the T cell antigen is a universal T cell antigen or T helper cell antigen. In a further embodiment, the one or more antigens comprise a B cell antigen and/or a T cell antigen and a universal T cell antigen or T helper cell antigen. In one embodiment, the B cell antigen is nicotine. In yet another embodiment, the compositions provided, including those of the methods provided, do not comprise an antigen.

In one embodiment, of the compositions provided, including those of the methods provided, the first adjuvant and/or second adjuvant comprises a mineral salt, gel-type adjuvant, a microbial adjuvant, an oil-emulsion or emulsifier-based adjuvant, a particulate adjuvant, a synthetic adjuvant, a phosphate adjuvant, a polymer, a liposome, a microcarrier, an immunostimulatory nucleic acid, alum, a saponin, an interleukin, an interferon, a cytokine, a toll-like receptor (TLR) agonist, an imidazoquinoline, a cytokine receptor agonist, a CD40 agonist, an Fc receptor agonist, a complement receptor agonist, QS21, vitamin E, squalene, tocopherol, Quil A, ISCOMs, ISCOMATRIX, Ribi Detox, CRL-1005, L-121, tetrachlorodecaoxide, alum, MF59, AS02, AS15, cholera toxin, monophosphoryl lipid A, incomplete Freund's adjuvant, complete Freund's adjuvant, muramyl dipeptide or montanide. In one embodiment, the immunostimulatory nucleic acid comprises a CpG-containing nucleic acid. In another embodiment, the imidazoquinoline comprises resiquimod or imiquimod. In still another embodiment, the first and/or second adjuvant comprises alum. In one embodiment, when the first adjuvant comprises a CpG-containing nucleic acid, the second

adjuvant comprises an imidazoquinoline or alum. In another embodiment, the imidazoquinoline is resiquimod. In still another embodiment, when the first adjuvant comprises an imidazoquinoline, the second adjuvant comprises a CpG-containing nucleic acid or alum. In a further embodiment, the imidazoquinoline is resiquimod. In one embodiment, when the first adjuvant comprises alum, the second adjuvant comprises an imidazoquinoline or a CpG-containing nucleic acid. In another embodiment, the imidazoquinoline is resiquimod. In a further embodiment, the TLR agonist comprises a TLR-1, TLR-2, TLR-3, TLR-4, TLR-5, TLR-6, TLR-7, TLR-8, TLR-9, TLR-10, TLR-11 agonist or a combination thereof. In another embodiment, the first adjuvant and/or second adjuvant does not comprise a TLR agonist. In yet another embodiment, the first adjuvant and/or second adjuvant does not comprise a TLR-3, TLR-7, TLR-8 or TLR-9 agonist. In one embodiment, the second adjuvant is coupled to the synthetic nanocarriers and comprises resiquimod.

In one embodiment, the synthetic nanocarriers of the compositions provided herein, including those of the methods provided, comprise lipid nanoparticles, polymeric nanoparticles, metallic nanoparticles, surfactant-based emulsions, dendrimers, buckyballs, nanowires, virus-like particles, peptide or protein particles, nanoparticles that comprise a combination of nanomaterials, spheroidal nanoparticles, cuboidal nanoparticles, pyramidal nanoparticles, oblong nanoparticles, cylindrical nanoparticles or toroidal nanoparticles.

In one embodiment, the synthetic nanocarriers comprise one or more polymers. In another embodiment, the one or more polymers comprise a polyester. In yet another embodiment, the one or more polymers comprise or further comprise a polyester coupled to a hydrophilic polymer. In a further embodiment, the polyester comprises a poly(lactic acid), poly(glycolic acid), poly(lactic-co-glycolic acid), or polycaprolactone. In still a further embodiment, the hydrophilic polymer comprises a polyether. In another embodiment, the polyether comprises polyethylene glycol.

In another embodiment, the one or more antigens of the compositions provided herein, including those of the methods provided, comprise nicotine and a universal T cell antigen or T-helper cell antigen, each of which are coupled to the synthetic nanocarriers.

In a further embodiment, the universal T cell antigen or T helper cell antigen of the compositions provided herein, including those of the methods provided, is coupled by encapsulation. In still another embodiment, the T-helper cell antigen comprises a peptide

obtained or derived from ovalbumin. In a further embodiment, the peptide obtained or derived from ovalbumin comprises the sequence as set forth in SEQ ID NO: 1.

In another aspect, a method comprising administering any of the compositions provided herein to a subject is provided. In one embodiment, the subject is a human.

In a further aspect, a method comprising administering any of the compositions provided and a second adjuvant to a subject, wherein the second adjuvant is administered at a time different from the administration of the composition, is provided. In one embodiment, the subject is a human. In another embodiment, the composition and second adjuvant are coadministered. In yet another embodiment, the composition and second adjuvant are not coadministered. In still another embodiment, the second adjuvant is administered prior to the composition.

In one embodiment, any of the methods provided further comprises administering one or more antigens. In another embodiment, any of the the compositions provided, including those of the methods provided, further comprises one or more antigens. In one embodiment, the one or more antigens are coadministered.

In one embodiment, the subject of any of the methods provided or to which any of the compositions provided is administered is in need of an inflammatory response. In another embodiment, the subject is in need of a Th1 immune response. In yet another embodiment, the subject is in need of a humoral immune response. In still another embodiment, the subject is in need of a specific local cytotoxic T lymphocyte response. In a further embodiment, the subject has or is at risk of having cancer. In still a further embodiment, the subject has or is at risk of having an infection or infectious disease. In yet a further embodiment, the subject has or is at risk of having an atopic condition, asthma, COPD or a chronic infection.

In one embodiment, any of the compositions can be for use in therapy or prophylaxis. In another embodiment, any of the compositions can be for use in any of the methods provided. In yet another embodiment, any of the compositions can be for use in a method of inducing an inflammatory response in a subject. In still another embodiment, any of the compositions can be for use in a method of inducing a Th1 immune response in a subject. In yet another embodiment, any of the compositions can be for use in a method of inducing a humoral immune response in a subject. In a further embodiment, any of the compositions can be for use in a method of inducing a specific local cytotoxic T lymphocyte response in a subject. In still a further embodiment, any of the compositions can be for use in a method of treating or

preventing cancer. In yet a further embodiment, any of the compositions can be for use in a method of treating or preventing infection or infectious disease. In another embodiment, any of the compositions can be for use in a method of treating or preventing an atopic condition, asthma, COPD or a chronic infection.

In another aspect, a use of any of the compositions provided for the manufacture of a medicament for use in any of the methods provided is provided herein.

BRIEF DESCRIPTION OF FIGURES

Fig. 1 shows the systemic cytokine production in mice after nanocarrier (NC) inoculation. **Fig. 1A**, **Fig. 1B** and **Fig. 1C** – TNF-α, IL-6, and IL-12 production in experimental groups shown, respectively. Sera from groups of three mice were pooled and analyzed by ELISA.

Fig. 2 demonstrates the systemic IFN-γ production in mice after NC inoculation. Sera from groups of three mice were pooled and analyzed by ELISA.

Fig. 3 demonstrates that utilization of entrapped R848 within NCs generates an immune response, which is superior to one induced by NC without R848.

Fig. 4 shows anti-nicotine antibody titers in mice immunized with NC containing surface nicotine and T-helper ovalbumin-derived peptide OP-II (NC-Nic) without adjuvant or with the same NC-Nic admixed with 20 μg of free R848 (5 animals/group; s.c., 100 μg of NC per injection, 3 times with 4-wk intervals). Titers for days 26 and 40 after the first immunization are shown (ELISA against polylysine-nicotine) (group 1: immunized with NC[Nic,Ø(i.e., no adjuvant),OP-II] (2.2% of OP-II); group 2: immunized with NC[Nic,Ø,OP-II] admixed with 20 μg of free R848).

Fig. 5 shows anti-nicotine antibody titers in mice immunized with NC containing surface nicotine and T-helper ovalbumin-derived peptide OP-II (NC-Nic) with R848 adjuvant or with the same NC-Nic admixed with 80 μg of free alum or 25 μg of free CpG-1826 (5 animals/group; s.c., 100 μg of NC per injection, 3 times with 4-wk intervals). Titers for days 26 and 40 after the first immunization are shown (ELISA against polylysine-nicotine) (all groups: immunized with NC[Nic,R848,OP-II]; group 2: NC admixed with 80 μg of free alum; group 3: admixed with 25 μg of free CpG-1826).

Fig. 6 shows anti-nicotine antibody titers in mice immunized with NC containing surface nicotine, R848 and T-helper ovalbumin-derived peptide OP-II NC[Nic,R848,OP-II] or with the same NC-Nic admixed with 80 μg of free alum (5 animals/group; s.c., 100 μg of

NC per injection, 3 times with 4-wk intervals). Titers for days 40 and 70 after the first immunization are shown (ELISA against polylysine-nicotine) (group 1: immunized with NC[Nic,R848,OP-II] (3.1% of R848, 1.5% of OP-II); group 2: immunized with NC[Nic,R848,OP-II] admixed with 80 μg of free alum).

Fig. 7 shows specific local CTL response in mice immunized with NC containing ovalbumin or free ovalbumin. Mice were immunized once (s.c., 100 μg of NC, containing 2.8% of OVA, or with 2.5 μg of OVA; both immunogens admixed with 10 μg of free 1826-CpG).

Fig. 8 shows anti-nicotine antibody titers in mice immunized with NC containing surface nicotine and T-helper ovalbumin-derived peptide OP-II (NC-Nic) (no adjuvant within NC) admixed with 20 μg of free CpG (PS) or 20 μg of free CpG (PO) (5 animals/group; s.c., 100 μg of NC per injection, 3 times with 2-wk intervals). Control mice received PBS alone. Titers for days 26 and 40 are shown (ELISA against polylysine-nicotine) (group 1: immunized with NC-Nic (no adjuvant) + free CpG (PS); group 2: immunized with NC-Nic (no adjuvant) + free CpG (PO); group 3: immunized with PBS only).

Fig. 9 shows anti-ovalbumin (OVA) antibody titers in mice immunized with NC containing surface OVA (NC-OVA) (no adjuvant within NC) admixed with 20 μg of free R848 or CpG (PS) (5 animals/group; s.c., 100 μg of NC per injection, 3 times with 2-wk intervals). Control mice were immunized with 2.5 μg of soluble OVA admixed with 20 μg of CpG (PS). Titers for days 26 and 44 are shown (ELISA against OVA protein) (group 1: immunized with NC-OVA (no adjuvant) + free R848; group 2: immunized with NC-OVA (no adjuvant) + free CpG (PS); group 3: immunized with soluble OVA + CpG (PS)).

Fig. 10 shows anti-nicotine antibody titers in mice injected with CpG (20 μg per injection, 2 times with 2-wk intervals) followed by immunization at day 35 with NC containing surface nicotine and T-helper ovalbumin-derived peptide OP-II (NC-Nic) either with or without NC-contained R848 (5 animals/group; s.c., 100 μg of NC per injection, 2 times with 2-wk intervals). Titers for days 12, 26, and 40 after immunization with NC are shown (ELISA against polylysine-nicotine) (group 1: immunized with CpG followed by NC-Nic (R848 + OP-II); group 2: immunized with CpG followed by NC-Nic (OP-II only)).

DETAILED DESCRIPTION OF THE INVENTION

Before describing the present invention in detail, it is to be understood that this invention is not limited to particularly exemplified materials or process parameters as such

may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting of the use of alternative terminology to describe the present invention.

All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety for all purposes.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the content clearly dictates otherwise. For example, reference to "a polymer" includes a mixture of two or more such molecules, reference to "a solvent" includes a mixture of two or more such solvents, reference to "an adhesive" includes mixtures of two or more such materials, and the like.

INTRODUCTION

The inventors have unexpectedly and surprisingly discovered that the problems and limitations noted above can be overcome by practicing the invention disclosed herein. The inventors have unexpectedly and surprisingly discovered that the administration of a population of synthetic nanocarriers and an adjuvant that is not coupled to any of the synthetic nanocarriers provides stronger and more rapid immune responses. In particular, the inventors have unexpectedly discovered that it is possible to provide compositions and methods that relate to a composition comprising a dosage form that comprises (1) a population of synthetic nanocarriers, (2) a first adjuvant that is not coupled to any synthetic nanocarriers, and (3) a pharmaceutically acceptable excipient.

In embodiments, the administration of adjuvant separate from synthetic nanocarriers leads to a rapid and strong systemic induction of pro-inflammatory cytokines, such as TNF-α, IL-6 and/or IL-12. The dose of the adjuvant or adjuvants in the compositions in some embodiments, therefore, is a systemic dose. In embodiments, the systemic dose results in the release of TNF-α, IL-6 or IL-12. In other embodiments, the systemic dose results in the systemic release of TNF-α, IL-6 and IL-12. As such cytokines are pro-inflammatory, the administration of compositions provided herein can be beneficial to subjects where an inflammatory response is desired. In some embodiments, therefore, the compositions provided are administered to such subjects. In embodiments, such subjects have or are at risk of having cancer. In other embodiments, such subjects have or are at risk of having an

infection or an infectious disease. Methods for the administration of the compositions to such subjects are also provided.

In other embodiments, the administration of adjuvant separate from synthetic nanocarriers leads to a rapid and strong systemic induction of cytokines that are important for a Th1 immune response, such as IFN-γ, IL-12 and/or IL-18. Therefore, the dose of the adjuvant or adjuvants in the compositions in some embodiments is a systemic dose that results in the systemic release of IFN-γ, IL-12 and/or IL-18. As such cytokines are important for a Th1 immune response, the administration of compositions provided herein can be beneficial to subjects where a Th1 immune response is desired. In some embodiments, the compositions provided are administered to such subjects. In embodiments, such subjects have or are at risk of having an atopic condition, asthma, chronic obstructive pulmonary disease (COPD) or a chronic infection. Methods for the administration of the compositions to such subjects are also provided.

The inventors have also unexpectedly discovered that it is possible to administer a second adjuvant with the aforementioned compositions to provide a strong humoral response. The aforementioned compositions, therefore, can further comprise a second adjuvant. In some embodiments, the second adjuvant is coupled to the synthetic nanocarriers. In other embodiments, the second adjuvant is not coupled to any synthetic nanocarriers. In still other embodiments, the second adjuvant is coupled to another population of synthetic nanocarriers. In some embodiments, however, the second adjuvant is administered to a subject at a time different from when the composition that comprises a population of synthetic nanocarriers and a first adjuvant that is not coupled to any synthetic nanocarriers is administered. In some embodiments, the second adjuvant is administered at a different time but is coadministered. In other embodiments, the second adjuvant is not coadministered. In still other embodiments, the second adjuvant is administered prior to or after the administration of the composition that comprises a population of synthetic nanocarriers and a first adjuvant that is not coupled to any synthetic nanocarriers. In some embodiments, the second adjuvant is also not coupled to any synthetic nanocarriers. In other embodiments, the second adjuvant is coupled to another population of synthetic nanocarriers. The compositions provided herein, therefore, can be beneficial to subjects where a humoral immune response is desired. In some embodiments, the compositions provided are administered to such subjects. In embodiments,

such subjects have or are at risk of having cancer, an infection or infectious disease. Methods for the administration of the compositions to such subjects are also provided.

In further embodiments, it is demonstrated that the administration of one or more antigens with the compositions provided above provides a strong specific local cytotoxic T lymphocyte (CTL) response. In embodiments, the antigen(s) are coadministered with the compositions provided. In some embodiments, the antigen(s) are coupled to the synthetic nanocarriers. In other embodiments, the antigen(s) are not coupled to the synthetic nanocarriers but to another population of synthetic nanocarriers. The antigen(s) can comprise a B cell or T cell antigen. In some embodiments, the T cell antigen is a T helper cell antigen. In other embodiments, the antigen(s) comprise a B cell or T cell antigen as well as a T helper cell antigen. Therefore, the compositions provided can be beneficial to subjects where a specific local CTL response is desired. In some embodiments, the compositions provided are administered to such subjects. Methods for the administration of the compositions to such subjects are also provided.

The present invention will now be described in more detail.

DEFINITIONS

"Adjuvant" means an agent that does not constitute a specific antigen, but boosts the strength and longevity of immune response to a concomitantly administered antigen. Such adjuvants may include, but are not limited to stimulators of pattern recognition receptors, such as Toll-like receptors, RIG-1 and NOD-like receptors (NLR), mineral salts, such as alum, alum combined with monphosphoryl lipid (MPL) A of Enterobacteria, such as Escherihia coli, Salmonella minnesota, Salmonella typhimurium, or Shigella flexneri or specifically with MPL® (AS04), MPL A of above-mentioned bacteria separately, saponins, such as QS-21,Quil-A, ISCOMs, ISCOMATRIX™, emulsions such as MF59™, Montanide® ISA 51 and ISA 720, AS02 (QS21+squalene+ MPL®), AS15, liposomes and liposomal formulations such as AS01, synthesized or specifically prepared microparticles and microcarriers such as bacteria-derived outer membrane vesicles (OMV) of N. gonorrheae, Chlamydia trachomatis and others, or chitosan particles, depot-forming agents, such as Pluronic® block co-polymers, specifically modified or prepared peptides, such as muramyl dipeptide, aminoalkyl glucosaminide 4-phosphates, such as RC529, or proteins, such as bacterial toxoids or toxin fragments.

In embodiments, adjuvants comprise agonists for pattern recognition receptors (PRR), including, but not limited to Toll-Like Receptors (TLRs), specifically TLRs 2, 3, 4, 5, 7, 8, 9 and/or combinations thereof. In other embodiments, adjuvants comprise agonists for Toll-Like Receptors 3, agonists for Toll-Like Receptors 7 and 8, or agonists for Toll-Like Receptor 9; preferably the recited adjuvants comprise imidazoquinolines; such as R848; adenine derivatives, such as those disclosed in US patent 6,329,381 (Sumitomo Pharmaceutical Company), US Published Patent Application 2010/0075995 to Biggadike et al., or WO 2010/018132 to Campos et al.; immunostimulatory DNA; or immunostimulatory RNA. In specific embodiments, synthetic nanocarriers incorporate as adjuvants compounds that are agonists for toll-like receptors (TLRs) 7 & 8 ("TLR 7/8 agonists"). Of utility are the TLR 7/8 agonist compounds disclosed in US Patent 6,696,076 to Tomai et al., including but not limited to imidazoquinoline amines, imidazopyridine amines, 6,7-fused cycloalkylimidazopyridine amines, and 1,2-bridged imidazoquinoline amines. Preferred adjuvants comprise imiquimod and resiquimod (also known as R848). In specific embodiments, an adjuvant may be an agonist for the DC surface molecule CD40. In certain embodiments, to stimulate immunity rather than tolerance, a synthetic nanocarrier incorporates an adjuvant that promotes DC maturation (needed for priming of naive T cells) and the production of cytokines, such as type I interferons, which promote antibody immune responses. In embodiments, adjuvants also may comprise immunostimulatory RNA molecules, such as but not limited to dsRNA, poly I:C or poly I:poly C12U (available as Ampligen ®, both poly I:C and poly I:polyC12U being known as TLR3 stimulants), and/or those disclosed in F. Heil et al., "Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8" Science 303(5663), 1526-1529 (2004); J. Vollmer et al., "Immune modulation by chemically modified ribonucleosides and oligoribonucleotides" WO 2008033432 A2; A. Forsbach et al., "Immunostimulatory oligoribonucleotides containing specific sequence motif(s) and targeting the Toll-like receptor 8 pathway" WO 2007062107 A2; E. Uhlmann et al., "Modified oligoribonucleotide analogs with enhanced immunostimulatory activity" U.S. Pat. Appl. Publ. US 2006241076; G. Lipford et al., "Immunostimulatory viral RNA oligonucleotides and use for treating cancer and infections" WO 2005097993 A2; G. Lipford et al., "Immunostimulatory G,U-containing oligoribonucleotides, compositions, and screening methods" WO 2003086280 A2. In some embodiments, an adjuvant may be a TLR-4 agonist, such as bacterial lipopolysacccharide (LPS), VSV-G, and/or HMGB-1. In some embodiments, adjuvants may comprise TLR-5

agonists, such as flagellin, or portions or derivatives thereof, including but not limited to those disclosed in US Patents 6,130,082, 6,585,980, and 7,192,725. In specific embodiments, synthetic nanocarriers incorporate a ligand for Toll-like receptor (TLR)-9, such as immunostimulatory DNA molecules comprising CpGs, which induce type I interferon secretion, and stimulate T and B cell activation leading to increased antibody production and cytotoxic T cell responses (Krieg et al., CpG motifs in bacterial DNA trigger direct B cell activation. Nature. 1995. 374:546-549; Chu et al. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J. Exp. Med. 1997. 186:1623-1631; Lipford et al. CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. Eur. J. Immunol. 1997. 27:2340-2344; Roman et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. Nat. Med. 1997. 3:849-854; Davis et al. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J. Immunol. 1998. 160:870-876; Lipford et al., Bacterial DNA as immune cell activator. Trends Microbiol. 1998. 6:496-500; US Patent 6,207,646 to Krieg et al.; US Patent 7,223,398 to Tuck et al.; US Patent 7,250,403 to Van Nest et al.; or US Patent 7,566,703 to Krieg et al.

In some embodiments, adjuvants may be proinflammatory stimuli released from necrotic cells (e.g., urate crystals). In some embodiments, adjuvants may be activated components of the complement cascade (e.g., CD21, CD35, etc.). In some embodiments, adjuvants may be activated components of immune complexes. The adjuvants also include complement receptor agonists, such as a molecule that binds to CD21 or CD35. In some embodiments, the complement receptor agonist induces endogenous complement opsonization of the synthetic nanocarrier. In some embodiments, adjuvants are cytokines, which are small proteins or biological factors (in the range of 5 kD – 20 kD) that are released by cells and have specific effects on cell-cell interaction, communication and behavior of other cells. In some embodiments, the cytokine receptor agonist is a small molecule, antibody, fusion protein, or aptamer.

In embodiments, at least a portion of the dose of adjuvant is not coupled to any synthetic nanocarriers, preferably, all of the dose of adjuvant is not coupled to any synthetic nanocarriers. In embodiments, the dose of adjuvant comprises two or more types of adjuvants, and at least a portion of at least one of the types of adjuvant is not coupled to any synthetic nanocarriers. For instance, and without limitation, adjuvants that act on different

receptors, such as different TLR receptors, may be combined. As an example, in an embodiment a TLR 7/8 agonist may be combined with a TLR 9 agonist. In another embodiment, a TLR 7/8 agonist may be combined with a TLR 4 agonist. In yet another embodiment, a TLR 9 agonist may be combined with a TLR 3 agonist.

"Administering" or "administration" means providing a substance (e.g., a drug) to a subject in a manner that is pharmacologically useful.

An "allergy" also referred to herein as an "allergic condition", is any condition where there is an undesired immune response to an allergen (i.e., allergic reaction). Allergies or allergic conditions include, but are not limited to, allergic asthma, hay fever, hives, eczema, plant allergies, bee sting allergies, pet allergies, latex allergies, mold allergies, cosmetic allergies, food allergies, allergic rhinitis or coryza, topic allergic reactions, anaphylaxis, atopic dermatitis, hypersensitivity reactions and other allergic conditions. The allergic reaction may be the result of an immune reaction to any allergen.

"Amount effective" is any amount of a composition provided herein that produces one or more desired immune responses. This amount can be for in vitro or in vivo purposes. For in vivo purposes, the amount can be one that a clinician would believe may have a clinical benefit for a subject in need of an inflammatory, a Th1, a humoral or specific local CTL immune response. Such subjects include those that have or are at risk of having cancer, an infection or infectious disease, an atopic condition, asthma, chronic obstructive pulmonary disease (COPD) or a chronic infection.

Amounts effective include those that involve the systemic release of one or more cytokines. In embodiments, the amounts effective include those that involve the production of a systemic cytokine release profile. In some embodiments, the one or more cytokines or cytokine release profile comprises the systemic release of TNF-α, IL-6 and/or IL-12. In other embodiments, the one or more cytokines or cytokine release profile comprises the systemic release of IFN-γ, IL-12 and/or IL-18. This can be monitored by routine methods. An amount that is effective to produce one or more desired immune responses can also be an amount of a composition provided herein that produces a desired therapeutic endpoint or a desired therapeutic result.

Amounts effective will depend, of course, on the particular subject being treated; the severity of a condition, disease or disorder; the individual patient parameters including age, physical condition, size and weight; the duration of the treatment; the nature of concurrent

therapy (if any); the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

In general, doses of the compositions of the invention can range from about 10 μg/kg to about 100,000 μg/kg. In some embodiments, the doses can range from about 0.1 mg/kg to about 100 mg/kg. In still other embodiments, the doses can range from about 0.1 mg/kg to about 25 mg/kg, about 25 mg/kg to about 50 mg/kg, about 50 mg/kg to about 75 mg/kg or about 75 mg/kg to about 100 mg/kg. Alternatively, the dose can be administered based on the number of synthetic nanocarriers. For example, useful doses include greater than 10^6, 10^7, 10^8, 10^9 or 10^{10} synthetic nanocarriers per dose. Other examples of useful doses include from about $1x10^6$ to about $1x10^{10}$, about $1x10^7$ to about $1x10^9$ or about $1x10^8$ to about $1x10^9$ synthetic nanocarriers per dose. In some embodiments, the doses of the compositions provided are systemic doses.

"Antigen" means a B cell antigen or T cell antigen. In embodiments, antigens are coupled to the synthetic nanocarriers. In other embodiments, antigens are not coupled to the synthetic nanocarriers. In embodiments, antigens are coadministered with the synthetic nanocarriers. In other embodiments, antigens are not coadministered with the synthetic nanocarriers. "Type(s) of antigens" means molecules that share the same, or substantially the same, antigenic characteristics. In embodiments, antigens of the compositions provided are associated with the disease or condition that is being treated. For examples, the antigen can be an allergen (for the treatment of an allergy or allergic condition), a cancer-associated antigen (for the treatment of cancer or a tumor), an infectious agent antigen (for the treatment of an infection, an infectious disease or a chronic infectious disease), etc.

"At least a portion of the dose" means at least some part of the dose, ranging up to including all of the dose.

An "at risk" subject is one in which a health practitioner believes has a chance of having as disease or condition as provided herein.

"B cell antigen" means any antigen that is recognized by a B cell, and triggers an immune response in a B cell (e.g., an antigen that is specifically recognized by a B cell receptor on a B cell). In some embodiments, an antigen that is a T cell antigen is also a B cell antigen. In other embodiments, the T cell antigen is not also a B cell antigen. B cell antigens include, but are not limited to proteins, peptides, small molecules, and carbohydrates. In some embodiments, the B cell antigen comprises a non-protein antigen (i.e., not a protein or peptide antigen). In some embodiments, the B cell antigen comprises a carbohydrate associated with an infectious agent. In some embodiments, the B cell antigen comprises a glycoprotein or glycopeptide associated with an infectious agent. The infectious agent can be a bacterium, virus, fungus, protozoan, parasite or prion. In some embodiments, the B cell antigen comprises a poorly immunogenic antigen. In some embodiments, the B cell antigen comprises an abused substance or a portion thereof. In some embodiments, the B cell antigen comprises an addictive substance or a portion thereof. Addictive substances include, but are not limited to, nicotine, a narcotic, a cough suppressant, a tranquilizer, and a sedative. In some embodiments, the B cell antigen comprises a toxin, such as a toxin from a chemical weapon or natural source, or a pollutant. The B cell antigen may also comprise a hazardous environmental agent. In other embodiments, the B cell antigen comprises an alloantigen, an allergen, a contact sensitizer, a degenerative disease antigen, a hapten, an infectious disease antigen, a cancer antigen, an atopic disease antigen, an autoimmune disease antigen, an addictive substance, a xenoantigen, or a metabolic disease enzyme or enzymatic product thereof.

"Coadministered" means administering two or more substances to a subject in a manner that is correlated in time, preferably sufficiently correlated in time so as to provide a modulation in an immune response. In embodiments, coadministration may occur through administration of two or more substances in the same dosage form. In other embodiments, coadministration may encompass administration of two or more substances in different dosage forms, but within a specified period of time, preferably within 1 month, more preferably within 1 week, still more preferably within 1 day, and even more preferably within 1 hour.

"Couple" or "Coupled" or "Couples" (and the like) means to chemically associate one entity (for example a moiety) with another. In some embodiments, the coupling is covalent, meaning that the coupling occurs in the context of the presence of a covalent bond between the two entities. In non-covalent embodiments, the non-covalent coupling is mediated by

non-covalent interactions including but not limited to charge interactions, affinity interactions, metal coordination, physical adsorption, host-guest interactions, hydrophobic interactions, TT stacking interactions, hydrogen bonding interactions, van der Waals interactions, magnetic interactions, electrostatic interactions, dipole-dipole interactions, and/or combinations thereof. In embodiments, encapsulation is a form of coupling. In embodiments, at least a portion of a dose of adjuvant(s) is not coupled to any synthetic nanocarriers, preferably all of a dose of adjuvant(s) is not coupled to any synthetic nanocarriers.

"Derived" means taken from a source and subjected to substantial modification. For instance, a peptide or nucleic acid with a sequence with only 50% identity to a natural peptide or nucleic acid, preferably a natural consensus peptide or nucleic acid, would be said to be derived from the natural peptide or nucleic acid. Substantial modification is modification that significantly affects the chemical or immunological properties of the material in question. Derived peptides and nucleic acids can also include those with a sequence with greater than 50% identity to a natural peptide or nucleic acid sequence if said derived peptides and nucleic acids have altered chemical or immunological properties as compared to the natural peptide or nucleic acid. These chemical or immunological properties comprise hydrophilicity, stability, affinity, and ability to couple with a carrier such as a synthetic nanocarrier.

"Dosage form" means a pharmacologically and/or immunologically active material in a medium, carrier, vehicle, or device suitable for administration to a subject.

"Encapsulate" means to enclose within a synthetic nanocarrier, preferably enclose completely within a synthetic nanocarrier. Most or all of a substance that is encapsulated is not exposed to the local environment external to the synthetic nanocarrier. Encapsulation is distinct from absorption, which places most or all of a substance on a surface of a synthetic nanocarrier, and leaves the substance exposed to the local environment external to the synthetic nanocarrier.

"Humoral response" means any immune response that results in the production or stimulation of B cells and/or the production of antibodies. Preferably, the humoral immune response is specific to an antigen comprised within an inventive composition or administered during the practice of an inventive method. Methods for assessing whether a humoral response is induced are known to those of ordinary skill in the art. Examples of such methods are provided below in the Examples.

An "infection" or "infectious disease" is any condition or disease caused by a microorganism, pathogen or other agent, such as a bacterium, fungus, prion or virus. Examples of infectious disease include, but are not limited to, viral infectious diseases, such as AIDS, Chickenpox (Varicella), Common cold, Cytomegalovirus Infection, Colorado tick fever, Dengue fever, Ebola hemorrhagic fever, Hand, foot and mouth disease, Hepatitis, Herpes simplex, Herpes zoster, HPV, Influenza (Flu), Lassa fever, Measles, Marburg hemorrhagic fever, Infectious mononucleosis, Mumps, Norovirus, Poliomyelitis, Progressive multifocal leukencephalopathy, Rabies, Rubella, SARS, Smallpox (Variola), Viral encephalitis, Viral gastroenteritis, Viral meningitis, Viral pneumonia, West Nile disease and Yellow fever; bacterial infectious diseases, such as Anthrax, Bacterial Meningitis, Botulism, Brucellosis, Campylobacteriosis, Cat Scratch Disease, Cholera, Diphtheria, Epidemic Typhus, Gonorrhea, Impetigo, Legionellosis, Leprosy (Hansen's Disease), Leptospirosis, Listeriosis, Lyme disease, Melioidosis, Rheumatic Fever, MRSA infection, Nocardiosis, Pertussis (Whooping Cough), Plague, Pneumococcal pneumonia, Psittacosis, Q fever, Rocky Mountain Spotted Fever (RMSF), Salmonellosis, Scarlet Fever, Shigellosis, Syphilis, Tetanus, Trachoma, Tuberculosis, Tularemia, Typhoid Fever, Typhus and Urinary Tract Infections; parasitic infectious diseases, such as African trypanosomiasis, Amebiasis, Ascariasis, Babesiosis, Chagas Disease, Clonorchiasis, Cryptosporidiosis, Cysticercosis, Diphyllobothriasis, Dracunculiasis, Echinococcosis, Enterobiasis, Fascioliasis, Fasciolopsiasis, Filariasis, Free-living amebic infection, Giardiasis, Gnathostomiasis, Hymenolepiasis, Isosporiasis, Kala-azar, Leishmaniasis, Malaria, Metagonimiasis, Myiasis, Onchocerciasis, Pediculosis, Pinworm Infection, Scabies, Schistosomiasis, Taeniasis, Toxocariasis, Toxoplasmosis, Trichinellosis, Trichinosis, Trichuriasis, Trichomoniasis and Trypanosomiasis; fungal infectious disease, such as Aspergillosis, Blastomycosis, Candidiasis, Coccidioidomycosis, Cryptococcosis, Histoplasmosis, Tinea pedis (Athlete's Foot) and Tinea cruris; prion infectious diseases, such as Alpers' disease, Fatal Familial Insomnia, Gerstmann-Sträussler-Scheinker syndrome, Kuru and Variant Creutzfeldt-Jakob disease.

"Inflammatory response" means any immune response involved in the body's innate immune defense system that operates in response to, for example, exposure to an infectious agent, cell injury, etc. In embodiments, the inflammatory response includes the systemic release of cytokines, such as TNF-α, IL-6 and/or IL-12. Methods for assessing whether an inflammatory response is induced, such as an assessment of the production of pro-

inflammatory cytokines, are known to those of ordinary skill in the art. Examples of such methods are provided below in the Examples.

"Isolated nucleic acid" means a nucleic acid that is separated from its native environment and present in sufficient quantity to permit its identification or use. An isolated nucleic acid may be one that is (i) amplified in vitro by, for example, polymerase chain reaction (PCR); (ii) recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical synthesis. An isolated nucleic acid is one which is readily manipulable by recombinant DNA techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known or for which polymerase chain reaction (PCR) primer sequences have been disclosed is considered isolated but a nucleic acid sequence existing in its native state in its natural host is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or expression vector is not pure in that it may comprise only a tiny percentage of the material in the cell in which it resides. Such a nucleic acid is isolated, however, as the term is used herein because it is readily manipulable by standard techniques known to those of ordinary skill in the art. Any of the nucleic acids provided herein may be isolated. In some embodiments, the antigens in the compositions provided herein are present in the form of an isolated nucleic acid, such as an isolated nucleic acid that encodes an antigenic peptide, polypeptide or protein.

"Isolated peptide, polypeptide or protein" means the polypeptide (or peptide or protein) is separated from its native environment and present in sufficient quantity to permit its identification or use. This means, for example, the polypeptide (or peptide or protein) may be (i) selectively produced by expression cloning or (ii) purified as by chromatography or electrophoresis. Isolated peptides, proteins or polypeptides may be, but need not be, substantially pure. Because an isolated peptide, polypeptide or protein may be admixed with a pharmaceutically acceptable carrier in a pharmaceutical preparation, the polypeptide (or peptide or protein) may comprise only a small percentage by weight of the preparation. The polypeptide (or peptide or protein) is nonetheless isolated in that it has been separated from the substances with which it may be associated in living systems, i.e., isolated from other proteins (or peptides or polypeptides). Any of the peptides, polypeptides or proteins provided herein may be isolated. In some embodiments, the antigens in the compositions provided herein are in the form of isolated peptides, polypeptides or proteins.

"Maximum dimension of a synthetic nanocarrier" means the largest dimension of a nanocarrier measured along any axis of the synthetic nanocarrier. "Minimum dimension of a synthetic nanocarrier" means the smallest dimension of a synthetic nanocarrier measured along any axis of the synthetic nanocarrier. For example, for a spheriodal synthetic nanocarrier, the maximum and minimum dimension of a synthetic nanocarrier would be substantially identical, and would be the size of its diameter. Similarly, for a cuboidal synthetic nanocarrier, the minimum dimension of a synthetic nanocarrier would be the smallest of its height, width or length, while the maximum dimension of a synthetic nanocarrier would be the largest of its height, width or length. In an embodiment, a minimum dimension of at least 75%, preferably at least 80%, more preferably at least 90%, of the synthetic nanocarriers in a sample, based on the total number of synthetic nanocarriers in the sample, is greater than 100 nm. In an embodiment, a maximum dimension of at least 75%, preferably at least 80%, more preferably at least 90%, of the synthetic nanocarriers in a sample, based on the total number of synthetic nanocarriers in the sample, is equal to or less than 5 μm. Preferably, a minimum dimension of at least 75%, preferably at least 80%, more preferably at least 90%, of the synthetic nanocarriers in a sample, based on the total number of synthetic nanocarriers in the sample, is greater than 110 nm, more preferably greater than 120 nm, more preferably greater than 130 nm, and more preferably still greater than 150 nm. Aspects ratios of the maximum and minimum dimensions of inventive synthetic nanocarriers may vary depending on the embodiment. For instance, aspect ratios of the maximum to minimum dimensions of the synthetic nanocarriers may vary from 1:1 to 1,000,000:1, preferably from 1:1 to 100,000:1, more preferably from 1:1 to 1000:1, still preferably from 1:1 to 100:1, and yet more preferably from 1:1 to 10:1. Preferably, a maximum dimension of at least 75%, preferably at least 80%, more preferably at least 90%, of the synthetic nanocarriers in a sample, based on the total number of synthetic nanocarriers in the sample is equal to or less than 3 μm, more preferably equal to or less than 2 μm, more preferably equal to or less than 1 μm, more preferably equal to or less than 800 nm, more preferably equal to or less than 600 nm, and more preferably still equal to or less than 500 nm. In preferred embodiments, a maximum dimension of at least 75%, preferably at least 80%, more preferably at least 90%, of the synthetic nanocarriers in a sample, based on the total number of synthetic nanocarriers in the sample, is equal to or greater than 100nm, more preferably equal to or greater than 120nm, more preferably equal to or greater than 130 nm, more

preferably equal to or greater than 140 nm, and more preferably still equal to or greater than 150 nm. Measurement of synthetic nanocarrier sizes is obtained by suspending the synthetic nanocarriers in a liquid (usually aqueous) media and using dynamic light scattering (e.g. using a Brookhaven ZetaPALS instrument).

"Obtained" means taken from a source without substantial modification. Substantial modification is modification that significantly affects the chemical or immunological properties of the material in question. For example, as a non-limiting example, a peptide or nucleic acid with a sequence with greater than 90%, preferably greater than 95%, preferably greater than 97%, preferably greater than 98%, preferably greater than 99%, preferably 100%, identity to a natural peptide or nucleotide sequence, preferably a natural consensus peptide or nucleotide sequence, and chemical and/or immunological properties that are not significantly different from the natural peptide or nucleic acid would be said to be obtained from the natural peptide or nucleotide sequence. These chemical or immunological properties comprise hydrophilicity, stability, affinity, and ability to couple with a carrier such as a synthetic nanocarrier.

"Pharmaceutically acceptable carrier or excipient" means a pharmacologically inactive material used together with the recited synthetic nanocarriers to formulate the inventive compositions. Pharmaceutically acceptable carriers or excipients comprise a variety of materials known in the art, including but not limited to, saccharides (such as glucose, lactose and the like), preservatives such as antimicrobial agents, reconstitution aids, colorants, saline (such as phosphate buffered saline) and buffers. In some embodiments, pharmaceutically acceptable carriers or excipients comprise calcium carbonate, calcium phosphate, various diluents, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

"Specific local cytotoxic T lymphocyte (CTL) response" means any stimulation, induction or proliferation of cytotoxic T cells, preferably cytotoxic T cells that are specific to an antigen. In embodiments, the antigen is associated with any of the diseases or conditions provided herein. In some embodiments, the antigen is comprised within an inventive composition or is administered in an inventive method provided herein. Methods for assessing CTL response are known to those of skill in the art. An examples of such a method is provided in the Examples.

"Subject" means animals, including warm blooded mammals such as humans and primates; avians; domestic household or farm animals such as cats, dogs, sheep, goats, cattle,

horses and pigs; laboratory animals such as mice, rats and guinea pigs; fish; reptiles; zoo and wild animals; and the like.

"Synthetic nanocarrier(s)" means a discrete object that is not found in nature, and that possesses at least one dimension that is less than or equal to 5 microns in size. Albumin nanoparticles are generally included as synthetic nanocarriers, however in certain embodiments the synthetic nanocarriers do not comprise albumin nanoparticles. In embodiments, inventive synthetic nanocarriers do not comprise chitosan.

A synthetic nanocarrier can be, but is not limited to, one or a plurality of lipid-based nanoparticles(e.g. liposomes) (also referred to herein as lipid nanoparticles, i.e., nanoparticles where the majority of the material that makes up their structure are lipids), polymeric nanoparticles, metallic nanoparticles, surfactant-based emulsions, dendrimers, buckyballs, nanowires, virus-like particles(i.e., particles that are primarily made up of viral structural proteins but that are not infectious or have low infectivity), peptide or protein-based particles (also referred to herein as protein particles, i.e., particles where the majority of the material that makes up their structure are peptides or proteins) (such as albumin nanoparticles) and/or nanoparticles that are developed using a combination of nanomaterials such as lipid-polymer nanoparticles. Synthetic nanocarriers may be a variety of different shapes, including but not limited to spheroidal, cuboidal, pyramidal, oblong, cylindrical, toroidal, and the like. Synthetic nanocarriers according to the invention comprise one or more surfaces, including but not limited to internal surfaces (surfaces generally facing an interior portion of the synthetic nanocarrier) and external surfaces (surfaces generally facing an external environment of the synthetic nanocarrier). Exemplary synthetic nanocarriers that can be adapted for use in the practice of the present invention comprise: (1) the biodegradable nanoparticles disclosed in US Patent 5,543,158 to Gref et al., (2) the polymeric nanoparticles of Published US Patent Application 20060002852 to Saltzman et al., (3) the lithographically constructed nanoparticles of Published US Patent Application 20090028910 to DeSimone et al., (4) the disclosure of WO 2009/051837 to von Andrian et al., (5) the nanoparticles disclosed in Published US Patent Application 2008/0145441 to Penades et al., (6) the protein nanoparticles disclosed in Published US Patent Application 20090226525 to de los Rios et al., (7) the virus-like particles disclosed in published US Patent Application 20060222652 to Sebbel et al., (8) the nucleic acid coupled virus-like particles disclosed in published US Patent Application 20060251677 to Bachmann et al., (9) the virus-like particles disclosed in WO2010047839A1 or WO2009106999A2, or (10) the nanoprecipitated nanoparticles

disclosed in P. Paolicelli et al., "Surface-modified PLGA-based Nanoparticles that can Efficiently Associate and Deliver Virus-like Particles" Nanomedicine. 5(6):843-853 (2010). In embodiments, synthetic nanocarriers may possess an aspect ratio greater than 1:1, 1:1.2, 1:1.5, 1:2, 1:3, 1:5, 1:7, or greater than 1:10.

Synthetic nanocarriers according to the invention that have a minimum dimension of equal to or less than about 100 nm, preferably equal to or less than 100 nm, do not comprise a surface with hydroxyl groups that activate complement or alternatively comprise a surface that consists essentially of moieties that are not hydroxyl groups that activate complement. In a preferred embodiment, synthetic nanocarriers according to the invention that have a minimum dimension of equal to or less than about 100 nm, preferably equal to or less than 100 nm, do not comprise a surface that substantially activates complement or alternatively comprise a surface that consists essentially of moieties that do not substantially activate complement. In a more preferred embodiment, synthetic nanocarriers according to the invention that have a minimum dimension of equal to or less than about 100 nm, preferably equal to or less than 100 nm, do not comprise a surface that activates complement or alternatively comprise a surface that consists essentially of moieties that do not activate complement. In embodiments, synthetic nanocarriers may possess an aspect ratio greater than 1:1, 1:1.2, 1:1.5, 1:2, 1:3, 1:5, 1:7, or greater than 1:10.

"Systemic dose" means a dose of an adjuvant that provides a particular systemic cytokine release, preferably a particular systemic cytokine release profile. In some embodiments, the particular systemic cytokine release, preferably a particular systemic cytokine release profile, is in a human. In embodiments, the compositions and methods provided herein (where at least a portion of a dose of adjuvant is not coupled to any nanocarriers) result in a particular systemic cytokine release profile in a subject. The term "separately" is also used to mean adjuvant that is not coupled to any synthetic nanocarriers. Additionally, "systemic cytokine release profile" means a pattern of systemic cytokine release, wherein the pattern comprises cytokine levels measured for several different systemic cytokines. In some embodiments, the particular systemic cytokine release profile comprises the systemic release of TNF-α, IL-6 and/or IL-12. In other embodiments, the particular systemic cytokine release profile comprises the systemic release of IFN-γ, IL12 and/or IL-18.

"T cell antigen" means any antigen that is recognized by and triggers an immune response in a T cell (e.g., an antigen that is specifically recognized by a T cell receptor on a T

cell or an NKT cell via presentation of the antigen or portion thereof bound to a Class I or Class II major histocompatability complex molecule (MHC), or bound to a CD1 complex.) In some embodiments, an antigen that is a T cell antigen is also a B cell antigen. In other embodiments, the T cell antigen is not also a B cell antigen. T cell antigens generally are proteins, polypeptides or peptides. T cell antigens may be an antigen that stimulates a CD8+ T cell response, a CD4+ T cell response, or both. The nanocarriers, therefore, in some embodiments can effectively stimulate both types of responses.

In some embodiments the T cell antigen is a 'universal' T cell antigen, or T cell memory antigen, (i.e., one to which a subject has a pre-existing memory and that can be used to boost T cell help to an unrelated antigen, for example an unrelated B cell antigen). Universal T cell antigens include tetanus toxoid, as well as one or more peptides derived from tetanus toxoid, Epstein-Barr virus, or influenza virus. Universal T cell antigens also include a components of influenza virus, such as hemagglutinin, neuraminidase, or nuclear protein, or one or more peptides derived therefrom. In some embodiments, the universal T cell antigen is not one that is presented in a complex with a MHC molecule. In some embodiments, the universal T cell antigen is not complexed with a MHC molecule for presentation to a T helper cell. Accordingly, in some embodiments, the universal T cell antigen is not a T helper cell antigen. However, in other embodiments, the universal T cell antigen is a T helper cell antigen.

In embodiments, a T-helper cell antigen may comprise one or more peptides obtained or derived from tetanus toxoid, Epstein-Barr virus, influenza virus, respiratory syncytial virus, measles virus, mumps virus, rubella virus, cytomegalovirus, adenovirus, diphtheria toxoid, or a PADRE peptide (known from the work of Sette et al. US Patent 7,202,351). In other embodiments, a T-helper cell antigen may comprise ovalbumin or a peptide obtained or derived therefrom. Preferably, the ovalbumin comprises the amino acid sequence as set forth in Accession No. AAB59956, NP_990483.1, AAA48998, or CAA2371. In other embodiments, the peptide obtained or derived from ovalbumin comprises the following amino acid sequence: H-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-OH (SEQ ID NO: 1). In other embodiments, a T-helper cell antigen may comprise one or more lipids, or glycolipids, including but not limited to: α-galactosylceramide (α -GalCer), α -linked glycosphingolipids (from Sphingomonas spp.), galactosyl diacylglycerols (from Borrelia burgdorferi), lypophosphoglycan (from Leishmania donovani), and phosphatidylinositol tetramannoside (PIM4) (from Mycobacterium leprae). For additional

lipids and/or glycolipids useful as T-helper cell antigen, see V. Cerundolo et al., "Harnessing invariant NKT cells in vaccination strategies." Nature Rev Immun, 9:28-38 (2009).

In embodiments, CD4+ T-cell antigens may be derivatives of a CD4+ T-cell antigen that is obtained from a source, such as a natural source. In such embodiments, CD4+ T-cell antigen sequences, such as those peptides that bind to MHC II, may have at least 70%, 80%, 90%, or 95% identity to the antigen obtained from the source. In embodiments, the T cell antigen, preferably a universal T cell antigen or T-helper cell antigen, may be coupled to, or uncoupled from, a synthetic nanocarrier. In some embodiments, the universal T cell antigen or T-helper cell antigen is encapsulated in the synthetic nanocarriers of the inventive compositions.

"Th1 immune response" means any immune response that results in the production of Th1 cells and Th1-associated cytokines, IFN-γ, IL-12 and/or IL-18, or that counteracts the differentiation of Th2 cells and the action of Th2 cytokines. Methods for assessing whether a Th1 immune response is induced are known to those of ordinary skill in the art. Examples of such methods are provided below in the Examples.

"Time different from administration" or "a time different from a time when the composition is administered" means a time more than about 30 seconds either before or after administration, preferably more than about 1 minute either before or after administration, more preferably more than 5 minutes either before or after administration, still more preferably more than 1 day either before or after administration, still more preferably more than 2 days either before or after administration, still more preferably more than 1 week either before or after administration, still more preferably more than 2 weeks either before or after administration, still more preferably more than 3 weeks either before or after administration, still more preferably more than 1 month either before or after administration, and still more preferably more than 2 months either before or after administration.

"Vaccine" means a composition of matter that improves the immune response to a particular pathogen or disease. A vaccine typically contains factors that stimulate a subject's immune system to recognize a specific antigen as foreign and eliminate it from the subject's body. A vaccine also establishes an immunologic 'memory' so the antigen will be quickly recognized and responded to if a person is re-challenged. Vaccines can be prophylactic (for example to prevent future infection by any pathogen), or therapeutic (for example a vaccine against a tumor specific antigen for the treatment of cancer or against an antigen derived from an infectious agent for the treatment of an infection or infectious disease). In embodiments, a

vaccine may comprise dosage forms according to the invention. Preferably, in some embodiments, these vaccines comprise an adjuvant not coupled to any synthetic nanocarriers.

In specific embodiments, the inventive compositions incorporate adjuvants that comprise agonists for toll-like receptors (TLRs) 7 & 8 ("TLR 7/8 agonists"). Of utility are the TLR 7/8 agonist compounds disclosed in U.S. Patent 6,696,076 to Tomai et al., including but not limited to imidazoquinoline amines, imidazopyridine amines, 6,7-fused cycloalkylimidazopyridine amines, and 1,2-bridged imidazoquinoline amines. Preferred adjuvants comprise imiquimod and R848.

In specific embodiments, the inventive compositons incorporate adjuvants that comprise a ligand for Toll-like receptor (TLR)-9, such as immunostimulatory DNA molecules comprising CpGs, which induce type I interferon secretion, and stimulate T and B cell activation leading to increased antibody production and cytotoxic T cell responses (Krieg et al., CpG motifs in bacterial DNA trigger direct B cell activation. Nature. 1995. 374:546-549; Chu et al. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J. Exp. Med. 1997. 186:1623-1631; Lipford et al. CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. Eur. J. Immunol. 1997. 27:2340-2344; Roman et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. Nat. Med. 1997. 3:849-854; Davis et al. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J. Immunol. 1998. 160:870-876; Lipford et al., Bacterial DNA as immune cell activator. Trends Microbiol. 1998. 6:496-500. In embodiments, CpGs may comprise modifications intended to enhance stability, such as phosphorothioate linkages, or other modifications, such as modified bases. See, for example, U.S. Patents, 5,663,153, 6,194,388, 7,262,286, or 7,276,489. In certain embodiments, to stimulate immunity rather than tolerance, a composition provided herein incorporates an adjuvant that promotes DC maturation (needed for priming of naive T cells) and the production of cytokines, such as type I interferons, which promote antibody responses and anti-viral immunity. In some embodiments, the adjuvant comprises a TLR-4 agonist, such as bacterial lipopolysacharide (LPS), VSV-G, and/or HMGB-1. In some embodiments, adjuvants comprise cytokines, which are small proteins or biological factors (in the range of 5 kD – 20 kD) that are released by cells and have specific effects on cell-cell interaction, communication and behavior of other cells. In some embodiments, adjuvants comprise proinflammatory stimuli released from necrotic cells (e.g., urate crystals). In some

embodiments, adjuvants comprise activated components of the complement cascade (e.g., CD21, CD35, etc.). In some embodiments, adjuvants comprise activated components of immune complexes. The adjuvants also include those that comprise complement receptor agonists, such as a molecule that binds to CD21 or CD35. In some embodiments, the complement receptor agonist induces endogenous complement opsonization of the nanocarrier. Adjuvants also include those that comprise cytokine receptor agonists, such as a cytokine.

In some embodiments, the cytokine receptor agonist is a small molecule, antibody, fusion protein, or aptamer. In embodiments, adjuvants also may comprise immunostimulatory RNA molecules, such as but not limited to dsRNA or poly I:C (a TLR3 stimulant), and/or those disclosed in F. Heil et al., "Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8" Science 303(5663), 1526-1529 (2004); J. Vollmer et al., "Immune modulation by chemically modified ribonucleosides and oligoribonucleotides" WO 2008033432 A2; A. Forsbach et al., "Immunostimulatory oligoribonucleotides containing specific sequence motif(s) and targeting the Toll-like receptor 8 pathway" WO 2007062107 A2; E. Uhlmann et al., "Modified oligoribonucleotide analogs with enhanced immunostimulatory activity" U.S. Pat. Appl. Publ. US 2006241076; G. Lipford et al., "Immunostimulatory viral RNA oligonucleotides and use for treating cancer and infections" WO 2005097993 A2; G. Lipford et al., "Immunostimulatory G,U-containing oligoribonucleotides, compositions, and screening methods" WO 2003086280 A2.

In some embodiments, the adjuvants comprise gel-type adjuvants (e.g., aluminum hydroxide, aluminum phosphate, calcium phosphate, etc.), microbial adjuvants (e.g., immunomodulatory DNA sequences that include CpG motifs; immunostimulatory RNA molecules; endotoxins such as monophosphoryl lipid A; exotoxins such as cholera toxin, E. coli heat labile toxin, and pertussis toxin; muramyl dipeptide, etc.); oil-emulsion and emulsifier-based adjuvants (e.g., Freund's Adjuvant, MF59 [Novartis], SAF, etc.); particulate adjuvants (e.g., liposomes, biodegradable microspheres, saponins, etc.); synthetic adjuvants (e.g., nonionic block copolymers, muramyl peptide analogues, polyphosphazene, synthetic polynucleotides, etc.), and/or combinations thereof.

SYNTHETIC NANOCARRIER COMPOSITIONS

A wide variety of synthetic nanocarriers can be used according to the invention. In some embodiments, synthetic nanocarriers are spheres or spheroids. In some embodiments,

synthetic nanocarriers are flat or plate-shaped. In some embodiments, synthetic nanocarriers are cubes, cuboidal or cubic. In some embodiments, synthetic nanocarriers are ovals or ellipses. In some embodiments, synthetic nanocarriers are cylinders, cones, or pyramids.

In some embodiments, it is desirable to use a population of synthetic nanocarriers that is relatively uniform in terms of size, shape, and/or composition so that each synthetic nanocarrier has similar properties. For example, at least 80%, at least 90%, or at least 95% of the synthetic nanocarriers, based on the total number of synthetic nanocarriers, may have a minimum dimension or maximum dimension that falls within 5%, 10%, or 20% of the average diameter or average dimension of the synthetic nanocarriers. In some embodiments, a population of synthetic nanocarriers may be heterogeneous with respect to size, shape, and/or composition.

Synthetic nanocarriers can be solid or hollow and can comprise one or more layers. In some embodiments, each layer has a unique composition and unique properties relative to the other layer(s). To give but one example, synthetic nanocarriers may have a core/shell structure, wherein the core is one layer (e.g. a polymeric core) and the shell is a second layer (e.g. a lipid bilayer or monolayer). Synthetic nanocarriers may comprise a plurality of different layers.

In some embodiments, synthetic nanocarriers may optionally comprise one or more lipids. In some embodiments, a synthetic nanocarrier may comprise a liposome. In some embodiments, a synthetic nanocarrier may comprise a lipid bilayer. In some embodiments, a synthetic nanocarrier may comprise a lipid monolayer. In some embodiments, a synthetic nanocarrier may comprise a micelle. In some embodiments, a synthetic nanocarrier may comprise a core comprising a polymeric matrix surrounded by a lipid layer (e.g., lipid bilayer, lipid monolayer, etc.). In some embodiments, a synthetic nanocarrier may comprise a non-polymeric core (e.g., metal particle, quantum dot, ceramic particle, bone particle, viral particle, proteins, nucleic acids, carbohydrates, etc.) surrounded by a lipid layer (e.g., lipid bilayer, lipid monolayer, etc.).

In some embodiments, synthetic nanocarriers can comprise one or more polymers or polymeric matrices. In some embodiments, such a polymer or polymeric matrix can be surrounded by a coating layer (e.g., liposome, lipid monolayer, micelle, etc.). In some embodiments, various elements of the synthetic nanocarriers can be coupled with the polymer or polymeric matrix.

In some embodiments, an element, such as a targeting moiety, oligonucleotide, antigen, adjuvant, etc. can be covalently associated with a polymeric matrix. In some embodiments, covalent association is mediated by a linker. In some embodiments, an element can be noncovalently associated with a polymeric matrix. For example, in some embodiments, an element can be encapsulated within, surrounded by, and/or dispersed throughout a polymeric matrix. Alternatively or additionally, an element can be associated with a polymeric matrix by hydrophobic interactions, charge interactions, van der Waals forces, etc.

A wide variety of polymers and methods for forming polymeric matrices therefrom are known conventionally. In general, a polymeric matrix comprises one or more polymers. Polymers may be natural or unnatural (synthetic) polymers. Polymers may be homopolymers or copolymers comprising two or more monomers. In terms of sequence, copolymers may be random, block, or comprise a combination of random and block sequences. Typically, polymers in accordance with the present invention are organic polymers.

Examples of polymers suitable for use in the present invention include, but are not limited to polyethylenes, polycarbonates (e.g. poly(1,3-dioxan-2one)), polyanhydrides (e.g. poly(sebacic anhydride)), polypropylfumerates, polyamides (e.g. polycaprolactam), polyacetals, polyethers, polyesters (e.g., polylactide, polyglycolide, polylactide-co-glycolide, polycaprolactone, polyhydroxyacid (e.g., poly(β-hydroxyalkanoate)), poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polyureas, polystyrenes, polyamines, polylysine, polylysine-PEG copolymers, and poly(ethyleneimine), poly(ethylene imine)-PEG copolymers.

In some embodiments, polymers in accordance with the present invention include polymers which have been approved for use in humans by the U.S. Food and Drug Administration (FDA) under 21 C.F.R. § 177.2600, including but not limited to polyesters (e.g., polylactic acid, poly(lactic-co-glycolic acid), polycaprolactone, polyvalerolactone, poly(1,3-dioxan-2one)); polyanhydrides (e.g., poly(sebacic anhydride)); polyethers (e.g., polyethylene glycol); polyurethanes; polymethacrylates; polyacrylates; and polycyanoacrylates.

In some embodiments, polymers can be hydrophilic. For example, polymers may comprise anionic groups (e.g., phosphate group, sulphate group, carboxylate group); cationic groups (e.g., quaternary amine group); or polar groups (e.g., hydroxyl group, thiol group, amine group). In some embodiments, a synthetic nanocarrier comprising a hydrophilic

polymeric matrix generates a hydrophilic environment within the synthetic nanocarrier. In some embodiments, polymers can be hydrophobic. In some embodiments, a synthetic nanocarrier comprising a hydrophobic polymeric matrix generates a hydrophobic environment within the synthetic nanocarrier. Selection of the hydrophilicity or hydrophobicity of the polymer may have an impact on the nature of materials that are incorporated (e.g. coupled) within the synthetic nanocarrier.

In some embodiments, polymers may be modified with one or more moieties and/or functional groups. A variety of moieties or functional groups can be used in accordance with the present invention. In some embodiments, polymers may be modified with polyethylene glycol (PEG), with a carbohydrate, and/or with acyclic polyacetals derived from polysaccharides (Papisov, 2001, ACS Symposium Series, 786:301). Certain embodiments may be made using the general teachings of U.S. Patent No. 5543158 to Gref et al., or WO publication WO2009/051837 by Von Andrian et al.

In some embodiments, polymers may be modified with a lipid or fatty acid group. In some embodiments, a fatty acid group may be one or more of butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, arachidic, behenic, or lignoceric acid. In some embodiments, a fatty acid group may be one or more of palmitoleic, oleic, vaccenic, linoleic, alpha-linoleic, gamma-linoleic, arachidonic, gadoleic, arachidonic, eicosapentaenoic, docosahexaenoic, or erucic acid.

In some embodiments, polymers may be polyesters, including copolymers comprising lactic acid and glycolic acid units, such as poly(lactic acid-co-glycolic acid) and poly(lactide-co-glycolide), collectively referred to herein as "PLGA"; and homopolymers comprising glycolic acid units, referred to herein as "PGA," and lactic acid units, such as poly-L-lactic acid, poly-D-lactic acid, poly-D,L-lactic acid, poly-L-lactide, poly-D-lactide, and poly-D,L-lactide, collectively referred to herein as "PLA." In some embodiments, exemplary polyesters include, for example, polyhydroxyacids; PEG copolymers and copolymers of lactide and glycolide (e.g., PLA-PEG copolymers, PGA-PEG copolymers, PLGA-PEG copolymers, and derivatives thereof. In some embodiments, polyesters include, for example, poly(caprolactone), poly(caprolactone)-PEG copolymers, poly(L-lactide-co-L-lysine), poly(serine ester), poly(4-hydroxy-L-proline ester), poly[α-(4-aminobutyl)-L-glycolic acid], and derivatives thereof.

In some embodiments, a polymer may be PLGA. PLGA is a biocompatible and biodegradable co-polymer of lactic acid and glycolic acid, and various forms of PLGA are

characterized by the ratio of lactic acid:glycolic acid. Lactic acid can be L-lactic acid, D-lactic acid, or D,L-lactic acid. The degradation rate of PLGA can be adjusted by altering the lactic acid:glycolic acid ratio. In some embodiments, PLGA to be used in accordance with the present invention is characterized by a lactic acid:glycolic acid ratio of approximately 85:15, approximately 75:25, approximately 60:40, approximately 50:50, approximately 40:60, approximately 25:75, or approximately 15:85.

In some embodiments, polymers may be one or more acrylic polymers. In certain embodiments, acrylic polymers include, for example, acrylic acid and methacrylic acid copolymers, methyl methacrylate copolymers, ethoxyethyl methacrylates, cyanoethyl methacrylate, aminoalkyl methacrylate copolymer, poly(acrylic acid), poly(methacrylic acid), methacrylic acid alkylamide copolymer, poly(methyl methacrylate), poly(methacrylic acid anhydride), methyl methacrylate, polymethacrylate, poly(methyl methacrylate) copolymer, polyacrylamide, aminoalkyl methacrylate copolymer, glycidyl methacrylate copolymers, polycyanoacrylates, and combinations comprising one or more of the foregoing polymers. The acrylic polymer may comprise fully-polymerized copolymers of acrylic and methacrylic acid esters with a low content of quaternary ammonium groups.

In some embodiments, polymers can be cationic polymers. In general, cationic polymers are able to condense and/or protect negatively charged strands of nucleic acids (e.g. DNA, or derivatives thereof). Amine-containing polymers such as poly(lysine) (Zauner et al., 1998, Adv. Drug Del. Rev., 30:97; and Kabanov et al., 1995, Bioconjugate Chem., 6:7), poly(ethylene imine) (PEI; Boussif et al., 1995, Proc. Natl. Acad. Sci., USA, 1995, 92:7297), and poly(amidoamine) dendrimers (Kukowska-Latallo et al., 1996, Proc. Natl. Acad. Sci., USA, 93:4897; Tang et al., 1996, Bioconjugate Chem., 7:703; and Haensler et al., 1993, Bioconjugate Chem., 4:372) are positively-charged at physiological pH, form ion pairs with nucleic acids, and mediate transfection in a variety of cell lines. In embodiments, the inventive synthetic nanocarriers may not comprise (or may exclude) cationic polymers.

In some embodiments, polymers can be degradable polyesters bearing cationic side chains (Putnam et al., 1999, Macromolecules, 32:3658; Barrera et al., 1993, J. Am. Chem. Soc., 115:11010; Kwon et al., 1989, Macromolecules, 22:3250; Lim et al., 1999, J. Am. Chem. Soc., 121:5633; and Zhou et al., 1990, Macromolecules, 23:3399). Examples of these polyesters include poly(L-lactide-co-L-lysine) (Barrera et al., 1993, J. Am. Chem. Soc., 115:11010), poly(serine ester) (Zhou et al., 1990, Macromolecules, 23:3399), poly(4-hydroxy-L-proline ester) (Putnam et al., 1999, Macromolecules, 32:3658; and Lim et al.,

1999, J. Am. Chem. Soc., 121:5633), and poly(4-hydroxy-L-proline ester) (Putnam et al., 1999, Macromolecules, 32:3658; and Lim et al., 1999, J. Am. Chem. Soc., 121:5633).

The properties of these and other polymers and methods for preparing them are well known in the art (see, for example, U.S. Patents 6,123,727; 5,804,178; 5,770,417; 5,736,372; 5,716,404; 6,095,148; 5,837,752; 5,902,599; 5,696,175; 5,514,378; 5,512,600; 5,399,665; 5,019,379; 5,010,167; 4,806,621; 4,638,045; and 4,946,929; Wang et al., 2001, J. Am. Chem. Soc., 123:9480; Lim et al., 2001, J. Am. Chem. Soc., 123:2460; Langer, 2000, Acc. Chem. Res., 33:94; Langer, 1999, J. Control. Release, 62:7; and Uhrich et al., 1999, Chem. Rev., 99:3181). More generally, a variety of methods for synthesizing certain suitable polymers are described in Concise Encyclopedia of Polymer Science and Polymeric Amines and Ammonium Salts, Ed. by Goethals, Pergamon Press, 1980; Principles of Polymerization by Odian, John Wiley & Sons, Fourth Edition, 2004; Contemporary Polymer Chemistry by Allcock et al., Prentice-Hall, 1981; Deming et al., 1997, Nature, 390:386; and in U.S. Patents 6,506,577, 6,632,922, 6,686,446, and 6,818,732.

In some embodiments, polymers can be linear or branched polymers. In some embodiments, polymers can be dendrimers. In some embodiments, polymers can be substantially cross-linked to one another. In some embodiments, polymers can be substantially free of cross-links. In some embodiments, polymers can be used in accordance with the present invention without undergoing a cross-linking step. It is further to be understood that inventive synthetic nanocarriers may comprise block copolymers, graft copolymers, blends, mixtures, and/or adducts of any of the foregoing and other polymers. Those skilled in the art will recognize that the polymers listed herein represent an exemplary, not comprehensive, list of polymers that can be of use in accordance with the present invention.

In some embodiments, the synthetic nanocarriers comprise one or more polymers. The polymeric synthetic nanocarriers, therefore, can also include those described in WO publication WO2009/051837 by Von Andrian et al., including, but not limited to those, with one or more hydrophilic components. Preferably, the one or more polymers comprise a polyester, such as a poly(lactic acid), poly(glycolic acid), poly(lactic-co-glycolic acid), or polycaprolactone. More preferably, the one or more polymers comprise or further comprise a polyester coupled to a hydrophilic polymer, such as a polyether. In embodiments, the polyether comprises polyethylene glycol. Still more preferably, the one or more polymers comprise a polyester and a polyester coupled to a hydrophilic polymer, such as a polyether.

In other embodiments, the one or more polymers are coupled to one or more antigens and/or one or more adjuvants. In embodiments, at least some of the polymers are coupled to the antigen(s) and/or at least some of the polymers are coupled to the adjuvant(s). Preferably, when there are more than one type of polymer, one of the types of polymer is coupled to the antigen(s). In embodiments, one of the other types of polymer is coupled to the adjuvant(s). For example, in embodiments, when the nanocarriers comprise a polyester and a polyester coupled to a hydrophilic polymer, such as a polyether, the polyester is coupled to the adjuvant, while the polyester coupled to the hydrophilic polymer, such as a polyether, is coupled to the antigen(s). In embodiments, where the nanocarriers comprise a T helper cell antigen, the T helper cell antigen can be encapsulated in the nanocarrier.

In some embodiments, synthetic nanocarriers may not comprise a polymeric component. In some embodiments, synthetic nanocarriers may comprise metal particles, quantum dots, ceramic particles, etc. In some embodiments, a non-polymeric synthetic nanocarrier is an aggregate of non-polymeric components, such as an aggregate of metal atoms (e.g., gold atoms).

In some embodiments, synthetic nanocarriers may optionally comprise one or more amphiphilic entities. In some embodiments, an amphiphilic entity can promote the production of synthetic nanocarriers with increased stability, improved uniformity, or increased viscosity. In some embodiments, amphiphilic entities can be associated with the interior surface of a lipid membrane (e.g., lipid bilayer, lipid monolayer, etc.). Many amphiphilic entities known in the art are suitable for use in making synthetic nanocarriers in accordance with the present invention. Such amphiphilic entities include, but are not limited to, phosphoglycerides; phosphatidylcholines; dipalmitoyl phosphatidylcholine (DPPC); dioleylphosphatidyl ethanolamine (DOPE); dioleyloxypropyltriethylammonium (DOTMA); dioleoylphosphatidylcholine; cholesterol; cholesterol ester; diacylglycerol; diacylglycerolsuccinate; diphosphatidyl glycerol (DPPG); hexanedecanol; fatty alcohols such as polyethylene glycol (PEG); polyoxyethylene-9-lauryl ether; a surface active fatty acid, such as palmitic acid or oleic acid; fatty acids; fatty acid monoglycerides; fatty acid diglycerides; fatty acid amides; sorbitan trioleate (Span®85) glycocholate; sorbitan monolaurate (Span®20); polysorbate 20 (Tween®20); polysorbate 60 (Tween®60); polysorbate 65 (Tween®65); polysorbate 80 (Tween®80); polysorbate 85 (Tween®85); polyoxyethylene monostearate; surfactin; a poloxomer; a sorbitan fatty acid ester such as sorbitan trioleate; lecithin; lysolecithin; phosphatidylserine;

phosphatidylinositol;sphingomyelin; phosphatidylethanolamine (cephalin); cardiolipin; phosphatidic acid; cerebrosides; dicetylphosphate; dipalmitoylphosphatidylglycerol; stearylamine; dodecylamine; hexadecyl-amine; acetyl palmitate; glycerol ricinoleate; hexadecyl sterate; isopropyl myristate; tyloxapol; poly(ethylene glycol)5000-phosphatidylethanolamine; poly(ethylene glycol)400-monostearate; phospholipids; synthetic and/or natural detergents having high surfactant properties; deoxycholates; cyclodextrins; chaotropic salts; ion pairing agents; and combinations thereof. An amphiphilic entity component may be a mixture of different amphiphilic entities. Those skilled in the art will recognize that this is an exemplary, not comprehensive, list of substances with surfactant activity. Any amphiphilic entity may be used in the production of synthetic nanocarriers to be used in accordance with the present invention.

In some embodiments, synthetic nanocarriers may optionally comprise one or more carbohydrates. Carbohydrates may be natural or synthetic. A carbohydrate may be a derivatized natural carbohydrate. In certain embodiments, a carbohydrate comprises monosaccharide or disaccharide, including but not limited to glucose, fructose, galactose, ribose, lactose, sucrose, maltose, trehalose, cellbiose, mannose, xylose, arabinose, glucoronic acid, galactoronic acid, mannuronic acid, glucosamine, galatosamine, and neuramic acid. In certain embodiments, a carbohydrate is a polysaccharide, including but not limited to pullulan, cellulose, microcrystalline cellulose, hydroxypropyl methylcellulose (HPMC), hydroxycellulose (HC), methylcellulose (MC), dextran, cyclodextran, glycogen, starch, hydroxyethylstarch, carageenan, glycon, amylose, chitosan, N,O-carboxylmethylchitosan, algin and alginic acid, starch, chitin, heparin, inulin, konjac, glucommannan, pustulan, heparin, hyaluronic acid, curdlan, and xanthan. In embodiments, the inventive synthetic nanocarriers do not comprise (or specifically exclude) carbohydrates, such as a polysaccharide. In certain embodiments, the carbohydrate may comprise a carbohydrate derivative such as a sugar alcohol, including but not limited to mannitol, sorbitol, xylitol, erythritol, maltitol, and lactitol.

Compositions according to the invention comprise inventive synthetic nanocarriers in combination with pharmaceutically acceptable excipients, such as preservatives, buffers, saline, or phosphate buffered saline. The compositions may be made using conventional pharmaceutical manufacturing and compounding techniques to arrive at useful dosage forms. In an embodiment, inventive synthetic nanocarriers are suspended in sterile saline solution for injection together with a preservative.

In embodiments, when preparing synthetic nanocarriers as carriers for agents (e.g., antigen or adjuvant) for use in vaccines methods for coupling the agents to the synthetic nanocarriers may be useful. If the agent is a small molecule it may be of advantage to attach the agent to a polymer prior to the assembly of the synthetic nanocarriers. In embodiments, it may also be an advantage to prepare the synthetic nanocarriers with surface groups that are used to couple the agent to the synthetic nanocarrier through the use of these surface groups rather than attaching the agent to a polymer and then using this polymer conjugate in the construction of synthetic nanocarriers. A variety of reactions can be used for the purpose of attaching agents to synthetic nanocarriers.

In certain embodiments, the coupling can be a covalent linker. In embodiments, peptides according to the invention can be covalently coupled to the external surface via a 1,2,3-triazole linker formed by the 1,3-dipolar cycloaddition reaction of azido groups on the surface of the nanocarrier with antigen or adjuvant containing an alkyne group or by the 1,3-dipolar cycloaddition reaction of alkynes on the surface of the nanocarrier with antigens or adjuvants containing an azido group. Such cycloaddition reactions are preferably performed in the presence of a Cu(I) catalyst along with a suitable Cu(I)-ligand and a reducing agent to reduce Cu(II) compound to catalytic active Cu(I) compound. This Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) can also be referred as the click reaction.

Additionally, the covalent coupling may comprise a covalent linker that comprises an amide linker, a disulfide linker, a thioether linker, a hydrazone linker, a hydrazide linker, an imine or oxime linker, an urea or thiourea linker, an amidine linker, an amine linker, and a sulfonamide linker.

An amide linker is formed via an amide bond between an amine on one component such as the antigen or adjuvant with the carboxylic acid group of a second component such as the nanocarrier. The amide bond in the linker can be made using any of the conventional amide bond forming reactions with suitably protected amino acids or antigens or adjuvants and activated carboxylic acid such N-hydroxysuccinimide-activated ester.

A disulfide linker is made via the formation of a disulfide (S-S) bond between two sulfur atoms of the form, for instance, of R_1-S-S-R_2. A disulfide bond can be formed by thiol exchange of an antigen or adjuvant containing thiol/mercaptan group(-SH) with another activated thiol group on a polymer or nanocarrier or a nanocarrier containing thiol/mercaptan groups with a antigen or adjuvant containing activated thiol group.

A triazole linker, specifically a 1,2,3-triazole of the form , wherein R_1 and R_2 may be any chemical entities, is made by the 1,3-dipolar cycloaddition reaction of an azide attached to a first component such as the nanocarrier with a terminal alkyne attached to a second component such as the peptide. The 1,3-dipolar cycloaddition reaction is performed with or without a catalyst, preferably with Cu(I)-catalyst, which links the two components through a 1,2,3-triazole function. This chemistry is described in detail by Sharpless et al., Angew. Chem. Int. Ed. 41(14), 2596, (2002) and Meldal, et al, Chem. Rev., 2008, 108(8), 2952-3015 and is often referred to as a "click" reaction or CuAAC.

In embodiments, a polymer containing an azide or alkyne group, terminal to the polymer chain is prepared. This polymer is then used to prepare a synthetic nanocarrier in such a manner that a plurality of the alkyne or azide groups are positioned on the surface of that nanocarrier. Alternatively, the synthetic nanocarrier can be prepared by another route, and subsequently functionalized with alkyne or azide groups. The antigen or adjuvant is prepared with the presence of either an alkyne (if the polymer contains an azide) or an azide (if the polymer contains an alkyne) group. The antigen or adjuvant is then allowed to react with the nanocarrier via the 1,3-dipolar cycloaddition reaction with or without a catalyst which covalently couples the antigen or adjuvant to the particle through the 1,4-disubstituted 1,2,3-triazole linker.

A thioether linker is made by the formation of a sulfur-carbon (thioether) bond in the form, for instance, of R_1-S-R_2. Thioether can be made by either alkylation of a thiol/mercaptan (-SH) group on one component such as the antigen or adjuvant with an alkylating group such as halide or epoxide on a second component such as the nanocarrier. Thioether linkers can also be formed by Michael addition of a thiol/mercaptan group on one component such as a antigen or adjuvant to an electron-deficient alkene group on a second component such as a polymer containing a maleimide group or vinyl sulfone group as the Michael acceptor. In another way, thioether linkers can be prepared by the radical thiol-ene reaction of a thiol/mercaptan group on one component such as a antigen or adjuvant with an alkene group on a second component such as a polymer or nanocarrier.

A hydrazone linker is made by the reaction of a hydrazide group on one component such as the antigen or adjuvant with an aldehyde/ketone group on the second component such as the nanocarrier.

A hydrazide linker is formed by the reaction of a hydrazine group on one component such as the antigen or adjuvant with a carboxylic acid group on the second component such as the nanocarrier. Such reaction is generally performed using chemistry similar to the formation of amide bond where the carboxylic acid is activated with an activating reagent.

An imine or oxime linker is formed by the reaction of an amine or N-alkoxyamine (or aminooxy) group on one component such as the antigen or adjuvant with an aldehyde or ketone group on the second component such as the nanocarrier.

An urea or thiourea linker is prepared by the reaction of an amine group on one component such as the antigen or adjuvant with an isocyanate or thioisocyanate group on the second component such as the nanocarrier.

An amidine linker is prepared by the reaction of an amine group on one component such as the antigen or adjuvant with an imidoester group on the second component such as the nanocarrier.

An amine linker is made by the alkylation reaction of an amine group on one component such as the antigen or adjuvant with an alkylating group such as halide, epoxide, or sulfonate ester group on the second component such as the nanocarrier. Alternatively, an amine linker can also be made by reductive amination of an amine group on one component such as the antigen or adjuvant with an aldehyde or ketone group on the second component such as the nanocarrier with a suitable reducing reagent such as sodium cyanoborohydride or sodium triacetoxyborohydride.

A sulfonamide linker is made by the reaction of an amine group on one component such as the antigen or adjuvant with a sulfonyl halide (such as sulfonyl chloride) group on the second component such as the nanocarrier.

A sulfone linker is made by Michael addition of a nucleophile to a vinyl sulfone. Either the vinyl sulfone or the nucleophile may be on the surface of the nanoparticle or attached to the antigen or adjuvant.

The antigen or adjuvant can also be conjugated to the nanocarrier via non-covalent conjugation methods. For examples, a negative charged antigen or adjuvant can be conjugated to a positive charged nanocarrier through electrostatic adsorption. An antigen or

adjuvant containing a metal ligand can also be conjugated to a nanocarrier containing a metal complex via a metal-ligand complex.

In embodiments, the antigen or adjuvant can be attached to a polymer, for example polylactic acid-block-polyethylene glycol, prior to the assembly of the synthetic nanocarrier or the synthetic nanocarrier can be formed with reactive or activatible groups on its surface. In the latter case, the antigen or adjuvant is prepared with a group which is compatible with the attachment chemistry that is presented by the synthetic nanocarriers' surface. In other embodiments, agents, such as a peptide antigen, can be attached to VLPs or liposomes using a suitable linker. A linker is a compound or reagent that capable of coupling two molecules together. In an embodiment, the linker can be a homobifuntional or heterobifunctional reagent as described in Hermanson 2008. For example, an VLP or liposome synthetic nanocarrier containing a carboxylic group on the surface can be treated with a homobifunctional linker, adipic dihydrazide (ADH), in the presence of EDC to form the corresponding synthetic nanocarrier with the ADH linker. The resulting ADH linked synthetic nanocarrier is then conjugated with an agent containing an acid group via the other end of the ADH linker on the NC to produce the corresponding VLP or liposome peptide conjugate.

For detailed descriptions of available conjugation methods, see Hermanson G T "Bioconjugate Techniques", 2nd Edition Published by Academic Press, Inc., 2008. In addition to covalent attachment the antigen or adjuvant can be coupled by adsorbtion to a pre-formed synthetic nanocarrier or it can be coupled by encapsulation during the formation of the synthetic nanocarrier.

METHODS OF MAKING AND USING THE COMPOSITIONS AND RELATED METHODS

Synthetic nanocarriers may be prepared using a wide variety of methods known in the art. For example, synthetic nanocarriers can be formed by methods as nanoprecipitation, flow focusing using fluidic channels, spray drying, single and double emulsion solvent evaporation, solvent extraction, phase separation, milling, microemulsion procedures, microfabrication, nanofabrication, sacrificial layers, simple and complex coacervation, and other methods well known to those of ordinary skill in the art. Alternatively or additionally, aqueous and organic solvent syntheses for monodisperse semiconductor, conductive, magnetic, organic, and other nanomaterials have been described (Pellegrino et al., 2005,

Small, 1:48; Murray et al., 2000, Ann. Rev. Mat. Sci., 30:545; and Trindade et al., 2001, Chem. Mat., 13:3843). Additional methods have been described in the literature (see, e.g., Doubrow, Ed., "Microcapsules and Nanoparticles in Medicine and Pharmacy," CRC Press, Boca Raton, 1992; Mathiowitz et al., 1987, J. Control. Release, 5:13; Mathiowitz et al., 1987, Reactive Polymers, 6:275; and Mathiowitz et al., 1988, J. Appl. Polymer Sci., 35:755, US Patents 5578325 and 6007845; P. Paolicelli et al., "Surface-modified PLGA-based Nanoparticles that can Efficiently Associate and Deliver Virus-like Particles" Nanomedicine. 5(6):843-853 (2010)).

Various materials may be encapsulated into synthetic nanocarriers as desirable using a variety of methods including but not limited to C. Astete et al., "Synthesis and characterization of PLGA nanoparticles" J. Biomater. Sci. Polymer Edn, Vol. 17, No. 3, pp. 247–289 (2006); K. Avgoustakis "Pegylated Poly(Lactide) and Poly(Lactide-Co-Glycolide) Nanoparticles: Preparation, Properties and Possible Applications in Drug Delivery" Current Drug Delivery 1:321-333 (2004); C. Reis et al., "Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles" Nanomedicine 2:8– 21 (2006); P. Paolicelli et al., "Surface-modified PLGA-based Nanoparticles that can Efficiently Associate and Deliver Virus-like Particles" Nanomedicine. 5(6):843-853 (2010). Other methods suitable for encapsulating materials, such as oligonucleotides, into synthetic nanocarriers may be used, including without limitation methods disclosed in United States Patent 6,632,671 to Unger October 14, 2003.

In certain embodiments, synthetic nanocarriers are prepared by a nanoprecipitation process or spray drying. Conditions used in preparing synthetic nanocarriers may be altered to yield particles of a desired size or property (e.g., hydrophobicity, hydrophilicity, external morphology, "stickiness," shape, etc.). The method of preparing the synthetic nanocarriers and the conditions (e.g., solvent, temperature, concentration, air flow rate, etc.) used may depend on the materials to be coupled to the synthetic nanocarriers and/or the composition of the polymer matrix.

If particles prepared by any of the above methods have a size range outside of the desired range, particles can be sized, for example, using a sieve.

Elements of the inventive synthetic nanocarriers (such as targeting moieties, polymeric matrices, antigens, adjuvants, and the like), may be coupled to the synthetic nanocarrier, e.g., by one or more covalent bonds, or may be coupled by means of one or more linkers. Additional methods of functionalizing synthetic nanocarriers may be adapted from

Published U.S. Patent Application 2006/0002852 to Saltzman et al., Published U.S. Patent Application 2009/0028910 to DeSimone et al., or Published International Patent Application WO/2008/127532 A1 to Murthy et al.

Alternatively or additionally, synthetic nanocarriers can be coupled to an element, such as targeting moieties, adjuvants, various antigens, etc., directly or indirectly via non-covalent interactions. In non-covalent embodiments, the non-covalent coupling is mediated by non-covalent interactions including but not limited to charge interactions, affinity interactions, metal coordination, physical adsorption, host-guest interactions, hydrophobic interactions, TT stacking interactions, hydrogen bonding interactions, van der Waals interactions, magnetic interactions, electrostatic interactions, dipole-dipole interactions, and/or combinations thereof. Such couplings may be arranged to be on an external surface or an internal surface of an inventive synthetic nanocarrier. In embodiments, encapsulation and/or absorption is a form of coupling.

In embodiments, the inventive synthetic nanocarriers can be combined with other adjuvants by admixing in the same vehicle or delivery system. Such adjuvants may include, but are not limited to mineral salts, such as alum, alum combined with monphosphoryl lipid (MPL) A of Enterobacteria, such as *Escherihia coli*, *Salmonella minnesota*, *Salmonella typhimurium*, or *Shigella flexneri* or specifically with MPL® (AS04), MPL A of above-mentioned bacteria separately, saponins, such as QS-21,Quil-A, ISCOMs, ISCOMATRIX™, emulsions such as MF59™, Montanide® ISA 51 and ISA 720, AS02 (QS21+squalene+ MPL®), AS15, liposomes and liposomal formulations such as AS01, synthesized or specifically prepared microparticles and microcarriers such as bacteria-derived outer membrane vesicles (OMV) of *N. gonorrheae*, *Chlamydia trachomatis* and others, or chitosan particles, depot-forming agents, such as Pluronic® block co-polymers, specifically modified or prepared peptides, such as muramyl dipeptide, aminoalkyl glucosaminide 4-phosphates, such as RC529, or proteins, such as bacterial toxoids or toxin fragments. Additional useful adjuvants may be found in WO 2002/032450; US 7,357,936 "Adjuvant Systems and Vaccines"; US 7,147,862 "Vaccine composition containing adjuvants"; US 6,544,518 "Vaccines"; US 5,750,110 "Vaccine composition containing adjuvants." The doses of such other adjuvants can be determined using conventional dose ranging studies. In embodiments, adjuvant that is not coupled to the recited synthetic nanocarriers may be the same or different from adjuvant that is coupled to the synthetic nanocarriers, if any. In other embodiments, the doses of such adjuvants may also be the same or different.

In embodiments, any adjuvant coupled to the inventive synthetic nanocarriers can be different, similar or identical to those not coupled to any nanocarriers. The adjuvants (coupled and not coupled) can be administered separately at a different time-point and/or at a different body location and/or by a different immunization route. Additionally, the separate adjuvant and population of nanocarriers can be administered separately at a different time-point and/or at a different body location and/or by a different immunization route.

Populations of synthetic nanocarriers may be combined to form pharmaceutical dosage forms according to the present invention using traditional pharmaceutical mixing methods. These include liquid-liquid mixing in which two or more suspensions, each containing one or more subset of nanocarriers, are directly combined or are brought together via one or more vessels containing diluent. As synthetic nanocarriers may also be produced or stored in a powder form, dry powder-powder mixing could be performed as could the re-suspension of two or more powders in a common media. Depending on the properties of the nanocarriers and their interaction potentials, there may be advantages conferred to one or another route of mixing.

Typical inventive compositions that comprise synthetic nanocarriers may comprise inorganic or organic buffers (e.g., sodium or potassium salts of phosphate, carbonate, acetate, or citrate) and pH adjustment agents (e.g., hydrochloric acid, sodium or potassium hydroxide, salts of citrate or acetate, amino acids and their salts) antioxidants (e.g., ascorbic acid, alpha-tocopherol), surfactants (e.g., polysorbate 20, polysorbate 80, polyoxyethylene9-10 nonyl phenol, sodium desoxycholate), solution and/or cryo/lyo stabilizers (e.g., sucrose, lactose, mannitol, trehalose), osmotic adjustment agents (e.g., salts or sugars), antibacterial agents (e.g., benzoic acid, phenol, gentamicin), antifoaming agents (e.g., polydimethylsilozone), preservatives (e.g., thimerosal, 2-phenoxyethanol, EDTA), polymeric stabilizers and viscosity-adjustment agents (e.g., polyvinylpyrrolidone, poloxamer 488, carboxymethylcellulose) and co-solvents (e.g., glycerol, polyethylene glycol, ethanol).

Compositions according to the invention comprise inventive synthetic nanocarriers in combination with pharmaceutically acceptable excipients. The compositions may be made using conventional pharmaceutical manufacturing and compounding techniques to arrive at useful dosage forms. Techniques suitable for use in practicing the present invention may be found in Handbook of Industrial Mixing: Science and Practice, Edited by Edward L. Paul, Victor A. Atiemo-Obeng, and Suzanne M. Kresta, 2004 John Wiley & Sons, Inc.; and Pharmaceutics: The Science of Dosage Form Design, 2nd Ed. Edited by M. E. Auten, 2001,

Churchill Livingstone. In an embodiment, inventive synthetic nanocarriers are suspended in sterile saline solution for injection together with a preservative.

It is to be understood that the compositions of the invention can be made in any suitable manner, and the invention is in no way limited to compositions that can be produced using the methods described herein. Selection of an appropriate method may require attention to the properties of the particular moieties being associated.

In some embodiments, inventive synthetic nanocarriers are manufactured under sterile conditions or are terminally sterilized. This can ensure that resulting composition are sterile and non-infectious, thus improving safety when compared to non-sterile compositions. This provides a valuable safety measure, especially when subjects receiving synthetic nanocarriers have immune defects, are suffering from infection, and/or are susceptible to infection. In some embodiments, inventive synthetic nanocarriers may be lyophilized and stored in suspension or as lyophilized powder depending on the formulation strategy for extended periods without losing activity.

The inventive compositions may be administered by a variety of routes of administration, including but not limited to subcutaneous, intramuscular, intradermal, oral, intranasal, transmucosal, sublingual, rectal, ophthalmic, transdermal, transcutaneous or by a combination of these routes.

Doses of dosage forms contain varying amounts of populations of synthetic nanocarriers and/or varying amounts of adjuvants and/or antigens, according to the invention. The amount of synthetic nanocarriers and/or adjuvants and/or antigens present in the inventive dosage forms can be varied according to the nature of the adjuvants and/or antigens, the therapeutic benefit to be accomplished, and other such parameters. In some embodiments, the doses of the dosage forms are systemic doses. In embodiments, dose ranging studies can be conducted to establish optimal therapeutic amount of the population of synthetic nanocarriers and/or the amount of adjuvants and/or antigens to be present in the dosage form. In embodiments, the synthetic nanocarriers and/or the adjuvants and/or antigens are present in the dosage form in an amount effective to generate an immune response upon administration to a subject. It may be possible to determine amounts of the adjuvants and/or antigens effective to generate an immune response using conventional dose ranging studies and techniques in subjects. Inventive dosage forms may be administered at a variety of frequencies. In a preferred embodiment, at least one administration of the dosage form is sufficient to generate a pharmacologically relevant response. In more preferred

embodiment, at least two administrations, at least three administrations, or at least four administrations, of the dosage form are utilized to ensure a pharmacologically relevant response.

The compositions and methods described herein can be used to induce, enhance, stimulate, modulate, direct or redirect an immune response. The compositions and methods described herein can be used in the diagnosis, prophylaxis and/or treatment of conditions such as cancers, infectious diseases, metabolic diseases, degenerative diseases, autoimmune diseases, inflammatory diseases, immunological diseases, or other disorders and/or conditions. The compositions and methods described herein can also be used for the prophylaxis or treatment of an addiction, such as an addiction to nicotine or a narcotic. The compositions and methods described herein can also be used for the prophylaxis and/or treatment of a condition resulting from the exposure to a toxin, hazardous substance, environmental toxin, or other harmful agent.

In embodiments, the compositions provided can be used to induce a rapid and strong systemic induction of pro-inflammatory cytokines, such as TNF-α, IL-6 and/or IL-12. The compositions provided, therefore, can be administered to subjects in need of an inflammatory response, preferably a systemic inflammatory response. In other embodiments, the compositions provided can be used for the rapid and strong systemic induction of cytokines that are important for a Th1 immune response, such as IFN-γ, IL-12 and/or IL-18. The compositions provided, therefore, can be administered to subjects in need of a Th1 response, preferably a systemic Th1 response. In still other embodiments, the compositions provided can be used to induce a strong humoral response. The compositions provided, therefore, can be administered to subjects in need of a humoral response. In still further embodiments, the compositions provided can be used to induce a strong specific local CTL response. The compositions provided, therefore, can be administered to subjects in need of a specific local CTL response. Such a response can be specific to any of the antigens provided herein, preferably to one or more antigens in an inventive composition or that is administered according to an inventive method provided herein.

The subjects provided herein can have or be at risk of having cancer. Cancers include, but are not limited to, breast cancer; biliary tract cancer; bladder cancer; brain cancer including glioblastomas and medulloblastomas; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; hematological neoplasms

including acute lymphocytic and myelogenous leukemia, e.g., B Cell CLL; T-cell acute lymphoblastic leukemia/lymphoma; hairy cell leukemia; chronic myelogenous leukemia, multiple myeloma; AIDS-associated leukemias and adult T-cell leukemia/lymphoma; intraepithelial neoplasms including Bowen's disease and Paget's disease; liver cancer; lung cancer; lymphomas including Hodgkin's disease and lymphocytic lymphomas; neuroblastomas; oral cancer including squamous cell carcinoma; ovarian cancer including those arising from epithelial cells, stromal cells, germ cells and mesenchymal cells; pancreatic cancer; prostate cancer; rectal cancer; sarcomas including leiomyosarcoma, rhabdomyosarcoma, liposarcoma, fibrosarcoma, and osteosarcoma; skin cancer including melanoma, Merkel cell carcinoma, Kaposi's sarcoma, basal cell carcinoma, and squamous cell cancer; testicular cancer including germinal tumors such as seminoma, non-seminoma (teratomas, choriocarcinomas), stromal tumors, and germ cell tumors; thyroid cancer including thyroid adenocarcinoma and medullar carcinoma; and renal cancer including adenocarcinoma and Wilms tumor.

The subjects provided herein can have or be at risk of having an infection or infectious disease. Infections or infectious diseases include, but are not limited to, viral infectious diseases, such as AIDS, Chickenpox (Varicella), Common cold, Cytomegalovirus Infection, Colorado tick fever, Dengue fever, Ebola hemorrhagic fever, Hand, foot and mouth disease, Hepatitis, Herpes simplex, Herpes zoster, HPV, Influenza (Flu), Lassa fever, Measles, Marburg hemorrhagic fever, Infectious mononucleosis, Mumps, Norovirus, Poliomyelitis, Progressive multifocal leukencephalopathy, Rabies, Rubella, SARS, Smallpox (Variola), Viral encephalitis, Viral gastroenteritis, Viral meningitis, Viral pneumonia, West Nile disease and Yellow fever; bacterial infectious diseases, such as Anthrax, Bacterial Meningitis, Botulism, Brucellosis, Campylobacteriosis, Cat Scratch Disease, Cholera, Diphtheria, Epidemic Typhus, Gonorrhea, Impetigo, Legionellosis, Leprosy (Hansen's Disease), Leptospirosis, Listeriosis, Lyme disease, Melioidosis, Rheumatic Fever, MRSA infection, Nocardiosis, Pertussis (Whooping Cough), Plague, Pneumococcal pneumonia, Psittacosis, Q fever, Rocky Mountain Spotted Fever (RMSF), Salmonellosis, Scarlet Fever, Shigellosis, Syphilis, Tetanus, Trachoma, Tuberculosis, Tularemia, Typhoid Fever, Typhus and Urinary Tract Infections; parasitic infectious diseases, such as African trypanosomiasis, Amebiasis, Ascariasis, Babesiosis, Chagas Disease, Clonorchiasis, Cryptosporidiosis, Cysticercosis, Diphyllobothriasis, Dracunculiasis, Echinococcosis, Enterobiasis, Fascioliasis, Fasciolopsiasis, Filariasis, Free-living amebic infection, Giardiasis, Gnathostomiasis,

Hymenolepiasis, Isosporiasis, Kala-azar, Leishmaniasis, Malaria, Metagonimiasis, Myiasis, Onchocerciasis, Pediculosis, Pinworm Infection, Scabies, Schistosomiasis, Taeniasis, Toxocariasis, Toxoplasmosis, Trichinellosis, Trichinosis, Trichuriasis, Trichomoniasis and Trypanosomiasis; fungal infectious disease, such as Aspergillosis, Blastomycosis, Candidiasis, Coccidioidomycosis, Cryptococcosis, Histoplasmosis, Tinea pedis (Athlete's Foot) and Tinea cruris; prion infectious diseases, such as Alpers' disease, Fatal Familial Insomnia, Gerstmann-Sträussler-Scheinker syndrome, Kuru and Variant Creutzfeldt-Jakob disease.

Subject provided here also include those that have or are at risk of having an atopic condition, such as but not limited to allergy, allergic asthma, or atopic dermatitis; asthma; chronic obstructive pulmonary disease (COPD, e.g. emphysema or chronic bronchitis); and chronic infections due to chronic infectious agents such as chronic leishmaniasis, candidiasis or schistosomiasis and infections caused by plasmodia, Toxoplasma gondii, mycobacteria, HIV, HBV, HCV EBV or CMV, or any one of the above, or any subset of the above. Other indications treatable using the inventive compositions include but are not limited to indications in which a subject's Th1 response is suboptimal and/or ineffective. Use of the present invention can enhance a subject's Th1 immune response with an adjuvant that can stimulate a Th1 immune response. The subjects, therefore, also include those that have or are at risk of having cancer, subjects with compromised or suboptimal immunity, such as infants, the elderly, cancer patients, individuals receiving immunosuppressive drugs or irradiation, hemodialysis patients and those with genetic or idiopathic immune dysfunction.

EXAMPLES

Example 1: Administration of Nanocarrier and Admixed R848 Adjuvant Results in Strong Systemic Production of Inflammatory Cytokines

Materials for NC-R848-1 Nanocarrier Formulations

Ovalbumin peptide 323-339 amide acetate salt, was purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Part # 4065609.) PLGA-R848 conjugate of 75/25 lactide/glycolide monomer composition and of approximately 4100 Da molecular weight having 5.2% w/w R848 content was synthesized. PLA-PEG-Nicotine with a nicotine-terminated PEG block of approximately 3,500 Da and DL-PLA block of

approximately 15,000 Da was synthesized Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for NC-R848-1 Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 70mg/mL was prepared in 0.13N hydrochloric acid at room temperature.

Solution 2: PLGA-R848 @ 75 mg/mL and PLA-PEG-Nicotine @ 25 mg/mL in dichloromethane was prepared by dissolving PLGA-R848 at 100 mg/mL in dichloromethane and PLA-PEG-Nicotine at 100 mg/mL in dichloromethane, then combining 3 parts of the PLGA-R848 solution to 1 part of the PLA-PEG-Nicotine solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in 100mM phosphate buffer, pH 8.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.1 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (2.0 mL) to the primary emulsion and sonicating at 30% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to an open 50 mL beaker containing 70 mM phosphate buffer solution (30 mL) and stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and the nanocarriers to form in suspension. A portion of the suspended nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube, spinning at 13800 rcf for 60 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in phosphate buffered saline to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 1: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier ID	Effective Diameter (nm)	TLR Agonist, % w/w	T-cell helper peptide, % w/w

NC-R848-1	220	R848, 1.3	Ova 323-339, 2.0

Materials for NC-R848-2 Formulations

Ovalbumin peptide 323-339 amide acetate salt was purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Part # 4065609.) PLGA-R848 conjugate of 75/25 lactide/glycolide monomer composition and of approximately 4100 Da molecular weight having 5.2% w/w R848 content was synthesized. PLA-PEG-Nicotine with a nicotine-terminated PEG block of approximately 5,000 Da and DL-PLA block of approximately 17,000 Da was synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for NC-R848-2 Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 70mg/mL was prepared in 0.13N hydrochloric acid at room temperature.

Solution 2: PLGA-R848 @ 75 mg/mL and PLA-PEG-Nicotine @ 25 mg/mL in dichloromethane was prepared by dissolving PLGA-R848 at 100 mg/mL in dichloromethane and PLA-PEG-Nicotine at 100 mg/mL in dichloromethane, then combining 3 parts of the PLGA-R848 solution to 1 part of the PLA-PEG-Nicotine solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in 100mM phosphate buffer, pH 8.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.1 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (2.0 mL) to the primary emulsion and sonicating at 30% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to an open 50 mL beaker containing 70 mM phosphate buffer solution (30 mL) and stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and the nanocarriers to form in suspension. A portion of the suspended nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube, spinning at 13800 rcf for 60 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in phosphate buffered saline to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 2: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier ID	Effective Diameter (nm)	TLR Agonist, % w/w	T-cell helper peptide, % w/w
NC-R848-2	229	R848, 3.3	Ova 323-339, 1.6

Materials for NC Only Formulations

Ovalbumin peptide 323-339 amide acetate salt, was purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Part # 4065609.) PLGA with 73% lactide and 27% glycolide content and an inherent viscosity of 0.12 dL/g was purchased from SurModics Pharmaceuticals (756 Tom Martin Drive, Birmingham, AL 35211. Product Code 7525 DLG 1A.) PLA-PEG-Nicotine with a nicotine-terminated PEG block of approximately 3,500 Da and DL-PLA block of approximately 15,000 Da was synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for NC Only Production

Solutions were prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 70mg/mL was prepared in 0.13N hydrochloric acid at room temperature.

Solution 2: PLGA @ 75 mg/mL and PLA-PEG-Nicotine @ 25 mg/mL in dichloromethane was prepared by dissolving PLGA at 100 mg/mL in dichloromethane and PLA-PEG-Nicotine at 100 mg/mL in dichloromethane, then combining 3 parts of the PLGA solution to 1 part of the PLA-PEG-Nicotine solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in 100mM phosphate buffer, pH 8.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.1 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (2.0 mL) to the primary emulsion and sonicating at 30% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to an open 50 mL beaker containing 70 mM phosphate buffer solution
(30 mL) and stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and the nanocarriers to form in suspension. A portion of the suspended nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube, spinning at 13800 rcf for
60 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in

phosphate buffered saline to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 3: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier	Effective Diameter (nm)	TLR Agonist, % w/w	T-cell helper peptide, % w/w
NC only	176	None	1.1

Results

Groups of mice were injected subcutaneously into hind limbs with 100 μg of nanocarriers (NC) coupled, non-coupled or admixed with small molecule nucleoside analogue and known TLR7/8 agonist and adjuvant, R848. R848 amounts in nanocarrier were 2-3% resulting in
2-3 μg of coupled R848 per injection; amount of free R848 used was 20 μg per injection. Mouse serum was taken by terminal bleed and systemic cytokine production in serum was measured at different time-points by ELISA (BD Biosciences). As seen in Figs. 1A-1C, strong systemic production of major pro-inflammatory cytokines TNF-α, IL-6 and IL-12 was observed when admixed R848 (NC + R848) was used, while no expression of TNF-α, IL-6 and IL-12 was detected when two separate preparations of NC coupled with R848 (NC-R848-1 and NC-R848-2) were used. The difference in peak cytokine expression levels was > 100-fold for TNF-α and IL-6, and > 50-fold for IL-12. NC not coupled to R848 (labeled as NC only) did not induce any systemic cytokines when used without admixed R848.

Example 2: Coupling of Nanocarrier to R848 Adjuvant does not Inhibit Systemic Production of Immune Cytokine IFN-γ

Materials for NC-R848 Nanocarrier Formulations

Ovalbumin peptide 323-339 amide acetate salt was purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Part # 4065609.) PLGA-R848 conjugate of 75/25 lactide/glycolide monomer composition and of approximately 4100 Da molecular weight having 5.2% w/w R848 content was synthesized. PLA-PEG-Nicotine with a nicotine-terminated PEG block of approximately 3,500 Da and DL-PLA block of approximately

15,000 Da was synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) purchased from J.T. Baker (Part Number U232-08).

Methods for NC-R848 Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 70mg/mL was prepared in 0.13N hydrochloric acid at room temperature.

Solution 2: PLGA-R848 @ 75 mg/mL and PLA-PEG-Nicotine @ 25 mg/mL in dichloromethane was prepared by dissolving PLGA-R848 at 100 mg/mL in dichloromethane and PLA-PEG-Nicotine at 100 mg/mL in dichloromethane, then combining 3 parts of the PLGA-R848 solution to 1 part of the PLA-PEG-Nicotine solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in 100mM phosphate buffer, pH 8.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.1 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (2.0 mL) to the primary emulsion and sonicating at 30% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to an open 50 mL beaker containing 70 mM phosphate buffer solution
(30 mL) and stirred at room temperature for 2 hours to allow for the dichloromethane to evaporate and for the nanocarriers to form in suspension. A portion of the suspended nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube, spinning at 13800 rcf for 60 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in phosphate buffered saline to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 4: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier ID	Effective Diameter	TLR Agonist, % w/w	T-cell helper peptide, % w/w

	(nm)		
NC-R848	213	R848, 2.6	Ova 323-339, 0.9

Materials for NC Only Nanocarrier Formulations

Ovalbumin peptide 323-339 amide acetate salt was purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Part # 4065609.) PLGA with 73% lactide and 27% glycolide content and an inherent viscosity of 0.12 dL/g was purchased from SurModics Pharmaceuticals (756 Tom Martin Drive, Birmingham, AL 35211. Product Code 7525 DLG 1A.) PLA-PEG-Nicotine with a nicotine-terminated PEG block of approximately 3,500 Da and DL-PLA block of approximately 15,000 Da was synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for NC Only Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 70mg/mL was prepared in 0.13N hydrochloric acid at room temperature.

Solution 2: PLGA @ 75 mg/mL and PLA-PEG-Nicotine @ 25 mg/mL in dichloromethane was prepared by dissolving PLGA at 100 mg/mL in dichloromethane and PLA-PEG-Nicotine at 100 mg/mL in dichloromethane, then combining 3 parts of the PLGA solution to 1 part of the PLA-PEG-Nicotine solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in 100mM phosphate buffer, pH 8.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.1 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (2.0 mL) to the primary emulsion and sonicating at 30% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to an open 50 mL beaker containing 70 mM phosphate buffer solution (30 mL) and stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and the nanocarriers to form in suspension. A portion of the suspended

nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube, spinning at 13800 rcf for 60 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in phosphate buffered saline to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 5: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier	Effective Diameter (nm)	TLR Agonist, % w/w	T-cell helper peptide, % w/w
NC Only	176	None	Ova 323-339, 1.1

Results

While early proinflammatory cytokines are mostly associated with side effects during immunization, the production of other cytokines, such as immune IFN-γ is known to be involved in the induction of effective immune response. Therefore, systemic production of immune cytokine IFN-γ after injection with NC was measured 0-24 hours after inoculation. Briefly, groups of mice were injected subcutaneously into hind limbs with 100 μg of NCs coupled or admixed with small molecule nucleoside analogue and known TLR7/8 agonist and adjuvant, R848. R848 amounts in nanocarrier were 2% resulting in 2 μg of coupled R848 per injection; amount of free R848 used was 20 μg per injection. Mouse serum was taken by terminal bleed and systemic cytokine production in serum was measured at different time-points by ELISA (BD Biosciences). IFN-γ, which is important for Th1 immune response, was seen with both NC-R848 (containing 2 μg of R848) and NC with admixed R848 (20 μg) (Fig. 2). Furthermore, higher levels of IFN- γ by NC with admixed R848 occurred earlier.

Example 3: Addition of Free Adjuvant Augments Immune Response

Materials for NC-Nic w/o R848 Nanocarrier Formulations

Ovalbumin peptide 323-339 amide TFA salt was purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Part # 4064565.) PLA with an inherent viscosity of 0.19 dL/g was purchased from Boehringer Ingelheim (Ingelheim Germany. Product Code R202H). PLA-PEG-Nicotine with a nicotine-terminated PEG block of

approximately 3,500 Da and DL-PLA block of approximately 15,000 Da was synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for NC-Nic w/o R848 Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 69mg/mL was prepared in 0.13N hydrochloric acid at room temperature.

Solution 2: PLA @ 75 mg/mL and PLA-PEG-Nicotine @ 25mg/mL in dichloromethane was prepared by dissolving PLA @ 100 mg/mL in dichloromethane and PLA-PEG-Nicotine at 100mg/mL in dichloromethane, then combining 3 parts of the PLA solution to 1 part of the PLA-PEG-Nicotine solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in deionized water.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.1 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (2.0 mL) to the primary emulsion and sonicating at 35% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to a beaker containing 70 mM phosphate buffer solution (30 mL) in an open 50ml beaker and stirred at room temperature for 2 hours to allow for the dichloromethane to evaporate and for the nanocarriers to form in suspension. A portion of the suspended nanocarriers were washed by transferring the nanocarrier suspension to centrifuge tubes, spinning at 5300 rcf for 60 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in phosphate buffered saline to achieve nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 6: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier	Effective Diameter (nm)	TLR Agonist, % w/w	T-cell helper peptide, % w/w

NC-Nic w/o R848	248	None	Ova 323-339, 2.2

Materials for NC-Nic w/ Entrapped R848 Nanocarrier Formulations

Ovalbumin peptide 323-339 amide TFA salt was purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Part # 4064565.) R848 (Resiquimod) of approximately 98-99% purity was synthesized and purified. PLA with an inherent viscosity of 0.19 dL/g was purchased from Boehringer Ingelheim (Ingelheim Germany. Product Code R202H). PLA-PEG-Nicotine with a nicotine-terminated PEG block of approximately 3,500 Da and DL-PLA block of approximately 15,000 Da was synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for NC-Nic w/ Entrapped R848 Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 69mg/mL was prepared in 0.13N hydrochloric acid at room temperature.

Solution 2: PLA @ 75 mg/mL, R848 @ 7.5 mg/mL, and PLA-PEG-Nicotine @ 25mg/mL in dichloromethane was prepared by dissolving PLA @ 100 mg/mL in dicholoromethane and adding R848 at 10mg/mL, also dissolving PLA-PEG-Nicotine at 100mg/mL in dichloromethane, then combining 3 parts of the PLA/R848 solution to 1 part of the PLA-PEG-Nicotine.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in deionized water.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.1 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (2.0 mL) to the primary emulsion and sonicating at 35% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to a beaker containing 70 mM phosphate buffer solution (30 mL) in an open 50ml beaker and stirred at room temperature for 2 hours to allow for the dichloromethane to evaporate and for the nanocarriers to form in suspension. A portion of the suspended nanocarriers were washed by transferring the nanocarrier

suspension to centrifuge tubes, spinning at 5300 rcf for 60 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in phosphate buffered saline to achieve nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 7: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier ID	Effective Diameter (nm)	TLR Agonist, % w/w	T-cell helper peptide, % w/w
NC-Nic w/ Entrapped R848	207	R848, 0.8	Ova 323-339, 1.6

Results

Mice were immunized with NC-Nic (nanocarrier exhibiting nicotine on the outer surface) carrying entrapped (non-conjugated) R848 with or without second adjuvant. Groups of five mice were immunized three times (subcutaneously, hind limbs) at 2-week intervals (days 0, 14 and 28) with 100 μg of NC-Nic. Serum anti-nicotine antibodies were then measured on days 26 and 40. EC_{50} for anti-nicotine antibodies were measured by standard ELISA against polylysine-nicotine (Fig. 3) (Group 1: NC-Nic w/o entrapped R848; group 2: NC-Nic w. 1.5% of entrapped R848; group 3: NC-Nic w. 1.5% of entrapped R848 + 80 μg of alum; group 4: NC-Nic w. 1.5% of entrapped R84 + 25 μg of CpG-1826). This demonstrates that utilization of entrapped R848 (Th1 adjuvant, TLR7/8 agonist) within the nanocarriers (NC) generates an immune response, which is superior to one induced by NC without R848 (group 2 > group 1). Moreover, addition of free Th2 adjuvant (alum) to NC-Nic with R848 further augments humoral immune response (group 3 > group 2). At the same time, addition of another free Th1 adjuvant (CpG, TLR9 agonist; group 4) also augments immune response, but is less potent in this combination than alum (group 4 > group 2 versus 4 < group 3).

Example 4: Addition of Free Adjuvant Augments Immune Response to NC without Adjuvant

Materials for NC-Nic Nanocarrier Formulations

Ovalbumin peptide 323-339 amide TFA salt, was purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Part # 4064565.) PLA with an inherent viscosity of 0.19 dL/g was purchased from Boehringer Ingelheim (Ingelheim Germany. Product Code R202H). PLA-PEG-Nicotine with a nicotine-terminated PEG block of approximately 3,500 Da and DL-PLA block of approximately 15,000 Da was synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for NC-Nic Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 69mg/mL was prepared in 0.13N hydrochloric acid at room temperature.

Solution 2: PLA @ 75 mg/mL and PLA-PEG-Nicotine @ 25mg/mL in dichloromethane was prepared by dissolving PLA @ 100 mg/mL in dichloromethane and PLA-PEG-Nicotine at 100mg/mL in dichloromethane, then combining 3 parts of the PLA solution to 1 part of the PLA-PEG-Nicotine solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in deionized water.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.1 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (2.0 mL) to the primary emulsion and sonicating at 35% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to a beaker containing 70 mM phosphate buffer solution (30 mL) in an open 50ml beaker and stirred at room temperature for 2 hours to allow for the dichloromethane to evaporate and for the nanocarriers to form in suspension. A portion of the suspended nanocarriers were washed by transferring the nanocarrier suspension to centrifuge tubes, spinning at 5300 rcf for 60 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in phosphate buffered saline to achieve nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 8: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier ID	Effective Diameter (nm)	TLR Agonist, % w/w	T-cell helper peptide, % w/w
NC-Nic	248	None	Ova 323-339, 2.2

Materials for NC-Nic-R848 Nanocarrier Formulations

Ovalbumin peptide 323-339 amide TFA salt, was purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Part # 4064565.) R848 (Resiquimod) of approximately 98-99% purity was synthesized and purified. PLA with an inherent viscosity of 0.19 dL/g was purchased from Boehringer Ingelheim (Ingelheim Germany. Product Code R202H). PLA-PEG-Nicotine with a nicotine-terminated PEG block of approximately 3,500 Da and DL-PLA block of approximately 15,000 Da was synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for NC-Nic-R848 Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 69mg/mL was prepared in 0.13N hydrochloric acid at room temperature.

Solution 2: PLA @ 75 mg/mL, R848 @ 7.5 mg/mL, and PLA-PEG-Nicotine @ 25mg/mL in dichloromethane was prepared by dissolving PLA @ 100 mg/mL in dicholoromethane and adding R848 at 10mg/mL, also dissolving PLA-PEG-Nicotine at 100mg/mL in dichloromethane, then combining 3 parts of the PLA/R848 solution to 1 part of the PLA-PEG-Nicotine.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in deionized water.

Solution 4: 70 mM phosphate buffer, pH 8

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.1 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (2.0 mL) to the primary emulsion and sonicating at 35% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to a beaker containing 70 mM phosphate buffer solution (30 mL) in an open 50ml beaker and stirred at room temperature for 2 hours

to allow for the dichloromethane to evaporate and for the nanocarriers to form in suspension.

A portion of the suspended nanocarriers were washed by transferring the nanocarrier suspension to centrifuge tubes, spinning at 5300 rcf for 60 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in phosphate buffered saline to achieve nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 9: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier ID	Effective Diameter (nm)	TLR Agonist, % w/w	T-cell helper peptide, % w/w
NC-Nic-R848	207	R848, 0.8	Ova 323-339, 1.6

Results

Mice were immunized with NC-Nic (nanocarrier exhibiting nicotine on the outer surface) that did not have adjuvant in the NC with or without admixed R848. Groups of five mice were immunized three times (subcutaneously, hind limbs) at 2-week intervals (days 0, 14 and 28) with 100 μg of NC-Nic. Serum anti-nicotine antibodies were then measured on days 26 and 40. EC_{50} for anti-nicotine antibodies were measured by standard ELISA against polylysine-nicotine (Fig. 4) (group 1: NC-Nic w/o entrapped R848; group 2: NC-Nic w/o entrapped R848 + 20 μg of free R848). This demonstrates that admixing of free R848 (Th1 adjuvant, TLR7/8 agonist) to antigen-carrying NCs generates immune response, which is superior to one induced by NC without admixed R848 (group 2 > group 1).

Similarly, the immune response to NC-carried antigen in mice immunized with NC-Nic carrying encapsulated R848 adjuvant (NC-Nic-R848) admixed with free Th1 adjuvant CpG-1826 (Enzo) or Th2 adjuvant alum (Pierce) was augmented. Groups of five mice were immunized three times (s.c., hind limbs) at 2-week intervals (days 0, 14 and 28) with 100 μg of NC-Nic-R848. Serum anti-nicotine antibodies were then measured on days 26 and 40. EC_{50} for anti-nicotine antibodies as measured in standard ELISA against polylysine-nicotine (Fig. 5) (group 1: NC-Nic-R848; group 2: NC-NicR848 + 80 μg of free alum; group 3: NC-NicR848 + 25 μg of free CpG-1826). This demonstrates that admixing of a free adjuvant to antigen/adjuvant-carrying NCs generates immune response, which is superior to one induced by the same NC without admixed adjuvant (group 2 > group 1; group 3> group 1).

Example 5: Addition of Free Adjuvant Augments Immune Response to NC Containing Encapsulated Adjuvant

Materials for Nanocarrier Formulations

Ovalbumin peptide 323-339 amide acetate salt, was purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Part # 4065609.) R848 (Resiquimod) of approximately 98-99% purity was synthesized and purified. PLA-R848 conjugate having molecular weight of approximately 1300 Da and R848 content of approximately 9% by weight was synthesized at by a ring-opening process. PLA-PEG-Nicotine with a nicotine-terminated PEG block of approximately 3,500 Da and DL-PLA block of approximately 15,000 Da was synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 70 mg/mL was prepared in 0.13N hydrochloric acid at room temperature.

Solution 2: PLA-R848 @ 75 mg/mL, PLA-PEG-Nicotine @ 25 mg/mL, and R848 @ 1.9 mg/mL in dichloromethane was prepared by dissolving the polymers at 100 mg/mL, adding the R848 to the PLA-PEG-Nicotine solution, and then combining 3 parts of the PLA-R848 solution to 1 part of the PLA-PEG-Nicotine/R848 solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in deionized water.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.1 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (2.0 mL) to the primary emulsion and sonicating at 35% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to a beaker containing 70 mM phosphate buffer solution (30 mL) in an open 50ml beaker and stirred at room temperature for 2 hours to allow for the dichloromethane to evaporate and for the nanocarriers to form in suspension. A portion of the suspended nanocarriers were washed by transferring the nanocarrier

suspension to a centrifuge tube, spinning at 13,800 rcf for 60 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in phosphate buffered saline to achieve nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 10: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier	Effective Diameter (nm)	TLR Agonist, % w/w	T-cell helper peptide, % w/w
	231	R848, 3.3	Ova 323-339, 1.5

Results

Mice were immunized with the nanocarriers, NC-Nic (nanocarrier exhibiting nicotine on the outer surface) which carried R848 and OP-II helper peptide, with or without admixed alum. Groups of five mice were immunized three times (subcutaneously, hind limbs) at 2-week intervals (days 0, 14 and 28) with 100 μg of NC[Nic,R848,OP-II] +/- 80 μg of admixed alum (Pierce). Serum anti-nicotine antibodies were then measured on days 40 and 70. EC_{50} for anti-nicotine antibodies were measured by standard ELISA against polylysine-nicotine (Fig. 6) (Group 1: NC[Nic,R848,OP-II]; group 2: NC[Nic,R848,OP-II] + 80 μg of admixed alum). This demonstrates that admixing of free alum (Th2 adjuvant) to antigen-carrying adjuvant-containing NCs generates immune response, which is superior to one induced by the same NC without admixed alum (group 2 > group 1).

Example 6: NC-encapsulated Antigen Generates a Stronger Cellular Immune Response than Free Antigen (Free Adjuvant Admixed)

Materials for Nanocarrier Formulations

Ovalbumin protein, was purchased from Worthington Biochemical Corporation (730 Vassar Avenue, Lakewood, NJ 08701. Product Code 3048.) PLA with an inherent viscosity of 0.21 dL/g was purchased from SurModics Pharmaceuticals (756 Tom Martin Drive, Birmingham, AL 35211. Product Code 100 DL 2A.) PLA-PEG-OMe block co-polymer with a methyl ether terminated PEG block of approximately 2,000 Da and PLA block of

approximately 19,000 Da was synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin protein @ 20mg/mL was prepared in phosphate buffered saline at room temperature.

Solution 2: PLA @ 75 mg/mL and PLA-PEG-OMe @ 25 mg/mL in dichloromethane was prepared by dissolving PLA at 100 mg/mL in dichloromethane and PLA-PEG-OMe at 100 mg/mL in dichloromethane, then combining 3 parts of the PLA solution to 1 part of the PLA-PEG-OMe solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in 100mM phosphate buffer, pH 8.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.2 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (3.0 mL) to the primary emulsion, vortexing to create a course dispersion, and then sonicating at 30% amplitude for 60 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to an open 50 mL beaker containing 70 mM phosphate buffer solution (30 mL) and stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and the nanocarriers to form in suspension. A portion of the suspended nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube, spinning at 21,000 rcf for 45 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in phosphate buffered saline to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 11: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier	Effective Diameter (nm)	TLR Agonist, % w/w	Antigen, % w/w
	228	None	OVA protein, 2.8

Results

Mice were immunized either with the nanocarriers, NC-OVA (nanocarrier carrying encapsulated ovalbumin protein), or with free ovalbumin (OVA) with a free adjuvant admixed. Groups of 3 mice were immunized once (s.c., hind limbs) with 100 µg of NC-OVA (2.8% OVA) or with 2.5 µg of free OVA admixed with 10 µg of free 1826-CpG (TLR9 agonist). Draining popliteal lymph nodes were taken at day 4 after immunization, meshed, incubated in vitro for 4 days in complete RPMI medium supplemented with 10 units/ml of IL-2, and specific CTL (cytotoxic T cell) activity was determined (as % of lysis of ovalbumin-expressing cell line EG.7-OVA – % of lysis of its parental cell line EL-4) at different effector/target (E:T) ratios (Fig. 7). This demonstrates that utilization of NC-encapsulated antigen results in the generation of a stronger local cellular immune response than immunization with a free antigen.

Example 7: Addition of Free Adjuvant Augments Immune Response to NC without Adjuvant

Materials for Nanocarrier Formulations

Ovalbumin peptide 323-339 amide acetate salt was purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Product code 4065609.) PLA with an inherent viscosity of 0.19 dL/g was purchased from Boehringer Ingelheim (Ingelheim Germany. Product Code R202H). PLA-PEG-Nicotine with a nicotine-terminated PEG block of approximately 5,000 Da and DL-PLA block of approximately 17,000 Da was synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 17.5 mg/mL in dilute hydrochloric acid aqueous solution. The solution was prepared by dissolving ovalbumin peptide in 0.13N hydrochloric acid solution at room temperature.

Solution 2: 0.19-IV PLA @ 75 mg/mL and PLA-PEG-nicotine @ 25 mg/ml in dichloromethane. The solution was prepared by separately dissolving PLA @ 100 mg/mL in dichloromethane and PLA-PEG-nicotine @ 100 mg/mL in dichloromethane, then mixing the solutions by adding 3 parts PLA solution for each part of PLA-PEG-nicotine solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM pH 8 phosphate buffer.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.1 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (2.0 mL) to the primary emulsion and then sonicating at 30% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion was then added to an open 50 mL beaker containing 70mM pH 8 phosphate buffer solution (30 mL) and stirred at room temperature for 2 hours to evaporate dichloromethane and to form nanocarriers in aqueous suspension. A portion of the nanocarriers was washed by transferring the suspension to a centrifuge tube and spinning at 13,800g for one hour, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. The washing procedure was repeated, and the pellet was re-suspended in phosphate buffered saline for a final nanocarrier dispersion of about 10 mg/mL. The amounts of oligonucleotide and peptide in the nanocarrier were determined by HPLC analysis. The total dry-nanocarrier mass per mL of suspension was determined by a gravimetric method and was adjusted to 5 mg/mL.

Table 12: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier	Effective Diameter (nm)	TLR Agonist, % w/w	T-cell helper peptide, % w/w
	211	None	Ova 323-339, 0.7

Results

Mice were immunized with NC-Nic (nanocarrier exhibiting nicotine on the outer surface and containing OP-II helper peptide, no adjuvant in the NC) admixed with CpG in either the phosphodiester (PO) or phosphorothioate (PS) form. The PO form is degraded by nucleases and, therefore, is not stable once injected into mice. The PS form is nuclease-resistant and, therefore, stable once injected into mice. As a negative control, mice were immunized with PBS only. Groups of five mice were immunized three times (subcutaneously, hind limbs) at 2-week intervals (days 0, 14 and 28) with 100 μg of NC-Nic + 20 μg of CpG (PS or PO) or PBS. Serum anti-nicotine antibody titers were measured on days 26 and 40. Anti-nicotine antibody titers (EC_{50}) were measured by ELISA against polylysine-nicotine (Fig. 8) (group 1: NC-Nic (no adjuvant) + free CpG (PS); group 2: NC-Nic (no adjuvant) + free CpG (PO); group 3: PBS only). This demonstrates that admixing of free CpG (PS) (Th1 adjuvant, TLR9 agonist) to antigen-carrying NCs generates an immune response which is superior to those induced by NC with admixed CpG (PO) or with PBS (group 1 > group 2 > group 3).

Example 8: Addition of Free Adjuvant Augments Immune Response to NC without Adjuvant

Materials for Nanocarrier Formulations

Ovalbumin protein was purchased from Worthington Biochemical Corporation (730 Vassar Avenue, Lakewood, NJ 08701. Product Code 3048.) PLA with an inherent viscosity of 0.21 dL/g was purchased from SurModics Pharmaceuticals (756 Tom Martin Drive, Birmingham, AL 35211. Product Code 100 DL 2A.) PLA-PEG-OMe block co-polymer with a methyl ether terminated PEG block of approximately 2,000 Da and PLA block of approximately 19,000 Da was synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin protein @ 20mg/mL was prepared in phosphate buffered saline at room temperature.

Solution 2: PLA @ 75 mg/mL and PLA-PEG-OMe @ 25 mg/mL in dichloromethane was prepared by dissolving PLA at 100 mg/mL in dichloromethane and PLA-PEG-OMe at 100 mg/mL in dichloromethane, then combining 3 parts of the PLA solution to 1 part of the PLA-PEG-OMe solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in 100mM phosphate buffer, pH 8.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.2 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (3.0 mL) to the primary emulsion, vortexing to create a course dispersion, and then sonicating at 30% amplitude for 60 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to an open 50 mL beaker containing 70 mM phosphate buffer solution (30 mL) and stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and the nanocarriers to form in suspension. A portion of the suspended nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube, spinning at 21,000 rcf for 45 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in phosphate buffered saline to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 13: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier	Effective Diameter (nm)	TLR Agonist, % w/w	Antigen, % w/w
NC-OVA	228	None	OVA protein, 2.8

Results

Mice were immunized with NC-OVA (nanocarrier exhibiting ovalbumin (OVA) on the outer surface, no adjuvant in the NC) admixed with either 20 μg of R848 or CpG (PS; nuclease-resistant). Control mice received 2.5 μg of soluble antigen (OVA) admixed with 20 μg of CpG (PS). Groups of five mice were immunized three times (subcutaneously, hind limbs) at 2-week intervals (days 0, 14 and 28) with 100 μg of NC-OVA + 20 μg of R848 or CpG (PS) or 2.5 μg of soluble OVA + 20 μg of CpG (PS). Serum anti-OVA antibody titers were measured on days 26 and 44. Anti-OVA antibody titers (EC_{50}) were measured by ELISA against OVA protein (Fig. 9) (group 1: NC-OVA (no adjuvant) + free R848; group 2: NC-OVA (no adjuvant) + free CpG (PS); group 3: soluble OVA + CpG (PS)). This demonstrates that admixing of free R848 (Th1 adjuvant, TLR7/8 agonist) or CpG (PS) (Th1 adjuvant, TLR9 agonist) to antigen-carrying NCs generates an immune response, which is superior to those induced by soluble antigen admixed with adjuvant (CpG (PS)) (groups 1 and 2 > group 3).

Example 9: Addition of Free Adjuvant Augments Immune Response to NC with Adjuvant

Materials for Group 1 Nanocarrier Formulations

Ovalbumin peptide 323-339 amide acetate salt was purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Part # 4065609.) PLGA-R848 conjugate of 75/25 lactide/glycolide monomer composition and of approximately 4100 Da molecular weight having 5.2% w/w R848 content was synthesized. PLA-PEG-Nicotine with a nicotine-terminated PEG block of approximately 3,500 Da and DL-PLA block of approximately 15,000 Da was synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for Group 1 Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 70mg/mL was prepared in 0.13N hydrochloric acid at room temperature.

Solution 2: PLGA-R848 @ 75 mg/mL and PLA-PEG-Nicotine @ 25 mg/mL in dichloromethane was prepared by dissolving PLGA-R848 at 100 mg/mL in dichloromethane

and PLA-PEG-Nicotine at 100 mg/mL in dichloromethane, then combining 3 parts of the PLGA-R848 solution to 1 part of the PLA-PEG-Nicotine solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in 100mM phosphate buffer, pH 8.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.1 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (2.0 mL) to the primary emulsion and sonicating at 30% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to an open 50 mL beaker containing 70 mM phosphate buffer solution (30 mL) and stirred at room temperature for 2 hours to allow for the dichloromethane to evaporate and for the nanocarriers to form in suspension. A portion of the suspended nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube, spinning at 13800 rcf for 60 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in phosphate buffered saline to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 14: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier	Effective Diameter (nm)	TLR Agonist, % w/w	T-cell helper peptide, % w/w
Group 1 NC	213	R848, 2.6	Ova 323-339, 0.9

Materials for Group 2 Nanocarrier Formulations

Ovalbumin peptide 323-339 amide acetate salt was purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Part # 4065609.) PLGA with 73% lactide and 27% glycolide content and an inherent viscosity of 0.12 dL/g was purchased from SurModics Pharmaceuticals (756 Tom Martin Drive, Birmingham, AL 35211. Product Code 7525 DLG 1A.) PLA-PEG-Nicotine with a nicotine-terminated PEG block of approximately 3,500 Da and DL-PLA block of approximately 15,000 Da was synthesized. Polyvinyl

alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) was purchased from J.T. Baker (Part Number U232-08).

Methods for Group 2 Nanocarrier Production

Solutions were prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 70mg/mL was prepared in 0.13N hydrochloric acid at room temperature.

Solution 2: PLGA @ 75 mg/mL and PLA-PEG-Nicotine @ 25 mg/mL in dichloromethane was prepared by dissolving PLGA at 100 mg/mL in dichloromethane and PLA-PEG-Nicotine at 100 mg/mL in dichloromethane, then combining 3 parts of the PLGA solution to 1 part of the PLA-PEG-Nicotine solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in 100mM phosphate buffer, pH 8.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion was first created using Solution 1 & Solution 2. Solution 1 (0.1 mL) and Solution 2 (1.0 mL) were combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion was then formed by adding Solution 3 (2.0 mL) to the primary emulsion and sonicating at 30% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion was added to an open 50 mL beaker containing 70 mM phosphate buffer solution (30 mL) and stirred at room temperature for 2 hours to allow the dichloromethane to evaporate and the nanocarriers to form in suspension. A portion of the suspended nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube, spinning at 13800 rcf for 60 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure was repeated, and then the pellet was re-suspended in phosphate buffered saline to achieve a nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension was stored frozen at -20°C until use.

Table 15: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier	Effective Diameter (nm)	TLR Agonist, % w/w	T-cell helper peptide, % w/w

Group 2 NC	176	None	Ova 323-339, 1.1

Mice were injected with 20 μg of CpG twice (subcutaneously, hind limbs) at 2-week intervals (days 0 and 14). At days 35 and 49, mice were immunized with 100 μg of NC-Nic (containing 2.6% R848 and 0.9% OP-II peptide) or 100 μg of NC-Nic (containing 1.1% OP-II peptide only). Serum anti-nicotine antibody titers were measured at days 12, 26, and 40 after immunization with NC. Anti-nicotine antibody titers (EC_{50}) were measured by ELISA against polylysine-nicotine (Fig. 10 (group 1: NC-Nic (R848 + OP-II); group 2: NC-Nic (OP-II only)). This demonstrates that mice immunized with a combination of CpG followed at a later date by NC-Nic that contain R848 generate higher antibody titers to nicotine than mice immunized with CpG followed at a later date by NC-Nic that do not contain R848 (group 1 > group 2).

Example 10: Addition of Two Free Adjuvants Augments Immune Response to NC-Nic (Prophetic)

Materials for NC-Nic Nanocarrier Formulations

Ovalbumin peptide 323-339 amide acetate salt is purchased from Bachem Americas Inc. (3132 Kashiwa Street, Torrance CA 90505. Part # 4064565.) PLA with an inherent viscosity of 0.19 dL/g is purchased from SurModics Pharmaceuticals (756 Tom Martin Drive, Birmingham, AL 35211 (Product Code 100 DL 2A). PLA-PEG-Nicotine with a nicotine-terminated PEG block of approximately 5,000 Da and DL-PLA block of approximately 20,000 Da is synthesized. Polyvinyl alcohol (Mw = 11,000 – 31,000, 87-89% hydrolyzed) is purchased from J.T. Baker (Part Number U232-08).

Methods for NC-Nic Nanocarrier Production

Solutions are prepared as follows:

Solution 1: Ovalbumin peptide 323 – 339 @ 20mg/mL is prepared in 0.13N hydrochloric acid at room temperature.

Solution 2: PLA @ 75 mg/mL and PLA-PEG-Nicotine @ 25mg/mL in dichloromethane is prepared by dissolving PLA @ 100 mg/mL in dichloromethane and PLA-PEG-Nicotine at 100mg/mL in dichloromethane, then combining 3 parts of the PLA solution to 1 part of the PLA-PEG-Nicotine solution.

Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM in deionized water.

Solution 4: 70 mM phosphate buffer, pH 8.

A primary (W1/O) emulsion is first created using Solution 1 & Solution 2. Solution 1 (0.2 mL) and Solution 2 (1.0 mL) are combined in a small glass pressure tube and sonicated at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary (W1/O/W2) emulsion is then formed by adding Solution 3 (2.0 mL) to the primary emulsion and sonicating at 30% amplitude for 40 seconds using the Branson Digital Sonifier 250. The secondary emulsion is added to a beaker containing 70 mM phosphate buffer solution (30 mL) in an open 50ml beaker and stirred at room temperature for 2 hours to allow for the dichloromethane to evaporate and for the nanocarriers to form in suspension. A portion of the suspended nanocarriers are washed by transferring the nanocarrier suspension to centrifuge tubes, spinning at 21,000 rcf for 45 minutes, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. This washing procedure is repeated, and then the pellet is re-suspended in phosphate buffered saline to achieve nanocarrier suspension having a nominal concentration of 10 mg/mL on a polymer basis. The suspension is stored frozen at -20°C until use.

Table 16: Characterization of the Nanocarriers Produced According to the Above

Nanocarrier	Effective Diameter (nm)	TLR Agonist, % w/w	T-cell helper peptide, % w/w
	200	None	Ova 323-339, 1.5

Results

Mice are immunized with NC-Nic (nanocarrier exhibiting nicotine on the outer surface) admixed with a first (R848) and second adjuvant (alum). Groups of five mice are immunized three times (subcutaneously, hind limbs) at 2-week intervals (days 0, 14 and 28) with 100 μg of NC-Nic. Serum anti-nicotine antibodies are then measured on days 26 and 40. EC_{50} for anti-nicotine antibodies are measured by standard ELISA against polylysine-nicotine.

CLAIMS

What is claimed is:

1. A composition comprising:
a dosage form that comprises
(1) a population of synthetic nanocarriers,
(2) a first adjuvant that is not coupled to any synthetic nanocarriers, and
(3) a pharmaceutically acceptable excipient.

2. The composition of claim 1, wherein the composition comprises a systemic dose of the first adjuvant.

3. The composition of claim 1, wherein the composition further comprises a second adjuvant.

4. The composition of claim 3, wherein the first adjuvant and second adjuvant are different.

5. The composition of claim 3 or 4, wherein the composition comprises a systemic dose of the first adjuvant and/or second adjuvant.

6. The composition of claim 2 or 5, wherein the systemic dose results in the systemic release of TNF-α, IL-6 and/or IL-12.

7. The composition of claim 2 or 5, wherein the systemic dose results in the systemic release of IFN-γ, IL-12 and/or IL-18.

8. The composition of any of claims 3-7, wherein the second adjuvant is coupled to the synthetic nanocarriers.

9. The composition of any of claims 3-7, wherein the second adjuvant is not coupled to any synthetic nanocarriers.

10. The composition of any of claims 3-7, wherein the second adjuvant is coupled to another population of synthetic nanocarriers.

11. The composition of any of claims 1-10, further comprising one or more antigens.

12. The composition of claim 11, wherein the one or more antigens are coupled to the synthetic nanocarriers.

13. The composition of claim 11, wherein the one or more antigens are coupled to another population of synthetic nanocarriers.

14. The composition of claim 11, wherein the one or more antigens are not coupled to any synthetic nanocarriers.

15. The composition of any of claims 11-14, wherein the one or more antigens comprise a B cell antigen and/or a T cell antigen.

16. The composition of claim 15, wherein the T cell antigen is a T helper cell antigen.

17. The composition of any of claims 11-14, wherein the one or more antigens comprise a B cell antigen and/or a T cell antigen and a T helper cell antigen.

18. The composition of any of claims 1-10, wherein the composition does not comprise an antigen.

19. The composition of any of claims 1-18, wherein the first adjuvant and/or second adjuvant comprises a mineral salt, gel-type adjuvant, a microbial adjuvant, an oil-emulsion or emulsifier-based adjuvant, a particulate adjuvant, a synthetic adjuvant, a phosphate adjuvant, a polymer, a liposome, a microcarrier, an immunostimulatory nucleic acid, alum, a saponin, an interleukin, an interferon, a cytokine, a toll-like receptor (TLR) agonist, an

imidazoquinoline, a cytokine receptor agonist, a CD40 agonist, an Fc receptor agonist, a complement receptor agonist, QS21, vitamin E, squalene, tocopherol, Quil A, ISCOMs, ISCOMATRIX, Ribi Detox, CRL-1005, L-121, tetrachlorodecaoxide, alum, MF59, AS02, AS15, cholera toxin, monophosphoryl lipid A, incomplete Freund's adjuvant, complete Freund's adjuvant, muramyl dipeptide or montanide.

20. The composition of claim 19, wherein the immunostimulatory nucleic acid comprises a CpG-containing nucleic acid.

21. The composition of claim 19, wherein the imidazoquinoline comprises resiquimod or imiquimod.

22. The composition of claim 19, wherein the first and/or second adjuvant comprises alum.

23. The composition of claim 20, wherein when the first adjuvant comprises a CpG-containing nucleic acid, the second adjuvant comprises an imidazoquinoline or alum.

24. The composition of claim 23, wherein the imidazoquinoline is resiquimod.

25. The composition of claim 19, wherein when the first adjuvant comprises an imidazoquinoline, the second adjuvant comprises a CpG-containing nucleic acid or alum.

26. The composition of claim 25, wherein the imidazoquinoline is resiquimod.

27. The composition of claim 22, wherein when the first adjuvant comprises alum, the second adjuvant comprises an imidazoquinoline or a CpG-containing nucleic acid.

28. The composition of claim 27, wherein the imidazoquinoline is resiquimod.

29. The composition of claim 19, wherein the TLR agonist comprises a TLR-1, TLR-2, TLR-3, TLR-4, TLR-5, TLR-6, TLR-7, TLR-8, TLR-9, TLR-10, TLR-11 agonist or a combination thereof.

30. The composition of any of claims 1-18, wherein the first adjuvant and/or second adjuvant does not comprise a TLR agonist.

31. The composition of any of claims 1-18, wherein the first adjuvant and/or second adjuvant does not comprise a TLR-3, TLR-7, TLR-8 or TLR-9 agonist.

32. The composition of any of claims 1-31, wherein the synthetic nanocarriers comprise lipid nanoparticles, polymeric nanoparticles, metallic nanoparticles, surfactant-based emulsions, dendrimers, buckyballs, nanowires, virus-like particles, peptide or protein particles, nanoparticles that comprise a combination of nanomaterials, spheroidal nanoparticles, cuboidal nanoparticles, pyramidal nanoparticles, oblong nanoparticles, cylindrical nanoparticles or toroidal nanoparticles.

33. The composition of claim 32, wherein the second adjuvant is coupled to the synthetic nanocarriers and comprises resiquimod.

34. The composition of claim 33, wherein the one or more antigens comprise nicotine and a T-helper cell antigen, each of which are coupled to the synthetic nanocarriers.

35. The composition of claim 34, wherein the T-helper cell antigen comprises a peptide obtained or derived from ovalbumin.

36. The composition of claim 35, wherein the peptide obtained or derived from ovalbumin comprises the sequence as set forth in SEQ ID NO: 1.

37. The composition of any of claims 15-17 and 34-36, wherein the T helper cell antigen is coupled by encapsulation.

38. The composition of any of claims 32-37, wherein the synthetic nanocarriers comprise one or more polymers.

39. The composition of claim 38, wherein the one or more polymers comprise a polyester.

40. The composition of claim 38 or 39, wherein the one or more polymers comprise or further comprise a polyester coupled to a hydrophilic polymer.

41. The composition of claim 39 or 40, wherein the polyester comprises a poly(lactic acid), poly(glycolic acid), poly(lactic-co-glycolic acid), or polycaprolactone.

42. The composition of claim 40 or 41, wherein the hydrophilic polymer comprises a polyether.

43. The composition of claim 42, wherein the polyether comprises polyethylene glycol.

44. A method comprising:

administering the composition of any of claims 1-43 to a subject.

45. The method of claim 44, wherein the subject is a human.

46. A method comprising:

administering the composition of claim 1 and a second adjuvant to a subject, wherein the second adjuvant is administered at a time different from the administration of the composition.

47. The method of claim 46, wherein the subject is a human.

48. The method of claim 46 or 47, wherein the composition and second adjuvant are coadministered.

49. The method of claim 46 or 47, wherein the composition and second adjuvant are not coadministered.

50. The method of any of claims 46-49, wherein the second adjuvant is administered prior to the composition.

51. The method of any of claims 46-50, wherein the second adjuvant is not coupled to any synthetic nanocarriers.

52. The method of any of claims 46-50, wherein the second adjuvant is coupled to another population of synthetic nanocarriers.

53. The method of any of claims 44-52, wherein the method further comprises administering one or more antigens.

54. The method of claim 53, wherein the composition further comprises one or more antigens.

55. The method of claim 54, wherein the one or more antigens are coupled to the synthetic nanocarriers.

56. The method of claim 54, wherein the one or more antigens are coupled to another population of synthetic nanocarriers.

57. The method of claim 54, wherein the one or more antigens are not coupled to any synthetic nanocarriers.

58. The method of claim 53, 56 or 57, wherein the one or more antigens are coadministered.

59. The method of any of claims 53-58, wherein the one or more antigens comprise a B cell antigen and/or a T cell antigen.

60. The method of claim 59, wherein the T cell antigen is a T helper cell antigen.

61. The method of any of claims 53-58, wherein the one or more antigens comprise a B cell antigen and/or a T cell antigen and a T helper cell antigen.

62. The method of any of claims 59-61, wherein the B cell antigen is nicotine.

63. The method of any of claims 60-62, wherein the T helper cell antigen comprises a peptide obtained or derived from ovalbumin.

64. The method of claim 63, wherein the peptide obtained or derived from ovalbumin comprises the sequence as set forth in SEQ ID NO: 1.

65. The method of any of claims 60-64, wherein the T helper cell antigen is coupled by encapsulation.

66. The method of any of claims 44-65, wherein the synthetic nanocarriers comprise one or more polymers.

67. The method of claim 66, wherein the one or more polymers comprise a polyester.

68. The method of claim 66 or 67, wherein the one or more polymers comprise or further comprise a polyester coupled to a hydrophilic polymer.

69. The method of claim 67 or 68, wherein the polyester comprises a poly(lactic acid), poly(glycolic acid), poly(lactic-co-glycolic acid), or polycaprolactone.

70. The method of claim 68 or 69, wherein the hydrophilic polymer comprises a polyether.

71. The method of claim 70, wherein the polyether comprises polyethylene glycol.

72. The method of any of claims 44-71, wherein the subject is in need of an inflammatory response.

73. The method of any of claims 44-71, wherein the subject is in need of a Th1 immune response.

74. The method of any of claims 44-71, wherein the subject is in need of a humoral immune response.

75. The method of any of claims 44-71, wherein the subject is in need of a specific local cytotoxic T lymphocyte response.

76. The method of any of claims 44-75, wherein the subject has or is at risk of having cancer.

77. The method of any of claims 44-75, wherein the subject has or is at risk of having an infection or infectious disease.

78. The method of any of claims 44-71 and 73, the subject has or is at risk of having an atopic condition, asthma, COPD or a chronic infection.

79. The composition of any one of claims 1-43 for use in therapy or prophylaxis.

80. The composition of any one of claims 1-43 for use in a method as defined in any one of claims 44-78.

81. The composition of any one of claims 1-43 for use in a method of inducing an inflammatory response in a subject.

82. The composition of any one of claims 1-43 for use in a method of inducing a Th1 immune response in a subject.

83. The composition of any one of claims 1-43 for use in a method of inducing a humoral immune response in a subject.

84. The composition of any one of claims 1-43 for use in a method of inducing a specific local cytotoxic T lymphocyte response in a subject.

85. The composition of any one of claims 1-43 for use in a method of treating or preventing cancer.

86. The composition of any one of claims 1-43 for use in a method of treating or preventing infection or infectious disease.

87. The composition of any one of claims 1-43 for use in a method of treating or preventing an atopic condition, asthma, COPD or a chronic infection.

88. Use of the composition of any one of claims 1-43 for the manufacture of a medicament for use in a method as defined in any one of claims 44-78 or 81-87.

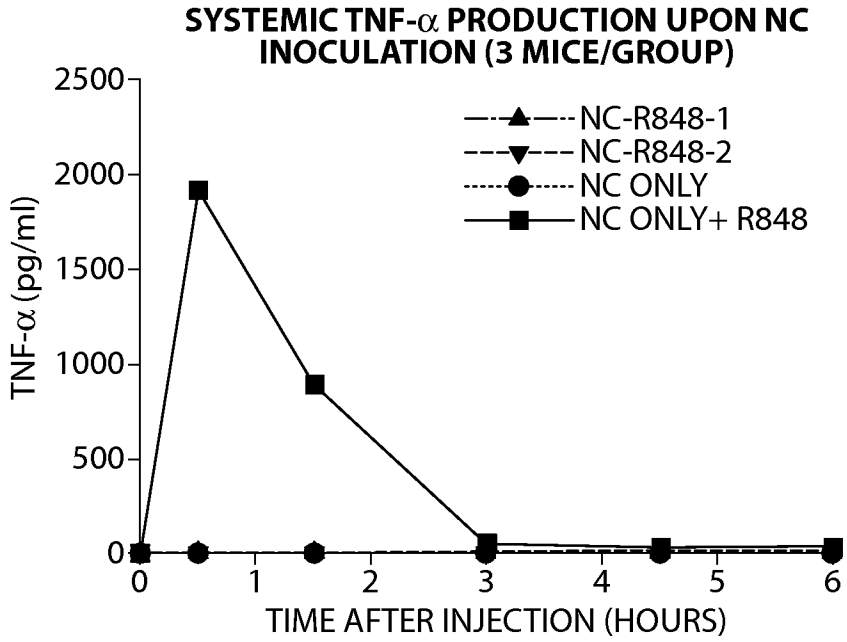
SYSTEMIC TNF-α PRODUCTION UPON NC INOCULATION (3 MICE/GROUP)
TNF-α (pg/ml)
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6
TIME AFTER INJECTION (HOURS)
NC-R848-1
NC-R848-2
NC ONLY
NC ONLY+ R848

Fig. 1A

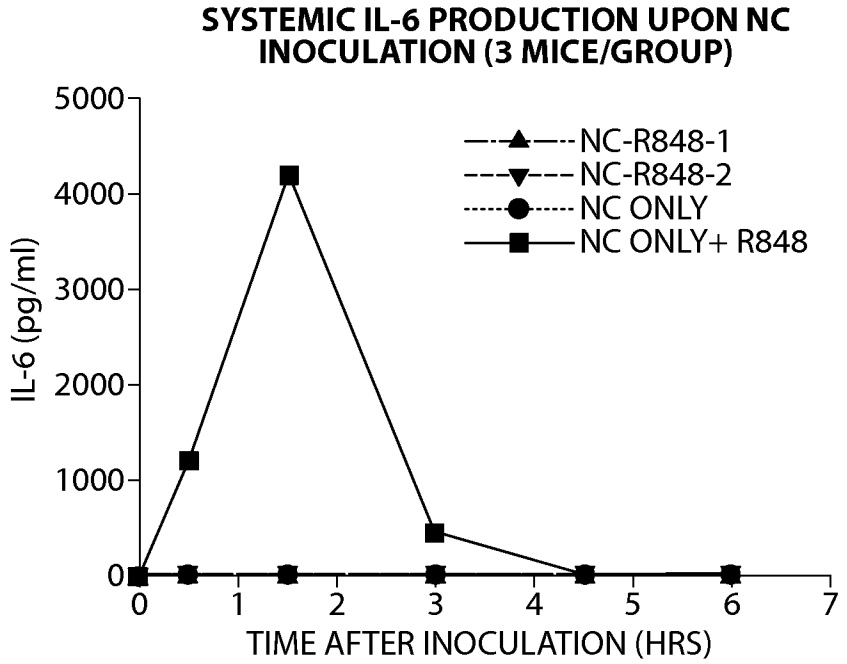
SYSTEMIC IL-6 PRODUCTION UPON NC INOCULATION (3 MICE/GROUP)
IL-6 (pg/ml)
5000
4000
3000
2000
1000
0
0 1 2 3 4 5 6 7
TIME AFTER INOCULATION (HRS)
NC-R848-1
NC-R848-2
NC ONLY
NC ONLY+ R848

Fig. 1B

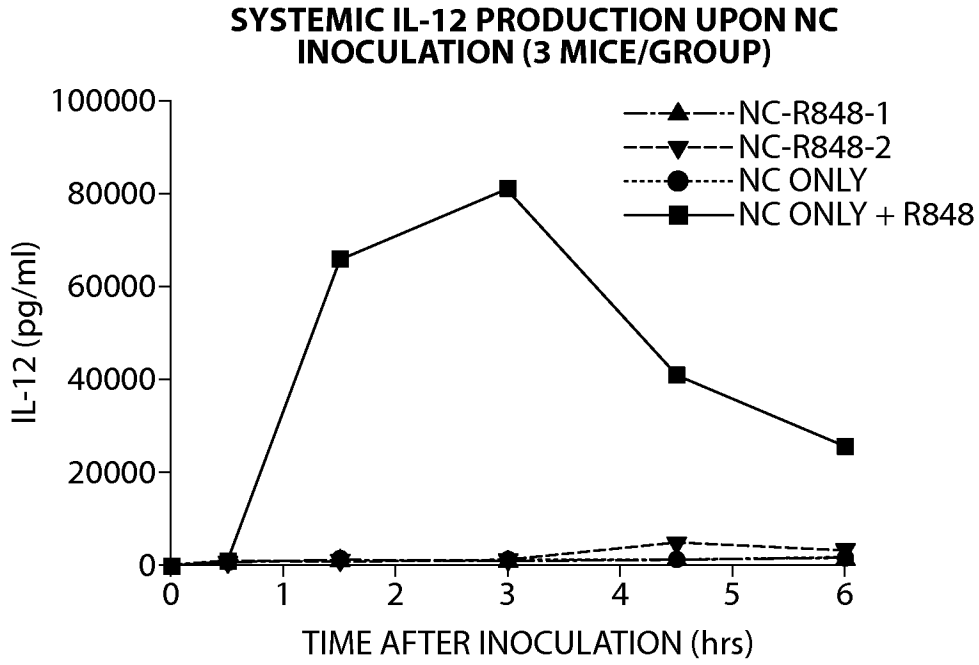
SYSTEMIC IL-12 PRODUCTION UPON NC INOCULATION (3 MICE/GROUP)
IL-12 (pg/ml)
100000
80000
60000
40000
20000
0
0
1
2
3
4
5
6
TIME AFTER INOCULATION (hrs)
NC-R848-1
NC-R848-2
NC ONLY
NC ONLY + R848

Fig. 1C

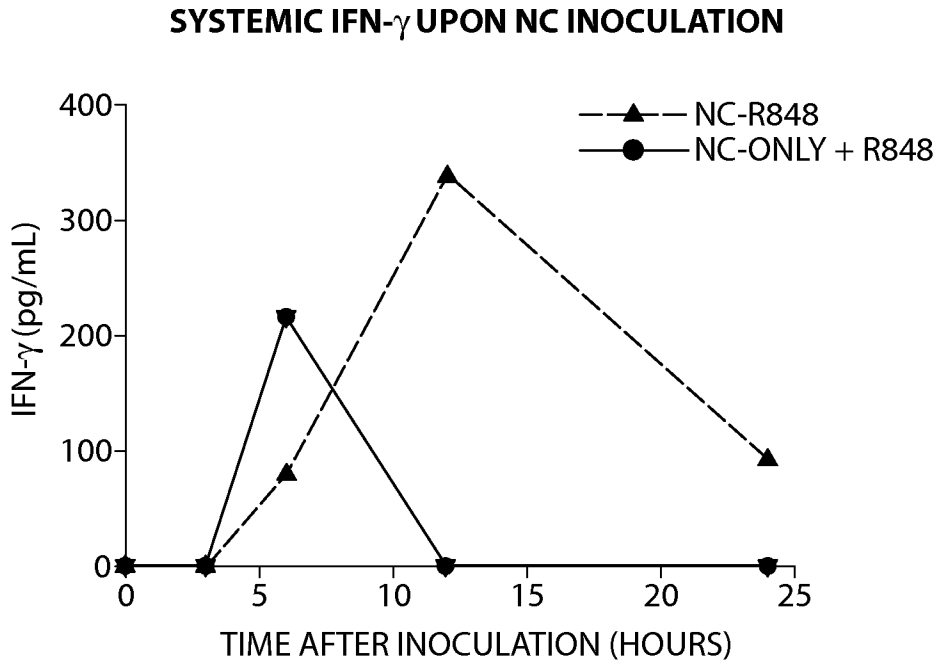
SYSTEMIC IFN-γ UPON NC INOCULATION
NC-R848
NC-ONLY + R848
IFN-γ (pg/mL)
400
300
200
100
0
0
5
10
15
20
25
TIME AFTER INOCULATION (HOURS)

Fig. 2

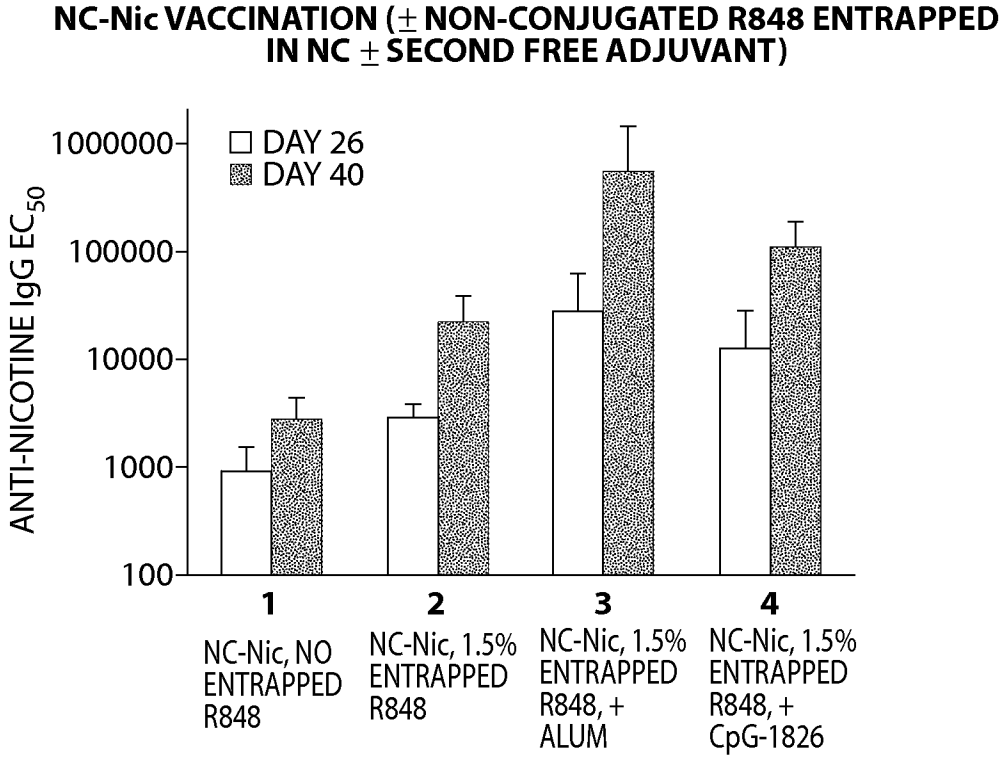
NC-Nic VACCINATION (± NON-CONJUGATED R848 ENTRAPPED IN NC ± SECOND FREE ADJUVANT)
DAY 26
DAY 40
ANTI-NICOTINE IgG EC50
1000000
100000
10000
1000
100
1
NC-Nic, NO ENTRAPPED R848
2
NC-Nic, 1.5% ENTRAPPED R848
3
NC-Nic, 1.5% ENTRAPPED R848, + ALUM
4
NC-Nic, 1.5% ENTRAPPED R848, + CpG-1826

Fig. 3

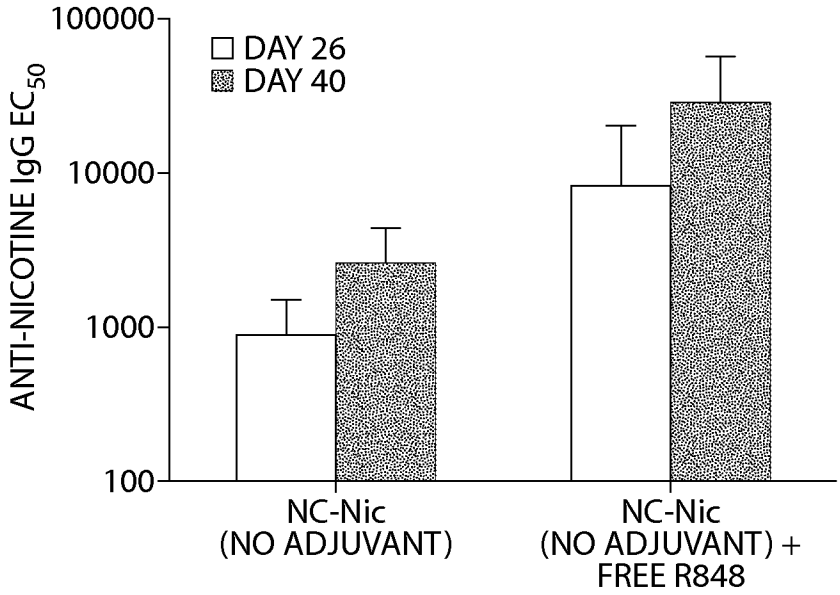
100000
10000
1000
100
ANTI-NICOTINE IgG EC50
DAY 26
DAY 40
NC-Nic
(NO ADJUVANT)
NC-Nic
(NO ADJUVANT) +
FREE R848

Fig. 4

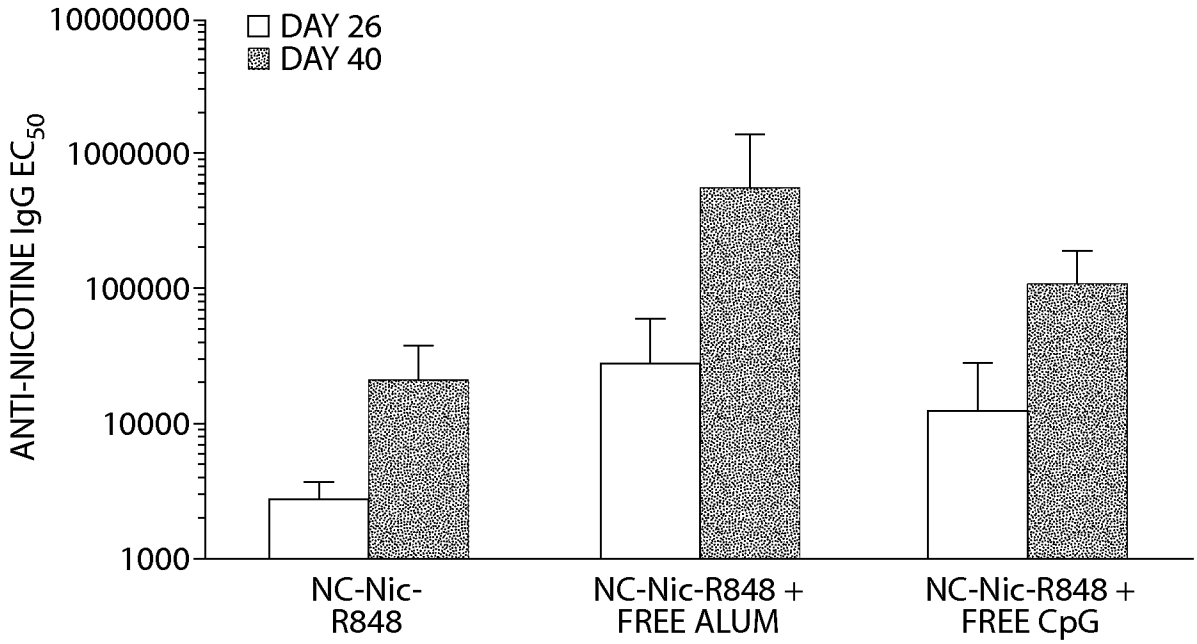
10000000
1000000
100000
10000
1000
ANTI-NICOTINE IgG EC50
DAY 26
DAY 40
NC-Nic-
R848
NC-Nic-R848 +
FREE ALUM
NC-Nic-R848 +
FREE CpG

Fig. 5

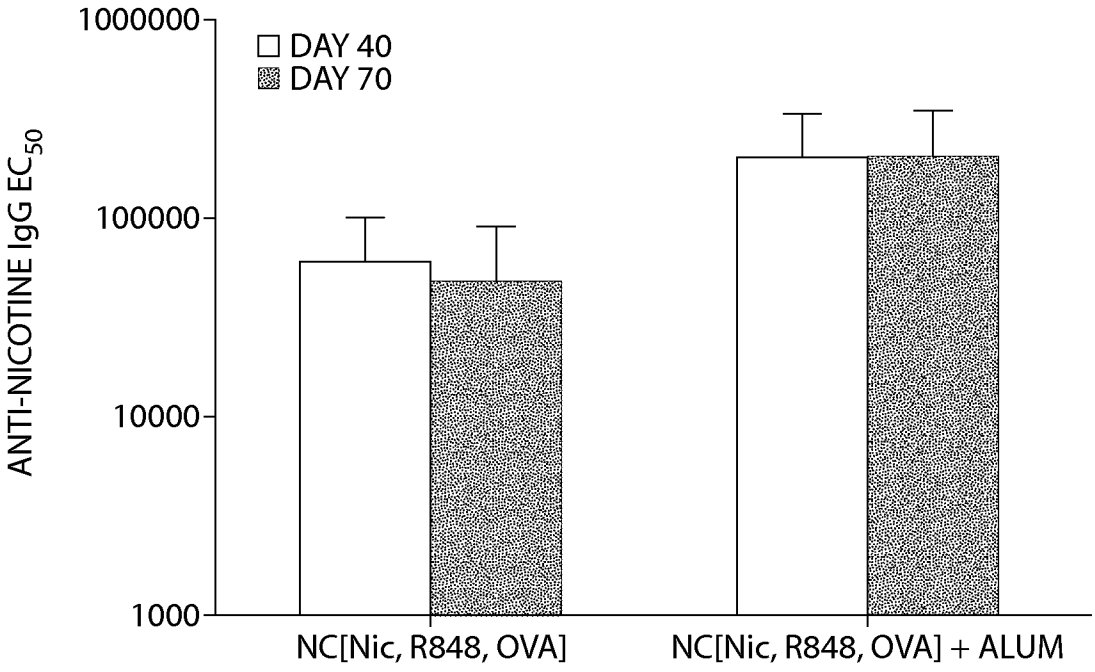
1000000
100000
10000
1000
DAY 40
DAY 70
ANTI-NICOTINE IgG EC50
NC[Nic, R848, OVA]
NC[Nic, R848, OVA] + ALUM

Fig. 6

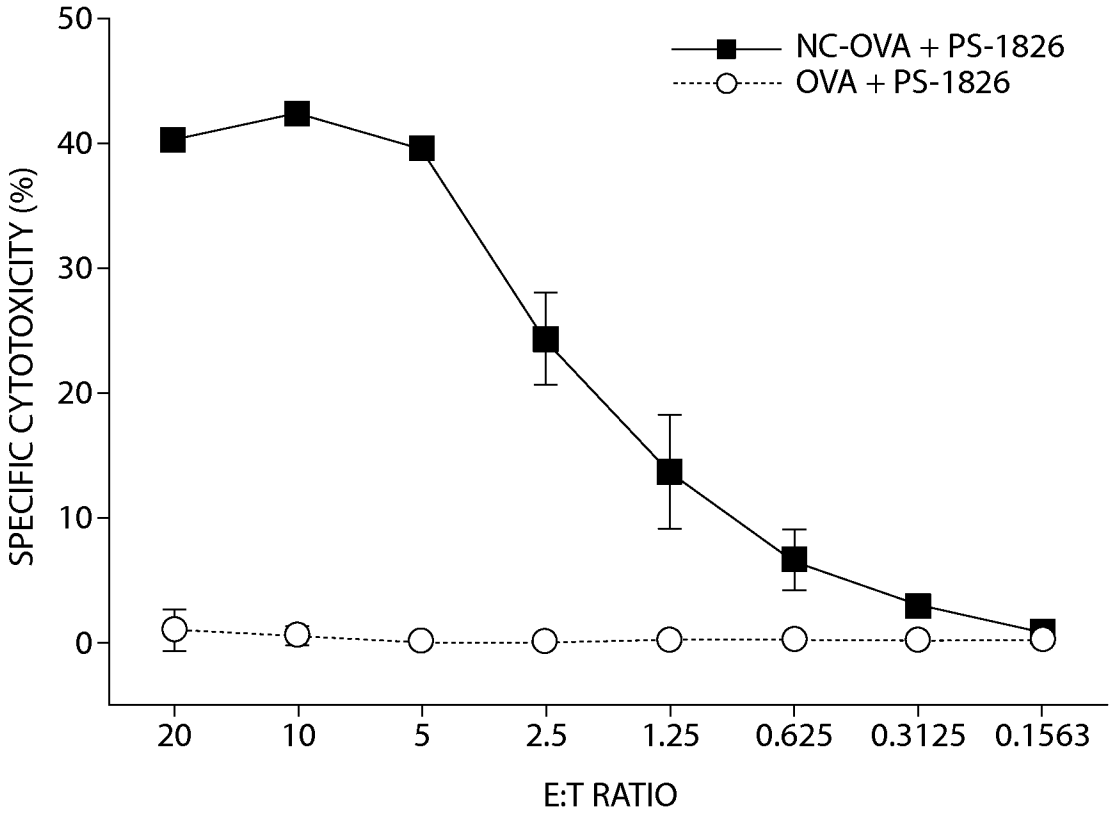
50
40
30
20
10
0
NC-OVA + PS-1826
OVA + PS-1826
SPECIFIC CYTOTOXICITY (%)
20
10
5
2.5
1.25
0.625
0.3125
0.1563
E:T RATIO

Fig. 7

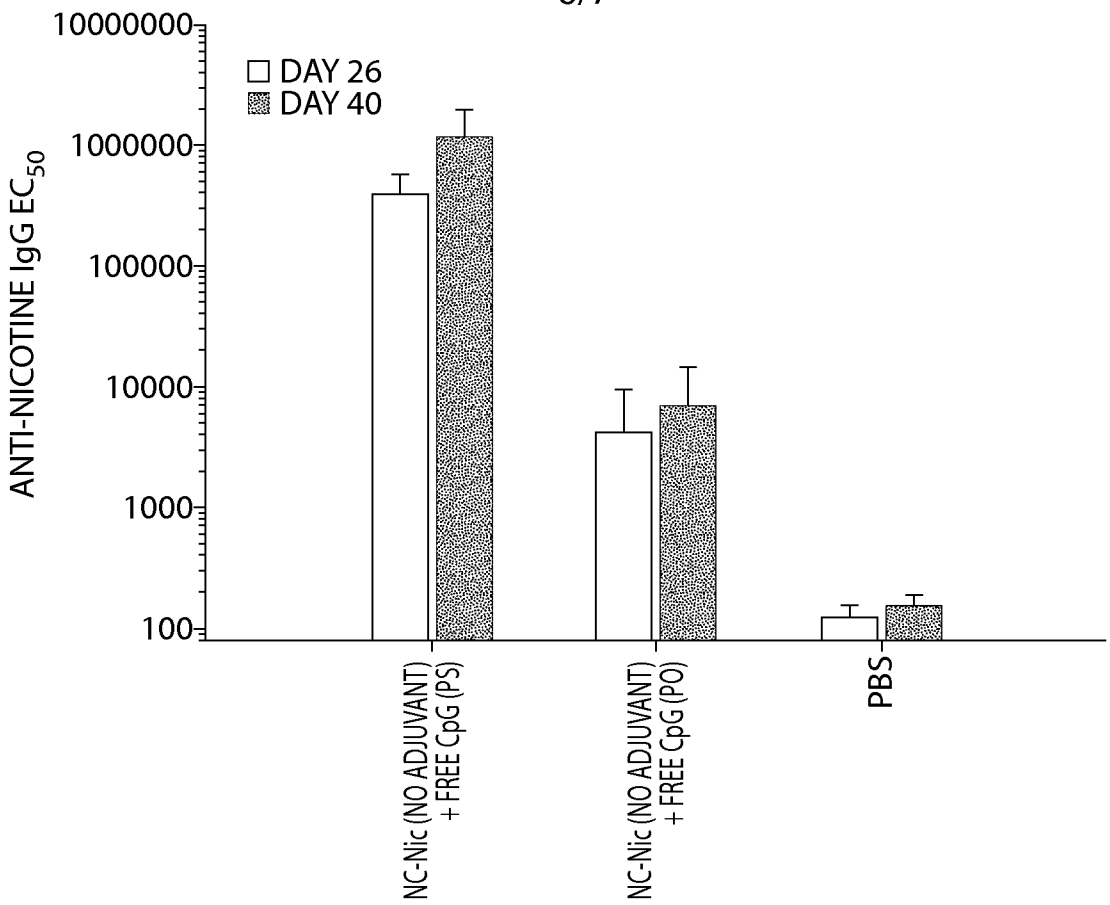
10000000
1000000
100000
10000
1000
100
ANTI-NICOTINE IgG EC50
DAY 26
DAY 40
NC-Nic (NO ADJUVANT) + FREE CpG (PS)
NC-Nic (NO ADJUVANT) + FREE CpG (PO)
PBS

Fig. 8

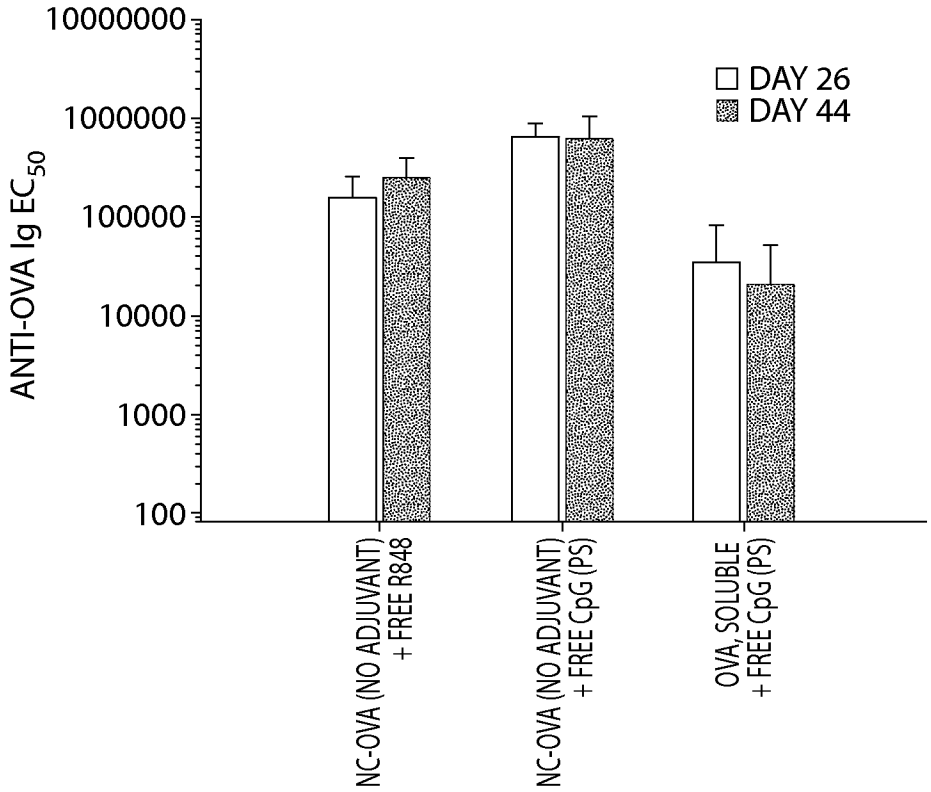
10000000
1000000
100000
10000
1000
100
ANTI-OVA Ig EC50
DAY 26
DAY 44
NC-OVA (NO ADJUVANT) + FREE R848
NC-OVA (NO ADJUVANT) + FREE CpG (PS)
OVA, SOLUBLE + FREE CpG (PS)

Fig. 9

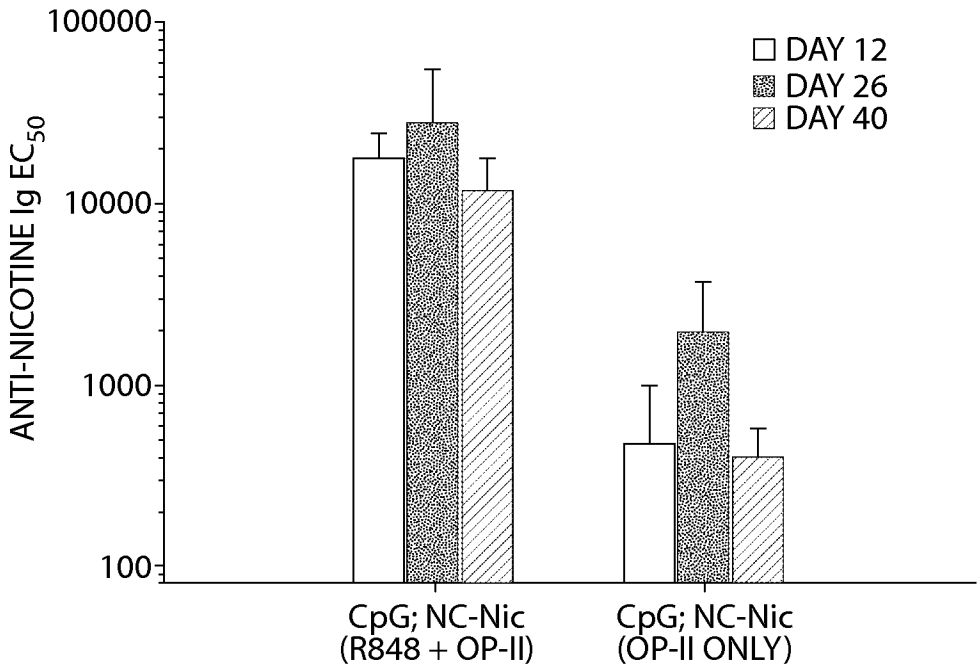
100000
10000
1000
100
ANTI-NICOTINE Ig EC50
DAY 12
DAY 26
DAY 40
CpG; NC-Nic
(R848 + OP-II)
CpG; NC-Nic
(OP-II ONLY)

Fig. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/38190

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 45/00, 47/00 (2011.01)
USPC - 977/917, 773; 424/278.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 977/917, 773; 424/278.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 977/917, 773; 424/278.1, 184.1 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases : PubWEST (PGPB, USPT, EPAB, JPAB); Google Scholar
Search terms: Nanocarrier, nanoparticle, vaccine, vaccine nanotechnology, composition, adjuvant, second adjuvant, immunostimulatory agent, imidazoquinoline, R848, alum, CpG, Freund's,administ$6

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	WO 2009/051837 A2 (VON ANDRIAN et al.) 23 April 2009 (23.04.2009). Especially claims 54, 55, 58, 66; para [0033], [00066]. [0095], [00119], [00289], [00290], [00456], [00459], [00460], [00561], [00562], [00566], sheet 48 fig 28B.	1-5 -------- 46-49
Y	WO 2004/098509 A2 (O'HAGAN et al.) 18 November 2004 (18.11.2004). Especially para [00016], [00070], [00118].	46-49

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 August 2011 (11.08.2011)	26 AUG 2011
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/38190

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-45 and 50-88
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.