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(57) Abstract:

METHODS AND COMPOSITIONS FOR ENHANCING CD4+ REGULATORY T CELLS

RELATED APPLICATIONS

5 This application claims the benefit under 35 U.S.C. §119 of United States provisional applications 61/819517, filed May 3, 2013; 61/881851, filed September 24, 2013; 61/881913, filed September 24, 2013; 61/881921, filed September 24, 2013; 61/907177, filed November 21, 2013; 61/948313, filed March 5, 2014; and 61/948384, filed March 5, 2014, the entire contents of each of which are incorporated herein by reference.

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FIELD OF THE INVENTION

This invention relates to administering immunosuppressants and therapeutic macromolecules for enhancing CD4+ regulatory T cells, such as those specific to the therapeutic macromolecules. The methods and compositions provided herein allow for a 15 shift to tolerogenic immune response development, in particular CD4+ regulatory T cell production or development. Accordingly, the methods and compositions provided can be used to generate tolerogenic immune responses in a subject in which the administration of a therapeutic macromolecule may result in undesired immune responses. The method and compositions are preferably used for subjects that would benefit from the enhancement of 20 CD4+ regulatory T cells.

BACKGROUND OF THE INVENTION

Therapeutic treatments, such as protein or enzyme replacement therapies, often result in undesired immune responses to the particular therapeutic. Such undesired immune 25 responses may be reduced through the use of immunosuppressant drugs. Conventional immunosuppressant drugs, however, are broad-acting. Additionally, in order to maintain immunosuppression, immunosuppressant drug therapy is generally a life-long proposition. Unfortunately, the use of broad-acting immunosuppressants are associated with a risk of severe side effects, such as tumors, infections, nephrotoxicity and metabolic disorders. 30 Accordingly, new tolerogenic therapies would be beneficial.

SUMMARY OF THE INVENTION

In one aspect, a method comprising enhancing the number or percentage (or ratio) of CD4+ regulatory T cells, such as those specific to a therapeutic macromolecule, by administering to a subject synthetic nanocarriers attached to immunosuppressants, and 5 therapeutic macromolecules, wherein the therapeutic macromolecules are not co-formulated with the synthetic nanocarriers attached to immunosuppessants prior to administration, is provided.

In one embodiment of any one of the methods provided herein, the synthetic nanocarriers attached to immunosuppressants and therapeutic macromolecules are 10 administered concomitantly to the subject.

In another embodiment of any one of the methods provided herein, the administration is according to a protocol previously demonstrated to result in enhanced numbers or percentage (or ratio) of CD4+ regulatory T cells when the therapeutic macromolecules are not co-formulated with the synthetic nanocarriers prior to administration. In another 15 embodiment of any one of the methods provided herein, the method further comprises determining the protocol.

In another embodiment of any one of the methods provided herein, the method further comprises assessing the the number or percentage (or ratio) of CD4+ regulatory T cells in the subject prior to and/or after the administration.

20 In another embodiment of any one of the methods provided herein, the enhanced number or percentage (or ratio) of CD4+ regulatory T cells is an increase of at least 2-fold, 3-fold, 4-fold, 5-fold or 6-fold as compared to the number or percentage of CD4+ regulatory T cells prior to the administration.

In another embodiment of any one of the methods provided herein, the administering 25 is by intravenous, intraperitoneal or subcutaneous administration.

In another embodiment of any one of the methods provided herein, the method further comprises recording an increase in the number or percentage (or ratio) of CD4+ regulatory T cells following the administration.

In another embodiment of any one of the methods provided herein, the 30 immunosuppressant comprises a statin, an mTOR inhibitor, a TGF-β signaling agent, a corticosteroid, an inhibitor of mitochondrial function, a P38 inhibitor, an NF-κB inhibitor, an adenosine receptor agonist, a prostaglandin E2 agonist, a phosphodiesterase 4 inhibitor, an

HDAC inhibitor or a proteasome inhibitor. In another embodiment of any one of the methods provided herein, the mTOR inhibitor is rapamycin.

In another embodiment of any one of the methods provided herein, the therapeutic macromolecule is a therapeutic protein or a therapeutic polynucleotide. In another
5 embodiment of any one of the methods provided herein, the therapeutic protein is for protein replacement of protein supplementation therapy. In another embodiment of any one of the methods provided herein, the therapeutic protein comprises a/an infusible or injectable therapeutic protein, enzyme, enzyme cofactor, hormone, blood or blood coagulation factor, cytokine, interferon, growth factor, monoclonal antibody, polyclonal antibody, or protein
10 associated with Pompe's disease. In another embodiment of any one of the methods provided herein, the infusible or injectable therapeutic protein comprises Tocilizumab, alpha-1 antitrypsin, Hematide, albinterferon alfa-2b, Thucin, tesamorelin, ocrelizumab, belimumab, pegloticase, taliglucerase alfa, agalsidase alfa, or velaglucerase alfa. In another embodiment of any one of the methods provided herein, the enzyme comprises an ocidforeductase,
15 transferase, hydrolase, lysase, isomerase or ligase. In another embodiment of any one of the methods provided herein, the enzyme comprises an enzyme for enzyme replacement therapy for a lysosomal storage disorder. In another embodiment of any one of the methods provided herein, the enzyme for replacement therapy for a lysosomal storage disorder comprises imiglucerase, a-galactosidase A (a-gal A), agalsidase beta, acid α-glucosidase (GAA),
20 alglucosidase alfa, LUMIZYME, MYOZYME, arylsulfatase B, laronidase, ALDURAZYME, idursulfase, ELAPRASE, arylsulfatase B, pegloticase, pegsiticase or NAGLAZYME. In another embodiment of any one of the methods provided herein, the cytokine comprises a lymphokine, interleukin, chemokine, type 1 cytokine or a type 2 cytokine. In another embodiment of any one of the methods provided herein, the blood or blood coagulation factor
25 comprises Factor I, Factor II, tissue factor, Factor V, Factor VII, Factor VIII , Factor IX, Factor X, Factor Xa, Factor XII, Factor XIII, von Willebrand factor, prekallikrein, high-molecular weight kininogen, fibronectin, antithrombin III, heparin cofactor II, protein C, protein S, protein Z, protein Z-related protease inhibitor (ZPI), plasminogen, alpha 2-antiplasmin, tissue plasminogen activator (tPA), urokinase, plasminogen activator inhibitor-1
30 (PAI1), plasminogen activator inhibitor-2 (PAI2), cancer procoagulant or epoetin alfa.

In another embodiment of any one of the methods provided herein, a load of immunosuppressant attached to the synthetic nanocarriers, on average across the synthetic

nanocarriers, is between 0.1% and 50%. In another embodiment of any one of the methods provided herein, the load is between 0.1% and 20%.

In another embodiment of any one of the methods provided herein, the synthetic nanocarriers comprise lipid nanoparticles, polymeric nanoparticles, metallic nanoparticles, surfactant-based emulsions, dendrimers, buckyballs, nanowires, virus-like particles or peptide or protein particles. In another embodiment of any one of the methods provided herein, the synthetic nanocarriers comprise lipid nanoparticles. In another embodiment of any one of the methods provided herein, the synthetic nanocarriers comprise liposomes. In another embodiment of any one of the methods provided herein, the synthetic nanocarriers comprise metallic nanoparticles. In another embodiment of any one of the methods provided herein, the metallic nanoparticles comprise gold nanoparticles. In another embodiment of any one of the methods provided herein, the synthetic nanocarriers comprise polymeric nanoparticles. In another embodiment of any one of the methods provided herein, the polymeric nanoparticles comprise polymer that is a non-methoxy-terminated, pluronic polymer. In another embodiment of any one of the methods provided herein, the polymeric nanoparticles comprise a polyester, polyester attached to a polyether, polyamino acid, polycarbonate, polyacetal, polyketal, polysaccharide, polyethyloxazoline or polyethyleneimine. In another embodiment of any one of the methods provided herein, the polyester comprises a poly(lactic acid), poly(glycolic acid), poly(lactic-co-glycolic acid) or polycaprolactone. In another embodiment of any one of the methods provided herein, the polymeric nanoparticles comprise a polyester and a polyester attached to a polyether. In another embodiment of any one of the methods provided herein, the polyether comprises polyethylene glycol or polypropylene glycol.

In another embodiment of any one of the methods provided herein, the mean of a particle size distribution obtained using dynamic light scattering of the synthetic nanocarriers is a diameter greater than 100nm. In another embodiment of any one of the methods provided herein, the diameter is greater than 150nm. In another embodiment of any one of the methods provided herein, the diameter is greater than 200nm. In another embodiment of any one of the methods provided herein, the diameter is greater than 250nm. In another embodiment of any one of the methods provided herein, the diameter is greater than 300nm.

In another embodiment of any one of the methods provided herein, an aspect ratio of the synthetic nanocarriers is greater than 1:1, 1:1.2, 1:1.5, 1:2, 1:3, 1:5, 1:7 or 1:10.

In another aspect, a method of manufacturing any one of the compositions or kits provided herein is provided. In one embodiment, the method of manufacturing comprises producing a dose or dosage form of a therapeutic macromolecule and producing a dose or dosage form of an immunosuppressant. In another embodiment of any one of the methods of manufacturing provided, the step of producing a dose or dosage form of an immunosuppressant comprises attaching the immunosuppressant to synthetic nanocarriers. In another embodiment of any one of the methods of manufacturing provided, the method further comprises combining the dose or dosage form of the immunosuppressant and dose or dosage form of the therapeutic macromolecule in a kit. In another embodiment of any one of the methods of manufacture provided herein, the therapeutic macromolecules are not co-formulated with the immunosuppessants.

In another aspect, a use of any one of the compositions or kits provided herein for the manufacture of a medicament for enhancing the number or percentage (or ratio) of CD4+ regulatory T cells, such as therapeutic macromolecule-specific CD4+ regulatory T cells, in a subject is provided. In one embodiment, the composition or kit comprises an immunosuppressant and a therapeutic macromolecule, wherein the immunosuppressant and therapeutic macromolecule are not co-formulated. In another embodiment of any one of the uses provided herein, the immunosuppressant is attached to synthetic nanocarriers.

In another aspect, any one of the compositions provided herein is provided for use in any one of the methods provided herein. In one embodiment, the method comprises administering to a subject immunosuppressants and therapeutic macromolecules, wherein the therapeutic macromolecules are not co-formulated with the immunosuppessants prior to administration. In another embodiment, the immunosuppressant is attached to synthetic nanocarriers. In yet another embodiment, the administration is concomitant administration.

In another aspect, a method of manufacturing a medicament intended for enhancing the number or percentage (or ratio) of CD4+ regulatory T cells, such as therapeutic macromolecule-specific CD4+ regulatory T cells, is provided. In one embodiment, the medicament comprises an immunosuppressant and a therapeutic macromolecule, wherein the immunosuppressant and therapeutic macromolecule are not co-formulated. In another embodiment of any one of the methods of manufacturing provided herein, the immunosuppressant is attached to synthetic nanocarriers.

BRIEF DESCRIPTION OF FIGURES

Fig. 1 shows the percentage of CD4+ and CD25+Fox3p+ (regulatory T cells) as assessed by flow cytometry following administration of the indicated treatments.

Fig. 2 shows the reduction in antibody formation to polyethylene glycol (PEG) following administration of the indicated treatments.

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 or a mixture of differing molecular weights of a single polymer species, reference to "a synthetic nanocarrier" includes a mixture of two or more such synthetic nanocarriers or a plurality of such synthetic nanocarriers, reference to “a RNA molecule” includes a mixture of two or more such RNA molecules or a plurality of such RNA molecules, reference to "an immunosuppressant" includes a mixture of two or more such materials or a plurality of such immunosuppressant molecules, and the like.

As used herein, the term “comprise” or variations thereof such as “comprises” or “comprising” are to be read to indicate the inclusion of any recited integer (e.g. a feature, element, characteristic, property, method/process step or limitation) or group of integers (e.g. features, element, characteristics, properties, method/process steps or limitations) but not the exclusion of any other integer or group of integers. Thus, as used herein, the term “comprising” is inclusive and does not exclude additional, unrecited integers or method/process steps.

In embodiments of any one of the compositions and methods provided herein, “comprising” may be replaced with “consisting essentially of” or “consisting of”. The phrase “consisting essentially of” is used herein to require the specified integer(s) or steps as well as

those which do not materially affect the character or function of the claimed invention. As used herein, the term "consisting" is used to indicate the presence of the recited integer (e.g. a feature, element, characteristic, property, method/process step or limitation) or group of integers (e.g. features, element, characteristics, properties, method/process steps or
5 limitations) alone.

A. INTRODUCTION

As previously mentioned, current conventional immunosuppressants are broad-acting and generally result in an overall systemic downregulation of the immune system. The
10 methods and compositions provided herein allow for more targeted immune effects and, in particular, surprisingly, the enhancement in the production of CD4+ regulatory T cells, such as therapeutic macromolecule-specific CD4+ regulatory T cells. It has been found that an enhanced number or percentage (or ratio) of therapeutic macromolecule-specific CD4+ regulatory T cells can be achieved by practicing the methods described, or administering the
15 compositions provided herein. Accordingly, such methods and compositions can result in a decrease in undesired immune responses associated with administration of therapeutic macromolecules and/or can be beneficial for subjects in need of treatment with therapeutic macromolecules.

The inventors have unexpectedly and surprisingly discovered that the problems and
20 limitations noted above can be overcome by practicing the invention disclosed herein. The present invention is illustrated in the Examples below.

The invention will now be described in more detail below.

B. DEFINITIONS

25 "Administering" or "administration" or "administer" means providing a material to a subject in a manner that is pharmacologically useful. The term is intended to include "causing to be administered " in some embodiments. "Causing to be administered" means causing, urging, encouraging, aiding, inducing or directing, directly or indirectly, another party to administer the material.

30 "Amount effective" in the context of a composition or dosage form for administration to a subject refers to an amount of the composition or dosage form that produces one or more desired immune responses in the subject, for example, the generation of a tolerogenic immune response, such as enhancement in the production or development of CD4+

regulatory T cells, such as those specific to a therapeutic macromolecule. Therefore, in some embodiments, an amount effective is the amount of a composition provided herein that produces one or more desired immune responses, such as an increase in the number or percentage (or ratio) of CD4+ regulatory T cells. The amount effective can be for *in vitro* or
5 *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 that may experience undesired immune responses as a result of administration of a therapeutic macromolecule.

Amounts effective can involve reducing the level of an undesired immune response, although in some embodiments, it involves preventing an undesired immune response
10 altogether. Amounts effective can also involve delaying the occurrence of an undesired immune response. An amount that is effective can also be an amount of a composition provided herein that produces an increase in the production or development of CD4+ regulatory T cells, such as therapeutic macromolecule-specific CD4+ regulatory T cells. Specifically, the increase in the production or development can be an increase in the number
15 of percentage (or ratio) of such cells. An amount effective can also be an amount that results in a desired therapeutic endpoint or a desired therapeutic result. Amounts effective, preferably, result in a tolerogenic immune response in a subject to an antigen, such as a therapeutic macromolecule. The achievement of any of the foregoing can be monitored by routine methods.

20 In some embodiments of any one of the compositions and methods provided, the amount effective is one in which the desired immune response persists in the subject for at least 1 week, at least 2 weeks or at least 1 month. In other embodiments of any one of the compositions and methods provided, the amount effective is one which produces a measurable desired immune response, for example, a measurable decrease in an immune
25 response (e.g., to a specific antigen), for at least 1 week, at least 2 weeks or at least 1 month.

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
30 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 reason.

In general, doses of the immunosuppressants and/or therapeutic macromolecules in the compositions of the invention refer to the amount of the immunosuppressants and/or therapeutic macromolecules. Alternatively, the dose can be administered based on the number of synthetic nanocarriers that provide the desired amount of immunosuppressants and/or antigens.

"Antigen-specific" refers to any immune response that results from the presence of the antigen, or portion thereof, or that generates molecules that specifically recognize or bind the antigen. For example, where the immune response is antigen-specific antibody production, antibodies are produced that specifically bind the antigen. As another example, the immune response is the production of CD4+ regulatory T cells, which may be CD4+regulatory T cells that bind to a therapeutic macromolecule antigen-presenting cell (APC) presentable antigen when presented by an APC.

"Assessing an immune response" refers to any measurement or determination of the level, presence or absence, reduction, increase in, etc. of an immune response *in vitro* or *in vivo*. Such measurements or determinations may be performed on one or more samples obtained from a subject. Such assessing can be performed with any of the methods provided herein or otherwise known in the art. The assessing may be assessing the number or percentage of CD4+ regulatory T cells, such as those specific to a therapeutic macromolecule, such as in a sample from a subject.

"Attach" or "Attached" or "Couple" or "Coupled" (and the like) means to chemically associate one entity (for example a moiety) with another. In some embodiments, the attaching is covalent, meaning that the attachment occurs in the context of the presence of a covalent bond between the two entities. In non-covalent embodiments, the non-covalent attaching 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 attaching. In embodiments, therapeutic macromolecules and immunosuppressants are not attached to one another, meaning that the therapeutic macromolecules and immunosuppressants are not subjected to a process specifically intended to chemically

associate one with another. In embodiments, therapeutic macromolecules and/or immunosuppressants are not attached to synthetic nanocarriers, meaning that the therapeutic macromolecules (and/or immunosuppressants) and synthetic nanocarriers are not subjected to a process specifically intended to chemically associate one with another.

5 "Average", as used herein, refers to the arithmetic mean unless otherwise noted.

"Co-formulated" means that the indicated materials are processed so as to produce a filled and finished pharmaceutical dosage form wherein the materials are in intimate physical contact or are chemically attached covalently or non-covalently. As used herein, "not co-formulated" means that the indicated materials (*e.g.*, therapeutic macromolecules and
10 immunosuppressants (or synthetic nanocarriers attached to the immunosuppressants)) are not in intimate physical contact and are not chemically attached. In some embodiments, the therapeutic macromolecules and immunosuppressants (or synthetic nanocarriers attached to the immunosuppressants) as described herein are not co-formulated prior to administration to a subject.

15 "Combination", as applied to two or more materials and/or agents (also referred to herein as the *components*), is intended to define material in which the two or more materials/agents are associated. Components may be separately identified, e.g. first component, second component, third component, etc. The terms "combined" and "combining" in this context are to be interpreted accordingly.

20 The association of the two or more materials /agents in a combination may be physical or non-physical. Examples of physically associated combined materials/agents include:

- compositions (*e.g.* unitary formulations) comprising the two or more materials/agents in admixture (for example within the same unit dose);
- 25 compositions comprising material in which the two or more materials/agents are chemically/physicochemically linked (for example by crosslinking, molecular agglomeration or binding to a common vehicle moiety);
- compositions comprising material in which the two or more materials/agents are chemically/physicochemically co-packaged (for example, disposed on or within lipid
30 vesicles, particles (*e.g.* micro- or nanoparticles) or emulsion droplets);
- pharmaceutical kits, pharmaceutical packs or patient packs in which the two or more materials/agents are co-packaged or co-presented (*e.g.* as part of an array of unit doses);

Examples of non-physically associated combined materials/agents include:

- material (*e.g.* a non-unitary formulation) comprising at least one of the two or more materials/agents together with instructions for the extemporaneous association of the at least one compound/agent to form a physical association of the two or more materials/agents;
- material (*e.g.* a non-unitary formulation) comprising at least one of the two or more materials/agents together with instructions for combination therapy with the two or more materials/agents;
- material comprising at least one of the two or more materials/agents together with instructions for administration to a patient population in which the other(s) of the two or more materials/agents have been (or are being) administered;
- material comprising at least one of the two or more materials/agents in an amount or in a form which is specifically adapted for use in combination with the other(s) of the two or more materials/agents.

As used herein, the term "combination therapy" is intended to define therapies which comprise the use of a combination of two or more materials/agents (as defined above). Thus, references to "combination therapy", "combinations" and the use of materials/agents "in combination" in this application may refer to materials/agents that are administered as part of the same overall treatment regimen. As such, the posology of each of the two or more materials/agents may differ: each may be administered at the same time or at different times. It will therefore be appreciated that the materials/agents of the combination may be administered sequentially (e.g. before or after) or simultaneously, either in the same pharmaceutical formulation (i.e. together), or in different pharmaceutical formulations (*i.e.* separately). Simultaneously in the same formulation is as a unitary formulation whereas simultaneously in different pharmaceutical formulations is non-unitary. The posologies of each of the two or more materials/agents in a combination therapy may also differ with respect to the route of administration.

"Concomitantly" means administering two or more materials/agents 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, and even more preferably the two or more materials/agents are administered in combination. In embodiments, concomitant

administration may encompass administration of two or more materials/agents 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. In embodiments, the materials/agents may be repeatedly administered concomitantly; that is concomitant administration on more than one occasion, such as provided in the Examples.

"Determining" or "determine" means to ascertain a factual relationship. Determining may be accomplished in a number of ways, including but not limited to performing experiments, or making projections. For instance, a dose of an immunosuppressant or therapeutic macromolecule may be determined by starting with a test dose and using known scaling techniques (such as allometric or isometric scaling) to determine the dose for administration. Such may also be used to determine a protocol as provided herein. In another embodiment, the dose may be determined by testing various doses in a subject, i.e. through direct experimentation based on experience and guiding data. In embodiments, "determining" or "determine" comprises "causing to be determined." "Causing to be determined" means causing, urging, encouraging, aiding, inducing or directing or acting in coordination with an entity for the entity to ascertain a factual relationship; including directly or indirectly, or expressly or impliedly.

"Dosage form" means a pharmacologically and/or immunologically active material in a medium, carrier, vehicle, or device suitable for administration to a subject. Any one of the compositions or doses provided herein may be in a dosage form.

"Dose" refers to a specific quantity of a pharmacologically and/or immunologically active material for administration to a subject for a given time.

"Encapsulate" means to enclose at least a portion of a substance within a synthetic nanocarrier. In some embodiments, a substance is enclosed completely within a synthetic nanocarrier. In other embodiments, most or all of a substance that is encapsulated is not exposed to the local environment external to the synthetic nanocarrier. In other embodiments, no more than 50%, 40%, 30%, 20%, 10% or 5% (weight/weight) is exposed to the local environment. 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.

"Enhancing the number or percentage of CD4+ regulatory T cells" refers to increasing the number or percentage (or ratio) (of the total number of a type of cells, such as the total number of T cells or CD4+ T cells) of said cells in a subject or subjects, as determined by

taking samples from a subject or subjects and then assaying the samples using appropriate test methods. In some embodiments, by practicing the methods provided herein or following administration of the compositions described herein, the percentage of CD4+ regulatory T cells, such as those specific to a therapeutic macromolecule, increases by at least 2-, 3-, 4-, 5-, or 6-fold or more.

CD4+ regulatory T cells can be characterized as CD4+CD25+Fox3p+ cells. The number or percentage of CD4+ regulatory T cells can be assessed by any method described herein or known in the art. For example, the CD4+ regulatory T cells in the peripheral blood of a subject can be quantified by obtaining a sample of peripheral blood from the subject, assessing the gene expression, protein presence, and/or localization of one or more molecules associated with CD4+ regulatory T cells, including without limitation CD25, Foxp3, CCR4, CCR8, CCR5, CTLA4, CD134, CD39, and/or GITR. Any of the foremetioned molecules can be assessed by transcriptional analysis, such as quantitative RT-PCR, northern blotting, microarray, fluorescence *in situ* hybridization, or RNAseq; proteins can be detected by western blotting, immunofluorescence microscopy, flow cytometry, or ELISA. Cell surface molecules such as CD25, CCR4, CCR8, CCR5, CTLA4, CD134, CD39 and/or GITR can be evaluated by methods such as flow cytometry, cell surface staining, immunofluorescence microscopy, ELISAs, etc. In some embodiments, CD4+ regulatory T cells are detected based on an anergic phenotype (*e.g.*, lack of proliferation following TCR stimulation). In some embodiments, CD4+regulatory T cells are identified based on resistance to activation-induced cell death or sensitivity to death induced by cytokine deprivation. In some embodiments, CD4+ regulatory T cells can be identified based on the methylation state of the gene encoding Foxp3; for example, in CD4+ regulatory T cells, a portion of the Foxp3 gene has been found to be demethylated, which can be detected by DNA methylation analysis such as by PCR or other DNA-based methods. CD4+ regulatory T cells can be further identified or quantified based on the production of immunosuppressive cytokines including IL-9, IL-10, or TGF-β. Therapeutic macromolecule-specific CD4+ regulatory T cells can be identified and quantified by any method known in the art, for example, by stimulating cells *ex vivo* with an antigen-presenting cell loaded with an antigen derived from a therapeutic macromolecule and assessing activation of CD4+ regulatory T cells, or evaluating the T cell receptors of CD4+ regulatory T cells. The number or percentage (or ratio) of therapeutic macromolecule-specific CD4+ regulatory T cells can be indirectly quantified by assessing one or more function or activity of activated CD4+

regulatory T cells following administration of a therapeutic macromolecule or antigenic portion thereof.

"Generating" means causing an action, such as an immune response (e.g., a tolerogenic immune response) to occur, either directly oneself or indirectly.

5 "Identifying a subject" is any action or set of actions that allows a clinician to recognize a subject as one who may benefit from the methods, compositions or kits provided herein. Preferably, the identified subject is one who is in need of a tolerogenic immune response as provided herein, such as a subject in need of enhanced CD4+ regulatory T cell production or development, such as therapeutic macromolecule-specific CD4+ regulatory T 10 cell production or development. The action or set of actions may be either directly oneself or indirectly. In one embodiment of any one of the methods provided herein, the method further comprises identifying a subject in need of a method, composition or kit as provided herein.

"Immunosuppressant" means a compound that causes an APC to have an immunosuppressive effect (e.g., tolerogenic effect) or a T or B cell to be suppressed. An 15 immunosuppressive effect generally refers to the production or expression of cytokines or other factors by the APC that reduces, inhibits or prevents an undesired immune response or that promotes a desired immune response, such as a regulatory immune response (e.g., the production or development of CD4+ regulatory T cells). When the APC acquires an immunosuppressive function (under the immunosuppressive effect) on immune cells that 20 recognize an antigen presented by this APC, the immunosuppressive effect is said to be specific to the presented antigen. Without being bound by any particular theory, it is thought that the immunosuppressive effect is a result of the immunosuppressant being delivered to the APC, preferably in the presence of an antigen. In one embodiment, the immunosuppressant is one that causes an APC to promote a regulatory phenotype in one or more immune effector 25 cells. For example, the regulatory phenotype may be characterized by the inhibition of the production, induction, stimulation or recruitment of antigen-specific CD4+ T cells or B cells, the inhibition of the production of antigen-specific antibodies, the production, induction, stimulation or recruitment of Treg cells (e.g., CD4+CD25highFoxP3+ Treg cells), etc. This may be the result of the conversion of CD4+ T cells or B cells to a regulatory phenotype. 30 This may also be the result of induction of FoxP3 in other immune cells, such as CD8+ T cells, macrophages and iNKT cells. In one embodiment, the immunosuppressant is one that affects the response of the APC after it processes an antigen. In another embodiment, the immunosuppressant is not one that interferes with the processing of the antigen. In a further

embodiment, the immunosuppressant is not an apoptotic-signaling molecule. In another embodiment, the immunosuppressant is not a phospholipid.

Immunosuppressants include, but are not limited to, statins; mTOR inhibitors, such as rapamycin or a rapamycin analog; TGF-β signaling agents; TGF-β receptor agonists; histone deacetylase inhibitors, such as Trichostatin A; corticosteroids; inhibitors of mitochondrial function, such as rotenone; P38 inhibitors; NF-κβ inhibitors, such as 6Bio, Dexamethasone, TCPA-1, IKK VII; adenosine receptor agonists; prostaglandin E2 agonists (PGE2), such as Misoprostol; phosphodiesterase inhibitors, such as phosphodiesterase 4 inhibitor (PDE4), such as Rolipram; proteasome inhibitors; kinase inhibitors; G-protein coupled receptor agonists; G-protein coupled receptor antagonists; glucocorticoids; retinoids; cytokine inhibitors; cytokine receptor inhibitors; cytokine receptor activators; peroxisome proliferator-activated receptor antagonists; peroxisome proliferator-activated receptor agonists; histone deacetylase inhibitors; calcineurin inhibitors; phosphatase inhibitors; PI3KB inhibitors, such as TGX-221; autophagy inhibitors, such as 3-Methyladenine; aryl hydrocarbon receptor inhibitors; proteasome inhibitor I (PSI); and oxidized ATPs, such as P2X receptor blockers. Immunosuppressants also include IDO, vitamin D3, cyclosporins, such as cyclosporine A, aryl hydrocarbon receptor inhibitors, resveratrol, azathiopurine (Aza), 6-mercaptopurine (6-MP), 6-thioguanine (6-TG), FK506, sanglifehrin A, salmeterol, mycophenolate mofetil (MMF), aspirin and other COX inhibitors, niflumic acid, estriol and triptolide. In embodiments, the immunosuppressant may comprise any of the agents provided herein.

The immunosuppressant can be a compound that directly provides the immunosuppressive effect on APCs or it can be a compound that provides the immunosuppressive effect indirectly (i.e., after being processed in some way after administration). Immunosuppressants, therefore, include prodrug forms of any of the compounds provided herein.

In embodiments of any one of the methods, compositions or kits provided herein, the immunosuppressants provided herein are attached to synthetic nanocarriers. In preferable embodiments, the immunosuppressant is an element that is in addition to the material that makes up the structure of the synthetic nanocarrier. For example, in one embodiment, where the synthetic nanocarrier is made up of one or more polymers, the immunosuppressant is a compound that is in addition and attached to the one or more polymers. As another example, in one embodiment, where the synthetic nanocarrier is made up of one or more lipids, the immunosuppressant is again in addition and attached to the one or more lipids. In

embodiments, such as where the material of the synthetic nanocarrier also results in an immunosuppressive effect, the immunosuppressant is an element present in addition to the material of the synthetic nanocarrier that results in an immunosuppressive effect.

Other exemplary immunosuppressants include, but are not limited, small molecule drugs, natural products, antibodies (e.g., antibodies against CD20, CD3, CD4), biologics-based drugs, carbohydrate-based drugs, nanoparticles, liposomes, RNAi, antisense nucleic acids, aptamers, methotrexate, NSAIDs; fingolimod; natalizumab; alemtuzumab; anti-CD3; tacrolimus (FK506), etc. Further immunosuppressants, are known to those of skill in the art, and the invention is not limited in this respect.

In embodiments of any one of the methods, compositions or kits provided herein, the immunosuppressant is in a form, such as a nanocrystalline form, whereby the form of the immunosuppressant itself is a particle or particle-like. In embodiments, such forms mimic a virus or other foreign pathogen. Many drugs have been nanonized and appropriate methods for producing such drug forms would be known to one of ordinary skill in the art. Drug nanocrystals, such as nanocrystalline rapamycin, are known to those of ordinary skill in the art (Katteboinaa, et al. 2009, International Journal of PharmTech Resesarch; Vol. 1, No. 3; pp682-694. As used herein, a "drug nanocrystal" refers to a form of a drug (e.g., an immunosuppressant) that does not include a carrier or matrix material. In some embodiments, drug nanocrystals comprise 90%, 95%, 98%, or 99% or more drug. Methods for producing drug nanocrystals include, without limitation, milling, high pressure homogenization, precipitation, spray drying, rapid expansion of supercritical solution (RESS), Nanoedge® technology (Baxter Healthcare), and Nanocrystal Technology™ (Elan Corporation). In some embodiments, a surfactant or a stabilizer may be used for steric or electrostatic stability of the drug nanocrystal. In some embodiments, the nanocrystal or nanocrytalline form of an immunosuppressant may be used to increase the solubility, stability, and/or bioavailability of the immunosuppressant, particularly immunosuppressants that are insoluble or labile. In some embodiments, concomitantly administering a therapeutic macromolecule with an immunosuppressant in nanocrystalline form results in enhancing the number or percentage (or ratio) of CD4+ regulatory T cells, such as those specific to a therapeutic macromolecule.

"Load", when attached to a synthetic nanocarrier, is the amount of the immunosuppressant and/or therapeutic macromolecule attached to the synthetic nanocarrier based on the total dry recipe weight of materials in an entire synthetic nanocarrier

(weight/weight). Generally, such a load is calculated as an average across a population of synthetic nanocarriers. In one embodiment, the load on average across the synthetic nanocarriers is between 0.1% and 99%. In another embodiment, the load is between 0.1% and 50%. In another embodiment, the load is between 0.1% and 20%. In a further embodiment, the load is between 0.1% and 10%. In still a further embodiment, the load is between 1% and 10%. In still a further embodiment, the load is between 7% and 20%. In yet another embodiment, the load is at least 0.1%, at least 0.2%, at least 0.3%, at least 0.4%, at least 0.5%, at least 0.6%, at least 0.7%, at least 0.8%, at least 0.9%, at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, at least 12%, at least 13%, at least 14%, at least 15%, at least 16%, at least 17%, at least 18%, at least 19%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% on average across the population of synthetic nanocarriers. In yet a further embodiment, the load is 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% or 20% on average across the population of synthetic nanocarriers. In some embodiments of the above embodiments, the load is no more than 25% on average across a population of synthetic nanocarriers. In embodiments, the load is calculated as may be described in the Examples or as otherwise known in the art.

In some embodiments, when the form of the immunosuppressant is itself a particle or particle-like, such as a nanocrystalline immunosuppressant, the load of immunosuppressant is the amount of the immunosuppressant in the particles or the like (weight/weight). In such embodiments, the load can approach 97%, 98%, 99% or more.

"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 spheroidal 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 equal to or 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 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 10,000:1, more preferably from 1:1 to 1000:1, still more 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 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 equal to or greater than 100 nm, more preferably equal to or greater than 120 nm, 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 dimensions (e.g., effective diameter) may be obtained, in some embodiments, by suspending the synthetic nanocarriers in a liquid (usually aqueous) media and using dynamic light scattering (DLS) (e.g. using a Brookhaven ZetaPALS instrument). For example, a suspension of synthetic nanocarriers can be diluted from an aqueous buffer into purified water to achieve a final synthetic nanocarrier suspension concentration of approximately 0.01 to 0.1 mg/mL. The diluted suspension may be prepared directly inside, or transferred to, a suitable cuvette for DLS analysis. The cuvette may then be placed in the DLS, allowed to equilibrate to the controlled temperature, and then scanned for sufficient time to acquire a stable and reproducible distribution based on appropriate inputs for viscosity of the medium

and refractive indicies of the sample. The effective diameter, or mean of the distribution, is then reported. Determining the effective sizes of high aspect ratio, or non-spheroidal, synthetic nanocarriers may require augmentative techniques, such as electron microscopy, to obtain more accurate measurements. "Dimension" or "size" or "diameter" of synthetic
5 nanocarriers means the mean of a particle size distribution, for example, obtained using dynamic light scattering.

"Non-methoxy-terminated polymer" means a polymer that has at least one terminus that ends with a moiety other than methoxy. In some embodiments, the polymer has at least two termini that ends with a moiety other than methoxy. In other embodiments, the polymer
10 has no termini that ends with methoxy. "Non-methoxy-terminated, pluronic polymer" means a polymer other than a linear pluronic polymer with methoxy at both termini. Polymeric nanoparticles as provided herein can comprise non-methoxy-terminated polymers or non-methoxy-terminated, pluronic polymers.

"Pharmaceutically acceptable excipient" or "pharmaceutically acceptable carrier"
15 means a pharmacologically inactive material used together with a pharmacologically active material to formulate the compositions. Pharmaceutically acceptable 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.

20 "Protocol" means a pattern of administering to a subject and includes any dosing regimen of one or more substances to a subject. Protocols are made up of elements (or variables); thus a protocol comprises one or more elements. Such elements of the protocol can comprise dosing amounts, dosing frequency, routes of administration, dosing duration, dosing rates, interval between dosing, combinations of any of the foregoing, and the like. In
25 some embodiments, such a protocol may be used to administer one or more compositions of the invention to one or more test subjects. Immune responses in these test subjects can then be assessed to determine whether or not the protocol was effective in generating a desired or desired level of an immune response or therapeutic effect. Any therapeutic and/or immunologic effect may be assessed. One or more of the elements of a protocol may have
30 been previously demonstrated in test subjects, such as non-human subjects, and then translated into human protocols. For example, dosing amounts demonstrated in non-human subjects can be scaled as an element of a human protocol using established techniques such as alimetric scaling or other scaling methods. Whether or not a protocol had a desired effect

can be determined using any of the methods provided herein or otherwise known in the art. For example, a sample may be obtained from a subject to which a composition provided herein has been administered according to a specific protocol in order to determine whether or not specific immune cells, cytokines, antibodies, etc. were reduced, generated, activated, etc. In preferable embodiments, the number or percentage (or ratio) of CD4+ regulatory T cells, such as those therapeutic macromolecule-specific, is determined. An exemplary protocol is one previously demonstrated to result in enhanced numbers or percentage (or ratio) of CD4+ regulatory T cells when the therapeutic macromolecules are not co-formulated with the immunosuppressants (immunosuppressants attached to synthetic nanocarriers) prior to administration (as compared to the number or percentage of CD4+ regulatory T cells prior to the administration according to the protocol in the subject or one or more test subjects). Useful methods for detecting the presence and/or number of immune cells include, but are not limited to, flow cytometric methods (e.g., FACS), ELISpot, proliferation responses, cytokine production, and immunohistochemistry methods. Antibodies and other binding agents for specific staining of immune cell markers, are commercially available. Such kits typically include staining reagents for antigens that allow for FACS-based detection, separation and/or quantitation of a desired cell population from a heterogeneous population of cells. In embodiments, a number of compositions as provided herein are administered to another subject using one or more or all or substantially all of the elements of which the protocol is comprised. In some embodiments, the protocol has been demonstrated to result in the development or production of CD4+ regulatory T cells with the immunosuppressants, such as those therapeutic macromolecule-specific, and therapeutic macromolecules as provided herein.

"Providing" means an action or set of actions that an individual performs that supply a needed item or set of items or methods for practicing of the present invention. The action or set of actions may be taken either directly oneself or indirectly.

"Providing a subject" is any action or set of actions that causes a clinician to come in contact with a subject and administer a composition provided herein thereto or to perform a method provided herein thereupon. Preferably, the subject is one who is in need of antigen-specific tolerance or enhanced production or development of CD4+ regulatory T cells, such as those therapeutic macromolecule-specific. The action or set of actions may be taken either directly oneself or indirectly. In one embodiment of any one of the methods provided herein, the method further comprises providing a subject.

"Recording" means noting, or causing directly or indirectly activities in the expectation that such noting would take place, in any written or electronic form, that a method or composition provided herein achieved enhanced production or development of CD4+ regulatory T cells, such as those therapeutic macromolecule-specific. In some embodiments, the recording occurs when immunosuppressants in combination with therapeutic macromolecules are administered to a subject according to a method as provided herein or at some point thereafter. "Written form", as used herein, refers to any recordation on a medium such as paper. "Electronic form", as used herein, refers to any recordation on electronic media.

"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, synthetic nanocarriers do not comprise chitosan. In other embodiments, synthetic nanocarriers are not lipid-based nanoparticles. In further embodiments, synthetic nanocarriers do not comprise a phospholipid.

A synthetic nanocarrier can be, but is not limited to, one or a plurality of lipid-based nanoparticles (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. 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
5 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 attached virus-like particles disclosed in published US Patent Application 20060251677 to Bachmann et al., (9)
10 the virus-like particles disclosed in WO2010047839A1 or WO2009106999A2, (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), (11) apoptotic cells, apoptotic bodies or the synthetic or semisynthetic mimics disclosed in U.S. Publication 2002/0086049, or (12) those of Look et
15 al., Nanogel-based delivery of mycophenolic acid ameliorates systemic lupus erythematosus in mice" J. Clinical Investigation 123(4):1741-1749(2013). 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
20 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
25 100 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
30 alternatively comprise a surface that consists essentially of moieties that do not activate complement. In embodiments, synthetic nanocarriers exclude virus-like particles. 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.

A "therapeutic macromolecule" refers to any protein, carbohydrate, lipid or nucleic acid that may be administered to a subject and have a therapeutic effect. In some embodiments, administration of the therapeutic macromolecule to a subject may result in an undesired immune response. As described herein, administration of a therapeutic macromolecule concomitantly with an immunosuppressant can enhance the production or development of CD4+ regulatory T cells, such as those therapeutic macromolecule-specific, and/or therapeutic effectiveness of the therapeutic macromolecule, such as by reducing undesired immune responses thereto. In some embodiments, the therapeutic macromolecule may be a therapeutic polynucleotide or therapeutic protein.

"Therapeutic polynucleotide" means any polynucleotide or polynucleotide-based therapy that may be administered to a subject and have a therapeutic effect. Such therapies include gene silencing. Examples of such constructs for such therapy are known in the art, and include, but are not limited to, naked RNA (including messenger RNA, modified messenger RNA, and forms of RNAi). Examples of other therapeutic polynucleotides are provided elsewhere herein. Therapeutic polynucleotides may be produced in, on or by cells and also may be obtained using cell free or from fully synthetic in vitro methods. Subjects, therefore, include any subject that is in need of treatment with any of the foregoing. Such subject include those that will receive any of the foregoing.

"Therapeutic protein" means any protein or protein-based therapy that may be administered to a subject and have a therapeutic effect. Such therapies include protein replacement and protein supplementation therapies. Such therapies also include the administration of exogenous or foreign proteins, antibody therapies, and cell or cell-based therapies. Therapeutic proteins comprise, but are not limited to, enzymes, enzyme cofactors, hormones, blood clotting factors, cytokines, growth factors, monoclonal antibodies, antibody-drug conjugates, and polyclonal antibodies. Examples of other therapeutic proteins are provided elsewhere herein. Therapeutic proteins may be produced in, on or by cells and may be obtained from such cells or administered in the form of such cells. In embodiments, the therapeutic protein is produced in, on or by mammalian cells, insect cells, yeast cells, bacteria cells, plant cells, transgenic animal cells, transgenic plant cells, etc. The therapeutic protein may be recombinantly produced in such cells. The therapeutic protein may be produced in, on or by a virally transformed cell. Subjects, therefore, include any subject that is in need of treatment with any of the foregoing. Such subject include those that will receive any of the foregoing.

"Therapeutic macromolecule APC presentable antigen" means an antigen that is associated with a therapeutic macromolecule (i.e., the therapeutic macromolecule or a fragment thereof that can generate an immune response against the therapeutic macromolecule (e.g., the production of anti-therapeutic macromolecule-specific antibodies)).
5 Generally, therapeutic macromolecule antigen-presenting cell (APC) presentable antigens can be presented for recognition by the immune system (e.g., cells of the immune system, such as presented by antigen presenting cells, including but not limited to dendritic cells, B cells or macrophages). The therapeutic macromolecule APC presentable antigen can be presented for recognition by, for example, T cells. Such antigens may be recognized by and trigger an
10 immune response in a T cell via presentation of an epitope of the antigen bound to Class I or Class II major histocompatability complex molecule (MHC). Therapeutic macromolecule APC presentable antigens generally include proteins, polypeptides, peptides, polynucleotides, lipoproteins, or are contained or expressed in, on or by cells. The therapeutic macromolecule antigens, in some embodiments, comprise MHC Class I-restricted epitopes and/or MHC
15 Class II-restricted epitopes and/or B cell epitopes. Preferably, tolerogenic immune responses specific to the therapeutic macromolecule result with the methods, compositions or kits provided herein. In embodiments, populations of the synthetic nanocarriers comprise no added therapeutic macromolecule APC presentable antigens, meaning that no substantial amounts of therapeutic macromolecule APC presentable antigens are intentionally added to
20 the synthetic nanocarriers during the manufacturing thereof.

"Undesired immune response" refers to any undesired immune response that results from exposure to an antigen, promotes or exacerbates a disease, disorder or condition provided herein (or a symptom thereof), or is symptomatic of a disease, disorder or condition provided herein. Such immune responses generally have a negative impact on a subject's
25 health or is symptomatic of a negative impact on a subject's health.

C. COMPOSITIONS

Provided herein are compositions for use in administering immunosuppressants and therapeutic macromolecules for enhancing the production or development of CD4+
30 regulatory T cells, such as those specific to a therapeutic macromolecule, and related methods and kits. Such compositions, kits, and methods are useful for subjects in need of therapeutic macromolecule therapy, such as those that will receive therapeutic macromolecule therapy.

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 or cubic. In some embodiments, synthetic nanocarriers are ovals or ellipses. In
5 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 or shape 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
10 dimension or maximum dimension that falls within 5%, 10%, or 20% of the average diameter or average dimension of the synthetic nanocarriers.

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
15 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
20 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-
25 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 other embodiments, synthetic nanocarriers may comprise metal particles, quantum dots, ceramic particles, etc. In some embodiments, a non-polymeric synthetic nanocarrier is
30 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, hydroxyethylstarch, carageenan, glycon, amylose, chitosan, N,O-carboxylmethylchitosan, algin and alginic acid, starch, chitin, inulin, konjac, glucommannan, pustulan, heparin, hyaluronic acid, curdlan, and xanthan. In embodiments, the 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.

In some embodiments, synthetic nanocarriers can comprise one or more polymers. In some embodiments, the synthetic nanocarriers comprise one or more polymers that is a non-methoxy-terminated, pluronic polymer. In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 99% (weight/weight) of the polymers that make up the synthetic nanocarriers are non-methoxy-terminated, pluronic polymers. In some embodiments, all of the polymers that make up the synthetic nanocarriers are non-methoxy-terminated, pluronic polymers. In some embodiments, the synthetic nanocarriers comprise one or more polymers that is a non-methoxy-terminated polymer. In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 99% (weight/weight) of the polymers that make up the synthetic nanocarriers are non-methoxy-terminated polymers. In some embodiments, all of the polymers that make up the synthetic nanocarriers are non-methoxy-terminated polymers. In some embodiments, the synthetic nanocarriers comprise one or more polymers that do not comprise pluronic polymer. In some embodiments, at least 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 99% (weight/weight) of the polymers that make up the synthetic nanocarriers do not comprise pluronic polymer. In some embodiments, all of the polymers that make up the synthetic nanocarriers do not comprise pluronic polymer. In some embodiments, such a polymer 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 attached to the polymer.

The immunosuppressants can be attached to the synthetic nanocarriers by any of a number of methods. Generally, the attaching can be a result of bonding between the immunosuppressants and the synthetic nanocarriers. This bonding can result in the immunosuppressants being attached to the surface of the synthetic nanocarriers and/or
5 contained (encapsulated) within the synthetic nanocarriers. In some embodiments, however, the immunosuppressants are encapsulated by the synthetic nanocarriers as a result of the structure of the synthetic nanocarriers rather than bonding to the synthetic nanocarriers. In preferable embodiments, the synthetic nanocarrier comprises a polymer as provided herein, and the immunosuppressants are attached to the polymer.

10 When attaching occurs as a result of bonding between the immunosuppressants and synthetic nanocarriers, the attaching may occur via a coupling moiety. A coupling moiety can be any moiety through which an immunosuppressant is bonded to a synthetic nanocarrier. Such moieties include covalent bonds, such as an amide bond or ester bond, as well as separate molecules that bond (covalently or non-covalently) the immunosuppressant to the
15 synthetic nanocarrier. Such molecules include linkers or polymers or a unit thereof. For example, the coupling moiety can comprise a charged polymer to which an immunosuppressant electrostatically binds. As another example, the coupling moiety can comprise a polymer or unit thereof to which it is covalently bonded.

In preferred embodiments, the synthetic nanocarriers comprise a polymer as provided
20 herein. These synthetic nanocarriers can be completely polymeric or they can be a mix of polymers and other materials.

In some embodiments, the polymers of a synthetic nanocarrier associate to form a polymeric matrix. In some of these embodiments, a component, such as an immunosuppressant, can be covalently associated with one or more polymers of the
25 polymeric matrix. In some embodiments, covalent association is mediated by a linker. In some embodiments, a component can be noncovalently associated with one or more polymers of the polymeric matrix. For example, in some embodiments, a component can be encapsulated within, surrounded by, and/or dispersed throughout a polymeric matrix. Alternatively or additionally, a component can be associated with one or more polymers of a
30 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.

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.

In some embodiments, the polymer comprises a polyester, polycarbonate, polyamide, or polyether, or unit thereof. In other embodiments, the polymer comprises poly(ethylene glycol) (PEG), polypropylene glycol, poly(lactic acid), poly(glycolic acid), poly(lactic-co-glycolic acid), or a polycaprolactone, or unit thereof. In some embodiments, it is preferred that the polymer is biodegradable. Therefore, in these embodiments, it is preferred that if the polymer comprises a polyether, such as poly(ethylene glycol) or polypropylene glycol or unit thereof, the polymer comprises a block-co-polymer of a polyether and a biodegradable polymer such that the polymer is biodegradable. In other embodiments, the polymer does not solely comprise a polyether or unit thereof, such as poly(ethylene glycol) or polypropylene glycol or unit thereof.

Other 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, and 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
5 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. attached) 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
10 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 US Patent No. 5543158 to Gref et al., or WO publication WO2009/051837 by Von Andrian et al.

15 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,
20 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
25 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,
30 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. 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. In embodiments, the 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).

5 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.
10 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
15 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
20 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 the 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
25 comprehensive, list of polymers that can be of use in accordance with the present invention.

In some embodiments, synthetic nanocarriers do 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
30 atoms (e.g., gold atoms).

Compositions according to the invention can comprise elements, such as immunosuppressants, 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, compositions, such as those comprising immunosuppressants, are suspended in sterile saline solution for injection together with a preservative.

5 In embodiments, when preparing synthetic nanocarriers as carriers, methods for attaching components to the synthetic nanocarriers may be useful. If the component is a small molecule it may be of advantage to attach the component 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 attach the component
10 to the synthetic nanocarrier through the use of these surface groups rather than attaching the component to a polymer and then using this polymer conjugate in the construction of synthetic nanocarriers.

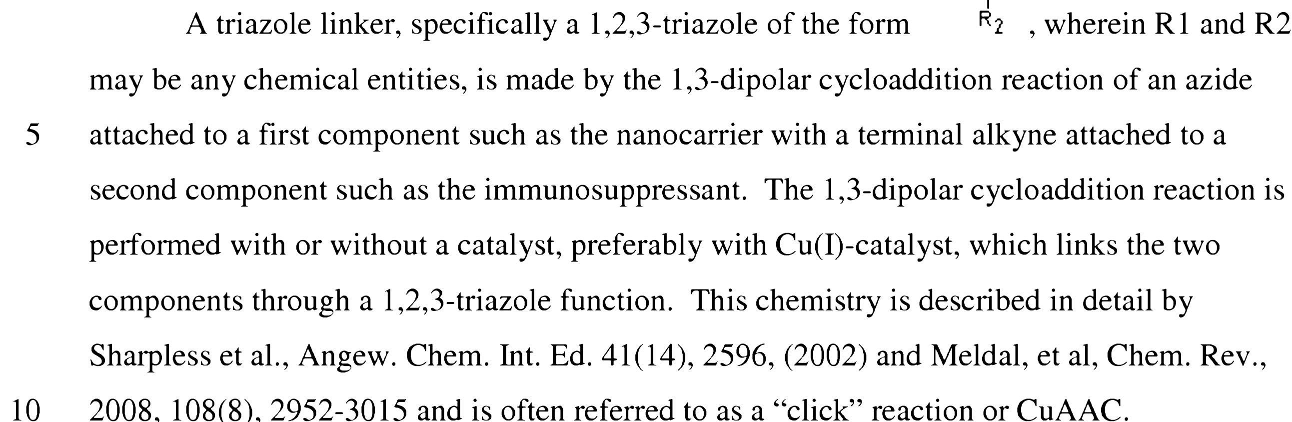
In certain embodiments, the attaching can be a covalent linker. In embodiments, immunosuppressants according to the invention can be covalently attached to the external
15 surface via a 1,2,3-triazole linker formed by the 1,3-dipolar cycloaddition reaction of azido groups on the surface of the nanocarrier with immunosuppressant containing an alkyne group or by the 1,3-dipolar cycloaddition reaction of alkynes on the surface of the nanocarrier with immunosuppressants 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
20 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.

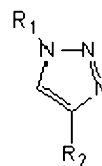
Additionally, covalent coupling may comprise a covalent linker that comprises an amide linker, a disulfide linker, a thioether linker, a hydrazone linker, a hydrazide linker, an imine or oxime linker, an urea or thiourea linker, an amidine linker, an amine linker, and a
25 sulfonamide linker.

An amide linker is formed via an amide bond between an amine on one component such as an immunosuppressant 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 and activated carboxylic
30 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 R1-S-S-R2. A disulfide bond can be formed by thiol exchange of a component containing thiol/mercaptan group(-SH) with another activated

thiol group on a polymer or nanocarrier or a nanocarrier containing thiol/mercaptan groups with a component containing activated thiol group.



A triazole linker, specifically a 1,2,3-triazole of the form 

, wherein R1 and R2 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 immunosuppressant. 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 component 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 component is then allowed to react with the nanocarrier via the 1,3-dipolar cycloaddition reaction with or without a catalyst which covalently attaches the component 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 R1-S-R2. Thioether can be made by either alkylation of a thiol/mercaptan (-SH) group on one component with an alkylating group such as halide or epoxide on a second component. Thioether linkers can also be formed by Michael addition of a thiol/mercaptan group on one component to an electron-deficient alkene group on a second component 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 with an alkene group on a second component.

A hydrazone linker is made by the reaction of a hydrazide group on one component with an aldehyde/ketone group on the second component.

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

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

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

10 An amidine linker is prepared by the reaction of an amine group on one component with an imidoester group on the second component.

An amine linker is made by the alkylation reaction of an amine group on one component with an alkylating group such as halide, epoxide, or sulfonate ester group on the second component. Alternatively, an amine linker can also be made by reductive amination 15 of an amine group on one component with an aldehyde or ketone group on the second component 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 with a sulfonyl halide (such as sulfonyl chloride) group on the second component.

20 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 nanocarrier or attached to a component.

The component can also be conjugated to the nanocarrier via non-covalent conjugation methods. For example, a negative charged immunosuppressant can be conjugated 25 to a positive charged nanocarrier through electrostatic adsorption. A component containing a metal ligand can also be conjugated to a nanocarrier containing a metal complex via a metal-ligand complex.

In embodiments, the component can be attached to a polymer, for example polylactic acid-block-polyethylene glycol, prior to the assembly of the synthetic nanocarrier or the 30 synthetic nanocarrier can be formed with reactive or activatible groups on its surface. In the latter case, the component may be prepared with a group which is compatible with the attachment chemistry that is presented by the synthetic nanocarriers' surface. In other embodiments, a peptide component 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,
5 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 a peptide component containing an acid group via the other end of the ADH linker on nanocarrier to produce the corresponding VLP or liposome peptide conjugate.

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

Any immunosuppressant as provided herein can be used in the methods or
15 compositions provided and can be, in some embodiments, attached to synthetic nanocarriers. Immunosuppressants include, but are not limited to, statins; mTOR inhibitors, such as rapamycin or a rapamycin analog; TGF-β signaling agents; TGF-β receptor agonists; histone deacetylase (HDAC) inhibitors; corticosteroids; inhibitors of mitochondrial function, such as rotenone; P38 inhibitors; NF-κβ inhibitors; adenosine receptor agonists; prostaglandin E2
20 agonists; phosphodiesterase inhibitors, such as phosphodiesterase 4 inhibitor; proteasome inhibitors; kinase inhibitors; G-protein coupled receptor agonists; G-protein coupled receptor antagonists; glucocorticoids; retinoids; cytokine inhibitors; cytokine receptor inhibitors; cytokine receptor activators; peroxisome proliferator-activated receptor antagonists; peroxisome proliferator-activated receptor agonists; histone deacetylase inhibitors;
25 calcineurin inhibitors; phosphatase inhibitors and oxidized ATPs. Immunosuppressants also include IDO, vitamin D3, cyclosporine A, aryl hydrocarbon receptor inhibitors, resveratrol, azathiopurine, 6-mercaptopurine, aspirin, niflumic acid, estriol, tripolide, interleukins (e.g., IL-1, IL-10), cyclosporine A, siRNAs targeting cytokines or cytokine receptors and the like.

Examples of statins include atorvastatin (LIPITOR®, TORVAST®), cerivastatin,
30 fluvastatin (LESCOL®, LESCOL® XL), lovastatin (MEVACOR®, ALTOCOR®, ALTOPREV®), mevastatin (COMPACTIN®), pitavastatin (LIVALO®, PIAVA®), rosuvastatin (PRAVACHOL®, SELEKTINE®, LIPOSTAT®), rosuvastatin (CRESTOR®), and simvastatin (ZOCOR®, LIPEX®).

Examples of mTOR inhibitors include rapamycin and analogs thereof (e.g., CCL-779, RAD001, AP23573, C20-methallylrapamycin (C20-Marap), C16-(S)-butylsulfonamidorapamycin (C16-BSrap), C16-(S)-3-methylindolerapamycin (C16-iRap) (Bayle et al. Chemistry & Biology 2006, 13:99-107)), AZD8055, BEZ235 (NVP-BEZ235),
5 chrysophanic acid (chrysophanol), deforolimus (MK-8669), everolimus (RAD0001), KU-0063794, PI-103, PP242, temsirolimus, and WYE-354 (available from Selleck, Houston, TX, USA).

Examples of TGF-β signaling agents include TGF-β ligands (e.g., activin A, GDF1, GDF11, bone morphogenic proteins, nodal, TGF-βs) and their receptors (e.g., ACVR1B,
10 ACVR1C, ACVR2A, ACVR2B, BMPR2, BMPR1A, BMPR1B, TGFβRI, TGFβRII), R-SMADS/co-SMADS (e.g., SMAD1, SMAD2, SMAD3, SMAD4, SMAD5, SMAD8), and ligand inhibitors (e.g, follistatin, noggin, chordin, DAN, lefty, LTBP1, THBS1, Decorin).

Examples of inhibitors of mitochondrial function include atractyloside (dipotassium salt), bongkrekic acid (triammonium salt), carbonyl cyanide m-chlorophenylhydrazone,
15 carboxyatractyloside (e.g., from *Atractylis gummifera*), CGP-37157, (-)-Deguelin (e.g., from *Mundulea sericea*), F16, hexokinase II VDAC binding domain peptide, oligomycin, rotenone, Ru360, SFK1, and valinomycin (e.g., from *Streptomyces fulvissimus*) (EMD4Biosciences, USA).

Examples of P38 inhibitors include SB-203580 (4-(4-Fluorophenyl)-2-(4-
20 methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole), SB-239063 (trans-1-(4hydroxycyclohexyl)-4-(fluorophenyl)-5-(2-methoxy-pyrimidin-4-yl) imidazole), SB-220025 (5-(2amino-4-pyrimidinyl)-4-(4-fluorophenyl)-1-(4-piperidinyl)imidazole)), and ARRY-797.

Examples of NF (e.g., NK-κβ) inhibitors include IFRD1, 2-(1,8-naphthyridin-2-yl)-
25 Phenol, 5-aminosalicylic acid, BAY 11-7082, BAY 11-7085, CAPE (Caffeic Acid Phenethylester), diethylmaleate, IKK-2 Inhibitor IV, IMD 0354, lactacystin, MG-132 [Z-Leu-Leu-Leu-CHO], NFκB Activation Inhibitor III, NF-κB Activation Inhibitor II, JSH-23, parthenolide, Phenylarsine Oxide (PAO), PPM-18, pyrrolidinedithiocarbamic acid ammonium salt, QNZ, RO 106-9920, rocaglamide, rocaglamide AL, rocaglamide C,
30 rocaglamide I, rocaglamide J, rocaglaol, (R)-MG-132, sodium salicylate, triptolide (PG490), and wedelolactone.

Examples of adenosine receptor agonists include CGS-21680 and ATL-146e.

Examples of prostaglandin E2 agonists include E-Prostanoid 2 and E-Prostanoid 4.

Examples of phosphodiesterase inhibitors (non-selective and selective inhibitors) include caffeine, aminophylline, IBMX (3-isobutyl-1-methylxanthine), paraxanthine, pentoxifylline, theobromine, theophylline, methylated xanthines, vinpocetine, EHNA (erythro-9-(2-hydroxy-3-nonyl)adenine), anagrelide, enoximone (PERFAN™), milrinone, levosimendon, mesembrine, ibudilast, piclamilast, luteolin, drotaverine, roflumilast (DAXAS™, DALIRESP™), sildenafil (REVATION®, VIAGRA®), tadalafil (ADCIRCA®, CIALIS®), vardenafil (LEVITRA®, STAXYN®), udenafil, avanafil, icariin, 4-methylpiperazine, and pyrazolo pyrimidin-7-1.

Examples of proteasome inhibitors include bortezomib, disulfiram, epigallocatechin-3-gallate, and salinosporamide A.

Examples of kinase inhibitors include bevacizumab, BIBW 2992, cetuximab (ERBITUX®), imatinib (GLEEVEC®), trastuzumab (HERCEPTIN®), gefitinib (IRESSA®), ranibizumab (LUCENTIS®), pegaptanib, sorafenib, dasatinib, sunitinib, erlotinib, nilotinib, lapatinib, panitumumab, vandetanib, E7080, pazopanib, and mubritinib.

Examples of glucocorticoids include hydrocortisone (cortisol), cortisone acetate, prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone, beclometasone, fludrocortisone acetate, deoxycorticosterone acetate (DOCA), and aldosterone.

Examples of retinoids include retinol, retinal, tretinoin (retinoic acid, RETIN-A®), isotretinoin (ACCUTANE®, AMNESTEEM®, CLARAVIS®, SOTRET®), alitretinoin (PANRETIN®), etretinate (TEGISON™) and its metabolite acitretin (SORIATANE®), tazarotene (TAZORAC®, AVAGE®, ZORAC®), bexarotene (TARGRETIN®), and adapalene (DIFFERIN®).

Examples of cytokine inhibitors include IL1ra, IL1 receptor antagonist, IGFBP, TNF-BF, uromodulin, Alpha-2-Macroglobulin, Cyclosporin A, Pentamidine, and Pentoxifylline (PENTOPAK®, PENTOXIL®, TRENTAL®).

Examples of peroxisome proliferator-activated receptor antagonists include GW9662, PPARγ antagonist III, G335, and T0070907 (EMD4Biosciences, USA).

Examples of peroxisome proliferator-activated receptor agonists include pioglitazone, ciglitazone, clofibrate, GW1929, GW7647, L-165,041, LY 171883, PPARγ activator, Fmoc-Leu, troglitazone, and WY-14643 (EMD4Biosciences, USA).

Examples of histone deacetylase inhibitors include hydroxamic acids (or hydroxamates) such as trichostatin A, cyclic tetrapeptides (such as trapoxin B) and

depsipeptides, benzamides, electrophilic ketones, aliphatic acid compounds such as phenylbutyrate and valproic acid, hydroxamic acids such as vorinostat (SAHA), belinostat (PXD101), LAQ824, and panobinostat (LBH589), benzamides such as entinostat (MS-275), CI994, and mocetinostat (MGCD0103), nicotinamide, derivatives of NAD, dihydrocoumarin, 5 naphthopyranone, and 2-hydroxynaphaldehydes.

Examples of calcineurin inhibitors include cyclosporine, pimecrolimus, voclosporin, and tacrolimus.

Examples of phosphatase inhibitors include BN82002 hydrochloride, CP-91149, calyculin A, cantharidic acid, cantharidin, cypermethrin, ethyl-3,4-dephostatin, fostriecin 10 sodium salt, MAZ51, methyl-3,4-dephostatin, NSC 95397, norcantharidin, okadaic acid ammonium salt from prorocentrum concavum, okadaic acid, okadaic acid potassium salt, okadaic acid sodium salt, phenylarsine oxide, various phosphatase inhibitor cocktails, protein phosphatase 1C, protein phosphatase 2A inhibitor protein, protein phosphatase 2A1, protein phosphatase 2A2, and sodium orthovanadate.

15 In some embodiments of any one of the methods, compositions or kits provided herein, the therapeutic macromolecules as described herein are also attached to synthetic nanocarriers. In other embodiments, the therapeutic macromolecules are not attached to any synthetic nanocarriers. In some embodiments of either of these situations, the therapeutic macromolecules may be delivered in the form of the therapeutic macromolecule itself, or 20 fragments or derivatives thereof.

Therapeutic macromolecules can include therapeutic proteins or therapeutic polynucleotides. Therapeutic proteins include, but are not limited to, infusible therapeutic proteins, enzymes, enzyme cofactors, hormones, blood clotting factors, cytokines and interferons, growth factors, monoclonal antibodies, and polyclonal antibodies (e.g., that are 25 administered to a subject as a replacement therapy), and proteins associated with Pompe's disease (e.g., acid glucosidase alfa, rhGAA (e.g., Myozyme and Lumizyme (Genzyme)). Therapeutic proteins also include proteins involved in the blood coagulation cascade. Therapeutic proteins include, but are not limited to, Factor VIII, Factor VII, Factor IX, Factor V, von Willebrand Factor, von Heldebrant Factor, tissue plasminogen activator, insulin, 30 growth hormone, erythropoietin alfa, VEGF, thrombopoietin, lysozyme, antithrombin and the like. Therapeutic proteins also include adipokines, such as leptin and adiponectin. Other examples of therapeutic proteins are as described below and elsewhere herein.

Examples of therapeutic proteins used in enzyme replacement therapy of subjects having a lysosomal storage disorder include, but are not limited to, imiglucerase for the treatment of Gaucher's disease (e.g., CEREZYME™), a-galactosidase A (a-gal A) for the treatment of Fabry disease (e.g., agalsidase beta, FABRYZYME™), acid α-glucosidase (GAA) for the treatment of Pompe disease (e.g., acid glucosidase alfa, LUMIZYME™, MYOZYME™), arylsulfatase B for the treatment of Mucopolysaccharidoses (e.g., laronidase, ALDURAZYME™, idursulfase, ELAPRASE™, arylsulfatase B, NAGLAZYME™)), pegloticase (KRYSTEXXA) and pegsiticase.

Examples of enzymes include oxidoreductases, transferases, hydrolases, lyases, isomerases, asparaginases, uricases, glycosidases, asparaginases, uricases, proteases, nucleases, collagenases, hyaluronidases, heparinases, heparanases, lysins, and ligases.

Therapeutic proteins may also include any enzyme, toxin, or other protein or peptide isolated or derived from a bacterial, fungal, or viral source.

Examples of hormones include Melatonin (N-acetyl-5-methoxytryptamine), Serotonin, Thyroxine (or tetraiodothyronine) (a thyroid hormone), Triiodothyronine (a thyroid hormone), Epinephrine (or adrenaline), Norepinephrine (or noradrenaline), Dopamine (or prolactin inhibiting hormone), Antimullerian hormone (or mullerian inhibiting factor or hormone), Adiponectin, Adrenocorticotropic hormone (or corticotropin), Angiotensinogen and angiotensin, Antidiuretic hormone (or vasopressin, arginine vasopressin), Atrial-natriuretic peptide (or atriopeptin), Calcitonin, Cholecystokinin, Corticotropin-releasing hormone, Erythropoietin, Follicle-stimulating hormone, Gastrin, Ghrelin, Glucagon, Glucagon-like peptide (GLP-1), GIP, Gonadotropin-releasing hormone, Growth hormone-releasing hormone, Human chorionic gonadotropin, Human placental lactogen, Growth hormone, Inhibin, Insulin, Insulin-like growth factor (or somatomedin), Leptin, Luteinizing hormone, Melanocyte stimulating hormone, Orexin, Oxytocin, Parathyroid hormone, Prolactin, Relaxin, Secretin, Somatostatin, Thrombopoietin, Thyroid-stimulating hormone (or thyrotropin), Thyrotropin-releasing hormone, Cortisol, Aldosterone, Testosterone, Dehydroepiandrosterone, Androstenedione, Dihydrotestosterone, Estradiol, Estrone, Estriol, Progesterone, Calcitriol (1,25-dihydroxyvitamin D3), Calcidiol (25-hydroxyvitamin D3), Prostaglandins, Leukotrienes, Prostacyclin, Thromboxane, Prolactin releasing hormone, Lipotropin, Brain natriuretic peptide, Neuropeptide Y, Histamine, Endothelin, Pancreatic polypeptide, Renin, and Enkephalin.

Examples of blood or blood coagulation factors include Factor I (fibrinogen), Factor II (prothrombin), tissue factor, Factor V (proaccelerin, labile factor), Factor VII (stable factor, proconvertin), Factor VIII (antihemophilic globulin), Factor IX (Christmas factor or plasma thromboplastin component), Factor X (Stuart-Prower factor), Factor Xa, Factor XI, Factor XII (Hageman factor), Factor XIII (fibrin-stabilizing factor), von Willebrand factor, prekallikrein (Fletcher factor), high-molecular weight kininogen (HMWK) (Fitzgerald factor), fibronectin, fibrin, thrombin, antithrombin III, heparin cofactor II, protein C, protein S, protein Z, protein Z-related protease inhibitot (ZPI), plasminogen, alpha 2-antiplasmin, tissue plasminogen activator (tPA), urokinase, plasminogen activator inhibitor-1 (PAI1), plasminogen activator inhibitor-2 (PAI2), cancer procoagulant, and epoetin alfa (Epogen, Procrit).

Examples of cytokines include lymphokines, interleukins, and chemokines, type 1 cytokines, such as IFN-γ, TGF-β, and type 2 cytokines, such as IL-4, IL-10, and IL-13.

Examples of growth factors include Adrenomedullin (AM), Angiopoietin (Ang), Autocrine motility factor, Bone morphogenetic proteins (BMPs), Brain-derived neurotrophic factor (BDNF), Epidermal growth factor (EGF), Erythropoietin (EPO), Fibroblast growth factor (FGF), Glial cell line-derived neurotrophic factor (GDNF), Granulocyte colony-stimulating factor (G-CSF), Granulocyte macrophage colony-stimulating factor (GM-CSF), Growth differentiation factor-9 (GDF9), Hepatocyte growth factor (HGF), Hepatoma-derived growth factor (HDGF), Insulin-like growth factor (IGF), Migration-stimulating factor, Myostatin (GDF-8), Nerve growth factor (NGF) and other neurotrophins, Platelet-derived growth factor (PDGF), Thrombopoietin (TPO), Transforming growth factor alpha(TGF-α), Transforming growth factor beta(TGF-β), Tumour_necrosis_factor-alpha(TNF-α), Vascular endothelial growth factor (VEGF), Wnt Signaling Pathway, placental growth factor (PlGF), (Foetal Bovine Somatotrophin) (FBS), IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, and IL-7.

Examples of monoclonal antibodies include Abagovomab, Abciximab, Adalimumab, Adecatumumab, Afelimomab, Afutuzumab, Alacizumab pegol, ALD, Alemtuzumab, Altumomab pentetate, Anatumomab mafenatox, Anrukinzumab, Anti-thymocyte globin, Apolizumab, Arcitumomab, Aselizumab, Atlizumab (tocilizumab), Atorolimumab, Bapineuzumab, Basiliximab, Bavituximab, Bectumomab, Belimumab, Benralizumab, Bertilimumab, Besilesomab, Bevacizumab, Biciromab, Bivatuzumab mertansine, Blinatumomab, Brentuximab vedotin, Briakinumab, Canakinumab, Cantuzumab mertansine, Capromab pendetide, Catumaxomab, Cedelizumab, Certolizumab pegol, Cetuximab,

Citatuzumab bogatox, Cixutumumab, Clenoliximab, Clivatuzumab tetraxetan, Conatumumab, Dacetuzumab, Daclizumab, Daratumumab, Denosumab, Detumomab, Dorlimomab aritox, Dorlixizumab, Ecromeximab, Eculizumab, Edobacomab, Edrecolomab, Efalizumab, Efungumab, Elotuzumab, Elsilimomab, Enlimomab pegol, Epitumomab cituxetan, Epratuzumab, Erlizumab, Ertumaxomab, Etaracizumab, Exbivirumab, Fanolesomab, Faralimomab, Farletuzumab, Felvizumab, Fezakinumab, Figitumumab, Fontolizumab , Foravirumab, Fresolimumab, Galiximab, Gantenerumab, Gavilimomab, Gemtuzumab ozogamicin, GC1008, Girentuximab, Glembatumumab vedotin, Golimumab, Gomiliximab, Ibalizumab, Ibritumomab tiuxetan, Igovomab, Imciromab, Infliximab, Intetumumab, Inolimomab, Inotuzumab ozogamicin, Ipilimumab, Iratumumab, Keliximab, Labetuzumab, Lebrikizumab, Lemalesomab, Lerdelimumab, Lexatumumab, Libivirumab, Lintuzumab, Lorvotuzumab mertansine, Lucatumumab, Lumiliximab, Mapatumumab, Maslimomab, Matuzumab, Mepolizumab, Metelimumab, Milatuzumab, Minretumomab, Mitumomab, Morolimumab, Motavizumab, Muromonab-CD3, Nacolomab tafenatox, Naptumomab estafenatox, Natalizumab, Nebacumab, Necitumumab, Nerelimomab, Nimotuzumab, Nofetumomab merpentan, Ocrelizumab, Odulimomab, Ofatumumab, Olaratumab, Omalizumab, Oportuzumab monatox, Oregovomab, Otelixizumab, Pagibaximab, Palivizumab, Panitumumab, Panobacumab, Pascolizumab, Pemtumomab, Pertuzumab, Pexelizumab, Pintumomab, Priliximab, Pritumumab, Rafivirumab, Ramucirumab, Ranibizumab, Raxibacumab, Regavirumab Reslizumab, Rilotumumab, Rituximab, Robatumumab, Rontalizumab, Rovelizumab, Ruplizumab, Satumomab pendetide, Sevirumab, Sibrotuzumab, Sifalimumab, Siltuximab, Siplizumab, Solanezumab, Sonepcizumab, Sontuzumab, Stamulumab, Sulesomab, Tacatuzumab tetraxetan, Tadocizumab, Talizumab, Tanezumab, Taplitumomab paptox, Tefibazumab, Telimomab aritox, Tenatumomab, Teneliximab, Teplizumab, Ticilimumab (tremelimumab), Tigatuzumab, Tocilizumab (atlizumab), Toralizumab, Tositumomab, Trastuzumab, Tremelimumab, Tucotuzumab celmoleukin, Tuvirumab, Urtoxazumab, Ustekinumab, Vapaliximab, Vedolizumab, Veltuzumab, Vepalimomab, Visilizumab, Volociximab, Votumumab, Zalutumumab, Zanolimumab, Ziralimumab, and Zolimomab aritox. Monoclonal antibodies further include anti-TNF-α antibodies.

Examples of infusion therapy or injectable therapeutic proteins include, for example, Tocilizumab (Roche/Actemra®), alpha-1 antitrypsin (Kamada/AAT), Hematide® (Affymax and Takeda, synthetic peptide), albinterferon alfa-2b (Novartis/Zalbin™), Rhucin®

(Pharming Group, C1 inhibitor replacement therapy), tesamorelin (Theratechnologies/Egrifta, synthetic growth hormone-releasing factor), ocrelizumab (Genentech, Roche and Biogen), belimumab (GlaxoSmithKline/Benlysta®), pegloticase (Savient Pharmaceuticals/Krystexxa™), pegsiticase, taliglucerase alfa (Protalix/Uplyso), agalsidase alfa (Shire/Replagal®), velaglucerase alfa (Shire), and Keyhole Limpet Hemocyanin (KLH).

Additional therapeutic proteins include, for example, engineered proteins, such as Fc fusion proteins, bispecific antibodies, multi-specific antibodies, nanobodies, antigen-binding proteins, antibody fragments, and protein conjugates, such as antibody drug conjugates.

Therapeutic polynucleotides include, but are not limited to nucleic acid aptamers such as Pegaptanib (Macugen, a pegylated anti-VEGF aptamer), antisense therapeutics such as antisense poly- or oligonucleotides (e.g., antiviral drug Fomivirsen, or Mipomersen, an antisense therapeutic that targets the messenger RNA for apolipoprotein B for reduction of cholesterol level); small interfering RNAs (siRNAs) (e.g., dicer substrate siRNA molecules (DsiRNAs) which are 25-30 base pair asymmetric double-stranded RNAs that mediate RNAi with extremely high potency); or modified messenger RNAs (mmRNAs) such as those disclosed in US Patent application 2013/0115272 to de Fougerolles et al. and in Published US Patent application 2012/0251618 to Schrum et al.

Additional therapeutic macromolecules useful in accordance with aspects of this invention will be apparent to those of skill in the art, and the invention is not limited in this respect.

In some embodiments, a component, such as a therapeutic macromolecule or immunosuppressant, may be isolated. Isolated refers to the element being separated from its native environment and present in sufficient quantities to permit its identification or use. This means, for example, the element may be (i) selectively produced by expression cloning or (ii) purified as by chromatography or electrophoresis. Isolated elements may be, but need not be, substantially pure. Because an isolated element may be admixed with a pharmaceutically acceptable excipient in a pharmaceutical preparation, the element may comprise only a small percentage by weight of the preparation. The element 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 lipids or proteins. Any of the elements provided herein may be isolated and included in the compositions or used in the methods in isolated form.

D. METHODS OF MAKING AND USING THE COMPOSITIONS AND RELATED METHODS

Aspects of the invention relate to determining a protocol for the methods of administration as provided herein. A protocol can be determined by varying the frequency, dosage amount and other aspects of administration of the therapeutic macromolecule and the immunosuppressant and subsequently assessing the number or percentage (or ratio) of CD4+ regulatory T cells, such as those therapeutic macromolecule-specific, and/or any desired or undesired immune response based on such variation. A preferred protocol for practice of the invention enhances the number or percentage (or ratio) of CD4+ regulatory T cells, such as therapeutic macromolecule-specific CD4+ regulatory T cells.

Synthetic nanocarriers may be prepared using a wide variety of methods known in the art. For example, synthetic nanocarriers can be formed by methods such 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 into synthetic nanocarriers may be used, including without limitation methods disclosed in United States Patent 6,632,671 to Unger issued 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 attached to the synthetic nanocarriers and/or the composition of the polymer matrix.

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

Elements (i.e., components) of the synthetic nanocarriers may be attached to the overall synthetic nanocarrier, e.g., by one or more covalent bonds, or may be attached by means of one or more linkers. Additional methods of functionalizing synthetic nanocarriers may be adapted from Published US Patent Application 2006/0002852 to Saltzman et al., Published US 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 attached to components directly or indirectly via non-covalent interactions. In non-covalent embodiments, the non-covalent attaching 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 attachments may be arranged to be on an external surface or an internal surface of a synthetic nanocarrier. In embodiments, encapsulation and/or absorption is a form of attaching. In embodiments, the synthetic nanocarriers can be combined with an antigen by admixing in the same vehicle or delivery system.

Compositions provided herein 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,
5 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).

10 Compositions according to the invention may comprise 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
15 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, compositions are suspended in sterile saline solution for injection with a preservative.

It is to be understood that the compositions of the invention can be made in any
20 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 of manufacture may require attention to the properties of the particular moieties being associated.

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

30 Administration according to the present invention may be by a variety of routes, including but not limited to subcutaneous, intravenous, intraperitoneal, intramuscular, transmucosal, transdermal, transcutaneous or intradermal routes. In a preferred embodiment, administration is via a subcutaneous route of administration. The compositions referred to

herein may be manufactured and prepared for administration, in some embodiments concomitant administration, using conventional methods.

The compositions of the invention can be administered in effective amounts, such as the effective amounts described elsewhere herein. Doses of dosage forms may contain varying amounts of immunosuppressants and/or therapeutic macromolecule, according to the invention. The amount of immunosuppressants and/or therapeutic macromolecule present in the dosage forms can be varied according to the nature of the therapeutic macromolecules and/or immunosuppressants, the therapeutic benefit to be accomplished, and other such parameters. In embodiments, dose ranging studies can be conducted to establish optimal therapeutic amount of the immunosuppressants and/or therapeutic macromolecules to be present in dosage forms. In embodiments, the immunosuppressants and/or therapeutic macromolecules are present in dosage forms in an amount effective to generate a tolerogenic immune response to the therapeutic macromolecules upon administration to a subject. In preferable embodiments, the immunosuppressants and/or therapeutic macrocmolecules are present in dosage forms in an amount effective enhance the production or development of CD4+ regulatory T cells, such as when concomitantly administered to a subject. It may be possible to determine amounts of the immunosuppressants and/or therapeutic macromolecules effective to generate desired immune responses using conventional dose ranging studies and techniques in subjects. Dosage forms may be administered at a variety of frequencies.

In some embodiments, administration of immunosuppressants, such as those attached to synthetic nanocarriers, with a therapeutic macromolecule is undertaken e.g., prior to subsequent further administration of the therapeutic macromolecule.

Another aspect of the disclosure relates to kits. In some embodiments, the kit comprises an immunosuppressant, in some embodiments attached to synthetic nanocarriers, and a therapeutic macromolecule. The immunosuppressant and therapeutic macromolecule can be contained within separate containers in the kit. In some embodiments, the container is a vial or an ampoule. In some embodiments, the therapeutic macromolecule or immunosuppressant are contained within a solution separate from the container, such that the therapeutic macromolecule or immunosuppressant may be added to the container at a subsequent time. In preferred embodiments, the therapeutic macromolecule is not co-formulated with the immunosuppressant prior to administration. In some embodiments, the therapeutic macromolecule or immunosuppressant are in lyophilized form each in a separate

container, such that they may be reconstituted at a subsequent time. In some embodiments, the kit further comprises instructions for reconstitution, mixing, administration, etc. In some embodiments, the instructions include a description of the methods described herein. Instructions can be in any suitable form, e.g., as a printed insert or a label. In some
5 embodiments, the kit further comprises one or more syringes or other means for administering the immunosuppressant and therapeutic macromolecule.

EXAMPLES

10 **Example 1: Polymeric Nanocarrier Containing Polymer-Rapamycin Conjugate (Prophetic)**

Preparation of PLGA-rapamycin conjugate:

PLGA polymer with acid end group (7525 DLG1A, acid number 0.46 mmol/g, Lakeshore Biomaterials; 5 g, 2.3 mmol, 1.0 eq) is dissolved in 30 mL of dichloromethane
15 (DCM). N,N-Dicyclohexylcarbodimide (1.2 eq, 2.8 mmol, 0.57 g) is added followed by rapamycin (1.0 eq, 2.3 mmol, 2.1 g) and 4-dimethylaminopyridine (DMAP) (2.0 eq, 4.6 mmol, 0.56 g). The mixture is stirred at rt for 2 days. The mixture is then filtered to remove insoluble dicyclohexylurea. The filtrate is concentrated to ca. 10 mL in volume and added to 100 mL of isopropyl alcohol (IPA) to precipitate out the PLGA-rapamycin conjugate. The
20 IPA layer is removed and the polymer is then washed with 50 mL of IPA and 50 mL of methyl t-butyl ether (MTBE). The polymer is then dried under vacuum at 35 C for 2 days to give PLGA-rapamycin as a white solid (ca. 6.5 g).

Nanocarrier containing PLGA-rapamycin is prepared as follows:

Solutions for nanocarrier formation are prepared as follows:

25 Solution 1: PLGA-rapamycin @ 100 mg/mL in methylene chloride. The solution is prepared by dissolving PLGA-rapamycin in pure methylene chloride. Solution 2: PLA-PEG @ 100 mg/mL in methylene chloride. The solution is prepared by dissolving PLA-PEG in pure methylene chloride. Solution 3: Polyvinyl alcohol @ 50 mg/mL in 100 mM pH 8 phosphate buffer.

30 A primary water-in-oil emulsion is prepared first. W1/O1 is prepared by combining solution 1 (0.75 mL), and solution 2 (0.25 mL) in a small pressure tube and sonicating at 50% amplitude for 40 seconds using a Branson Digital Sonifier 250. A secondary emulsion (W1/O1/W2) is then prepared by combining solution 3 (3.0 mL) with the primary W1/O1

emulsion, vortexing for 10 s, and sonicating at 30% amplitude for 60 seconds using the Branson Digital Sonifier 250. The W1/O1/W2 emulsion is added to a beaker containing 70 mM pH 8 phosphate buffer solution (30 mL) and stirred at room temperature for 2 hours to allow the methylene chloride to evaporate and for the nanocarriers to form. A portion of the nanocarriers is washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 75,600×g and 4 °C for 35 min, removing the supernatant, and re-suspending the pellet in phosphate buffered saline. The washing procedure is repeated, and the pellet is re-suspended in phosphate buffered saline for a final nanocarrier dispersion of about 10 mg/mL.

Example 2: Preparation of Gold Nanocarriers (AuNCs) Containing Rapamycin (Prophetic)

Preparation of HS-PEG-rapamycin:

A solution of PEG acid disulfide (1.0 eq), rapamycin (2.0-2.5 eq), DCC (2.5 eq) and DMAP (3.0 eq) in dry DMF is stirred at rt overnight. The insoluble dicyclohexylurea is removed by filtration and the filtrate is added to isopropyl alcohol (IPA) to precipitate out the PEG-disulfide-di-rapamycin ester and washed with IPA and dried. The polymer is then treated with tris(2-carboxyethyl)phosphine hydrochloride in DMF to reduce the PEG disulfide to thiol PEG rapamycin ester (HS-PEG-rapamycin). The resulting polymer is recovered by precipitation from IPA and dried as previously described and analyzed by H NMR and GPC.

Formation of Gold NCs (AuNCs):

An aq. solution of 500 mL of 1 mM HAuCl4 is heated to reflux for 10 min with vigorous stirring in a 1 L round-bottom flask equipped with a condenser. A solution of 50 mL of 40 mM of trisodium citrate is then rapidly added to the stirring solution. The resulting deep wine red solution is kept at reflux for 25-30 min and the heat is withdrawn and the solution is cooled to room temperature. The solution is then filtered through a 0.8 μm membrane filter to give the AuNCs solution. The AuNCs are characterized using visible spectroscopy and transmission electron microscopy. The AuNCs are ca. 20 nm diameter capped by citrate with peak absorption at 520 nm.

AuNCs conjugate with HS-PEG-rapamycin:

A solution of 150 μl of HS-PEG-rapamycin (10 μM in 10 mM pH 9.0 carbonate buffer) is added to 1 mL of 20 nm diameter citrate-capped gold nanocarriers (1.16 nM) to

produce a molar ratio of thiol to gold of 2500:1. The mixture is stirred at room temperature under argon for 1 hour to allow complete exchange of thiol with citrate on the gold nanocarriers. The AuNCs with PEG-rapamycin on the surface is then purified by centrifuge at 12,000g for 30 minutes. The supernatant is decanted and the pellet containing AuNC-S-
5 PEG-rapamycin is then pellet washed with 1x PBS buffer. The purified Gold-PEG-rapamycin nanocarriers are then resuspend in suitable buffer for further analysis and bioassays.

Example 3: Mesoporous Silica Nanoparticles with Attached Ibuprofen (Prophetic)

Mesoporous SiO2 nanoparticle cores are created through a sol-gel process.
10 Hexadecyltrimethyl-ammonium bromide (CTAB) (0.5 g) is dissolved in deionized water (500 mL), and then 2 M aqueous NaOH solution (3.5 mL) is added to the CTAB solution. The solution is stirred for 30 min, and then Tetraethoxysilane (TEOS) (2.5 mL) is added to the solution. The resulting gel is stirred for 3 h at a temperature of 80 °C. The white precipitate which forms is captured by filtration, followed by washing with deionized water and drying
15 at room temperature. The remaining surfactant is then extracted from the particles by suspension in an ethanolic solution of HCl overnight. The particles are washed with ethanol, centrifuged, and redispersed under ultrasonication. This wash procedure is repeated two additional times.

The SiO2 nanoparticles are then functionalized with amino groups using (3-
20 aminopropyl)-triethoxysilane (APTMS). To do this, the particles are suspended in ethanol (30 mL), and APTMS (50 μL) is added to the suspension. The suspension is allowed to stand at room temperature for 2 h and then is boiled for 4 h, keeping the volume constant by periodically adding ethanol. Remaining reactants are removed by five cycles of washing by centrifugation and redispersing in pure ethanol.

25 In a separate reaction, 1-4 nm diameter gold seeds are created. All water used in this reaction is first deionized and then distilled from glass. Water (45.5 mL) is added to a 100 mL round-bottom flask. While stirring, 0.2 M aqueous NaOH (1.5 mL) is added, followed by a 1% aqueous solution of tetrakis(hydroxymethyl)phosphonium chloride (THPC) (1.0 mL). Two minutes after the addition of THPC solution, a 10 mg/mL aqueous solution of
30 chloroauric acid (2 mL), which has been aged at least 15 min, is added. The gold seeds are purified through dialysis against water.

To form the core-shell nanocarriers, the amino-functionalized SiO2 nanoparticles formed above are first mixed with the gold seeds for 2 h at room temperature. The gold-

decorated SiO2 particles are collected through centrifugation and mixed with an aqueous solution of chloroauric acid and potassium bicarbonate to form the gold shell. The particles are then washed by centrifugation and redispersed in water. Ibuprofen is loaded by suspending the particles in a solution of sodium ibuprofen (1 mg/L) for 72 h. Free ibuprofen is then washed from the particles by centrifugation and redispersing in water.

Example 4: Liposomes Containing Cyclosporine A (Prophetic)

The liposomes are formed using thin film hydration. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (32 μmol), cholesterol (32 μmol), and cyclosporin A (6.4 μmol) are dissolved in pure chloroform (3 mL). This lipid solution is added to a 50 mL round-bottom flask, and the solvent is evaporated on a rotary evaporator at a temperature of 60 °C. The flask is then flushed with nitrogen gas to remove remaining solvent. Phosphate buffered saline (2 mL) and five glass beads are added to the flask, and the lipid film is hydrated by shaking at 60 °C for 1 h to form a suspension. The suspension is transferred to a small pressure tube and sonicated at 60 °C for four cycles of 30s pulses with a 30 s delay between each pulse. The suspension is then left undisturbed at room temperature for 2 h to allow for complete hydration. The liposomes are washed by centrifugation followed by resuspension in fresh phosphate buffered saline.

Example 5: Synthetic Nanocarriers Containing Rapamycin

Materials

Rapamycin was purchased from TSZ CHEM (185 Wilson Street, Framingham, MA 01702; Product Catalogue # R1017). PLGA with 76% lactide and 24% glycolide content and an inherent viscosity of 0.69 dL/g was purchased from SurModics Pharmaceuticals (756 Tom Martin Drive, Birmingham, AL 35211. Product Code 7525 DLG 7A.) PLA-PEG block co-polymer with a PEG block of approximately 5,000 Da and PLA block of approximately 40,000 Da was purchased from SurModics Pharmaceuticals (756 Tom Martin Drive, Birmingham, AL 35211; Product Code 100 DL mPEG 5000 5CE). Polyvinyl alcohol (85-89% hydrolyzed) was purchased from EMD Chemicals (Product Number 1.41350.1001).

Method

Solutions were prepared as follows:

Solution 1: PLGA at 75 mg/mL and PLA-PEG at 25 mg/mL in methylene chloride. The solution was prepared by dissolving PLGA and PLA-PEG in pure methylene chloride.

5 Solution 2: Rapamycin at 100 mg/mL in methylene chloride. The solution was prepared by dissolving rapamycin in pure methylene chloride.

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

An oil-in-water emulsion was used to prepare the nanocarriers. The O/W emulsion was prepared by combining solution 1 (1 mL), solution 2 (0.1 mL), and solution 3 (3 mL) in a 10 small pressure tube and sonicating at 30% amplitude for 60 seconds using a Branson Digital Sonifier 250. The O/W emulsion was added to a beaker containing 70 mM pH 8 phosphate buffer solution (30 mL) and stirred at room temperature for 2 hours to allow the methylene chloride to evaporate and for the nanocarriers to form. A portion of the nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 15 75,000×g and 4 °C for 35 min, 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.

Nanocarrier size was determined by dynamic light scattering. The amount rapamycin in the nanocarrier was determined by HPLC analysis. The total dry-nanocarrier mass per mL 20 of suspension was determined by a gravimetric method.

Effective Diameter (nm)	Rapamycin Content (% w/w)
227	6.4

Example 6: Synthetic Nanocarriers Containing GSK1059615

25 **Materials**

GSK1059615 was purchased from MedChem Express (11 Deer Park Drive, Suite 102D Monmouth Junction, NJ 08852), product code HY-12036. PLGA with a lactide:glycolide ratio of 1:1 and an inherent viscosity of 0.24 dL/g was purchased from Lakeshore Biomaterials (756 Tom Martin Drive, Birmingham, AL 35211), product code 30 5050 DLG 2.5A. PLA-PEG-OMe block co-polymer with a methyl ether terminated PEG

block of approximately 5,000 Da and an overall inherent viscosity of 0.26 DL/g was purchased from Lakeshore Biomaterials (756 Tom Martin Drive, Birmingham, AL 35211; Product Code 100 DL mPEG 5000 5K-E). Cellgro phosphate buffered saline 1X pH 7.4 (PBS 1X) was purchased from Corning (9345 Discovery Blvd. Manassas, VA 20109),
5 product code 21-040-CV.

Method

Solutions were prepared as follows:

Solution 1: PLGA (125 mg), and PLA-PEG-OMe (125 mg), were dissolved in 10 mL
10 of acetone. Solution 2: GSK1059615 was prepared at 10 mg in 1 mL of N-methyl-2-pyrrolidinone (NMP).

Nanocarriers were prepared by combining Solution 1 (4 mL) and Solution 2 (0.25 mL) in a small glass pressure tube and adding the mixture drop wise to a 250 mL round bottom flask containing 20 mL of ultra-pure water under stirring. The flask was mounted
15 onto a rotary evaporation device, and the acetone was removed under reduced pressure. A portion of the nanocarriers was washed by transferring the nanocarrier suspension to centrifuge tubes and centrifuging at 75,600 rcf and 4 °C for 50 minutes, removing the supernatant, and re-suspending the pellet in PBS 1X. The washing procedure was repeated, and the pellet was re-suspended in PBS 1X to achieve a nanocarrier suspension having a
20 nominal concentration of 10 mg/mL on a polymer basis. The washed nanocarrier solution was then filtered using 1.2μm PES membrane syringe filters from Pall, part number 4656. An identical nanocarrier solution was prepared as above, and pooled with the first after the filtration step. The homogenous suspension was stored frozen at -20°C.

Nanocarrier size was determined by dynamic light scattering. The amount of
25 GSK1059615 in the nanocarrier was determined by UV absorption at 351nm. The total dry-nanocarrier mass per mL of suspension was determined by a gravimetric method.

Effective Diameter (nm)	GSK1059615 Content (% w/w)
143	1.02

Example 7: Induction of CD4+ Regulatory T Cells with Synthetic Nanocarriers

Materials

Rapamycin was purchased from TSZ CHEM (185 Wilson Street, Framingham, MA 01702; Product Code R1017). PLGA with a lactide:glycolide ratio of 3:1 and an inherent viscosity of 0.75 dL/g was purchased from SurModics Pharmaceuticals (756 Tom Martin Drive, Birmingham, AL 35211; Product Code 7525 DLG 7A). PLA-PEG-OMe block co-polymer with a methyl ether terminated PEG block of approximately 5,000 Da and an overall inherent viscosity of 0.5 DL/g was purchased from Lakeshore Biochemicals (756 Tom Martin Drive, Birmingham, AL 35211; Product Code 100 DL mPEG 5000 5CE). EMPROVE® Polyvinyl Alcohol 4-88, USP (85-89% hydrolyzed, viscosity of 3.4-4.6 mPa·s) was purchased from EMD Chemicals Inc. (480 South Democrat Road Gibbstown, NJ 08027. Product Code 1.41350).

Method

Solutions were prepared as follows:

Solution 1: PLGA at 75 mg/mL, PLA-PEG-OMe at 25 mg/mL, and rapamycin at 12.5 mg/mL in methylene chloride. The solution was prepared by dissolving PLGA, PLA-PEG-OMe, and rapamycin in pure methylene chloride. Solution 2: Polyvinyl alcohol @ 50 mg/mL in 100 mM pH 8 phosphate buffer.

An oil-in-water emulsion was used to prepare the nanocarriers. The O/W emulsion was prepared by combining Solution 1 (1.0 mL) and Solution 2 (3.0 mL) in a small pressure tube and sonicating at 30% amplitude for 60 seconds using a Branson Digital Sonifier 250. The O/W emulsion was added to a beaker containing 70 mM pH 8 phosphate buffer solution and stirred at room temperature for 2 hours to allow the methylene chloride to evaporate and for the nanocarriers to form. A portion of the nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 75,600×g and 4 °C for 50 min, 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.

Nanocarrier size was determined by dynamic light scattering. The amount of rapamycin in the nanocarrier was determined by HPLC analysis. The total dry-nanocarrier mass per mL of suspension was determined by a gravimetric method.

Effective Diameter (nm)	Rapamycin Content (% w/w)
241	11.1

In order to monitor the determine the effect of immunosuppressant attached to synthetic nanocarriers on the development of CD4+ regulatory T cells, CD4+ T cells were
5 purified from the transgenic mouse strain OTII using negative selection by magnetic-activated cell sorting (MACS). OTII mice express a T cell receptor specific for the chicken ovalbumin peptide $OVA_{323-339}$. Following isolation, $4x10^6$ CD4+ OTII cells were transferred into SJL mice congenically expressing the pan-leukocyte marker CD45.1 (*Ptprc*[a]) (SJL-*Prprc*[a]/BoyAiTac). The recipient animals were left untreated (PBS injection) or treated on
10 days 1 and 5 by subcutaneous injection in the hind limbs with rapamycin containing nanocarriers alone (NP[Rapa]), free $OVA_{323-339}$ peptide (fOPII.323) or a combination of both rapamycin containing nanocarriers and $OVA_{323-339}$ peptide.

On day 10, 5 days following the second administration of the indicated treatment, the animlas were sacrified and the popliteal lymph nodes that drain the site of injection were
15 harvested to analyze developmental status and quantify the CD4+ OTII cells that had been transferred into the SJL mice by flow cytometry.

As shown in **Fig. 1**, the animals that were untreated (PBS) or only received rapamycin containing nanocarriers (NP[Rapa]) had few to undetectable levels of OTII cells that had acquired the regulatory T cell phenotype (CD25+Fox3p+) characterized by staining with
20 anti-CD25 and anti-Foxp3 antibodies. Administration of free OVA peptide (fOPII.323) resulted in a detectable but not statisitically significant increase in the proportion of CD25+Fox3p+ cells. In contrast, administration of rapamycin containing nanocarriers concomitantly with OVA peptide resulted in a robust population of CD25+ Fox3p+ cells indicating that the combination treatment induced a large proportion of the transferred CD4+
25 OTII to develop into regulatory T cells (Tregs).

These results show that immunosuppressants provided herein, when administered concomitantly with antigen can induced the formation of regulatory immune responses, such as an enhancement in the percentage of CD4+ regulatory T cells specific to the antigen.

Example 8: Evaluating Anti-PEG Immune Responses

Materials

Rapamycin was purchased from TSZ CHEM (185 Wilson Street, Framingham, MA 01702; Product Code R1017). PLGA with a lactide:glycolide ratio of 3:1 and an inherent viscosity of 0.75 dL/g was purchased from SurModics Pharmaceuticals (756 Tom Martin Drive, Birmingham, AL 35211; Product Code 7525 DLG 7A). PLA-PEG-OMe block co-polymer with a methyl ether terminated PEG block of approximately 5,000 Da and an overall inherent viscosity of 0.5 DL/g was purchased from Lakeshore Biochemicals (756 Tom Martin Drive, Birmingham, AL 35211; Product Code 100 DL mPEG 5000 5CE). EMPROVE® Polyvinyl Alcohol 4-88, USP (85-89% hydrolyzed, viscosity of 3.4-4.6 mPa•s) was purchased from EMD Chemicals Inc. (480 South Democrat Road Gibbstown, NJ 08027. Product Code 1.41350).

Method

Solutions were prepared as follows:

Solution 1: PLGA at 75 mg/mL, PLA-PEG-OMe at 25 mg/mL, and rapamycin at 12.5 mg/mL in methylene chloride. The solution was prepared by dissolving PLGA, PLA-PEG-OMe, and rapamycin in pure methylene chloride. Solution 2: Polyvinyl alcohol @ 50 mg/mL in 100 mM pH 8 phosphate buffer.

An oil-in-water emulsion was used to prepare the nanocarriers. The O/W emulsion was prepared by combining Solution 1 (1.0 mL) and Solution 2 (3.0 mL) in a small pressure tube and sonicating at 30% amplitude for 60 seconds using a Branson Digital Sonifier 250. The O/W emulsion was added to a beaker containing 70 mM pH 8 phosphate buffer solution and stirred at room temperature for 2 hours to allow the methylene chloride to evaporate and for the nanocarriers to form. A portion of the nanocarriers was washed by transferring the nanocarrier suspension to a centrifuge tube and centrifuging at 75,600×g and 4 °C for 50 min, 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.

Nanocarrier size was determined by dynamic light scattering. The amount of rapamycin in the nanocarrier was determined by HPLC analysis. The total dry-nanocarrier mass per mL of suspension was determined by a gravimetric method.

Effective Diameter (nm)	Rapamycin Content (% w/w)
238	10.6

C57BL/6 age-matched (5-6 weeks) females were injected i.v. in the tail vein weekly (days 0, 7, 14, 21, 28, 35, 42, 49) with 250μg of a conjugate of keyhole limpet hemocyanin
5 and polyethylene glycol (KLH-PEG) with (days 0, 7, 14, 21, 28) or without 0.47mg of rapamycin-containing nanocarriers for the first 5 injections (NP[Rapa], 50μg of rapamycin content). The following 3 injections consisted of just KLH-PEG in the same amounts. The IgM antibody response to PEG was monitored weekly (days 12, 20, 34, 40, 47, 54) in the blood of these animals.
10 As shown in **Fig. 2**, 5 doses of synthetic nanocarriers administered concomitantly with KLH-PEG in the same solution i.v. were effective in preventing antibody formation to PEG. These results show that rapamycin-containing nanocarriers administered concomitantly with a PEGylated protein can reduce or prevent antibody formation to PEG. Thus, use of a method of treatment as provided herein can be used to reduced an undesired immune
15 response against a therapeutic protein, such as pegylated therapeutic proteins. The data also demonstrate an administration schedule for achieving such effect.

Example 9: Enhancing Factor VIII-Specific CD4+ Tregs Using Synthetic Nanocarriers (Prophetic)

20 A pilot trial is performed on non-human primate subjects using soluble Factor VIII and the synthetic nanocarriers of Example 1, with the Factor VIII and synthetic nanocarriers not co-formulated prior to adminstration. 50 non-human primate subjects are randomly assigned to 5 arms: placebo, and then four dose levels of synthetic nanocarriers chosen for dose ranging. The dose ranging is established to select optimal enhancement of CD4+ Tregs
25 (CD4+ regulatory T cells) that will be Factor VIII-specific. On day zero, the subjects in each active arm all are administered the dose of synthetic nanocarriers subcutaneously, and within 24 hours of the synthetic nanocarrier dose get an infusion of a standard infusion dose of Factor VIII. Two weeks later, each animal is challenged with a standard dose of soluble Factor VIII, and the number or percentage of Factor VIII-specific CD4+ Tregs is measured
30 using standard techniques. The lowest dose of synthetic nanocarriers from among the four

active arms that shows significant enhancement in Factor VIII-specific CD4+ Tregs is selected as the test dose.

The test dose of synthetic nanocarriers is then allometrically scaled for administration to human subjects, and is used in a human clinical trial to determine a range of administration dose levels of synthetic nanocarriers used with standard doses of soluble Factor VIII. Again, the Factor VIII and synthetic nanocarriers are not co-formulated prior to administration. Non-coformulated administration doses of synthetic nanocarriers and Factor VIII are then made available for regular clinical practice.

Example 10: Enhancing Factor VIII-Specific CD4+ Tregs Using Synthetic Osmotic Pumps (Prophetic)

A pilot trial is performed on non-human primate subjects using soluble Factor VIII and osmotic pumps (prepared generally according to Example 6, but substituting GSK1059615 for the rapamycin of Example 6), with the Factor VIII and the osmotic pumps not co-formulated prior to adminstration. 50 non-human primate subjects are randomly assigned to 5 arms: placebo, and then four dose levels of GSK1059615 delivered by osmotic pump and chosen for dose ranging. The dose ranging is established to select optimal enhancement of CD4+ Tregs that will be Factor VIII-specific. On day zero, the subjects in each active arm all are administered the dose of synthetic nanocarriers subcutaneously, and within 24 hours of the synthetic nanocarrier dose get an infusion of a standard infusion dose of Factor VIII. Two weeks later, each animal is challenged with a standard dose of soluble Factor VIII, and the number or percentage of Factor VIII-specific CD4+ Tregs is measured using standard techniques. The lowest dose of GSK1059615 delivered by osmotic pump from among the four active arms that shows significant enhancement in Factor VIII-specific CD4+ Tregs is selected as the test dose.

The test dose of GSK1059615 delivered by osmotic pump is then allometrically scaled for administration to human subjects, and is used in a human clinical trial to determine a range of administration dose levels of GSK1059615 delivered by osmotic pump used with standard doses of soluble Factor VIII. Again, the Factor VIII and the osmotic pumps are not co-formulated prior to administration. Non-coformulated administration doses of GSK1059615 delivered by osmotic pump and Factor VIII are then made available for regular clinical practice.

Example 11: Enhancing Specific CD4+ Tregs Using Therapeutic Polynucleotides (Prophetic)

A pilot trial is performed on non-human primate subjects using asparaginase mmRNA (made generally according to prepared according to US Patent application 2013/0115272 to de Fougerolles et al. ("mmRNA")) and the synthetic nanocarriers of Example 1, with the mmRNA and the synthetic nanocarriers not co-formulated prior to adminstration. 50 non-human primate subjects are randomly assigned to 5 arms: placebo, and then four doses of synthetic nanocarriers chosen for dose ranging. The dose ranging is established to select optimal enhancement of CD4+ Tregs that will be mmRNA specific. On day zero, the subjects in each active arm all are administered the dose of synthetic nanocarriers subcutaneously, and within 24 hours of the synthetic nanocarrier dose get an infusion of a standard infusion dose of Factor VIII. Two weeks later, each animal is challenged with a standard dose of mmRNA, and the number or percentage of mmRNA specific CD4+ Tregs is measured using standard techniques. The lowest dose of synthetic nanocarriers from among the four active arms that shows significant enhancement in mmRNA-specific CD4+ Tregs is selected as the test dose.

The test dose of synthetic nanocarriers is then allometrically scaled for administration to human subjects, and is used in a human clinical trial to determine a range of administration dose of synthetic nanocarriers used with standard dose levels of mmRNA. Again, the mmRNA and the synthetic nanocarriers are not co-formulated prior to adminstration. Non-coformulated administration doses of synthetic nanocarriers and mmRNA are then made available for regular clinical practice.

Example 12: Evaluating Anti-PEG Immune Responses (Prophetic)

C57BL/6 age-matched (5-6 weeks) females are injected i.v. in the tail vein weekly (days 0, 7, 14, 21, 28, 35, 42, 49) with 250μg of a conjugate of keyhole limpet hemocyanin and polyethylene glycol (KLH-PEG) with (days 0, 7, 14, 21, 28) or without 0.47mg of nanocrystalline rapamycin for the first 5 injections. The following 3 injections consist of just KLH-PEG in the same amounts. The IgM antibody response to PEG is monitored weekly (days 12, 20, 34, 40, 47, 54) in the blood of these animals.

A reduction in the titer of KLH-specific IgM antibodies in the animals that receive doses of nanocrystalline rapamycin concomitantly with KLH-PEG as compared to animals that only receive KLH-PEG (and no nanocrystalline rapamycin) indicates that the

nanocrystalline form of rapamycin is able to reduce or prevent antibody formation when administered concomitantly with a PEGylated protein.

Example 13: Enhancing Factor VIII-Specific CD4+ Tregs Using Synthetic
5 **Nanocarriers (Prophetic)**

A pilot trial is performed on non-human primate subjects using soluble Factor VIII and the nanocrystalline rapamycin, with the Factor VIII and nanocrystalline rapamycin not co-formulated prior to adminstration. 50 non-human primate subjects are randomly assigned to 5 arms: placebo, and then four dose levels of nanocrystalline rapamycin chosen for dose
10 ranging. The dose ranging is established to select optimal enhancement of CD4+ Tregs (CD4+ regulatory T cells) that will be Factor VIII-specific. On day zero, the subjects in each active arm all are administered the dose of nanocrystalline rapamycin subcutaneously, and within 24 hours of the nanocrystalline rapamycin dose get an infusion of a standard infusion dose of Factor VIII. Two weeks later, each animal is challenged with a standard dose of
15 soluble Factor VIII, and the number or percentage of Factor VIII-specific CD4+ Tregs is measured using standard techniques. The lowest dose of nanocrystalline rapamycin from among the four active arms that shows significant enhancement in Factor VIII-specific CD4+ Tregs is selected as the test dose.

The test dose of nanocrystalline rapamycin is then allometrically scaled for administration to
20 human subjects, and is used in a human clinical trial to determine a range of administration dose levels of nanocrystalline rapamycin used with standard doses of soluble Factor VIII. Again, the Factor VIII and nanocrystalline rapamycin are not co-formulated prior to administration. Non-coformulated administration doses of nanocrystalline rapamycin and Factor VIII are then made available for regular clinical practice.

25

What is claimed is:

CLAIMS

1. A method comprising:

5 enhancing the number or percentage of CD4+ regulatory T cells by administering to a subject

(i) synthetic nanocarriers attached to immunosuppressants, and

(ii) therapeutic macromolecules,

wherein the therapeutic macromolecules are not co-formulated with the synthetic
10 nanocarriers attached to immunosuppressants prior to administration.

2. The method of claim 1, wherein the synthetic nanocarriers attached to immunosuppressants and therapeutic macromolecules are administered concomitantly to the subject.

15

3. The method of claim 1 or 2, wherein the administration is according to a protocol previously demonstrated to result in enhanced numbers or percentage of CD4+ regulatory T cells when the therapeutic macromolecules are not co-formulated with the synthetic nanocarriers prior to administration.

20

4. The method of any one of claims 1-3, wherein the method further comprises determining the protocol.

5. The method of any one of claims 1-4, wherein the method further comprises assessing
25 the the number or percentage of CD4+ regulatory T cells in the subject prior to and/or after the administration.

6. The method of any one of claims 1-5, wherein the enhanced number or percentage of CD4+ regulatory T cells is an increase of at least 2-fold, 3-fold, 4-fold, 5-fold or 6-fold as
30 compared to the number or percentage of CD4+ regulatory T cells prior to the administration.

7. The method of any one of claims 1-6, wherein the administering is by intravenous, intraperitoneal or subcutaneous administration.

8. The method of any one of claims 1-7, wherein the method further comprises recording an increase in the number or percentage of CD4+ regulatory T cells following the administration.

5

9. The method of any one of the preceding claims, wherein the immunosuppressant comprises a statin, an mTOR inhibitor, a TGF-β signaling agent, a corticosteroid, an inhibitor of mitochondrial function, a P38 inhibitor, an NF-κB inhibitor, an adenosine receptor agonist, a prostaglandin E2 agonist, a phosphodiesterase 4 inhibitor, an HDAC inhibitor or a
10 proteasome inhibitor.

10. The method of claim 9, wherein the mTOR inhibitor is rapamycin.

11. The method of any one of the preceding claims, wherein the therapeutic
15 macromolecule is a therapeutic protein or a therapeutic polynucleotide.

12. The method of claim 11, wherein the therapeutic protein is for protein replacement of protein supplementation therapy.

20 13. The method of claim 11, wherein the therapeutic protein comprises a/an infusible or injectable therapeutic protein, enzyme, enzyme cofactor, hormone, blood or blood coagulation factor, cytokine, interferon, growth factor, monoclonal antibody, polyclonal antibody, or protein associated with Pompe's disease.

25 14. The method of claim 13, wherein the infusible or injectable therapeutic protein comprises Tocilizumab, alpha-1 antitrypsin, Hematide, albinterferon alfa-2b, Thucin, tesamorelin, ocrelizumab, belimumab, pegloticase, taliglucerase alfa, agalsidase alfa, or velaglucerase alfa.

30 15. The method of claim 13, wherein the enzyme comprises an ocidforeductase, transferase, hydrolase, lysase, isomerase or ligase.

16. The method of claim 13, wherein the enzyme comprises an enzyme for enzyme replacement therapy for a lysosomal storage disorder.

17. The method of claim 16, wherein the enzyme for replacement therapy for a lysosomal storage disorder comprises imiglucerase, a-galactosidase A (a-gal A), agalsidase beta, acid α-glucosidase (GAA), alglucosidase alfa, LUMIZYME, MYOZYME, arylsulfatase B, laronidase, ALDURAZYME, idursulfase, ELAPRASE, arylsulfatase B, pegloticase, pegsiticase or NAGLAZYME.

18. The method of claim 13, wherein the cytokine comprises a lymphokine, interleukin, chemokine, type 1 cytokine or a type 2 cytokine.

19. The method of claim 13, wherein the blood or blood coagulation factor comprises Factor I, Factor II, tissue factor, Factor V, Factor VII, Factor VIII , Factor IX, Factor X, Factor Xa, Factor XII, Factor XIII, von Willebrand factor, prekallikrein, high-molecular weight kininogen, fibronectin, antithrombin III, heparin cofactor II, protein C, protein S, protein Z, protein Z-related protease inhibitor (ZPI), plasminogen, alpha 2-antiplasmin, tissue plasminogen activator (tPA), urokinase, plasminogen activator inhibitor-1 (PAI1), plasminogen activator inhibitor-2 (PAI2), cancer procoagulant or epoetin alfa.

20. The method of any one of the preceding claims, wherein a load of immunosuppressant attached to the synthetic nanocarriers, on average across the synthetic nanocarriers, is between 0.1% and 50%.

21. The method of claim 20, wherein the load is between 0.1% and 20%.

22. The method of any one of the preceding claims, wherein the synthetic nanocarriers comprise lipid nanoparticles, polymeric nanoparticles, metallic nanoparticles, surfactant-based emulsions, dendrimers, buckyballs, nanowires, virus-like particles or peptide or protein particles.

23. The method of claim 22, wherein the synthetic nanocarriers comprise lipid nanoparticles.

24. The method of claim 22, wherein the synthetic nanocarriers comprise liposomes.

25. The method of claim 22, wherein the synthetic nanocarriers comprise metallic
5 nanoparticles.

26. The method of claim 25, wherein the metallic nanoparticles comprise gold nanoparticles.

10 27. The method of claim 22, wherein the synthetic nanocarriers comprise polymeric nanoparticles.

28. The method of claim 27, wherein the polymeric nanoparticles comprise polymer that is a non-methoxy-terminated, pluronic polymer.

15

29. The method of claim 27 or 28, wherein the polymeric nanoparticles comprise a polyester, polyester attached to a polyether, polyamino acid, polycarbonate, polyacetal, polyketal, polysaccharide, polyethyloxazoline or polyethyleneimine.

20 30. The method of claim 29, wherein the polyester comprises a poly(lactic acid), poly(glycolic acid), poly(lactic-co-glycolic acid) or polycaprolactone.

31. The method of claim 29 or 30, wherein the polymeric nanoparticles comprise a polyester and a polyester attached to a polyether.

25

32. The method of any one of claims 29-31, wherein the polyether comprises polyethylene glycol or polypropylene glycol.

33. The method of any one of the preceding claims, wherein the mean of a particle size
30 distribution obtained using dynamic light scattering of the synthetic nanocarriers is a diameter greater than 100nm.

34. The method of claim 33, wherein the diameter is greater than 150nm.

35. The method of claim 34, wherein the diameter is greater than 200nm.

36. The method of claim 35, wherein the diameter is greater than 250nm.

5

37. The method of claim 36, wherein the diameter is greater than 300nm.

38. The method of any one of the preceding claims, wherein an aspect ratio of the synthetic nanocarriers is greater than 1:1, 1:1.2, 1:1.5, 1:2, 1:3, 1:5, 1:7 or 1:10.

10

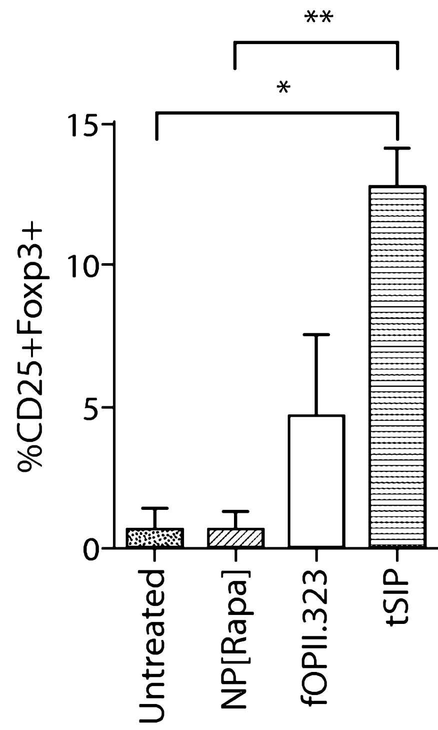


Fig. 1

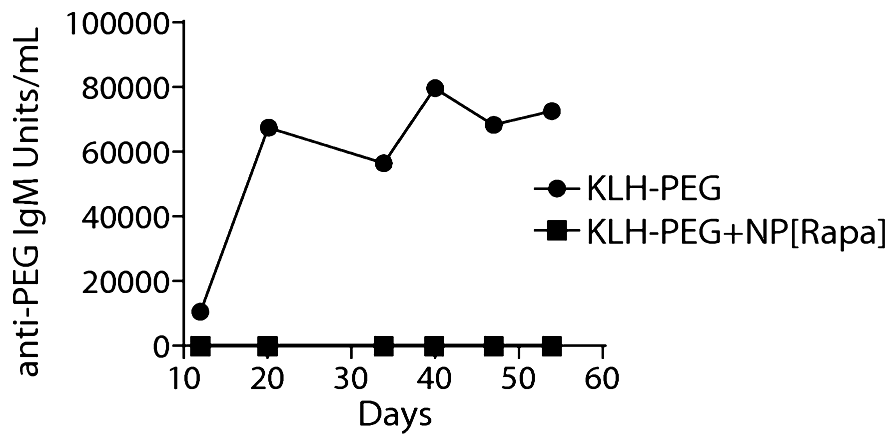


Fig. 2