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(71) Applicants: **THE CURATORS OF THE UNIVERSITY OF MISSOURI** [US/US]; 316 University Hall, Columbia, Missouri 65211 (US). **CORNELL UNIVERSITY** [US/US]; Center for Technology Licensing ("CTL") at Cornell University, 395 Pine Tree Road, Suite 310, Ithaca, New York 14850 (US).

(72) Inventors: **SHIRWAN, Haval**; c/o THE CURATORS OF THE UNIVERSITY OF MISSOURI, 316 University Hall, Columbia, Missouri 65211 (US). **YOLCU, Esma S.**; c/o THE CURATORS OF THE UNIVERSITY OF MISSOURI, 316 University Hall, Columbia, Missouri 65211 (US). **WANG, Yadong**; c/o CORNELL UNIVERSITY, 134 Hollister Dr., Ithaca, New York 14853 (US). **WRIGHT, Nathaniel S.**; c/o CORNELL UNIVERSITY, 290 Kimball Hall, Ithaca, New York 14850 (US).

(74) Agent: **BRINCKERHOFF, Courtenay C.** et al.; FOLEY & LARDNER LLP, 3000 K Street NW, Suite 600, Washington, District of Columbia 20007-5109 (US).

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(54) **Title:** CONTROLLED RELEASE OF THERAPEUTIC PROTEINS FOR IMMUNOMODULATION

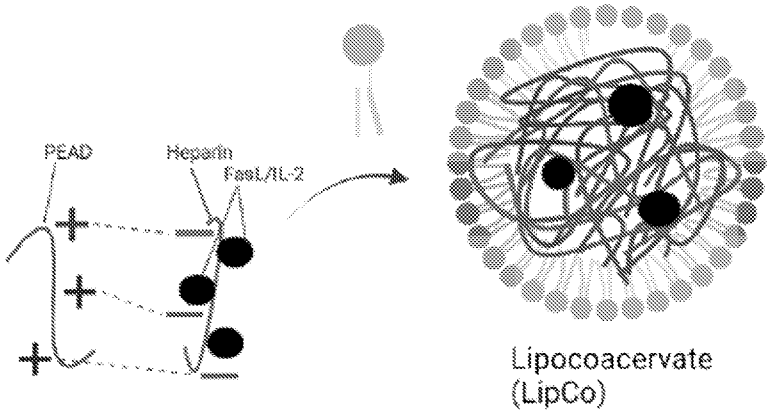

FIG. 1

(57) **Abstract:** Described herein are compositions and methods for inducing immune tolerance comprising lipocoacervates loaded with a FasL species and IL-2 protein for controlled release of biologically active FasL and IL-2 over an extended time period.

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[Continued on next page]

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— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

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CONTROLLED RELEASE OF THERAPEUTIC PROTEINS FOR IMMUNOMODULATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional application 63/469,260, filed May 26, 2023, the entire contents of which are incorporated herein by reference.

SEQUENCE LISTING

[0002] A Sequence Listing in XML format, named 136937-0103_SL, created April 22, 2024, with a size of 5031 bytes, is incorporated herein by reference.

GOVERNMENT GRANT SUPPORT CLAUSE

[0003] This invention was made with government support under W81XWH-21-1-0527 and W81XWH-21-1-0526 awarded by the Medical Research and Development Command. The government has certain rights in the invention.

TECHNICAL FIELD

[0004] Described herein are pharmaceutical compositions and their use for modulating the immune system, such as for the treatment of autoimmune diseases including Type 1 diabetes, and foreign graft rejection.

BACKGROUND

[0005] Type 1 diabetes (T1D) is a chronic, incurable autoimmune disorder impacting over 1.5 million people in the U.S. Exogenous insulin treatment is the standard of care for T1D, but is ineffective in preventing recurrent hyperglycemic episodes and associated chronic complications. Allogeneic islet transplantation (*e.g.*, transplantation of pancreatic islets from cadaver donors) is another treatment modality that has been shown to improve metabolic control and quality of life for diabetics. However, long-term graft survival requires continuous use of immunosuppressants to control rejection, which is not always effective and often has significant long-term adverse effects on the graft recipients, including higher incidence of cancer and infections. Induction of immune tolerance to allogeneic islets or beta cell products, such as stem cells or induced pluripotent stem cell (PSC)-derived products, offers a promise of significant advantages

including the possibility of obviating the need for chronic immunosuppression. Inventors Shirwan and Yolcu previously demonstrated that transient display of SA-FasL on the surface of biological membranes or biomaterials provides an effective means of immunomodulation, and also have shown that systemic administration of SA-FasL and IL-2 as soluble biologics has an immunomodulatory efficacy in the setting of islet and heart transplantation to induce specific immune tolerance. *See, e.g.,* WO 2018/165547 and U.S. Pre-Grant Publication 2018-0318394. However, systemic delivery requires repeated treatments with a potential for off-target effects.

[0006] Thus, there remains a need for pharmaceutical compositions of FasL and IL-2 that can be used in the treatment of autoimmune diseases including T1D and foreign graft rejection.

SUMMARY

[0007] Described herein are lipocoacervates and compositions comprising lipocoacervates, wherein the lipocoacervates are loaded with a chimeric FasL protein and/or an IL-2 protein, wherein the lipocoacervates comprises a lipid membrane encapsulating a coacervate and the chimeric FasL protein and/or IL-2 protein. The compositions may comprise lipocoacervates as described herein and a pharmaceutically acceptable carrier.

[0008] The chimeric FasL protein may comprise a FasL moiety and a streptavidin or avidin moiety. The chimeric FasL protein may comprise a FasL moiety comprising the extracellular domain of human FasL and a streptavidin moiety. The chimeric FasL protein may comprise a FasL moiety comprising the amino acid sequence of SEQ ID NO: 1. The chimeric FasL protein may comprise the amino acid sequence of SEQ ID NO: 2.

[0009] The IL-2 protein may comprise the amino acid sequence of SEQ ID NO: 3.

[0010] The lipid membrane may comprise a phospholipid, cholesterol, and a glycolipid. The lipid membrane comprises 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol), and cholesterol, optionally at a weight ratio of 5:1:4 DOPC:DSPG:cholesterol. The lipid membrane may comprise 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG), and cholesterol.

[0011] The coacervate may comprise polyelectrolytes, such as polycations and/or polyanions, such as poly(ethylene argininylaspartate diglyceride) (PEAD). The coacervate may comprise PEAD and heparin, optionally at a weight ratio of from 3:1 to 5:1, further optionally at a weight ratio of from 3.5:1 to 4.5:1, further optionally at a weight ratio of from 3.6:1 to 4.4:1, further optionally at a weight ratio of about 4:1 PEAD:heparin.

[0012] The polyelectrolyte (e.g., PEAD) may be biotinylated, in which case the streptavidin or avidin moiety of the chimeric FasL protein may be bound to the polyelectrolyte (e.g., PEAD) through a biotin-streptavidin (or biotin-avidin) linkage.

[0013] The lipocoacervate may comprises lipid membrane components comprising 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG), and cholesterol, optionally at a weight ratio of 5:1:4 DOPC:DSPG:cholesterol, and coacervate components comprising poly(ethylene argininylaspartate diglyceride) (PEAD) and heparin, optionally at a weight ratio of 3.6:1 PEAD:heparin. The chimeric FasL protein and/or IL-2 protein may be dispersed in the coacervate.

[0014] The composition may exhibit controlled release of the chimeric FasL protein and/or IL-2 protein, such as controlled release of the chimeric FasL protein over a period of time of 25 days, 30 days, or longer, and/or controlled release of the IL-2 protein over a period of time of 14 days, or longer.

[0015] FasL protein and/or IL-2 protein released from the lipocoacervate may exhibit biological activity, such as retaining at least 50% biological activity, or at least 75% biological activity.

[0016] Also described are lipocoacervates prepared by a process comprising (a) preparing a mixture comprising (i) one or both of the chimeric FasL protein and the IL-2 protein and (ii) heparin, and adding a polyelectrolyte such as a polycation such as PEAD (or biotinylated PEAD) to the mixture to obtain a coacervate mixture, and (b) adding to the coacervate mixture a lipid membrane composition comprising a phospholipid, cholesterol, and a glycolipid, to obtain a lipocoacervate suspension. One or both of the heparin and polyelectrolyte (e.g., PEAD or biotinylated PEAD) may be provided in saline. The lipid membrane composition of (b) may further comprise ethanol, and wherein the process further comprises centrifuging the

lipocoacervate suspension to remove excess ethanol in the supernatant, and, optionally resuspending the lipocoacervate.

[0017] Also described are processes for preparing a lipocoacervate loaded with one or both of a chimeric FasL protein and an IL-2 protein, comprising: (a) preparing a mixture comprising (i) one or both of the chimeric FasL protein and the IL-2 protein and (ii) heparin, and adding a polyelectrolyte such as a polycation such as PEAD (or biotinylated PEAD) to the mixture to obtain a coacervate mixture, and (b) adding to the coacervate mixture a lipid membrane composition comprising lipid membrane components comprising a phospholipid, cholesterol, and a glycolipid, to obtain a lipocoacervate suspension. One or both of the heparin and a polyelectrolyte (e.g., PEAD or biotinylated PEAD) may be provided in saline. The lipid membrane composition of (b) may further comprise ethanol, and wherein the process further comprises centrifuging the lipocoacervate suspension to remove excess ethanol in the supernatant, and, optionally resuspending the lipocoacervate. The lipid membrane components may comprise 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG), and cholesterol, optionally at a weight ratio of 5:1:4 DOPC:DSPG:cholesterol. The lipid membrane comprises 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG), and cholesterol. The lipocoacervate may be loaded with both a chimeric FasL protein and an IL-2 protein.

[0018] Also described are methods of inducing immune tolerance in a subject in need thereof, comprising administering to the subject a lipocoacervate or lipocoacervate composition as described herein.

[0019] Also described are compositions as described herein for use in inducing immune tolerance in a subject in need thereof, or treating Type 1 diabetes in a subject in need thereof.

[0020] Also described are uses of a composition as described herein in the preparation of a medicament for inducing immune tolerance in a subject in need thereof, or treating Type 1 diabetes in a subject in need thereof.

[0021] With regard to the methods, compositions for use, and uses, a composition comprising a lipocoacervate loaded with a chimeric FasL protein and a separate composition comprising a lipocoacervate loaded with IL-2 protein may be administered, or a composition comprising a lipocoacervate loaded with a chimeric FasL protein and a separate lipocoacervate loaded with IL-2 protein may be administered, or a composition comprising a lipocoacervate loaded with both a chimeric FasL protein and IL-2 protein may be administered.

[0022] With regard to the methods, compositions for use, and uses, the subject may be suffering from Type 1 diabetes. The subject may be suffering from an autoimmune condition. The subject may be an allograft or xenograft transplant patient, a patient being treated with a stem cell-derived product, insulin-producing beta cells, hepatocytes, or hematopoietic stem cells, or a subject at risk of or being treated for graft-versus-host disease.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] FIG. 1 is a schematic of a lipocoacervate vesicle as described herein, encapsulating a FasL species and/or IL-2.

[0024] FIGS. 2A and 2B shows release of IL-2 and SA-FasL, respectively, from lipocoacervate (grey) as described herein and from coacervate (black).

[0025] FIGS. 3A and 3B shows *in vitro* apoptotic activity of SA-FasL released from lipocoacervate as described herein (Lipo) as compared to coacervate (Cos). Data for soluble SA-FasL (not formulated in lipocoacervate or coacervate) (Ctrl) and cells alone also are shown (Cells/PBS). FIG. 3A shows results of SA-FasL released on day 1; FIG. 3B shows results of SA-FasL released on day 9.

[0026] FIG. 4 shows *in vitro* activity of IL-2 released from lipocoacervate on day 9 (in triplicate) as compared to IL-2 not formulated in lipocoacervate (control).

[0027] FIG. 5 illustrates an experimental design for *in vivo* assessment of immunomodulatory activity of lipocoacervate as described herein on alloreactive immune responses.

[0028] FIGS. 6A-6C show results of *in vivo* assessment of the immunomodulatory activity of lipocoacervate as described herein on alloreactive immune responses.

[0029] FIGS. 7A-7B show results of *in vivo* assessment of the tracking and release of SA-FasL from LipCo in BALB/C mice.

[0030] FIGS. 8A-8B show results of *in vivo* assessment of markers for liver, renal, or muscle damage after treatment with SA-FasL from LipCo in BALB/C mice.

DETAILED DESCRIPTION

[0031] The present disclosure relates to the surprising discovery that encapsulating Fas ligand (FasL) and IL-2 in lipocoacervate provides controlled release of FasL and IL-2 over an extended period of time, wherein the released FasL and IL-2 exhibit high potency (*e.g.*, retained biological activity) effective for long-term and specific immunosuppression, such as for the treatment of autoimmune diseases such as T1D, and prevention of foreign graft rejection, and also exhibit reduced toxicity.

[0032] $CD8^+$ and $CD4^+$ T effector cells, in particular $CD4^+$ T cells, play a critical role in the initiation and perpetuation of various autoimmune diseases, including T1D, and in foreign graft rejection, including rejection of allogeneic and xenogeneic grafts. Under normal physiological conditions, T effector cells (Teff) are kept in check by another class of T cells, designated as T regulatory cells (Treg). Accumulating scientific evidence implicates a dysregulated balance between Teff and protective Treg cells as the underlying cause of both T1D and allogeneic islet graft rejection. When activated, alloreactive Teff cells upregulate Fas on their surface and become sensitive to Fas ligand (FasL)-mediated apoptosis, which is critical to the induction of self-tolerance and maintenance. In contrast, Treg cells are relatively refractive to FasL-mediated apoptosis. IL-2 is required for the development and expansion of Treg cells.

[0033] Inventors Shirwan and Yolcu previously described the use of a soluble form of Fas ligand (SA-FasL) in combination with IL-2 to induce long-term and specific immunosuppression for the treatment of autoimmune diseases including T1D and foreign graft rejection. However, given the potency and pleiotropic effects of these two immune modulators, their systemic delivery may have off-target effects. The compositions described herein address this issue and provide pharmaceutical compositions comprising SA-FasL and/or IL-2 that can be used in methods for

modulating the immune system, such as for the treatment of autoimmune diseases including T1D and prevention of foreign graft rejection.

[0034] In the discussion that follows, specific embodiments of different aspects of the invention are described. It should be understood that any specific embodiment of one aspect may be used in conjunction with any specific embodiment of another aspect, even if every possible permutation and combination of specific embodiments is not expressly set forth individually. It also should be understood that each embodiment can be operated with every other embodiment, unless expressly stated otherwise. Particular details of various embodiments are set forth below to illustrate certain aspects, but not to limit the scope of the disclosure.

Definitions

[0035] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the presently-disclosed subject matter belongs.

[0036] For the purposes of the present disclosure, the following terms have these definitions:

[0037] As used herein "a," "an," "the," and "said" mean one or more, unless specifically indicated to mean only one.

[0038] As used herein, "about" when used with a numerical value means the numerical value stated as well as plus or minus 10% of the numerical value. For example, "about 10" should be understood as both "10" and "9-11".

[0039] As used herein, a phrase in the form "A/B" or in the form "A and/or B" means (A), (B), or (A and B); a phrase in the form "at least one of A, B, and C" means (A), (B), (C), (A and B), (A and C), (B and C), or (A, B, and C).

[0040] As used herein, "subject" denotes any mammal, including humans.

[0041] The terms "administer," "administration," and "administering" as used herein refer to providing, giving, dosing and/or prescribing, such as by a health professional or his or her authorized agent or under his or her direction, and putting into, taking, or consuming, such as by a health professional or the subject.

[0042] The terms "treat," "treating," and "treatment" as used herein include alleviating, abating, or ameliorating a disease or condition or one or more symptoms thereof, whether or not the disease or condition is considered to be "cured" or "healed," and whether or not all symptoms are resolved.

[0043] "Immune cell" as used herein includes any cell that is involved in the generation, regulation, or effect of the acquired or innate immune system. Immune cells include T cells such as CD4+ cells, CD8+ cells and various other T cell subsets, B cells, natural killer cells, macrophages, monocytes and dendritic cells, and neutrophils.

[0044] "Autoantigen" means a self antigen, that despite being a normal tissue constituent, is the target of a humoral or cell-mediated immune response by the host, as in autoimmune disease.

[0045] "Binding pair" refers to two molecules which interact with each other through any of a variety of molecular forces including, for example, ionic, covalent, hydrophobic, van der Waals, and hydrogen bonding, so that the pair have the property of binding specifically to each other. Specific binding means that the binding pair members exhibit binding to each other under conditions where they do not bind to another molecule. Examples of binding pairs are biotin-streptavidin, biotin-avidin, and the like. An exemplary binding pair is biotin and streptavidin (SA) or avidin. As used herein "biotin" includes biotin-containing moieties that are able to bind to surfaces, such as cell surfaces (including tumor cell surfaces), such as NHS-biotin and EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce). Such protein reactive forms of biotin are available commercially. The interaction between biotin and its binding partner, avidin or streptavidin, offers several advantages in the present context. For example, biotin has an extremely high affinity for both streptavidin (10^{13} M^{-1}) and avidin (10^{15} M^{-1}). Conjugates comprising streptavidin or avidin can be further complexed with conjugates comprising biotin. Additionally, both streptavidin and avidin are tetrameric polypeptides that each bind four molecules of biotin. Conjugates comprising streptavidin or avidin therefore have a tendency to form tetramers and higher structures, and can form complexes with multiple biotin-containing moieties.

[0046] SA or avidin fragments which retain substantial binding activity for biotin, such as at least 50% or more of the binding affinity of native SA or avidin, respectively, also may be used. Such fragments include "core streptavidin" ("CSA"), a truncated version of the full-length streptavidin polypeptide which may include streptavidin residues 13-138, 14-138, 13-139 or 14-

139. *See, e.g.*, Pahler et al., 1987, *J. Biol. Chem.*, 262: 13933-37. Other truncated forms of streptavidin and avidin that retain strong binding to biotin also may be used. *See, e.g.* Sano et al., 1995, *J. Biol. Chem.* 270(47): 28204-09 (describing core streptavidin variants 16-133 and 14-138) (U.S. patent no. 6,022,951). Mutants of streptavidin and core forms of streptavidin which retain substantial biotin binding activity or increased biotin binding activity also may be used. *See, e.g.*, Chilcoti et al., 1995, *Proc Natl Acad Sci U S A.* 92(5): 1754-58; Reznik et al., 1996, *Nat Biotechnol.* 14(8): 1007-11. For example, mutants with reduced immunogenicity, such as mutants mutated by site-directed mutagenesis to remove potential T cell epitopes or lymphocyte epitopes, can be used. *See* Meyer et al., 2001, *Protein Sci.* 10: 491-503. Likewise, mutants of avidin and core forms of avidin which retain substantial biotin binding activity or increased biotin binding activity also may be used. *See* Hiller et al., 1991, *J. Biochem.* 278: 573-85; Livnah et al., 1993, *Proc Natl Acad Sci USA* 90: 5076-80 (1993). For convenience, in the present description, the terms "avidin" and "streptavidin" as used herein are intended to encompass biotin-binding fragments, mutants and core forms of these binding pair members. Avidin and streptavidin are available from commercial suppliers. Moreover, the nucleic acid sequences encoding streptavidin and avidin and the streptavidin and avidin amino acid sequences can be found, for example, in GenBank Accession Nos. X65082; X03591; NM_205320; X05343; Z21611; and Z21554.

[0047] As used herein, unless otherwise indicated, the term "pharmaceutically acceptable" refers to those components and dosage forms that are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans or animals without undesirable or excessive toxicity, irritation, or other problem or complication, which may be commensurate with a reasonable benefit/risk ratio.

[0048] Other terms used herein may be defined elsewhere herein.

FasL and IL-2

[0049] Described herein are compositions and methods for inducing immune tolerance, using FasL and IL-2 loaded in lipocoacervate. The lipocoacervate compositions provide sustained release of biologically active FasL and IL-2 that exhibit reduced toxicity.

[0050] The FasL species may be a chimeric protein The chimeric protein may comprise at least a functional portion of a member of a binding pair, such as SA (or CSA) or avidin, operably linked

to at least a functional portion of FasL. As used herein, the term "functional portion" of FasL means an apoptosis-inducing portion. As used herein, "FasL moiety" means at least the apoptosis-inducing moiety of FasL. As used herein, "functional portion" of a member of a binding pair (such as SA or avidin) means a portion that retains substantial binding activity for its binding partner (such as biotin), such as at least 50% or more of the binding affinity of native SA (or CSA) or avidin for biotin, respectively.

[0051] The FasL moiety may be wild-type FasL (wtFasL) which includes any mammalian FasL, including rat, mouse, or human wild-type FasL, modified FasL (mFasL) which includes any mammalian FasL modified to be stably expressed on a cell surface, or soluble (sFasL) which includes the extracellular domain of any mammalian FasL. It should be noted that "soluble FasL" as used herein contains the apoptotic extracellular moiety of FasL, and is distinct from an unbound molecule derived from FasL that is unable to induce apoptosis.

[0052] In some embodiments, the FasL moiety comprises a human FasL sequence, such as an apoptosis-inducing portion of a human wild-type FasL, such as an extracellular domain of a human wild-type FasL.

[0053] In particular embodiments, a chimeric protein comprises the extracellular domain of FasL as the apoptosis-inducing molecule, *i.e.*, the FasL moiety. Independently, in particular embodiments, the chimeric protein comprises SA (or core streptavidin) as the binding pair member. In specific embodiments, a chimeric protein comprises an SA (or CSA) moiety and a FasL moiety. In some embodiments, the FasL moiety is positioned C-terminal relative to the binding pair member moiety. In other embodiments, the FasL moiety is positioned N-terminal relative to the binding pair member moiety. In some embodiments, the chimeric FasL protein forms tetramers and/or oligomers.

[0054] In some embodiments, the chimeric FasL protein comprises an extracellular domain of human FasL, such as SEQ ID NO:1):

I G H P S P P P E K K E L R K V A H L T G K S N S R S M P L E W E D T Y G I V L L S G V K Y K K G G L V I N E T G L Y F V Y S K V Y F R G Q S C N N L P L S H K V Y M R N S K Y P Q D L V M M E G K M M S Y C T T G Q M W A R S S Y L G A V F N L T S A D H L Y V N V S E L S L V N F E E S Q T F F G L Y K L (SEQ ID NO:1)

[0055] In some embodiments, the chimeric FasL protein has the amino acid sequence of SEQ ID NO: 2, which comprises the extracellular domain of human FasL C-terminus to a modified form of core streptavidin:
R S D Y K D D D D K G T I T G T W Y N Q L G S T F I V T A G A D G A L T G T Y E S A V G N A E S R Y V L T G R Y D S A P A T D G S G T A L G W T V A W K N N Y R N A H S A T T W S G Q Y V G G A E A R I N T Q W L L T S G A T E A N A W K S T L V G H D T F T K V K P S A A S S G G G G S G G G G S G E F I G H P S P P P E K K E L R K V A H L T G K S N S R S M P L E W E D T Y G I V L L S G V K Y K K G G L V I N E T G L Y F V Y S K V Y F R G Q S C N N L P L S H K V Y M R N S K Y P Q D L V M M E G K M M S Y C T T G Q M W A R S S Y L G A V F N L T S A D H L Y V N V S E L S L V N F E E S Q T F F G L Y K L (SEQ ID NO:2). This construct is referred to herein as SA-FasL.

[0056] As used herein, a chimeric protein that is not bound to a surface may be said to be "soluble." Thus, SA-FasL may be a soluble chimeric protein.

[0057] The IL-2 species may be isolated IL-2, recombinant IL-2, a chimeric form of IL-2, or another polypeptide or protein that contain an IL-2 moiety. An "IL-2 moiety" as used herein contains at least the part of IL-2 sufficient for binding to the IL-2 receptor and causing signaling.

[0058] The IL-2 moiety may be a wt IL-2 (including any mammalian IL-2, including rat, mouse, or human wild-type IL-2 molecule), mIL-2 (including any mammalian IL-2 modified to be stably expressed on the cell surface), or sIL-2 (including the soluble extracellular portion of any mammalian IL-2). In some embodiments, a construct comprising an IL-2 moiety is capable of binding through the IL-2 moiety to a cell expressing an IL-2 receptor.

[0059] In some embodiments, the IL-2 species is a soluble IL-2 species. As used herein, "soluble IL-2" or "sIL-2" means that the IL-2 is not bound to a surface (such as a cell). The soluble IL-2 may be a chimeric IL-2, or may be non-chimeric IL-2.

[0060] In some embodiments, the IL-2 species comprises the human IL-2 amino acid sequence of SEQ ID NO:3:

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCL EEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNR WITFCQSIISTLT (SEQ ID NO:3)

[0061] In some embodiments, the IL-2 species comprises SEQ ID NO:4, which includes additional C-terminal amino acids, including a His tag and an RGD peptide: APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCL EEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNR WITFCQSIISTLTGGPGHHHHHHGRGDNP (SEQ ID NO:4)

[0062] It is to be understood that these constructs are representative, and not limiting of chimeric proteins that can be used in accordance with the present disclosure.

[0063] A chimeric protein as described herein can be made by constructing a chimeric cDNA and expressing the chimeric cDNA in an appropriate host cell. As an example, a cell for the production of chimeric proteins as described herein may be the *Drosophila* system that is commercially available. Those skilled in the art of producing chimeric proteins will recognize that other expression systems and vectors are suitable for production of the chimeric proteins described herein, such as *Escherichia coli,* yeast, and mammalian cell cultures.

Lipocoacervates and Compositions

[0064] FIG. 1 is a schematic of a lipocoacervate vesicle as described herein. In the context of the present disclosure, lipocoacervates are lipid vesicles comprising a lipid membrane encapsulating a coacervate suitable for use as a vehicle for FasL and/or IL-2. In some embodiments, the lipid vesicles also encapsulate one or both of a FasL species (*e.g.*, SA-FasL) and IL-2 with the coacervate. As illustrated in the examples, the lipocoacervate may offer many specific advantages, such as protecting the FasL species and/or IL-2 species and preserving biological activity, providing controlled release of the FasL species and/or IL-2 species, increasing biocompatibility of the FasL species and/or IL-2 species, and decreasing off-target effects and toxicity associated with the FasL species and/or IL-2 species.

[0065] A lipocoacervate ("LipCo") (e.g., a LipCo droplet, a LipCo vesicle, or the like) typically comprises a coacervate phase (e.g., a droplet, a vesicle, or the like), and one or more lipid(s),

wherein the lipid(s) may be disposed on at least a portion of an exterior surface (or substantially all or all of the exterior surfaces) of the coacervate phase.

[0066] In the context of the present disclosure, the coacervate is an aqueous phase material with a high concentration of macromolecules (*e.g.*, polymers and proteins) that form liquid-liquid phase separated dense phase droplets dispersed in a dilute phase. The coacervate phase may be formed through charge interactions of polymers and/or proteins. The coacervate may include one or more polyelectrolytes, such as polycations and/or polyanions. In some embodiments, the coacervate comprises one or more cationic polymers, such as polycations synthesized from or comprising amino acids, such as poly(ethylene argininylaspartate diglyceride) (PEAD), and one or more proteins or anionic polymers, such as heparin, hyaluronic acid, chondroitin sulfate, dermatan sulfate, etc. The coacervate phase may have a predetermined surface charge, which may be a neutral charge, an approximately neutral charge, a positive charge, or a negative charge. Polyelectrolyte(s) and protein(s) may be used in any suitable ratio, such as a ratio that provides the predetermined net surface charge. In some embodiments, the coacervate comprises one or more cationic polymers and a protein, such as heparin. In some embodiments, the coacervate comprises PEAD and heparin. In some embodiments, the coacervate comprises PEAD and heparin in a weight ratio from about 3:1 to about 5:1 PEAD:heparin, such as a weight ratio from about 3.5:1 to about 4.5:1, including a weight ratio of about 3.6:1 to 4.4:1, including a weight ratio of about 4:1 PEAD:heparin. When a different polyelectrolyte and/or a different protein is used, the weight ratio of anionic polymer to protein can be adjusted to achieve a predetermined net surface charge.

[0067] In some embodiments, the LipCo includes a coacervate phase that is a simple coacervate phase including a single component (such as, for example, a multidomain polymer comprising two or more differently (e.g., oppositely) charged domains or the like). In other embodiments, the LipCo includes a coacervate phase that includes one or more cationic component(s) and one or more anionic component(s). For example, a cationic component may be a non-naturally-occurring polycation (e.g., synthetic polycation or the like) or a naturally-occurring polycation (such as, for example, chitosan or the like). The cationic component may be chosen from polycations (such as, for example, poly(ethylene argininylaspartate diglceride), chitosan, spermine, spermidine, positively-charged proteins, and the like, and any combination thereof),

and the like, or any combination thereof. For example, an anionic component may be a naturally-occurring polyanion or a biomolecule. In various embodiments, an anionic component may be a therapeutic component. The anionic component may be chosen from polyanions (such as, for example, glycosaminoglycans (e.g., heparin, hyaluronic acid, and the like), nucleic acids (such, as for example, RNAs, DNAs, and the like), negatively charged proteins, and the like, and any combination thereof), and the like, and any combination thereof. The mass ratio of cationic component to anionic component may be about 4 to about 1 to about 1 to about 4, including all 0.1 mass ratio values and ranges therebetween.

[0068] In some embodiments, the LipCo includes a coacervate phase that includes a biotinylated species, such as a biotinylated polyelectrolyte, such as biotinylated PEAD. In such cases, the biotinylated species, such as biotinylated PEAD, may be bound to an SA, CSA, or avidin moiety of the chimeric FasL protein. For example, the biotin moiety of biotinylated PEAD may bind to/be bound to the SA moiety of a chimeric SA-FasL protein. These embodiments may provide longer retention of the FasL species in the LipCo and/or slower release of the FasL species from the LipCo.

[0069] When present in the LipCo, the FasL species and/or IL-2 may be dispersed in the coacervate, as schematically illustrated in FIG. 1. As noted above, in some embodiments, a chimeric FasL species may be bound to a biotinylated component of the coacervate, such as biotinylated PEAD, through a biotin-streptavidin (or biotin-avidin) linkage.

[0070] The lipids may form a shell, membrane, or lipid membrane on at least a portion of the exterior surface of the coacervate phase forming the LipCo. In some embodiments, the shell, membrane, or lipid membrane encapsulates the coacervate phase. The shell or lipid membrane generally includes lipids (e.g., phospholipids), cholesterol, proteins, and glycolipids. Lipids may include one or more unsaturated lipid(s), one or more saturated lipid(s), or the like or any combination thereof. For example, a LipCo may include a shell, membrane, or lipid membrane including one or more unsaturated lipid(s) and one or more saturated lipid(s).

[0071] As used herein, "lipids" includes organic molecules such as fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterols, saccharolipids, and polyketides. Amphiphilic properties of lipids may promote formation of structures such as vesicles, liposomes, and membranes. The lipid(s) may be chosen from fats, oils, waxes, vitamins (such as, for example,

vitamin A, vitamin D, vitamin E, vitamin K, and the like), hormones, phospholipids (Phosphatidylglycerol (PG) including 18:0 PG, 17:0 PG, 16:0 PG, 18:2 PG, 20:4 PG etc, Phosphatidylserine (PS) including DOPS, 18:2 PS, 22:6 PS, 18:0 PS, 17:0 PS etc., Phosphatidic acid (PA) including 18:0 PA, 18:1 PA, 18:2 PA, 17:0 PA, 16:0 PA etc), glycerolipids, glycerophospholipids (Phosphatidylethanolamine (PE) including 18:0 PE, 17:0 PE, 10:0 PE, 16:1 PE, 18:2 PE etc. phosphatidylcholine (PC) including 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC), 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG)), sphingolipids (such as, for example, sphingomyelin, ceramides, and the like), sterols (such as, for example, cholesterol, or the like), and the like, and any combination thereof. Phospholipids include anionic phospholipids.

[0072] The lipid components may be selected to promote a desired vesicle rigidity, surface charge, or other properties of the LipCo. For example, DOPC exhibits high fluidity at body temperature and contributes to rigidity of the lipid vesicle. Additionally or alternatively, DPPC is a suitable component. In some embodiments, the lipid membrane comprises one or more or all of the following components: DOPC, DSPG, and DPPC, or a salt of any thereof. In some embodiments, the lipid membrane comprises one or more or all of the following components: DOPC, DSPG, and cholesterol. In some embodiments, the lipid membrane comprises one or more or all of the following components: DPPC, DSPG, and cholesterol. In some embodiments, a LipCo (or a LipCo shell) comprises cholesterol or the like. Without intending to be bound by any particular theory, it is considered cholesterol provides an increase in the of lipid packing, stabilizing the lipid shell (e.g., against structural damage or the like), or both.The components may be present in the lipid membrane in any suitable ratio, such as a ratio that achieves a lipid membrane with a neutral or approximately neutral charge. For example, in some embodiments, the lipid membrane comprises DOPC, DSPG and cholesterol at a molar ratio of 5:1:4 DOPC:DSPG:cholesterol.

[0073] A LipCo may include a coacervate to lipid(s) mass ratio of about 95:5 to about 99.9997:0.0003, including all 0.0001 mass ratio values and ranges therebetween (e.g., about 98:2

to about 99.9997:0.0003, about 99:1 to about 99.9997:0.0003, about 99.9:0.1 to about 99.9997:0003, about 99.99:0.01 to about 99.9997:0.0003, 98:2 to about 99.9995:0.0005, about 99:1 to about 99.9995:0.0005, about 99.9:0.1 to about 99.9995:0.0005, about 99.99:0.01 to about 99.9995:0.0005, 98:2 to about 99.999:0.001, about 99:1 to about 99.999:0.001, about 99.9:0.1 to about 99.999:0.001, or about 99.99:0.01 to about 99.999:0.001).

[0074] While not particularly limited, in some embodiments, the LipCo has a longest linear dimension (such as, for example, a diameter or the like) of about 100 nanometers (nm) to about 20 micrometers (μm), including all 0.1 nanometer values and ranges therebetween. For example, the lipocoacervate vesicles may have a diameter from about 0.01 μm to about 100 μm. While not particularly limited, in some embodiments, the lipocoacervate vesicles may have a substantially spherical shape (e.g., spherical) or the like. The longest linear dimension and vesicle shape may be determined by optical microscopy, electron microscopy, light scattering, or the like, or any combination thereof,

[0075] The LipCo may have a zeta potential of from about −4 millivolts (mV) to about +0.5 mV, including all 0.1 mV values and ranges therebetween. The LipCo may have a zeta potential of about 0 mV.

[0076] The LipCo may be stable. In various embodiments, a stable LipCo does not exhibit substantial or any aggregation (observable aggregation (e.g., by optical microscopy, electron microscopy, light scattering, or the like, or any combination thereof) or the like), a substantial change or any change (such as, for example, an observable change (e.g., by optical microscopy, electron microscopy, light scattering, or the like, or any combination thereof)) in size (such as, for example, a longest linear dimension (such as, for example, a diameter or the like) for at least one-week or more (e.g., at least two-weeks or more, at least three-weeks or more, at least four-weeks or more) at a temperature of about 4 degrees Celsius (°C), or both. In various embodiments, a stable composition does not exhibit a change (observable change (e.g., by optical microscopy, electron microscopy, light scattering, or the like, or any combination thereof) in LipCo size (such as, for example, average of the LipCos size) (such as, for example, a longest linear dimension (such as, for example, a diameter or the like) of greater than about 5%, greater than about 4%, greater than about 3%, greater than about 2%, greater than about 1%, greater than about 0.5%, or greater than about 0.1% ,or greater, 2% or greater, of the LipCos for at least one-

week or more (e.g., at least two-weeks or more, at least three-weeks or more, at least four-weeks or more) at a temperature of about 4 degrees Celsius (°C) or both.

[0077] As noted above and illustrated below, the LipCo can be loaded with a FasL species (*e.g.*, SA-FasL) and/or IL-2. "Loading" as used herein with respect to a therapeutic payload such as FasL or IL-2 refers to incorporation into the lipocoacervate vesicle, e.g., being encapsulated by the lipid membrane, such as being dispersed in the coacervate. Incorporation may be affected as the vesicle is formed, e.g., as a lipid membrane is formed around the coacervate components, as outlined below. While not particularly limited, the amount of FasL species and/or IL-2 species loaded in the lipocoacervate vesicles can range from a weight ratio of 0.001:1 to 4:1 FasL/IL-2 to heparin.

[0078] Thus, a LipCo as described herein may be loaded with a chimeric FasL protein, wherein the LipCo comprises a lipid membrane encapsulating a coacervate and the chimeric FasL protein (e.g., wherein the chimeric FasL protein is dispersed in the coacervate) wherein the chimeric FasL protein comprises a FasL moiety and a streptavidin or avidin moiety, such as SA-FasL. In some embodiments, the LipCo also encapsulates an IL-2 protein (e.g., wherein IL-2 also is dispersed in the coacervate). Additionally or alternatively, a LipCo as described herein may comprise a lipid membrane encapsulating a coacervate and IL-2 protein (e.g., wherein the chimeric IL-2 protein is dispersed in the coacervate). In various embodiments, one or both of chimeric FasL protein and chimeric IL-2 protein are sequestered or the like in the coacervate phase. A composition as described herein may comprise one or more or all of these types of LipCos.

[0079] In various embodiments, a LipCo further comprises a localization tag, which can be used to determine the location of a LipCo. In various emobdiments, a localization tag is a fluorescent tag (comprising one or more fluorophore(s) or the like) or the like.

[0080] In general, a method of forming a lipocoacervate (LipCo) comprises forming a lipid membrane around a coacervate such the lipid membrane encapsulates the coacervate. Thus, a method of making LipCos (or a LipCo composition) may comprise providing a coacervate composition (e.g., a coacervate solution or the like) and contacting the coacervate composition with a lipid composition, whereby the LipCos (or the LipCo composition) is formed.

[0081] The coacervate can be prepared by mixing its components (such as PEAD and heparin, and optionally a FasL species and/or an IL-2 species) in an aqueous vehicle. The aqueous vehicle may be, for example, water, saline, or phosphate-buffered saline (PBS). For example, a coacervate can be prepared comprising PEAD and heparin at a suitable ratio (such as a 4:1 weight ratio) in 0.9% normal saline. For encapsulation of the FasL species and/or IL-2, the FasL species and/or IL-2 may be added to the coacervate or coacervate components. For example, the FasL species and/or IL-2 may be combined with the heparin to form a mixture, and then that mixture may be combined with the PEAD to form a coacervate. Similar processes can be followed to prepare a coacervate that comprised biotinylated PEAD. Biotinylated PEAD can be prepared by any suitable method, such as by biotinylating PEAD at the amine group of the arginine residue of PEAD, e.g., by forming an amide bond between the carbonyl group of biotin and the amine group of the arginine. Following synthesis, biotinylated PEAD can be purified by any suitable method, such as dialysis. Similar processes can be followed to prepare biotinylated forms of other coacervate components.

[0082] In general, the lipid membrane is formed via self-assembly, such as may be driven by electrostatic interaction between negatively charged lipids and coacervate with a positive surface charge. In that regard, the coacervate may have a positive surface charge which may be tuned by adjusting the ratio of polyelectrolyte(s) and protein(s) (e.g., the ratio of PEAD:heparin). To form the lipid membrane, the lipid membrane components may be mixed with the coacervate. For example, a lipid mixture comprising DOPC, DSPG, and cholesterol in a suitable molar ratio (such as 5:1:4 DOPC:DSPG:cholesterol) may be added to the coacervate and mixed to form a lipid membrane. The resulting lipid membrane may have the same or substantially similar weight ratio of components as the lipid mixture.

[0083] As a non-limiting example, a lipocoacervate as described herein may be prepared by combining 400 μL of coacervate (5 mg/mL) and 10 μL of lipid components (10 mg/mL). This leads to a lipocoacervate with a 20:1 weight ratio and 40:1 volume ratio of lipid membrane components to coacervate.

[0084] As noted above, the FasL species (*e.g.*, SA-FasL) and IL-2 may be encapsulated in the same lipocoacervate, or may be encapsulated in different lipocoacervates. That is, a lipocoacervate may be loaded with one or both of a FasL species (*e.g.*, SA-FasL) and IL-2. A

composition as described herein may include one or more or all of (i) lipocoacervate loaded with a FasL species only (*e.g.*, SA-FasL), (ii) lipocoacervate loaded with IL-2 only, and (iii) lipocoacervate loaded with a FasL species (*e.g.*, SA-FasL) and IL-2. As noted above, while not particularly limited, the amount of FasL species and/or IL-2 species loaded in the lipocoacervate vesicles can range from a weight ratio of 0.001:1 to 4:1 FasL/IL-2 to heparin.

[0085] The lipocoacervates (LipCos) described herein may be formulated in a composition. The composition may be a solution (such as, for example, a saline solution or the like), an aqueous dispersion, or the like. For example, the composition may be a pharmaceutical composition. The composition may further include a pharmaceutically acceptable carrier for the intended route of administration, such as to prepare a pharmaceutical composition suitable for administration to a subject in need thereof. The composition may further comprise one or more additional pharmaceutically acceptable components, such as one or more conventional pharmaceutical additives such as buffers, tonicity agents, preservatives, excipients, etc. LipCos may be present in the composition at about 1 wt.% to about 80 wt.% (based on the total weight of the composition), including all 0.1 values and ranges therebetween (e.g., about l wt.% to about 75 wt.%, or about 5 wt.% to about 60 wt.%). In some embodiments, the composition does not include a hydrogel.

[0086] Some non-limiting examples of materials which can be used as pharmaceutically acceptable components in a composition include sugars, such as, for example, lactose, glucose, sucrose, and the like; starches, such as, for example, corn starch, potato starch, and the like; cellulose, and its derivatives, such as, for example, sodium carboxymethyl cellulose, ethyl cellulose, cellulose acetate, and the like; powdered tragacanth; malt; gelatin; talc; excipients, such as, for example, cocoa butter, suppository waxes, and the like; oils, such as, for example, peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil, soybean oil, and the like; glycols, such as, for example, propylene glycol and the like; polyols, such as, for example, glycerin, sorbitol, mannitol, polyethylene glycol, and the like; esters, such as, for example, ethyl oleate, ethyl laurate, and the like; agar; buffering agents, such as, for example, magnesium hydroxide, aluminum hydroxide, and the like; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations. (See, e.g., REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)).

[0087] In some embodiments, a composition as described herein may further comprise an alloantigen and/or an autoantigen. In some embodiments, a composition as described herein is administered with an alloantigen and/or an autoantigen, such as in methods comprising administration of a composition as described herein and transplantation of an alloantigen and/or an autoantigen, or in methods comprising cotransplantation of a composition as described herein and an alloantigen and/or an autoantigen. In some embodiments, the alloantigen is a cell. In particular embodiments, the cell is a mammalian cell. In certain embodiments, the alloantigen is a pancreatic islet cell, a tissue, or an organ. In other embodiments, the cell of the pharmaceutical composition is selected from the group consisting of islet cells, bone marrow cells, hematopoietic stem cells, stem cells, induced pluripotent stem cells, human beta cell products, hepatocytes, dendritic cells, peripheral blood mononuclear cells (PBMCs), macrophages, endothelial cells, mesenchymal stem cell, and immune cells, including T cells. In even further embodiments, the cell is part of a tissue or organ.

Methods of Treatment

[0088] Also described are uses of lipocoacervate compositions as described herein in methods for inducing immune tolerance that comprise administering FasL and IL-2 formulated in a lipocoacervate composition as described herein to a subject in need thereof.

[0089] As noted above and illustrated in the examples, administering FasL and IL-2 formulated in a lipocoacervate composition as described herein offers several advantages, including controlled release over a period of time on the order of 14 days or longer while maintaining biological activity at effective levels, while at the same time reducing toxicity. Indeed, as illustrated in the examples, SA-FasL formulated in the coacervate showed high toxicity that was not observed when SA-FasL was formulated in lipocoacervate. On the other hand, SA-FasL released from lipocoacervate after 9 days maintained greater biological activity than SA-FasL released from coacervate, while IL-2 released from lipocoacervate after 9 days did not show any loss of activity. Importantly, in an *in vivo* study, SA-FasL and IL-2 loaded lipocoacervate tilted the balance of alloreactive T cell responses in favor of Treg cells, supporting efficacy for inducing tolerance to auto and alloantigens. The unprecedented biocompatibility of lipocoacervate loaded with high doses of SA-FasL and IL-2 combined with its demonstrated efficacy in modulating alloreactive responses by physically depleting Teff cells and increasing Treg cells promises significant therapeutic advantages.

[0090] In some embodiments, the use or method comprises administering a chimeric FasL protein and IL-2 by administering a composition comprising a lipocoacervate loaded with (i) a chimeric FasL protein comprising a FasL moiety and a streptavidin or avidin moiety and/or (ii) an IL-2 protein. In some embodiments, the FasL protein and the IL-2 protein are loaded in the same lipocoacervate. In other embodiments, a lipocoacervate is loaded with only either the FasL species or IL-2. In some embodiments, a composition comprises lipocoacervate loaded with only either the FasL species or IL-2. In other embodiments, a composition comprises both FasL-loaded lipocoacervate and IL-2-loaded lipocoacervate (where the FasL and IL-2 may be loaded in the same or different lipocoacervates). When formulated in separate compositions, FasL-loaded lipocoacervate and IL-2-loaded lipocoacervate may be administered at substantially the same time or at different times, in any order.

[0091] The compositions may be administered by any suitable route of administration, including by subcutaneous injection, or by intraperitoneal, intramuscular, or intrapleural injection, or by administration into lymph nodes or pancreatic septa.

[0092] The uses and method described herein can be used to treat any mammal having a condition which is alleviated by apoptosis of activated pathogenic lymphocytes. In certain embodiments, the mammal has a condition alleviated by the apoptosis of activated pathogenic lymphocytes and the induction/expansion of protective lymphocytes, such as T regulatory cells. In other embodiments, the mammal has a genetically inherited hematopoietic metabolic disorder or cancer. In certain embodiments, the mammal has a condition selected from asthma, allergy, food poisoning, autoimmunity, and transplantation of allogeneic or xenogeneic cells, tissues, and organs. For example, bone marrow transplantation is a critical tool in the treatment of leukemia and other cancers. Stem cell transplants show promise to repair damaged or degenerated tissue, and therefore tolerance to donor stem cells is advantageous. Other situations where tolerance to donor tissues, organs or cells is advantageous include heart transplants, kidney transplants, liver transplants, vascular transplants, skin transplants, and blood transfusions. The methods described herein can be used in such contexts. In general, the uses and method described herein are useful in the context of allo- and xeno-cellular transplantation (e.g., bone marrow transplantation, hematopoietic stem cells transplantation, preventing or treating graft-vs-host disease,

administration of stem cell-derived beta cells, administration of hepatocytes, etc.), transplantation of tissues and solid organs, and treating other autoimmune diseases.

[0093] In more specific embodiments, the mammal has an autoimmune condition selected from Type 1 diabetes, multiple sclerosis, lupus erythematosis, sarcoidosis, Sjögren's syndrome, polymyalgia rheumatica, ankylosing spondylitis, alopecia areata, and rheumatoid arthritis. autoimmune hematological disorders (including e.g. hemolytic anaemia, aplastic anaemia, pure red cell anaemia and idiopathic thrombocytopenia), systemic lupus erythematosus, polychondritis, scleroderma, Wegener granulomatosis, dermatomyositis, chronic active hepatitis, myasthenia gravis, psoriasis, Steven-Johnson syndrome, idiopathic sprue, (autoimmune) inflammatory bowel disease (including e.g. ulcerative colitis and Crohn's disease), endocrine ophthalmopathy, Graves' disease, sarcoidosis, multiple sclerosis, primary biliary cirrhosis, juvenile diabetes (diabetes mellitus type I), uveitis (anterior and posterior), keratoconjunctivitis sicca and vernal keratoconjunctivitis, interstitial lung fibrosis, psoriatic arthritis, glomerulonephritis (with and without nephrotic syndrome, e.g. including idiopathic nephrotic syndrome or minimal change nephropathy) and juvenile dermatomyositis. Accumulating data indicate that many chronic diseases, such as Type 2 diabetes atherosclerosis, may be caused by immune dysregulation, i.e. pathogenic/regulatory cell imbalance. Autoimmune and inflammatory conditions of the skin are also considered to be amenable to treatment and prevention using the synergistic combination of the invention, e.g., psoriasis, contact dermatitis, atopic dermatitis, alopecia areata, erythema multiforma, dermatitis herpetiformis, scleroderma, vitiligo, hypersensitivity angiitis, urticaria, bullous pemphigoid, lupus erythematosus, pemphigus, epidermolysis bullosa acquisita, and other inflammatory or allergic conditions of the skin, as are inflammatory conditions of the lungs and airways including asthma, allergies, and pneumoconiosis.

[0094] The uses and methods described herein are particularly useful for treating T1D, as shown by the animal model examples below. The uses and methods described herein also are useful for the treatment or prevention of allograft or xenograft rejection.

[0095] In any methods, the subject also may be administered an alloantigen and/or an autoantigen as discussed above, which may be formulated in the same composition as the lipocoacervate, or which may be administered in a separate composition, including

administration by transplantation. As discussed above, in some embodiments, the alloantigen is a cell, such as a mammalian cell, including a pancreatic islet cell, a tissue, or an organ, such as islet cells, bone marrow cells, hematopoietic stem cells, stem cells, induced pluripotent stem cells, human beta cell products, hepatocytes, dendritic cells, peripheral blood mononuclear cells (PBMCs), macrophages, endothelial cells, mesenchymal stem cell, and immune cells, including T cells, or a part of a tissue or organ.

[0096] In any embodiments, the use or method may further comprise administering an effective amount of an immunomodulatory drug, such as an immunosuppressant. Exemplary immunomodulatory drugs include rapamycin and cyclophosphamide. Other non-limiting examples include busulfan, fludarabine, methotrexate, sulfasalazine, hydroxychloroquine, azathioprine, tocilizumab, etanercept, adalimumab, anakinra, abatacept, rituximab, certolizumab, golimumab, cyclosporine, dexamethasone, methylprednisolone, prednisone, and triamcinolone.

[0097] Absolute dosages of the FasL and IL-2 will vary depending on the individual, the route of administration, and the nature and severity of the condition to be treated. Generally speaking, the dosages of FasL and IL-2 reported in the examples, as used in mice, can be converted to human dosages according to the following table:

TO

FROM	**Mouse 20 g**	**Rat 150 g**	**Monkey 3 kg**	**Dog 8 kg**	**Man 60 kg**
Mouse	1	1/2	1/4	1/6	1/12
Rat	2	1	1/2	1/4	1/7
Monkey	4	2	1	3/5	1/3
Dog	6	4	1 2/3	1	1/2
Man	12	7	3	2	1

[0098] Generally speaking, the methods described herein using FasL may include administering FasL doses of from less than about 0.2 μg/day/patient to at least about 10 μg/day/patient, or more, based on the FasL moiety. For example, methods described herein may be carried out

using daily doses of FasL at amounts of less than about 0.2 μg/day/patient, about 0.2 μg/day/patient, about 0.5 μg/day/patient, about 1 μg/day/patient, about 1.5 μg/day/patient, about 2 μg/day/patient, about 2.5 μg/day/patient, about 3 μg/day/patient, about 3.5 μg/day/patient, about 4 μg/day/patient, about 4.5 μg/day/patient, about 5 μg/day/patient, or more.

[0099] Generally speaking, the methods described herein using IL-2 may include administering IL-2 at daily doses ranging from less than about 5000 IU/day/patient to 25000 IU/day/patient, or more, based on the IL-2 moiety. For example, methods described herein may be carried out using daily IL-2 doses of less than about 5000 IU/day/patient, of about 5000 IU/day/patient, about 10000 IU/day/patient, about 15000 IU/day/patient, about 25000 IU/day/patient, or more. Typically, doses < 25000 IU/day/patient will be used.

[0100] As noted above, these dosages are illustrative only. The dosage and schedule of administration may vary within and even from these ranges depending on the aim of the treatment, the disease to be treated, the specific subject, etc.

[0101] In some embodiments, the use or method comprises repeated administrations over an extended period of time, such as administration once weekly, once every other week, once every three weeks, or once a month over an extended period of time of one month, three months, 6 months, 9, months, 12 months, or longer. As with dosage, the duration of treatment may vary within and even from these ranges depending on the aim of the treatment, the disease to be treated, the specific subject, etc.

Kits

[0102] In another aspect, the present disclosure provides kits. Non-limiting examples of kits are provided herein.

[0103] In various embodiments, a kit comprises a lipocoacervate or lipocoacervates and/or composition(s) of the present disclosure and/or one or more starting material(s) for any of same. In various embodiments, a kit includes a closed or sealed package that comprises the lipocoacervate or lipocoacervates and/or the composition(s). In various embodiments, the package comprises one or more closed or sealed vials, bottles, blister (bubble) packs, or any other suitable packaging for the sale, distribution, or use of the lipocoacervate(s) and/or the composition(s) and/or starting material(s). The printed material may include printed information.

The printed information may be provided on a label, on a paper insert, printed on a packaging material, or the like. The printed information may include information that identifies the lipocoacervate(s) and/or the composition(s) and/or starting material(s) in the package, the amounts and types of other active and/or inactive ingredients in the lipocoacervate(s) and/or the composition(s) and/or starting material(s), and instructions for taking (e.g., administration or the like) the lipocoacervate(s) and/or the composition(s) and/or starting material(s). The instructions may include information, such as, for example, the number of doses to take over a given period of time, and/or information directed to a pharmacist and/or another health care provider, such as, for example, a physician or the like, or a patient. The printed material may include an indication or indications that the lipocoacervate(s) and/or the composition(s) and/or starting material(s) and/or any other agent provided therein is for treatment of an individual. In various examples, the kit includes a label describing the contents of the kit and providing indications and/or instructions regarding use of the contents of the kit to treat an individual.

Exemplary Embodiments

[0104] Embodiment 1. A composition comprising a lipocoacervate loaded with a chimeric FasL protein, wherein the lipocoacervate comprises a lipid membrane encapsulating a coacervate and the chimeric FasL protein, wherein the chimeric FasL protein comprises a FasL moiety and a streptavidin or avidin moiety.

[0105] Embodiment 2. The composition according to Embodiment 1, wherein the chimeric FasL protein comprises a FasL moiety and a streptavidin moiety.

[0106] Embodiment 3. The composition according to Embodiment 1 or Embodiment 2, wherein the chimeric FasL protein comprises a FasL moiety comprising the extracellular domain of human FasL and a streptavidin moiety.

[0107]Embodiment 4. The composition according to any one of Embodiments 1-3, wherein the chimeric FasL protein comprises a FasL moiety comprising the amino acid sequence of SEQ ID NO: 1.

[0108] Embodiment 5. The composition according to any one of Embodiments 1-4, wherein the chimeric FasL protein comprises the amino acid sequence of SEQ ID NO: 2.

[0109] Embodiment 6. The composition according to any one of Embodiments 1-5, further comprising an IL-2 protein encapsulated within the lipid membrane.

[0110] Embodiment 7. A composition comprising a lipocoacervate loaded with an IL-2 protein, wherein the lipocoacervate comprises a lipid membrane encapsulating a coacervate and the IL-2 protein.

[0111] Embodiment 8. The composition according to Embodiment 6 or Embodiment 7, wherein the IL-2 protein comprises the amino acid sequence of SEQ ID NO: 3.

[0112] Embodiment 9. The composition according to any one of the preceding Embodiments, wherein the lipid membrane comprises a phospholipid, cholesterol, and a glycolipid.

[0113] Embodiment 10. The composition according to any one of the preceding Embodiment, wherein the lipid membrane comprises 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol), and cholesterol, optionally at a weight ratio of 5:1:4 DOPC:DSPG:cholesterol.

[0114] Embodiment 11. The composition according to any one of Embodiments 1-9, wherein the lipid membrane comprises 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG), and cholesterol.

[0115] Embodiment 12. The composition according to any one of the preceding Embodiment, wherein the coacervate comprises poly(ethylene argininylaspartate diglyceride) (PEAD) and heparin, optionally at a weight ratio of from 3:1 to 5:1, further optionally at a weight ratio of from 3.5:1 to 4.5:1, further optionally at a weight ratio of from 3.6:1 to 4.4:1, further optionally at a weight ratio of about 4:1 PEAD:heparin.

[0116] Embodiment 13. The composition according to any one of the preceding Embodiments, wherein the coacervate comprises a biotinylated component, such as biotinylated PEAD, optionally wherein the streptavidin or avidin moiety of the chimeric FasL protein is bound to the biotinylated component, such as biotinylated PEAD, through a biotin-streptavidin or biotin-avidin linkage, respectively.

[0117] Embodiment 14. The composition according to any one of Embodiments 1-10 or 12-13, wherein the lipocoacervate comprises: (i) lipid membrane components comprising 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG), and cholesterol, optionally at a weight ratio of 5:1:4 DOPC:DSPG:cholesterol, and (ii) coacervate components comprising poly(ethylene argininylaspartate diglyceride) (PEAD) or biotinylated PEAD and heparin, optionally at a weight ratio of 3.6:1 PEAD:heparin or 4:1 PEAD:heparin..

[0118] Embodiment 15. The composition according to Embodiment 14, wherein the chimeric FasL protein and/or IL-2 protein are dispersed in the coacervate. **[0119]** Embodiment 16. The composition according to any one of the preceding Embodiments, further comprising a pharmaceutically acceptable carrier.

[0120] Embodiment 17. The composition according to any one of the preceding Embodiments, wherein the composition exhibits controlled release of the chimeric FasL protein and/or IL-2 protein, optionally wherein the composition exhibits controlled release of the chimeric FasL protein over a period of time of 25 days, further optionally wherein the composition exhibits controlled release of the IL-2 protein over a period of time of 14 days or more.

[0121] Embodiment 18. The composition according to any one of the preceding Embodiments, wherein the FasL protein and/or IL-2 protein released from the lipocoacervate exhibits biological activity, optionally wherein FasL protein and/or IL-2 protein released from the lipocoacervate retains at least 50% of its biological activity, optionally wherein FasL protein and/or IL-2 protein released from the lipocoacervate retains at least 75% of its biological activity.

[0122] Embodiment 19. The composition according to any one of the preceding Embodiments, prepared by a process comprising: (a) preparing a mixture comprising (i) one or both of the chimeric FasL protein and the IL-2 protein and (ii) heparin, and adding PEAD to the mixture to obtain a coacervate mixture, and (b) adding to the coacervate mixture a lipid membrane composition comprising a phospholipid, cholesterol, and a glycolipid, to obtain a lipocoacervate suspension, optionally wherein the PEAD is biotinylated PEAD.

[0123] Embodiment 20. The composition according to Embodiment 19, wherein one or both of the heparin and PEAD (or biotinylated PEAD) are provided in saline.

[0124] Embodiment 21. The composition according to Embodiment 19 or 20, wherein the lipid membrane composition of (b) further comprises ethanol, and wherein the process further comprises centrifuging the lipocoacervate suspension to remove excess ethanol in the supernatant, and, optionally resuspending the lipocoacervate.

[0125] Embodiment 22. A process for preparing a lipocoacervate loaded with one or both of a chimeric FasL protein and an IL-2 protein, comprising (a) preparing a mixture comprising (i) one or both of the chimeric FasL protein and the IL-2 protein and (ii) heparin, and adding PEAD to the mixture to obtain a coacervate mixture, optionally wherein the PEAD is biotinylated PEAD, and (b) adding to the coacervate mixture a lipid membrane composition comprising lipid membrane components comprising a phospholipid, cholesterol, and a glycolipid, to obtain a lipocoacervate suspension.

[0126] Embodiment 23. The process according to Embodiment 22, wherein one or both of the heparin and PEAD (or biotinylated PEAD) are provided in saline.

[0127] Embodiment 24. The process according to Embodiment 22 or Embodiment 23, wherein the lipid membrane composition of (b) further comprises ethanol, and wherein the process further comprises centrifuging the lipocoacervate suspension to remove excess ethanol in the supernatant, and, optionally resuspending the lipocoacervate.

[0128] Embodiment 25. The process according to any one of Embodiments 22-24, wherein the lipid membrane components comprise 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG), and cholesterol, optionally at a weight ratio of 5:1:4 DOPC:DSPG:cholesterol.

[0129] Embodiment 26. The process according to any one of Embodiments 22-25, wherein the lipid membrane comprises 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG), and cholesterol.

[0130] Embodiment 27. The process according to any one of Embodiments 22-26, wherein the lipocoacervate is loaded with both a chimeric FasL protein and an IL-2 protein.

[0131] Embodiment 28. A method of inducing immune tolerance in a subject in need thereof, comprising administering to the subject a composition according to any one of Embodiments 1-21.

[0132] Embodiment 29. The method according to Embodiment 28, wherein the method comprises administering a composition comprising a lipocoacervate loaded with a chimeric FasL protein and administering a composition comprising a lipocoacervate loaded with IL-2 protein.

[0133] Embodiment 30. The method according to Embodiment 28, wherein the method comprises administering a composition comprising a lipocoacervate loaded with a chimeric FasL protein and a lipocoacervate loaded with IL-2 protein.

[0134] Embodiment 31. The method according to Embodiment 28, wherein the method comprises administering a composition comprising a lipocoacervate loaded with a chimeric FasL protein and IL-2 protein.

[0135] Embodiment 32. The method according to any one of Embodiments 28-31, wherein the subject is suffering from Type 1 diabetes.

[0136] Embodiment 33. The method according to any one of Embodiment 28-31, wherein the subject is suffering from an autoimmune condition.

[0137] Embodiment 34. The method according to any one of Embodiments 28-31, wherein the subject is an allograft or xenograft transplant patient, a patient being treated with a stem cell-derived product, insulin-producing beta cells, hepatocytes, or hematopoietic stem cells, or a subject at risk of or being treated for graft-versus-host disease.

[0138] Embodiment 35. A composition according to any one of Embodiments 1-21, for use in inducing immune tolerance in a subject in need thereof, optionally wherein the subject is suffering from Type 1 diabetes, the subject is suffering from an autoimmune condition, the subject is an allograft or xenograft transplant patient, the subject is a patient being treated with a

stem cell-derived product, insulin-producing beta cells, hepatocytes, or hematopoietic stem cells, or the subject is at risk of or being treated for graft-versus-host disease.

[0139] Embodiment 36. A composition according to any one of claims Embodiments 1-21, for use in treating Type 1 diabetes.

[0140] Embodiment 37. Use of a composition according to any one of Embodiments 1-21, in the preparation of a medicament for inducing immune tolerance in a subject in need thereof, optionally wherein the subject is suffering from Type 1 diabetes, the subject is suffering from an autoimmune condition, the subject is an allograft or xenograft transplant patient, the subject is a patient being treated with a stem cell-derived product, insulin-producing beta cells, hepatocytes, or hematopoietic stem cells, or the subject is at risk of or being treated for graft-versus-host disease.

EXAMPLES

[0141] The following examples are given by way of illustration and are in no way intended to limit the scope of the present disclosure.

EXAMPLE 1: Construction and Expression of SA-FasL

[0142] The SA-FasL construct having SEQ ID NO:2 was prepared as outlined below.

[0143] The construct was designed to include a 5' BglII site followed by a FLAG tag, KpnI restriction site, core streptavidin, a linker sequence, EcoRI site, extra cellular domain of human FasL, and a 3' Xho restriction site. Genomic DNA was isolated from *Streptomyces avidinii* (ATCC Cat.# 27419) and 0.2 μg of this DNA was used as template for PCR amplification using primers specific for the 5'-end and 3'-end of core streptavidin. The construct was cloned in frame with the *Drosophila* secretion signal (BiP) in vector pMT/BiP/V5-His for expression in the DES™ system (Invitrogen) as a secreted protein and purified using immunoaffinity columns.

[0144] *Drosophila* S2 cells were transfected with 1-2 μg of expression vector pMT/BiP/V5-His containing the SA-FasL recombinant gene in frame with the BiP secretion signal using the CellFectin II Transfection kit according to the manufacturer's instructions (Gibco). Stable transfectants were established by cotransfection with 0.1 μg of vector pCoHYGRO and

maintained in the presence of 300 μg/ml of hygromycin. SA-FasL expression was induced with 0.6-1 mM copper sulfate. Supernatant was collected 1-4 days after induction, purified using FLAG-tag immunoaffinity columns (QIAGEN, Valencia, CA), and dialyzed against PBS. The concentration of purified SA-FasL was determined by the Bradford method.

EXAMPLE 2: Preparation and formulation of coacervates and lipocoacervates

[0145] Coacervate was prepared by mixing poly(ethylene argininylaspartate diglyceride) (PEAD) and heparin. Desired amounts of SA-FasL and IL-2 proteins were mixed with heparin for loading into coacervate formulations. Coacervate was formed at a 3.6:1 mass ratio of PEAD and heparin, respectively.

[0146] Lipocoacervate was prepared by mixing lipids with the coacervate. Lipocoacervate was formulated at a 4.0:1 weight ratio with PEAD and heparin and a lipid mixture (5:1:4, DOPC:DSPG:Cholesterol, 10 mg/mL) at a 40:1 volume ratio, respectively. The formulations were incubated at room temperature for 1 hour while rotated, followed by centrifugation to pellet the lipocoacervates. Pellets were then resuspended prior to use.

EXAMPLE 3: Release of SA-FasL and IL-2 from lipocoacervate

[0147] Coacervate and lipocoacervate were prepared as described above loaded with 5 μg of IL-2 and 15 μg of SA-FasL, and incubated in triplicate at 37°C in 200 μl saline supplemented with 0.1% BSA. Coacervates and lipocoacervates were pelleted by centrifugation to collect the supernatant every 24 hours and resuspended in fresh saline. Amounts of released protein were assessed using ELISA specific for each protein. Results are reported in FIGS. 2A and 2B.

[0148] As seen in the figures, the lipocoacervate and coacervate formulations showed comparable sustained release of SA-FasL and IL-2 over 25 days. IL-2 had a faster release kinetics with 80% of the protein released within 2 weeks (in FIG. 2A, the data points in the top row after 10 days relate to the IL-2 coacervate formulation, and the data points in the bottom row after 10 days relate to the IL-2 lipocoacervate formulation), whereas SA-FasL showed slower kinetics with about 18% of total protein being released by day 25 (in FIG. 2B, the data points in the top row after 20 days relate to the SA-FasL lipocoacervate formulation, and the data points in the bottom row after 20 days relate to the SA-FasL coacervate formulation).

EXAMPLE 4: Apoptotic activity of SA-FasL released from lipocoacervate

[0149] SA-FasL released from coacervate or lipocoacervate on various days was assessed for apoptotic function using a human T cell line (Jurkat). Cell culture was supplemented with the indicated doses of SA-FasL released from coacervate (Cos) or lipocoacervate (Lipo) formulations, incubated in a CO_2 incubator at 37°C for 18 hours, and analyzed for live and dead cells using flow cytometry to determine the percentage of apoptotic cells. Cultures without protein (Cells alone) and those with SA-FasL (Ctrl (SA-FAsL)) were used as controls. Results are shown in FIGS. 3A (Day 1) and 3B (Day 9). As seen in the figures, SA-FasL released from coacervate exhibited 50% loss of activity, while SA-FasL released from lipocoacervate exhibited a 25% loss of activity.

EXAMPLE 5: Proliferative function of IL-2 released from lipocoacervate

[0150] IL-2 released from lipocoacervate was assessed for proliferative function using CTLL-2 cells that require this cytokine for growth. CTLL-2 cells were seeded in 96-titer plates in triplicates with the indicated units of IL-2 control and IL-2 released from lipocoacervate on day 9. Units were calculated based on the amount of IL-2 protein released. Cells were incubated in a CO_2 incubator at 37°C for 20 hours and pulsed with ^{3}H-thymidine for 7.5 additional hours. Cells were harvested and DNA associated radioactivity was determined using a scintillation count and graphed as count per minute ± SEM of triplicate wells. Results are shown in FIG. 4. As seen in the figure, the IL-2 released from lipocoacervate on day 9 showed no loss of activity as compared with the control IL-2.

EXAMPLE 6: Toxicity profile of lipocoacervate formulations *in vivo*.

[0151] Coacervate and lipocoacervate formulations were loaded with amounts of SA-FasL and IL-2 as indicated in the following table and resuspended in 100 µL of fibrinogen (100 mg/mL) and 5 µL thrombin (5 mg/mL), and immediately injected intraperitoneally into mice. Animals were monitored for signs of toxicity and adverse events.

Group	IL-2 (µg)	FasL (µg)	Lipid	Survival
1	0	0	N/A	1/1
2	5	0	N/A	2/2
3	5	10	N/A	0/1

4	5	12	N/A	0/1
5	5	15	N/A	0/1
6	5	10	DOPC	1/1
7	5	12	DOPC	2/2
8	5	15	DOPC	1/1

[0152] Mice injected with coacervate loaded with 10 µg of SA-FasL and 5 µg of IL-2 expired due to acute toxicity. In marked contrast, animals injected with lipocoacervate loaded with the same amount of IL-2 and 15 µg of SA-FasL survived without visible adverse effects. Injection of 4 µg of soluble SA-FasL and 6 µg of IL-2 (not formulated in coacervate or lipocoacervate) resulted in acute toxicity with 2 treated mice expiring within 48 hours.

EXAMPLE 7: Immunosuppressant activity of SA-FasL and IL-2 loaded lipocoacervate

[0153] FIG. 5 depicts an experimental design for *in vivo* assessment of immunomodulatory activity of lipocoacervate loaded with SA-FasL and IL-2 on alloreactive immune responses. C57BL/6.FoxP3^{DTRGFP} mice expressing GFP under the control of FoxP3 were challenged subcutaneously with 20 million live allogeneic BALB/c splenocytes and 24 hours later were treated subcutaneously with lipocoacervate loaded with 4 µg SA-FasL and 6 µg IL-2, or with blank lipocoacervate. A group without splenocyte challenge was treated with PBS as control. Mice were euthanized 4 days after lipocoacervate treatment to collect spleen, injection site draining lymph nodes (dLN) and contralateral lymph nodes (cLN). Spleens from BALB/c mice were harvested and processed into single-cell suspension using frosted slides. Red blood cells were lysed using a home-made buffered ammonium chloride solution. Single cells prepared from these tissues were stained with fluorescence labeled antibodies to various cell surface markers and analyzed using flow cytometry. Percentage of $CD4^+$ Teff ($CD4^+CD44^{hi}CD62L^{lo}$), $CD8^+$ Teff ($CD8^+CD44^{hi}CD62L^{lo}$), and Treg ($CD4^+CD25^{high}GFP^+$) and ratios of Treg:Teff are graphed. Data expressed as mean ± SEM of 3 independent experiments and compared using one-way ANOVA. *$p < 0.05$, **$p < 0.001$. Results are shown in FIGS. 6A-6C.

[0154] As seen in the figures, there was a significant reduction in the percentage of $CD4^+$ Teff ($CD44^{hi}CD62L^{lo}$) cells in the spleen and lymph nodes of mice treated with lipocoacervate loaded with SA-FasL and IL-2 as compared to blank lipocoacervate. There was also reduction in CD8+ Teff ($CD44^{hi}CD62L^{lo}$) cells in the spleen and lymph nodes, the difference in spleen being statistically significant. Importantly, lipocoacervate loaded with both proteins increased the

percentage of Treg ($CD4^{-}CD25^{high}$ GFP^{+}) cells in the spleen and lymph nodes that translated to higher ratio of Treg to both $CD4^{+}$ Teff and $CD8^{+}$ Teff for all 3 tissues.

EXAMPLE 8: Tracking and release of SA-FasL from LipCo *in vivo*

[0155] FIGS. 7A-7B show results of *in vivo* assessment of the tracking and release of SA-FasL from LipCo in BALB/C mice. FIG. 7A displays representative images of *in vivo* tracking data using cyanine-5 labeled heparin and Alexa Fluor 750 labeled SA-FasL. Fibrin hydrogel was injected subcutaneously into the flank of BALB/C mice with labeled markers of the protein SA-FasL and lipocoacervate. In FIG. 7A, mouse 1 was a control injected with unlabeled fibrin hydrogel components. The lipocoacervate included PEAD, heparin, 15 μg of SA-FasL, and 5 μg of IL-2. The lipocoacervate vesicles were formed and pelleted to remove excess supernatant, and then the lipocoacervate vesicles were resuspended in a fibrinogen in PBS solution. Before injection, thrombin was added to crosslink fibrinogen upon injection into the flank of the mice. An IVIS Spectrum *in vivo* imaging system was used for *in vivo* tracking of compounds. For comparison across days, radian efficiency was used to account for fluctuations of laser intensity, emission time, and exposure of fluorophores.

[0156] FIG. 7B is a graph quantifying *in vivo* decay of FasL and heparin signals at the injection site over 21 days. Both SA-FasL and heparin were tracked over the 21-day time course revealing differential release profiles of the two fluorophores from the site of injection. Cyanine-5 labeled heparin *had* a linear decay rate over the three-week observation period. SA-FasL labeled with Alexa Fluor 750 had a large burst release within the first few days of hydrogel injection followed by a slower release rate characteristic of controlled release of therapeutics. On the fourth day after hydrogel injection nearly 70% of the initial SA-FasL at the site of injection had been released. Concurrently, only 15% of heparin had been released. At the conclusion of the study over 90% of SA-FasL was released from the initial site of injection while only 70% of the heparin was released.

[0157] These results demonstrate different release profiles of lipocoacervate from the fibrin gel and SA-FasL from lipocoacervate. Additionally, there was a shift of the release of SA-FasL from lipocoacervate in contrast to the initial results of a slow release of SA-FasL *in vitro*. In addition to the data shown in FIGS. 7A and 7B, images of the mouse chest were taken to look

for any accumulation with the organs over the time course of the experiment. No significant signal was detected in the kidneys or liver, with the only significant signal in the bladder of the animals. *Ex vivo* imaging was also conducted post-sacrifice of the animals to look for accumulations of either heparin or SA-FasL in major organs at the endpoint of this study (Day 21). No such accumulations were seen.

EXAMPLE 9: Assessing markers for liver, renal, and muscle damage after treatment with SA-FasL loaded LipCo *in vivo*

[0158] FIGS. 8A-8B show results of *in vivo* assessment of markers for liver, renal, or muscle damage after treatment with SA-FasL from LipCo in BALB/C mice. Because of the risk that treatment with SA-FasL and IL-2 may also activate and cause the proliferation of other immune cells, which may cause damage, high dosage treatments of SA-FasL loaded LipCo were conducted to assess potential risk of kidney, liver, and muscle damage as an off-target effect. The protein markers of kidney, liver, and muscle damage assessed were ALT, GLDH, urea nitrogen, creatinine, LDH, creatine kinase, and AST.

[0159] BALB/c mice were treated with a subcutaneous injection of SA-FasL and IL-2 encapsulated in the lipocoacervate/fibrin gel formulation (5 μg/mouse IL-2 and 15 μg/mouse SA-FasL). Blood was collected from the mice on days 2, 4, and 14 post-injection and the serum was isolated. Additionally, blood from untreated mice was collected and used as a negative control for this study. As shown in FIGS. 8A and 8B, there were no significant differences in the amounts of these protein markers after treatment with SA-FasL loaded LipCo as compared to an untreated negative control group.

[0160] The panel of protein markers included a variety of commonly measured markers for liver, renal, and muscle damage. The subsequent analysis revealed high tolerance of treatment over the markers observed. All markers, ALT, GLDH, urea nitrogen, creatinine, LDH, creatine kinase, and AST, were observed as within the normal ranges following injection of IL-2 and SA-FasL. These results were compared to healthy untreated mice as a baseline for blood measurements.

What is claimed is:

1. A composition comprising a lipocoacervate loaded with a chimeric FasL protein, wherein the lipocoacervate comprises a lipid membrane encapsulating a coacervate and the chimeric FasL protein, wherein the chimeric FasL protein comprises a FasL moiety and a streptavidin or avidin moiety.

2. The composition according to claim 1, wherein the chimeric FasL protein comprises a FasL moiety and a streptavidin moiety.

3. The composition according to claim 1, wherein the chimeric FasL protein comprises a FasL moiety comprising the extracellular domain of human FasL and a streptavidin moiety.

4. The composition according to claim 1, wherein the chimeric FasL protein comprises a FasL moiety comprising the amino acid sequence of SEQ ID NO: 1.

5. The composition according to claim 1, wherein the chimeric FasL protein comprises the amino acid sequence of SEQ ID NO: 2.

6. The composition according to claim 1, further comprising an IL-2 protein encapsulated within the lipid membrane.

7. A composition comprising a lipocoacervate loaded with an IL-2 protein, wherein the lipocoacervate comprises a lipid membrane encapsulating a coacervate and the IL-2 protein.

8. The composition according to claim 6, wherein the IL-2 protein comprises the amino acid sequence of SEQ ID NO: 3.

9. The composition according to claim 1, wherein the lipid membrane comprises a phospholipid, cholesterol, and a glycolipid.

10. The composition according claim 1, wherein the lipid membrane comprises 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol), and cholesterol, optionally at a weight ratio of 5:1:4 DOPC:DSPG:cholesterol.

11. The composition according to claim 1, wherein the lipid membrane comprises 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG), and cholesterol.

12. The composition according to claim 1, wherein the coacervate comprises poly(ethylene argininylaspartate diglyceride) (PEAD) and heparin, optionally at a weight ratio of from 3:1 to 5:1, further optionally at a weight ratio of from 3.5:1 to 4.5:1, further optionally at a weight ratio of from 3.6:1 to 4.4:1, further optionally at a weight ratio of about 4:1 PEAD:heparin.

13. The composition according to claim 12, wherein the PEAD is biotinylated PEAD, optionally wherein the chimeric FasL protein is bound to the biotinylated PEAD through a biotin-streptavidin or biotin-avidin linkage via the streptavidin or avidin moiety of the chimeric FasL protein.

14. The composition according to claim 1, wherein the lipocoacervate comprises:

lipid membrane components comprising 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG), and cholesterol, and

coacervate components comprising poly(ethylene argininylaspartate diglyceride) (PEAD) and heparin.

15. The composition according to claim 14, wherein the chimeric FasL protein is dispersed in the coacervate.

16. The composition according to claim 1, further comprising a pharmaceutically acceptable carrier.

17. The composition according to claim 1, wherein the composition exhibits controlled release of the chimeric FasL protein and/or IL-2 protein, optionally wherein the composition exhibits controlled release of the chimeric FasL protein over a period of time of 25 days, further optionally wherein the composition exhibits controlled release of the IL-2 protein over a period of time of 14 days.

18. The composition according to claim 1, wherein the FasL protein and/or IL-2 protein released from the lipocoacervate exhibits biological activity, optionally wherein FasL protein

and/or IL-2 protein released from the lipocoacervate retains at least 50% of its biological activity, optionally wherein FasL protein and/or IL-2 protein released from the lipocoacervate retains at least 75% of its biological activity.

19. The composition according to claim 1, prepared by a process comprising:

(a) preparing a mixture comprising (i) FasL protein and (ii) heparin, and adding PEAD to the mixture to obtain a coacervate mixture, optionally wherein the PEAD is biotinylated PEAD, and

(b) adding to the coacervate mixture a lipid membrane composition comprising a phospholipid, cholesterol, and a glycolipid, to obtain a lipocoacervate suspension.

20. The composition according to claim 19, wherein one or both of the heparin and PEAD are provided in saline.

21. The composition according to claim 19, wherein the lipid membrane composition of (b) further comprises ethanol, and wherein the process further comprises centrifuging the lipocoacervate suspension to remove excess ethanol in the supernatant, and, optionally resuspending the lipocoacervate.

22. A process for preparing a lipocoacervate loaded with one or both of a chimeric FasL protein and an IL-2 protein, comprising:

(a) preparing a mixture comprising (i) one or both of the chimeric FasL protein and the IL-2 protein and (ii) heparin, and adding PEAD to the mixture to obtain a coacervate mixture, and

(b) adding to the coacervate mixture a lipid membrane composition comprising lipid membrane components comprising a phospholipid, cholesterol, and a glycolipid, to obtain a lipocoacervate suspension.

23. The process according to claim 22, wherein one or both heparin and PEAD are provided in saline.

24. The process according to claim 22, wherein the lipid membrane composition of (b) further comprises ethanol, and wherein the process further comprises centrifuging the

lipocoacervate suspension to remove excess ethanol in the supernatant, and, optionally resuspending the lipocoacervate.

25. The process according to claim 22, wherein the lipid membrane components comprise 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG), and cholesterol, optionally at a weight ratio of 5:1:4 DOPC:DSPG:cholesterol.

26. The process according to claim 22, wherein the lipid membrane comprises 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DSPG), and cholesterol.

27. The process according to claim 22, wherein the lipocoacervate is loaded with both a chimeric FasL protein and an IL-2 protein.

28. A method of inducing immune tolerance in a subject in need thereof, comprising administering to the subject a composition according to claim 1, optionally wherein the composition further comprises IL-2 protein.

29. The method according to claim 28, wherein the method comprises administering a composition comprising a lipocoacervate loaded with a chimeric FasL protein and administering a composition comprising a lipocoacervate loaded with IL-2 protein.

30. The method according to claim 28, wherein the method comprises administering a composition comprising a lipocoacervate loaded with a chimeric FasL protein and a lipocoacervate loaded with IL-2 protein.

31. The method according to claim 28, wherein the method comprises administering a composition comprising a lipocoacervate loaded with a chimeric FasL protein and IL-2 protein.

32. The method according to claim 28, wherein the subject is suffering from Type 1 diabetes.

33. The method according to claim 28, wherein the subject is suffering from an autoimmune condition.

34. The method according to claim 28, wherein the subject is an allograft or xenograft transplant patient, a patient being treated with a stem cell-derived product, insulin-producing beta cells, hepatocytes, or hematopoietic stem cells, or a subject at risk of or being treated for graft-versus-host disease.

35. A composition according to claim 1, for use in inducing immune tolerance in a subject in need thereof, optionally wherein the subject is suffering from Type 1 diabetes, the subject is suffering from an autoimmune condition, the subject is an allograft or xenograft transplant patient, the subject is a patient being treated with a stem cell-derived product, insulin-producing beta cells, hepatocytes, or hematopoietic stem cells, or the subject is at risk of or being treated for graft-versus-host disease, optionally wherein the composition further comprises IL-2 protein.

36. A composition according to claim 1, for use in treating Type 1 diabetes, optionally wherein the composition further comprises IL-2 protein.

37. Use of a composition according to claim 1, in the preparation of a medicament for inducing immune tolerance in a subject in need thereof, optionally wherein the subject is suffering from Type 1 diabetes, the subject is suffering from an autoimmune condition, the subject is an allograft or xenograft transplant patient, the subject is a patient being treated with a stem cell-derived product, insulin-producing beta cells, hepatocytes, or hematopoietic stem cells, or the subject is at risk of or being treated for graft-versus-host disease, optionally wherein the composition further comprises IL-2 protein.

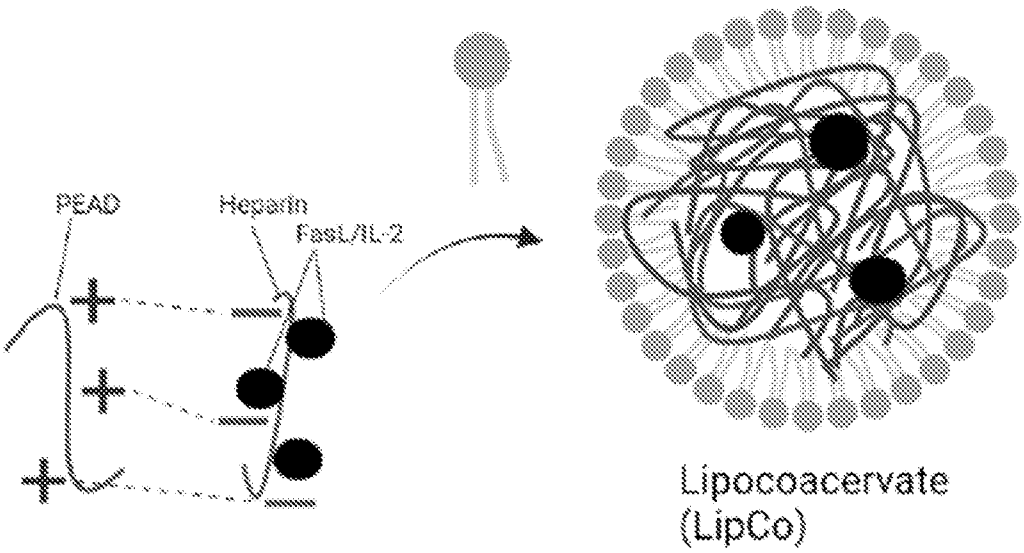

FIG. 1

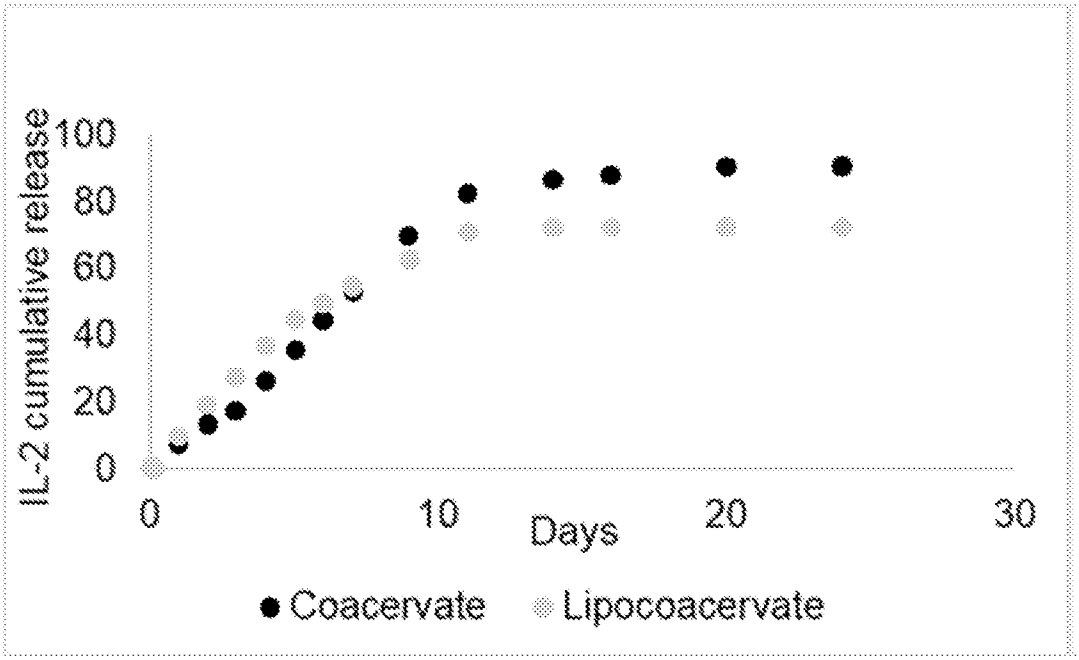
IL-2 cumulative release
100
80
60
40
20
0
0
10
20
30
Days
Coacervate
Lipocoacervate

FIG. 2A

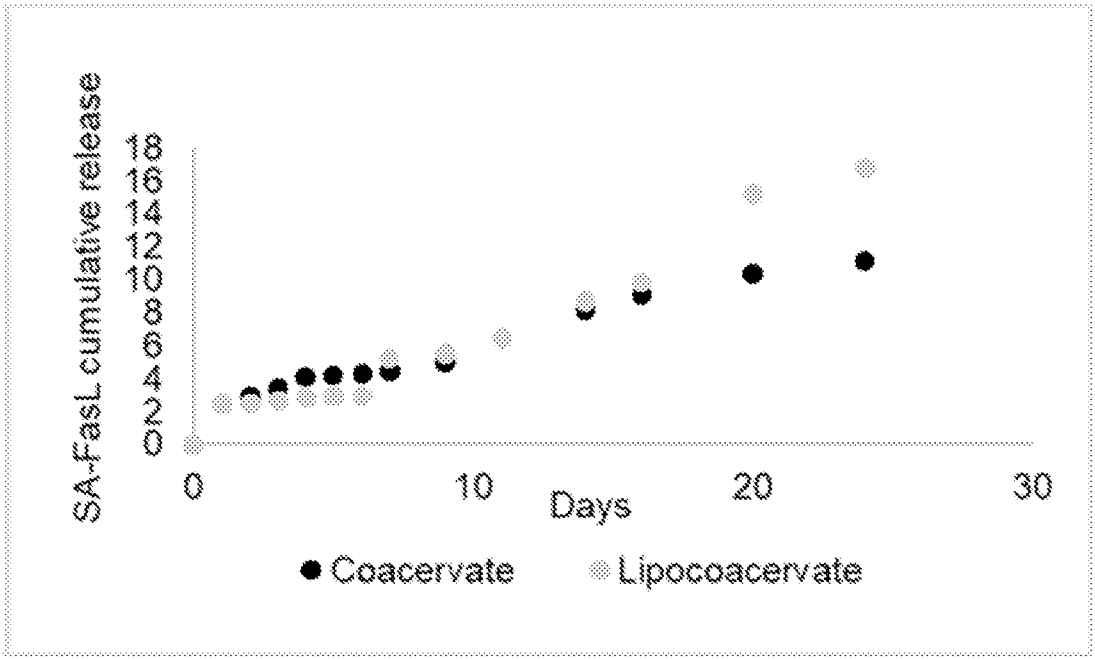
SA-FasL cumulative release
18
16
14
12
10
8
6
4
2
0
0
10
20
30
Days
Coacervate
Lipocoacervate

FIG. 2B

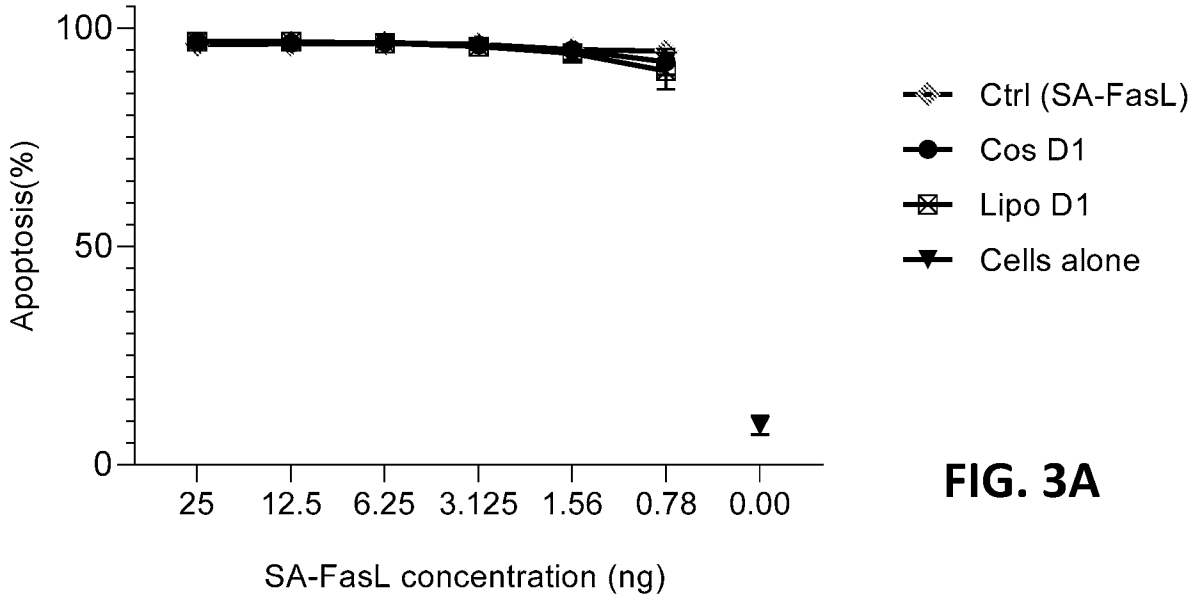
100
50
0
Apoptosis(%)
25
12.5
6.25
3.125
1.56
0.78
0.00
SA-FasL concentration (ng)
Ctrl (SA-FasL)
Cos D1
Lipo D1
Cells alone

FIG. 3A

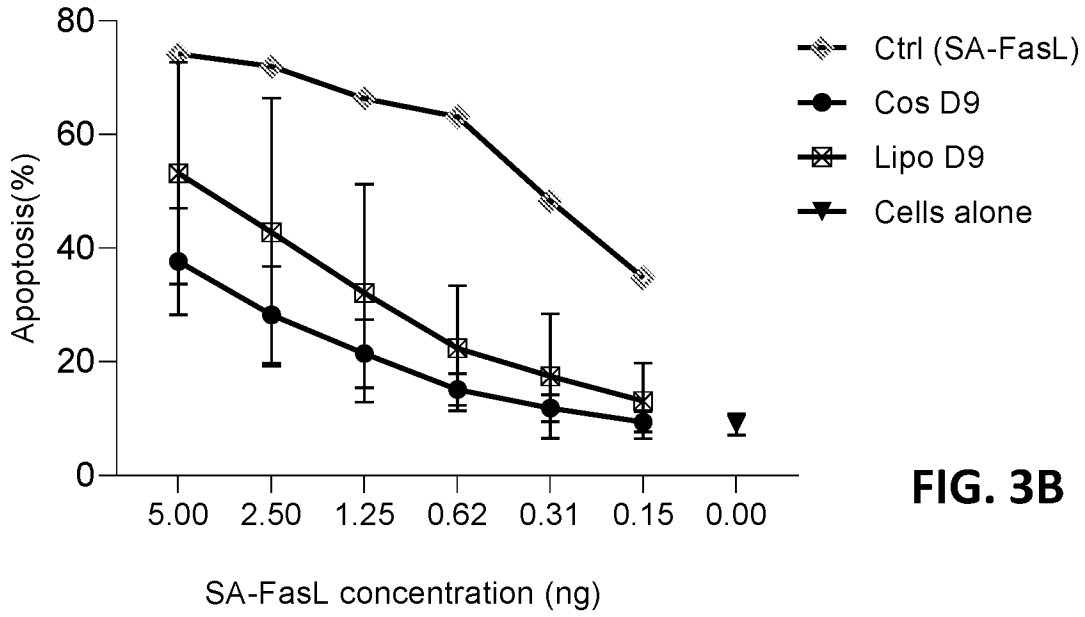
80
60
40
20
0
Apoptosis(%)
5.00
2.50
1.25
0.62
0.31
0.15
0.00
SA-FasL concentration (ng)
Ctrl (SA-FasL)
Cos D9
Lipo D9
Cells alone

FIG. 3B

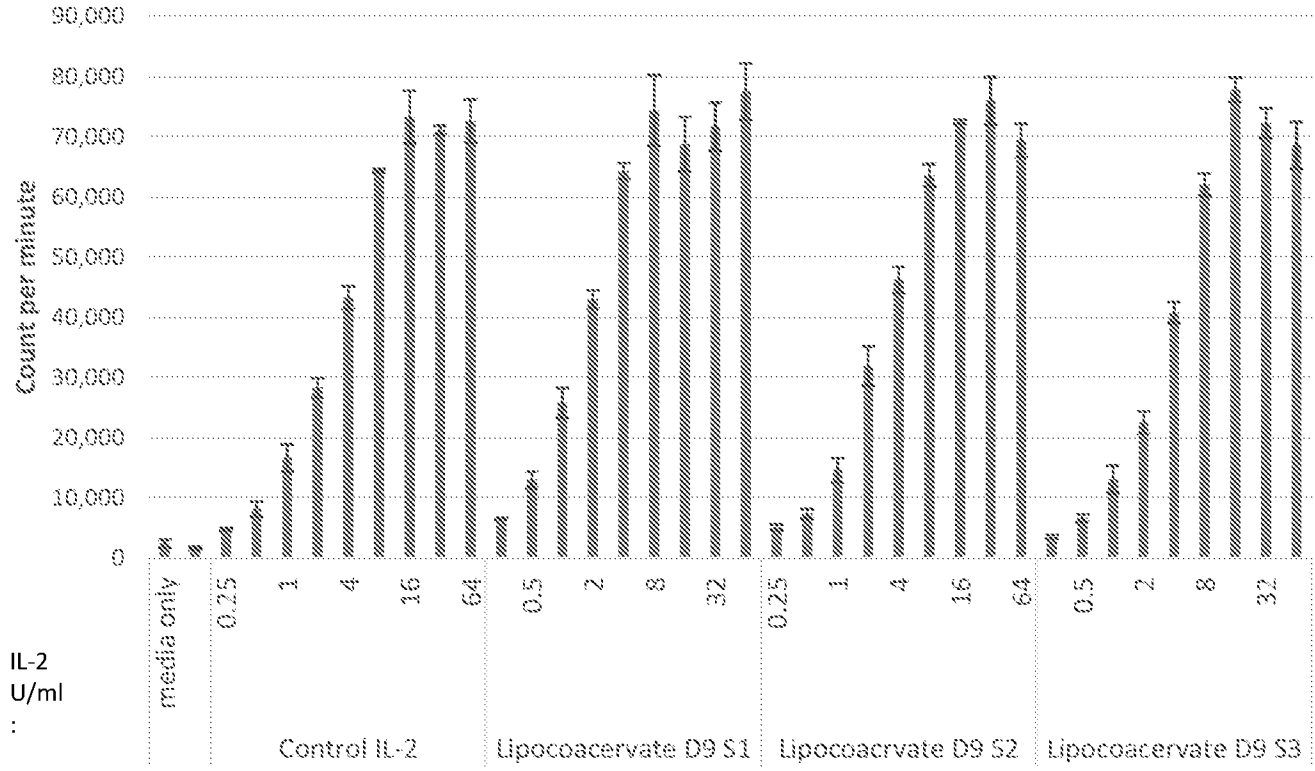
90,000
80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
Count per minute
IL-2 U/ml :
media only
0.25
1
4
16
64
0.5
2
8
32
0.25
1
4
16
64
0.5
2
8
32
Control IL-2
Lipocoacervate D9 S1
Lipocoacrvate D9 S2
Lipocoacervate D9 S3

FIG. 4

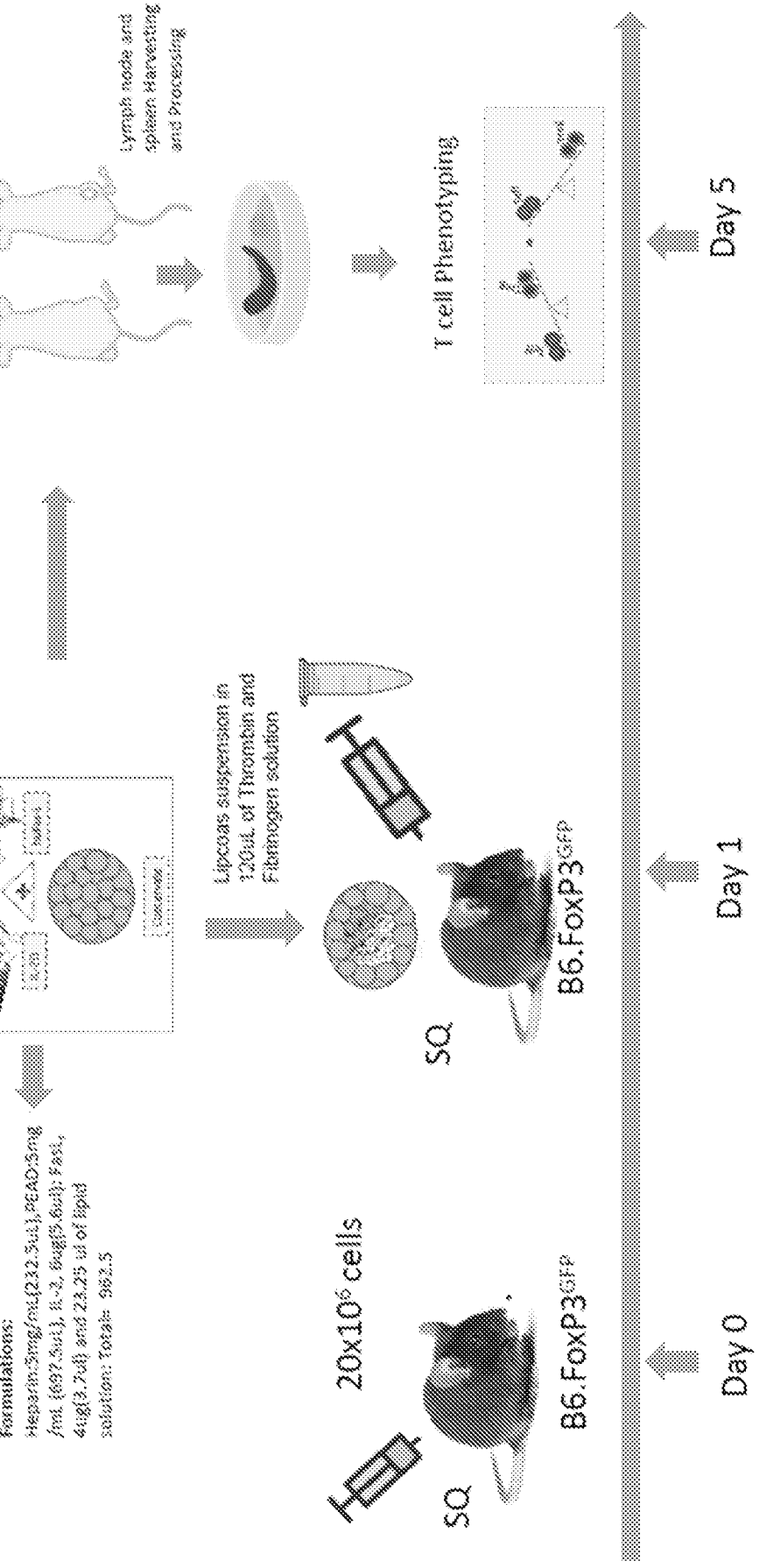
Day 0
Day 1
Day 5
B6.FoxP3GFP
20x10^6 cells
SQ
B6.FoxP3GFP
SQ
B6.FoxP3GFP
Lipoas suspension in 120uL of Thrombin and Fibrinogen solution
Formulations:
Heparin:5mg/mL(232.5uL),PEAO:5mg/mL (697.5uL), IL-2, 8ug(5.6ul), Fas, 4ug(3.7ul) and 23.25 ul of lipid solution: Total= 962.5
Lymph node and spleen Harvesting and Processing
T cell Phenotyping

FIG. 5

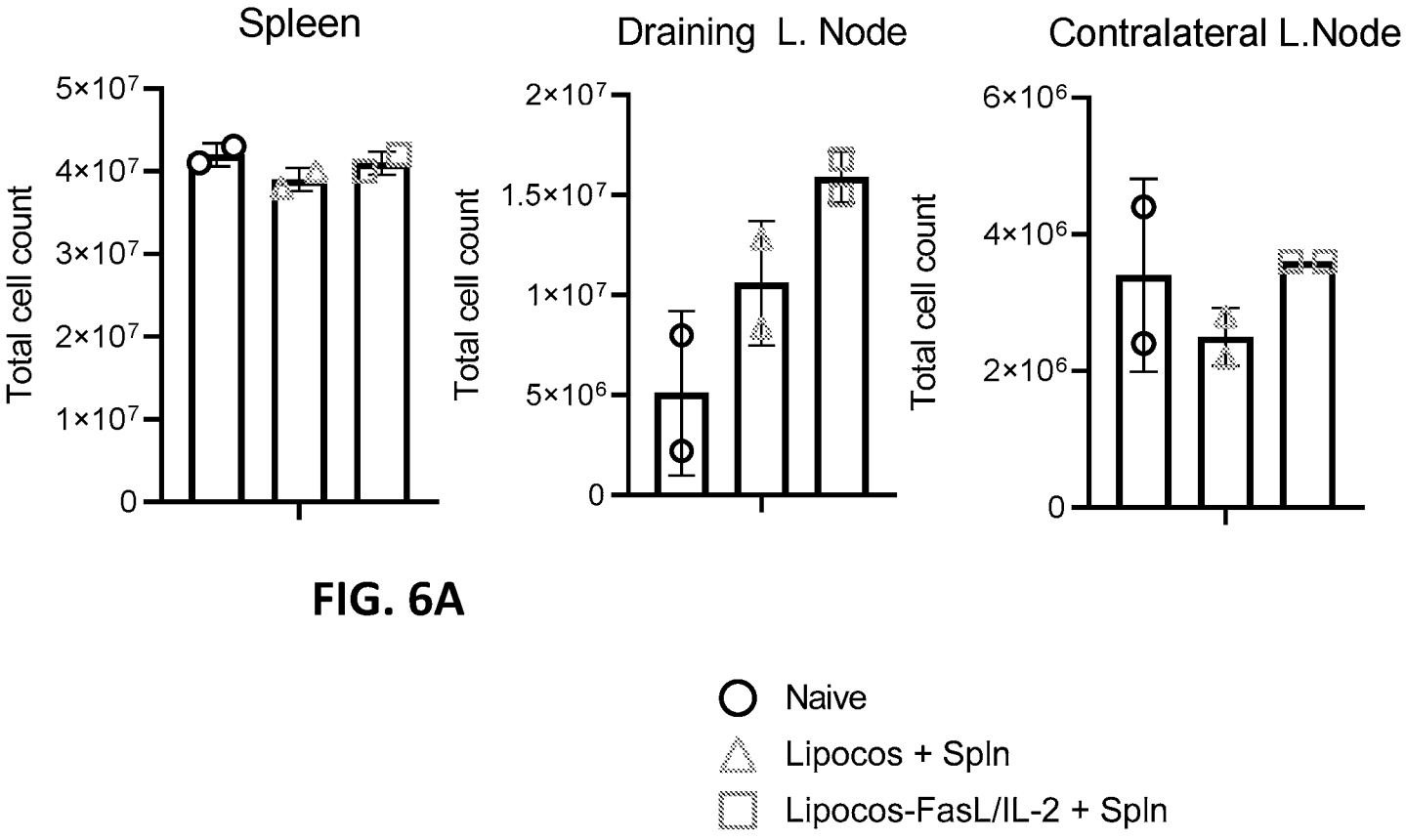
Spleen
Draining L. Node
Contralateral L.Node
Total cell count
5×10^7
4×10^7
3×10^7
2×10^7
1×10^7
0
2×10^7
1.5×10^7
1×10^7
5×10^6
0
6×10^6
4×10^6
2×10^6
0
Naive
Lipocos + Spln
Lipocos-FasL/IL-2 + Spln

FIG. 6A

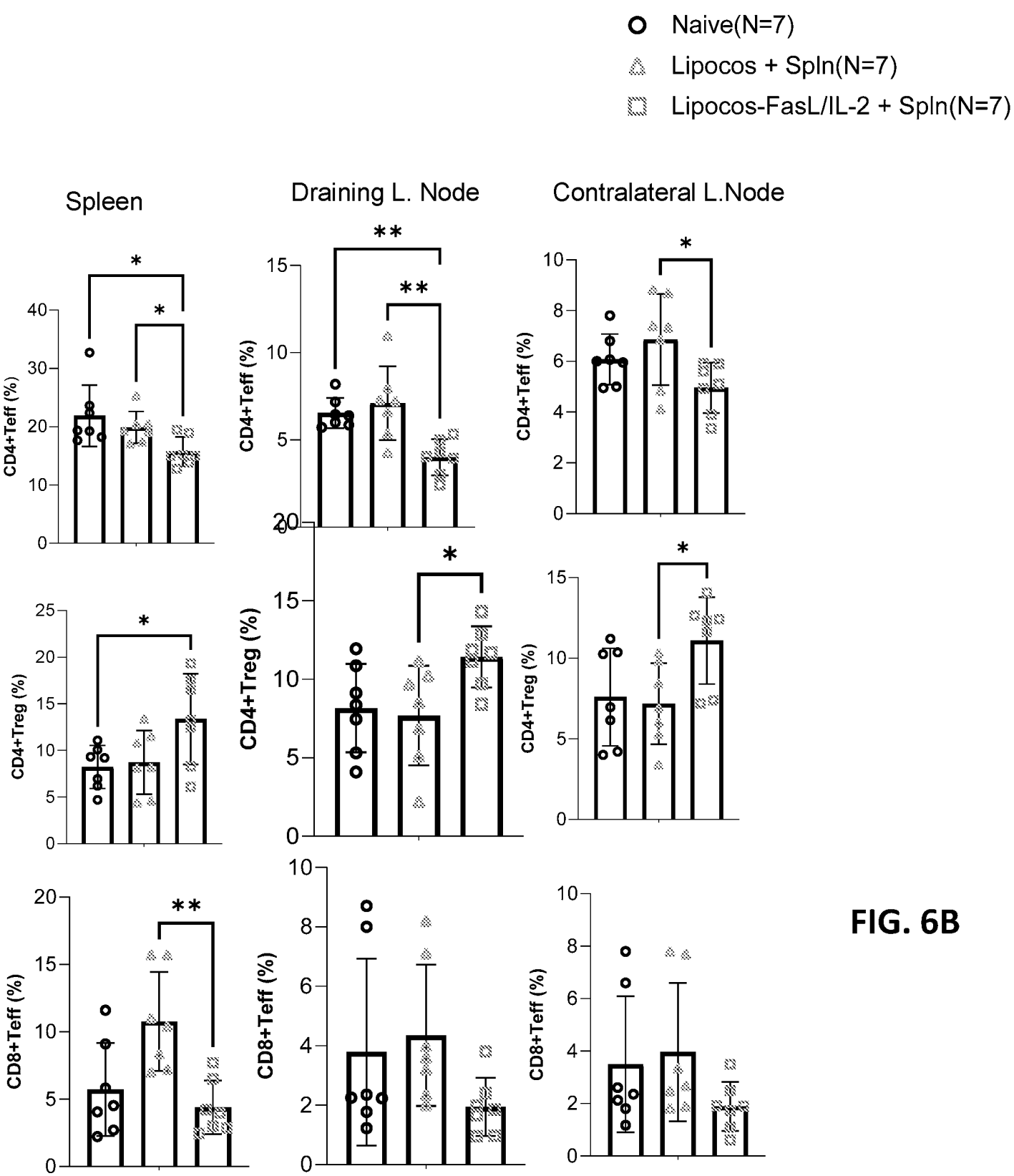
Naive(N=7)
Lipocos + Spln(N=7)
Lipocos-FasL/IL-2 + Spln(N=7)
Spleen
Draining L. Node
Contralateral L.Node
CD4+Teff (%)
CD4+Treg (%)
CD8+Teff (%)
*
**

FIG. 6B

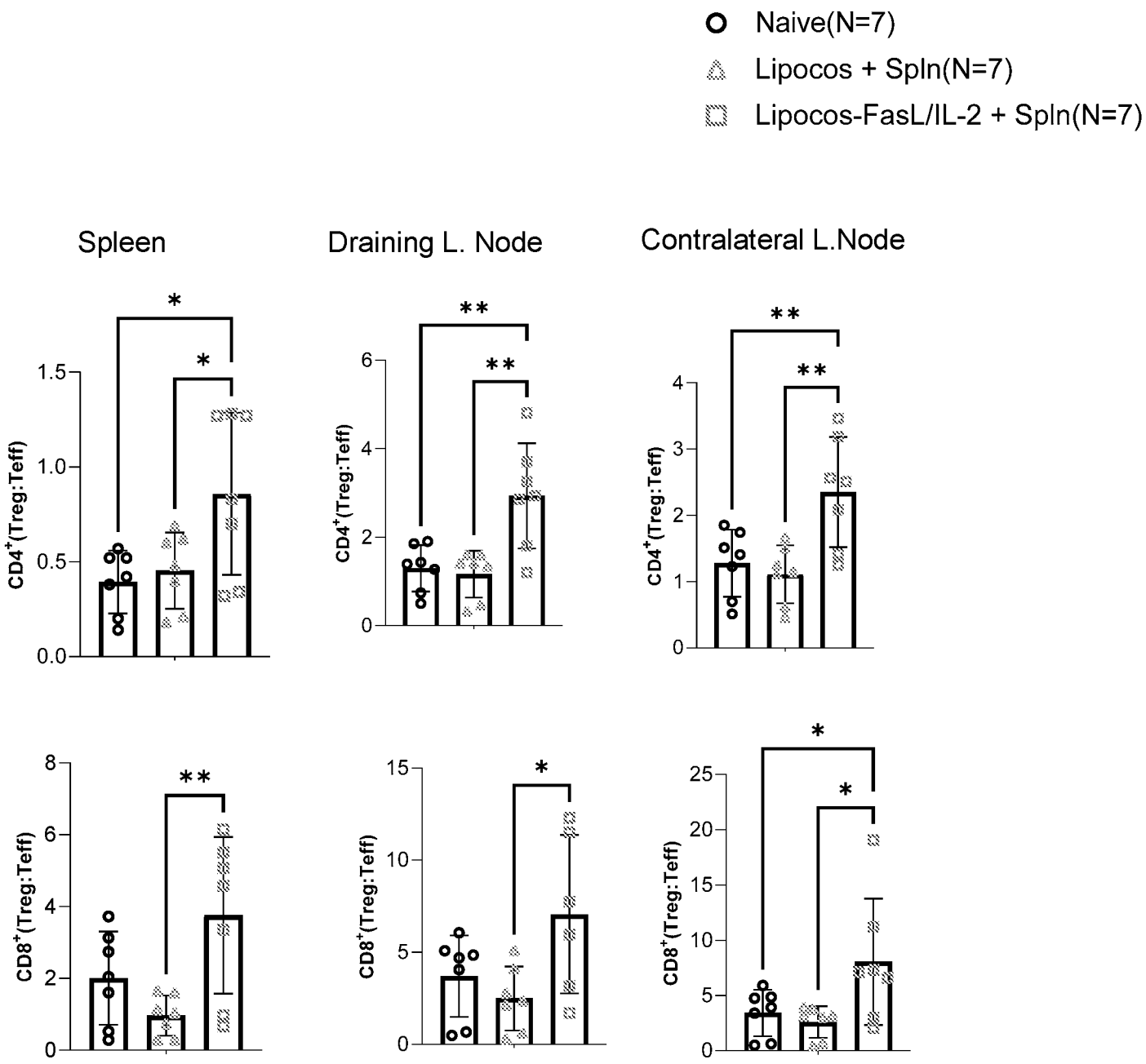
Naive(N=7)
Lipocos + Spln(N=7)
Lipocos-FasL/IL-2 + Spln(N=7)
Spleen
Draining L. Node
Contralateral L.Node
CD4+(Treg:Teff)
CD8+(Treg:Teff)

FIG. 6C

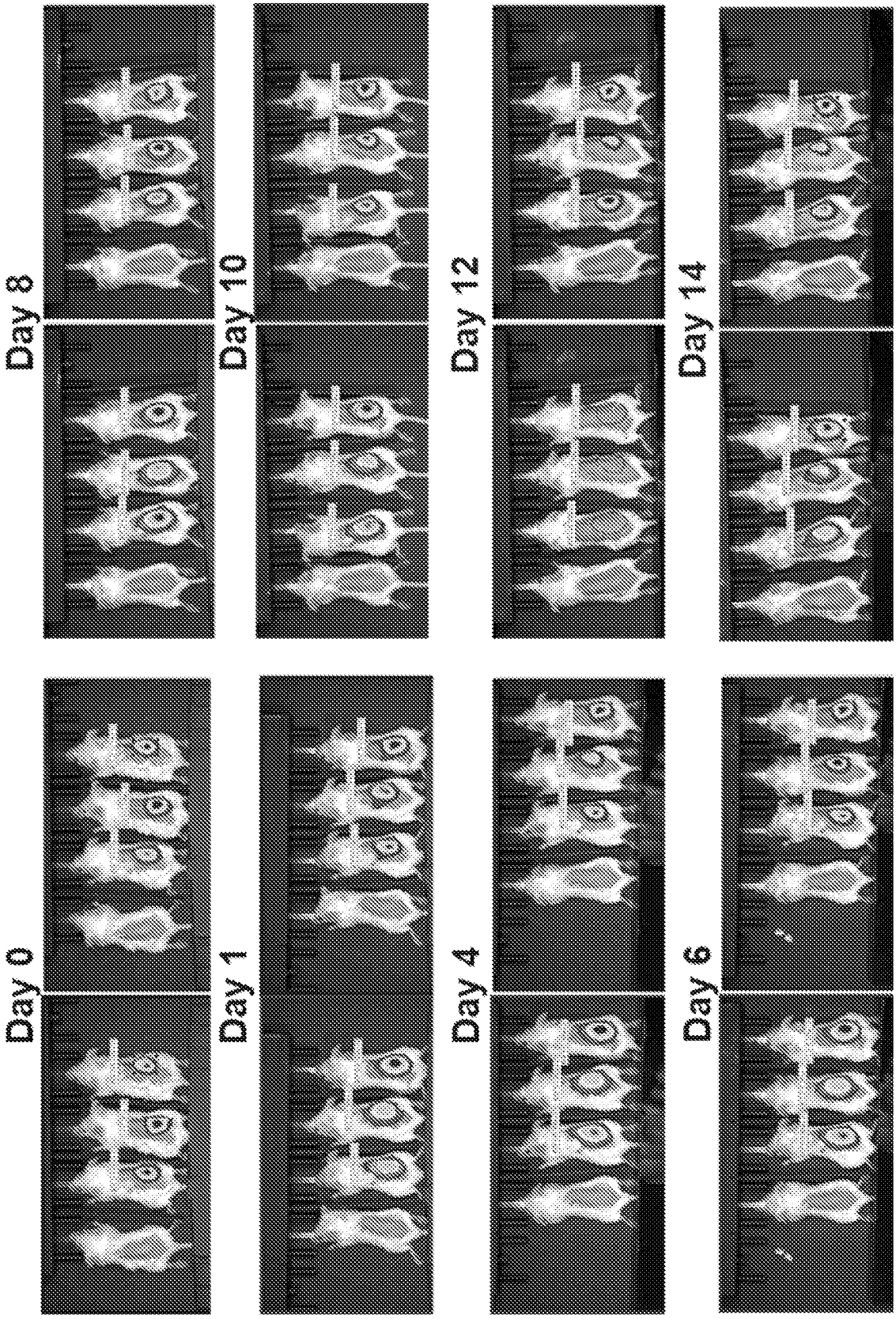

FIG. 7A

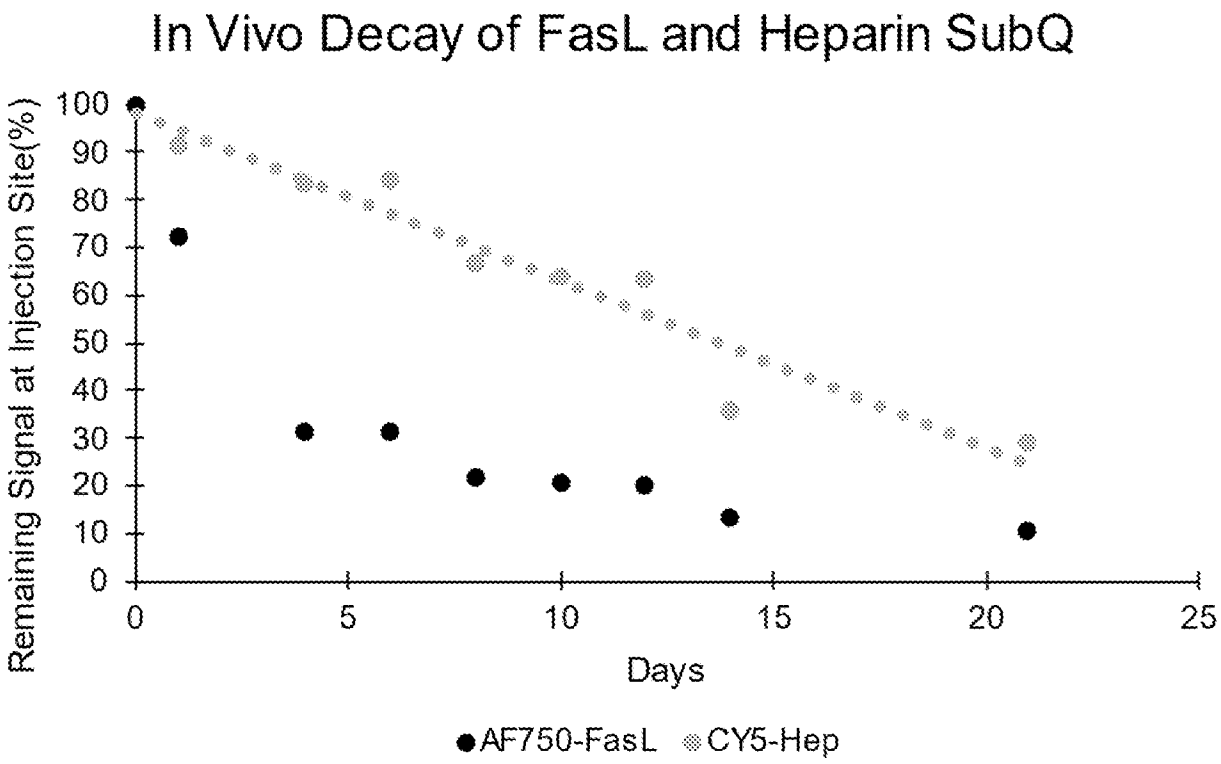
In Vivo Decay of FasL and Heparin SubQ
Remaining Signal at Injection Site(%)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Days
AF750-FasL
CY5-Hep

FIG. 7B

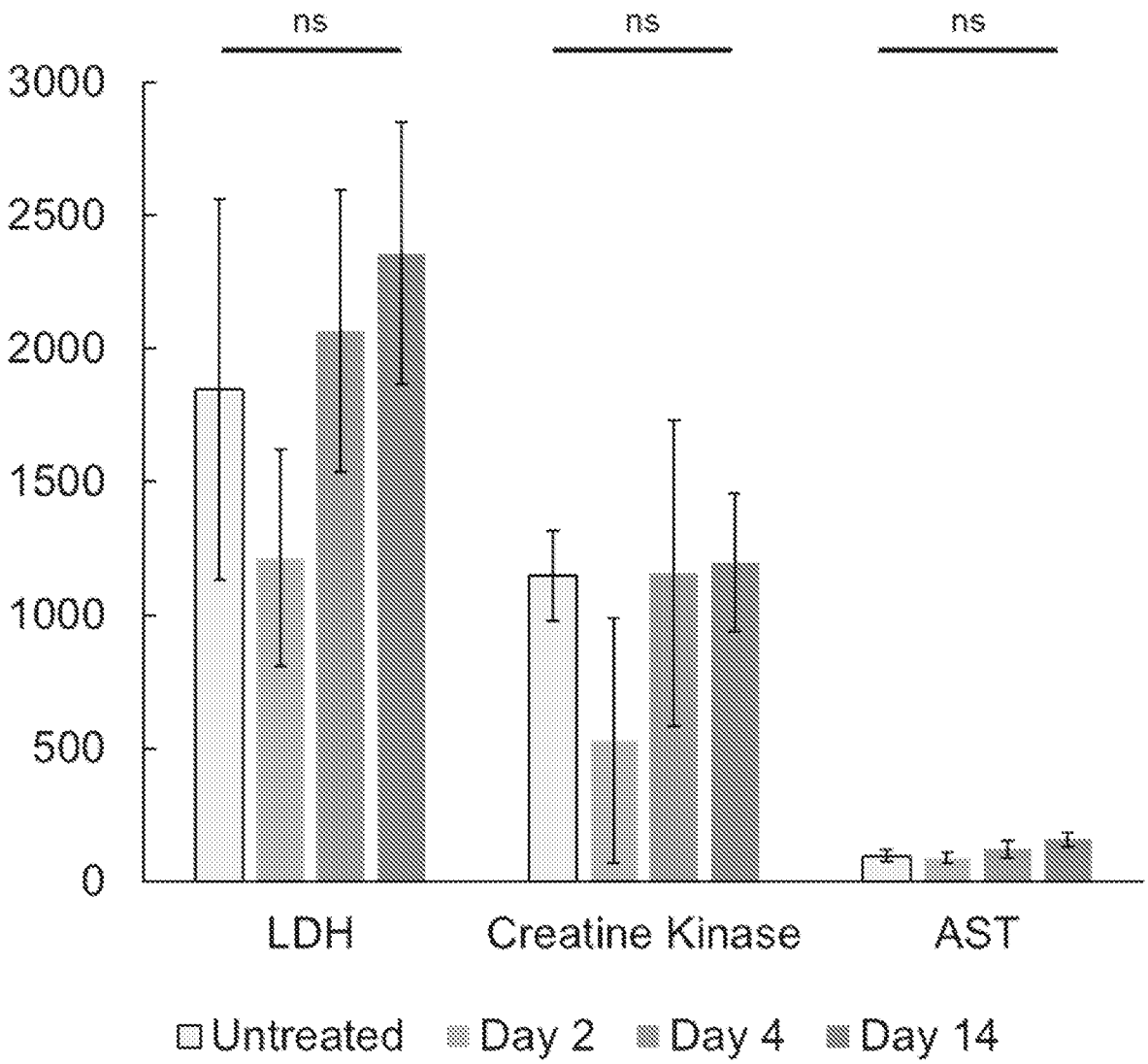
ns
ns
ns
3000
2500
2000
1500
1000
500
0
LDH
Creatine Kinase
AST
Untreated
Day 2
Day 4
Day 14

FIG. 8A

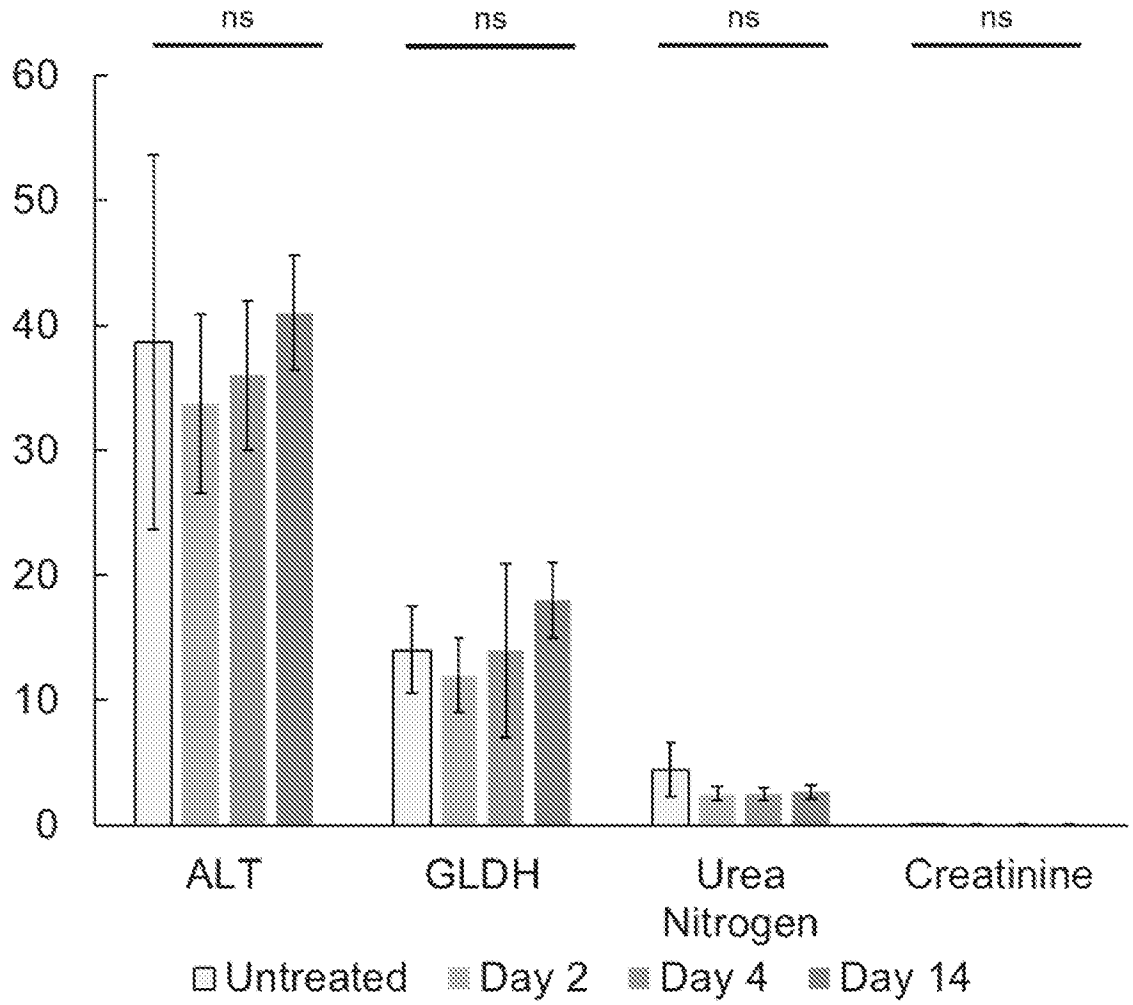
ns
ns
ns
ns
60
50
40
30
20
10
0
ALT
GLDH
Urea Nitrogen
Creatinine
Untreated
Day 2
Day 4
Day 14

FIG. 8B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/030961

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***C07K 14/705*** (2024.01); ***C07K 19/00*** (2024.01); ***A61K 38/17*** (2024.01); ***A61K 38/20*** (2024.01); ***A61K 9/127*** (2024.01); ***A61P 3/10*** (2024.01); ***A61P 37/06*** (2024.01); ***C07K 14/55*** (2024.01); *A61K 47/69* (2024.01)

CPC: ***C07K 14/70575; A61K 38/177; A61K 38/2013; A61K 9/127; A61P 3/10; A61P 37/06; C07K 14/55; C07K 19/00;*** *A61K 47/6911; C07K 2319/74*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2018/0318394 A1 (UNIVERSITY OF LOUISVILLE RESEARCH FOUNDATION INC.) 08 November 2018 (08.11.2018) entire document	1-6, 8, 16-18, 28-37
Y	US 2008/0089927 A1 (MALININ) 17 April 2008 (17.04.2008) entire document	1-6, 8-12, 14-18, 28-37
Y	US 2020/0046780 A1 (UNIVERSITY OF LOUISVILLE RESEARCH FOUNDATION INC. et al.) 13 February 2020 (13.02.2020) entire document	1, 9-12, 14, 15
Y	US 2016/0304574 A1 (SHARMA et al.) 20 October 2016 (20.10.2016) entire document	8
Y	US 2021/0290770 A1 (UNIVERSITY OF PITTSBURGH - OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION) 23 September 2021 (23.09.2021) entire document	12, 14, 15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
26 July 2024 (26.07.2024)	**23 September 2024 (23.09.2024)**

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US **Commissioner for Patents** **P.O. Box 1450, Alexandria, VA 22313-1450**	**MATOS** **TAINA**
Facsimile No. **571-273-8300**	Telephone No. **571-272-4300**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/030961

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed.
   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),
      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/030961

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

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

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I: claims 1-6, 8-21, and 28-37 are drawn to compositions comprising a lipocoacervate loaded with a chimeric FasL protein, methods of inducing immune tolerance in a subject in need thereof, and uses of a composition.

Group II: claim 7 is drawn to a composition comprising a lipocoacervate loaded with an IL-2 protein.

Group III: claims 22-27 are drawn to processes for preparing a lipocoacervate loaded with one or both of a chimeric FasL protein and an IL-2 protein.

The inventions listed in Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The special technical features of Group I, compositions comprising a lipocoacervate loaded with a chimeric FasL protein, methods of inducing immune tolerance in a subject in need thereof, and uses of a composition, are not present in Groups II and III; the special technical features of Group II, a composition comprising a lipocoacervate loaded with an IL-2 protein, are not present in Groups I and III; and the special technical features of Group III, processes for preparing a lipocoacervate loaded with one or both of a chimeric FasL protein and an IL-2 protein, are not present in Groups I and II.

Additionally, even if Groups I-III were considered to share the technical features of compositions comprising a lipocoacervate wherein the lipocoacervate comprises a lipid membrane encapsulating a coacervate, FasL, and IL-2, these shared technical features do not represent a contribution over the prior art.

Specifically, US 2008/0089927 to Malinin teaches compositions comprising a lipocoacervate wherein the lipocoacervate comprises a lipid membrane encapsulating a coacervate (the unstable coacervate is exposed to a high concentration lipid solution. It is believed that a nucleation effect results where the coacervate seeds the precipitation of the lipids. The lipids form a bilayer encapsulating the coacervate, Para. [0056]; The lipid antiinfective formulations, Para. [0117]).

Further, US 2020/0046780 to University of Louisville Research Foundation Inc. et al. teaches FasL (FasL-engineered biomaterials, Abstract).

Further, US 2021/0290770 to University of Pittsburgh - of The Commonwealth System of Higher Education teaches IL-2 (In forming the composition (e.g., coacervate), the cationic polycationic polymer is complexed with a polyanionic polymer, such as heparin or heparan sulfate, which is further complexed with an active agent ... Specific examples of active agents include interleukins (IL), such as IL-2, Para. [0052]).

The inventions listed in Groups I-III therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/030961

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

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

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

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

4. ☑ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-6, 8-21, 28-37**

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.