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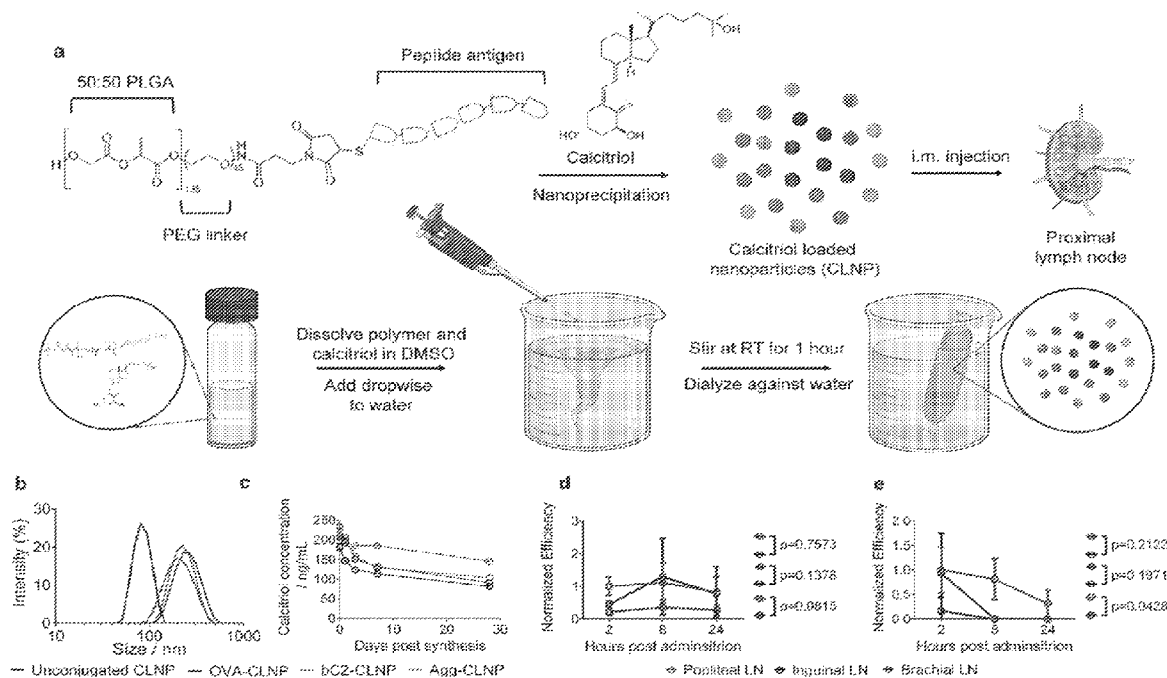


FIG. 1

(57) Abstract: Described herein are compositions and methods for reducing the severity of or preventing arthritic flares that are effective systemically without causing generalized immunosuppression when administered intramuscularly.

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PCT PATENT APPLICATION
FOR
IMMUNOMODULATORY NANOPARTICLES FOR MODULATING ARTHRITIS FLARES

5 CROSS REFERENCE

This application claims the benefit of U.S. Provisional Application No. 63/538,708 filed September 15, 2023, which application is incorporated herein by reference in its entirety

SEQUENCE LISTING

- 10 The instant application contains a Sequence Listing which has been submitted electronically in XML format and is hereby incorporated by reference in its entirety. Said XML copy, created on September 11, 2024, is named 24978-0952_SL.xml and is 5,182 bytes in size.

GOVERNMENT SPONSORSHIP

- 15 This invention was made with government support under grant numbers T32AR064194, F31AR079921, F31HL164055, P30AR073761, R01AR081887, P30CA23100, and UL1TR001442 awarded by the National Institutes of Health and grant number DMR-2011924 awarded by the National Science Foundation. The government has certain rights in the invention.

20 BACKGROUND

- Rheumatoid arthritis (RA) is a common chronic autoimmune joint disorder that, when left uncontrolled, leads to progressive destruction of articular structures, including cartilage and bone.^{1,2} Disease modifying anti-rheumatic drugs (DMARDs) have greatly improved treatment options for RA. However, recurring disease fluctuations in the joints, referred to as flares, can be
25 a common experience in otherwise adequate DMARD responders. Flares vary in their presentation and repeated flares can lead to joint damage even in the context of overall good disease control.³⁻⁶ Current treatment of flares focuses on symptom management with corticosteroids or non-steroidal anti-inflammatory drugs (NSAID).^{7,8} However, symptomatic control is ineffective at stemming joint damage and flare recurrence.⁹⁻¹¹ In addition, for patients
30 that have achieved durable disease control, tapering of the DMARD dose is appealing to reduce

the risk of chronic medication. However, flares are common when tapering is attempted.¹²⁻¹⁴ There is an unmet need for durable flare control agents.

SUMMARY OF THE INVENTION

5 Although RA is a systemic disease, flares typically recur in a restricted subset of joints.¹⁵⁻¹⁸ While the pathogenesis can be complex, antigen-presenting cells are key mediators, of which conventional dendritic cells (DC) are the major subset.¹⁹⁻²¹ Preclinical mouse models have established that activated DC contribute to the development of RA pathology.²²⁻²⁴ DC are thought to uptake autoantigen and/or activating stimuli in the periphery, such as from toll-like
10 receptor (TLR) ligands and proinflammatory cytokines, and subsequently migrate to lymph nodes to prime autoreactive T cells, which have been associated with RA flares.^{20,25 28} DC also infiltrate synovial tissue and fluid in response to locally produced cytokines and chemokines or differentiate locally from progenitors and are often associated with tertiary lymphoid structures.²⁹⁻³⁵ DC in the lymph node and affected joint express classical activation markers such
15 as major histocompatibility class 2 (MHC2), CD80/CD86, and present joint autoantigens to polarize T cells into pro-inflammatory phenotypes.³⁶⁻³⁸ The role of DC in CIA is further supported by previous work which demonstrated that *Batf3*^{-/-} mice, which have defective DC and exhibit impaired antigen cross-presentation, are resistant to collagen-induced arthritis (CIA).³⁹ On the other hand, experiments using adoptive transfer of autoantigen-primed DC in mice have
20 been shown to expand disease-specific T cells in the joint-draining lymph node.⁴⁰ Immunomodulatory DC have been shown to impart immunoregulation through the induction of autoimmune-protective regulatory T cells (T_{reg}) and by inducing T cell anergy.^{41 45} Disease modifying drugs have improved the management of rheumatoid arthritis, but flares of disease continue to be commonly experienced which tend to recur in the same joints and lead to
25 progressive damage. This work reports the development and application of flare-modulating poly(lactic-co-glycolic acid)-poly(ethylene glycol)-maleimide (PLGA-PEG-MAL)-based nanoparticles conjugated with joint-relevant (aggrecan⁸⁹⁻¹⁰³ and type 2 bovine collagen²⁷¹⁻²⁸⁵) N-cysteine terminated peptide antigens via thiol-Michael addition. Peptide-conjugated PLGA-PEG-MAL nanoparticles encapsulated calcitriol, the active form of vitamin D and an
30 immunoregulatory agent, and were termed calcitriol loaded nanoparticles (CLNP). CLNP were ~200 nm in diameter with low a polydispersity index.

In vitro, CLNP induced phenotypic changes in bone marrow derived dendritic cells (DC), reducing costimulatory molecule, major histocompatibility complex II, and proinflammatory cytokine expression. Bulk RNA sequencing of DC revealed that CLNP enhanced expression of the inhibitory ligand-associated gene *Ctla4*, which is associated with rheumatoid arthritis. In vivo, CLNP accumulated in the proximal lymph nodes after intramuscular injection. In the collagen induced arthritis and SKG mouse models of arthritis, CLNP reduced clinical scores, prevented bone erosion, and preserved cartilage proteoglycan as assessed by high-resolution microcomputed tomography and histology. These disease protective effects were associated with increased CTLA-4 expression in joint-localized CD4⁺ T cells. Local administration of the CLNP promoted potent joint-protective effects, underscoring their potential as therapeutic agents for arthritis flares. CLNP offer a promising strategy for preventing arthritis flares by modulating DC without generalized immunosuppression.

Now, therefore, the instant disclosure provides in one aspect a method of treating inflammatory arthritis in a patient, comprising administering to the patient a therapeutically effective amount of a composition comprising nanoparticles made of biodegradable polymers conjugated to a joint-relevant peptide antigen, wherein the nanoparticles encapsulate a dendritic cell modulator. In embodiments, treating the inflammatory arthritis comprises treating arthritis flares.

In embodiments, the biodegradable polymers comprise poly(ethylene glycol) (PEG), poly(lactic-co-glycolic acid) (PLGA), or a combination thereof. In embodiments, the biodegradable polymers comprise a copolymer of PLGA and PEG. In embodiments, the biodegradable polymers comprise a structure of PLGA-PEG. In embodiments, the composition is administered intramuscularly. In embodiments, the nanoparticles have an average hydrodynamic diameter of from about 50 nm to about 300 nm. In embodiments, the nanoparticles have a size which permits accumulation of the nanoparticles in lymph nodes of the patient.

In embodiments, the joint-relevant peptide antigen is conjugated to biodegradable nanoparticles by a reaction between a cysteine residue on the joint-relevant peptide antigen and a maleimide on the biodegradable polymers. In embodiments, the joint-relevant peptide antigen is a peptide derived from one or more cartilage tissue components. In embodiments, the cartilage tissue component is selected from a cartilage proteoglycan. In embodiments, the cartilage proteoglycan is selected from aggrecan, versican, link protein, biglycan (dermatan sulfate proteoglycan (DS-PG)I), decorin (DS-PGII), epiphygan (DS-PGIII), fibromodulin, lumican, perlecan, and lubricin.

In embodiments, the cartilage tissue component is collagen. In embodiments, the cartilage tissue component is a cartilage protein. In embodiments, the cartilage protein is selected from cartilage oligomeric matrix protein (COMP) (Thrombospondin-5), Thrombospondin-1, Thrombospondin-3, CMP (cartilage matrix protein) (Matrilin-1), Matrilin-3, cartilage intermediate layer protein (CILP), C-type lectin, Fibronectin, PRELP (proline- and arginine-rich end leucine-rich repeat protein), Chondroadherin, Tenascin-C, Fibrillin, Elastin, gp (glycoprotein)-39/YKL-40, Matrix gla (gamma-carboxyglutamic acid) protein/MGP, Pleiotrophin, Chondromodulin-I, cartilage-derived retinoic acid responsive protein (CD-RAP), Chondrocalcin, and PARP (proline- and arginine-rich protein). In embodiments, the joint-relevant peptide antigen comprises a stretch of at least 8, 10, 12 or 14 amino acids from the cartilage tissue component. In embodiments, the joint-relevant peptide antigen is an immunodominant peptide derived from the cartilage protein.

In embodiments, the dendritic cell modulator induces an immunomodulatory phenotype of dendritic cells. In embodiments, the dendritic cell modulator reduces expression of one or more of MHC2, CD80, CD86, IL-6, or TNF α in dendritic cells. In embodiments, the dendritic cell modulator increases the expression of one or more of CTLA-4 and MERTK (Mer tyrosine kinase) in dendritic cells. In embodiments, the dendritic cell modulator is calcitriol, or a pharmaceutically acceptable salt thereof.

In embodiments, administering the composition is effective to modulate dendritic cells in a joint and/or lymph node of the patient. In embodiments, the administering does not cause systemic immunosuppression in the patient. In embodiments, the administering reduces severity of or prevents an arthritis flare.

In embodiments, the patient is currently under treatment with a disease modifying antirheumatic drug (DMARD). In embodiments, the patient is currently tapering a dose of the DMARD.

In another aspect herein is a composition comprising nanoparticles made of biodegradable polymers conjugated to a joint-relevant peptide antigen, wherein the nanoparticles encapsulate a therapeutically effective amount of a dendritic cell modulator. In embodiments, the biodegradable polymers comprise poly(ethylene glycol) (PEG), poly(lactic-co-glycolic acid) (PLGA), or a combination thereof. In embodiments, the biodegradable polymers comprise a copolymer of PLGA and PEG. In embodiments, the biodegradable polymers comprise a structure of PLGA-PEG. In embodiments, the nanoparticles have an average hydrodynamic diameter of from about 50 nm to about 300 nm. In embodiments, the joint-relevant peptide antigen is

conjugated to biodegradable nanoparticles by a reaction between a cysteine residue on the joint-relevant peptide antigen and a maleimide on the biodegradable polymers.

In embodiments, the joint-relevant peptide antigen is a peptide derived from a cartilage tissue component. In embodiments, the cartilage proteoglycan is selected from aggrecan, versican, link protein, biglycan (dermatan sulfate proteoglycan (DS-PG)I), decorin (DS-PGII), epiphygan (DS-PGIII), fibromodulin, lumican, perlecan, and lubricin. In embodiments, the cartilage tissue component is collagen. In embodiments, the cartilage tissue component is a cartilage protein selected from cartilage oligomeric matrix protein (COMP) (Thrombospondin-5), Thrombospondin-1, Thrombosponin-3, CMP (cartilage matrix protein) (Matrilin-1), Matrilin-3, cartilage intermediate layer protein (CILP), C-type lectin, Fibronectin, PRELP (proline- and arginine-rich end leucine-rich repeat protein), Chondroadherin, Tenascin-C, Fibrillin, Elastin, gp (glycoprotein)-39/YKL-40, Matrix gla (gamma-carboxyglutamic acid) protein/MGP, Pleiotrophin, Chondromodulin-I, cartilage-derived retinoic acid responsive protein (CD-RAP), Chondrocalcin, and PARP (proline- and arginine-rich protein). In embodiments, the joint-relevant peptide antigen comprises a stretch of at least 8, 10, 12 or 14 amino acids from the cartilage tissue component. In embodiments, the joint-relevant peptide antigen is an immunodominant peptide derived from the cartilage protein.

In embodiments, the dendritic cell modulator induces an immunomodulatory phenotype of dendritic cells. In embodiments, the dendritic cell modulator increases expression of one or more of CTLA-4 and MERTK in dendritic cells. In embodiments, the dendritic cell modulator is calcitriol, or a pharmaceutically acceptable salt thereof.

Also provided herein is a pharmaceutical composition comprising a nanoparticle composition as described herein, and a pharmaceutically acceptable carrier or excipient. In embodiments, the composition is formulated for intramuscular administration.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Synthesis, characterization, and lymph node accumulation of CLNP formulations. (Panel a) Chemical structure of calcitriol loaded nanoparticle and schematic of synthesis. (Panel b) Size measurements using dynamic light scattering of CLNP formulations. (Panel c) HPLC-based quantification of calcitriol up to 4-weeks post-formulation. (Panels d, e) Normalized Cy5 fluorescence measurements over 24 hours in inguinal, popliteal and brachial lymph nodes; Cy5-

PEG-PLGA in (Panel d) and Cy5-Calcitriol in (Panel e). Data in panels b-c are means $\pm$ SD of three technical replicates. Data in panels d-e are means $\pm$ SD of $n=3$ mice per timepoint. Statistical analyses in panels d-e were performed using two-way ANOVA. Schematic in panel a was composed using BioRender and ChemDraw.

5 **Figure 2.** CLNP induces immunoregulatory DC in vitro. (Panel a) Experimental schematic of in vitro effects of CLNP on DC phenotype. Expression of CD80 (Panel b), CD86 (Panel c), major histocompatibility complex II (MHC2) (Panel d), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Panel e) by CD11c⁺ DC under the specified treatment conditions. Cytokine concentrations in cell culture supernatant measured focused on RA-relevant IL-6 (Panel f) and
10 TNF (Panel g). Data in panels b-g are means $\pm$ SD ($n = 6$) from a representative experiment. Statistical analyses in panels b-g were performed using ANOVA with Dunnett's multiple comparison test. Schematic in panel a was composed using BioRender.

Figure 3. Calcitriol modulates RA relevant targets in DC. (Panel a) Pathway enrichment analysis based on top 50 DEG with RA boxed. (Panel b) Volcano plot with RA-associated genes
15 highlighted in red. (Panel c) Heatmap and dendrogram comparing the top 50 DEG from bulk RNA-seq of +/- Calcitriol DC, *Ctla4* and *Tgfb3* are highlighted. Data analyzed by quantile and TMM normalization. In panel b, the blue line represents a p value <0.05 and red lines indicate a $\log_2$ fold change $>1.5x$ by absolute value. In panel c each column represents a replicate of the respective condition and data represents the signal across each gene ranked as z-scores using data
20 across each row.

Figure 4. bC2-CLNP reduce disease severity and bone erosions in CIA mice. (Panel a) Schematic of experimental procedure. (Panel b) Clinical scores of mice treated with either bolus calcitriol (10ng calcitriol/biceps femoris/day, $n=4$) or bC2-CLNP (33 μ g bC2-CLNP/biceps femoris/day, $n=3$). (Panel c) Representative micro-CT images of hind paws from calcitriol and
25 bC2-CLNP treated joints. Magnified images were used for treatment-blinded quantification of bone loss in the metacarpophalangeal (Panel d) and ankle joints (Panel e) as well as bone erosion scoring (Panel f). Statistical analyses were performed using two-way ANOVA (panel b), unpaired Student's t -test with Welch's correction (panels d-e) and Mann-Whitney test (panel f). Schematic in panel a was composed using BioRender.

30 **Figure 5.** bC2-CLNP reduce synovitis and cartilage destruction in CIA mice. Representative H&E- (Panel a) and toluidine blue-stained (Panel b) ankle sections from mice that received either

calcitriol or bC2-CLNP. Regions of synovitis (arrows) and proteoglycan loss (arrows) are identified. Treatment-blinded synovitis (panel c), bone erosion (panel d) and proteoglycan loss (panel e) scores. Data in panels c-e represents means $\pm$ SD. Data in panels c-e are from n=8 sections from 4 mice for the calcitriol-treated and n=6 sections from 3 mice for the bC2-CLNP-treated mice. Statistical analyses were performed using Mann-Whitney test in panels c-e. Scale bar in panels a and b is 2 μ m.

Figure 6. Agg-CLNP modulate autoimmune arthritis in SKG mice. (Panel a) Schematic and timeline of experimental procedure. (Panel b) Clinical scores of mice with no treatment (untreated, $n = 5$) and treated with bolus calcitriol (10 ng calcitriol/biceps femoris/day, $n = 4$), OVA-CLNP (33 μ g OVA-CLNP/biceps femoris/day, $n = 6$), bC2-CLNP (33 μ g bC2-CLNP/biceps femoris/day, $n = 6$), or Agg-CLNP (33 μ g Agg-CLNP/ biceps femoris/day, $n = 6$). (Panel c) Quantification of CTLA-4+CD4+ T cells isolated from the ankle 14 days post-mannan. (Panel d) Quantification of CTLA-4+ CD11c+ DC isolated from the ankle 14 days post-mannan. qPCR relative quantification of *Tnf* (Panel e), *Il6* (Panel f), *Mmp3* (Panel g), and *Mmp13* (Panel h). Representative (i) H&E- (panel i) and safranin O-stained (panel j) histological ankle sections from mice that received either no treatment or Agg-CLNP depicting synovitis (arrows) and proteoglycan loss (red arrows). Treatment-blinded synovitis (panel k), bone erosion (panel l), and proteoglycan loss (panel m) scores from H&E, H&E, and safranin O histological sections, respectively. Data in panel b represent means $\pm$ SEM and in panels c– h, k– m represent means $\pm$ SD. Data for immune cell counts, bone erosion, and proteoglycan loss are from $n = 4$ sections from 2 mice for the untreated group and $n = 6$ sections from 3 mice for the Agg-CLNP group. Statistical analyses were performed using two-way ANOVA (panel b), unpaired Students *t*-test with Welsch's correction (panels c-h, k), and Mann–Whitney test (panels l, m). Scale bar in panels i, j is 2 μ m. Schematic in panel a was composed using BioRender.

Figure 7. Peptide names, sequences, molecular weights, and hydrophobic residue counts. Shown in Figure 7 are the sequence of N-cysteine ovalbumin (CISQAVHAAHAEINEAGR, SEQ ID NO: 3), N-cysteine bovine 2 collagen (CGEPGIAGFKGEQGPK, SEQ ID NO: 4), and N-cysteine aggrecan (CATEGVRVNSAYQDK, SEQ ID NO: 5).

Figure 8. Representative flow cytometry analysis of CD11c⁺ dendritic cells.

Figure 9. Representative flow cytometry analysis of CD4⁺ T cells.

Figure 10. Representative TEM images and sizing histograms of CLNP (a) unconjugated CLNP (Panel a), OVA-CLNP (Panel b), bC2-CLNP (Panel c), and Agg-CLNP (Panel d).

Figure 11. Biodistribution of CLNP components. (Panel a) IVIS images of target organs after Cy5-PEG-PLGA injection 2-, 8- and 24 hours post injection. (Panel b) Plots of Cy5 efficiency normalized to the 2-hour liver datapoint over 24 hours in liver, kidneys and spleen. (Panel c) IVIS images of target organs after Cy5-calcitriol injection 2-, 8- and 24 hours post injection. (Panel d) Plots of Cy5 efficiency normalized to the 2-hour kidney datapoint over 24 hours in liver, kidneys, and spleen. Data in panels b, d are means $\pm$ SD of three experimental replicates.

Figure 12. Representative flow cytometry analysis of CD11b⁺ immune cell subsets. Samples were divided and stained for different markers.

Figure 13. Quantification of innate immune cell subsets in the ankle. (Panel a) F4-80⁺ CD11b⁺ (Panel b) Ly6G⁺ CD11b⁺ cells.

Figure 14. Characterization of generalized immunosuppression in Agg-CLNP treated mice. (Panel a) Experimental timeline/schematic and (Panel b) anti-OVA IgG1 titers from whole blood of mice vaccinated against OVA and vaccinated against OVA with concurrent Agg-CLNP treatment; n=4 OVA vaccination only and n=5 OVA vaccination with Agg-CLNP. Statistical analysis was performed using the log values of the titers and a Mann-Whitney test. Schematic in panel a was composed using BioRender.

Figure 15. Peripheral blood immune cell and cytokine analysis. (Panel a) Experimental timeline. (Panel b) Ratio of B220⁺, CD11c⁺, F4-80⁺, Ly6G⁺, and TCR β ⁺ as a percentage of CD45⁺ cells. (Panel c) B220⁺, (Panel d) CD11c⁺, (Panel e) F4-80⁺, (Panel f) Ly6G⁺, and (Panel g) TCR β ⁺ CD45⁺ peripheral blood cells collected on day 7. (Panel h) IFN- γ , (Panel i) IL-4, (Panel j) IL-6, and (Panel k) TNF concentrations in serum collected on day 3. Data in panels c-k are means $\pm$ SD of four experimental replicates for each group. Data below the limit of detection or greater than three standard deviations were excluded in panels h-k. Statistical analyses in panels c-k performed with unpaired Student's *t*-test with Welsch's correction.

Figure 16. Synthesis, characterization and murine DC culture comparison of Agg-CLNP formulations. (Panel a) Experimental schematic and chemical structure of aggrecan peptide conjugated calcitriol loaded nanoparticle synthesis. (Panel b) Dynamic light scattering by intensity graph of old and new Agg-CLNP formulations. (Panels c, d, and e) Costimulatory molecules (CD80 and CD86) and major histocompatibility complex II (MHC2) positivity on

CD11c⁺ DC after culture with LPS, and both formulations of Agg-CLNP as measured by CD80 (panel c), CD86 (panel d), and MHC2 (panel e). Data in panels c-e are means $\pm$ SD of six experimental replicates. Statistical analyses in panels c-e were performed using ANOVA with Tukey's multiple comparison test. Schematic in panel a was composed using BioRender and ChemDraw.

Figure 17. Agg-CLNP imparts a tolerogenic phenotype on human DC *in vitro*. (Panel a) Experimental schematic of DC culture. (Panels b, c, d, e, f and g) Costimulatory molecules (CD40, CD80 and CD86), human leukocyte antigen II (HLA-2), MERTK and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) positivity on CD11c⁺ DC after culture with LPS, free calcitriol, free dexamethasone, Agg-CLNP and a combination of dexamethasone with Agg-CLNP as measured by CD40 (panel b), CD80 (panel c), CD86 (panel d), HLA-2 (panel e), MERTK (panel f), and CTLA-4 (panel g). Data in panels b-g are means $\pm$ SD of six technical replicates from representative experiments. Statistical analyses in panels b-g were performed using ANOVA with Tukey's multiple comparison test. Schematic in panel a was composed using BioRender.

Figure 18. Agg-CLNP modulate arthritis flare in SKG mice. (Panel a) Schematic and timeline of experimental procedure. (Panel b) Clinical scores of mice treated with either bolus dexamethasone (25 μ g/day, n=10) or bolus dexamethasone followed by Agg-CLNP (33 μ g Agg-CLNP/biceps femoris/day, n=12). (Panel c) Ankle thickness deltas of the mice clinically scored in panel b. Statistical analyses were performed using two-way ANOVA (panels b and c). Schematic in panel a was composed using BioRender.

Figure 19. Agg-CLNP locally reduces pathogenic Th17 cells in SKG mice. (Panel a) Schematic and timeline of experimental procedure. (Panel b) Clinical scores of mice treated with either bolus dexamethasone (25 μ g/day on days 5-7, 125 μ g/day on days 8-10, n=10) or bolus dexamethasone followed by Agg-CLNP (33 μ g Agg-CLNP/biceps femoris/day, n=11). (Panel c) Ankle thickness deltas of the mice clinically scored in panel b. (Panel d) Quantification of CD4⁺CD45⁺ T cells isolated from the spleen fourteen-days post mannan. (Panel e) Quantification of IL-17⁻ CD4⁺ Th17 cells isolated from the spleen fourteen-days post mannan. (Panel f) Quantification of CD4⁺CD45⁺ T cells isolated from the popliteal and inguinal lymph nodes fourteen-days post mannan. (Panel g) Quantification of IL-17⁺ CD4⁺ Th17 cells isolated from the inguinal and popliteal lymph nodes fourteen-days post mannan. (Panel h) Quantification of

CD4⁺CD45⁺ T cells isolated from the forepaws fourteen-days post mannan. (Panel i) Quantification of IL-17⁺ CD4⁺ T_H17 cells isolated from the forepaws fourteen-days post mannan. (Panel j) Quantification of CD4⁺CD45⁺ T cells isolated from the hind paws fourteen-days post mannan. (Panel k) Quantification of IL-17⁺ CD4⁺ T_H17 cells isolated from the hind paws
5 fourteen-days post mannan. Statistical analyses were performed using two-way ANOVA (panels b and c) and Student's *t*-test with Welsch's correction (panels d-k). Schematic in panel a was composed using BioRender.

DETAILED DESCRIPTION

10 All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

Unless defined otherwise, all technical and scientific terms and any acronyms used herein have the same meanings as commonly understood by one of ordinary skill in the art in the field of the
15 invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the exemplary methods, devices, and materials are described herein.

Definitions

As used herein, the terms “comprises,” “comprising,” “includes,” “including,” “has,” “having,”
20 “contains,” “containing,” “characterized by,” or any other variation thereof, are intended to encompass a non-exclusive inclusion, subject to any limitation explicitly indicated otherwise, of the recited components. For example, a fusion protein, a pharmaceutical composition, and/or a method that “comprises” a list of elements (e.g., components, features, or steps) is not necessarily limited to only those elements (or components or steps), but may include other
25 elements (or components or steps) not expressly listed or inherent to the fusion protein, pharmaceutical composition and/or method.

As used herein, the transitional phrases “consists of” and “consisting of” exclude any element, step, or component not specified. For example, “consists of” or “consisting of” used in a claim would limit the claim to the components, materials or steps specifically recited in the claim
30 except for impurities ordinarily associated therewith (i.e., impurities within a given component). When the phrase “consists of” or “consisting of” appears in a clause of the body of a claim,

rather than immediately following the preamble, the phrase “consists of” or “consisting of” limits only the elements (or components or steps) set forth in that clause; other elements (or components) are not excluded from the claim as a whole.

As used herein, the transitional phrases “consists essentially of” and “consisting essentially of” are used to define a fusion protein, pharmaceutical composition, and/or method that includes materials, steps, features, components, or elements, in addition to those literally disclosed, provided that these additional materials, steps, features, components, or elements do not materially affect the basic and novel characteristic(s) of the claimed invention. The term “consisting essentially of” occupies a middle ground between “comprising” and “consisting of”.

When introducing elements of the present invention or the preferred embodiment(s) thereof, the articles “a”, “an”, “the” and “said” are intended to mean that there are one or more of the elements. The terms “comprising”, “including” and “having” are intended to be inclusive and mean that there may be additional elements other than the listed elements.

The term “and/or” when used in a list of two or more items, means that any one of the listed items can be employed by itself or in combination with any one or more of the listed items. For example, the expression “A and/or B” is intended to mean either or both of A and B, i.e. A alone, B alone or A and B in combination. The expression “A, B and/or C” is intended to mean A alone, B alone, C alone, A and B in combination, A and C in combination, B and C in combination or A, B, and C in combination.

It is understood that aspects and embodiments of the invention described herein include “consisting” and/or “consisting essentially of” aspects and embodiments.

It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range. Values or ranges may be also be expressed herein as “about,” from “about” one particular value, and/or to “about” another particular value. When such values or ranges are expressed, other embodiments disclosed include the specific value

recited, from the one particular value, and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another embodiment. It will be further understood that there are a number of values disclosed therein, and that each value is also herein disclosed as “about” that particular value in addition to the value itself. In embodiments, “about” can be used to mean, for example, within 10% of the recited value, within 5% of the recited value, or within 2% of the recited value.

The term “at least” prior to a number or series of numbers is understood to include the number adjacent to the term “at least”, and all subsequent numbers or integers that could logically be included, as clear from context. When at least (or similar such term, such as at most, etc.) is present before a series of numbers or a range, it is understood that “at least” can modify each of the numbers in the series or range.

As used herein, “patient” or “subject” means a human or animal subject to be treated.

As used herein the term “pharmaceutical composition” refers to pharmaceutically acceptable compositions, wherein the composition comprises a pharmaceutically active agent, and in some embodiments further comprises a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition may be a combination of pharmaceutically active agents and carriers.

The term “combination” refers to either a fixed combination in one dosage unit form, or a kit of parts for the combined administration where one or more active compounds and a combination partner (e.g., another drug as explained below, also referred to as “therapeutic agent” or “co-agent”) may be administered independently at the same time or separately within time intervals. In some circumstances, the combination partners show a cooperative, e.g., synergistic effect. The terms “co-administration” or “combined administration” or the like as utilized herein are meant to encompass administration of the selected combination partner to a single subject in need thereof (e.g., a patient), and are intended to include treatment regimens in which the agents are not necessarily administered by the same route of administration or at the same time. The term “pharmaceutical combination” as used herein means a product that results from the mixing or combining of more than one active ingredient and includes both fixed and non-fixed combinations of the active ingredients. The term “fixed combination” means that the active ingredients, e.g., a compound and a combination partner, are both administered to a patient

simultaneously in the form of a single entity or dosage. The term “non-fixed combination” means that the active ingredients, e.g., a compound and a combination partner, are both administered to a patient as separate entities either simultaneously, concurrently or sequentially with no specific time limits, wherein such administration provides therapeutically effective levels of the two compounds in the body of the patient. The latter also applies to cocktail therapy, e.g., the administration of three or more active ingredients.

As used herein the term “pharmaceutically acceptable” means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopoeia, other generally recognized pharmacopoeia in addition to other formulations that are safe for use in animals, and more particularly in humans and/or non-human mammals.

As used herein the term “pharmaceutically acceptable carrier” refers to an excipient, diluent, preservative, solubilizer, emulsifier, adjuvant, and/or vehicle with which demethylation compound(s), is administered. Such carriers may be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents. Antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; and agents for the adjustment of tonicity such as sodium chloride or dextrose may also be a carrier. Methods for producing compositions in combination with carriers are known to those of skill in the art. In some embodiments, the language “pharmaceutically acceptable carrier” is intended to include any and all solvents, dispersion media, coatings, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. See, e.g., Remington, The Science and Practice of Pharmacy, 20th ed., (Lippincott, Williams & Wilkins 2003). Except insofar as any conventional media or agent is incompatible with the active compound, such use in the compositions is contemplated.

As used herein, “therapeutically effective amount” refers to an amount of a pharmaceutically active compound(s) that is sufficient to treat or ameliorate, or in some manner reduce the symptoms associated with diseases and medical conditions. When used with reference to a method, the method is sufficiently effective to treat or ameliorate, or in some manner reduce the symptoms associated with diseases or conditions. For example, an effective amount in reference

to diseases is that amount which is sufficient to block or prevent onset; or if disease pathology has begun, to palliate, ameliorate, stabilize, reverse or slow progression of the disease, or otherwise reduce pathological consequences of the disease. In any case, an effective amount may be given in single or divided doses.

5 As used herein, the terms “treat,” “treatment,” or “treating” embraces at least an amelioration of the symptoms associated with diseases in the patient, where amelioration is used in a broad sense to refer to at least a reduction in the magnitude of a parameter, e.g. a symptom associated with the disease or condition being treated. As such, “treatment” also includes situations where the disease, disorder, or pathological condition, or at least symptoms associated therewith, are
10 completely inhibited (e.g. prevented from happening) or stopped (e.g. terminated) such that the patient no longer suffers from the condition, or at least the symptoms that characterize the condition.

As used herein, and unless otherwise specified, the terms "prevent," "preventing" and "prevention" refer to the prevention of the onset, recurrence or spread of a disease or disorder, or
15 of one or more symptoms thereof. In certain embodiments, the terms refer to the treatment with or administration of a compound or dosage form provided herein, with or without one or more other additional active agent(s), prior to the onset of symptoms, particularly to subjects at risk of disease or disorders provided herein. The terms encompass the inhibition or reduction of a symptom of the particular disease. In certain embodiments, subjects with familial history of a
20 disease are potential candidates for preventive regimens. In certain embodiments, subjects who have a history of recurring symptoms are also potential candidates for prevention. In this regard, the term "prevention" may be interchangeably used with the term "prophylactic treatment."

As used herein, and unless otherwise specified, a "prophylactically effective amount" of a compound is an amount sufficient to prevent a disease or disorder, or prevent its recurrence. A
25 prophylactically effective amount of a compound means an amount of therapeutic agent, alone or in combination with one or more other agent(s), which provides a prophylactic benefit in the prevention of the disease. The term "prophylactically effective amount" can encompass an amount that improves overall prophylaxis or enhances the prophylactic efficacy of another prophylactic agent.

30 As used herein, and unless otherwise specified, the term "subject" is defined herein to include animals such as mammals, including, but not limited to, primates (e.g., humans), cows, sheep,

goats, horses, dogs, cats, rabbits, rats, mice, and the like. In specific embodiments, the subject is a human. The terms "subject" and "patient" are used interchangeably herein in reference, for example, to a mammalian subject, such as a human.

As used herein, the term "sustained release" refers to an agent-containing formulation, such as a composition or scaffold as described herein, in which complete release of the agent is not immediate, i.e., with a "sustained release" formulation, administration does not result in immediate release of the entirety of the agent. The term is used interchangeably with "nonimmediate release" as defined in Remington: The Science and Practice of Pharmacy, Nineteenth Ed. (Easton, PA: Mack Publishing Company, 1995). The sustained release may be a slower release of a portion of the relevant agent (e.g., an antigen and/or adjuvant as described herein) following an initial quick release of a portion of the agent (i.e., following a "burst" phase). As such, only a portion of the agent within the scaffold need be released gradually over an extended period of time.

"Injection" includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, sub capsular, subarachnoid, intraspinal, intracerebro spinal, and intrasternal injection and infusion. In preferred embodiments, the compositions are administered by injection, e.g., subcutaneous injection.

A "linker" refers to a chemical moiety that covalently or non-covalently attaches a compound or substituent group to another moiety. Linkers are typically at least bifunctional chemical moieties. Generally a linker has no specific biological activity other than to, e.g., join chemical species together or to preserve some minimum distance or other spatial relationship between such species. However, the constituents of a linker may be selected to influence some property of the linked chemical species such as three-dimensional conformation, net charge, hydrophobicity, etc. Exemplary linkers include, e.g., oligopeptides, oligopolyamides, oligoethyleneglycerols, oligoacrylamides, alkyl chains, or the like.

The term "attached" or "conjugated" refers to interactions and/or states in which material or compounds are connected or otherwise joined with one another. These interactions and/or states are typically produced by, e.g., covalent bonding, ionic bonding, chemisorption, physisorption, and combinations thereof. In preferred embodiments, a conjugated product will have two separate moieties linked by covalent binding.

Nanoparticle Compositions

In one aspect provided herein are compositions of nanoparticles which can be useful in the treatment of arthritis or its associated symptoms. In certain embodiments, the nanoparticles described herein are particularly suited to the prevention, amelioration, treatment, and/or management of arthritis flares. In embodiments, a nanoparticle of the instant disclosure is comprises of a biodegradable polymer which is conjugated to a joint-relevant peptide antigen, and the nanoparticle at least partially encapsulates an active agent which is a dendritic cell modulator.

In embodiments, the nanoparticles described herein are made of biodegradable polymers. The biodegradable polymers are ones which is able to be broken down within the body of a subject. In embodiments, the breaking down of the biodegradable polymers allows for the release of the active agent (e.g., the dendritic cell modulator, such as calcitriol). In embodiments, the biodegradable polymer is selected such that it releases the active agent over a desired period of time. For example, in embodiments, the nanoparticle is made of biodegradable polymers which allows for the release of the active agent (e.g., the dendritic cell modulator) over a period of at least 7 days, at least 14 days, at least 21 days, or at least 28 days.

In embodiments, the nanoparticle is made from biodegradable polymers selected from poly(caprolactone) (PCL), ethylene vinyl acetate polymer (EVA), poly(lactic acid) (PLA), poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid)(PLGA), PLGA-poly(ethylene glycol) block copolymer; poly(L-lactic-T-glycolic acid) (PLLGA), poly(D,L-lactide) (PDLA), poly(D,L-lactide-co-caprolactone), poly(D,L-lactide-co-caprolactone-co-glycolide), poly(D,L-lactide-co-PEO-co-D,L-lactide), poly(D,L-lactide-co-PPO-co-D,L-lactide), polyhydroxylalcanoates, poly(hydroxybutyrate) (P4HB), poly-L-lysine (PLL), poly-L-glutamic acid, poly(hydroxy acids), polyanhydrides, poly(ester amides), polyamides, poly(ester ethers), polycarbonates, polyphosphates, polyphosphoesters, polyphosphazines, polydioxazones, polyurethanes, derivatized celluloses such as alkyl celluloses, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, carboxymethylcellulose, polyvinylalcohols, polyaminoacids, poly(butyric acid), poly(valeric acid), poly(levulinic acid), and combinations thereof, including block copolymers thereof and mixtures comprising block copolymers thereof combined with individual polymers thereof (or mixt with other block copolymers thereof). For example, the nanoparticles can be made from a block copolymer

portion (e.g., poly(lactic-co-glycolic acid)) attached to a single-polymer portion (e.g., a poly(ethylene glycol)). In embodiments, the biodegradable polymers comprise poly(ethylene glycol) (PEG), poly(lactic-co-glycolic acid) (PLGA), or a combination thereof.

In embodiments, the nanoparticles are made of biodegradable polymers which comprise a mixture of copolymer PLGA and PEG. In embodiments, the biodegradable polymers comprises a first portion which is a PLGA polymer and as second portion which is a PEG polymer. In embodiments, the biodegradable polymers comprise a structure PLGA-PEG. In embodiments, the PLGA portion has lactide:glycolic acid ratio of from about 90:10 to about 10:90 (mol/mol). In embodiments, the PLGA portion has a lactide:glycolic acid ratio of about 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, or 10:90 (mol/mol). In embodiments, the PLGA portion has a lactide:glycolic acid ratio of about 50:50 (mol/mol). In embodiments, the PLGA portion has a molecular weight of from about 1 kDa to about 50 kDa, from about 1 kDa to about 40 kDa, from about 1 kDa to about 30 kDa, from about 1 kDa to about 25 kDa, from about 1 kDa to about 20 kDa, from about 5 kDa to about 50 kDa, from about 5 kDa to about 40 kDa, from about 5 kDa to about 30 kDa, from about 5 kDa to about 25 kDa, from about 5 kDa to about 20 kDa, from about 10 kDa to about 50 kDa, from about 10 kDa to about 40 kDa, from about 10 kDa to about 30 kDa, from about 10 kDa to about 25 kDa, from about 10 kDa to about 20 kDa, from about 15 kDa to about 50 kDa, from about 15 kDa to about 40 kDa, from about 15 kDa to about 30 kDa, from about 15 kDa to about 25 kDa, from about 15 kDa to about 20 kDa, from about 20 kDa to about 50 kDa, from about 20 kDa to about 40 kDa, from about 20 kDa to about 30 kDa, or from about 20 kDa to about 25 kDa. In embodiments, the PLGA portion has a molecular weight of about 20 kDa. In embodiments, the PEG portion has a molecular weight of about 0.5 kDa to about 10 kDa, about 0.5 kDa to about 7.5 kDa, about 0.5 kDa to 5 kDa, about 0.5 kDa to about 3 kDa, about 0.5 kDa to about 2.5 kDa, about 0.5 kDa to about 2 kDa, about 1 kDa to about 10 kDa, about 1 kDa to about 7.5 kDa, about 1 kDa to 5 kDa, about 1 kDa to about 3 kDa, about 1 kDa to about 2.5 kDa, about 1 kDa to about 2 kDa, about 1.5 kDa to about 10 kDa, about 1.5 kDa to about 7.5 kDa, about 1.5 kDa to 5 kDa, about 1.5 kDa to about 3 kDa, about 1.5 kDa to about 2.5 kDa, about 1.5 kDa to about 2 kDa, about 0.5 kDa to about 10 kDa, about 2 kDa to about 7.5 kDa, about 2 kDa to 5 kDa, about 2 kDa to about 3 kDa, or about 2 kDa to about 2.5 kDa. In embodiments, the PEG portion has a molecular weight of about 2 kDa.

In embodiments, the nanoparticle can comprise additional materials forming its structure in addition to the biodegradable polymers. For example, the nanoparticle can incorporate additional materials which are not biodegradable, such as metal, plastic polymers, or silk polymers. However, in preferred embodiments, all or nearly all of the nanoparticle will be biodegradable (e.g., all or entirely made from biodegradable polymers). In embodiments, the nanoparticle structure is made up of at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the biodegradable polymers (w/w).

In embodiments, the nanoparticle composition is not a liposomal composition. In embodiments, the nanoparticle composition lacks phospholipids. In embodiments, the nanoparticle composition lacks esterified propoxylated glycerols. In embodiments, the nanoparticle composition does not comprise a lipid bilayer.

In embodiments, the nanoparticles are of a desired size. In embodiments, the nanoparticles are of a size which permits or favors accumulation of the nanoparticles in one or more lymph nodes of a patient upon administration. In embodiments, the nanoparticles are of a size which permits accumulation of the nanoparticles of one or more lymph nodes of the subject. In embodiments, the nanoparticles are of a size which allows for accumulation of the nanoparticles in an inguinal, popliteal, and/or brachial lymph node.

In embodiments, the nanoparticles have an average hydrodynamic diameter of from about 10 nm to about 500 nm. In embodiments, the nanoparticles have an average hydrodynamic diameter of from about 50 nm to about 500 nm. In embodiments, the nanoparticles have an average hydrodynamic diameter of from about 50 nm to about 300 nm. In embodiments, the nanoparticles have an average hydrodynamic diameter of from about 50 nm to about 500 nm, about 50 nm to about 400 nm, about 50 nm to about 300 nm, about 50 nm to about 250 nm, about 50 nm to about 200 nm, about 50 nm to about 150 nm, about 50 nm to about 100 nm, about 100 nm to about 500 nm, about 100 nm to about 400 nm, about 100 nm to about 300 nm, about 100 nm to about 250 nm, about 100 nm to about 200 nm, about 150 nm to about 500 nm, about 150 nm to about 400 nm, about 150 nm to about 300 nm, about 150 nm to about 250 nm, or about 150 nm to about 200 nm. In embodiments, the nanoparticles have an average hydrodynamic diameter of about 100 nm to about 300 nm. In embodiments, the nanoparticles have an average hydrodynamic diameter of about 50 nm, about 100 nm, about 150 nm, about 200 nm, about 250 nm, or about 300 nm. In embodiments, the nanoparticles have an average

hydrodynamic diameter of about 200 nm. In embodiments, the nanoparticles have an average hydrodynamic radius of at most about 500 nm, 450 nm, 400 nm, 350 nm, 300 nm, or 250 nm. In embodiments, the nanoparticles have an average hydrodynamic diameter of at most about 250 nm. In embodiments, the nanoparticles have an average hydrodynamic diameter of at least 10 nm, at least 25 nm, at least 50 nm, or at least 100 nm.

In embodiments, the nanoparticles have a zeta potential of from about -5 mV to about -50 mV. In embodiments, the nanoparticles have a zeta potential of from about -5 mV to about -50 mV, about -10 mV to about -40 mV, about -10 mV to about -30 mV, or about -10 mV to about -20 mV.

In embodiments, the nanoparticles have an average particle size diameter of about 10 nm to about 300 nm. In embodiments, the nanoparticles have an average particle size diameter of about 10 nm to about 300 nm, about 10 nm to about 250 nm, about 10 nm to about 200 nm, about 10 nm to about 150 nm, about 10 nm to about 100 nm, about 25 nm to about 300 nm, about 25 nm to about 250 nm, about 25 nm to about 200 nm, about 25 nm to about 150 nm, about 25 nm to about 100 nm, about 50 nm to about 300 nm, about 50 nm to about 250 nm, about 50 nm to about 200 nm, about 50 nm to about 150 nm, or about 50 nm to about 100 nm. In embodiments, the nanoparticles have an average particle size diameter of about 50 nm to about 150 nm. In embodiments, the nanoparticles have an average particle size diameter of at most about 300 nm, 250 nm, 200 nm, or 150 nm. In embodiments, the nanoparticles have an average particle size diameter of at most about 150 nm.

In embodiments, the nanoparticles are preferably of a relatively uniform size distribution (either measured as hydrodynamic diameter or particle size diameter). In embodiments, the nanoparticles have a polydispersity index of less than 0.25, less than 0.2, less than 0.15, or less than 0.1.

In embodiments, the nanoparticles comprise a joint-relevant peptide antigen conjugated to the nanoparticles. In embodiments, the joint-relevant peptide antigen is conjugated to the biodegradable polymers of the nanoparticle. In embodiments, the joint-relevant peptide antigen acts to modulate the immune system in order to reduce inflammation in arthritic tissue, thereby treating and/or preventing arthritis or its symptoms (e.g., flares).

The joint-relevant peptide antigen can be conjugated to the biodegradable polymers of the nanoparticle via a variety of chemistry and/or linkers. In preferred embodiments, the joint-

relevant peptide antigen is conjugated to the biodegradable polymers via a reaction with a cysteine residue of the joint-relevant peptide antigen with a suitable reactive group attached to the biodegradable polymers (e.g., a maleimide functionality), though other suitable chemistries for this purpose are well known in the art (e.g., CLICK chemistry). In embodiments, the joint-relevant peptide antigen comprises a cysteine residue which is added to a stretch of native sequence of the joint-relevant peptide, preferably to the N-terminus. The cysteine (or other suitable reactive group) can also be added to the joint-relevant peptide antigen could also be added to the joint-relevant peptide antigen amino acid sequence in a variety of ways and by a wide variety of linkers which are well known in the art (e.g., PEG linkers, alkyl-linkers, and the like). In embodiments, the joint-relevant peptide antigen is conjugated to biodegradable nanoparticles by a reaction between a cysteine residue on the joint-relevant peptide antigen and a maleimide on the biodegradable polymers.

In embodiments, the joint-relevant peptide antigen is derived from a protein which is at higher abundance in joints compared to other tissues. In embodiments, the joint-relevant peptide antigen is derived from a cartilage tissue component. In embodiments, the joint-relevant peptide antigen is derived from a cartilage protein. In embodiments, the joint-relevant peptide antigen is derived from a mammalian cartilage protein. In embodiments, the joint-relevant peptide antigen is derived from a human cartilage protein. In embodiments, the joint-relevant peptide antigen is derived from collagen, a cartilage proteoglycan, and elastin.

In embodiments, the joint-relevant peptide antigen is derived from collagen. In embodiments, the joint-relevant peptide antigen is derived from a mammalian collagen. In embodiments, the joint-relevant peptide antigen is derived from a mammalian Type II collagen. In embodiments, the joint-relevant peptide antigen is derived from a human or bovine collagen. In embodiments, the joint-relevant peptide antigen is derived from a bovine collagen. In embodiments, the joint-relevant peptide antigen is derived from bovine Type II collagen. In embodiments, the joint-relevant peptide antigen comprises the amino acid sequence set forth in SEQ ID NO: 1 (GEPLGIAGFKGEQGPK). In embodiments, the joint-relevant peptide antigen is derived from a human collagen. In embodiments, the joint-relevant peptide antigen is derived from a human Type II collagen.

In embodiments, the joint-relevant peptide antigen is derived from a cartilage proteoglycan. In embodiments, the cartilage proteoglycan is selected from aggrecan (e.g., UniProt ID P16112),

versican (e.g., UniProt ID P13611, Q86W61), link protein (e.g., UniProt ID P10915), biglycan (dermatan sulfate proteoglycan (DS-PG)I) (e.g., UniProt ID P21810), decorin (DS-PGII) (e.g., UniProt ID P07585), epiphykan (DS-PGIII) (e.g., UniProt ID Q99645), fibromodulin (e.g., UniProt ID Q06828), lumican (e.g., UniProt ID P51884), perlecan (e.g. UniProt ID P98160), and lubricin (e.g., UniProt ID Q92954). In embodiments, the joint-relevant peptide antigen is derived from aggrecan, biglycan, perlecan, agrin, fibromodulin, and lumican. In embodiments, the joint-relevant peptide antigen is derived from aggrecan or collagen. In embodiments, the joint-relevant peptide antigen is derived from aggrecan. In embodiments, the joint-relevant peptide antigen is derived from human aggrecan. In embodiments, the joint-relevant peptide antigen comprises the amino acid sequence set forth in SEQ ID NO: 2 (ATEGRVRVNSAYQDK).

In embodiments, the joint-relevant peptide antigen is derived from a cartilage protein. In embodiments, the cartilage protein is selected from cartilage oligomeric matrix protein (COMP) (a.k.a. Thrombospondin-5) (e.g., UniProt ID P49747), Thrombospondin-1 (e.g., UniProt ID P07996), Thrombosponin-3 (e.g., P49746), CMP (cartilage matrix protein) (a.k.a. Matrilin-1) (e.g., UniProt ID P21941), Matrilin-3 (e.g., UniProt ID O15232), cartilage intermediate layer protein (CILP) (e.g., UniProt ID O75339), C-type lectin (e.g., CLEC1A, CLEC1B, CLEC2A, CLEC2B, CLEC2C, CLEC2D, CLEC2L, CLEC3A, CLEC3B, CLEC4A, CLEC4C, CLEC4D, CLEC4E, CLEC4F, CLEC4G, CLEC4H1, CLEC4H2, CLEC4J, CLEC4K, CLEC4L, CLEC4M, CLEC5A, CLEC6A, CLEC7A, CLEC8A, CLEC9A, CLEC10A, CLEC11A, CLEC12A, CLEC12B, CLEC13A, CLEC13B, CLEC13C, CLEC13D, CLEC13E, CLEC14A, CLEC16A, or CLEC17A), Fibronectin (e.g., UniProt ID P02751), PRELP (proline- and arginine-rich end leucine-rich repeat protein) (e.g., UniProt ID P51888), Chondroadherin (e.g., UniProt ID O15335), Tenascin-C (e.g., P24821), Fibrillin (e.g., Fibrillin-1, Fibrillin-2, Fibrillin-3, or Fibrillin-4), Elastin (e.g., UniProt ID P15502), gp (glycoprotein)-39/YKL-40 (e.g., UniProt ID P36222), Matrix gla (gamma-carboxyglutamic acid) protein/MGP (e.g., P08493), Pleiotrophin (e.g., P21246), Chondromodulin-I (e.g., UniProt ID O75829), cartilage-derived retinoic acid sensitive protein (CD-RAP) (e.g., UniProt ID Q16674), Chondrocalcin (e.g., UniProt ID P02458), and PARP (proline- and arginine-rich protein).

In embodiments, the joint-relevant peptide antigen comprises a stretch of at least 8, 10, 12, or 14 amino acids of the protein from which it is derived. In embodiments, the joint-relevant peptide antigen comprises a stretch of at least 8 amino acids of the protein from which it is derived. In

embodiments, the joint-relevant peptide antigen comprises a stretch of at least 10 amino acids of the protein from which it is derived. In embodiments, the joint-relevant peptide antigen comprises a stretch of at least 12 amino acids of the protein from which it is derived. In embodiments, the joint-relevant peptide antigen comprises a stretch of at least 14 amino acids of the protein from which it is derived. In embodiments, the joint-relevant peptide antigen comprises a stretch of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids of the protein from which it is derived. In embodiments, the joint-relevant peptide antigen comprises a stretch of at most 50, 40, 30, 25, 20, 19, 18, 17, 16, 15, or 14 amino acids of the protein from which it is derived.

In embodiments, the joint-relevant peptide antigen comprises at least a portion of an immunodominant peptide of the protein from which the joint-relevant peptide antigen is derived. In embodiments, the joint-relevant peptide antigen comprises an immunodominant peptide of a cartilage protein and/or a cartilage proteoglycan as described herein.

In embodiments, the nanoparticle at least partially encapsulates a dendritic cell modulator. In embodiments, the dendritic cell modulator is one which induces an immunomodulatory phenotype of dendritic cells. In embodiments, the immunomodulatory phenotype of dendritic cells imparts immunoregulation through the induction of autoimmune-protective regulator T cells (T_{reg}) and/or induced T cell anergy. In embodiments, the immunomodulatory phenotype of dendritic cells is characterized by reduced expression by the dendritic cell of MHC2, CD80, CD86, IL-6, and/or TNF α in the dendritic cell. In embodiments, the immunomodulatory phenotype of dendritic cells is characterized by reduced expression by the dendritic cell of 2, 3, 4, or 5 of MHC2, CD80, CD86, IL-6, and TNF α . In embodiments, the immunomodulatory phenotype of dendritic cells is characterized by reduced expression by the dendritic cell of each of MHC2, CD80, CD86, IL-6, and TNF α . In embodiments, the reduced expression of one or more of MHC2, CD80, CD86, IL-6, and/or TNF α is a reduced expression following exposure of the dendritic cell to lipopolysaccharide (LPS) (e.g., the dendritic cells show reduced expression of MHC2, CD80, CD86, IL-6, and/or TNF α after incubation with LPS as compared to dendritic cells which are not of the immunomodulatory phenotype (e.g., those not treated with a dendritic cell modulator as described herein, such as calcitriol). In embodiments, the immunomodulatory phenotype is characterized by increased expression of CTLA-4, MERTK (myeloid-epithelial-reproductive tyrosine kinase) and/or TGFB3. In embodiments, the immunomodulatory

phenotype is characterized by increased expression of 1, 2, or 3 of CTLA-4, MERTK (myeloid-epithelial-reproductive tyrosine kinase) and/or TGFB3. In embodiments, the immunomodulatory phenotype is characterized by increased expression of CTLA-4 and/or MERTK. In embodiments, the immunomodulatory phenotype is characterized by increased expression of CTLA-4 and MERTK.

In embodiments, the dendritic cell modulator reduces expression of MHC2, CD80, CD86, IL-6, and/or TNF α in dendritic cells. In embodiments, the dendritic cell modulator reduces expression of 2, 3, 4, or 5 of MHC2, CD80, CD86, IL-6, and/or TNF α in dendritic cells. In embodiments, the dendritic cell modulator reduces expression of each of MHC2, CD80, CD86, IL-6, and TNF α in dendritic cells. In embodiments, the reduced expression of one or more of MHC2, CD80, CD86, IL-6, and/or TNF α is a reduced expression following exposure of the dendritic cell to lipopolysaccharide (LPS) (e.g., the dendritic cells show reduced expression of MHC2, CD80, CD86, IL-6, and/or TNF α after incubation with LPS as compared to dendritic cells which are not of the immunomodulatory phenotype (e.g., those not treated with a dendritic cell modulator as described herein, such as calcitriol).

In embodiments, the dendritic cell modulator increases expression of CTLA-4, MERTK (myeloid-epithelial-reproductive tyrosine kinase) and/or TGFB3 in dendritic cells. In embodiments, the dendritic cell modulator increases expression of 1, 2, or 3 of CTLA-4, MERTK (myeloid-epithelial-reproductive tyrosine kinase) and/or TGFB3 in dendritic cells. In embodiments, the dendritic cell modulator increases expression of CTLA-4 and/or MERTK in dendritic cells. In embodiments, the dendritic cell modulator increases expression of CTLA-4 and MERTK in dendritic cells.

In embodiments, the dendritic cell modulator is a small molecule compound. In embodiments, the dendritic cell modulator is calcitriol, or an analog thereof, or a pharmaceutically acceptable salt thereof. In embodiments, the dendritic cell modulator is calcitriol or a pharmaceutically acceptable salt thereof. In embodiments, the dendritic cell modulator is calcitriol.

In embodiments, after administration to a patient, the composition is effective to modulate dendritic cells in a joint and/or lymph node of the patient. In embodiments, the composition directly modulates dendritic cells in the joint and/or lymph node of the patient. In embodiments, the composition directly modulates dendritic cells in a lymph node of the patient, and the dendritic cells subsequently migrate to an arthritis affected joint. In embodiments, the

composition modulates dendritic cells in a local lymph node (i.e., one near the site of delivery of the composition). In embodiments, the nanoparticles localize predominantly in a local lymph node.

In embodiments, the nanoparticle composition does not cause systemic immunosuppression of the patient following administration. In embodiments, the nanoparticle composition imparts its effect by acting only locally to activate dendritic cells in lymph nodes and/or joints of the patient.

In embodiments, the nanoparticle composition does not substantially alter levels of at least one or more of B cells (B220⁺), dendritic cells (CD11C⁺), macrophages (F4-80⁺), neutrophils (Ly6G⁺), and/or T cells (TCRb⁺) in peripheral blood of the patient following administration.

In embodiments, the nanoparticle composition does not substantially increase levels of at least one or more of B cells (B220⁺), dendritic cells (CD11C⁺), macrophages (F4-80⁺), neutrophils (Ly6G⁺), and/or T cells (TCRb⁺) in peripheral blood of the patient following administration. In embodiments, the nanoparticle composition does not substantially alter levels of IFN- γ , IL-4, IL-6, and/or TNF in peripheral blood of the patient following administration.

The dendritic cell modulator can be present in the nanoparticle composition at any desired amount (e.g., up to 50% w/w) and the level of the dendritic cell modulator present in the composition will depend on the exact dendritic cell modulator and desired dose. For example, in embodiments wherein the dendritic cell modulator is calcitriol, the nanoparticle composition will comprise only a small percentage of calcitriol to biodegradable polymer (e.g., less than 1%, less than 0.1%, or even less than 0.01% w/w). In embodiments, the nanoparticle composition comprises about 0.001% to about 0.1% w/w of calcitriol.

In embodiments, administration of the nanoparticle composition does not release a concentration of the dendritic cell modulator into peripheral blood at a physiologically relevant level. In embodiments, administration of the nanoparticle composition does not result in a peripheral blood concentration of calcitriol released from the nanoparticles above 1 nM, 0.9 nM, 0.8 nM, 0.7 nM, 0.6 nM, 0.5 nM, 0.4 nM, 0.3 nM, 0.2 nM, 0.1 nM, 0.05 nM, or 0.01 nM.

In embodiments, a dose of calcitriol administered from the nanoparticle composition is at most about 1 mg, at most about 500 micrograms, at most about 100 micrograms, at most about 10 micrograms, at most about 5 micrograms, at most about 1 microgram, at most about 500 nanograms, at most about 100 nanograms, at most about 50 nanograms, at most about 10 nanograms, at most about 5 nanograms, or at most about 1 nanogram.

In embodiments, a dose of the nanoparticle composition (e.g., the total weight of nanoparticle composition, including mass of polymer, active ingredients, etc.) is from about 1 microgram to about 10 mg.

Pharmaceutical Compositions

5 In embodiments, the nanoparticle composition described herein can comprise the nanoparticles described herein and a pharmaceutically acceptable carrier. Pharmaceutical compositions containing the therapeutic agents described herein can be prepared by available procedures using available ingredients. The formulations can contain pharmaceutically acceptable carriers, vehicles, and adjuvants. For example, the therapeutic agents can be formulated with common
10 excipients, diluents, or carriers, and formed into tablets, capsules, solutions, suspensions, powders, aerosols, and the like. Examples of excipients, diluents, and carriers that are suitable for such formulations include buffers, as well as fillers and extenders such as starch, cellulose, sugars, mannitol, and silicic derivatives. Binding agents can also be included such as carboxymethyl cellulose, hydroxymethylcellulose, hydroxypropyl methylcellulose and other
15 cellulose derivatives, alginates, gelatin, and polyvinyl-pyrrolidone. Agents for retarding dissolution can also be included such as paraffin. Resorption accelerators such as quaternary ammonium compounds can also be included. Surface active agents such as cetyl alcohol and glycerol monostearate can be included. Adsorptive pharmaceutical carriers such as kaolin and bentonite can be added. Preservatives can also be added. The compositions of the invention can
20 also contain thickening agents such as cellulose and/or cellulose derivatives. They can also contain gums such as xanthan, guar or carbo gum or gum arabic, or alternatively polyethylene glycols, bentones and montmorillonites, and the like.

It is possible, for example, to prepare solutions using one or more aqueous or organic solvent(s) that is/are acceptable from the physiological standpoint, chosen, in addition to water, from
25 solvents such as acetone, ethanol, isopropyl alcohol, glycol ethers such as the products sold under the name "Dowanol," polyglycols and polyethylene glycols, C₁-C₄ alkyl esters of short-chain acids, ethyl or isopropyl lactate, fatty acid triglycerides such as the products marketed under the name "Miglyol," isopropyl myristate, animal, mineral and vegetable oils and polysiloxanes.

30 The nanoparticles can be formulated for intramuscular administration (e.g., by injection, for example, bolus injection or infusion into muscle tissue of the patient) and can be presented in

unit dose form in ampules, pre-filled syringes, small volume infusion containers or in multi-dose containers.

As noted above, preservatives can be added to help maintain the shelf life of the dosage form. The active agents and other ingredients can form suspensions, solutions, or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the therapeutic agents and other ingredients can be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

The compositions can also include antioxidants, surfactants, preservatives, film-forming, keratolytic or comedolytic agents. Antioxidants such as t-butylhydroquinone, butylated hydroxyanisole, butylated hydroxytoluene and α -tocopherol and its derivatives can be added.

The compositions can include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are available in the art. Examples of such substances include normal saline solutions such as physiologically buffered saline solutions and water. Specific non-limiting examples of the pharmaceutical carriers and/or diluents that are useful in the pharmaceutical formulations of the present invention include water and physiologically acceptable buffered saline solutions such as phosphate buffered saline solutions pH 7.0-8.0.

Methods of Treatment

In one aspect herein is a method of treating arthritis in a patient in need thereof. In embodiments, the method comprises administering to the patient a therapeutically effective amount of a composition comprising the nanoparticles described herein. In embodiments, the method is effective to treat and/or prevent one or more symptoms associated with the arthritis in the patient. In embodiments, the methods described herein are particularly useful in the management and/or prevention of arthritis flares. In embodiments, administering the nanoparticles to the patient reduces severity of or prevents arthritis flares in the patient. Such treatment or prevention of flares can take the form of, for example, reducing the risk of flares, reducing the number of flares, reducing the severity of flares, reducing the frequency of flares, and/or reducing the duration of flares.

In embodiments, the patient is also undergoing an additional therapy to treat arthritis. In embodiments, the patient is currently under treatment with a disease modifying antirheumatic

drug (DMARD). In embodiments, the patient is currently tapering a dose of the DMARD (e.g., the patient is undergoing a DMARD therapy regimen which reduces the overall dose of the DMARD therapy). In embodiments, the nanoparticle composition is co-administered with another anti-arthritis therapy.

- 5 In embodiments, the nanoparticle composition is administered to the patient via intramuscular administration. In embodiments, the composition is administered to a muscle in the vicinity of a desired lymph node.

In embodiments, the patient is currently suffering from arthritis. In embodiments, the patient has joints which are actively inflamed by arthritis. In embodiments, the joints of the patient are not
10 actively inflamed by arthritis. In embodiments, the arthritis is rheumatoid arthritis. In embodiments, the arthritis is an antigen-induced arthritis. In embodiments, the arthritis is autoimmune arthritis.

In embodiments, the nanoparticle composition is administered according to a regimen. In embodiments, due to the sustained-release properties of the nanoparticles, the nanoparticle
15 composition need not be delivered frequently (e.g., the nanoparticle composition is not needed to be administered daily, weekly, etc. in order to impart the therapeutic effects). In embodiments, the nanoparticle composition is not dosed more frequently than once every 4 weeks. In embodiments, the nanoparticle composition is administered once every 4 weeks, once every 5 weeks, once every 6 weeks, once every 7 weeks, once every 8 weeks, or less frequently.

20

EXAMPLES

EXAMPLE 1

Here, we test the hypothesis that in vivo modulation of DC in arthritis-affected joints and proximal lymph nodes induces an arthritis flare protective effect. Our approach is based on nanoparticles (NP) delivered via intramuscular injection that accumulate in lymph nodes proximal to the ankle joint. We show that DC take up NP loaded with a joint antigen-relevant peptide and an immunomodulatory agent which together induce an arthritis protective effect. The NP were formulated with a biodegradable poly(lactic-co-glycolic acid)-poly(ethylene glycol) (PLGA-PEG) co-polymer functionalized with maleimide for conjugation of N-terminal cysteine peptide antigens by the thiol-Michael addition. CLNP were ~200 nm in diameter with a low polydispersity index, a size that permitted accumulation in the proximal lymph nodes.⁴⁶ CLNP encapsulated 1,25-dihydroxycholecalciferol (calcitriol), the active form of vitamin D3, a well-known modulator of innate and adaptive immune responses which also induces an immunomodulatory DC phenotype.⁴⁷⁻⁴⁹ Calcitriol and CLNP induced immunomodulatory bone marrow-derived DC in vitro, characterized by reduced expression of CD80/CD86, MHC2 and proinflammatory cytokines (IL-6 and TNF). Calcitriol and CLNP also increased CTLA-4 expression in bone marrow-derived DC. Bulk RNA-seq analysis confirmed that calcitriol induces key genes associated with modulation of RA.^{50,51} CLNP formulations were tested in the collagen induced arthritis (CIA) and the SKG mouse models of RA. In CIA mice, disease-specific type 2 bovine collagen₂₇₁₋₂₈₅ peptide (bC2)-functionalized CLNP and in SKG mice, joint relevant aggrecan₈₉₋₁₀₃ peptide (Agg)-functionalized CLNP significantly reduced clinical scores, prevented bone erosion, and reduced cartilage proteoglycan loss.

Results

Calcitriol loaded nanoparticle formulations accumulate in the proximal lymph nodes

Calcitriol loaded nanoparticles (CLNP) were formulated with poly-(lactic-co-glycolic)-polyethylene glycol-maleimide (PLGA-PEG-MAL) and calcitriol by nanoprecipitation. (Figure 1 panel a). In a subset of formulations, N-terminus modified cysteine peptides were conjugated to the PLGA-PEG-MAL (Figure 7) prior to nanoprecipitation. Post conjugation BCA analysis of the particles showed a quantitative yield of peptide bound to the polymer. To estimate the hydrodynamic diameter, unconjugated CLNP, ovalbumin peptide-conjugated CLNP (OVA-CLNP), type 2 bovine collagen peptide-conjugate CLNP (bC2-CLNP) or aggrecan peptide-

conjugated CLNP (Agg-CLNP) were suspended in DI water were transferred to a cuvette and analyzed using dynamic light scattering (DLS). Unconjugated CLNP had a z-avg of 96.6nm with a PDI of 0.28, OVA-CLNP had a z-avg of 233nm with a PDI of 0.10, bC2-CLNP had a z-avg of 210nm with a PDI of 0.20, and Agg-CLNP had a z-avg of 214nm with a PDI of 0.07 (Figure 1 panel b).

To measure surface charge, unconjugated CLNP, OVA-CLNP, bC2-CLNP, or Agg-CLNP were suspended in DI water and transferred to a zeta potential cell. The zeta potentials were as follows: unconjugated CLNP: -25.4 mV, OVA-CLNP: -19.2 mV, bC2-CLNP: -18.9 mV, and Agg-CLNP: -16.5 mV (See Table below). The aforementioned CLNP formulations were imaged using transmission electron microscopy (TEM) to further characterize particle characteristics by negative stain preparation. All CLNP formulations were associated with a circular morphology. Using image analysis, the average diameters were as follows: unconjugated CLNP: 66 ± 16 nm (SD), OVA-CLNP: 160 ± 37 nm, bC2-CLNP: 129 ± 34 nm, and Agg-CLNP: 128 ± 28 nm (Figure 10 panels a-d).

Formulation	ζ potential / mV
Unconjugated CLNP	-25.4
OVA-CLNP	-19.2
bC2-CLNP	-18.9
Agg-CLNP	-16.5

Calcitriol release characterization was performed with ultra-high performance liquid chromatography (UHPLC) and a reverse phase column at a detection wavelength of 265 nm. UHPLC analysis showed encapsulation efficiencies (*ee*) of 9.1% for unconjugated CLNP, 10.5% for OVA-CLNP, 10.8% for Agg-CLNP, and 11.7% for bC2-CLNP. All formulations showed an initial burst release in the first three days post synthesis followed by a gradual release for at least 28 days post synthesis (Figure 1 panel c).

To assess biodistribution in vivo, PLGA-PEG-MAL or calcitriol were conjugated with cyanine 5 (Cy5) and used to prepare distinct Cy5-labeled CLNP formulations. Cy5-PEG-PLGA NPs were

injected intramuscularly (i.m.) into the biceps femoris of C57BL/6J (B6) mice. Inguinal, popliteal, and brachial lymph nodes were excised 2-, 8-, and 24-hours post injection and imaged on an In Vivo Imaging System (IVIS) (Figure 11 panel a). Image analysis showed that Cy5-PEGPLGA preferentially drained into the proximal (popliteal) lymph node (LN) at 2 h post administration. At 8 and 24 h post administration, the concentration of Cy5-PEG-PLGA was comparable in both the popliteal and inguinal LNs, but significantly lower, at the limit of detection, in the distal (brachial) LNs (Figure 1 panel d). Cy5-PEG-PLGA was associated with hepatic clearance (Figure 11 panel b). Biodistribution of Cy5-calcitriol encapsulated in CLNP was measured following the same procedure (Figure 11 panel c). Image analysis showed that Cy5-calcitriol, accumulates at a similar concentration into the popliteal and inguinal LNs at 2 h post administration. At 8 and 24 h post administration, Cy5-calcitriol is only detectable in the popliteal LNs (Figure 1 panel e). Cy5-calcitriol was associated with renal clearance (Figure 11 panel d).

CLNP modulate dendritic cells maturation

Monocyte derived dendritic cells (DC) were cultured in vitro with either free calcitriol (1nM) or CLNP (0.67 μ g and 6.7 μ g) (Figure 2 panel a). After overnight stimulation with LPS, DCs were analyzed by flow cytometry to quantify the expression of costimulatory molecules (CD80 and CD86), major histocompatibility complex 2 (MHCII) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). DC were identified as live CD45⁺CD11b⁺CD11c⁺ cells (Figure 8). The 6.7 μ g dose of CLNP (CLNP High) reduced the fraction of CD80^{hi} DC relative to LPS treated DC (7.2% $\pm$ 1.4% vs 11.3% $\pm$ 1.5%) (Figure 2 panel b). 1nM calcitriol, 0.67 μ g CLNP and 6.7 μ g CLNP significantly lowered the fraction of CD86^{hi} DC relative to LPS treated DC (5.7% $\pm$ 1.1% calcitriol, 6.5% $\pm$ 1.2% CLNP Low, 3.0% $\pm$ 0.6% CLNP High vs 13.8% $\pm$ 4.2% LPS control) (Figure 2 panel c). The same groups also significantly lowered the fraction of MHC2^{hi} DC relative to LPS treated DC (2.5% $\pm$ 0.5% calcitriol, 3.2% $\pm$ 0.9% CLNP Low, 1.1% $\pm$ 0.2% CLNP High vs 10% $\pm$ 4.0% LPS control) (Figure 2 panel d). 1nM calcitriol and 6.7 μ g CLNP also lowered the fraction of MHC2^{hi} DC relative to no LPS treated DC (4.5% $\pm$ 0.5% no LPS control). The 6.7 μ g dose of CLNP significantly increased the fraction of CTLA-4⁺ DC relative to LPS treated DC (27% $\pm$ 3.9% vs 17% $\pm$ 3.6%) (Figure 2 panel e).

The cell culture supernatant was analyzed for inflammatory cytokines (IL-6 and TNF) using a cytometric bead assay kit. All treatments significantly lowered the concentration of IL-6 relative

to LPS treated DCs (Figure 2 panel f). However, only the 6.7 μ g dose of CLNP reduced the concentration of TNF relative to LPS control (Figure 2 panel g)

Calcitriol modulates disease-relevant targets in dendritic cells

The induction of an immunomodulatory DC phenotype was correlated with Next Generation RNA sequencing (RNAseq). RNAseq profiles of DC cultured in 1nM calcitriol (+ Calcitriol) or without calcitriol (- Calcitriol) were compared. Pathway enrichment analysis of the top 50 differentially expressed genes (DEG), showed significant differences in RA-associated pathway (Figure 3 panel a). A volcano plot of RNAseq genes with those associated with RA-associated pathway highlighted in red was created; *Ctla4* and *Tgfb3*, which are associated with RA, were among the most induced genes (Figure 3 panel b). The log₂ fold change in differential expression of *Ctla4* and *Tgfb3* was greater than 1.5 in + Calcitriol DC. *Ctla4* and *Tgfb3* also were prominent in a heatmap of the top 50 DEG (Figure 3 panel c).

bC2-CLNP modulates clinical arthritis in collagen induced arthritis model

To test the concept of CLNP-mediated DC modulation in vivo, the collagen-induced arthritis (CIA) mouse model was used. The cysteine-modified immunodominant peptide of type 2 bovine collagen in the I-A^d haplotype was conjugated to the PLGA-PEG-MAL and precipitated into nanoparticles to form bC2-CLNP. DBA/1 mice were primed with an emulsion of complete Freund's adjuvant (CFA) and collagen. After 18 days, mice were treated with either free calcitriol or bC2-CLNP (33 μ g bC2-CLNP or 10 ng calcitriol/day) in both biceps femoris via i.m. injection once a day for three days before boost with an emulsion of incomplete Freund's adjuvant (IFA) and collagen (Figure 4 panel a). Clinical scores were monitored for 13 days post boost (Figure 4 panel b). bC2-CLNP-treated mice presented no clinical signs of arthritis on day 6 post-boost and had scores of 6 $\pm$ 2.6 at day 13. These scores were significantly lower than those of bolus calcitriol treated mice (1.75 $\pm$ 0.96 & 11.5 $\pm$ 1.3 at day 6 and day 13 respectively). Analysis of high-resolution micro-CT images (Figure 4 panel c) of the metacarpophalangeal (Figure 4 panel d) and ankle (Figure 4 panel e) joints confirmed strong protection against bone erosions by bC2-CLNP as assessed by treatment-blinded bone surface area to volume ratios and bone erosion scores. In the metacarpophalangeal joint, calcitriol treated mice had an average bone surface area/volume ratio of 21.3 mm⁻¹ $\pm$ 3.4 mm⁻¹ while bC2-CLNP treated mice had an average bone surface area/volume ratio of 14.4 mm⁻¹ $\pm$ 0.8 mm⁻¹. In the ankle joint, calcitriol treated mice had an average bone surface area/volume ratio of 27.6 mm⁻¹ $\pm$ 2.0 mm⁻¹ while bC2-CLNP treated mice

had an average bone surface area/volume ratio of $17.7 \text{ mm}^{-1} \pm 5.1 \text{ mm}^{-1}$. Bone erosion scoring based on micro-CT scans of calcitriol treated mice averaged 2.0 ± 1.1 and bC2-CLNP treated mice averaged 0.33 ± 0.5 , a significant difference (Figure 4 panel f).

Ankles were subsequently processed for histomorphometry and stained with hematoxylin and eosin (H&E) (Figure 5 panel a) as well as toluidine blue (Figure 5 panel b). Scoring of H&E stained sections confirmed lowered synovitis (Figure 5 panel c) and bone erosion (Figure 5 panel d) scores in bC2-CLNP treated mice. Calcitriol treated mice had an average synovitis score of 3.75 ± 0.46 while bC2-CLNP treated mice had an average synovitis score of 1.2 ± 1.5 . Calcitriol treated mice had an average bone erosion score of 3.13 ± 0.64 while bC2-CLNP treated mice had an average synovitis score of 0.66 ± 1.0 . Scoring of toluidine blue sections confirmed reduced proteoglycan (PG) loss scores in bC2-CLNP treated mice (Figure 5 panel e). Calcitriol treated mice had an average PG loss score of 3.63 ± 0.52 while bC2-CLNP treated mice had an average PG loss score of 1.33 ± 1.37 .

Agg-CLNP modulates clinical arthritis in SKG arthritis model

Unlike CIA mice, the induction of arthritis in SKG mice is not associated with a specific antigen, similar to RA. Therefore, the selection of the target for immunomodulation, aggrecan, was based on abundance in the joint. The cysteine-modified immunodominant peptide of aggrecan in the I-Ad haplotype was conjugated to the PLGA-PEG-MAL and precipitated into nanoparticles to form Agg-CLNP. Agg-CLNP, OVA-CLNP, bC2-CLNP, or free calcitriol were injected into each biceps femoris of SKG mice ($33 \mu\text{g}$ CLNP/day or 10 ng calcitriol/ day) for 3 days prior to arthritis flare synchronization with mannan (Figure 6 panel a). Clinical scores were monitored for 2 weeks post mannan injection (Figure 6 panel b). All groups started with clinical scores of 0. The clinical scores of Agg-CLNP-treated mice were 0.23 ± 0.091 and 0.90 ± 0.23 at days 7 and 14, respectively, significantly lower than those of untreated mice (1.4 ± 0.22 and 2.5 ± 0.47), calcitriol-treated mice (1.7 ± 0.77 and 2.85 ± 0.74), OVA-CLNP-treated mice (1.3 ± 0.36 and 2.3 ± 0.56), and bC2-CLNP-treated mice (0.88 ± 0.29 and 2.5 ± 0.47) at the same time points. The ankles were digested with collagenase to yield a single-cell suspension before staining for CD4⁺ T cells (Figure 9) and innate immune cells (Figure 12). CTLA-4⁺CD4⁺ T cells were significantly higher in Agg-CLNP ($47 \pm 35\%$) versus untreated ($2.5 \pm 0.8\%$) and OVA-CLNP ($3.7 \pm 1.8\%$) mice (Figure 6 panel c). CTLA-4⁺CD11c⁺ DC were higher in Agg-CLNP ($5.3 \pm 1.1\%$) compared to untreated ($2.8 \pm 0.6\%$) and OVA-CLNP ($3.2 \pm 0.4\%$) mice (Figure 6 panel

d). F4- 80+CD11b+ cells were lower in the ankles of Agg-CLNP-treated mice ($33 \pm 7.7\%$) compared to untreated mice ($46 \pm 2.8\%$) (Figure 13 panel a). Ly6G+CD11b+ cells were higher in the ankles of Agg-CLNP-treated mice ($36 \pm 3.4\%$) compared to untreated mice ($25 \pm 3.2\%$) (Figure 13 panel b). qPCR analysis showed that *Tnf* was significantly lower in the ankles of Agg-CLNP-treated mice (0.24 ± 0.16) compared to untreated mice (1.0 ± 0.33) (Figure 6 panel e). *Il6* was lower in the ankles of Agg-CLNP-treated mice (0.01 ± 0.01) compared to untreated mice (1.4 ± 1.4) (Figure 6 panel f). *Mmp3* was significantly reduced in the ankles of Agg-CLNP-treated mice (0.15 ± 0.18) compared to untreated mice (1.7 ± 0.92) (Figure 6 panel g). *Mmp13* was significantly reduced in the ankles of Agg-CLNP-treated mice (0.39 ± 0.42) compared to untreated mice (3.9 ± 1.3) (Figure 6 panel h). Following the guidelines recently published for Standardized Microscopic Arthritis Scoring of Histological sections (SMASH),⁵⁴ a computer-aided algorithm in the QuPath software was generated using default settings for tissue thresholding and cell detection/classification, to facilitate quantification of cell infiltrates of H&E-stained sections (Figure 6 panel i). Bone erosion (BE) and cartilage proteoglycan (PG) loss scoring were performed on safranin-O-stained ankle joint sections from untreated and Agg-CLNP-treated mice (Figure 6 panel j). Untreated mice ankle sections had significantly higher cell infiltrates compared with those from Agg-CLNP treated mice (Figure 6 panel k). BE scores for untreated mouse ankles (2.0 ± 0) were significantly higher than those for Agg-CLNP treated mouse ankles (1.0 ± 0.7) (Figure 6 panel l). The PG loss scores for untreated mouse ankles (1.75 ± 0.5) were significantly higher than those for AGG-CLNP-treated mouse ankles (0.6 ± 0.5) (Fig. 6m).

To measure if CLNP induced generalized immunosuppression, SKG mice were immunized by a priming injection of OVA/CFA and boosted with OVA/IFA (Figure 14 panel a). A subset of SKG mice received Agg-CLNP injections as previously described. Anti-OVA IgG1 Ab titers were comparable between Agg-CLNP-treated and untreated mice (Figure 14 panel b). To conduct a preliminary safety analysis, Agg-CLNP were injected into each biceps femoris of SKG mice ($33 \mu\text{g}$ Agg-CLNP/day) for 3 days prior to arthritis flare synchronization with mannan as above. Serum was collected just before mannan injection on day 3, and blood was collected on day 7 (Figure 15 panel a). Blood collected on day 7 was analyzed by flow cytometry to compare peripheral blood cellular composition consisting of B cells (B220+), dendritic cells (CD11c+),

macrophages (F4-80+), neutrophils (Ly6G+), and T cells (TCR β +) (Figure 15 panel b). There were no significant differences in the cellular composition between untreated and Agg-CLNP-treated mice (Figure 15 panels c–g). Serum collected on day 3 was analyzed with a Th1/Th2/Th17 CBA kit to quantify proinflammatory cytokines in the peripheral blood. No significant differences in IFN- γ , IL-4, IL-6, or TNF were found between untreated and Agg-CLNP-treated mice (Figure 15 panels h–k).

Discussion

Here, we demonstrate that joint antigen-relevant peptide-conjugated CLNP modulate arthritis severity and flare. CLNP regulate DC maturation which, in turn, suppresses disease-specific inflammation. Phenotypic modulation of DC by CLNP was confirmed by flow cytometry analysis and bulk next generation RNAseq, which showed a reduction in co-stimulatory CD80/CD86 expression and an increase in inhibitory CTLA-4, consistent with a tolerogenic DC phenotype. bC2-CLNP and Agg-CLNP, when administered i.m. in proximity to the joint, had potent local inflammation and flare controlling effects in the CIA and SKG mouse models arthritis. Importantly, unlike standard-of-care treatment, CLNP are not immunosuppressive. Moreover, bolus systemic delivery of calcitriol had no clinical benefit in control of arthritic inflammation supporting that both encapsulation of calcitriol and co-delivery with a disease-relevant peptide were necessary to achieve the benefit. These results support the utility of peptide conjugated CLNP as a flare control agent.

The choice of PLGA-PEG-MAL in the CLNP formulation was inspired by the long history of use of PLGA and PEG polymers in drug delivery formulations which have enabled safe and effective drug delivery.^{52–55} Maleimide-cysteine conjugation has a long history and provides an efficient method to conjugate peptides to polymers in mild conditions without the need for coupling reagents.^{56,57} This method of conjugation is also utilized in multiple FDA approved antibody drug conjugates.^{58–60} The active agent in the formulation, calcitriol, is the active form of vitamin D3, which regulates a range of physiological processes via nuclear factor kappa B through the vitamin D receptor.⁴⁸ Calcitriol has also been demonstrated to mediate physiologically relevant inhibition of DC maturation and thereby modulation of antigen-specific immune responses.⁴⁹ However, calcitriol is hydrophobic with a serum half-life of ~3–6 hours.^{61,62} Here, we encapsulated calcitriol in CLNP with a size ≤ 250 nm, which favors lymphatic drainage and is consistent with other reports.^{46,52,53,63} UHPLC analysis indicated encapsulation efficiencies

ranging from 9.1% to 11.7%, and degradation studies indicated an initial burst release of calcitriol prior to a slow degradation over 28 days for an average degradation of 50% between all formulations. While this *ee* is lower compared to other reports, the formulation reported here does not use surfactants/stabilizers, which likely contributes to the encapsulation and initial degradation.^{64–66} Analysis of proximal and distal lymph nodes after i.m. injection confirmed the localized accumulation of CLNP. The results support the notion that CLNP readily accumulates in the local LN, with minimal systemic exposure.

It is well-established that calcitriol promotes an immunomodulatory DC phenotype in vitro, which favors induction of regulatory T cells (T_{reg}).^{67–69} The tolerogenic DC phenotype is characterized by lower costimulatory molecule expression (CD80 and CD86) as well as lowered proinflammatory cytokine expression. Our studies clearly show that both free calcitriol and CLNP impart the tolerogenic phenotype on DC as measured by lower CD80, CD86, MHC2, IL-6 and TNF expression relative to LPS only stimulated control. RNAseq and KEGG pathway enrichment analysis of in vitro DC cultured with calcitriol confirmed that *Ctla4* was one of the most prominent differentially expressed genes, and flow cytometry analysis of DC treated with CLNP confirmed a significant increase in CTLA-4 expression. While CTLA-4 expression is generally associated with T cells,⁷⁰ CTLA-4 expressing DC have been shown to influence cell function and antigen presentation, resulting in a regulatory role.^{71,72} *CTLA4* polymorphisms are also a known risk factor for RA in humans.⁷³

Unlike disorders in which the predominant autoantigen is known, the identification of antigenic targets in RA is challenging as the complex pathology is not well-defined. RA involves a combination of genetic, environmental, and stochastic factors, and it is likely that multiple antigens are involved in disease initiation and progression. To this end, targeting multiple antigens utilizing an ex-vivo tolerogenic DC has been demonstrated.⁷⁴ In this work, the selection of the peptide for CLNP treatment in the CIA model was based on the known I-A^(q) restricted peptide sequence for bovine collagen II (bC2), which is a systemic target. On the other hand, the lack of a well-defined autoantigen target in SKG mice recapitulates human RA. Inspired by the results from the proteoglycan-induced inflammatory arthritis (PGIA) model in a BALB/c background mouse strain⁷⁵, we selected aggrecan, a cartilage-specific proteoglycan core protein that is enriched in the joint, with the goal of achieving joint specific immunomodulation. As the SKG mice used were on the BALB/c background, a strain that is susceptible to PGIA, we

utilized the known immunodominant peptide of aggrecan for the I-A^(d) haplotype. As a control, we used the I-A^(b) restricted immunodominant peptide of ovalbumin, in the SKG mice.

CLNP treatment reduced severity of CIA and flaring in the SKG mouse models of RA. In CIA, the study was designed to initially induce pathogenic collagen-specific T cells by the priming with bC2 emulsified in CFA and recapitulate progression from pre-RA to RA. Before boost, which rapidly increases joint inflammation, mice were treated with bC2-CLNP. Unlike the CIA mice, SKG mice have a known predisposition to inflammatory arthritis driven by an arthritogenic T cell receptor repertoire. The use of mannan, a fungal component, synchronizes arthritis and resembles a flare. By injecting CLNP prior to mannan injection, we assessed the flare prevention effect of CLNP.

The anti-inflammatory effects of CLNP were more pronounced in CIA compared to SKG mice, which we attribute to the use in CIA of a peptide that is derived from bC2, which is also used to induce the disease. In both models, the use of CLNP reduced inflammation relative to untreated and bolus calcitriol or irrelevant peptide CLNP formulations. Flow cytometry analysis of ankles from SKG mice showed a significant increase in CTLA-4⁺ T cells, supporting our hypothesis that CLNP modulate DC in the lymph nodes which, in turn, have a down-stream effect on T cells. These results support the utility of encapsulation of calcitriol and the use of disease relevant peptides in order to achieve a disease-modulating effect. The comparable anti-OVA antibody titers in Agg-CLNP treated mice and untreated OVA immunized mice show that the results are not due to generalized immunosuppression. This is a key distinction from increasing immunosuppression, which is often done for recurrent flares.

Ex vivo generated tolerogenic DC have been shown to reduce arthritis in rodent models of RA.^{76,77} However, despite infusion of high doses of DC in humans (up to $\sim 10^7$ cells), clinical benefit against RA progression or flares has remained elusive.^{74,78-80} Moreover, the complexity in the manufacturing of DC therapies presents a challenge to its widespread adoption. On the other hand, CLNP modulation of dendritic cells compares favorably to DC therapy and induces similar immunophenotypes. For example, the increase in CTLA-4⁺ T cells is consistent with the results from ex-vivo generated tolerogenic DC.⁸¹ The general approach of using antigen-specific immunomodulatory nanoparticles has been recently tested in clinical trials for RA which has demonstrated initial positive results.⁸²

In summary, an unmet need exists for durable flare control agents that potentially complement standard-of-care DMARDs. To this end, CLNP modulate DC to reduce flare progression and severity, as shown by lowered clinical score, bone erosion score, and proteoglycan loss score. CLNP may serve as an immunoregulatory adjuvant to treat patients that are experience recurrent local flares, potentially in combination with DMARDs without generalized immunosuppressive side effects

Methods

Study Design

The objective of this study was to develop an immunomodulatory agent to facilitate inflammation control during an arthritis flare. To this end, we formulated and characterized CLNP formulations. We validated the nanoprecipitation method to generate CLNP by synthesizing three initial lots for characterization by DLS. All material characterization studies (DLS and UHPLC) were conducted in triplicate with all formulations. All cell culture studies were performed with a minimum of three technical replicates. Three technical replicates were used for RNA-Seq analysis. For in vivo studies, outcomes were determined by assessing clinical scores, ankle thickness, flow cytometry assessments and histological appearances, unless otherwise noted. For SKG arthritis studies, littermate mice were injected with mannan to synchronize disease onset. The criteria for omission were (i) signs of arthritis on day 0 and (ii) failure to develop arthritis by day 17. All other animals were included in the data analysis. Endpoints for data collection were based on changes in and progression of clinical scores in the treatment groups. To achieve adequate power, all mouse arthritis studies were conducted by combining at least two age-matched litters. In general, statistical power for arthritis studies was based on prior reports of and our experience with arthritis mouse models. For CIA arthritis studies, littermate mice were injected with bC2/CFA on day 0 and bC2/IFA on day 21 to synchronize disease onset. The criteria for omission were (i) signs of arthritis on day 21 and (ii) failure to develop arthritis by day 35. All other animals were included in the data analysis. Sample size for each individual experiment is provided in the figure legends. Endpoints for data collection were based on changes in and progression of clinical scores in the treatment groups. All arthritis studies were conducted in at least two litters of mice. In general, statistical power for arthritis studies was based on prior reports of and our experience with CIA mice, and sample numbers for each individual experiment are provided in the figure legends.

Materials

Poly (lactide-co-glycolide)-polyethylene glycol-maleimide (50:50 L:G 20 kD PLGA, 2kD PEG SKU: 2794-20K-2000-1g, lots: 2794200202 & 2794200203) was purchased from NanoSoft polymers. Cy5-polyethylene glycol-thiol (2kD, FL078003-2K, lot: 20201217BL05,) was purchased from Biochempeg. Calcitriol (71820, lots: 0601887-33, 0601887-38, 0601887-53, 0601887-69) was purchased from Cayman Chemical. Dimethyl sulfoxide (DMSO, D128-500, lot:194474) and acetonitrile (A998-1, lot: 206498) were purchased from Fischer Chemical. Mannan (M7504-5G, lot:SLCF4977), lipopolysaccharide (LPS, L3012-5MG, lot: 0000091258), type VIII collagenase (C2139-1G, lot: 0000194156), DNase I (10104159001, lot: 60852700) and fetal bovine serum (FBS, F2442-500ML, 20K286) were purchased from Sigma-Aldrich. RPMI powder (31800-022, lot: 2338416) was purchased from Gibco. Incomplete Freund's Adjuvant + Ovalbumin (EK-0311, lots: 111, 115, 125), Complete Freund's Adjuvant + Ovalbumin (EK-0301, lots: 111, 115, 125), Incomplete Freund's Adjuvant + bovine collagen (EK-0221, lot: 102), Complete Freund's Adjuvant + bovine collagen (EK-0220, lot: 101) were purchased from Hooke Labs. Cy5-acid (BP-22274, lot: CY5-1G-1) was purchased from BroadPharm. 4-dimethylaminopyridine (148270250, lot: A0386855) was purchased from Acros Organics. N, N'-Dicyclohexylcarbodiimide (DCC, A10973, lot: 10209304) was purchased from Alfa Aesar. Dialysis sacs (12kD, D6191-25EA) were purchased from Millipore Sigma. 70 µm cell strainers (22-363-548, lot: 2021115) and 6-well culture plates (353046) were purchased from Fisher. GM-CSF (315-03-250UG, lot: 081955 L0821) was purchased from Peprotech. 96-well high affinity ELISA plates (3590) were purchased from Corning. Biotin anti-mouse IgG1 (406604, lot: B270354) was purchased from Biolegend. EDTA coated microtubes (365974, lot: 2181885) were purchased from BD. Ovalbumin (OVA, vac-stova, lot: 5823-43-01) was purchased from Invivogen.

25 Mouse models

All animal work was approved by the UCSD Institutional Animal Care and Use Committee (IACUC) under protocol # S17160 and followed the National Institutes of Health guidelines and relevant AALAC-approved procedures. C57BL/6J (B6, Jax #000664) and DBA/1JBomTac (DBA-1, Taconic Labs #DBA1BO-M) were purchased, BALB/c SKG mice were obtained through a Materials Transfer Agreement between UC San Diego and Kyoto University and

colonies are maintained at UCSD. BALB/c SKG mice used were both male and female. In each study, mice used were either all males or all females.

Arthritis in SKG mice was synchronized in 8–12-week-old SKG mice via intraperitoneal (i.p.) injection of 20 mg mannan dissolved in 200 μ L of sterile PBS. Disease severity was determined twice weekly using clinical scoring and measurement of hind paw swelling using calipers while mice were anesthetized. Fore and hind paws were assessed independently in each mouse and were assigned scores according to the following criterion: No visible swelling (0), mild to moderate swelling (0.5), severely swollen (1.0), as well as an additional for 0.1 for each swollen digit. Clinical scores reported are the aggregate of all paws (maximum of 5.8) from a single mouse unless otherwise noted. A score of 5.5 was considered the clinical endpoint and mice who attained this score before the end of the study were sacrificed according to IACUC guidelines.

Collagen induced arthritis (CIA) was induced in 8-week-old male DBA-1 mice by a prime injection of 50 μ L bovine collagen/CFA emulsion subcutaneously in the tail on day 0 and a boost injection of 50 μ L bovine collagen/IFA emulsion subcutaneously in the tail on day 21. After boost, mice were clinically scored 3x/week. Clinical scoring was performed for each limb (both wrists and both ankles) and each limb received a score of 0–4. No visible swelling/redness (0), mild but definite redness and swelling of the ankle or wrist, or apparent redness and swelling limited to individual digits, regardless of the number of affected digits (1), moderate redness and swelling of ankle or wrist (2), severe redness and swelling of the entire paw including digits (3), maximally inflamed limb with involvement of multiple joints (4). Clinical scores reported are the aggregate of all paws (maximum of 16) from a single mouse unless otherwise noted. A score of 16 was considered the clinical endpoint and mice who attained this score before the end of the study were sacrificed according to IACUC guidelines.

Calcitriol loaded nanoparticle (CLNP) synthesis

Cysteine-terminated peptides (Peptide 2.0) were added in a 1:1 molar ratio to 20 mg of PLGA-PEG-MAL and dissolved in 1 mL of DMSO. This mixture was agitated overnight. 60 μ L of 1 mg/mL calcitriol in DMSO was then added to the polymer solution. The polymer solution was then added dropwise to 30 mL of stirring MilliQ water and stirred for 1 hour. The nanoparticle solution was then transferred to a 12 kD dialysis bag and placed in a 6 L water bath. The water bath solution was changed every 3 hours for a total 9-hour dialysis against 18 L of water. The

nanoparticle solution was then stored in a 50 mL conical vial at 4°C until further use. Fresh nanoparticle solutions were prepared for each study.

CLNP characterization

An aliquot of neat nanoparticles was added to a cuvette and placed in a Malvern Zetasizer for dynamic light scattering analysis. Encapsulation of calcitriol in CLNP was determined on an UltiMate 3000 UHPLC (ThermoFischer Scientific). Briefly, nanoparticle suspensions were spun down at 21100g for 10 minutes in a centrifuge. The supernatant was aspirated, and the pellet dissolved in HPLC grade acetonitrile. The solutions were run in a Ascentis® Express 90Å C18 reverse phase column (MilliporeSigma, Cat#53825-U, lot: USWM003951) with a mobile phase of 100% acetonitrile at an isocratic flow rate of 0.1 mL/min. Area under the curve values were compared to a standard plot of known calcitriol concentrations run in the same conditions. The encapsulation efficiency (*ee*) of calcitriol in the formulations was calculated using the following equation:

$$ee(\%) = \frac{\text{Calcitriol concentration in particle}}{\text{Calcitriol concentration in reaction}} \times 100$$

Bicinchoninic acid (BCA) assay

A Micro BCA™ Protein Assay Kit was purchased from ThermoFischer (ref: 23235, lot: UD277184). The manual provided with the kit was used to determine conjugation efficiency of the thiol-Michael addition. Briefly, an albumin standard sample was prepared by serial dilution. Working reagent was prepared by mixing reagent MA, MB, and MC in a 25:24:1 ratio. Standard or unknown was added 1:1 in a flat bottom microplate and incubated at 37°C for 2 hours. Absorbance at 562nm was taken on a plate reader (ThermoFischer MultiskanFC, ref: 51119000, SN: 357-915576). Unknowns were compared to a standard curve to determine peptide concentration. Conjugation efficiency (*ce*) was calculated according to the following equation.

$$ce(\%) = \frac{\text{Total peptide in formulation}}{\text{Total peptide in conjugation}} \times 100$$

In vitro dendritic cell differentiation assay

BL6 mouse bone marrow cells were harvested by homogenizing the long bones using a mortar and pestle in complete 1640 RPMI media with 10% FBS and 20 ng/mL GM-CSF. The homogenate was strained through a 70 µm cell strainer. The strained solution was diluted to 2,000,000 cells/mL with media and 2 mL were added per well to a tissue culture treated 6-well

plate. In a subset of wells, calcitriol and/or nanoparticles were added at the concentrations as described in the results. Plates were incubated at 37°C at 5% CO₂. On day 3 of the culture, the wells were supplemented with 2 mL of fresh complete 1640 RPMI media containing GM-CSF and fresh calcitriol/CLNP to maintain experimental concentrations. On day 6 of the culture, half of the media was carefully removed and 2 mL of fresh media and fresh calcitriol/CLNP were added to maintain experimental concentrations. LPS was added at 50 ng/mL LPS for DC activation. After overnight activation, DC were analyzed by flow cytometry

RNA-seq of dendritic cells

DC were cultured as described above with SKG bone marrow. 1 nM calcitriol was added to a subset of DC. On day 7 of the culture, after overnight activation with LPS, the cells were transferred to a 15 mL conical tube. The cells were then sorted on a Sony SH800S cell sorter for AnnexinV⁻CD11c⁺ cells to isolate live DC. DC were spun down at 400 g for 5 minutes at 4°C to pellet the cells. Supernatant was removed and the cell pellets were flash frozen in liquid nitrogen before storage at -80°C. RNA was isolated and purified with a NEBNext®Ultra II Directional RNA Library Prep Kit for Illumina®, followed by 12 cycles of amplification. Sequencing was performed on an Illumina® MiSeq at the La Jolla Institute for Allergy and Immunology. Data was analyzed by quantile and TMM normalization in R.

Analysis of RNA-seq

Analysis of raw reads from sequencing of RNA-seq prepared libraries went as follows: FASTQ files were aligned to mm10 reference genome using STAR with parameters “--genomeDir mm10index_fp --readFilesIn input_files --readFilesCommand gunzip -c --outFileNamePrefix bamfile_out --outSAMtype BAM SortedByCoordinate”. Duplicate reads were removed using MarkDuplicates from Picard tools. Quantification through counting aligned sequencing reads counting reads within features using High-throughput sequence (HTSeq) with parameters ‘-f bam -r pos -s no -i gene_id -m intersection-nonempty’. Count normalization and differential RNA-seq analysis were then performed using quantile normalization and geTMM normalization after removing non-expressed genes. Differentially expressed genes (DEGs) were filtered using p-values ≤ 0.05 and log2 fold-change ≥ 1.5. KEGG enrichment to identify known pathways was performed using library clusterProfiler in R.

Ovalbumin Immunization

On day 0, two distinct 50 μ L subcutaneous injections of OVA/CFA emulsion were administered in the back of SKG mice. On day 10, mice were bled by the tail vein before receiving a subcutaneous booster immunization of 100 μ L of OVA/IFA emulsion in the back. On day 21, mice were bled by the tail vein before sacrifice. A subset of mice received 33 μ g/day Agg-CLNP on days 7-9 intramuscularly in the thigh. All blood was collected into EDTA coated tubes.

Anti-OVA IgG1 antibody titer assay

Anti-OVA IgG1 antibody titers were quantified using ELISA following established protocol. High-binding ELISA plates were coated with 1 μ g/mL OVA in PBS at 4 °C overnight.

Blood samples were diluted ranging from 1:3 to 1:65535 and incubated with the plates at room temperature for 1.5 hours before staining for mouse IgG1. The anti-OVA titer was defined as the serum dilution with an optical density value closest to 0.3.

Arthritis treatment with CLNP

To assess the efficacy of CLNP in SKG arthritis, 33 μ g/day of NP formulation (OVA-CLNP or Agg-CLNP) were injected into each biceps femoris of littermate SKG mice for 3 days prior to i.p. mannan injection on day 3. Clinical scores and ankle thickness of mice were tracked biweekly for two weeks to an endpoint on day 17. Endpoint immunological analysis of ankles was conducted with flow cytometry. In a replicate experiment, ankles were harvested for histological analysis.

To assess the efficacy of bC2-CLNP in CIA arthritis, 33 μ g/day of bC2-CLNP formulation were injected into each biceps femoris of DBA-1 mice for 3 days prior to bovine collagen/IFA injection on day 21. A separate cohort of mice were treated with 5ng/day of calcitriol. Mice were clinically scored for two weeks until sacrifice on day 35. Ankles were harvested for micro-CT and histological analysis.

In vivo biodistribution

To assess the biodistribution of the polymer and the encapsulation calcitriol, both were conjugated with Cy5. PLGA-PEG-MAL and thiol-PEG-Cy5 were dissolved in a 1:1 molar ratio in DMSO (20 mg of PLGA-PEG-MAL was used) and allowed to react overnight protected from light. The nanoparticle synthesis and dialysis purification were performed with the Cy5 conjugated material as described above. To conjugate Cy5 to calcitriol, Cy5 acid (1.4 mg, 2.7 μ mol), calcitriol (1 mg, 2.4 μ mol) and 4-dimethylaminopyridine (0.3 mg, 2.4 μ mol) in 0.3 mL of DCM were added to DCC (0.6 mg, 2.9 μ mol). The mixture was allowed to mix at room

temperature overnight before evaporating the solvent under a stream of air. The crude mixture was dissolved in 1 mL of DMSO and encapsulated in PLGA-PEG-MAL as described above.

In B6 mice, 33 μ g/bicep femoris of either Cy5-conjugated polymer NPs, or Cy5-calcitriol encapsulated NPs were injected. Mice were sacrificed at 2-, 8- and 24-hours post injection.

- 5 Inguinal lymph nodes (LN), popliteal LN, and brachial LN were harvested and organized on a plastic sheet for fluorescence quantification. Fluorescent images were taken with an In Vivo Imaging system (Xenogen). The radiant efficiency was quantified and plotted over time per organ normalized to the 2-hour popliteal lymph node datapoint.

Histological processing

- 10 After sacrifice, mouse hind limbs were excised below the knee joint. Muscle and skin was removed to the highest degree possible without damaging internal structures, and the limbs were fixed in 4% paraformaldehyde (PFA) for 48 hours. The fixed limbs were then transferred to a 70% ethanol solution. Samples were then sent to the University of Gothenburg (histology in Figure 5), or Inotiv (histology in Figure 6), where they were decalcified and embedded in
15 paraffin. Paraffin embedded limbs were sectioned to an appropriate depth according to SMASH guidelines and stained with hematoxylin and eosin, toluidine blue or safranin-O using standard tissue processing techniques. Stained slides were digitized using an Aperio AT2 Automated Digital Whole Slide Scanner or a Zeiss Axioscan 7 Slide Scanner.

Histomorphometry analysis

- 20 To quantify immune cell infiltration in histological sections, H&E sections were loaded into the QuPath software. Representative histological sections were used to train the software using built-in classification tools to broadly classify immune cells, muscle and tendon, and bone. Once trained, the software was then used to detect and classify types in sections from the metatarsals to part way up the tibia. Skin was excluded from the analysis to prevent misidentification of
25 dermal cells as immune cells. For proteoglycan loss scoring and bone erosion scoring, SMASH guidelines were followed. Briefly, histological sections were examined and proteoglycan loss was scored as follows: 0 – healthy intact cartilage consisting of fully stained cartilage layer with a smooth surface; 1 – Mild loss of staining in ~ 1/3 of the superficial cartilage zone, still predominantly red when stained with Safranin O; 2 – Moderate loss of Safranin O staining in up
30 to 2/3 of the superficial cartilage zone; 3 – Complete loss of Safranin O staining in the superficial cartilage zone. Bone erosion was scored as follows: 0 – Healthy, intact bone surface; 1 – Small,

superficial bone erosion at the outer surface of the bone, no breakage into marrow; 2 – Enhanced local bone erosions into subchondral space, partial or complete penetration of cortical bone; 3 – Massive enlarged subchondral bone erosion, extended synovial pannus invasion causing near-complete breakthrough of cortical bone to the marrow. Scoring was performed by two independent treatment-blinded operators.

Micro-computed tomography (micro-CT) analysis

Mouse ankles were placed in 4% PFA for 48 hours for fixation. After fixation, samples were transferred to 70% ethanol. Treatment-blinded scanning and analysis was performed at the University of Gothenburg. Before scanning, bones were transferred to PBS for 24 hours. Scanning was performed on a Skyscan1176 micro-CT (Bruker) with a voxel size of 9 μ m, at 55 kV/467 mA, with a 0.2-mm aluminum filter. Exposure time was 880 ms. The x-ray projections were obtained at 0.4° intervals with a scanning angular rotation of 180° and a combination of four average frames. The projection images were reconstructed into three-dimensional images using Nrecon software (version 1.6.9.8, Bruker) and aligned for further analysis in DataViewer (version 1.5.0.9, Bruker). Data were processed using CT-Analyzer software (version 1.14.4.1 Bruker), and images were generated using CTVox software (version 2.7, Bruker). Bone erosion was quantified as previously described.

Flow cytometry analysis

Anti-mouse antibodies against FoxP3 (PN: 11-5773-82, lot:2199652) were purchased from Invitrogen. Anti-mouse antibodies against CD4 (PN: 100428, Lot: B347337), CD45 (PN: 103130, lot: B349380), CD11b (PN: 101235, lot: B360998), CD11c (PN: 117346, lot: B325181), CD80 (PN: 104705, lot: B334893), CD86 (PN: 105115, lot: B315643), I-A/I-E (MHC2) (PN: 107628, lot: B350373), and CTLA-4 (PN: 106309, lot: B357050) were purchased from Biolegend. All cells were gated based on forward and side scatter characteristics to limit debris, including dead cells. The Zombie Aqua Fixable Viability Kit (Biolegend, lot:B333785) stain was used to separate live and dead cells. Antibodies were diluted 1:400. Gates were drawn based on fluorescence-minus-one controls, and the frequencies of positively stained cells for each marker were recorded. Intracellular/intranuclear stains were performed by first staining for surface markers according to manufacturer's protocols, then fixing and permeabilizing cells using the FoxP3 Fixation/Permeabilization Buffer Set (Invitrogen, 00-5523-00, lots:2333698, 2220750, 2203535). To quantify immune cell subsets in mouse ankles, ankles were harvested

after sacrificing mice, skin was removed, and ankles were harvested and incubated at 37°C in a solution of Complete RPMI, 1 mg/mL Type VIII collagenase and 0.1 mg/mL DNAase I for 50 minutes with constant gentle agitation. The supernatant was filtered through a 70 µm cell strainer and subsequently stained for flow cytometry. Flow cytometry was performed using an Attune®
5 NxT Acoustic Focusing cytometer analyzer (A24858) and data analyzed using FlowJo (BD) software.

Statistics

Sample sizes for animal studies were based on our prior work with SKG and CIA mice. Results were analyzed where indicated using unpaired Student's *t*-test, one or two- way ANOVA, and
10 Mann-Whitney rank test; each identified for each individual experiment in the figure legends. Data were analyzed using Graphpad Prism software.

Example 2

Further development of the CLNPs was performed. The results provided in this example describe additional efforts to optimize and characterize the CLNPs.

15 New formulation of Agg-CLNP modulates SKG dendritic cells

The Agg-CLNP synthesis was updated to achieve sterility and long-term storage ability (Figure 16 panel a) (referred to herein as the “new formulation”). The formulation described in Example 1 of Agg-CLNP (referred to as the “old formulation” elsewhere in this example) had a z-avg of 214nm with a PDI of 0.07 while new formulation Agg-CLNP had a z-avg of 71nm with a PDI of
20 0.16 (Figure 16 panel b). Old formulation Agg-CLNP possessed a calcitriol concentration of 215ng/mL for a 11% *ee* while new formulation Agg-CLNP possessed a calcitriol concentration of 273ng/mL for a 21% *ee*. Bone marrow derived dendritic cells (DC) were cultured *in vitro* with either old formulation Agg-CLNP or new formulation Agg-CLNP dose matched to 1nM calcitriol. After overnight stimulation with LPS, DC were analyzed by flow cytometry to
25 quantify the expression of costimulatory molecules (CD80 and CD86), and major histocompatibility complex 2 (MHCII). DC were identified as live CD45⁺CD11b⁺CD11c⁺ cells. Both Agg-CLNP formulations significantly reduced the fraction of CD80^{hi} DC relative to LPS treated DC (18.1%±8.7% old formulation, 19.4%±6.3% new formulation vs 31.1%±8.1% LPS control) (Figure 16 panel c). Both Agg-CLNP formulations significantly lowered the fraction of
30 CD86^{hi} DC relative to LPS treated DC (20.2%±4.3% old formulation, 24.2%±2.1% new formulation vs 36.4%±5.7% LPS control) (Figure 16 panel d). The same groups also lowered the

fraction of MHC2^{hi+} DC relative to LPS treated DC (30.4%±4.1% old formulation, 33.7%±3.0% new formulation vs 39.4%±6.3% LPS control) (Figure 16 panel e). There were no significant differences in dendritic cell modulation between the two formulations. All results in Example 2 utilize the new formulation of Agg-CLNP.

5 Agg-CLNP modulates human dendritic cells

Human dendritic cells differentiated from monocytes isolated from peripheral blood mononuclear cells were cultured *in vitro* with calcitriol (5nM), dexamethasone (dex, 1 µM), Agg-CLNP (5nM calcitriol dose matched), or a combination of dex and Agg-CLNP (at same doses previously listed) (Figure 17 panel a). After overnight stimulation with LPS, DC were
 10 analyzed by flow cytometry to quantify the expression of costimulatory molecules (CD40, CD80 and CD86), human leukocyte antigen 2 (HLA-II), MERTK, and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). DC were identified as live CD45⁺CD11b⁺CD11c⁺ cells. All treatments significantly reduced the fraction of CD40^{hi+} DC relative to LPS treated DC (9.2%±11.0% calcitriol, 7.0%±2.5% dex, 7.5%±4.4% Agg-CLNP, 2.4%±0.9% combo vs
 15 59.3%±11.0% LPS control) (Figure 17 panel b). All treatments significantly reduced the fraction of CD80^{hi+} DC relative to LPS treated DC (8.8%±11.4% calcitriol, 2.7%±1.0% dex, 21.9%±12.0% Agg-CLNP, 1.0%±0.9% combo vs 49.5%±12.6% LPS control) (Figure 17 panel c). All treatments significantly reduced the fraction of CD86^{hi+} DC relative to LPS treated DC (11.9%±8.1% calcitriol, 22.9%±4.7% dex, 15.6%±5.8% Agg-CLNP, 5.7%±1.6% combo vs
 20 42.2%±6.7% LPS control) (Figure 17 panel d). All treatments significantly reduced the fraction of HLA-2^{hi+} DC relative to LPS treated DC (12.7%±12.0% calcitriol, 41.0%±4.2% dex, 25.7%±11.7% Agg-CLNP, 11.2%±3.4% combo vs 62.7%±10.8% LPS control) (Figure 17 panel e). Dex and combo treatments significantly increased the fraction of MERTK⁺ DC relative to LPS treated DC (13.8%±10.7% calcitriol, 74.3%±18.5% dex, 13.0%±5.1% Agg-CLNP,
 25 73.3%±8.7% combo vs 15.1%±5.0% LPS control) (Figure 17 panel f). Calcitriol and Agg-CLNP treatments significantly increased the fraction of CTLA-4⁺ DC relative to LPS treated DC (70.2%±7.0% calcitriol, 88.8%±4.4% Agg-CLNP vs 14.9%±7.0% LPS control) (Figure 17 panel g).

Dexamethasone in combination with Agg-CLNP modulates subsequent arthritis flares in SKG
 30 mice

Corticosteroids are the standard treatment for flares, but do not prevent flare recurrence. Therefore Agg-CLNP were administered upon remission with dexamethasone to prevent flare recurrence. Dexamethasone was administered by daily i.p. injection on days 8-10, 22-24, and 36-38 (25 μ g/day) followed by daily i.m. Agg-CLNP injection on days 11-13, 25-27, and 39-41 into each biceps femoris (33 μ g/day) (Figure 18 panel a). Clinical scores (Figure 18 panel b) and ankle thickness deltas (Figure 18 panel c) were monitored for 42 days post mannan injection. All groups started with clinical scores and ankle thickness deltas of 0. The clinical scores of Agg-CLNP treated mice were 1.1 ± 0.20 , 1.8 ± 0.46 , and 1.9 ± 0.46 at days 14, 28, and 42 respectively, significantly lower than those of untreated mice (4.1 ± 0.45 , 3.9 ± 0.48 & 4.0 ± 0.67) and significantly lower than dexamethasone only treated mice (2.2 ± 0.34 , 3.4 ± 0.39 & 3.8 ± 0.40) at the same timepoints. The ankle thickness deltas of Agg-CLNP treated mice were 0.25 ± 0.04 mm, 0.45 ± 0.09 mm, and 0.38 ± 0.09 at days 14, 28 and 42 respectively, significantly lower than untreated treated mice (0.93 ± 0.15 mm, 0.99 ± 0.18 mm & 0.89 ± 0.15 mm), and dexamethasone only treated mice (0.52 ± 0.07 mm, 0.83 ± 0.07 mm & 0.76 ± 0.07 mm) at the same timepoints.

Dexamethasone in combination with Agg-CLNP reduces T_H17 counts in hind paws of SKG mice. T_H17 cells are the primary cell type responsible for arthritis induction and progression in SKG mice. Therefore Agg-CLNP were administered upon remission with dexamethasone to quantify T_H17 counts in various tissues. Dexamethasone was administered by daily i.p. injection on days 5-7 (25 μ g/day) and days 8-10 (125 μ g/day) followed by daily i.m. Agg-CLNP injection on days 11-13 into each biceps femoris (33 μ g/day) (Figure 19 panel a). Clinical scores (Figure 19 panel b) and ankle thickness deltas (Figure 19 panel c) were monitored for 14 days post mannan injection. All groups started with clinical scores and ankle thickness deltas of 0. The clinical scores of Agg-CLNP treated mice were 0.51 ± 0.15 and 0.88 ± 0.14 at days 11 and 14 respectively, lower than those of dexamethasone only treated mice (0.53 ± 0.14 & 2.8 ± 0.48) at the same timepoints. The ankle thickness deltas of Agg-CLNP treated mice were 0.06 ± 0.03 mm and 0.15 ± 0.03 at days 11 and 14 respectively, lower than dexamethasone only treated mice (0.20 ± 0.03 mm & 0.53 ± 0.07 mm) at the same timepoints. Spleens, popliteal and inguinal lymph nodes, forepaws, and hind paws were harvested and stained for T_H17 cells. Agg-CLNP treated mice had lower CD4 counts in the spleen relative to dexamethasone only treated mice (2216 ± 1813 vs 5057 ± 3535) (Figure 19 panel d). Agg-CLNP treated mice had lower T_H17 counts in the spleen relative to dexamethasone only treated mice (22 ± 32 vs 29 ± 26) (Figure 19 panel e).

Agg-CLNP treated mice had lower CD4 counts in the combined inguinal and popliteal lymph nodes relative to dexamethasone only treated mice (82950 ± 22279 vs 99112 ± 14144) (Figure 19 panel f). Agg-CLNP treated mice had lower T_H17 counts in the combined inguinal and popliteal lymph nodes relative to dexamethasone only treated mice (2820 ± 805 vs 4422 ± 1683) (Figure 19 panel g). Agg-CLNP treated mice had lower CD4 counts in the forepaws relative to dexamethasone only treated mice (1887 ± 596 vs 2912 ± 1793) (Figure 19 panel h). Agg-CLNP treated mice had lower T_H17 counts in the forepaws relative to dexamethasone only treated mice (190 ± 99 vs 274 ± 165) (Figure 19 panel i). Agg-CLNP treated mice had lower CD4 counts in the hind paws relative to dexamethasone only treated mice (21991 ± 8370 vs 53390 ± 27266) (Figure 19 panel j). Agg-CLNP treated mice had significantly lower T_H17 counts in the hind paws relative to dexamethasone only treated mice (530 ± 250 vs 990 ± 278) (Figure 19 panel k)

Methods

Study design

The objective of this study was to develop an immunomodulatory agent to modulate arthritis flares. To this end, we formulated Agg-CLNP. All cell culture studies were performed with a minimum of three technical replicates. For *in vivo* studies, outcomes were determined by assessing clinical scores and ankle thickness. For SKG arthritis studies, littermate mice were injected with mannan to synchronize disease onset. The criteria for omission were (i) signs of arthritis on day 0 and (ii) failure to develop arthritis by the first treatment timepoint. All other animals were included in the data analysis. Endpoints for data collection were based on changes in and progression of clinical scores in the treatment groups. Sample size for each individual experiment is provided in the figure legends. To achieve adequate power, all mouse arthritis studies were conducted by combining at least two age-matched litters. In general, statistical power for arthritis studies was based on prior reports of and our experience with arthritis mouse models.

Materials

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lot: SLCF4977), lipopolysaccharide (LPS, L3012-5MG, lot: 0000091258), and fetal bovine serum (FBS, F2442-500ML, 20K286) were purchased from Sigma-Aldrich. RPMI powder (31800-022, lot: 2338416) was purchased from Gibco. Dialysis sacs (12kD, D6191-25EA) were purchased from Millipore Sigma. 6-well culture plates (353046) were purchased from Fisher. GM-CSF (315-03-250UG, lot: 081955 L0821) was purchased from Peprotech. Dexamethasone (501012) was purchased from VetOne. Buffy coat was obtained from the Stanford Blood Bank.

Mouse models

All animal work was approved by the UCSD Institutional Animal Care and Use Committee (IACUC) under protocol # S17160 and followed the National Institutes of Health guidelines and relevant AALAC-approved procedures. BALB/c SKG mice were obtained through a Materials Transfer Agreement between UC San Diego and Kyoto University and colonies were maintained at UCSD. BALB/c SKG mice used were both male and female. In each study, mice used were either all males or all females.

Arthritis in SKG mice was synchronized in 8–12-week-old SKG mice *via* intraperitoneal (i.p.) injection of 20 mg mannan dissolved in 200 μ L of sterile PBS. Disease severity was determined twice weekly using clinical scoring and measurement of hind paw swelling using calipers while mice were anesthetized. Fore and hind paws were assessed independently in each mouse and were assigned scores according to the following criterion: no visible swelling (0), mild to moderate swelling (0.5), severely swollen (1.0), as well as an additional 0.1 for each swollen digit. Clinical scores reported are the aggregate of all paws (maximum of 5.8) from a single mouse unless otherwise noted. A score of 5.5 was considered the clinical endpoint and mice who attained this score before the end of the study were sacrificed according to IACUC guidelines.

Aggrecan calcitriol loaded nanoparticle (Agg-CLNP) synthesis

Old formulation: Cysteine-terminated peptides (Peptide 2.0) were added in a 1:1 molar ratio to 20 mg of PLGA-PEG-MAL and dissolved in 1 mL of DMSO. This mixture was agitated overnight. 60 μ L of 1 mg/mL calcitriol in DMSO was then added to the polymer solution. The polymer solution was then added dropwise to 30 mL of stirring MilliQ water and stirred for 1 hour. The nanoparticle solution was then transferred to a 12 kD dialysis bag and placed in a 6 L water bath. The water bath solution was changed every 3 hours for a total 9-hour dialysis against 18 L of water. The nanoparticle solution was then stored in a 50 mL conical vial at 4°C until further use. Fresh nanoparticle solutions were prepared for each study.

New formulation: Cysteine-terminated aggrecan peptide (Peptide 2.0) was added in a 1:1 molar ratio to 20 mg of PLGA-PEG-MAL and dissolved in 1 mL of DMSO. This mixture was agitated overnight. 60 µL of 1 mg/mL calcitriol in DMSO was then added to the polymer solution. The polymer solution was then diluted with 2 mL of DMSO and 3 mL of ethanol. The polymer solution was then added dropwise via syringe pump (SyringePump.com, Model 4000) to 40 mL of homogenizing MilliQ water at 3500 rpm (Silverson, L5M-A) and allowed to come to homogeneity for 10 seconds. The nanoparticle solution was then transferred to a 12 kD dialysis bag and placed in a 6 L water bath. The water bath solution was changed every 3 hours for a total 9-hour dialysis against 18 L of water. Sucrose was added to bring the total weight percent of sucrose to 10%. The Agg-CLNP solution was then sterile filtered, aliquoted, and stored at -20°C. Fresh aliquots were used for each study.

Agg-CLNP characterization

An aliquot of undiluted nanoparticles was added to a cuvette and placed in a Malvern Zetasizer Pro for dynamic light scattering analysis. An aliquot of undiluted nanoparticles was added to a zeta potential cell and placed in a Malvern Zetasizer Pro for zeta potential analysis. Measurements with the Zetasizer Pro utilize ZS XPLORER software. Encapsulation of calcitriol in CLNP was determined on an UltiMate 3000 UHPLC (ThermoFischer Scientific). Briefly, nanoparticle suspensions were spun down at 21100g for 10 minutes in a centrifuge. The supernatant was aspirated, and the pellet dissolved in HPLC grade acetonitrile. The solutions were run in a Ascentis® Express 90Å C18 reverse phase column (MilliporeSigma, Cat#53825-U, lot: USWM003951) with a mobile phase of 100% acetonitrile at an isocratic flow rate of 0.1 mL/min. Area under the curve values were compared to a standard plot of known calcitriol concentrations run in the same conditions. The encapsulation efficiency (*ee*) of calcitriol in the formulations was calculated using the following equation:

$$ee(\%) = \frac{\text{Calcitriol concentration measured in CLNP}}{\text{Calcitriol concentration in reaction}} \times 100$$

In vitro mouse dendritic cell differentiation assay

SKG mouse bone marrow cells were harvested by homogenizing the long bones using a mortar and pestle in complete 1640 RPMI media with 10% FBS and 20 ng/mL GM-CSF. The homogenate was strained through a 70 µm cell strainer. The strained solution was diluted to 2,000,000 cells/mL with media and 2 mL were added per well to a tissue culture treated 6-well plate. In a subset of wells, calcitriol and/or nanoparticles were added at the concentrations as

described in the results. Plates were incubated at 37°C at 5% CO₂. On day 3 of the culture, the wells were supplemented with 2 mL of fresh complete 1640 RPMI media containing GM-CSF and fresh Agg-CLNP to maintain experimental concentrations. On day 6 of the culture, half of the media was carefully removed and 2 mL of fresh media and fresh Agg-CLNP were added to maintain experimental concentrations. LPS was added at 50 ng/mL LPS for DC activation. After overnight activation, DC were analyzed by flow cytometry.

In vitro human dendritic cell differentiation assay

Human buffy coat was obtained and peripheral blood mononuclear cells were isolated by gradient centrifugation with Lymphopure (Biolegend). A human monocyte isolation kit (StemCell, PN: 19359, Lot: 1000168466) was utilized to enrich for monocytes. Monocytes were suspended at 1,000,000 cell/mL in complete 1640 RPMI with 10% FBS and 20ng/mL murine GM-CSF. 100µL of the monocyte solution was added per well to a 96-well tissue culture treated flat bottom plate and incubated at 37°C in 5% CO₂. On day 3, the supernatant was removed and fresh media was added. On day 4, calcitriol (5nM), dexamethasone (1µM), or Agg-CLNP (dose matched to 5nM calcitriol) was added. On day 5, LPS was added to each well to achieve 500ng/mL LPS, Agg-CLNP (dose matched to 5nM calcitriol) was added to the Dex+Agg-CLNP wells at this time. On day 7, the activated DC were analyzed by flow cytometry.

Multiple flare treatment with Agg-CLNP in combination with dexamethasone

To assess the efficacy of Agg-CLNP in SKG arthritis flare prevention post dexamethasone injection, littermate 8-12 week old female SKG mice were injected i.p. with 20 mg of mannan on day 0 to synchronize arthritis induction. On days 8-10, 22-24, and 36-38 a subset of mice was injected i.p. with dexamethasone (25 µg/day). On days 11-13, 25-27, and 39-41 a subset of dexamethasone treated mice were injected i.m. with Agg-CLNP into each biceps femoris (33 µg/day). Clinical scores and ankle thickness were assessed twice a week for 42 days post mannan injection. On day 42, mice were sacrificed and ankles were fixed in 4% paraformaldehyde.

Flare treatment with Agg-CLNP in combination with dexamethasone for TH17 assessment

To assess the effect of Agg-CLNP on TH17 counts in SKG post dexamethasone remission, littermate 8-12 week old female SKG mice were injected i.p. with 20 mg of mannan on day 0 to synchronize arthritis induction. On days 5-7, all mice were injected i.p. with dexamethasone (25 µg/day). On days 8-10, all mice were injected i.p. with dexamethasone (125 µg/day). On days

11-13 a subset of dexamethasone treated mice were injected i.m. with Agg-CLNP into each biceps femoris (33 µg/day). Clinical scores and ankle thickness were assessed twice a week for 14 days post mannan injection. On day 14, mice were sacrificed and spleens, inguinal lymph nodes, popliteal lymph nodes, forepaws, and hind paws were harvested for flow cytometry.

5 Flow cytometry analysis

Anti-mouse antibodies against CD4 (PN: 100428, clone: GK1.5, lot: B347337), CD45 (PN: 103130, clone: 30-F11, lot: B349380), CD11b (PN: 101235, clone: M1/70, lot: B360998), CD11c (PN: 117346, clone: N418, lot: B325181), CD80 (PN: 104705, clone: 16-10A1, lot: B334893), CD86 (PN: 105115, clone: GL-1, lot: B315643), I-A/I-E (MHC2) (PN: 107628, clone: M5/114.15.2, lot: B350373), CTLA-4 (PN: 106309, clone: UC10-4B9, lot: B357050), and IL-17 (PN: 506916, clone: TCC11-18H10.1, lot: B358441) were purchased from Biolegend. Anti-human antibodies against CD11c (PN: 337214, clone: Bu15, lot: B401287), CD40 (PN: 334320, clone: 5C3, lot: B383527), CD80 (PN: 305219, clone: 2D10, lot: B400367), HLA-2 (PN: 361715, clone: Tu39, lot: B423731), CTLA-4 (PN: 369633, clone: BNI3, lot: B352357), CD86 (PN: 367607, clone: 590H11, lot: B399839), and MERTK (PN: 367607, clone: Bu15, lot: B352175) were purchased from Biolegend. All cells were gated based on forward and side scatter characteristics to limit debris, including dead cells. The Zombie Aqua Fixable Viability Kit (Biolegend, lot: B333785) stain was used to separate live and dead cells. Antibodies were diluted 1:400. Gates were drawn based on fluorescence-minus-one controls, and the frequencies of positively stained cells for each marker were recorded. Intracellular/intranuclear stains were performed by first staining for surface markers according to manufacturer's protocols, then fixing and permeabilizing cells using a Fixation/Permeabilization Buffer Set (Invitrogen, 00-5523-00, lots: 2333698, 2220750, 2203535). To quantify immune cell subsets in mouse ankles, ankles were harvested after sacrificing mice, skin was removed, and ankles were harvested and incubated at 37°C in a solution of Complete RPMI, 1 mg/mL Type VIII collagenase and 0.1 mg/mL DNase I for 50 minutes with constant gentle agitation. The supernatant was filtered through a 70 µm cell strainer and subsequently stained for flow cytometry. To quantify immune cell subsets in the spleen, red blood cells (RBC) were first lysed with RBC lysis buffer before proceeding with staining. Flow cytometry was performed using an Attune® NxT Acoustic Focusing cytometer analyzer (A24858) and data analyzed using FlowJo (BD) software.

Statistics

Sample sizes for animal studies were based on prior work with SKG mice without use of additional statistical estimations. Results were analyzed where indicated using one- or two- way ANOVA; each identified for each individual experiment in the figure legends. Data were analyzed using Graphpad Prism software.

5 The specific methods, devices and compositions described herein are representative of preferred embodiments and are exemplary and not intended as limitations on the scope of the invention. Other objects, aspects, and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions
10 and modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention.

The invention illustratively described herein suitably can be practiced in the absence of any element or elements, or limitation or limitations, which is not specifically disclosed herein as essential. The methods and processes illustratively described herein suitably can be practiced in
15 differing orders of steps, and the methods and processes are not necessarily restricted to the orders of steps indicated herein or in the claims.

Under no circumstances can the patent be interpreted to be limited to the specific examples or embodiments or methods specifically disclosed herein. Under no circumstances can the patent be interpreted to be limited by any statement made by any Examiner or any other official or
20 employee of the Patent and Trademark Office unless such statement is specifically and without qualification or reservation expressly adopted in a responsive writing by Applicants.

The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intent in the use of such terms and expressions to exclude any equivalent of the features shown and described or portions thereof, but it is recognized that
25 various modifications are possible within the scope of the invention as claimed. Thus, it will be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed can be
5 resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims and
30 statements of the invention.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

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Tolerizing Immunotherapy with Peptide/Calcitriol Liposomes in ACPA+ Rheumatoid Arthritis.
JCI insight 2022, 7 (20).

What is claimed is:

1. A method of treating inflammatory arthritis in a patient, comprising:
administering to the patient a therapeutically effective amount of a composition comprising nanoparticles made of biodegradable polymers conjugated to a joint-relevant peptide antigen, wherein the nanoparticles encapsulate a dendritic cell modulator.
2. The method of claim 1, wherein treating the inflammatory arthritis comprises treating arthritis flares.
3. The method of claim 1, wherein the biodegradable polymers comprise poly(ethylene glycol) (PEG), poly(lactic-co-glycolic acid) (PLGA), or a combination thereof.
4. The method of claim 3, wherein the biodegradable polymers comprise a copolymer of PLGA and PEG.
5. The method of claim 3, wherein the biodegradable polymers comprise a structure of PLGA-PEG.
6. The method of claim 1, wherein the composition is administered intramuscularly.
7. The method of claim 1, wherein the nanoparticles have an average hydrodynamic diameter of from about 50 nm to about 300 nm.
8. The method of claim 1, wherein the nanoparticles have a size which permits accumulation of the nanoparticles in lymph nodes of the patient.
9. The method of claim 1, wherein the joint-relevant peptide antigen is conjugated to biodegradable nanoparticles by a reaction between a cysteine residue on the joint-relevant peptide antigen and a maleimide on the biodegradable polymers.
10. The method of claim 1, wherein the joint-relevant peptide antigen is a peptide derived from one or more cartilage tissue components.
11. The method of claim 10, wherein the cartilage tissue component is selected from a cartilage proteoglycan.
12. The method of claim 11, wherein the cartilage proteoglycan is selected from aggrecan, versican, link protein, biglycan (dermatan sulfate proteoglycan (DS-PG)I), decorin (DS-PGII), epiphycan (DS-PGIII), fibromodulin, lumican, perlecan, and lubricin.
13. The method of claim 10, wherein the cartilage tissue component is collagen.
14. The method of claim 10, wherein the cartilage tissue component is a cartilage protein selected from cartilage oligomeric matrix protein (COMP) (Thrombospondin-5),

Thrombospondin-1, Thrombosponin-3, CMP (cartilage matrix protein) (Matrilin-1), Matrilin-3, cartilage intermediate layer protein (CILP), C-type lectin, Fibronectin, PRELP (proline- and arginine-rich end leucine-rich repeat protein), Chondroadherin, Tenascin-C, Fibrillin, Elastin, gp (glycoprotein)-39/YKL-40, Matrix gla (gamma-carboxyglutamic acid) protein/MGP, Pleiotrophin, Chondromodulin-I, cartilage-derived retinoic acid responsive protein (CD-RAP), Chondrocalcin, and PARP (proline- and arginine-rich protein).

15. The method of claim 10, wherein the joint-relevant peptide antigen comprises a stretch of at least 8, 10, 12 or 14 amino acids from the cartilage tissue component.
16. The method of claim 10, wherein the joint-relevant peptide antigen is an immunodominant peptide derived from the cartilage protein.
17. The method of claim 1, wherein the dendritic cell modulator induces an immunomodulatory phenotype of dendritic cells.
18. The method of claim 1, wherein the dendritic cell modulator reduces expression of one or more of MHC2, CD80, CD86, IL-6, or TNF α in dendritic cells.
19. The method of claim 1, wherein the dendritic cell modulator increases the expression of one or more of CTLA-4 and MERTK (Mer tyrosine kinase) in dendritic cells.
20. The method of claim 1, wherein the dendritic cell modulator is calcitriol, or a pharmaceutically acceptable salt thereof.
21. The method of claim 1, wherein administering the composition is effective to modulate dendritic cells in a joint and/or lymph node of the patient.
22. The method of claim 1, wherein the administering does not cause systemic immunosuppression in the patient.
23. The method of claim 1, wherein the administering reduces severity of or prevents an arthritis flare.
24. The method of claim 1, wherein the patient is currently under treatment with a disease modifying antirheumatic drug (DMARD).
25. The method of claim 24, wherein the patient is currently tapering a dose of the DMARD.
26. A composition comprising nanoparticles made of biodegradable polymers conjugated to a joint-relevant peptide antigen, wherein the nanoparticles encapsulate a therapeutically effective amount of a dendritic cell modulator.

27. The composition of claim 26, wherein the biodegradable polymers comprise poly(ethylene glycol) (PEG), poly(lactic-co-glycolic acid) (PLGA), or a combination thereof.
28. The composition of claim 27, wherein the biodegradable polymers comprise a copolymer of PLGA and PEG.
29. The composition of claim 27, wherein the biodegradable polymers comprise a structure of PLGA-PEG.
30. The composition of claim 22, wherein the nanoparticles have an average hydrodynamic diameter of from about 50 nm to about 300 nm.
31. The composition of claim 26, wherein the joint-relevant peptide antigen is conjugated to biodegradable nanoparticles by a reaction between a cysteine residue on the joint-relevant peptide antigen and a maleimide on the biodegradable polymers.
32. The composition of claim 26, wherein the joint-relevant peptide antigen is a peptide derived from a cartilage tissue component.
33. The composition of claim 32, wherein the cartilage proteoglycan is selected from aggrecan, versican, link protein, biglycan (dermatan sulfate proteoglycan (DS-PG)I), decorin (DS-PGII), epiphygan (DS-PGIII), fibromodulin, lumican, perlecan, and lubricin.
34. The method of claim 32, wherein the cartilage tissue component is collagen.
35. The method of claim 32, wherein the cartilage tissue component is a cartilage protein selected from cartilage oligomeric matrix protein (COMP) (Thrombospondin-5), Thrombospondin-1, Thrombosponin-3, CMP (cartilage matrix protein) (Matrilin-1), Matrilin-3, cartilage intermediate layer protein (CILP), C-type lectin, Fibronectin, PRELP (proline- and arginine-rich end leucine-rich repeat protein), Chondroadherin, Tenascin-C, Fibrillin, Elastin, gp (glycoprotein)-39/YKL-40, Matrix gla (gamma-carboxyglutamic acid) protein/MGP, Pleiotrophin, Chondromodulin-I, cartilage-derived retinoic acid responsive protein (CD-RAP), Chondrocalcin, and PARP (proline- and arginine-rich protein).
36. The composition of claim 32, wherein the joint-relevant peptide antigen comprises a stretch of at least 8, 10, 12 or 14 amino acids from the cartilage tissue component.
37. The composition of claim 29, wherein the joint-relevant peptide antigen is an immunodominant peptide derived from the cartilage protein.

38. The composition of claim 26, wherein the dendritic cell modulator induces an immunomodulatory phenotype of dendritic cells.
39. The composition of claim 26, wherein the dendritic cell modulator reduces expression of one or more of MHC2, CD80, CD86, IL-6, or TNF α in dendritic cells.
40. The composition of claim 26, wherein the dendritic cell modulator increases expression of one or more of CTLA-4 and MERTK in dendritic cells.
41. The composition of claim 24, wherein the dendritic cell modulator is calcitriol, or a pharmaceutically acceptable salt thereof.
42. A pharmaceutical composition comprising the composition of claim 26, and a pharmaceutically acceptable carrier or excipient.
43. The pharmaceutical composition of claim 42, wherein the composition is formulated for intramuscular administration.

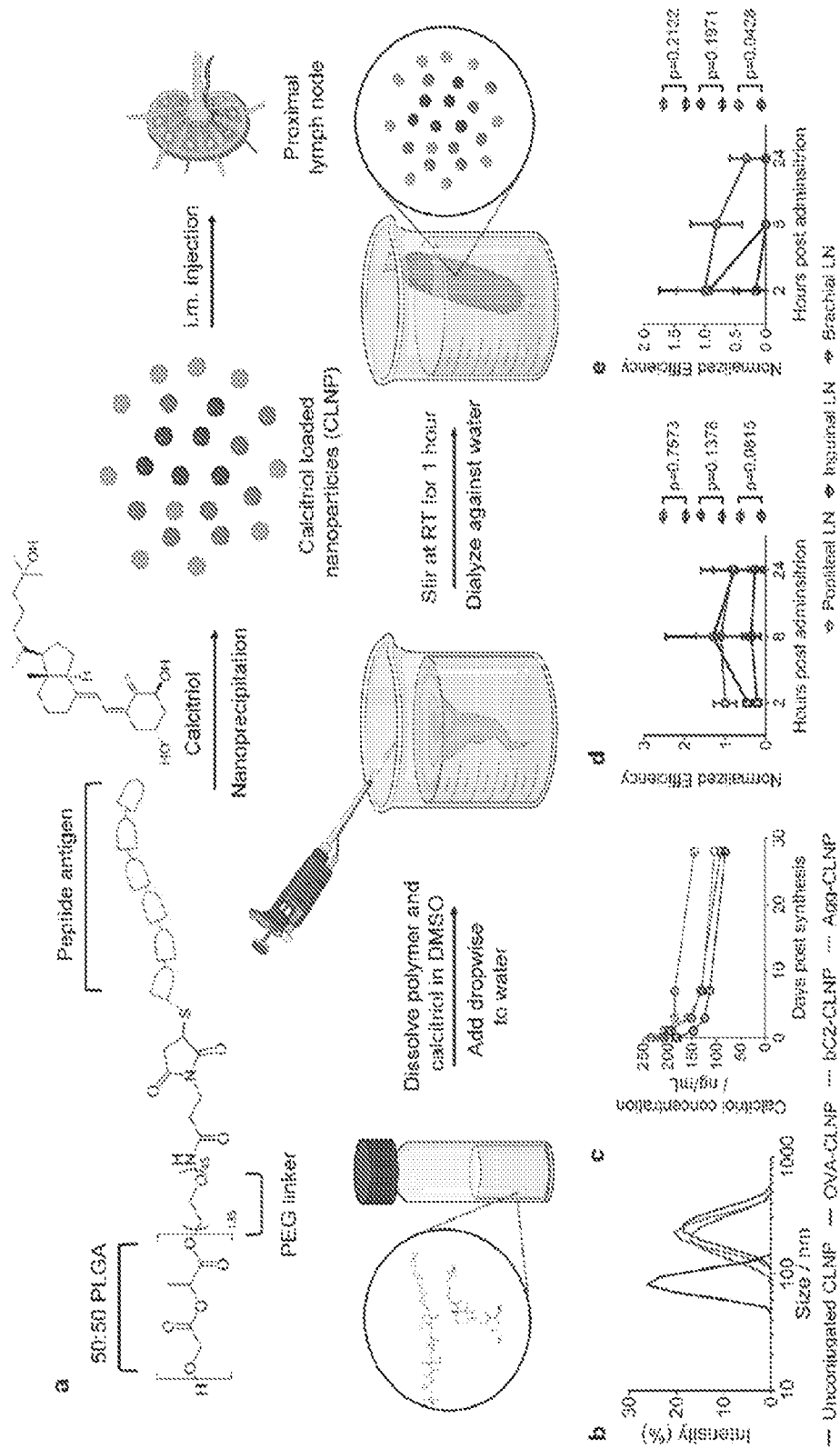


FIG. 1

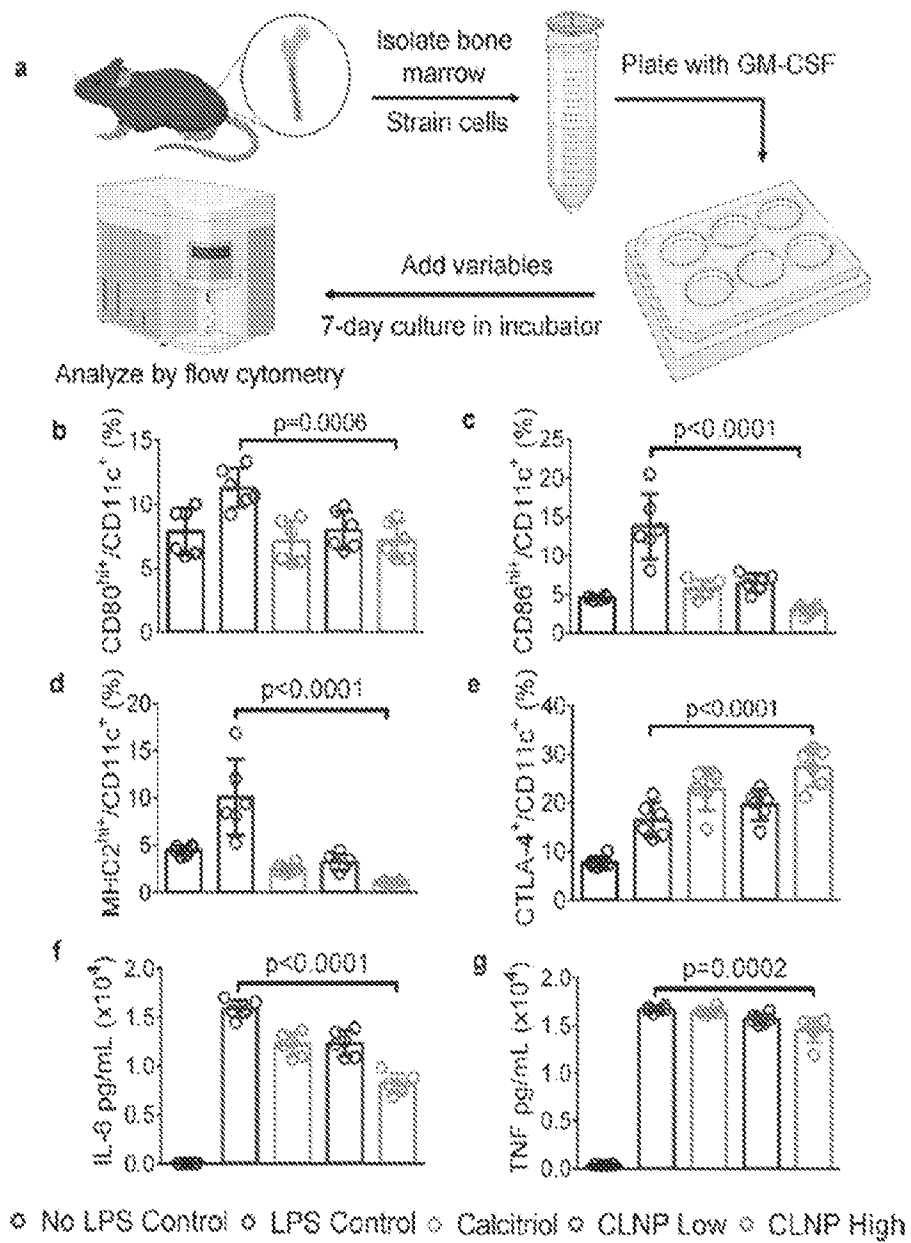


FIG. 2

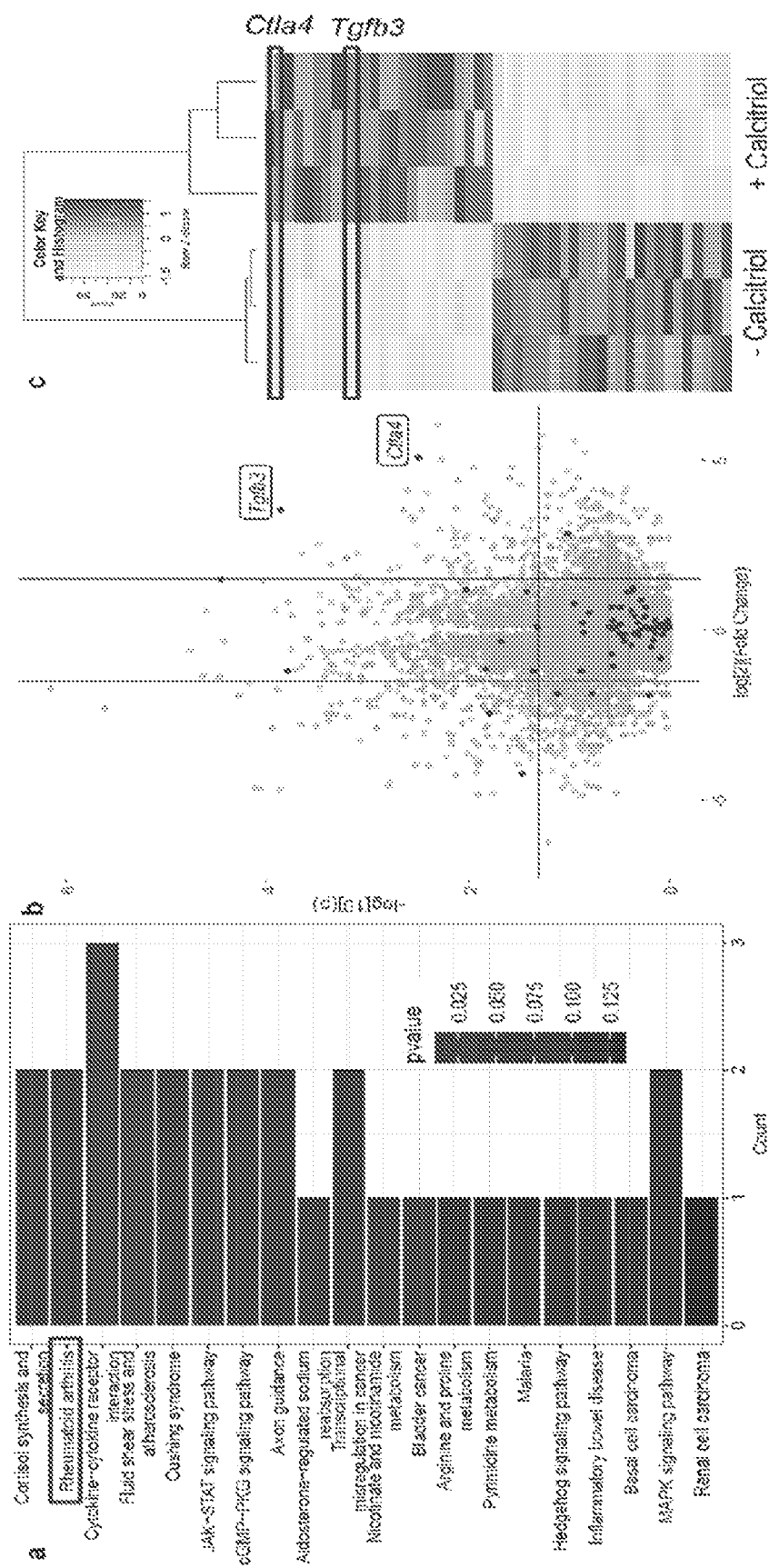


FIG. 3

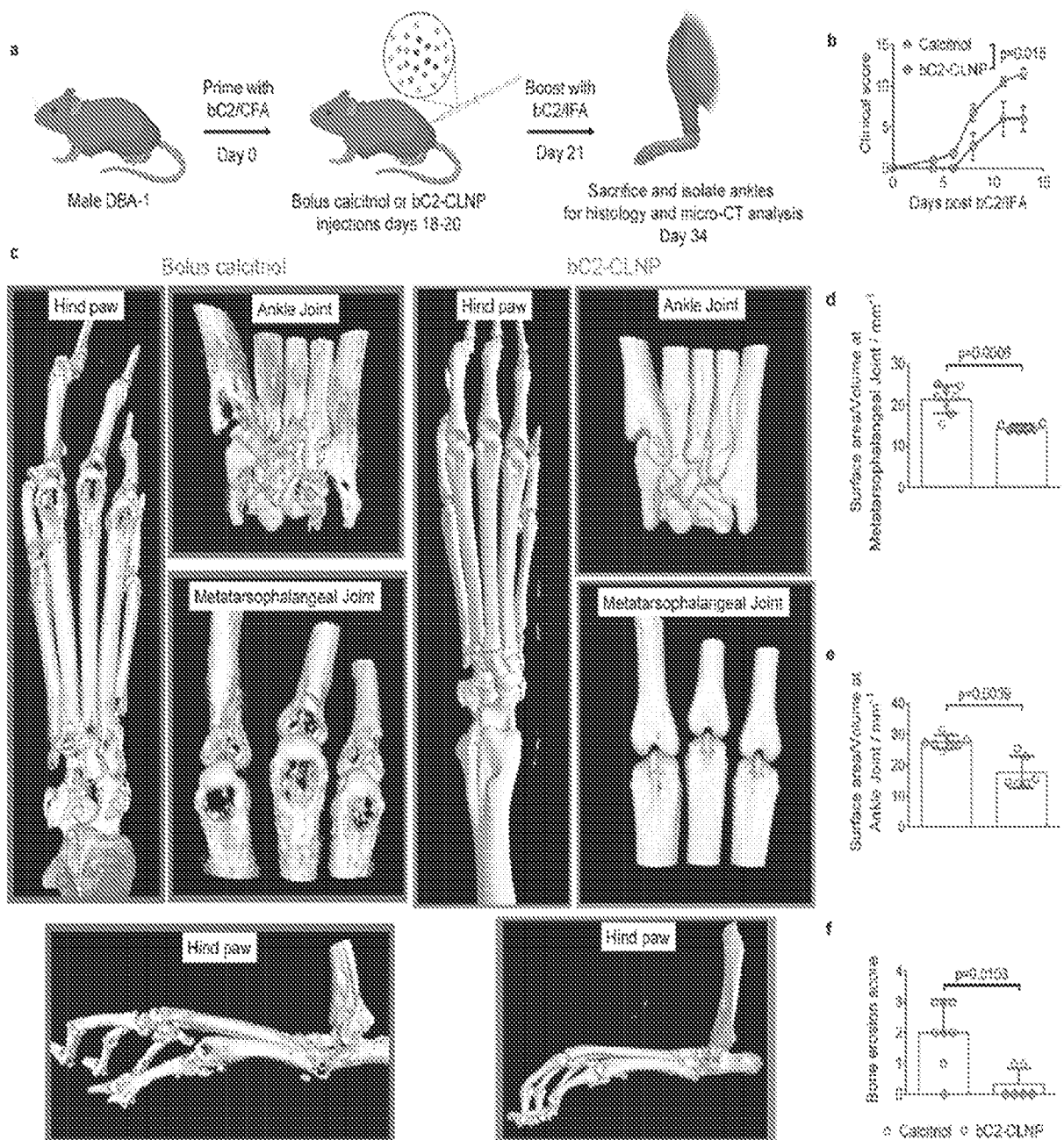


FIG. 4

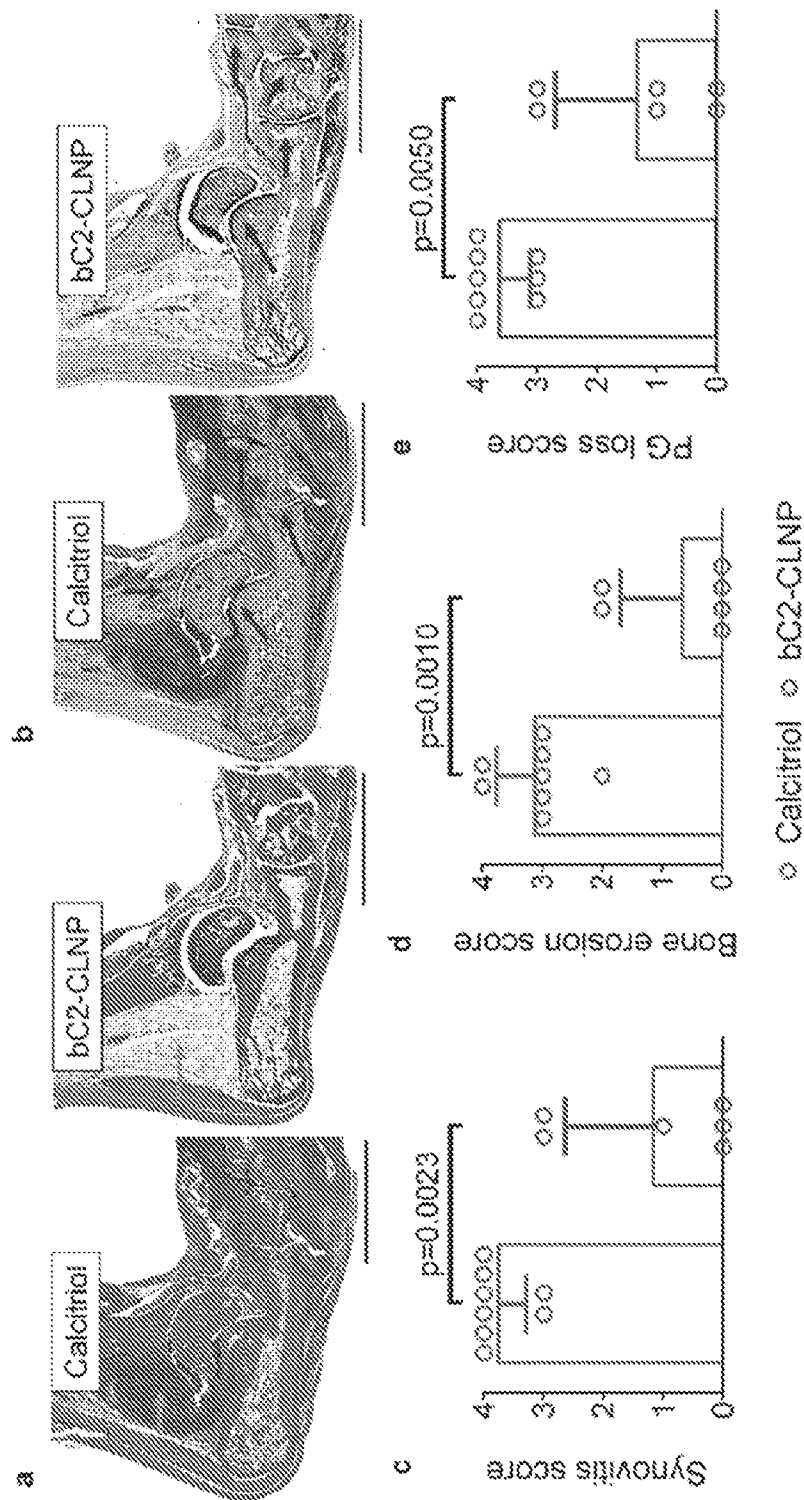


FIG. 5

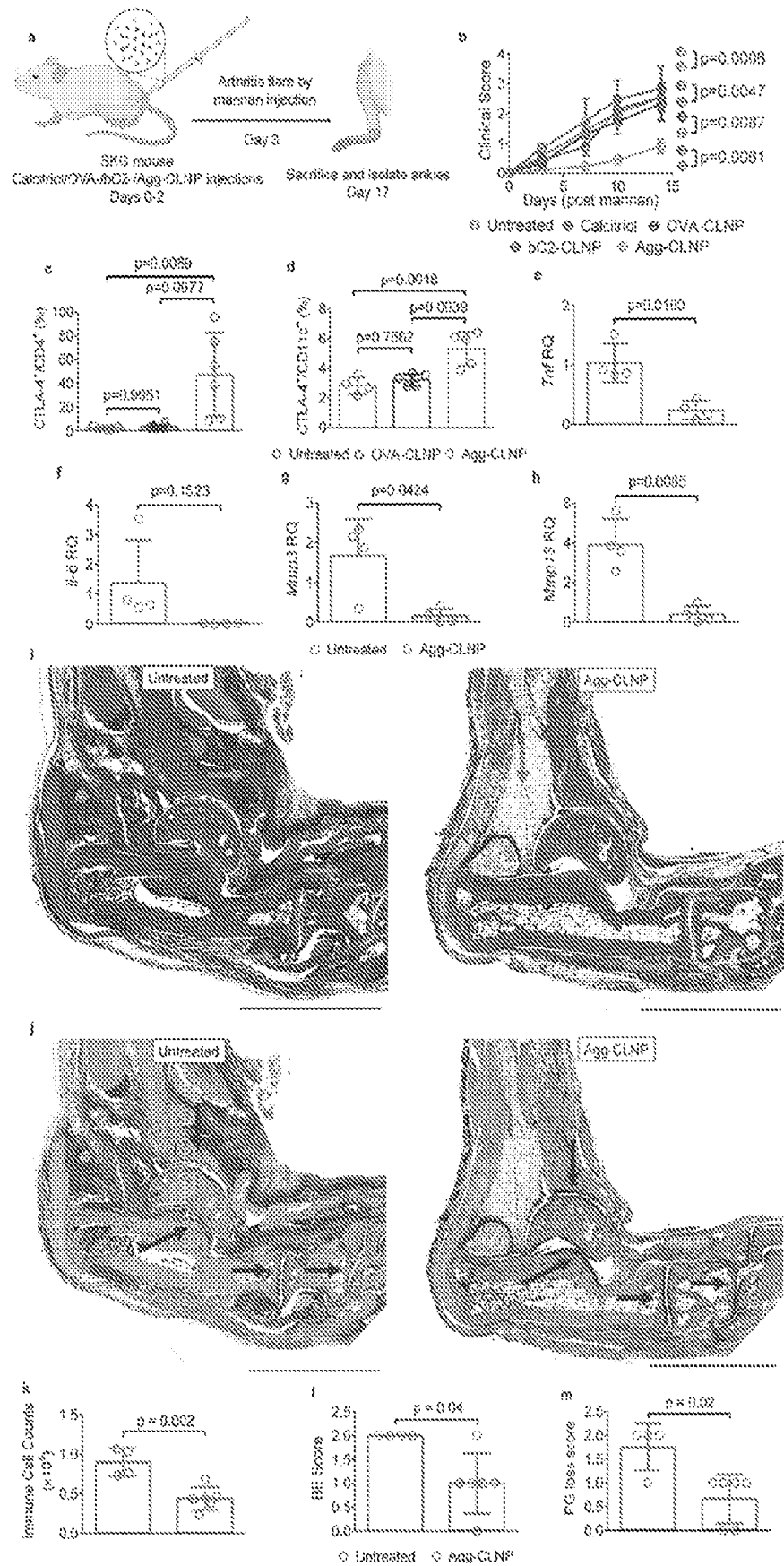


FIG. 6

Name	Sequence	Molecular weight (kDa)	Number of hydrophobic residues
N-cysteine ovalbumin ₃₂₀₋₃₂₈ (OVA)	CISQAVHAAHAEINEAGR (SEQ ID NO: 3)	1.88	8
N-cysteine bovine 2 collagen ₂₅₅₋₂₇₅ (bC2)	CGEFGIAGFKGEQGPK (SEQ ID NO: 4)	1.58	5
N-cysteine aggrecan ₇₀₋₈₄ (Agg)	CATEGRVRVNSAYQDK (SEQ ID NO: 5)	1.80	4

FIG. 7

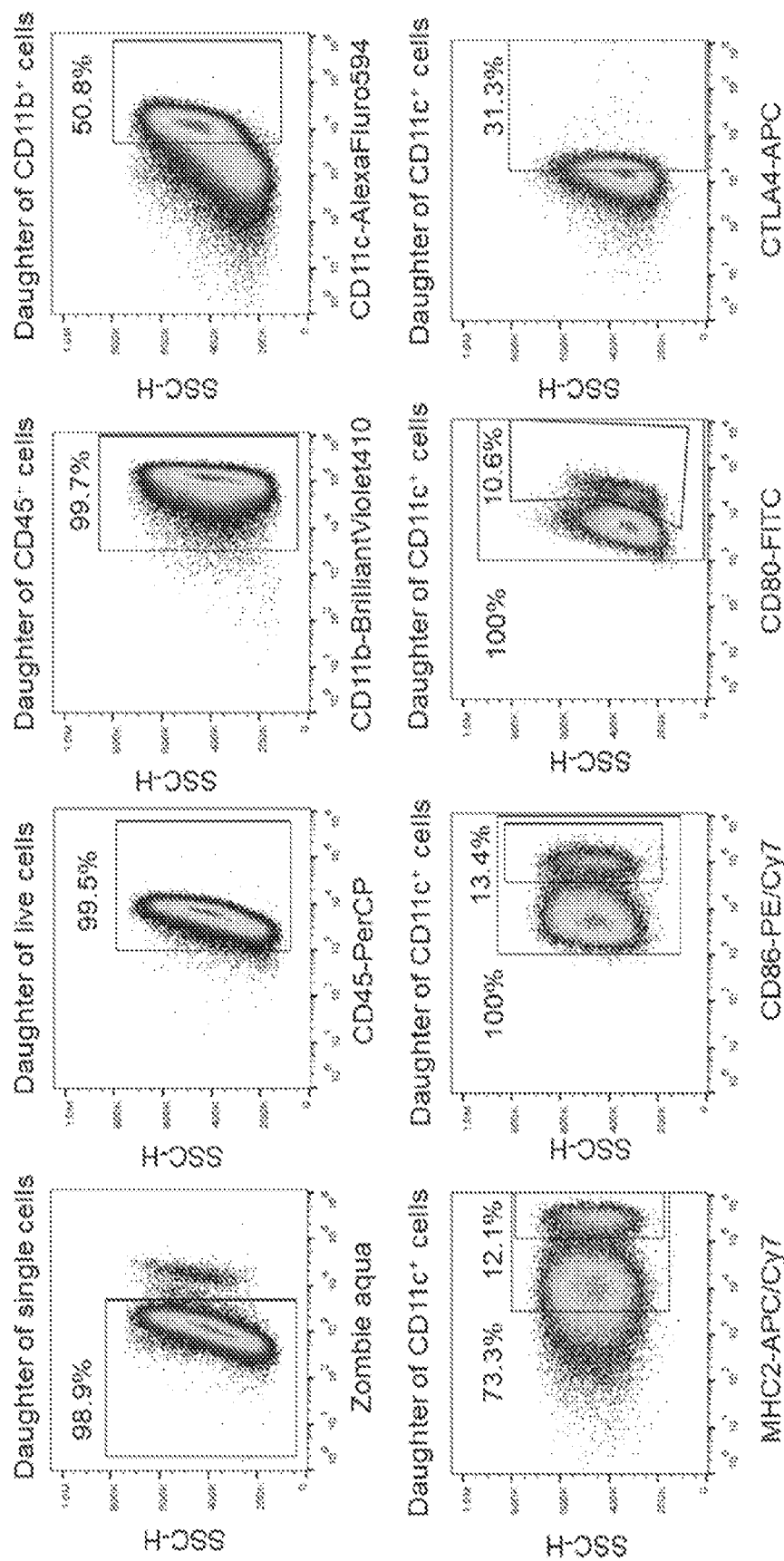


FIG. 8

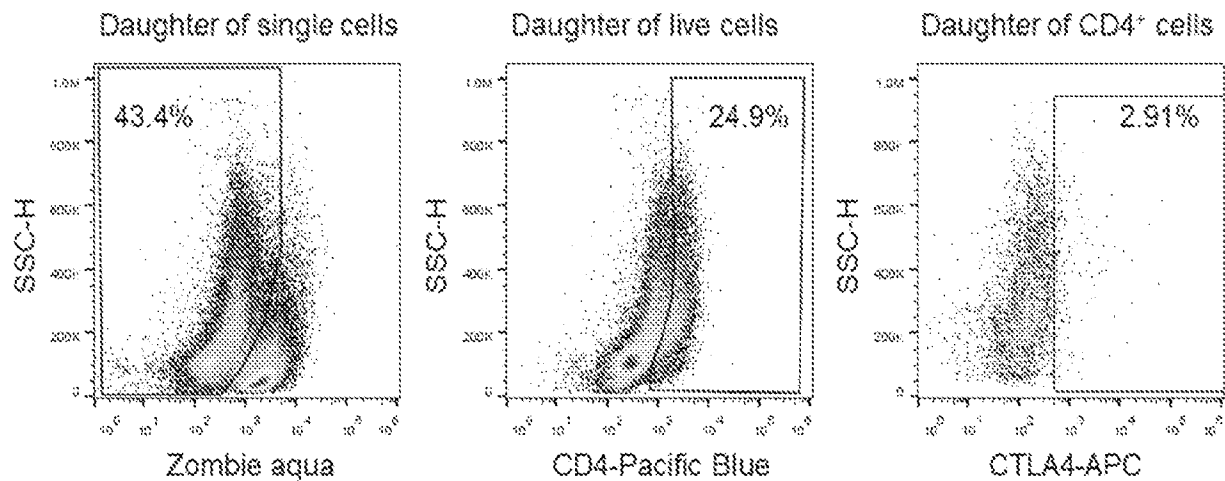


FIG. 9

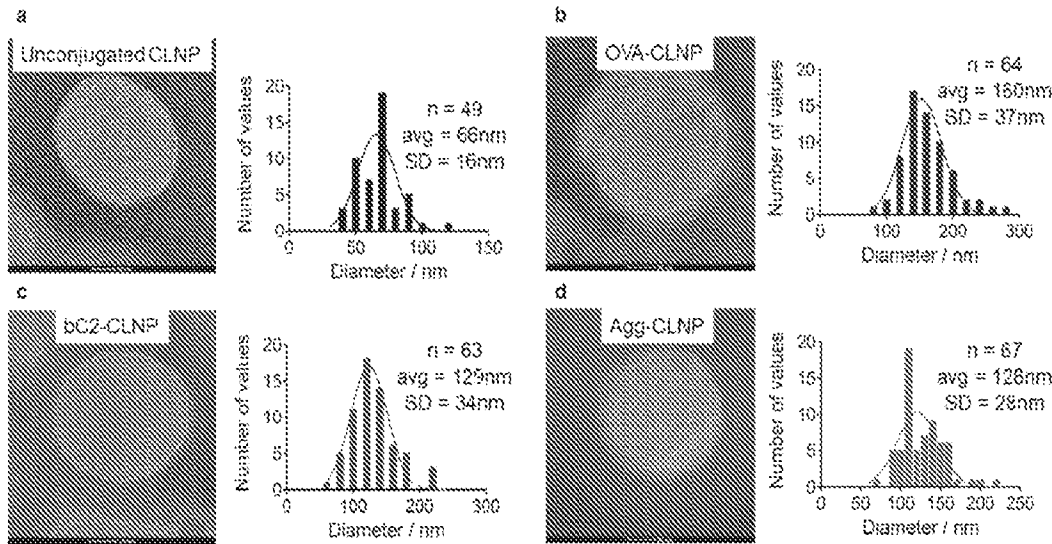


FIG. 10

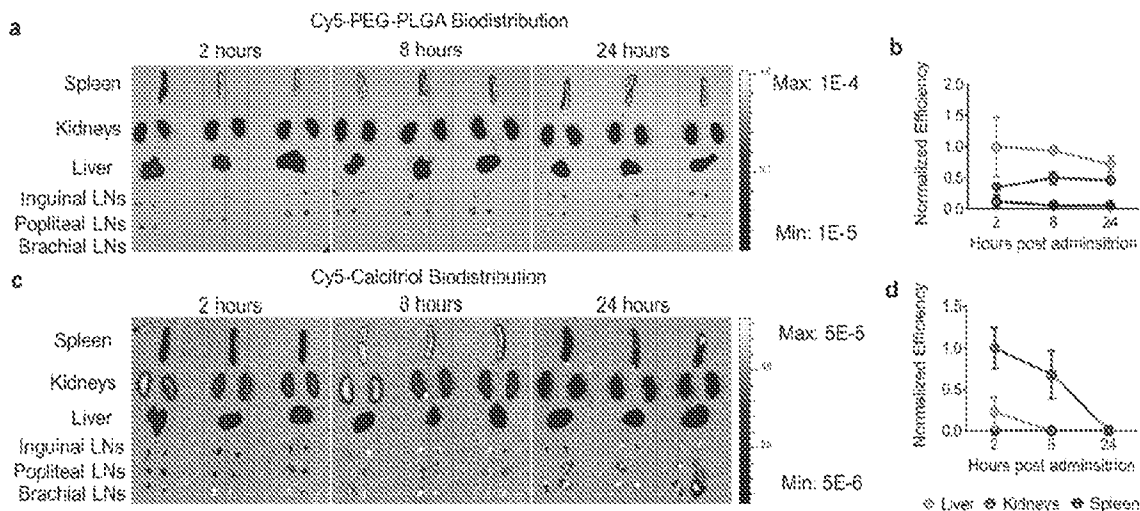


FIG. 11

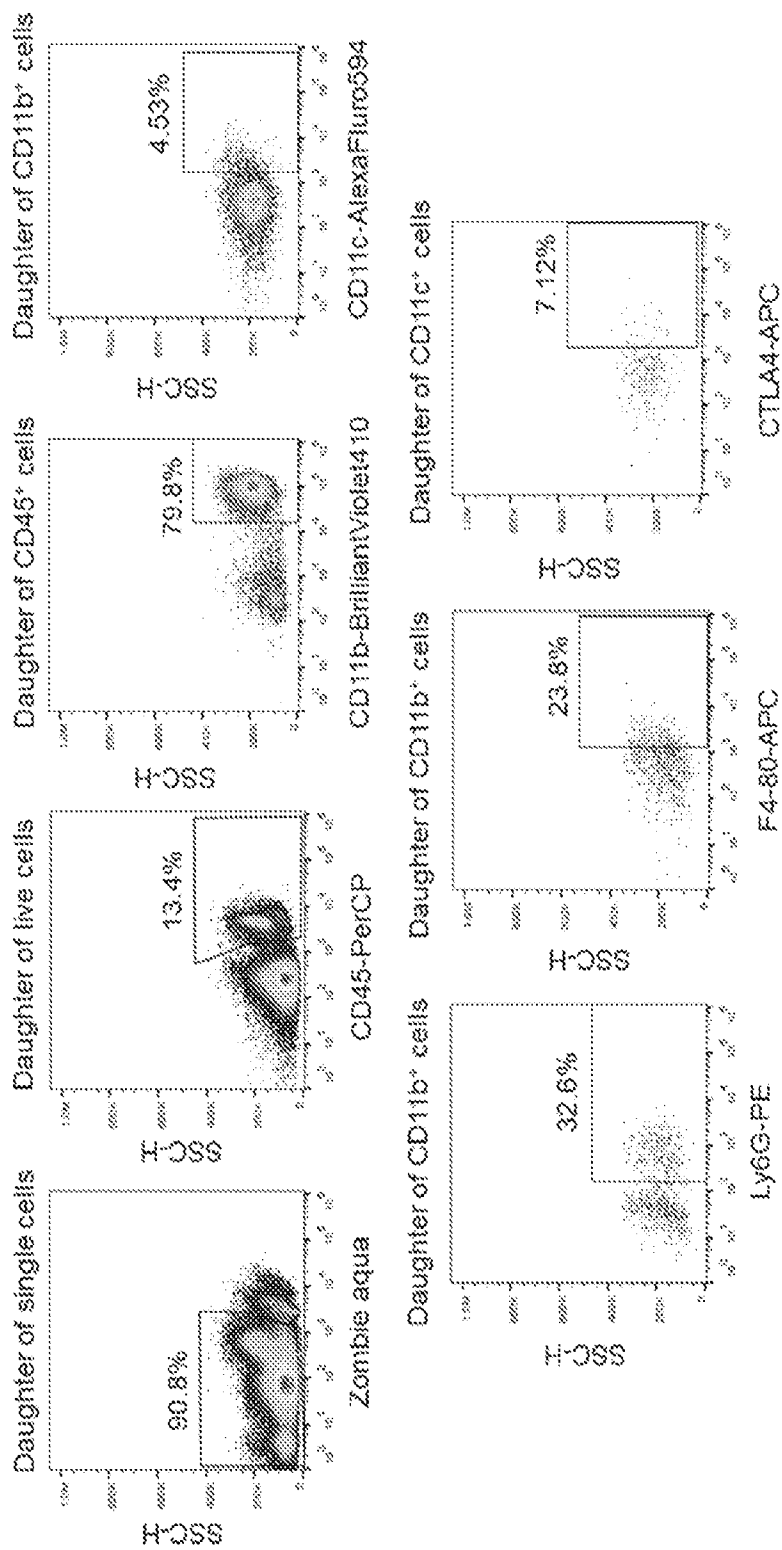


FIG. 12

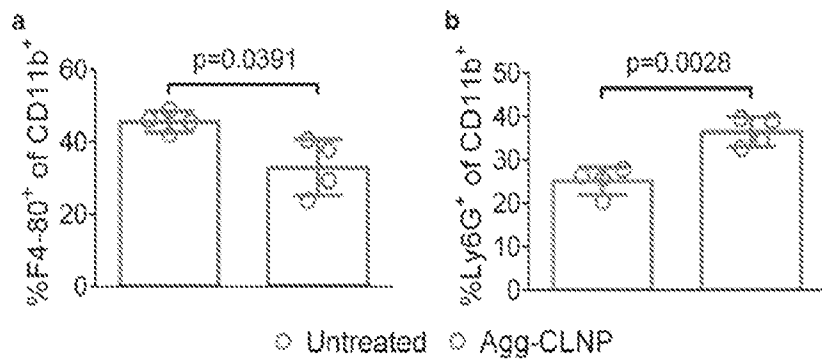


FIG. 13

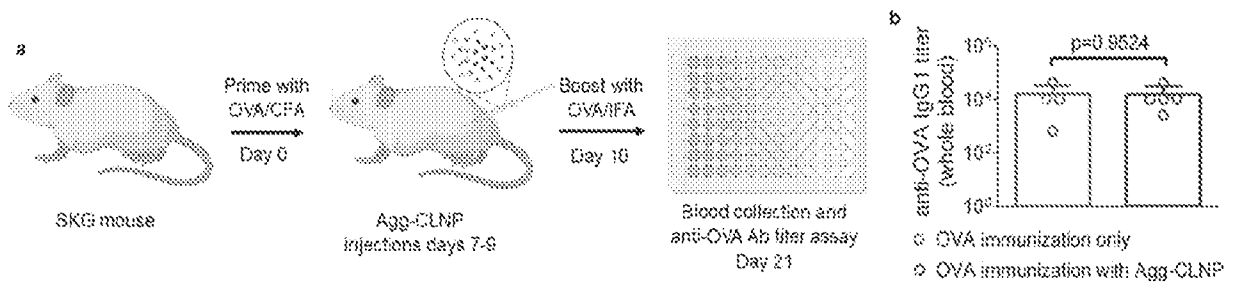


FIG. 14

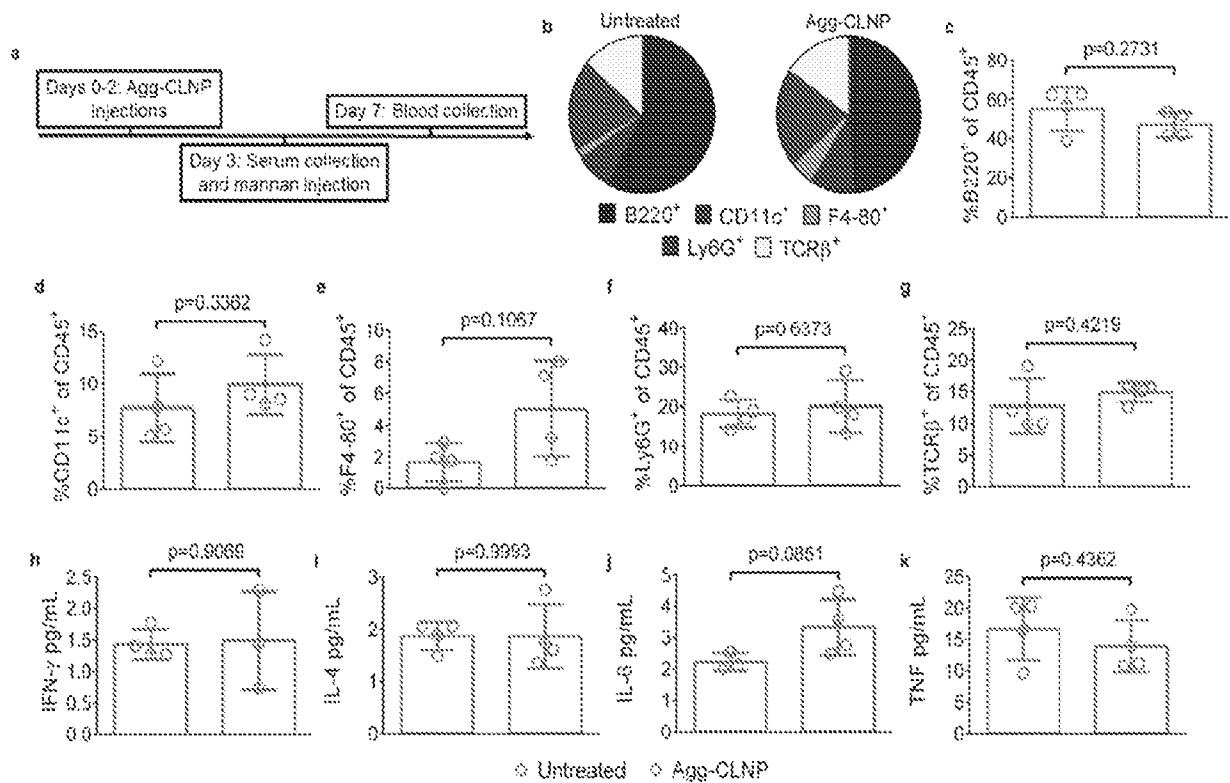


FIG. 15

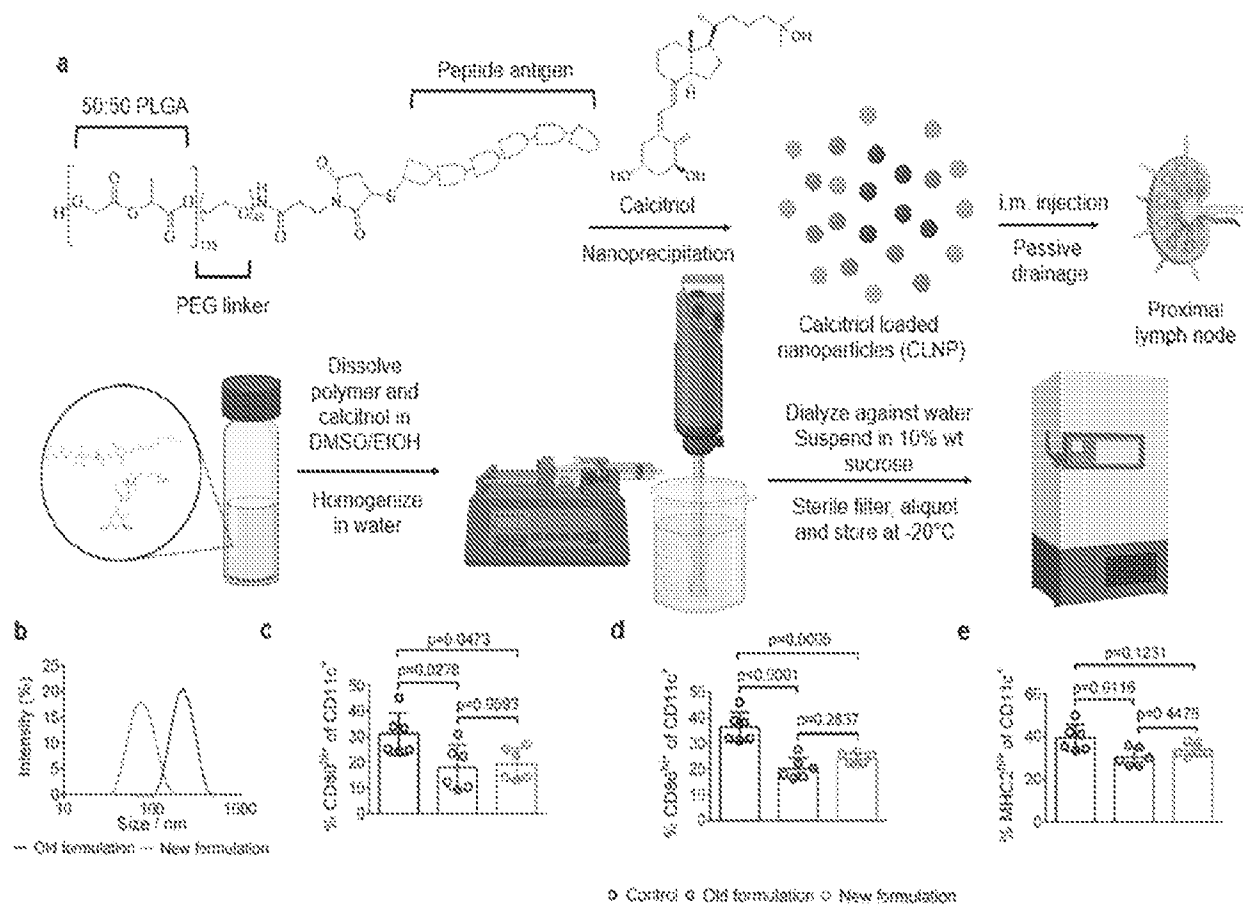


FIG. 16

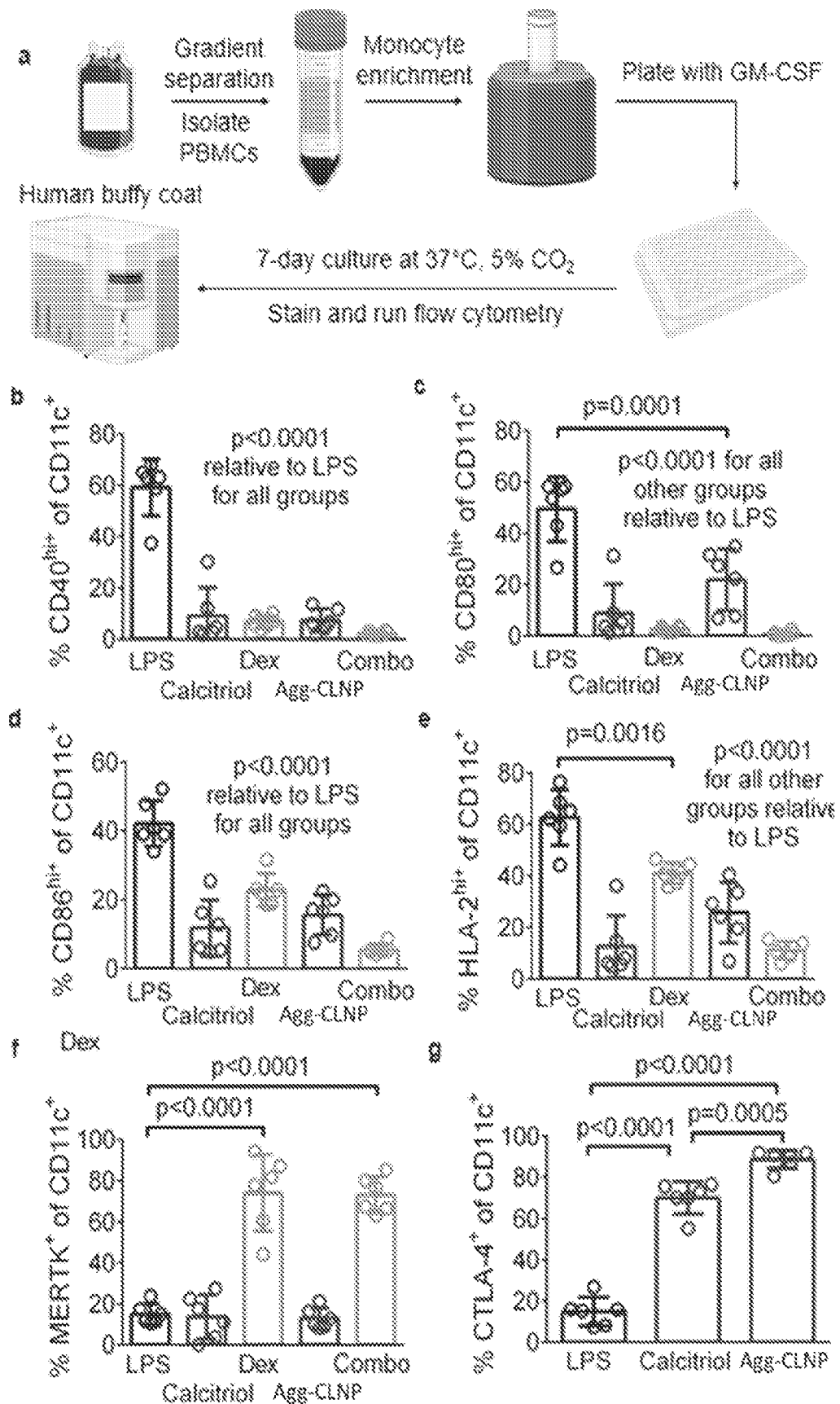


FIG. 17

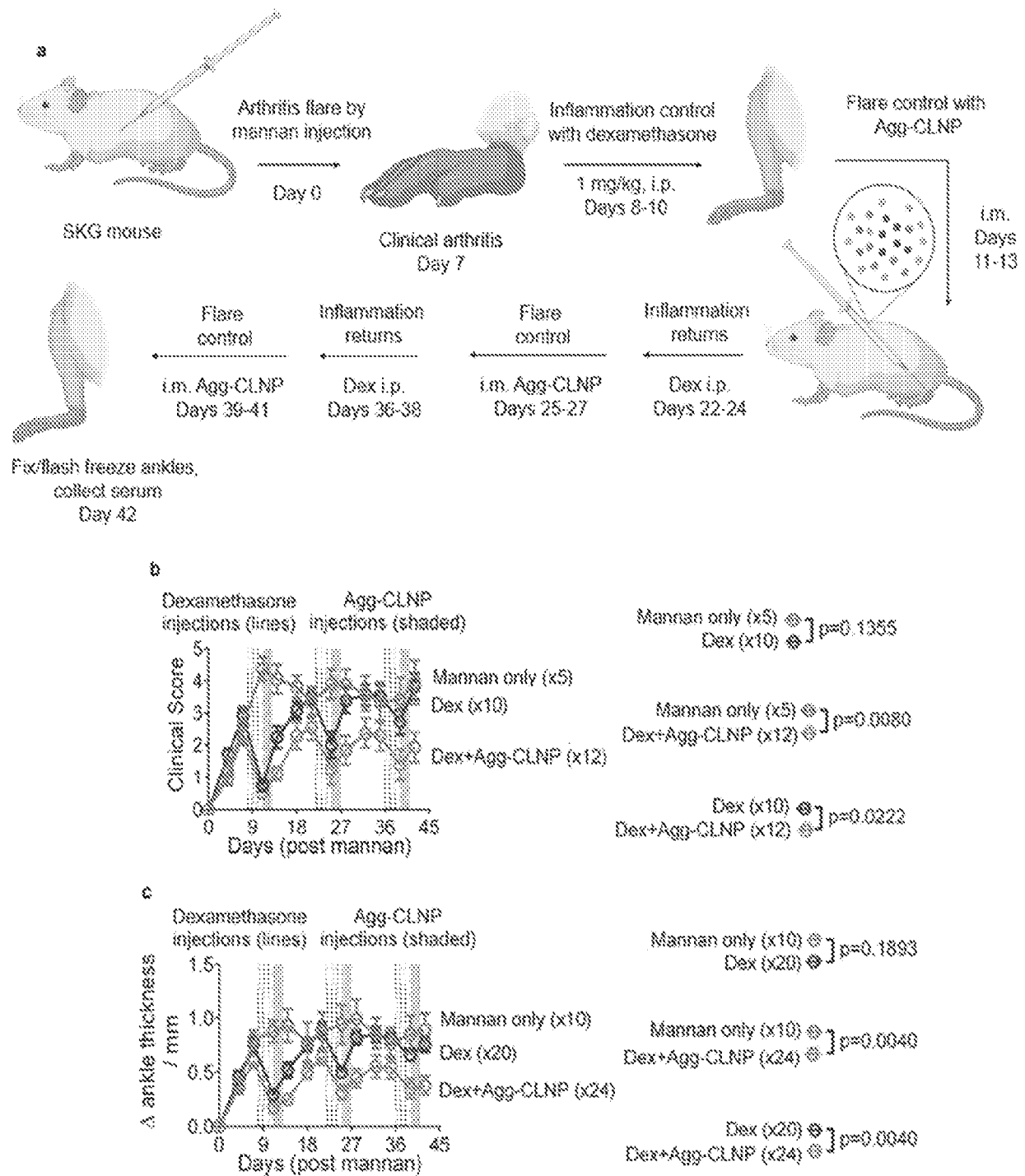


FIG. 18

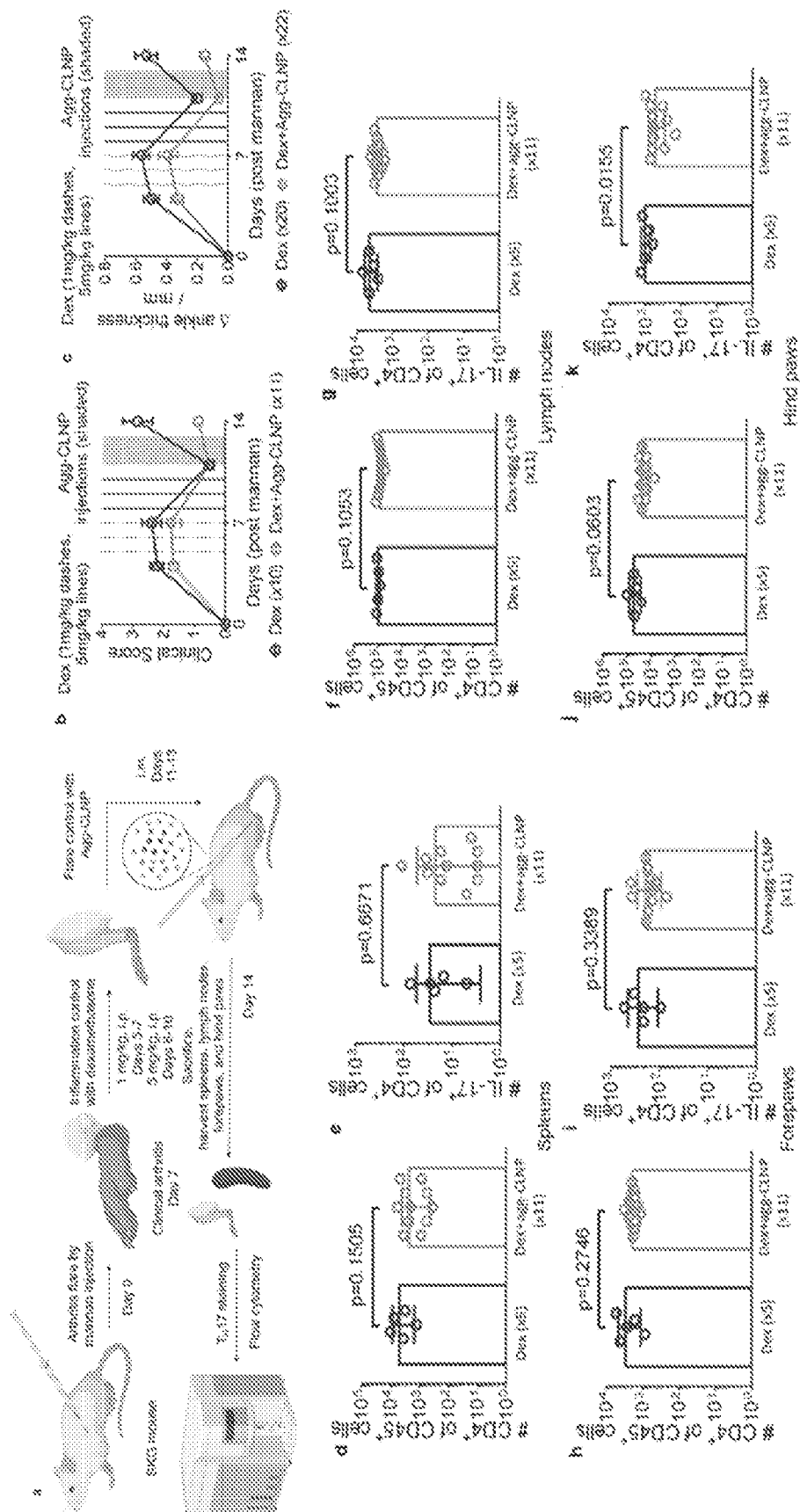


FIG. 19

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/046872

A. CLASSIFICATION OF SUBJECT MATTERIPC: **A61K 39/00** (2023.01); **A61K 47/69** (2023.01); **A61K 9/00** (2023.01); **A61P 19/02** (2023.01); **A61P 29/00** (2023.01); **A61K 31/573** (2023.01); **A61K 38/17** (2023.01)CPC: **A61K 39/46433**; **A61P 19/02**; **A61P 29/00**; **A61K 38/17**; **A61K 9/1271**; **A61K 31/573**; **A61K 47/6913**; A61K 2121/00

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.
X	KR 2022152512 A (SEOUL NATIONAL UNIVERSITY R & DB FOUNDATION) 16 November 2022 (16.11.2022) entire document	1, 3, 6, 8, 10, 13, 17, 18, 21, 22
Y	entire document	2, 4, 5, 7, 9, 11, 12, 14-16, 19, 20, 23-25
Y	US 2013/0236500 A1 (BIND BIOSCIENCES INC.) 12 September 2013 (12.09.2013) entire document	4, 5, 7
Y	US 2017/0095544 A1 (SANTAMARIA) 06 April 2017 (06.04.2017) entire document	9
Y	US 2015/0216955 A1 (THE UNIVERSITY OF QUEENSLAND) 06 August 2015 (06.08.2015) entire document	11, 12, 15, 16, 19



Further documents are listed in the continuation of Box C.



See patent family annex.

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“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

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Date of the actual completion of the international search

23 October 2024 (23.10.2024)

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/046872

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0038844 A1 (FOUSSAT et al.) 17 February 2011 (17.02.2011) entire document	14
Y	US 2016/0338955 A1 (PLS-DESIGN GMBH) 24 November 2016 (24.11.2016) entire document	20
Y	US 2017/0042972 A1 (KARYEKAR) 16 February 2017 (16.02.2017) entire document	24, 25
Y	MARTIN et al., Antigen-specific suppression of established arthritis in mice by dendritic cells deficient in NF-κB, Arthritis & Rheumatism, Vol. 56, Iss. 7, July 2007, [22.10.2024]. [Retrieved from internet: URL:https://doi.org/10.1002/art.22655]. Pgs. 2255-2266. entire document	2, 23

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/046872

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 13ter.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:

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 1-25 are drawn to methods of treating inflammatory arthritis in a patient.

Group II: claims 26-43 are drawn to compositions.

The inventions listed in Groups I and II 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, methods of treating inflammatory arthritis in a patient, are not present in Group II; and the special technical features of Group II, compositions, are not present in Group I.

Additionally, even if Groups I and II were considered to share the technical features of a composition comprising nanoparticles made of biodegradable polymers conjugated to a joint-relevant peptide antigen, wherein the nanoparticles encapsulate a dendritic cell modulator, these shared technical features do not represent a contribution over the prior art as disclosed by KR10-2022-0152512 A to Seoul National University R & DB Foundation (hereinafter, "SNU").

SNU teaches a composition (a pharmaceutical composition for preventing or treating autoimmune diseases, Para. [0034]) comprising nanoparticles (a pharmaceutical composition for preventing or treating autoimmune diseases, comprising, as active ingredients, the above-described antibody and lipid-antioxidant nanoparticles having self-antigen bound thereto, Para. [0034]) made of biodegradable polymers (the antioxidant nanoparticles according to the present disclosure may be biopolymer-based biopolymer nanoparticles, and may include a biopolymer or may be nanoparticles formed from a biopolymer. The biopolymer... has excellent biocompatibility, biodegradability, Para. [0059]) conjugated to a joint-relevant peptide antigen (the present invention provides uses of the lipid-antioxidant nanoparticles having the antibody and the autoantigen bound to the surface thereof for preventing or treating autoimmune diseases, Para. [0035]; the self-antigen can be, but is not limited to, a protein, a fragment thereof, Para. [0026]; the autoantigen is selected from the group consisting of collagen, Para. [0027]), wherein the nanoparticles encapsulate a dendritic cell modulator (the nanoparticle may further comprise, but is not limited to, an immunosuppressant, Para. [0030]; the immunosuppressant may be encapsulated in the lipid membrane, Para. [0031]; the immunosuppressant may be one or more selected from the group consisting of adrenocorticoids, calcineurin inhibitors, antimetabolites, mTOR inhibitors, and vitamin D3, Para. [0032], See instant disclosure, Pg. 34, Lns. 25-29, which states the active form of Vitamin D3 inhibits dendritic cell maturation).

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

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/046872

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.: **1-25**

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.