



(51) International Patent Classification:

A61K 9/14 (2006.01) A61K 31/195 (2006.01)
A61K 9/51 (2006.01) A61K 31/395 (2006.01)

(21) International Application Number:

PCT/US2017/056149

(22) International Filing Date:

11 October 2017 (11.10.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/406,468 11 October 2016 (11.10.2016) US

(71) Applicants: NEW YORK UNIVERSITY [US/US]; 70
Washington Square South, New York, NY 10012 (US).
ICAHN SCHOOL OF MEDICINE AT MOUNT SI-
NAI [US/US]; One Gustave L. Levy Place-box 1675, New
York, NY 10029-6574 (US).

(72) Inventors: **FAYAD, Zahi**; 1 Gustave L Levy Place - Floor
12, New York, NY 10029 (US). **SANCHEZ-GAYTAN,**

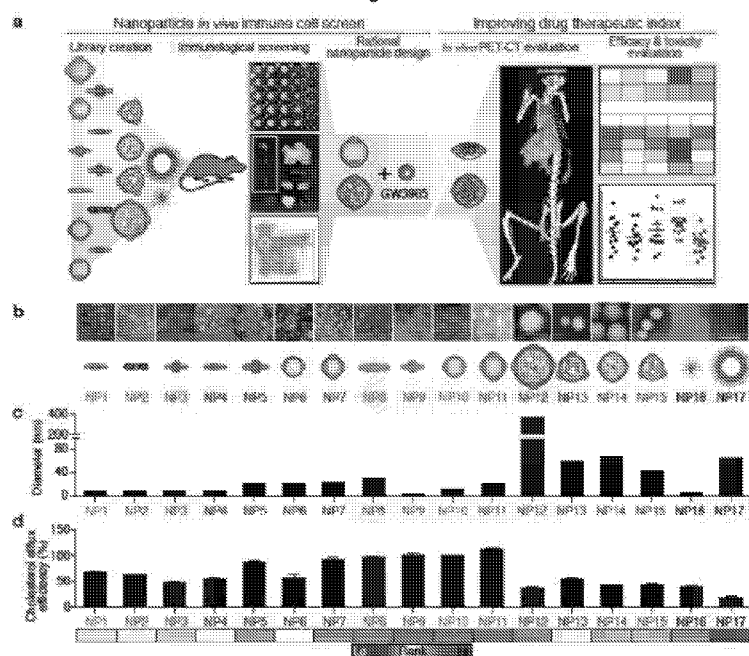
Brenda; 1295 5th Avenue, New York, NY 10029 (US).
MULDER, Willem; 1 Gustave L Levy Place - Floor 12,
New York, NY 10029 (US). **TANG, Jun**; 1 Gustave Levy
Place-floor 12, New York, NY 10029 (US). **FISHER,**
Edward, A.; 70 Washington Square South, New York, NY
10012 (US).

(74) Agent: **JACKSON, David, A.**; Klauber & Jackson LLC.,
25 East Spring Valley Avenue - Suite 160, Maywood, NJ
07607 (US).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,
KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(54) Title: NANOPARTICLES AND USES THEREOF

Figures 1A-D



(57) **Abstract:** Manmade nanoparticles possessing cell type specific targeting properties are disclosed. The nanoparticles may be prepared as pharmaceutical compositions and may be used for the prevention and treatment of a variety of conditions in mammals, including by way of non-limiting example, atherosclerosis, diabetes and its complications, cancer, ischemia/reperfusion injury, cardiovascular disease and other diseases related to macrophage-driven inflammatory immune responses.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

NANOPARTICLES AND USES THEREOF

GOVERNMENTAL SUPPORT

[0001] The research leading to the present invention was funded in part by National Institute of Health grants R01 HL118440, R01HL125703, R01 CA155432, and R01 EB009638. The United States government has certain rights in the invention.

FIELD OF THE INVENTION

[0002] Manmade nanoparticles capable of delivering therapeutic agents with specificity to particular cell types and/or organs are disclosed herein. More particularly, the present inventors have designed and generated recombinant, manmade nanoparticles that are capable of delivering therapeutic agents preferentially to macrophages involved in inflammatory immune responses. In a particular embodiment, the inflammatory immune responses are associated with the pathological progression of atherosclerosis, cancer, and/or diabetes. Also encompassed herein are compositions of the manmade nanoparticles (with or without a therapeutic agent payload), pharmaceutical compositions of the manmade nanoparticles (with or without a therapeutic agent payload), and methods for using same to treat diseases or conditions associated with macrophage driven inflammatory immune responses such as those exemplified by atherosclerosis, cancer, and/or diabetes.

BACKGROUND OF THE INVENTION

[0003] Research in the past decades has revealed the immune system's central role in the pathophysiology of cancer(1), diabetes(2), and atherosclerosis(3, 4). Because macrophages drive pathological progression of these diseases, immunomodulatory small-molecule compounds modulating macrophage function are promising therapeutic candidates for treating these maladies. However, their lack of cellular specificity necessitates a strategy for targeted delivery to harmful immune cells without negatively affecting beneficial immune cells. Despite numerous studies of nanoparticle-based delivery, rational attempts to screen meticulously designed nanoparticles for immune cell specificity *in vivo* have never been reported.

[0004] In view of the above, a need exists for nanoparticle-based therapeutic agents, and corresponding pharmaceutical compositions and related methods for treating conditions causally related to harmful immune cells, and it is toward the fulfillment and satisfaction of that need, that the present invention is directed.

SUMMARY OF THE INVENTION

[0005] The immune system plays an essential role in the pathophysiology of major diseases such as atherosclerosis, diabetes, and cancer, which has inspired the development of numerous small molecules to modulate immune cells, intending to create immunotherapies for these diseases. Tissue- and cell-specific delivery of these small molecules is the key to transform these compounds into safe, potent immunotherapies. To address this objective, the present inventors set forth a novel *in vivo* nanoparticle screening approach that involves designing and evaluating a library of nanoparticles with distinct immune cell targeting specificity. To the best of the present inventors' knowledge, this is the first study that carries out a systematic *in vivo* immune cell screening to create effective nanoparticle-based immunotherapy for modulating the activity of pathological immune cells involved in atherosclerosis, cancer, and respiratory diseases, such as, for example, asthma.

[0006] As detailed herein, the present inventors created a combinatorial library of hybrid lipoprotein-inspired nanoparticles with distinct physiochemical properties, particularly with respect to size and chemical composition, that possess differential immune cell specificity. The present inventors then chose atherosclerosis, a lipid-driven inflammatory process of the large arteries, as a model disease in which to evaluate the nanoparticle library. Atherosclerosis accounts for the majority of cardiovascular deaths worldwide(5) and macrophages are the major immune cells that drive the pathological inflammation associated with atherosclerotic plaques and progression thereof (6, 7). Atherosclerotic plaques, which are present throughout the vasculature, exhibit a complicated cellular composition(8). Nanoparticles described herein are envisioned as delivery vehicles that improve the therapeutic index of small-molecule immunomodulatory compounds and confer plaque macrophage-specific delivery, with minimal delivery to non-pathological cells in healthy tissues.

[0007] The present inventors used the *Apoe*^{-/-} mouse model of atherosclerosis to evaluate the nanoparticle library, because it accurately recapitulates many important immunological aspects of human atherosclerosis(9). Using a combination of optical methods, immunological techniques, and *in vivo* positron emission tomography (PET) imaging, the present inventors carefully selected candidate nanoparticles from the library for subsequent atherosclerosis drug delivery studies. As a proof of concept, the liver receptor X agonist GW3965, a therapeutic compound that did not reach clinical application due to its serious adverse effects on the liver(10, 11), was incorporated into two nanoparticles—one with favorable organ distribution and immune cell specificity and one without these features. In *Apoe*^{-/-} mice with advanced disease, the favorable nanoparticle identified in the library screen (NP10) was shown to abolish GW3965's liver toxicity while remaining effective with respect atherosclerotic plaque macrophages.

[0008] In accordance with discoveries detailed herein, manmade nanoparticles and manmade nanoparticles comprising therapeutic agents that are capable of specifically targeting macrophages and activity thereof are disclosed. By conferring targeting specificity for macrophages, nanoparticles described herein offer a delivery vehicle whereby therapeutic agents encapsulated or incorporated therein are deposited in a concentrated and localized fashion at macrophage-driven inflammatory sites. Accordingly, the manmade nanoparticles are envisioned for use in methods for treating diseases or conditions associated with macrophage-driven inflammation, such as atherosclerosis, cancer, and respiratory diseases, such as, for example, asthma.

[0009] In one aspect, a method is presented for preventing, treating or ameliorating in a mammal a disease or condition that is causally related to macrophage-driven inflammation.

[0010] In a further aspect, pharmaceutical compositions comprising manmade nanoparticles described herein and a pharmaceutical carrier, excipient or diluent are envisioned. In a particular aspect, the pharmaceutical composition may comprise one or more of the manmade nanoparticles described herein. Moreover, the manmade nanoparticles described herein as useful in the pharmaceutical compositions and treatment methods disclosed herein are all pharmaceutically acceptable as prepared and used.

[0011] In a further aspect, this invention provides a method for treating a mammal susceptible to or afflicted with a condition from among those listed herein, and particularly, such condition as may be associated with macrophage-driven inflammation. Such conditions include, without limitation, atherosclerosis, cancers, and diabetes and its complications.

[0012] Other objects and advantages will become apparent to those skilled in the art from a consideration of the ensuing detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] **Figure 1A-D. Study design and *in vitro* characterization of the nanoparticle library.** **A,** In the *in vivo* immune cell screen study (left panel), a combinatorial nanoparticle library was first created and then evaluated in atherosclerotic *Apoe*^{-/-} mice by using blood half-life determination, near infrared fluorescence imaging, and flow cytometry. The library screening data led to rational design of one GW3965-loaded nanoparticle with favorable characteristics and one without. In the second study, the two nanoparticles were radio- and fluorescence-labeled, and they were quantitatively and therapeutically evaluated by using PET-CT imaging, mRNA profiling, flow cytometry, and liver toxicity assays (right panel). **B,** Representative high-magnification transmission electron microscopy (TEM) images of negatively stained nanoparticles. Low-magnification images and a discussion of the different structures are

presented in supplementary figure 1. Scale bar is 50 nm. **C**, The size of nanoparticles as measured by dynamic laser scattering (DLS). **D**, Cholesterol efflux capacity of the nanoparticles in primary macrophages normalized to natural human HDL ($n = 6$). Error bars are standard deviations (SD). Color-coded bar in the bottom shows the relative rank of each nanoparticle, with the red indicating high cholesterol efflux efficiency and the blue indicating low efficiency.

[0014] Figure 2A-F. *In vivo* evaluation of the nanoparticle library. **A**, Relative nanoparticle plasma concentration in *Apoe*^{-/-} mice that were fed a 12-week high-cholesterol diet. The values were derived from the near infrared dye DiR incorporated in the nanoparticles. Heat map (bottom) ranks the blood half-lives, with the red indicating a long blood half-life and the blue a short blood half-life ($n = 5$ per nanoparticle). The values of blood half-lives are provided in supplementary table 2. **B**, Representative near infrared fluorescence images of nanoparticle accumulation in aorta, liver, and spleen. The heat map below the aorta images ranks the mean total fluorescent tissue signal, the heat map below the liver images ranks the total aorta-to-liver signal; the heat map below the spleen images ranks the mean aorta-to-spleen accumulation (Ao-to-sp) ratio, with the red indicating a high and the blue a low ratio ($n = 5$ for each nanoparticle). Bar graphs are provided in supplementary figure 2. **C**, Blood half-lives of 4 selected nanoparticles radiolabeled with ⁸⁹Zr ($n = 3$ per nanoparticle) were 7.0 hour for ⁸⁹Zr-NP10, 5.7 hour for ⁸⁹Zr-NP14, 7.1 hour for ⁸⁹Zr-NP15, and 6.6 hour for ⁸⁹Zr-NP17. Blood radioactivity (%ID/g) of all time points is normalized to that of the first time point—2 min after injection. **D**, Representative autoradiography images of aortas, livers, and spleens 24 hours after nanoparticle injection. Dashed windows indicate the aortic root and arch area analyzed in panel E. **E**, nanoparticle accumulation in aortic roots and arches as measured by percentage of injected dose (%ID) ($n = 3$ per nanoparticle). **F**, Relative accumulation of nanoparticles between aortas and liver or spleen. Arbitrary units (A.U.) were defined by the aortic accumulation (%ID/g) divided by the hepatic or splenic accumulation (%ID/g). Error bars are standard deviations (SD). Statistics were calculated with non-parametric two-tailed Student's *t*-test. N.S. means not statistically significant; * $P < 0.05$; ** $P < 0.01$.

[0015] Figure 3A-C. Nanoparticle immune cell specificity. **A**, The flow cytometry gating procedures to identify relevant immune cells in aorta, spleen, and blood. Black histograms on the right show representative signal distribution of different immune cells in the mice injected with nanoparticles compared to the cells from control animals injected with PBS (gray histogram on the left in each graph). **B**, Quantification of mean fluorescence intensity (MFI) of each immune cell type in different tissues. $n = 5$ for each nanoparticle, error bars are standard error of the means (S.E.M.) **C**, Heat map ranks targeting efficiency in key immune cells (aortic macrophages, spleen macrophages, and blood Ly-6C^{hi} monocytes), with red indicating a high and

the blue a low MFI in the first 3 rows. The last row shows the aortic-to-splenic macrophage MFI ratio (Ao-to-sp MΦ), and its quantitative values are provided in supplementary figure 3A.

[0016] Figure 4A-G. *In vivo* quantitative evaluation of GW3965-loaded nanoparticles. **A**, Schematic depictions of small Rx-HDL plaque macrophage-targeting and large polymer-hybrid Rx-PLGA-HDL nanoparticles. The two nanoparticles were either radiolabeled with ^{89}Zr or labeled with the near-infrared fluorescent dye DiR. **B**, Blood half-lives were determined in 3 mice per nanoparticle. **C**, Representative PET images of mice that received either the small or large nanoparticle at 0.5 and 24 hours after intravenous administration. 3D-rendered images are provided as supplementary movies 1 to 4 ($n = 5$ per nanoparticle). **D**, Quantification of radioactivity in the heart, liver, and spleen. **E**, Representative autoradiographic images of key organs. Full biodistribution of all organs are provided in supplementary figure 4E. **F**, Representative histograms of selected immune cell targeting specificity in the aorta, spleen, and blood. **G**, MFI quantification of relevant immune cells in the tissues ($n = 4$ per nanoparticle). Error bars are S.E.M. Statistics was calculated with non-parametric two-tailed student's *t*-test. * $P < 0.05$; ** $P < 0.01$.

[0017] Figure 5A-H. Evaluation of the toxicity and efficacy of GW3965 (Rx)-loaded nanoparticles. In panel **A** to **E**, *Apoe*^{-/-} mice received 4 intravenous administrations of nanoparticles or PBS on every other day ($n = 12$ per group). **A**, mRNA expression levels of 3 GW3965 target genes in liver homogenates. Triglyceride (**B**) and cholesterol (**C**) levels in the liver, the primary organ suffering from the toxic effects of GW3965. **D**, mRNA expression levels of 5 GW3965 target genes in aortic macrophages. **E**, mRNA expression levels of 19 inflammation-related genes in aortic macrophages. All gene expression was normalized to housekeeping gene *Hprt1*. The bar graph presentation of the heat maps is in supplementary figure 5 ($n = 12$ per group). In panel **F** to **H**, *Apoe*^{-/-} mice ($n = 10$ in PBS; $n = 9$ in Rx-HDL) received 12 intravenous injections in 6 weeks. Lipid levels of aortic cells were analyzed by flow cytometry. Cellular lipid levels were calculated by multiplying the mean fluorescence intensity of a cell type with the number of the cells per aorta ($\text{AU} = \text{MFI} \times \text{number of cells}$). The total lipid levels of aortic macrophages (**F**), monocytes (**G**), or non-immune CD45⁺ cells (**H**) per aorta in the mice are presented here. Gating procedure is provided in supplementary figure 6A and 6B. Error bars in all graphs are S.E.M. Statistics was calculated with non-parametric two-tailed Student's *t*-test.

[0018] Figure 6. Exemplary hydrophobic therapeutic agents for inclusion in nanoparticles. Molecular weight and hydrophobicity are the two key parameters that dictate a compounds' relative compatibility with various nanoparticles described herein. Using specialized software (ACD/Labs Percepta Predictors), the two key physiochemical properties of 5 tested (red

round dots) and 33 potentially compatible compounds (black triangle dots) are assessed. The details of the 38 compounds assessed are provided in Figure 7.

[0019] Figure 7: Table 1 presents information pertaining to the exemplary hydrophobic therapeutic agents of Figure 6.

[0020] Supplementary Figure 1A-B. Chemical characterization of the nanoparticle library. A, Low-magnification TEM images of the 17 nanoparticles, as well as the two therapeutic nanoparticles—Rx-HDL and Rx-PLGA-HDL. Scale bars are 100 nm. NP1-5 have a discoidal shape, as is typical for HDL nanoparticles reconstituted from phospholipids and APOA1. POPC based nanoparticles require a higher amount of APOA1 to force disc formation, as can be observed when comparing NP8 and NP9. The inclusion of triglycerides as a core material results in nanoparticles of ~20 nm (NP6, NP7, NP10, and NP11), while the inclusion of PLGA or PLA polymers allows fine-tuning of nanoparticle sizes, ranging from ~40 nm for NP15 to over 100 nm for NP12. B, Mass spectrometry of oxidized nanoparticle. A HDL-mimicking nanoparticle made mainly of DMPC and APOA1 (NP1) was oxidized to generate NP2, which was analyzed by mass spectrometry (MS). Mass spectrometry shows the increase of APOA1 M.W. by about 146.7 on average, indicating the oxidization of APOA1 (Ox-APOA1).

[0021] Supplementary Figure 2A-E. Representative and quantitative NIRF data. A, Representative NIRF images of key organs. Quantification of total signal of all organs (B) and aortas (C). D, The aortic-to-liver accumulation ratio is calculated by dividing the total signal of aorta with that of liver. E, The aorta-to-spleen accumulation ratio is calculated by dividing the total signal of aorta with that of spleen. n = 5 for per nanoparticle, and the error bars are SEM.

[0022] Supplementary Figure 3A-B. Macrophage targeting efficiency of the nanoparticle library. A, Aortic-to-splenic (Ao-to-sp) macrophage MFI ratio is calculated by dividing the MFI of aortic macrophages by that of splenic macrophages (n = 5 per nanoparticle). Error bars are SEM. B, Representative immunostaining images of aortic roots from the animals injected with the nanoparticles. CD68 was used to identify macrophages in the frozen section. Nanoparticles were identified by detecting their fluorescent label DiR.

[0023] Supplementary Figure 4A-F. In vivo evaluation of drug-loaded nanoparticles. A, The summary of the performance of the nanoparticle library in the immunological screening study, with the red showing the favorable and the blue the unfavorable performance according to the specific limitations of GW3965. High-rank nanoparticles show high cholesterol efflux capacity, long blood half-life, high relative aorta-to-liver accumulation ratio, and high relative aortic-to-splenic macrophage MFI ratio. NP10 was found to have the highest overall rank. B, Representative TEM images of negatively stained drug-loaded nanoparticles. Scale bar is 50 nm. Overview of the images can be found in supplementary figure 1A. C, Relative cholesterol efflux

capacity of drug-loaded nanoparticles ($n = 6$). Non-parametric student's t -test was used for statistics. **D**, Radioactive HPLC eluting profiles of drug-loaded nanoparticles. The black profile is the UV absorbance, which indicates the nanoparticle itself. The blue and red profiles are the ^{89}Zr radioactive signal. 2.5 min delay between UV and radioactive profiles is added on purpose in order to better present the profiles. **E**, Extensive biodistribution of radio-labeled and GW3965-loaded nanoparticles in relevant organs ($n = 5$ per nanoparticle). **F**, Representative flow cytometry histograms of relevant immune cells in different tissues. All error bars are SEM.

[0024] Supplementary Figure 5A-E. Efficacy of drug-loaded nanoparticles on aortic macrophages. **A**, Quantification of mRNA expression of the selected genes in atherosclerotic plaque macrophages. The relative expression of a gene is calculated by following these formulas: relative gene expression of gene X of Rx-HDL group = (expression of gene X in the Rx-HDL group / expression of gene X in the HDL group); relative gene expression of gene X of Rx-PLGA-HDL group = (expression of gene X in the Rx-PLGA-HDL group / expression of gene X in the PLGA-HDL group). **B**, Relative gene expression normalized to average expression both Rx-PLGA-HDL and Rx-HDL groups by following a formula: gene X expression in one group = (expression of gene X in the given group / average expression of gene X in both Rx-PLGA-HDL and Rx-HDL groups) **C**, Relative gene expression normalized to PBS group by following the following formula: gene X expression of a given group = (expression of gene X in the given group / expression of gene X in the PBS group). **D**, Representative CD68 immunostaining of aortic roots. CD68 was used as the macrophage marker in this experiment. Left panel is the original image and the right one is the mask generated by a MATLAB procedure. The dark pixels in the mask were calculated and used to calculate the CD68 positive area in the original immunostaining image. **E**, Quantification of macrophage levels in all treatment group. $n = 12$ per group. Non-parametric student's t -test was used to calculate statistics, and all error bars are SEM.

[0025] Supplementary Figure 6A-H. Cellular lipid measurement flow cytometry protocol and the efficacy evaluation of 6-week treatment. Aortic cells from one-year old C57BL/6 wild type (WT) mice ($n = 2$) and *Apoe*^{-/-} mice ($n = 2$) with 10 months of high-cholesterol diet were stained with BODIPY. Intracellular BODIPY signal was measured by a flow cytometry procedure used in the previous experiments (Figure 3A). **A**, Representative gating procedure shows the identification of CD45 negative (CD45⁻) non-immune cells, macrophages, and monocytes in the aortas. **B**, Representative BODIPY signal levels in non-immune cells (left), macrophages (middle), and monocytes (right) are shown in the histogram graphs. Grey histograms represent the non-BODIPY stained cells; green histograms represent cellular BODIPY levels of the cells from wild type mice; red histograms (Ctrl) represent cellular BODIPY levels of the cells from *Apoe*^{-/-} mice. Cells from *Apoe*^{-/-} mice show much higher

BODIPY levels than those from wild type mice, due to their higher cellular lipid levels than those in wild type mice. **C-E**, *Apoe*^{-/-} mice received oral treatment of either GW3965 (Oral Rx, n = 6) or PBS (Oral PBS, n = 5) twice per week for 6 weeks. Cellular lipid levels were calculated by multiplying the mean fluorescence intensity of a cell type with the number of the cells per aorta (AU = MFI X number of cells). **F-H**, Two batches of female *Apoe*^{-/-} mice with the same age and length of high-cholesterol diet were given either intravenous treatments (I.V. PBS or Rx-HDL, see Figure 5f-h) or oral treatments (Oral Rx or Oral PBS). Due to the batch and measurement difference, the cellular lipid levels of aortic cell populations treated with either Rx-HDL or Oral Rx were normalized to those from mice treated with either intravenous PBS or oral PBS, respectively. Error bars in all graphs are S.E.M. Statistics was calculated with non-parametric two-tailed Student's *t*-test.

[0026] Supplementary Figure 7A-L. A long-term Rx-HDL treatment does not cause toxic effects. Mice were treated with intravenous PBS (I.V. PBS, n = 10), intravenous Rx-HDL (Rx-HDL, n = 9), oral GW3965 (Oral Rx, n = 6), or oral PBS (Oral PBS, n = 5) two times per week for 6 weeks and key toxicity markers were measured. Blood cholesterol (**A**), triglyceride (**B**), and glucose (**C**) levels are metabolism markers. Gamma-glutamyl transaminase (**D**), alanine transaminase (**E**), and aspartate transaminase (**f**) and are liver damage markers. Creatine kinase (**G**) is a cardiac toxicity marker. Blood urea nitrogen (**H**) is a kidney damage marker. The red blood cell counts (**I**), hemoglobin levels (**J**), white blood cell counts (**K**), and the percentage of monocytes in blood white cells (**L**) are shown here too. No statistical significance was found in any measurements above. Non-parametric two-tailed student's *t*-test was used to calculate statistics between treatment and PBS control, and all error bars are Standard Deviation (SD).

[0027] Supplementary Figure 8. Table S1: Chemical composition of the nanoparticle library.

[0028] Supplementary Figure 9. Table S2: Blood half-lives of nanoparticle library.

DETAILED DESCRIPTION

[0029] Study design: The present inventors created a combinatorial library comprising 15 high-density lipoprotein-mimicking nanoparticles and two extensively studied nanoparticles, a PEGylated micellar and a long-circulating liposomal nanoparticle. The present inventors then combined *in vitro* assays and *in vivo* experiments in atherosclerotic *Apoe*^{-/-} mice to study the nanoparticle library's biological behavior by using near infrared fluorescence imaging (NIRF), flow cytometry, immunofluorescence, and radiolabeling. Based on the results of this library screening, two GW3965-loaded HDL nanoparticles were formulated with distinctly different immune cell specificity and organ distribution. The present inventors quantitatively studied the

pharmacokinetics, immune cell specificity, liver toxicity, and therapeutic effects of these drug-loaded nanoparticles in *Apoe*^{-/-} mice with advanced atherosclerosis. See Fig 1A.

[0030] *HDL nanoparticle library:* Previous studies indicate that the size, phospholipid composition, ratio of phospholipid to apolipoprotein A-1 (APOA1), and/or the inclusion of payloads can affect HDL-mimicking nanoparticles' *in vivo* performance(12-14). In the current study, the present inventors created a library containing HDL-mimicking nanoparticles that differ in size, shape, composition, and payload, all of which have been reported to affect nanoparticle's *in vivo* targeting efficiency(15). In order to fine-tune nanoparticle size and morphology, the present inventors added either triglyceride or polymers (poly-lactic-*co*-glycolic acid [PLGA] or polylactic acid [PLA]) to the HDL core (S Table 1). This inclusion facilitated modulation of nanoparticle size from about 10 nm (NP1, NP2, NP3, NP9) or 30 nm (NP6, NP7, NP10, and NP11) to over 100 nm (NP12) (Fig 1B and C). The present inventors observed that the inclusion of a core component, namely triglycerides or polymers, results in a nanoparticle shape change from discoidal to spherical, as can be clearly appreciated when comparing NP5 to NP7 and NP15. In addition to size and shape, the present inventors also varied phospholipid composition (S Table 1). Since oxidization greatly affects HDL function(16), the present inventors sought to test if this modification also changed the HDL-mimicking nanoparticles' drug delivery capability. The present inventors oxidized the phospholipids and APOA1 of NP1 to produce NP2 (S Fig 1B). The nanoparticle sizes were measured by dynamic light scattering, and their morphologies were visualized by transmission electron microscopy (TEM) (Fig 1B and C). Micellar(17) and liposomal(18) nanoparticles are established lipid-based platforms and served as references in this study.

[0031] *HDL nanoparticle library's cholesterol efflux efficiency:* Natural HDL facilitates reverse cholesterol transport, the intrinsic mechanism that removes cholesterol from macrophages in atherosclerotic plaques and protects against the atherosclerosis(19, 20). Cholesterol efflux capacity indicates the nanoparticles' biological similarity to native HDL. With this in mind, the present inventors measured the 17 nanoparticles' ability to induce cholesterol efflux from cholesterol-laden primary macrophages. The present inventors determined that 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)-based nanoparticles (NP8, 9, 10, and 11) are the most efficient nanoparticles tested for extracting cholesterol from macrophages. In contrast, polymer-core HDL nanoparticles produced the least cholesterol efflux (NP12, 13, 14, and 15). Liposomal and micellar nanoparticles, without APOA1 on their surface to bind the cholesterol efflux receptors Abca1 and Abcg1, performed similarly to polymer-core HDL nanoparticles (Fig 1D). These results demonstrate the essential role of APOA1, as well as the impact of phospholipid and core composition, in promoting cholesterol efflux. It has been

proposed that APOA1 changes conformation when interacting with its specific receptors to extract cholesterol(19). The artificial polymeric cores of NP12, NP13, NP14, and NP15 might limit conformational flexibility of APOA1, leading to impaired cholesterol efflux. On the other hand, POPC-based HDL nanoparticles display less rigidity, are more similar to natural HDL, and therefore result in more efficient cholesterol efflux.

[0032] *Nanoparticles' physiochemical properties affect their in vivo behavior:* The present inventors intravenously injected the library's DiR-labeled nanoparticles into atherosclerotic *Apoe*^{-/-} mice. NP1 and NP10, with diameters between 7 to 30 nm, exhibited the longest blood half-lives of 5.0 and 6.3 hours, respectively. NP12 and NP14, with diameters larger than 70 nm, had the shortest blood half-lives of 0.71 and 0.67 hour, respectively (Fig 2A). The difference between the longest and the shortest blood half-life was almost 10-fold (S Table 2).

[0033] Using near infrared fluorescence imaging (NIRF), the present inventors investigated nanoparticle accumulation in the heart, aorta, lung, liver, spleen, kidney, brain, and muscle 24 hours after intravenous administration (Fig 2B and S Fig 2A). Among all nanoparticles, liver accumulation was generally the highest, followed by spleen, kidney, and lung accumulation (S Fig 2B). Since the aorta is the primary target tissue while the liver and spleen are clearance organs, accumulation in the aorta was assessed relative to these two organs(21). These measurements showed a 3.4-fold difference between the highest and lowest aorta-to-liver accumulation ratios (NP5 vs NP12, $p < 0.01$, S Fig 2D), and a 4.7-fold difference between the aorta-to-spleen accumulation ratios (NP5 vs NP12, $p < 0.01$, S Fig 2E). For the nanoparticles' relative performance, see Fig 2B. The distribution of DiR-labeled nanoparticles in certain tissues is difficult to quantify *in vivo* due to the limited penetrating depth of light and varying absorbance rates among tissues (22). Radiolabeled nanoparticles' biodistribution and pharmacokinetics can, however, be quantitatively measured. Based on the initial optical imaging screen, the present inventors selected NP10, NP14, NP15, and NP17 to be labeled with ⁸⁹Zr through the hydrophobic chelator DFO-C34, which serves as a surrogate for hydrophobic payloads. In line with the optical imaging results, the smaller nanoparticles (NP10 and NP15) exhibited longer blood half-lives than the larger ones (NP14 and NP17, Fig 2C). The present inventors also observed that the HDL-based nanoparticles (NP10, NP14, and NP15), particularly NP10, accumulated more efficiently in atherosclerotic plaques than the liposomal nanoparticle (Fig 2D and E). Overall, NP10 displayed the most favorable performance among the 4 selected nanoparticles (Fig 2C-F).

[0034] *Distinct immune cell targeting patterns within the nanoparticle library:* Macrophages and monocytes are the key immune cells that drive atherosclerosis progression(4). In *Apoe*^{-/-} atherosclerotic mice, these cells mainly reside in atherosclerotic plaques, spleen, blood,

and bone marrow(4). Using a robust flow cytometry procedure adapted from previous studies(23, 24), the present inventors were able to identify macrophages, monocytes, and non-myeloid immune cells (Lin^+) in the aortas; macrophages, Ly-6C^{hi} monocytes, dendritic cells (DCs), and neutrophils in the spleens; and Ly-6C^{hi} monocytes, Ly-6C^{lo} monocytes, DCs, and neutrophils in the blood (Fig 3A).

[0035] In the aortas, all HDL-mimicking nanoparticles efficiently targeted macrophages and monocytes. The difference between the highest and lowest nanoparticle accumulations was 5.7-fold in macrophages (NP3 vs NP17, $p < 0.01$) and 2.7-fold in monocytes (NP3 vs NP7, $p < 0.001$; Fig 3B). In the spleens, all nanoparticles had the highest accumulation in macrophages, which do the bulk of nanoparticle clearance, with a 3.8-fold difference between the highest and lowest accumulation levels (NP12 vs NP17, $p < 0.01$, Fig 3B). In the blood, dendritic cells (DCs) and Ly-6C^{hi} monocytes displayed the highest nanoparticle association, with a 3.8-fold difference between the highest and lowest association levels (NP10 vs NP5, $p < 0.01$) in DCs and 3.79-fold difference in Ly-6C^{hi} monocytes (NP3 vs NP14, $p < 0.0001$, Fig 3B). In addition, the nanoparticles were far less effective in targeting Ly-6C^{lo} monocytes, the patrolling monocytes in the blood, when compared to Ly-6C^{hi} monocytes (Fig 3B).

[0036] While aortic macrophages are the main target of immunomodulatory nanoparticles, splenic macrophages, which clear nanoparticles from the blood and reduce their bioavailability to aortic macrophages(25), need to be avoided. The present inventors evaluated the ratios of nanoparticle accumulation in aortic macrophages versus splenic macrophages and found a 3.8-fold difference between the highest and lowest aortic-to-splenic ratios (NP16 vs NP12, $p < 0.0001$, S Fig 3A). This differential targeting specificity to atherosclerotic macrophages was confirmed by immunofluorescence in the aortic roots (S Fig 3B). Altogether, the flow cytometry data reveal that the distinct physiochemical nanoparticle properties within the library lead to drastically different immune cell targeting patterns (Fig 3C).

[0037] *GW3965-loaded nanoparticle development:* Liver X receptor (LXR) agonists promote cholesterol efflux from macrophages in atherosclerotic plaques have been proposed as novel immunomodulatory drugs for the disease(26). However, most experimental LXR agonists fail clinical translation or early-stage clinical trials due to poor safety profiles. For example, GW3965, an effective LXR agonist promoting cholesterol efflux from atherosclerotic macrophages(27, 28), did not reach the clinical phase due to its liver toxicity in hamsters and monkeys(10), as well as for human hepatocytes(11).

[0038] In the present nanoparticle library studies, NP10 was found to have high cholesterol efflux promotion efficiency, a long blood half-life, high relative aorta-to-liver accumulation, and a high relative aortic-to-splenic macrophage association ratio (S Fig 4A).

These features make NP10 a promising candidate for avoiding GW3965's liver toxicity and enhancing its efficacy on atherosclerotic plaque macrophages. Therefore, the present inventors replaced NP10's hydrophobic triglyceride cargo with hydrophobic GW3965 and created a GW3965-loaded nanoparticle (Rx-HDL) that was morphologically similar, but not identical due to the different nanoparticle composition, to NP10 in size (~30 nm), phospholipid composition (POPC-dominant), and morphology (S Fig 4B). Further, NP14 was identified as an unfavorable nanoparticle for GW3965 delivery due to the nanoparticle's poor cholesterol efflux efficiency, short blood half-life, and low relative aorta-to-liver accumulation (S Fig 4A). By loading GW3965 into the PLGA matrix of NP14, a PLGA-core GW3965-loaded nanoparticle was created (Rx-PLGA-HDL) with similar size, phospholipid composition, and morphology to NP14 (S Fig 4B). Notably, the size and cholesterol efflux capability of the two drug-loaded nanoparticles were drastically different (S Fig 4B and C).

[0039] *Quantitative evaluation of GW3965-loaded nanoparticles:* ⁸⁹Zr-labeled nanoparticles can be quantitatively characterized by *in vivo* positron emission tomography (PET) imaging as well as by *ex vivo* radioactivity counting (29). To accurately understand the *in vivo* performance of the two nanoparticles, the present inventors loaded the hydrophobic ⁸⁹Zr-DFO-C34 into Rx-HDL and Rx-PLGA-HDL (Fig 4A) and radioactive high-performance chromatography demonstrate that both Rx-HDL and Rx-PLGA-HDL were efficiently radiolabeled (S Fig 4D).

[0040] In *Apoe*^{-/-} atherosclerotic mice, Rx-HDL circulated in the blood much longer (weighted $t_{1/2}$ = 10.5 hour, n = 3) than Rx-PLGA-HDL (weighted $t_{1/2}$ = 5.0 hour, n = 3, Fig 4B) or its precursor NP10 ($t_{1/2}$ = 7.0 hour, Fig 2C), demonstrating its favorable features. The present inventors then used PET-computed tomography hybrid imaging (PET-CT) to measure the nanoparticle dynamic accumulation in the cardiac blood pool, the liver, and the spleen 30 minutes and 24 hours after intravenous administration (n = 5 per nanoparticle, Fig 4C). After 30 minutes, Rx-HDL had higher accumulation in the cardiac blood pool (29.9 vs 24.5 Max %ID/g, p < 0.05) but lower in the liver than Rx-PLGA-HDL (21.7 vs 29.7 Max %ID/g, p < 0.05). After 24 hours, Rx-HDL liver accumulation was still 36% lower than Rx-PLGA-HDL (26.5 vs 41.7 Max %ID/g, p < 0.05, Fig 4C and D). Autoradiography revealed that both nanoparticles displayed patchy aorta accumulation, in accordance with the heterogeneous distribution of atherosclerotic plaques in this tissue(30) (Fig 4E). Additionally, autoradiography confirmed the highest nanoparticle accumulation to be in the liver and spleen (Fig 4E), a result that was corroborated by an extensive biodistribution analysis (S Fig 4E).

[0041] Having labeled both nanoparticles with DiR (Fig 4A), the present inventors used their flow cytometry protocol (see Fig 3A) to quantify immune cell targeting specificity (S Fig

4F). Rx-HDL predominantly targeted macrophages in the aorta and its accumulation was 2-fold higher than Rx-PLGA-HDL ($p < 0.01$). In the spleen, Rx-HDL had 33% less accumulation in splenic macrophages than Rx-PLGA-HDL ($p < 0.05$). In the blood, Rx-HDL targeted Ly-6C^{hi} monocytes 2.4-fold more efficiently than Rx-PLGA-HDL ($p < 0.01$, Fig 4F and G). Collectively, these data show that, compared to Rx-PLGA-HDL, Rx-HDL has a longer blood half-life, lower accumulation in the liver, higher accumulation in atherosclerotic plaque macrophages, and lower accumulation in splenic macrophages.

[0042] *Nanoparticle abolishes liver toxicity and preserves efficacy of GW3965:* To test if Rx-HDL's optimal *in vivo* performance reduced GW3965's liver toxicity, the present inventors gave 4 intravenous injections (1 injection every 2 days, at a dose of 10 mg/kg GW3865) of Rx-HDL, its vehicle control (HDL), Rx-PLGA-HDL, its vehicle control (PLGA-HDL), or PBS to *Apoe*^{-/-} atherosclerotic mice ($n = 12$ per group).

[0043] In the liver, Rx-PLGA-HDL increased the expression of two of the three major GW3965-related toxicity genes, whereas Rx-HDL increased the expression of one gene (Figure 5A). Of note, the present inventors also measured hepatic triglyceride and cholesterol levels, which are the major biomarkers of GW3965-induced hepatic steatosis(10). The Rx-PLGA-HDL group had 35.4% more hepatic triglyceride than its control vehicle PLGA-HDL group ($p = 0.014$) and 21% more than the PBS group ($p = 0.12$). For hepatic cholesterol, the Rx-PLGA-HDL group had 31% higher levels than the PLGA-HDL group ($p = 0.018$) and 17% higher levels than the PBS group ($p = 0.014$). These results suggest that the high liver accumulation of Rx-PLGA-HDL caused severe liver toxicity (Fig 5B and C). On the other hand, the Rx-HDL group had 26.5% lower hepatic triglyceride levels than its vehicle HDL control group ($p = 0.06$) and 22.7% lower levels than the PBS group ($p = 0.076$). The Rx-HDL group also had 20% lower hepatic cholesterol levels than the HDL group ($p = 0.06$) and 33.3% lower levels than the PBS group ($p = 0.00043$). A recent study suggested that high HDL levels are associated with a lower degree of steatosis, which might explain the reduced hepatic triglyceride and cholesterol levels in mice treated with vehicle HDL nanoparticles(31). Furthermore, GW3965 has been reported to increase HDL levels in mice(32), likely explaining the additional hepatic benefits in Rx-HDL treated mice. Most importantly, compared to the Rx-PLGA-HDL group, the Rx-HDL group had 36.1% lower hepatic triglyceride ($p = 0.00083$) and 43.0% lower hepatic cholesterol levels ($p < 0.0001$). These results demonstrate that the two GW3965-loaded nanoparticles' distinct liver accumulations resulted in differential liver toxicity profiles (Fig 5A-C).

[0044] To measure the treatments' efficacies on aortic macrophages, cells were isolated from aortic roots by laser capture microdissection and their mRNA expression levels were measured by qPCR. The present inventors found that Rx-HDL increased expression of five

GW3965 target genes when compared to the vehicle HDL control, whereas Rx-PLGA-HDL increased only four genes when compared to the vehicle PLGA-HDL control (Fig 5D).

Furthermore, Rx-HDL produced elevated expression of the five target genes compared to Rx-PLGA-HDL (S Fig 5A and B). These results suggest successful GW3965 delivery to macrophages in atherosclerotic plaques. Clear effects of either nanoparticle on genes related to macrophage inflammation were not observed, however, since the number of genes with increased expression was almost equal to the number with decreased expression (Fig 5E, S Fig 5A and B). Similarly, the macrophage levels in aortic roots were the same among all groups (S Fig 5D and E).

[0045] Based on the favorable properties of Rx-HDL, its therapeutic effects were further evaluated in a six-week, long-term treatment regimen focusing on cellular lipid levels in aortas. Working from a BODIPY-based flow cytometry protocol(33), the present inventors developed a procedure to quantify the cellular lipid levels of macrophages, monocytes, and CD45 negative non-immune cells (CD45⁻) in the aortas (S Fig 6). Importantly, the present inventors found the 6-week treatment of Rx-HDL (10 mg/kg GW3965, 2 intravenous injections per week) resulted in 28% less total lipid in aortic macrophages ($p = 0.224$; Fig 5F). Further, the treatment reduced total lipid levels in monocytes by 43.0% ($p = 0.011$, Fig 5G) and in all CD45⁻ non-immune cells by 40.0% ($p = 0.021$, Fig 5H). Furthermore, no therapeutic effects were observed when the compound was given orally with the same 6-week treatment regimen (10 mg/kg GW3965, 2 gavages per week) when compared to placebo-treated group (S Fig 6C-H). Consistent with the liver toxicity results after the short-term Rx-HDL treatment, blood cholesterol and triglyceride levels were similar to those in mice receiving PBS treatment (S Fig 7A and B). In addition, the long-term treatment of Rx-HDL did not cause any observable toxicity in the liver, kidney, heart, and blood cells (S Fig 7C-L). Taken together, the results show that long-term Rx-HDL treatment produces significant therapeutic benefits without causing toxicity in major organs, most notably the liver, whereas long-term oral treatment at the same dose did not produce any therapeutic benefits.

[0046] In summary, the present inventors developed a rational library screen strategy to identify nanoparticles with favorable immune cell specificity and biodistribution in an atherosclerosis mouse model. On the basis of this nanoparticle screen, the present inventors optimized GW3965 delivery to plaque macrophages and, while preserving its efficacy on atherosclerotic plaques (Figure 5D-H), abolished GW3965 liver toxicity (Figure 5A-C; S Fig 7), a well-known adverse effect of LXR agonists.

[0047] Without wishing to be bound by theory, the present inventors postulate that NPs comprising both POPC and PHPC benefit from the ability of these molecules in combination to

form a monolayer of lipids with proper curvature that creates sufficient space inside the nanoparticle to hold inert hydrophobic filler or therapeutic compounds. The loosely packed POPC/PHPC monolayer may also lead to better compatibility with small molecules inside the core of nanoparticle.

[0048] With respect to hydrophobic core components, for very hydrophobic compounds (Log P > 3.8), incorporation into nanoparticles such as, for example, NP10, can be achieved without further modifications of the very hydrophobic compound. For other less hydrophobic compounds, they may need to be conjugated to a polymer (e.g., PLGA) and then loaded into nanoparticles as a drug-PLGA complex.

[0049] The size and circulating time of the nanoparticles can be modulated by controlling the ratio of lipids-to-APOA1 and the ratio of lipids-to-polymer.

[0050] To target immune cells in tissue with limited access to circulation, nanoparticles having long blood half-lives and small size (<50 nm) may be used to optimal effect. These scenarios include atherosclerotic plaques, myocardial infarcts, lymph nodes, solid tumors, and joints involved in rheumatoid arthritis.

[0051] To target immune cells in well-perfused tissues, nanoparticles having short blood half-lives and large size (about 100 nm) may be used to optimal effect. These tissues include spleen, liver, kidney, lungs, and bone marrow.

[0052] A target objective achieved after administration of nanoparticles described herein to a subject in need thereof is the reduction of cellular lipid levels in the subject. It is envisioned that intravenous nanoparticle treatment, for example, can be added to the current standard-of-care therapies that are administered to, for example, patients right after myocardial infarction. In that these patients have a high risk of recurrent coronary events, lowering their lipid levels quickly by, for example, intravenous dosing of nanoparticles described herein would reduce the lipid-driven inflammation of high-risk coronary arterial plaques, which in turn would reduce the risk of recurrence of coronary/vascular events in a patient immediately after a primary heart attack.

Definitions

[0053] When describing the compounds, pharmaceutical compositions containing such compounds and methods of using such compounds and compositions, the following terms have the following meanings unless otherwise indicated. It should also be understood that any of the moieties defined forth below may be substituted with a variety of substituents, and that the respective definitions are intended to include such substituted moieties within their scope. It should be further understood that the terms “groups” and “radicals” can be considered interchangeable when used herein.

[0054] “Pharmaceutically acceptable” means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopoeia or other generally recognized pharmacopoeia for use in animals, and more particularly in humans.

[0055] “Pharmaceutically acceptable salt” refers to a salt of a compound of the invention that is pharmaceutically acceptable and that possesses the desired pharmacological activity of the parent compound. Such salts include: (1) acid addition salts, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or formed with organic acids such as acetic acid, propionic acid, hexanoic acid, cyclopentanepropionic acid, glycolic acid, pyruvic acid, lactic acid, malonic acid, succinic acid, malic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, 3-(4-hydroxybenzoyl) benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, 1,2-ethane-disulfonic acid, 2-hydroxyethanesulfonic acid, benzenesulfonic acid, 4-chlorobenzenesulfonic acid, 2-naphthalenesulfonic acid, 4-toluenesulfonic acid, camphorsulfonic acid, 4-methylbicyclo[2.2.2]-oct-2-ene-1-carboxylic acid, glucoheptonic acid, 3-phenylpropionic acid, trimethylacetic acid, tertiary butylacetic acid, lauryl sulfuric acid, gluconic acid, glutamic acid, hydroxynaphthoic acid, salicylic acid, stearic acid, muconic acid, and the like; or (2) salts formed when an acidic proton present in the parent compound either is replaced by a metal ion, *e.g.*, an alkali metal ion, an alkaline earth ion, or an aluminum ion; or coordinates with an organic base such as ethanolamine, diethanolamine, triethanolamine, N-methylglucamine and the like. Salts further include, by way of example only, sodium, potassium, calcium, magnesium, ammonium, tetraalkylammonium, and the like; and when the compound contains a basic functionality, salts of non toxic organic or inorganic acids, such as hydrochloride, hydrobromide, tartrate, mesylate, acetate, maleate, oxalate and the like. The term “pharmaceutically acceptable cation” refers to a non toxic, acceptable cationic counter-ion of an acidic functional group. Such cations are exemplified by sodium, potassium, calcium, magnesium, ammonium, tetraalkylammonium cations, and the like.

[0056] “Pharmaceutically acceptable vehicle” or “Pharmaceutically acceptable carrier” refer to a pharmaceutically acceptable diluent, a pharmaceutically acceptable adjuvant, a pharmaceutically acceptable excipient, or a combination of any of the foregoing with which a composition provided by the present disclosure may be administered to a patient and which does not destroy the pharmacological activity thereof and which is non-toxic when administered in doses sufficient to provide a therapeutically effective amount of the composition. In addition to the adjuvants, excipients and diluents known to one skilled in the art, the vehicle or carrier includes nanoparticles of organic and inorganic nature.

[0057] “Preventing” or “prevention” refers to a reduction in risk of acquiring a disease or disorder (i.e., causing at least one of the clinical symptoms of the disease not to develop in a subject that may be exposed to or predisposed to the disease but does not yet experience or display symptoms of the disease).

[0058] “Prodrugs” refers to compounds, including derivatives of the compounds of the invention, which have cleavable groups and become by solvolysis or under physiological conditions the compounds of the invention which are pharmaceutically active *in vivo*. Such examples include, but are not limited to, choline ester derivatives and the like, N-alkylmorpholine esters and the like.

[0059] “Solvate” refers to forms of the compound that are associated with a solvent, usually by a solvolysis reaction. Conventional solvents include water, ethanol, acetic acid and the like. The compounds of the invention may be prepared *e.g.* in crystalline form and may be solvated or hydrated. Suitable solvates include pharmaceutically acceptable solvates, such as hydrates, and further include both stoichiometric solvates and non-stoichiometric solvates.

[0060] “Subject” includes all mammals and more particularly includes humans. The terms “human,” “patient” and “subject” may be used interchangeably herein.

[0061] “Therapeutically effective amount” means the amount of a compound that, when administered to a subject for treating a disease, is sufficient to effect such treatment for the disease. The “therapeutically effective amount” can vary depending on the compound, the disease and its severity, and the age, weight, etc., of the subject to be treated.

[0062] “Treating” or “treatment” of any disease or disorder refers, in one embodiment, to ameliorating the disease or disorder (*i.e.*, arresting or reducing the development of the disease or at least one of the clinical symptoms thereof). In another embodiment “treating” or “treatment” refers to ameliorating at least one physical parameter, which may not be discernible by the subject. In yet another embodiment, “treating” or “treatment” refers to modulating the disease or disorder, either physically, (*e.g.*, stabilization of a discernible symptom), physiologically, (*e.g.*, stabilization of a physical parameter), or both.

[0063] As used herein, the term “operably linked” refers to a regulatory sequence capable of mediating the expression of a coding sequence and which is placed in a DNA molecule (*e.g.*, an expression vector) in an appropriate position relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of coding sequences and transcription control elements (*e.g.* promoters, enhancers, and termination elements) in an expression vector. This definition is also sometimes applied to the arrangement of nucleic acid sequences of a first and a second nucleic acid molecule wherein a hybrid nucleic acid molecule is generated.

[0064] A "vector" is a replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element.

[0065] An "expression vector" or "expression operon" refers to a nucleic acid segment that may possess transcriptional and translational control sequences, such as promoters, enhancers, translational start signals (e.g., ATG or AUG codons), polyadenylation signals, terminators, and the like, and which facilitate the expression of a polypeptide coding sequence in a host cell or organism.

[0066] The terms "transform", "transfect", or "transduce", shall refer to any method or means by which a nucleic acid is introduced into a cell or host organism and may be used interchangeably to convey the same meaning. Such methods include, but are not limited to, transfection, electroporation, microinjection, PEG-fusion and the like.

[0067] The introduced nucleic acid may or may not be integrated (covalently linked) into nucleic acid of the recipient cell or organism. In bacterial, yeast, plant and mammalian cells, for example, the introduced nucleic acid may be maintained as an episomal element or independent replicon such as a plasmid. Alternatively, the introduced nucleic acid may become integrated into the nucleic acid of the recipient cell or organism and be stably maintained in that cell or organism and further passed on or inherited to progeny cells or organisms of the recipient cell or organism. In other applications, the introduced nucleic acid may exist in the recipient cell or host organism only transiently.

[0068] The phrase "consisting essentially of" when referring to a particular nucleotide or amino acid means a sequence having the properties of a given SEQ ID NO:. For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the basic and novel characteristics of the sequence.

[0069] Other derivatives of the compounds of this invention have activity in both their acid and acid derivative forms, but in the acid sensitive form often offers advantages of solubility, tissue compatibility, or delayed release in the mammalian organism (see, Bundgard, H., Design of Prodrugs, pp. 7-9, 21-24, Elsevier, Amsterdam 1985). Prodrugs include acid derivatives well known to practitioners of the art, such as, for example, esters prepared by reaction of the parent acid with a suitable alcohol, or amides prepared by reaction of the parent acid compound with a substituted or unsubstituted amine, or acid anhydrides, or mixed anhydrides. Simple aliphatic or aromatic esters, amides and anhydrides derived from acidic groups pendant on the compounds of this invention are preferred prodrugs. In some cases it is desirable to prepare double ester type prodrugs such as (acyloxy)alkyl esters or

((alkoxycarbonyl)oxy)alkylesters. Preferred are the C₁ to C₈ alkyl, C₂-C₈ alkenyl, aryl, C₇-C₁₂ substituted aryl, and C₇-C₁₂ arylalkyl esters of the compounds of the invention.

[0070] As used herein, the term “isotopic variant” refers to a compound that contains unnatural proportions of isotopes at one or more of the atoms that constitute such compound. For example, an “isotopic variant” of a compound can contain one or more non-radioactive isotopes, such as for example, deuterium (²H or D), carbon-13 (¹³C), nitrogen-15 (¹⁵N), or the like. It will be understood that, in a compound where such isotopic substitution is made, the following atoms, where present, may vary, so that for example, any hydrogen may be ²H/D, any carbon may be ¹³C, or any nitrogen may be ¹⁵N, and that the presence and placement of such atoms may be determined within the skill of the art. Likewise, the invention may include the preparation of isotopic variants with radioisotopes, in the instance for example, where the resulting compounds may be used for drug and/or substrate tissue distribution studies. The radioactive isotopes tritium, *i.e.* ³H, and carbon-14, *i.e.* ¹⁴C, are particularly useful for this purpose in view of their ease of incorporation and ready means of detection. Further, compounds may be prepared that are substituted with positron emitting isotopes, such as ¹¹C, ¹⁸F, ¹⁵O and ¹³N, and would be useful in Positron Emission Topography (PET) studies for examining substrate receptor occupancy.

[0071] All isotopic variants of the compounds provided herein, radioactive or not, are intended to be encompassed within the scope of the invention.

[0072] It is also to be understood that compounds that have the same molecular formula but differ in the nature or sequence of bonding of their atoms or the arrangement of their atoms in space are termed “isomers”. Isomers that differ in the arrangement of their atoms in space are termed “stereoisomers”.

[0073] Stereoisomers that are not mirror images of one another are termed “diastereomers” and those that are non-superimposable mirror images of each other are termed “enantiomers”. When a compound has an asymmetric center, for example, it is bonded to four different groups, a pair of enantiomers is possible. An enantiomer can be characterized by the absolute configuration of its asymmetric center and is described by the R- and S-sequencing rules of Cahn and Prelog, or by the manner in which the molecule rotates the plane of polarized light and designated as dextrorotatory or levorotatory (*i.e.*, as (+) or (-)-isomers respectively). A chiral compound can exist as either individual enantiomer or as a mixture thereof. A mixture containing equal proportions of the enantiomers is called a “racemic mixture”.

[0074] “Tautomers” refer to compounds that are interchangeable forms of a particular compound structure, and that vary in the displacement of hydrogen atoms and electrons. Thus, two structures may be in equilibrium through the movement of π electrons and an atom (usually

H). For example, enols and ketones are tautomers because they are rapidly interconverted by treatment with either acid or base. Another example of tautomerism is the aci- and nitro- forms of phenylnitromethane, that are likewise formed by treatment with acid or base.

[0075] Tautomeric forms may be relevant to the attainment of the optimal chemical reactivity and biological activity of a compound of interest.

[0076] Therapeutic agents described herein may possess one or more asymmetric centers; such compounds can therefore be produced as individual (R)- or (S)- stereoisomers or as mixtures thereof. Unless indicated otherwise, the description or naming of a particular compound in the specification and claims is intended to include both individual enantiomers and mixtures, racemic or otherwise, thereof. The methods for the determination of stereochemistry and the separation of stereoisomers are well-known in the art.

TREATMENT METHODS

[0077] In one embodiment, the conditions associated with macrophage-driven inflammation include, without limitation, atherosclerosis, diabetes, cancer, and respiratory diseases, such as, for example, asthma.

[0078] In one embodiment, with respect to the method, the compound is any one of the compounds listed in Figure 7 (Table 1).

[0079] In a further aspect, this invention provides a method of treating a mammal susceptible to or afflicted with a condition from among those listed herein, and particularly, such condition as may be associated with macrophage-driven inflammation. Such conditions include, without limitation, the disease or condition is selected from atherosclerosis, diabetes and its complications, peripheral vascular disease and associated complications, cancers, arthritis, acute and chronic inflammation, cardiovascular disease, tumor invasion and metastases, cardio- and cerebrovascular ischemia/reperfusion injury, heart attack, stroke, myocardial infarction, and ischemic cardiomyopathy.

[0080] In one embodiment, with respect to the method of treatment, the disease or condition is a diabetes associated complication.

[0081] In one embodiment, with respect to the method of treatment, the disease or condition is atherosclerosis.

[0082] In one embodiment, with respect to the method of treatment, the disease or condition is arthritis.

[0083] In certain aspects, the present invention provides prodrugs and derivatives of the compounds according to the formulae above. Prodrugs are derivatives of the compounds of the invention, which have metabolically cleavable groups and become by solvolysis or under physiological conditions the compounds of the invention, which are pharmaceutically active, *in*

vivo. Such examples include, but are not limited to, choline ester derivatives and the like, N-alkylmorpholine esters and the like.

[0084] Other derivatives of the compounds of this invention have activity in both their acid and acid derivative forms, but the acid sensitive form often offers advantages of solubility, tissue compatibility, or delayed release in the mammalian organism (see, Bundgard, H., Design of Prodrugs, pp. 7-9, 21-24, Elsevier, Amsterdam 1985). Prodrugs include acid derivatives well known to practitioners of the art, such as, for example, esters prepared by reaction of the parent acid with a suitable alcohol, or amides prepared by reaction of the parent acid compound with a substituted or unsubstituted amine, or acid anhydrides, or mixed anhydrides. Simple aliphatic or aromatic esters, amides and anhydrides derived from acidic groups pendant on the compounds of this invention are preferred prodrugs. In some cases it is desirable to prepare double ester type prodrugs such as (acyloxy)alkyl esters or ((alkoxycarbonyl)oxy)alkylesters. Preferred are the C₁ to C₈ alkyl, C₂-C₈ alkenyl, aryl, C₇-C₁₂ substituted aryl, and C₇-C₁₂ arylalkyl esters of the compounds of the invention.

PHARMACEUTICAL COMPOSITIONS

[0085] When employed as pharmaceuticals, the compounds of this invention are typically administered in the form of a pharmaceutical composition. Such compositions can be prepared in a manner well known in the pharmaceutical art and comprise at least one active compound.

[0086] Generally, the compounds of this invention are administered in a pharmaceutically effective amount. The amount of the compound actually administered will typically be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual compound -administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the like.

[0087] The pharmaceutical compositions of this invention can be administered by a variety of routes including oral, rectal, intraocular, transdermal, subcutaneous, intravenous, intramuscular, intraperitoneal, intradermal, directly into cerebrospinal fluid, intratracheal, and intranasal. Depending on the intended route of delivery, the compounds of this invention are preferably formulated as either injectable or oral compositions or as salves, as lotions or as patches all for transdermal administration.

[0088] The compositions for oral administration can take the form of bulk liquid solutions or suspensions, or bulk powders. More commonly, however, the compositions are presented in unit dosage forms to facilitate accurate dosing. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of active material calculated to produce the desired

therapeutic effect, in association with a suitable pharmaceutical excipient. Typical unit dosage forms include prefilled, premeasured ampules or syringes of the liquid compositions or pills, tablets, capsules or the like in the case of solid compositions. In such compositions, a compound as described herein is usually a minor component (from about 0.1 to about 50% by weight or preferably from about 1 to about 40% by weight) with the remainder being various vehicles or carriers and processing aids helpful for forming the desired dosing form.

[0089] Liquid forms suitable for oral administration may include a suitable aqueous or nonaqueous vehicle with buffers, suspending and dispensing agents, colorants, flavors and the like. Solid forms may include, for example, any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

[0090] Injectable compositions are typically based upon injectable sterile saline or phosphate-buffered saline or other injectable carriers known in the art. As before, the active compound in such compositions is typically a minor component, often being from about 0.05 to 10% by weight with the remainder being the injectable carrier and the like.

[0091] Transdermal compositions are typically formulated as a topical ointment or cream containing the active ingredient(s), generally in an amount ranging from about 0.01 to about 20% by weight, preferably from about 0.1 to about 20% by weight, preferably from about 0.1 to about 10% by weight, and more preferably from about 0.5 to about 15% by weight. When formulated as an ointment, the active ingredients will typically be combined with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredients may be formulated in a cream with, for example an oil-in-water cream base. Such transdermal formulations are well-known in the art and generally include additional ingredients to enhance the dermal penetration or stability of the active ingredients or the formulation. All such known transdermal formulations and ingredients are included within the scope of this invention.

[0092] Nanoparticles described herein can also be administered by a transdermal device. Accordingly, transdermal administration can be accomplished using a patch either of the reservoir or porous membrane type, or of a solid matrix variety.

[0093] The above-described components for orally administrable, injectable or topically administrable compositions are merely representative. Other materials as well as processing techniques and the like are set forth in Part 8 of Remington's Pharmaceutical Sciences, 17th

edition, 1985, Mack Publishing Company, Easton, Pennsylvania, which is incorporated herein by reference.

[0094] Nanoparticles described herein can also be administered locally to the eye for the treatment of diabetic neuropathy. Suitable compositions include those administrable by eye drops, injections or the like. In the case of eye drops, the composition can also optionally include, for example, ophthalmologically compatible agents such as isotonicizing agents, buffering agents, surfactants, stabilization agents, and other ingredients. For injection, nanoparticles described herein can be provided in an injection grade saline solution, in the form of an injectable liposome solution, slow-release polymer system or the like.

[0095] Nanoparticles described herein can also be administered in sustained release forms or from sustained release drug delivery systems. A description of representative sustained release materials can be found in Remington's Pharmaceutical Sciences.

[0096] The following formulation examples illustrate representative pharmaceutical compositions of this invention. The present invention, however, is not limited to the following pharmaceutical compositions.

Formulation 1 - Tablets

[0097] Nanoparticles described herein may be admixed as a dry powder with a dry gelatin binder in an approximate 1:2 weight ratio. A minor amount of magnesium stearate is added as a lubricant. The mixture is formed into 240-270 mg tablets (80-90 mg of active amide compound per tablet) in a tablet press.

Formulation 2 - Capsules

[0098] Nanoparticles described herein may be admixed as a dry powder with a starch diluent in an approximate 1:1 weight ratio. The mixture is filled into 250 mg capsules (125 mg of active amide compound per capsule).

Formulation 3 - Liquid

[0099] Nanoparticles described herein (125 mg), sucrose (1.75 g) and xanthan gum (4 mg) are blended, passed through a No. 10 mesh U.S. sieve, and then mixed with a previously made solution of microcrystalline cellulose and sodium carboxymethyl cellulose (11:89, 50 mg) in water. Sodium benzoate (10 mg), flavor, and color are diluted with water and added with stirring. Sufficient water is then added to produce a total volume of 5 mL.

Formulation 4 - Tablets

[00100] Nanoparticles described herein may be admixed as a dry powder with a dry gelatin binder in an approximate 1:2 weight ratio. A minor amount of magnesium stearate is added as a lubricant. The mixture is formed into 450-900 mg tablets (150-300 mg of active amide compound) in a tablet press.

Formulation 5 - Injection

[00101] Nanoparticles described herein may be dissolved or suspended in a buffered sterile saline injectable aqueous medium to a concentration of approximately 5 mg/ml.

Formulation 6 - Topical

[00102] Stearyl alcohol (250 g) and a white petrolatum (250 g) are melted at about 75°C and then a mixture of nanoparticles described herein (50 g), methylparaben (0.25 g), propylparaben (0.15 g), sodium lauryl sulfate (10 g), and propylene glycol (120 g) dissolved in water (about 370 g) is added and the resulting mixture is stirred until it congeals.

METHODS OF TREATMENT

[00103] Types 1 and 2 diabetes are on the rise in the United States and world-wide. The long-term consequences of diabetes ensue from the direct and indirect effects of hyperglycemia. Diabetes attacks the macro- and microvasculature and is well-established as a leading cause of heart attacks and stroke, blindness, renal failure, amputations, and peripheral neuropathies. Despite significant advances in the treatment of hyperglycemia, definitive means to prevent the complications of diabetes are not yet on the immediate horizon. Indeed, rigorous control of hyperglycemia, particularly in older individuals, may be fraught with significant sequelae, such as striking hypoglycemia, seizures, cardiac ischemia and death.

[00104] Diabetes and Diabetes Complications

[00105] Further to the above, the nanoparticles described herein are useful for managing and/or treating complications associated with diabetes.

[00106] Suitable animal models in which to study diabetes complications are known in the art and are described in, for example, Manigrasso et al. (2014, Trends in Endocrin Metab 25:15-22); Stirban et al. (2014, Molecular Metabolism 3:94-108); Johnson et al. (2014, EJNMMI Res 4:26); Tekabe et al. (2014, Int J Mol Imaging Article Id 695391); Kaida et al. (2013, Diabetes 62:3241-3250); Tekabe et al. (2013, EJNMMi Res 3:37); Calcutt et al. (2009, Nat Rev Drug Discov 8:417-429); Dauch et al. (2013, J Neuroinflammation 10:64); Juranek et al. (2013, Diabetes 62:931-943); Singh et al. (2014, Korean J Physiol Pharmacol 18:1-14); Ramasamy et al. (2012, Vascular Pharmacol 57: 160-167); Montagnani (2008, Br J Pharmacol 154:725-726); Nakamura et al. (1993, Am J Pathol 143:1649-1656); Lin et al. (2003, Atherosclerosis 168:213-220); Hofmann et al. (2002, Diabetes 51:2082-2089); Lin et al. (2002, Atherosclerosis 163:303-311); Vlassara et al. (1992, Proc Natl Acad Sci 89:12043-12047); Brownlee et al. (1986, Science 232:1629-1632); Li et al. (1996, Proc Natl Acad Sci 93:3902-3907); Park et al. (1998, Nature Med 4:1025-1031); Kislinger et al. (2001, Arteriosclerosis, Thrombosis, and Vascular Biology 21:905-910); Bucciarelli et al. (2002, Circulation 106:2827-2835); Wendt et al. (2006,

Atherosclerosis 185:70-77); the entire content of each of which is incorporated herein by reference.

[00107] Diabetic complications – heart

[00108] More particularly, animal models of human diabetes involving diabetic complications of the heart include ex vivo isolated perfused heart ischemia/reperfusion, left anterior descending coronary artery ligation, and cardiac autonomic neuropathy. References describing such models are known in the art and described in, for example, Stables et al. (2014, Autonom Neurosci 177: 746-80), Bucciarelli et al. (2000, Circulation (Supplement) 102: #563, II-117), and Aleshin et al. (2008, Am J Physiol Heart Circ Physiol 294: H1823-H1832); the entire content of each of which is incorporated herein by reference.

[00109] Diabetic complications – kidney

[00110] More particularly, animal models of human diabetes involving diabetic complications of the kidney include OVE26 mice, streptozotocin induced animals, Db/db mice, and nephrectomy. References describing such models are known in the art and described in, for example, Kaur et al. (2014, Inflammopharmacology 22:279-293), Reiniger et al. (2010, Diabetes 59: 2043-2054), and Wendt et al. (2003, American Journal of Pathology 162:1123-1137); the entire content of each of which is incorporated herein by reference.

[00111] Diabetic complications – retinopathy

[00112] More particularly, animal models of human diabetes involving diabetic complications leading to retinopathy include streptozotocin induced animals, Db/db mice, and Akita mice. References describing such models are known in the art and described in, for example, Lai et al. (2013, J Diabetes Res 013:106594) and Barile et al. (2005, Invest Ophthalmol Vis Sci 46:2916-2924); the entire content of each of which is incorporated herein by reference.

[00113] Diabetic complications – neuropathy

[00114] More particularly, animal models of human diabetes involving diabetic complications leading to neuropathy include Swiss Webster mice, Db/db mice, and Sciatic nerve transection/crush. References describing such models are known in the art and described in, for example, Juranek et al. (2010, Biochem Insights 2010:47-59), Juranek et al. (2013, Diabetes 62: 931-943), Islam (2013, J Diabetes Res 2013:149452); the entire content of each of which is incorporated herein by reference.

[00115] Animal models of diabetes in general include streptozotocin induced animals, Akita mice, Db/db mice, and Ob/ob mice. These animal models are known in the art and described in, for example, Park et al. (1998, Nature Medicine 4:1025-1031), Wendt et al. (2006, Atherosclerosis 185:70-77), Wang et al. (2014, Curr Diabetes Rev 10: 131-145), and Acharjee et

al. (2013, Can J Diabetes 37: 269-276); the entire content of each of which is incorporated herein by reference.

[00116] Cancer

[00117] Animal models for various forms of human cancers are known in the art and include those recapitulating aspects of human lung cancer, melanoma, colon cancer, pancreatic cancer, and breast cancer and bio-models of cancer for in silico screening. Such animal models are known in the art and are described in, for example, Taguchi et al. (2000, Nature 405:354-360), Arumugam et al. (2004, Journal of Biological Chemistry 279:5059-5065), Huang et al. (2006, Surgery 139:782-788), Huang et al. (2006, Surgery 139:782-788), Fuentes et al. (2007, Dis Colon Rectum 50:1230-1240), Arumugam et al. (2012, Clin Cancer Res 18: 4356-4364), Yu et al. (2014, J Gastric Cancer 14:67-86), Fleet (2014, Am J Physiol Gastrointest Liver Physiol. 307(3):G249-59), Lindner (2014, Semin Oncol 41: 146-155), Wang et al. (2014, Biofabrication 6(2):022001), Budhu et al. (2014, Curr Opin Genet Dev 24: 46-51, 2014); the entire content of each of which is incorporated herein by reference.

[00118] Ischemia/reperfusion Injury

[00119] Animal models human ischemia are known. See, for example, Tamarat et al. (2003, Proc Natl Acad Sci 100:14); Goova et al. (2001, Am J Pathol 159:513-525); Tekabe et al. (2010, J Nuc Med 51:92-97); Tekabe et al. (2013, EJNMMi Res 3:37); Bucciarelli et al. (2008, Diabetes 57:1941-1951); Shang et al. (2010, PLoS 5:e10092); Ma et al. (2009, J Cell Mol Med 13:1751-1764); the entire content of each of which is incorporated herein by reference.

[00120] Respiratory Diseases

[00121] In severe exacerbations of asthma there is an intense, mechanistically heterogeneous inflammatory response involving macrophage, neutrophil, and eosinophil accumulation and activation. Animal models for assessing the therapeutic potential of nanoparticles described herein are presented in, for example, Akirav et al. (2014, PLoS One 9:e95678); and Constant et al. (2002, J Clin Invest 110:1441-1448); the entire content of each of which is incorporated herein by reference.

[00122] Atherosclerosis

[00123] Examples of animal models of human atherosclerotic disease include apolipoprotein E null mice and Low Density Lipoprotein Receptor null mice. See, for example, Kapourchali et al. (2014, World J Clin Cases 2: 126-132), Harja et al. (2008, J. Clin. Invest. 118: 183-194), Nagareddy et al. (2013, Cell Metab 17: 695-708); the entire content of each of which is incorporated herein by reference.

[00124] In light of that which is understood in the art and described herein regarding the prominent role of macrophages in diseases/conditions characterized by acute and chronic

inflammation, methods are presented herein for treating such diseases/conditions, including but not limited to diabetic complications, ischemia, skin inflammation (e.g., psoriasis and atopic dermatitis), lung inflammation (e.g., asthma and chronic obstructive pulmonary disease), atherosclerosis, and tumor invasion and/or metastasis, which methods comprise administering to a subject in need thereof a nanoparticle described herein in a therapeutically effective amount. In a particular embodiment, at least one nanoparticle described herein is utilized, either alone or in combination with one or more known therapeutic agents. In a further particular embodiment, the present invention provides a method for treating macrophage-driven human diseases, wherein treatment alleviates one or more symptoms resulting from that disorder, the method comprising administration to a human in need thereof a therapeutically effective amount of a nanoparticle described herein.

[00125] In a method of treatment aspect, this invention provides a method of treating a mammal susceptible to or afflicted with a condition associated with diabetes complications, cancers, arthritis, acute and chronic inflammation, atherosclerosis, and tumor invasion and metastasis, and others, which method comprises administering an effective amount of one or more of the pharmaceutical compositions just described.

[00126] In additional method of treatment aspects, this invention provides methods of treating a mammal susceptible to or afflicted with an inflammatory condition causally related or attributable to macrophage activity. Such conditions and disorders include, without limitation, diabetes and its complications, peripheral vascular disease and associated complications, cancers, arthritis, acute and chronic inflammation, atherosclerosis, cardiovascular disease, tumor invasion and metastases, cardio- and cerebrovascular ischemia/reperfusion injury, heart attack, stroke, myocardial infarction, ischemic cardiomyopathy, renal ischemia, asthma, and skin disorders. Such methods comprise administering an effective condition-treating or condition-preventing amount of one or more of the pharmaceutical compositions just described.

[00127] As a further aspect of the invention, nanoparticles described herein are envisioned for use as a pharmaceutical especially in the treatment or prevention of the aforementioned conditions and diseases. Also provided herein is the use of nanoparticles described herein in the manufacture of a medicament for the treatment or prevention of one of the aforementioned conditions and diseases. Also provided herein are nanoparticles described herein for use in treating or preventing of one of the aforementioned conditions and diseases, wherein at least one of the nanoparticles described herein is administered to a subject in need thereof in a therapeutically effective amount sufficient to antagonize/reduce macrophage activity and thereby treat the condition or disease.

[00128] Injection dose levels range from about 0.1 mg/kg/hour to at least 10 mg/kg/hour, all for from about 1 to about 120 hours and especially 24 to 96 hours. A preloading bolus of from about 0.1 mg/kg to about 10 mg/kg or more may also be administered to achieve adequate steady state levels. The maximum total dose is not expected to exceed about 2 g/day for a 40 to 80 kg human patient.

[00129] For the prevention and/or treatment of long-term conditions, such as, e.g., arthritis, diabetes, or asthma, the regimen for treatment usually stretches over many months or years, so oral dosing is preferred for patient convenience and tolerance. With oral dosing, one to five and especially two to four and typically three oral doses per day are representative regimens. Using these dosing patterns, each dose provides from about 0.01 to about 20 mg/kg of the compound of the invention, with preferred doses each providing from about 0.1 to about 10 mg/kg and especially about 1 to about 5 mg/kg.

[00130] Transdermal doses are generally selected to provide similar or lower blood levels than are achieved using injection doses. Modes of administration suitable for mucosal sites are also envisioned herein and include without limitation: intra-anal swabs, enemas, intranasal sprays, and aerosolized or vaporized compounds and/or compositions for delivery to the lung mucosa. One of skill in the art would choose an appropriate delivery models based on a variety of parameters, including the organ or tissue site in a patient with a disease or condition that is most severely affected by the disease or condition.

[00131] When used to prevent the onset of an inflammatory condition, the compounds of this invention will be administered to a patient at risk for developing the condition or disorder, typically on the advice and under the supervision of a physician, at the dosage levels described above. Patients at risk for developing a particular condition generally include those that have a family history of the condition, or those who have been identified by genetic testing or screening to be particularly susceptible to developing the condition.

[00132] The compounds of this invention can be administered as the sole active agent or they can be administered in combination with other agents, including other compounds that demonstrate the same or a similar therapeutic activity and are determined to safe and efficacious for such combined administration.

GENERAL SYNTHETIC PROCEDURES

[00133] The therapeutic agents described herein may be purchased from various commercial sources or can be prepared from readily available starting materials using standard methods and procedures. It will be appreciated that where typical or preferred process conditions (i.e., reaction temperatures, times, mole ratios of reactants, solvents, pressures, etc.) are given,

other process conditions can also be used unless otherwise stated. Optimum reaction conditions may vary with the particular reactants or solvent used, but such conditions can be determined by one skilled in the art by routine optimization procedures.

[00134] The therapeutic agents described herein and nanoparticles comprising same may be prepared from known or commercially available starting materials and reagents by one skilled in the art of organic synthesis.

EXAMPLES

[00135] Immunological complexity in atherosclerosis warrants targeted treatment of specific inflammatory cells that aggravate the disease. With the initiation of large phase III trials investigating immunomodulatory drugs for atherosclerosis, cardiovascular disease treatment enters a new era. The present inventors herein propose a radically different approach: implementing and evaluating *in vivo* a combinatorial library of nanoparticles with distinct physiochemical properties and differential immune cell specificities. The library's nanoparticles are based on endogenous high-density lipoprotein (HDL), which can preferentially deliver therapeutic compounds to pathological macrophages in atherosclerosis. Using the *ApoE*^{-/-} mouse model of atherosclerosis, the present inventors quantitatively evaluated the library's immune cell specificity by combining immunological techniques and *in vivo* positron emission tomography (PET) imaging. Based on this screen, the present inventors formulated a liver receptor X agonist (GW3965) and abolished its liver toxicity, while still preserving its therapeutic function. Screening the immune cell specificity of nanoparticles can be employed to develop tailored therapies for atherosclerosis and other inflammatory diseases.

EXAMPLE 1

Materials and Methods

[00136] *Synthesis of library nanoparticles:* The composition of all NP synthesis materials is listed in supplementary table 1. The synthesis procedure for NP1 through NP11 was similar to a previous method(12). Briefly, phospholipids, DiR, and triglyceride were dissolved in a chloroform/methanol solvent, dried to form thin film, and then hydrated with human APOA1 solution. The homogenized solution was sonicated with a tip sonicator, and the aggregates and free lipids were removed by passing through a series of filters. The synthesis procedure for NP12 through NP15 was adapted from a previous microfluidics-based method(14). Briefly, a solution containing 0.79 mL of a PLGA or PLA solution in acetonitrile (100 mg/mL), 1.58 mL of a 3:1 molar ratio of DMPC/MHPC in ethanol (5 mg/mL), 0.36 mg of DiR, 7.9 mL of ethanol, and 14.5 mL of acetonitrile was prepared. The aforementioned solution was injected in the middle channel

of the microfluidic device at a rate of 2 mL/min, and a solution of APOA1 (0.01 mg/mL in PBS) was injected in the outer channels at a rate of 10 mL/min. The product was collected, washed with PBS, and concentrated using tangential filtration (100,000 Da molecular weight cut-off, MWCO) to remove acetonitrile, ethanol, and lipid-free APOA1. For micelle NP16, 2 mL chloroform solution containing 211 mg DSPE-PEG2000 and 0.4 mg DIR (0.5% mol) was slowly dripped into 10 mL PBS solution heated at 85 °C under vigorous stirring. After dripping, the solution was kept at 85 °C until all the chloroform was totally evaporated. The micelle solution was washed and concentrated in PBS using Millipore centrifugal filter (50,000 Da MWCO). For liposome NP17, a lipid film was first prepared by evaporating a chloroform solution containing 35.4 mg DPPC, 11.1 mg DSPE-PEG2000, 10.24 mg cholesterol, and 0.4 mg DIR (61.1%, 2%, 33.4%, 0.5% in molar percentage). The residue of chloroform was removed through blowing nitrogen gas. The resulting film was hydrated with 10 mL PBS, vortexed, and subsequently sonicated for 25 min. After centrifugation at 18 g for 10 min to remove aggregates, the liposome solution was washed and concentrated in PBS using Millipore centrifugal filter (100,000 Da MWCO). In order to oxidize NP1, a PBS solution containing NP1 was stirred at 37 °C in the presence of EDTA, α -tocopherol, and 2,2'-Azo-bis(2-amidinopropane) dihydrochloride (AAPH) for 20 hours. The resulting oxidized nanoparticle (NP2) was washed thoroughly with PBS.

[00137] *Synthesis of GW3965-loaded nanoparticles:* The synthesis procedure for Rx-HDL was similar to that used for NP10. In addition to phospholipids (POPC and PHPC, 2:1 by weight) and DiR, GW3965 was added to make up 26% by weight of the total starting materials. For Rx-PLGA-HDL, a solution containing 1 mL of GW3965 in DMSO (50 mg/mL), 2 mL of PLGA (100 mg/mL), 2 mL of lipids (10 mg/mL), 53 mL of acetonitrile, and 22 mL of ethanol was prepared. The organic solution was injected in the middle channel of the microfluidic device at a rate of 2 mL/min, while a solution of APOA1 (0.01 mg/mL in 1X PBS) was injected in the outer channels at a rate of 10 mL/min. The product was collected, washed with PBS, and concentrated to ~2 mg/mL using tangential and centrifugal filtration (100,000 Da MWCO and 300,000 Da MWCO, respectively). After synthesis, GW3965 was extracted from the final nanoparticles by acetonitrile, and the compound amount was measured by HPLC. The incorporation rate of Rx-HDL was above 90% and that for Rx-PLGA-HDL was about 50%. The GW3965 injecting dose was adjusted on the basis of the measured GW3965 concentration in the nanoparticle solution.

[00138] *Animals and treatment procedure:* All procedures and experiments were approved by the IACUC of Icahn School of Medicine at Mount Sinai. About 300 female *ApoE*^{-/-} mice (B6.129P2^{Apoetm1Unc/J}) were purchased from Jackson Laboratories and then fed a high fat diet (Harlan Teklad TD.88137, 42 % calories from fat) for 16 weeks. These mice developed advanced atherosclerosis in their aortic roots and aortas after 10 weeks of high fat diet and mimicked

advanced disease in humans after 16 weeks of the diet. Female C57BL/6 mice were fed on regular chow. All nanoparticles were intravenously injected into the lateral tail veins. Care was taken to ensure each injection was less than 150 μ l in volume. For the *in vivo* characterization study of the 17 nanoparticles, Rx-HDL, and Rx-PLGA-HDL, one intravenous injection was performed. No obvious toxicity or side effects were observed after the injections except for NP12. After NP12 injection, mice had slow movement, and 1 out of 5 died within 24 hours after the injection. For short-term toxicity study of Rx-HDL and Rx-PLGA-HDL, 4 injections in 8 days were given using a GW3965 dose of 10 mg/kg per injection. For the long-term treatment study, the mice received 2 intravenous injections of Rx-HDL (n = 12) per week for 6 weeks at the dose of 10 mg/kg GW3965 per injection or equal volume of PBS (n = 12). A separate cohort of female *Apoe*^{-/-} mice with the same duration of high-fat diet received 6-weeks of oral GW3965 (n = 6, 10 mg/kg GW3965, 2 times per week) or PBS (n = 5). No abnormal activities or deaths occurred during the treatment regimen.

[00139] *Micro-PET/CT imaging:* *Apoe*^{-/-} atherosclerotic mice were injected with either ⁸⁹Zr-labeled Rx-HDL or Rx-PLGA-HDL at about 200 μ Ci/mouse (n = 5). At 30 min and 24 hours after the injection, the mice were imaged on an Inveon PET/CT scanner (Siemens Healthcare Global, Erlangen, Germany) under isoflurane-induced (Baxter Healthcare, Deerfield, IL, USA) anesthesia. Whole body static PET scans recorded a minimum of 50 million coincident events in approximately 15 min. The energy and coincidence timing windows were 350-700keV and 6 ns, respectively. The image data were normalized to correct for non-uniform PET response, dead-time count losses, positron branching ratio, and physical decay to the time of injection, but no attenuation, scatter, or partial-volume averaging correction was applied. The counting rates in the reconstructed images were converted to activity concentrations (percentage injected dose [%ID] per gram of tissue [%ID/g]) via a system calibration factor derived from imaging a mouse-sized water-equivalent phantom containing ⁸⁹Zr. Images were analyzed using ASIPro VMTM software (Concorde Micro-systems). Activity concentration was quantified by averaging the maximal values of at least 10 ROIs drawn on consecutive slices of the chosen organs.

[00140] *Liver mRNA expression measurement:* Total RNA was obtained from snap-frozen liver tissue and homogenized in TRIzol reagent (Ambion, Austin, TX, USA). The homogenate was spun down to pellet tissue, and the aqueous Trizol supernatant was collected and processed using the Direct-Zol. RNA mini-prep kit (Zymo Research Corporation) was used for RNA purification. The RNA was then reverse-transcribed using the Verso cDNA kit (Thermo Scientific, Carlsbad, CA, USA) and diluted using RNase/DNase-free water. Quantitative real time PCR was performed with Taqman Gene Expression Master Mix (Applied Biosystems,

Foster City, CA, USA), and Taqman primer/probe mixes for *Abca1*, *Abcg1*, or *Srebp1c*. Gene expression was normalized to 18s ribosomal RNA (rRNA) expression.

[00141] *Aortic macrophage mRNA expression measurement:* The quality of RNA extracts from atherosclerotic plaques was measured by Agilent 2100 bioanalyzer (Agilent Technologies). High-quality (RIN > 7) RNA samples were amplified with a WT-Ovation Pico RNA amplification system (NuGen). The cDNA from the amplification reactions were used to run a microfluidics-based mRNA profiling chip (BioMark, Fluidigm, CA, USA), which had high reproducibility. *Hprt1* was used as the housekeeping gene. The following 24 genes were measured: *Abca1*, *Abcg1*, *Srebp1c*, *Nr1h2*, *Hr1h3*, *Ccl2*, *Ccr2*, *Icam1*, *Ifng*, *Il12a*, *Il1a*, *Il1b*, *Il6*, *Nos2*, *Mmp2*, *Mmp9*, *Tnfa*, *Vcam1*, *Ccr7*, *Cd206*, *Foxp3*, *Il10*, *Mrc2*, and *Tgfr1*. 12 biological repeats were included for each gene.

[00142] *Cellular lipid measurement with flow cytometry:* Single cells were prepared from aortas from either *Apoe*^{-/-} mice or wild type C57BL/6 mice and then stained with antibody cocktails as described above. On the basis of a previous procedure(33), the stained cells were washed with PBS 2 times at room temperature and then incubated with 250 μ l PBS containing 0.5 μ g/ml BODIPY (Molecular Probes, D-3922) for 15 min. The cells were washed with flow cytometry buffer 2 times and analyzed in a BD LSRRII flow cytometer.

[00143] *Statistics:* Data are presented as mean $\pm$ standard error of mean (SEM) unless otherwise noted. Two-tailed Student's *t*-test was used to calculate statistical significance. GraphPad Prism 5.0 for PC (GraphPad Software Inc.) was used for statistical analysis. *P* < 0.05 was regarded as significant. * denotes *P* value < 0.05 and ** denotes *P* value < 0.01.

Supporting Materials and Methods:

[00144] *Radiochemistry:* ⁸⁹Zr was produced at Memorial Sloan Kettering Cancer Center on an EBCO TR19/9 variable-beam energy cyclotron (Ebco Industries Inc., Richmond, BC, Canada) via the ⁸⁹Y(p,n)⁸⁹Zr reaction and purified with a method previously reported(46). Radioactivity was measured with a Capintec CRC-15R dose Calibrator (Capintec, Ramsey, NJ, USA).

[00145] *Synthesis of C34-DFO:* 2-Hexadecyl-octanoic acid (30 mg, 59 μ mol), (Benzotriazol-1-yloxy) tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 29 mg, 66 μ mol), and N,N-diisopropylethylamine (DIEA, 10 μ L) were dissolved in anhydrous dichloromethane (2 mL) and stirred at 40 °C for 10 min under nitrogen atmosphere. Next, a solution of deferoxamine mesylate (30 mg, 46 μ mol) and DIEA (10 μ L) in anhydrous DMSO (0.7 mL) was added and the resulting mixture stirred for 4 h at 40 °C under nitrogen. The cloudy suspension was allowed to cool to room temperature, and dichloromethane was removed under reduced pressure. Hydrochloric acid (0.1 M, 1 mL) was added, and the mixture was stirred for 10

min at room temperature. The solid was filtered with 0.1 M HCl (3x1 mL), DMSO (3x1 mL), water (3x1 mL), and, finally, dichloromethane (3x1 mL) and dried to yield a white solid (31 mg, 64 %). MS-ES⁺: 1074, (M+Na)⁺; MS-ES⁻: 1050 (M-H)⁻, 1086 (M+Cl)⁻. ¹H-NMR (CDCl₃/CD₃OD), δ in ppm: 0.55 (t, 6H); 0.92 (br, 60H); 1.06 (br, 6H); 1.27 (m, 6H); 1.32 (m, 6H); 1.76 (m, 1H); 1.80 (s, 3H); 2.15 (t, 4H); 2.46 (t, 4H); 2.87 (m, 6H); 3.29 (m, 6H). All chemicals were purchased from Sigma-Aldrich.

[00146] *Radiolabeling of library nanoparticles and GW3965-loaded nanoparticles:*

Nanoparticles were labeled using a slightly modified version of the synthesis procedure for regular screening nanoparticles or GW3965-loaded nanoparticles. Briefly, DiR of the library nanoparticles or 0.5% of GW3965 payload was replaced with C34-DFO while synthesizing radiolabeled nanoparticles. After synthesis and purification, the C34-DFO labeled nanoparticles were incubated with ⁸⁹Zr-oxalate in PBS (pH 7.1~7.4) at 37 °C for 2 h at an activity-to-APOA1 ratio of ~1 mCi/mg (or ~1 mCi/10mg lipids for NP17). The radiolabeled nanoparticles were purified by spin filtration by using 10,000 Da MWCO filter tubes. Radiochemical yields were in excess of 80%, and radiochemical purities, as determined by size exclusion chromatography, greater than 95% for all nanoparticles.

[00147] *Blood half-life determination:* In the library study, all nanoparticles were injected at a fixed DiR dose of 1 mg/kg. The theoretical 0 time point was calculated by assuming a mouse's blood accounts for 6% of its body weight and the density of mouse blood was 1.06 g/ml. The mice were bled 1, 6, and 24 hours after the injection. The DiR concentration was determined by comparing DiR fluorescence signal to a DiR standard curve. DiR signal was measured in an IVIS200 system (PerkinElmer, Waltham, MA, USA) at a 750 nm excitation wavelength and an 820 nm emission wavelength. Blood half-life was calculated by finding the X coordinate from the curve's intersection with a horizontal line at 50% signal of time 0. Five mice were used for each nanoparticle. Regarding the radiolabeled nanoparticles, animals were injected with ~20 μ Ci to ~30 μ Ci of the corresponding nanoparticles and blood samples (approximately 10 μ l each) were collected 2 min, 30 min, 1hr, 2hr, 4hr, 8hr, and 24hr after the intravenous injection. The blood was weighed, and its radioactivity was determined using a gamma counter (PerkinElmer, Waltham, MA, USA). Blood half-life was calculated via two-phase decay. Three mice were used for each nanoparticle.

[00148] *Autoradiography and biodistribution:* Upon sacrifice, animals were perfused with 20 ml PBS through cardiac puncture. Lung, liver, aorta, spleen, kidney, femur muscle, and heart were collected, and their radiotracer distribution was determined by placing the tissues in a film cassette against a phosphorimaging plate (BASMSM-2325, Fujifilm, Valhalla, NY, USA) for either 14 hours (mouse aortas) or 4 hours (all other organs). Phosphorimaging plates were read in

a Typhoon 7000IP plate reader (GE Healthcare, Pittsburg, PA, USA). Biodistribution in aorta, heart, liver, spleen, blood, lung, skin, brain, pancreas, stomach, small intestine, large intestine, kidney, muscle, and bone was determined by first weighing the tissues and then measuring their radioactivity in a gamma counter (PerkinElmer, Waltham, MA, USA). The relative activity per tissue is presented as percentage of injected dose per gram of tissue (%ID/g).

[00149] *Nanoparticles characterization:* The synthesized nanoparticles' size was measured by dynamic light scattering (DLS). To measure the DiR concentration in nanoparticles, DiR was first extracted from the nanoparticles with acetonitrile, and the amount of DiR was determined by measuring the signature absorbance of DiR at 750 nm. Nanoparticle morphology was determined by transition emission microscopy. Briefly, the original PBS solvent was replaced with an ammonium acetate buffer and then mixed with 2% sodium phosphotungstate (pH = 7.4) to negatively stain the nanoparticles. The solution was then added to a TEM grid and imaged with a Hitachi H7650 system linked to a Scientific Instruments and Applications digital camera controlled by Maxim CCD software. To measure nanoparticle APOA1 concentration, APOA1 was separated from the other nanoparticle components by acetonitrile precipitation. The pellets' protein component was then measured with a standard BCA assay (Thermo Scientific, Rockford, IL, USA).

[00150] *HPLC and Radio-HPLC:* HPLC was performed on a Shimadzu HPLC system equipped with two LC-10AT pumps and an SPD-M10AVP photodiode array detector. Radio-HPLC was performed using a Lablogic Scan-RAM Radio-TLC/HPLC detector. Size exclusion chromatography was performed on a Superdex 10/300 column (GE Healthcare Life Sciences, Pittsburgh, PA, USA) using PBS as an eluent at a flow rate of 1 mL/min. GW3965 detection was performed on a C18 column (Shimadzu, Kyoto, Japan) with an isocratic flow of water and acetonitrile at a flow rate of 1 ml/min.

[00151] *Cholesterol efflux assay:* Bone marrow cells were flushed from the tibia and femurs of 6-8 week old C57BL/6 mice and differentiated into macrophages by incubation in DMEM media supplemented with 10% FBS, 1% P/S, and 15% L929-conditioned media for 7 days. Bone marrow-derived macrophages (BMDMs) were subsequently incubated with media containing 0.5 $\mu\text{Ci/ml}$ [^3H]-cholesterol and acLDL (50 $\mu\text{g/mL}$) for 30 hours. Cells were washed twice with PBS and equilibrated overnight in media containing 2 mg/ml fatty acid-free albumin. Efflux to APOA1 (50 $\mu\text{g/mL}$) or various nanoparticles (50 $\mu\text{g/mL}$ normalized to APOA1 concentration) was carried out for 8 hours. Cell media was removed, and the cells were lysed in 0.1 M NaOH solution. [^3H]-cholesterol contents of media and cell lysates were measured by liquid scintillation counting. Efflux is expressed as a percentage of ^3H -cholesterol in medium by

using the following formula: $[\text{}^3\text{H-cholesterol in medium} / (\text{}^3\text{H-cholesterol in medium} + \text{}^3\text{H-cholesterol in cells})] \times 100\%$.

[00152] *Near infrared fluorescence imaging:* 24 hours after intravenous injection of DiR-containing nanoparticles, the mice were perfused with 20 ml PBS, and brain, lung, heart, aorta, spleen, liver, kidney, and femur muscle were collected. Their total DiR fluorescent signal was measured in an IVIS200 at a 750nm excitation wavelength and an 820nm emission wavelength. The radiant efficiency was calculated using LiveImaing (PerkinElmer, Waltham, MA, USA). For each nanoparticle, 5 mice were used.

[00153] *Flow cytometry:* A protocol similar to one previously reported was used (12). Briefly, at 24 hours after intravenous injection of DiR-loaded nanoparticles, blood was collected and the animals were perfused. Afterwards, aortas and spleens were collected, diced, and digested with a cocktail of enzymes, including liberase TH (Roche), hyaluronidase (Sigma-Aldrich), and DNase (Sigma-Aldrich) in a 37 °C oven for 1 hour. A single-cell suspension was made by removing tissue aggregates, extracellular matrix, and cell debris from the solution. Red blood cells were removed from the blood sample by lysis buffer. DiR was detected on the APC-Cy7 channel. To identify macrophages, monocytes, dendritic cells, neutrophils, and other immune cells, a lineage of antibodies (Lin) recognizing CD90 (clone 53.2.1), B220 (clone RA3-6B2), CD49b (clone DX5), NK1.1 (clone PK136), Ly-6G (clone 1A8), Ter-119 (clone TER-119), and antibodies recognizing Ly-6C (clone AL21), F4/80 (BM8), and CD11c (N418) were used. Antibodies were purchased from eBioscience, BD Biosciences, and Biolegend. Due to the large number of samples, 2 to 3 nanoparticles were measured per batch, each of which included 12 to 17 mice (10 to 15 from tested nanoparticles, 1 for reference DiR-nanoparticles, and 1 non-injected control to set up compensation). The APC-Cy7 channel of the flow cytometer was calibrated using APC-Cy7 calibration beads (Spherotech, Cat# ECFP-F7). Signal variation among batches was corrected by normalizing to the beads' signal. All samples were measured on an LSRII (BD Biosciences, San Jose, CA, USA) flow cytometer. Results were analyzed with FlowJo (Ashland, OR, USA) and statistics were calculated with Prism (GraphPad, La Jolla, CA USA).

[00154] *Hepatic triglyceride and cholesterol measurement:* Lipids were extracted from the livers, which were snap-frozen in -80 °C immediately after sacrifice. Portions of liver tissue (generally <100mg) were weighed, suspended in 2x weight volume of PBS, and homogenized. To analyze protein content, 10 µL of the total homogenate underwent a standard Lowry assay (BioRad). 50uL of the total homogenate was suspended in 3mL isopropanol and incubated overnight at 4°C while rocking to extract lipids. The solvent/lipid mixture was then centrifuged at 3000rpm for 10 min, and supernatant was collected in glass tubes and dried under a stream of

nitrogen. Following complete evaporation of the extraction isopropanol, lipids were stored at -20 °C until analysis. For quantification, dried lipids were re-suspended in 1mL Isopropanol, and analysis of triglycerides and total cholesterol was performed on diluted lipid solutions using standard colorimetric assays (Wako) as per manufacturer's protocol. The cholesterol and triglyceride concentrations were normalized to the measured protein concentration per sample.

[00155] *Immunostaining and laser capture microdissection:* As previously described(2), 6-µm thick frozen sections were made from the aortic roots of 60 mice. 6 evenly distributed sections were stained with CD68 (Serotec, clone MCA1957). The stained slides were digitally scanned using a slide scanner (3DHistech Panoramic Scanner, Budapest, Hungary). When CD68-positive area was quantified, the positively stained area was calculated using a MATLAB procedure developed previously by the present inventors(2). 360 sections in total were analyzed. For laser capture microdissection, 36 aortic roots sections per animal were used to extract atherosclerotic macrophages using Leica LDM6500. All LCM reagents were maintained, and procedures were done under RNase-free conditions. The sections were fixed in 70% ethanol for 1 min, washed in H₂O, stained with Mayer's hematoxylin (VWR Scientific) for 1 min, washed in H₂O, incubated in PBS (to develop blue color) for 15 seconds, washed in H₂O, partially dehydrated in 70% followed by 95% ethanol, stained in eosin Y (VWR Scientific) for 5 seconds, washed in 95% ethanol, and then completely dehydrated in 100% ethanol (30 seconds), xylene (30 seconds), and xylene (5 min). The sections were then air-dried for 10 min. Macrophages were identified under a microscope and verified by anti-CD68 staining on the guiding slides. Isolated macrophages were immediately lysed, and their RNA were extracted and stored in a -80 °C freezer. For laser capture microdissection, 2160 sections were used.

[00156] Results of the aforementioned experiments are described herein above and depicted in Figures 1-7 and Supplemental Figures 1-7.

Discussion

[00157] By fine-tuning the components and synthesis procedures, the present inventors created a combinatorial library of 17 nanoparticles with distinct composition, size, and morphology. These distinct physiochemical properties resulted in an approximately 6-fold difference in promoting cholesterol efflux from macrophages, 10-fold difference among blood half-lives, 3.4-fold difference in relative aorta-to-liver accumulation, and 3.8-fold difference in relative aortic-to-splenic macrophage accumulation. From this library screening, the present inventors identified the favorable lipid composition (POPC-dominant), pharmacokinetics (long blood half-life), size (around 30 nm), and morphology (spherical) to achieve optimal plaque macrophage-specific drug delivery. The present inventors hypothesize that the combination of long blood half-life and small size allows efficient and prolonged atherosclerotic plaque

penetration and subsequent macrophage accumulation. A favorable nanoparticle lipid composition and morphology increases stability and promotes delivery of the encapsulated small molecules to the targeted cells, as suggested by our recent study(34). In a proof-of-concept application, these guidelines were used to identify two nanoparticles from the library as the most and least favorable nanoparticles for delivering the liver-toxic compound GW3965. While the unfavorable nanoparticle Rx-PLGA-HDL caused severe GW3965-induced liver toxicity, the favorable nanoparticle Rx-HDL did not cause observable liver toxicity in treated animals, but did preserve the compound's therapeutic efficacy on the atherosclerotic plaques.

[00158] Despite the clinical introduction of antibody immunotherapies for atherosclerosis(35), such biological drugs can only modulate a limited number of extracellular targets, such as PCSK9(36, 37) or receptors on the cell surface(38). Intracellular entities present many more immunological targets that can be effectively controlled by immunomodulatory small molecules. Most experimental small molecules for atherosclerosis, however, have failed clinical trials due to their unfavorable toxicity profiles, generally caused by high accumulation in non-targeted tissues or in non-targeted cells within the targeted tissues(39). To convert these immunomodulatory small molecules into precision medicines for atherosclerosis, organ-specific and cell-specific delivery is highly desirable.

[00159] The major novelty of the present approach is the creation and immunological screening of a combinatorial nanoparticle library with distinct organ biodistribution and immune cell targeting specificities. By using PET imaging, NIRF, and flow cytometry to generate an extensive database detailing the *in vivo* performance of the library nanoparticles, the present inventors were able to rationally design a strategy that avoids the specific limitations of an immunomodulatory compound. Through this process, the present inventors successfully converted a liver-toxic immunomodulatory compound into a precision nanomedicine for atherosclerotic plaque macrophage treatment.

[00160] A polymer-core nanoparticle (NP13) in the library has similarly optimal performance relative to the best-performing NP10 (S Fig 4A). Polymer-based nanoparticles have been formulated with chemical compounds(40), peptides(41), and nucleic acids(42). This variety suggests that NP13 may be able to deliver a wide range of therapeutic molecules. In addition, a few nanoparticles (NP3, NP9, and NP10) in the library show high targeting specificity to Ly-6C^{hi} monocytes and dendritic cells (DCs), which are attractive targets in certain types of cancer(43), asthma(44), and diabetes(45). It would be interesting to apply the same library screening strategy to develop nanoparticle-based specific drug delivery to these immune cells in relevant diseases.

[00161] This novel nanoparticle library screening strategy in immune cells allowed us to improve the therapeutic index of an immunomodulatory molecule that causes hepatic toxicity and

has failed clinical translation. The precision nanomedicine strategy described herein is radically different from the current clinical therapeutics as well as those in experimental phases. Moreover, the approach's potential to deliver various compounds preferentially to other immune cells would expand its application to numerous immunologically implicated diseases, such as myocardial infarction, diabetes, and cancer.

[00162] From the foregoing description, various modifications and changes in the compositions and methods of this invention will occur to those skilled in the art. All such modifications coming within the scope of the appended claims are intended to be included therein.

[00163] All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

[00164] REFERENCES

- [00165]** 1. Fridman WH, Pages F, Sautes-Fridman C, & Galon J (2012) The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 12(4):298-306.
- [00166]** 2. Lehuen A, Diana J, Zaccane P, & Cooke A (2010) Immune cell crosstalk in type 1 diabetes. *Nat Rev Immunol* 10(7):501-513.
- [00167]** 3. Moore KJ, Sheedy FJ, & Fisher EA (2013) Macrophages in atherosclerosis: a dynamic balance. *Nat Rev Immunol* 13(10):709-721.
- [00168]** 4. Swirski FK & Nahrendorf M (2013) Leukocyte behavior in atherosclerosis, myocardial infarction, and heart failure. *Science* 339(6116):161-166.
- [00169]** 5. Libby P (2002) Inflammation in atherosclerosis. *Nature* 420(6917):868-874.
- [00170]** 6. Heidt T, *et al.* (2014) Chronic variable stress activates hematopoietic stem cells. *Nat Med* 20(7):754-758.
- [00171]** 7. Robbins CS, *et al.* (2013) Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nat Med* 19(9):1166-1172.
- [00172]** 8. Galkina E & Ley K (2009) Immune and inflammatory mechanisms of atherosclerosis (*). *Annu Rev Immunol* 27:165-197.
- [00173]** 9. Dutta P, *et al.* (2012) Myocardial infarction accelerates atherosclerosis. *Nature* 487(7407):325-329.
- [00174]** 10. Groot PH, *et al.* (2005) Synthetic LXR agonists increase LDL in CETP species. *J Lipid Res* 46(10):2182-2191.

- [00175] 11. Kotokorpi P, *et al.* (2007) Physiological differences between human and rat primary hepatocytes in response to liver X receptor activation by 3-[3-[N-(2-chloro-3-trifluoromethylbenzyl)-(2,2-diphenylethyl)amino]propyloxy]phenylacetic acid hydrochloride (GW3965). *Molecular pharmacology* 72(4):947-955.
- [00176] 12. Tang J, *et al.* (2015) Inhibiting macrophage proliferation suppresses atherosclerotic plaque inflammation. *Science advances* 1(3).
- [00177] 13. Duivenvoorden R, *et al.* (2014) A statin-loaded reconstituted high-density lipoprotein nanoparticle inhibits atherosclerotic plaque inflammation. *Nature communications* 5:3065.
- [00178] 14. Sanchez-Gaytan BL, *et al.* (2015) HDL-mimetic PLGA nanoparticle to target atherosclerosis plaque macrophages. *Bioconjug Chem* 26(3):443-451.
- [00179] 15. Cormode DP, *et al.* (2009) HDL as a contrast agent for medical imaging. *Clin Lipidol* 4(4):493-500.
- [00180] 16. Nagano Y, Arai H, & Kita T (1991) High density lipoprotein loses its effect to stimulate efflux of cholesterol from foam cells after oxidative modification. *Proc Natl Acad Sci U S A* 88(15):6457-6461.
- [00181] 17. Matsumura Y (2008) Poly (amino acid) micelle nanocarriers in preclinical and clinical studies. *Adv Drug Deliv Rev* 60(8):899-914.
- [00182] 18. Torchilin VP (2005) Recent advances with liposomes as pharmaceutical carriers. *Nat Rev Drug Discov* 4(2):145-160.
- [00183] 19. Tall AR, Yvan-Charvet L, Terasaka N, Pagler T, & Wang N (2008) HDL, ABC transporters, and cholesterol efflux: implications for the treatment of atherosclerosis. *Cell Metab* 7(5):365-375.
- [00184] 20. Tall AR & Yvan-Charvet L (2015) Cholesterol, inflammation and innate immunity. *Nat Rev Immunol* 15(2):104-116.
- [00185] 21. Schiener M, *et al.* (2014) Nanomedicine-based strategies for treatment of atherosclerosis. *Trends Mol Med* 20(5):271-281.
- [00186] 22. Weissleder R & Ntziachristos V (2003) Shedding light onto live molecular targets. *Nat Med* 9(1):123-128.
- [00187] 23. Galkina E, *et al.* (2007) CXCR6 promotes atherosclerosis by supporting T-cell homing, interferon-gamma production, and macrophage accumulation in the aortic wall. *Circulation* 116(16):1801-1811.
- [00188] 24. Galkina E, *et al.* (2006) Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *J Exp Med* 203(5):1273-1282.

- [00189] 25. Weissleder R, Nahrendorf M, & Pittet MJ (2014) Imaging macrophages with nanoparticles. *Nat Mater* 13(2):125-138.
- [00190] 26. Hong C & Tontonoz P (2014) Liver X receptors in lipid metabolism: opportunities for drug discovery. *Nat Rev Drug Discov* 13(6):433-444.
- [00191] 27. Joseph SB, *et al.* (2002) Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc Natl Acad Sci U S A* 99(11):7604-7609.
- [00192] 28. Naik SU, *et al.* (2006) Pharmacological activation of liver X receptors promotes reverse cholesterol transport in vivo. *Circulation* 113(1):90-97.
- [00193] 29. Deri MA, Zeglis BM, Francesconi LC, & Lewis JS (2013) PET imaging with (8)(9)Zr: from radiochemistry to the clinic. *Nuclear medicine and biology* 40(1):3-14.
- [00194] 30. Zhang SH, Reddick RL, Piedrahita JA, & Maeda N (1992) Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 258(5081):468-471.
- [00195] 31. Wu KT, *et al.* (2016) Nonalcoholic fatty liver disease severity is associated with the ratios of total cholesterol and triglycerides to high-density lipoprotein cholesterol. *J Clin Lipidol* 10(2):420-425 e421.
- [00196] 32. Brunham LR, *et al.* (2006) Tissue-specific induction of intestinal ABCA1 expression with a liver X receptor agonist raises plasma HDL cholesterol levels. *Circ Res* 99(7):672-674.
- [00197] 33. Herber DL, *et al.* (2010) Lipid accumulation and dendritic cell dysfunction in cancer. *Nat Med* 16(8):880-886.
- [00198] 34. Zhao Y, *et al.* (2016) Augmenting drug-carrier compatibility improves tumour nanotherapy efficacy. *Nature communications* 7:11221.
- [00199] 35. Mullard A (2015) PCSK9 inhibitors are go. *Nat Rev Drug Discov* 14(9):593.
- [00200] 36. Sabatine MS, *et al.* (2015) Efficacy and safety of evolocumab in reducing lipids and cardiovascular events. *N Engl J Med* 372(16):1500-1509.
- [00201] 37. Robinson JG, *et al.* (2015) Efficacy and safety of alirocumab in reducing lipids and cardiovascular events. *N Engl J Med* 372(16):1489-1499.
- [00202] 38. Chames P, Van Regenmortel M, Weiss E, & Baty D (2009) Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol* 157(2):220-233.
- [00203] 39. Cook D, *et al.* (2014) Lessons learned from the fate of AstraZeneca's drug pipeline: a five-dimensional framework. *Nat Rev Drug Discov* 13(6):419-431.
- [00204] 40. Mieszawska AJ, *et al.* (2013) Synthesis of polymer-lipid nanoparticles for image-guided delivery of dual modality therapy. *Bioconjug Chem* 24(9):1429-1434.

- [00205] 41. Fredman G, *et al.* (2015) Targeted nanoparticles containing the proresolving peptide Ac2-26 protect against advanced atherosclerosis in hypercholesterolemic mice. *Science translational medicine* 7(275):275ra220.
- [00206] 42. Choleris E, *et al.* (2007) Microparticle-based delivery of oxytocin receptor antisense DNA in the medial amygdala blocks social recognition in female mice. *Proc Natl Acad Sci U S A* 104(11):4670-4675.
- [00207] 43. Palucka K & Banchereau J (2012) Cancer immunotherapy via dendritic cells. *Nat Rev Cancer* 12(4):265-277.
- [00208] 44. Hammad H & Lambrecht BN (2008) Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* 8(3):193-204.
- [00209] 45. Mukherjee G & DiIorenzo TP (2010) The immunotherapeutic potential of dendritic cells in type 1 diabetes. *Clin Exp Immunol* 161(2):197-207.
- [00210] 46. Perez-Medina C, *et al.* (2015) PET Imaging of Tumor-Associated Macrophages with ⁸⁹Zr-Labeled High-Density Lipoprotein Nanoparticles. *J Nucl Med* 56(8):1272-1277.

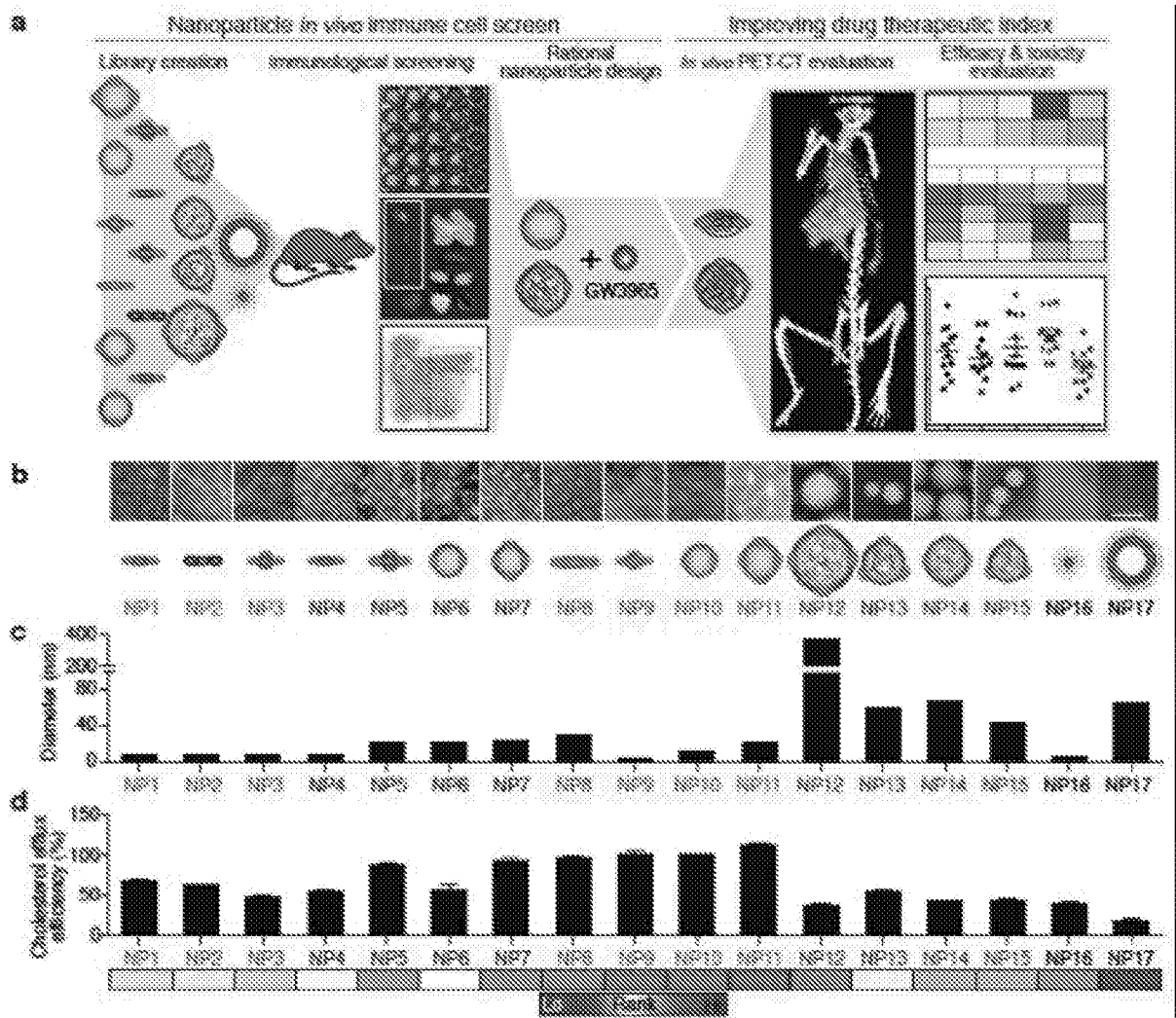
What is claimed is:

1. A nanoparticle comprising phospholipids, apolipoprotein A-1 (APOA1), and a hydrophobic core component, wherein the phospholipids comprise 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-hexadecyl-*sn*-glycero-3-phosphocholine (PHPC) and the ratio of POPC to PHPC is 10:1 to 1:1; the ratio of the phospholipids to APOA1 is 1:1 to 4:1; and the ratio of the phospholipids to the hydrophobic core component is 1:1 to 40:1; and wherein the nanoparticle has a diameter of 20 to 150 nm.
2. The nanoparticle of claim 1, wherein the ratio of POPC to PHPC is 5:1 to 1:1.
3. The nanoparticle of claim 1, wherein the ratio of the phospholipids to APOA1 is 2:1 to 4:1.
4. The nanoparticle of claim 1, wherein the ratio of the phospholipids to the hydrophobic core component is about 1:1 to 5:1.
5. The nanoparticle of claim 1, wherein the diameter is about 20 to 100 nm.
6. The nanoparticle of claim 1, wherein the diameter is about 30 nm.
7. The nanoparticle of claim 1, wherein the hydrophobic core component comprises a hydrophobic therapeutic agent, a therapeutic agent conjugated to poly(lactic-co-glycolic acid) (PLGA), triglycerides, or polymers.
8. The nanoparticle of claim 7, wherein the hydrophobic therapeutic agent comprises a hydrophobic small molecule compound or a hydrophobic polymer/compound.
9. The nanoparticle of claim 7, wherein the hydrophobic therapeutic agent is selected from the group consisting of GW3965, Simvastatin, Celecoxib, Rapamycin, PLX3397, Trametinib, Pioglitazone, Resolvin D3, Resolvin D2, 18S-Resolvin E1, AT-Resolvin D1, Resolvin E2, Linoleic Acid, Imiquimod, Resiquimod, Vemurafenib, Prednisolone, Epcadostat, NLG919, LM10 (inhibitor), NCX_4016 (Nitroaspirin), Tadalafil, AZ10606120(CHEMBL562308), SCH58261, SCH420814 (Preladenant), PSB1115, PF_04418948, RQ_15986(EP4A), PF_4136309, Maraviroc, 852A (UNII-6PJC3KPK6S), VTX_2337 (Motolimod), LY2157299, Vemurafenib, Dabrafenib, BMS_777607, BLZ945, PI_3065, and TG100_115.

10. The nanoparticle of claim 7, wherein the hydrophobic therapeutic agent is selected from the group consisting of GW3965, Simvastatin, Rapamycin, PLX3397, and Trametinib.
11. The nanoparticle of claim 7, wherein the hydrophobic therapeutic agent is selected from the group consisting of GW3965, PLX3397, and Trametinib.
12. The nanoparticle of claim 7, wherein the hydrophobic therapeutic agent is GW3965.
13. The nanoparticle of claim 7, wherein the hydrophobic therapeutic agent is GW3965 and the ratio of POPC to PHPC is about 3:1; the ratio of the phospholipids to APOA1 is about 2.5:1; and the ratio of the phospholipids to the GW3965 is about 2:1; and the nanoparticle has a diameter of about 30 nm
14. The nanoparticle of claim 7, wherein the hydrophobic therapeutic agent is PLX3397.
15. The nanoparticle of claim 7, wherein the hydrophobic therapeutic agent is Trametinib.
16. The nanoparticle of claim 7, wherein the therapeutic agent conjugated to poly(lactic-co-glycolic acid) (PLGA) is a small molecule, a small biologic, or a low molecular weight therapeutic .
17. A nanoparticle comprising phospholipids, apolipoprotein A-1 (APOA1), and a hydrophobic core component, wherein the phospholipids comprise 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1-myristoyl-2-hydroxy-*sn*-glycero-phosphocholine (MHPC) and the ratio of DMPC to MHPC is 10:1 to 1:1; the ratio of the phospholipids to APOA1 is 1:1 to 4:1; and the ratio of the phospholipids to the hydrophobic core component is 1:1 to 40:1.
18. A method for making the nanoparticle of any one of claims 1-17, the method comprising a) generating a homogenized solution comprising the phospholipids, apolipoprotein A-1 (APOA1), and the hydrophobic core component at the ratios indicated and under conditions such that nanoparticles form, and b) recovering the nanoparticles.

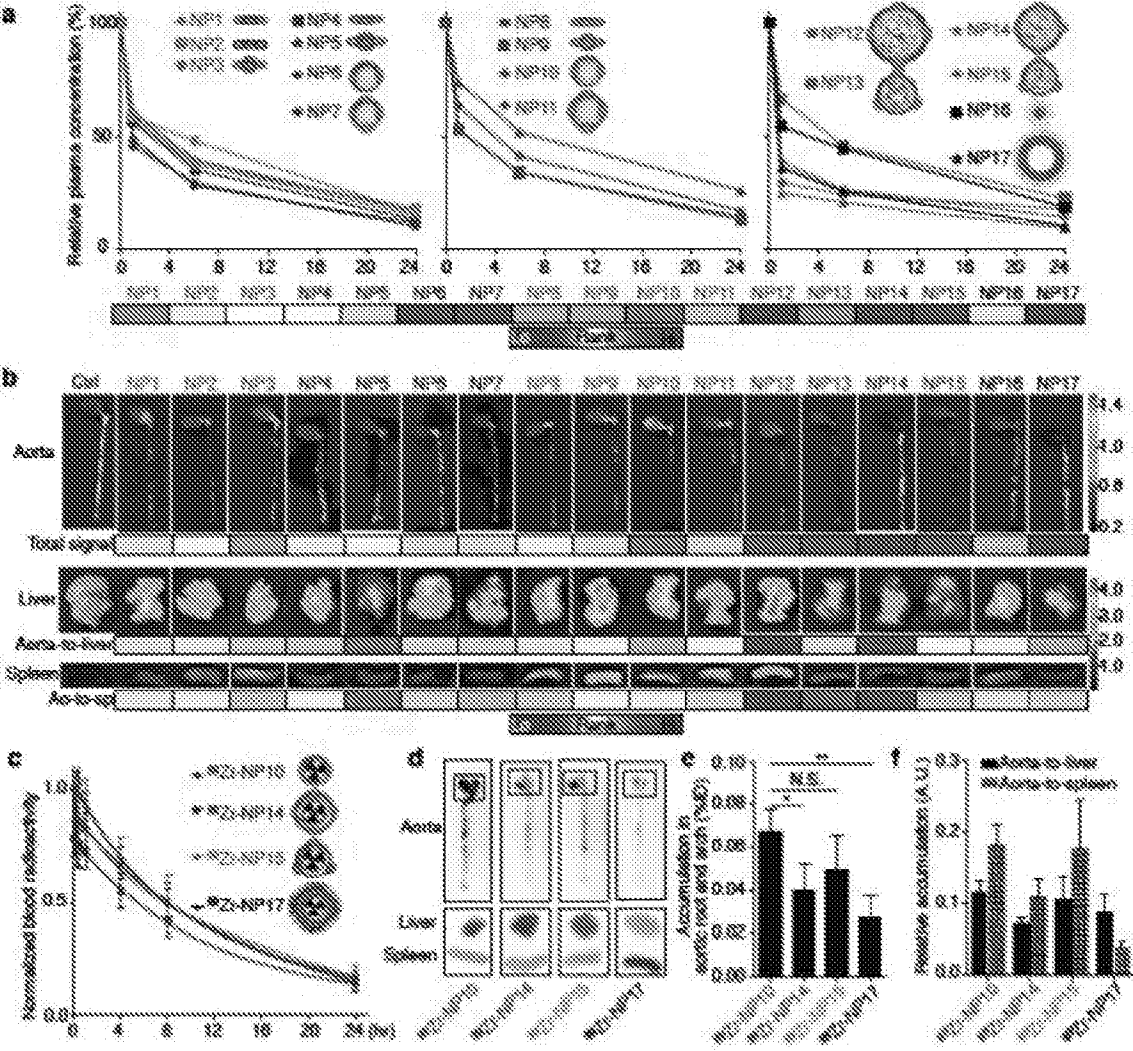
19. A method for treating a subject with atherosclerosis, the method comprising administering the nanoparticle of any one of claims 1-17 or the nanoparticle produced according to the method of claim 18 to the subject such that the nanoparticle promotes cholesterol efflux from macrophages in atherosclerotic plaques in the subject.
20. A nanoparticle comprising phospholipids, apolipoprotein A-1 (APOA1), a polymer, and a core component, wherein the phospholipids comprise 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1-myristoyl-2-hydroxy-*sn*-glycero-phosphocholine (MHPC) and the ratio of DMPC to MHPC is 10:1 to 1:1; the ratio of the phospholipids to APOA1 is 1:1 to 4:1; the ratio of the phospholipids to polymer is 0.5:1 to 1:10; and the ratio of the phospholipids to the core component is 1:1 to 40:1.
21. The nanoparticle of claim 20, wherein the polymer is polylactic acid (PLA).
22. The nanoparticle of claim 20, wherein the core component is a therapeutic agent.
23. The nanoparticle of claim 22, wherein the therapeutic agent is a small molecule, a small biologic, or a low molecular weight therapeutic .
24. A method for making the nanoparticle of any one of claims 20-23, the method comprising a) generating a homogenized solution comprising the phospholipids, apolipoprotein A-1 (APOA1), the polymer, and the core component at the ratios indicated and under conditions such that nanoparticles form, and b) recovering the nanoparticles.
25. A method for treating a subject with atherosclerosis, the method comprising administering the nanoparticle of any one of claims 20-23 or the nanoparticle produced according to the method of claim 24 to the subject such that the nanoparticle promotes cholesterol efflux from macrophages in atherosclerotic plaques in the subject.

Figures 1A-D

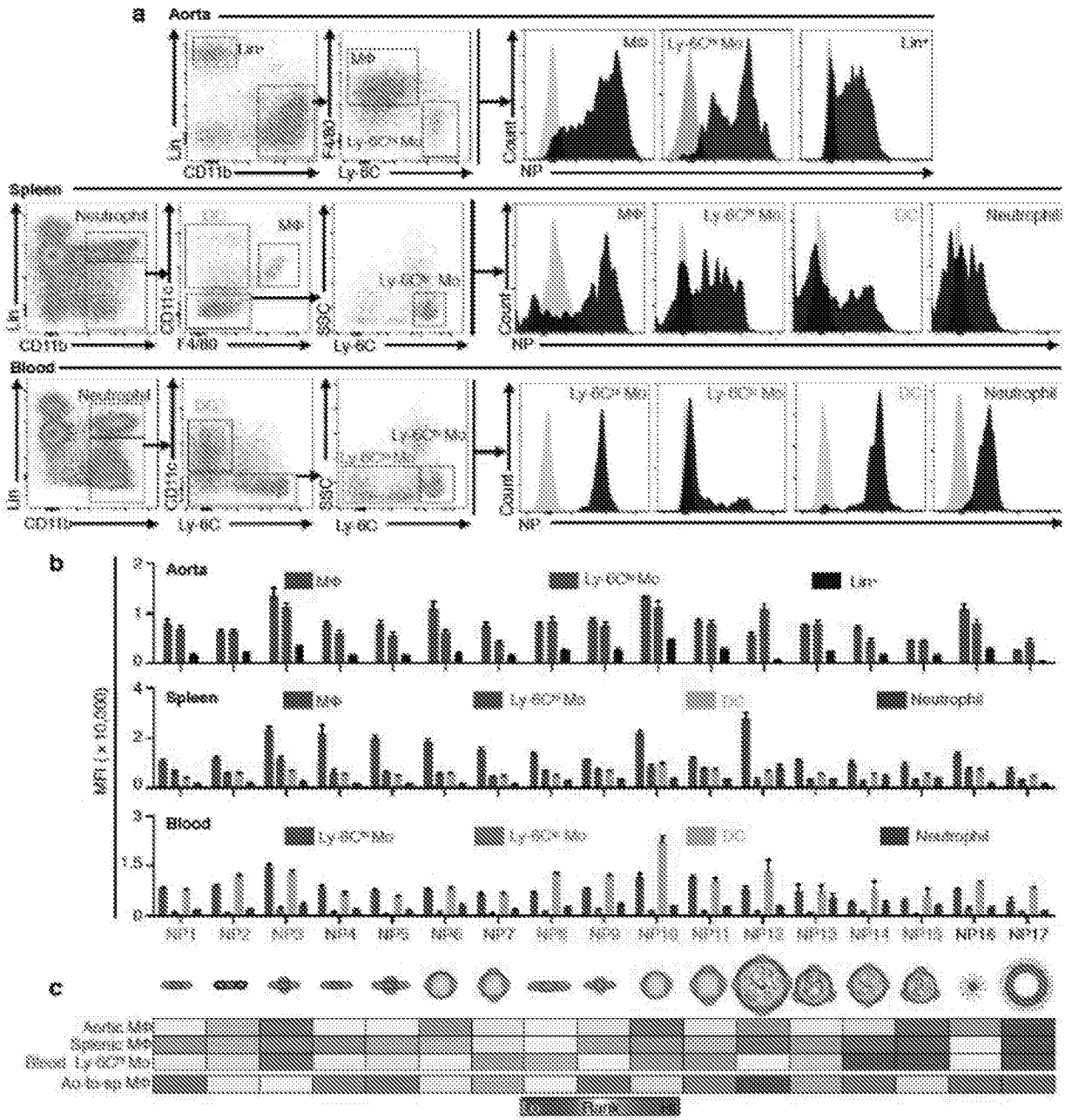


2/17

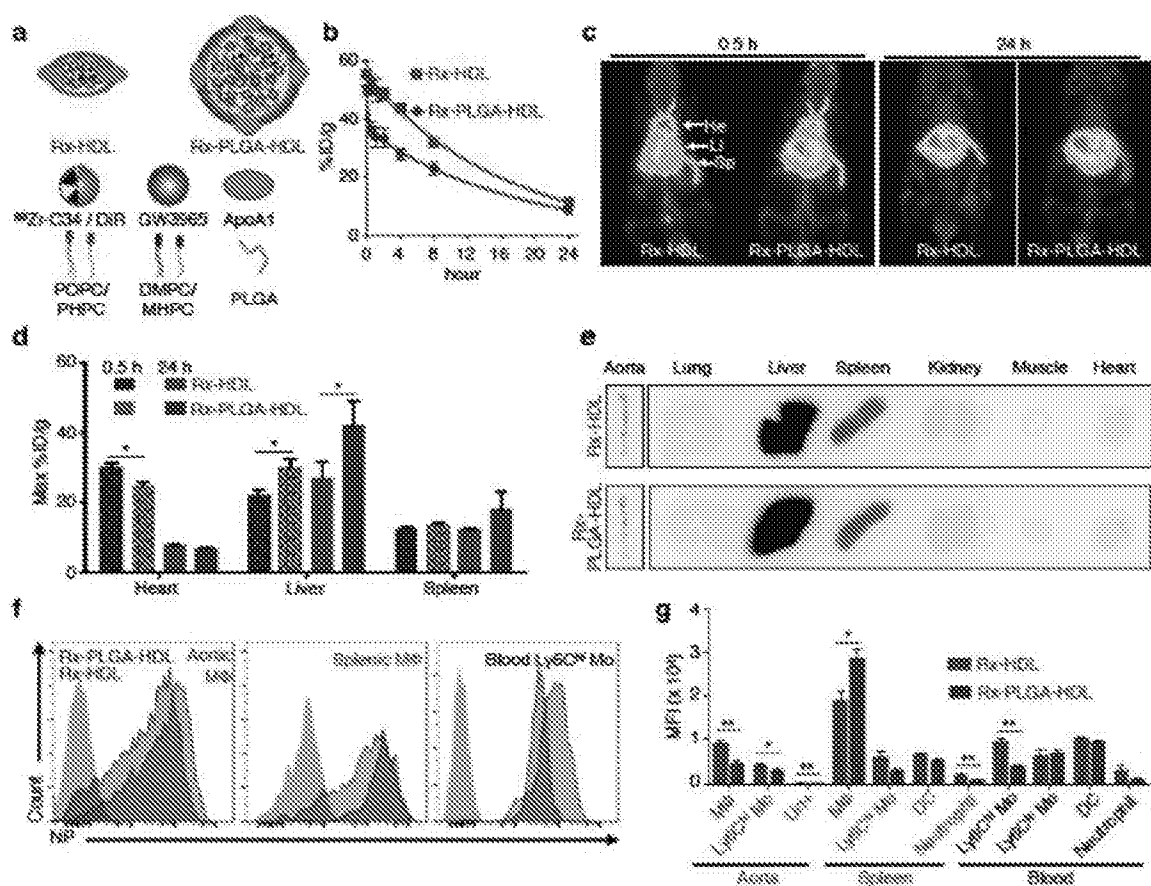
Figures 2A-F



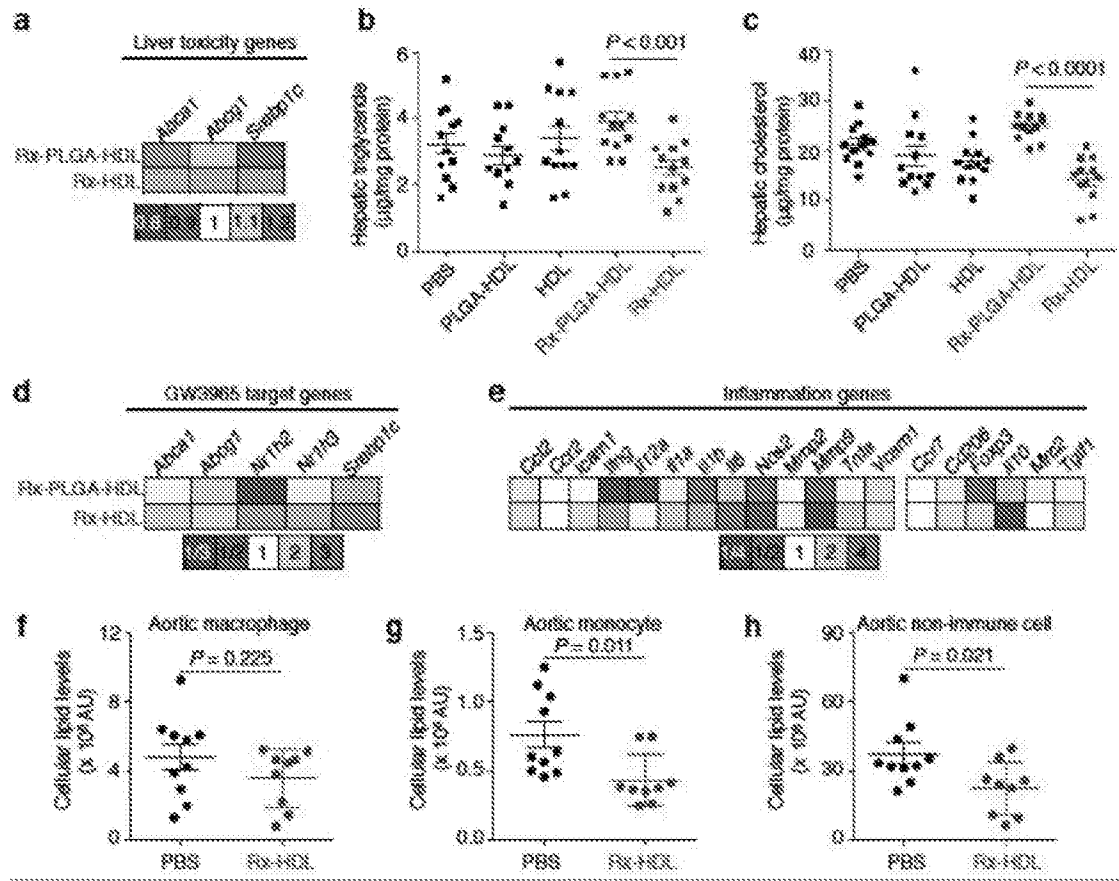
Figures 3A-C



Figures 4A-G



Figures 5A-H



6/17

Figure 6

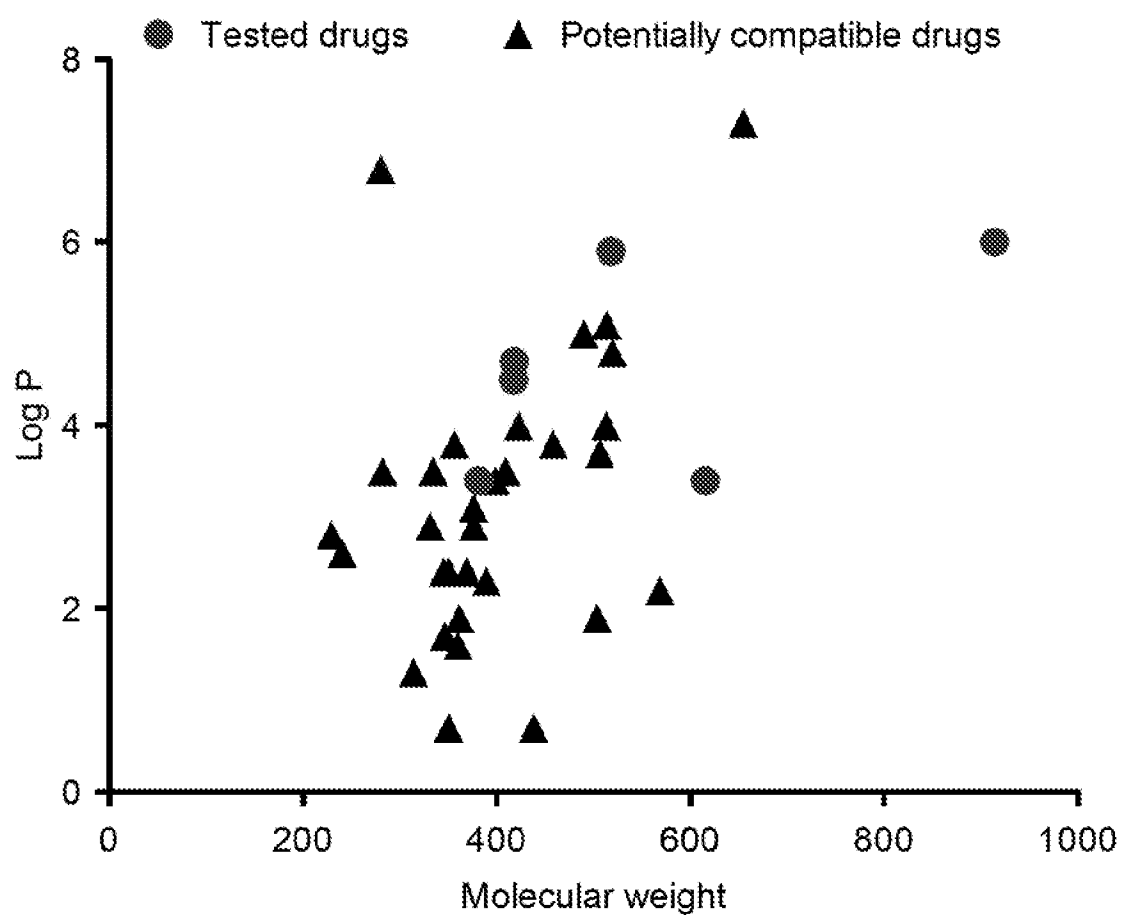


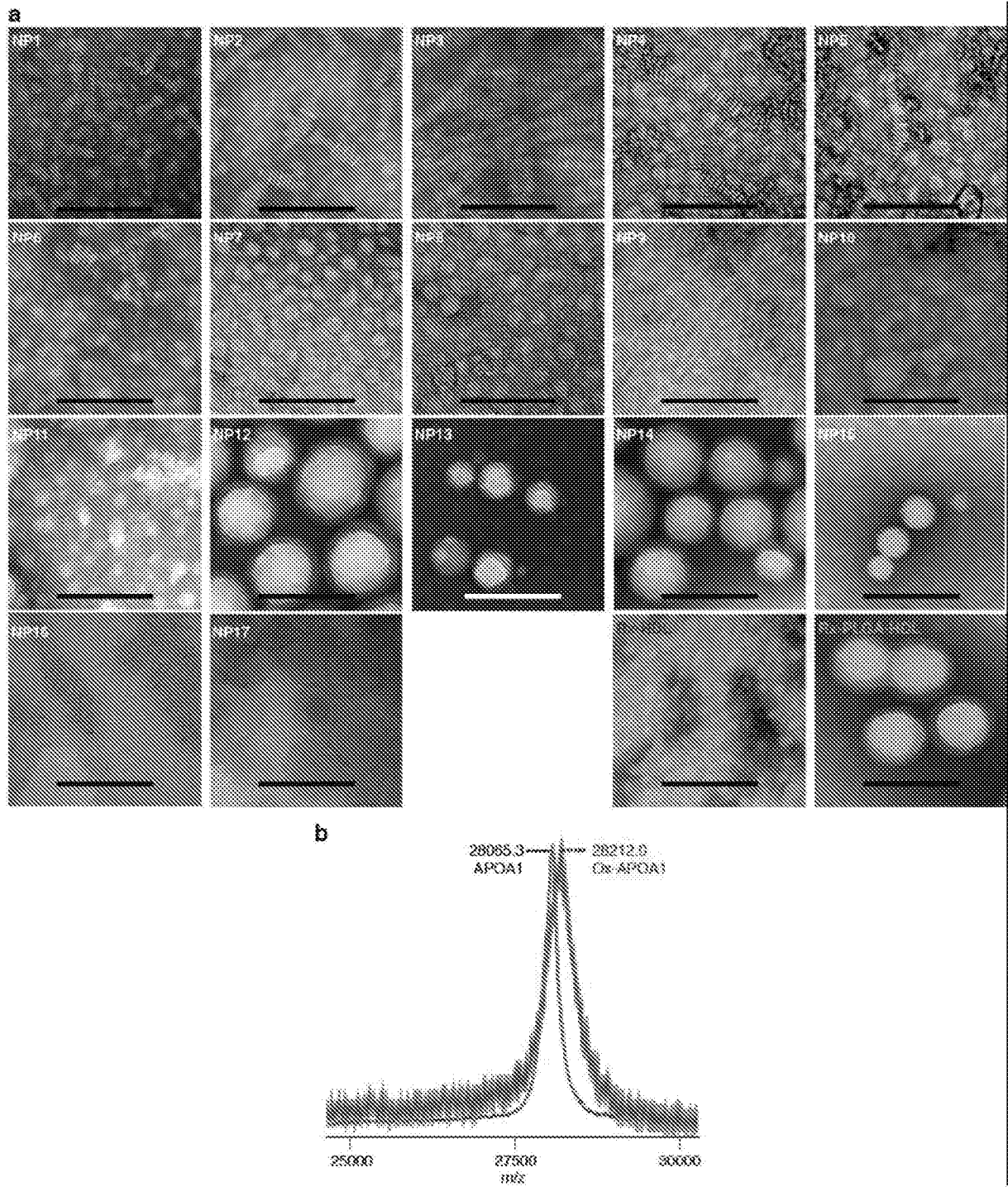
Figure 7: Table 1

Category	No	Name	Application	M.W.	LogP	log D (pH=7.4)	Serum	Link
Tested compound	1	Simvastatin	Cardiology	418.6	4.7	4.55		https://pubchem.ncbi.nlm.nih.gov/compound/54454#section=Chemical-and-Physical
	2	GW3965	Cardiology	518.2	5.9	4.29		https://pubchem.ncbi.nlm.nih.gov/compound/447905#section=Chemical-and-Physical
	3	Celecoxib	Cardiology	381.37	3.4	3.02		https://pubchem.ncbi.nlm.nih.gov/compound/2662#section=Top
	6	Rapamycin	Cardiology (heart transplant)	914.2	6	5.13		https://pubchem.ncbi.nlm.nih.gov/compound/25151352#section=Chemical-and-Physical
	4	PLX3397	Cancer immunotherapy	417.8	4.5	4.17		https://pubchem.ncbi.nlm.nih.gov/compound/11707110#section=Chemical-and-Physical
	5	Trametinib	Cancer immunotherapy	615.4	3.4	2.19		https://pubchem.ncbi.nlm.nih.gov/compound/4829#section=
	1	Pioglitazone	Cardiology	356.4	3.8	1.14		https://pubchem.ncbi.nlm.nih.gov/compound/53477497#section=Chemical-and-Physical
	2	Resolvin D3	Cardiology	376.5	2.9	-0.69		https://pubchem.ncbi.nlm.nih.gov/compound/11383310#section=Chemical-and-Physical
	3	Resolvin D2	Cardiology	376.5	3.1	-0.36		https://pubchem.ncbi.nlm.nih.gov/compound/53921890#section=Chemical-and-Physical
	4	18S-Resolvin E1	Cardiology	350.4	2.4	-0.95		https://pubchem.ncbi.nlm.nih.gov/compound/16126783#section=Chemical-and-Physical
	5	AT-Resolvin D1	Cardiology	376.5	3.1	-0.36		https://pubchem.ncbi.nlm.nih.gov/compound/16061125#section=Chemical-and-Physical
	6	Resolvin E2	Cardiology	334.4	3.5	0.09		https://pubchem.ncbi.nlm.nih.gov/compound/5280450#section=Chemical-and-Physical
	7	Linoleic Acid	Cardiology	280.4	6.8	3.95		https://pubchem.ncbi.nlm.nih.gov/compound/57469#section=Chemical-and-Physical
	8	Imiquimod	Cancer immunotherapy	240.3	2.6	2.1		https://pubchem.ncbi.nlm.nih.gov/compound/159603#section=Chemical-and-Physical
	9	Resiquimod	Cancer immunotherapy	314.4	1.3	2.43		https://pubchem.ncbi.nlm.nih.gov/compound/42611257
	10	Vemurafenib	Cancer immunotherapy	489.9	5	3.2		https://pubchem.ncbi.nlm.nih.gov/compound/5755#section=Chemical-and-Physical
	11	Prednisolone	Cancer immunotherapy	360.2	1.6	1.7		https://pubchem.ncbi.nlm.nih.gov/compound/74892459#section=Depositor-Supplied
	12	Epacadostat	Cancer immunotherapy	438.2	0.7	0.91		

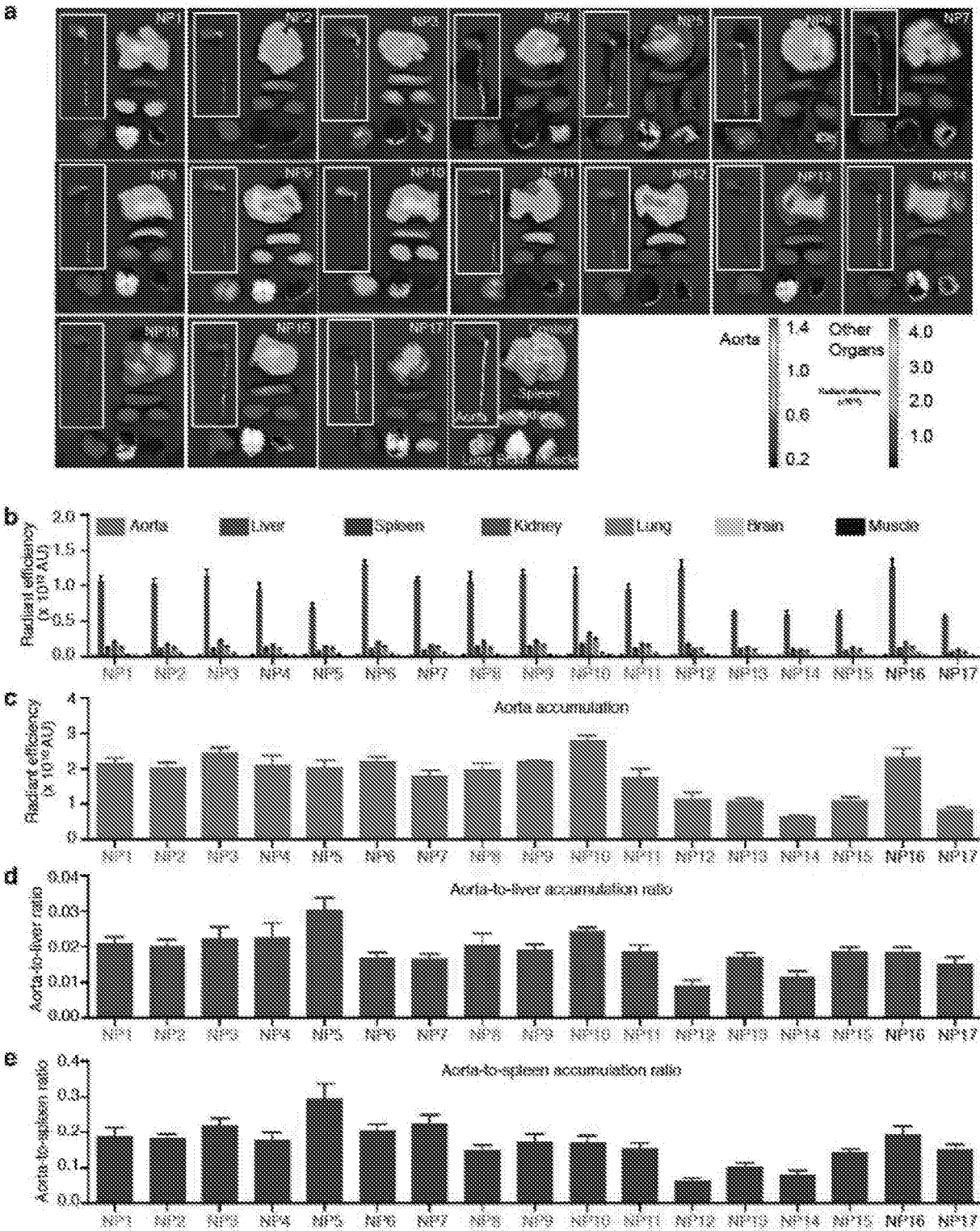
Figure 7: Table 1 (cont)

13 NLG919	Cancer Immunotherapy	282.38	3.5	3.59	https://pubchem.ncbi.nlm.nih.gov/compound/66558287#section=Chemical-and-Physical-https://pubchem.ncbi.nlm.nih.gov/compound/91885138#section=Chemical-and-Physical-https://pubchem.ncbi.nlm.nih.gov/compound/119032#section=Chemical-and-Physical-https://pubchem.ncbi.nlm.nih.gov/compound/110635#section=Chemical-and-Physical-https://pubchem.ncbi.nlm.nih.gov/compound/10310632#section=Chemical-and-Physical-https://pubchem.ncbi.nlm.nih.gov/compound/176408#section=Chemical-and-Physical-https://pubchem.ncbi.nlm.nih.gov/compound/10117987#section=Chemical-and-Physical-https://pubchem.ncbi.nlm.nih.gov/compound/5311479#section=Chemical-and-Physical-https://pubchem.ncbi.nlm.nih.gov/compound/25114442#section=Chemical-and-Physical-https://pubchem.ncbi.nlm.nih.gov/compound/5312129#section=Chemical-and-Physical-https://pubchem.ncbi.nlm.nih.gov/compound/11192346#section=InChI-Keyhttps://pubchem.ncbi.nlm.nih.gov/compound/3002977#section=Depositor-Supplied-https://pubchem.ncbi.nlm.nih.gov/compound/10309114#section=Depositor-Provided-https://pubchem.ncbi.nlm.nih.gov/compound/16049404#section=Tophttps://pubchem.ncbi.nlm.nih.gov/compound/10090485#section=Depositor-Supplied-https://pubchem.ncbi.nlm.nih.gov/compound/42611257#section=IUPAC-Namehttps://pubchem.ncbi.nlm.nih.gov/compound/44462760#section=InChIhttps://pubchem.ncbi.nlm.nih.gov/compound/24794418#section=Names-and-Identifiershttps://pubchem.ncbi.nlm.nih.gov/compound/46184986#section=Tophttps://pubchem.ncbi.nlm.nih.gov/compound/24937012#section=Depositor-Supplied-https://pubchem.ncbi.nlm.nih.gov/compound/10427712#section=Names-and-Identifiers
14 LM10 (inhibitor)	Cancer Immunotherapy	229.21	2.8	2.13	
15 NCX_4016 (Nitroaspirin)	Cancer Immunotherapy	331.27	2.9	3.68	
16 Tadalafil	Cancer Immunotherapy	389.4	2.3	2.62	
17 AZ10606120(CHEM81562308)	Cancer Immunotherapy	422.56	4	2.66	
18 SCH58261	Cancer Immunotherapy	345.358	2.4	1.65	
19 SCH420814 (Preladenant)	Cancer Immunotherapy	503.55	1.9	0.96	
20 PSB1115	Cancer Immunotherapy	350.3	0.7	-4.07	
21 PF_04418948	Cancer Immunotherapy	409.4	3.5	-0.65	
22 RQ_15986(EP4A)	Cancer Immunotherapy	654.7	7.3	3.39	
23 PF_4136309	Cancer Immunotherapy	568.59	2.2	1.18	
24 Maraviroc	Cancer Immunotherapy	513.66	5.1	1.95	
25 852A (UNIL-6PJC3KPK6S)	Cancer Immunotherapy	361.46	1.9	1.58	
26 VTX_2337 (Motolimod)	Cancer Immunotherapy	458.59	3.8	3.71	
27 LY2157299	Cancer Immunotherapy	369.1	2.4	3.66	
28 Vemurafenib	Cancer Immunotherapy	489.9	5	3.2	
29 Dabrafenib	Cancer Immunotherapy	519.56	4.8	3.21	
30 BMS_777607	Cancer Immunotherapy	512.89	4	3.07	
31 BI2945	Cancer Immunotherapy	398.47	3.4	2.08	
32 PI_3065	Cancer Immunotherapy	506.6	3.7	2.62	
33 TG100_115	Cancer Immunotherapy	346.3	1.7	1.88	

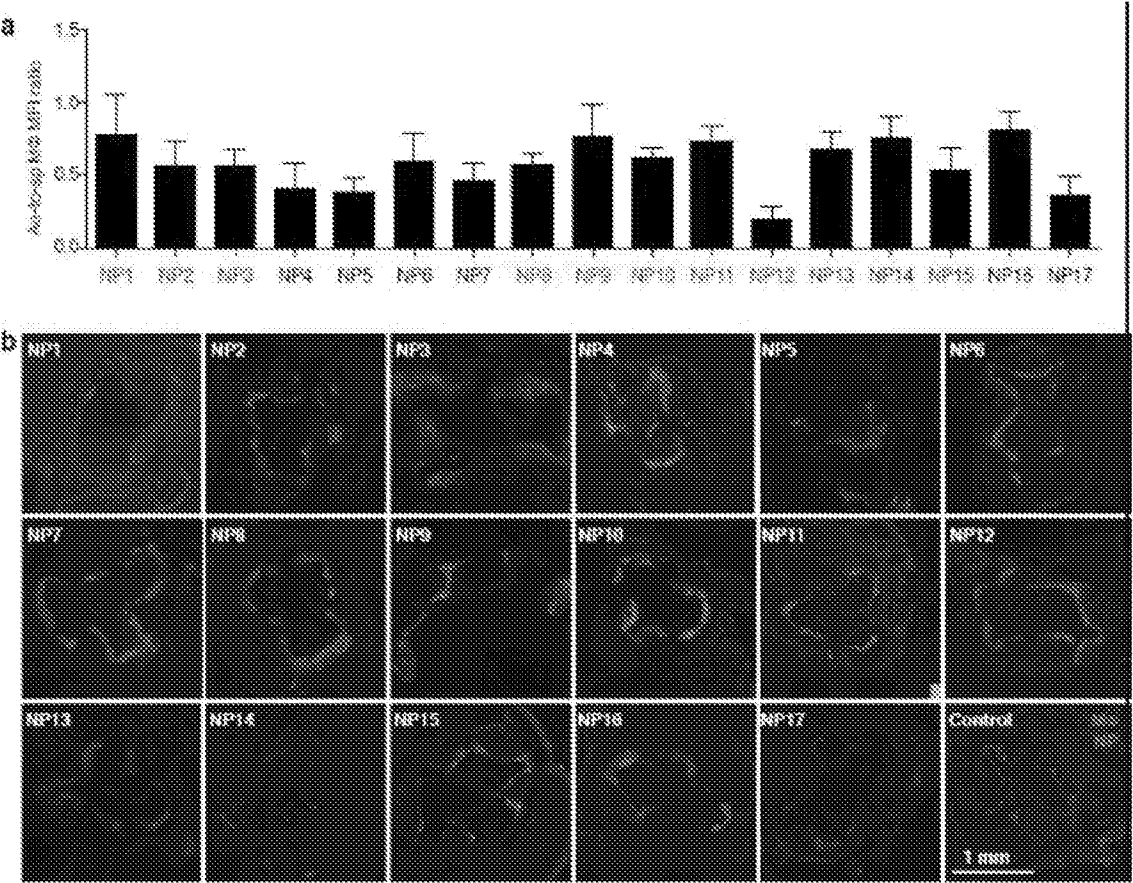
Supplementary Figure 1A-B



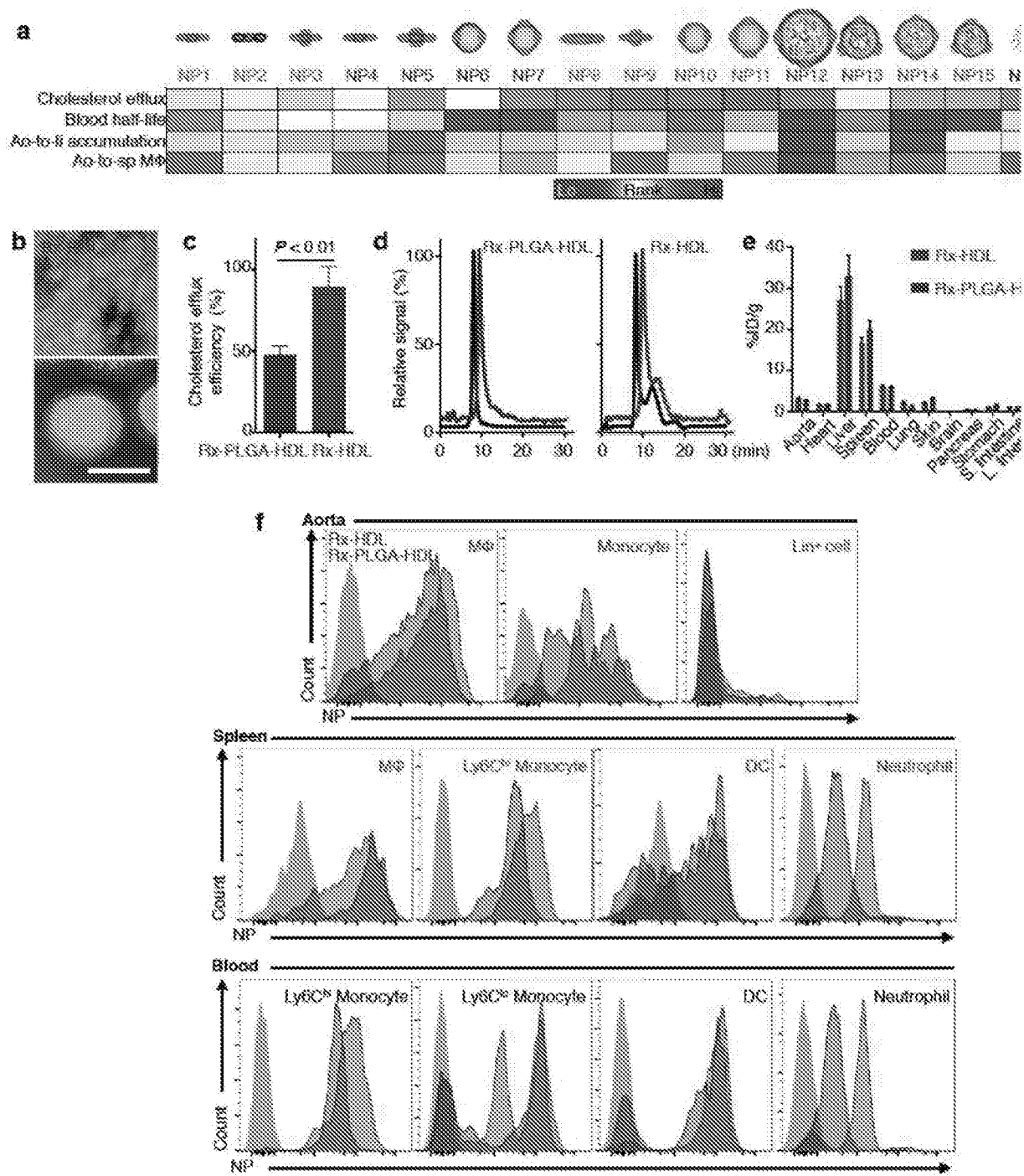
Supplementary Figure 2A-E



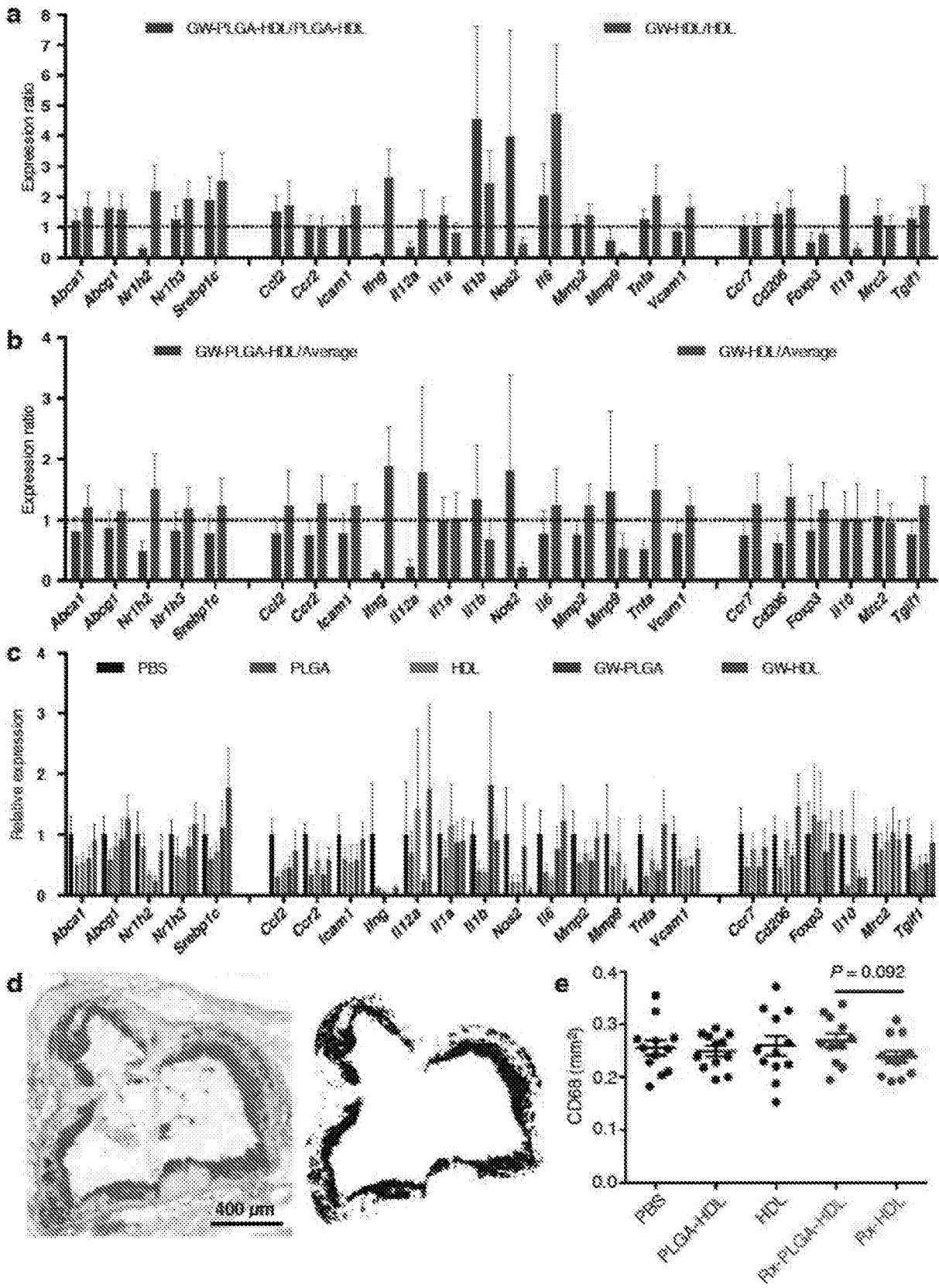
Supplementary Figure 3A-B



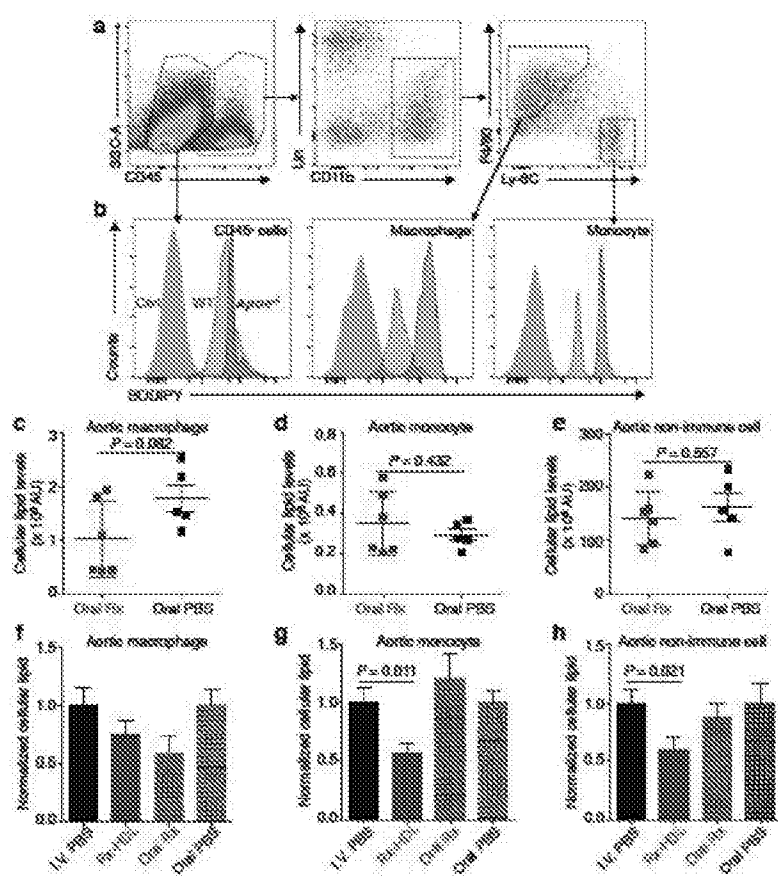
Supplementary Figure 4A-F



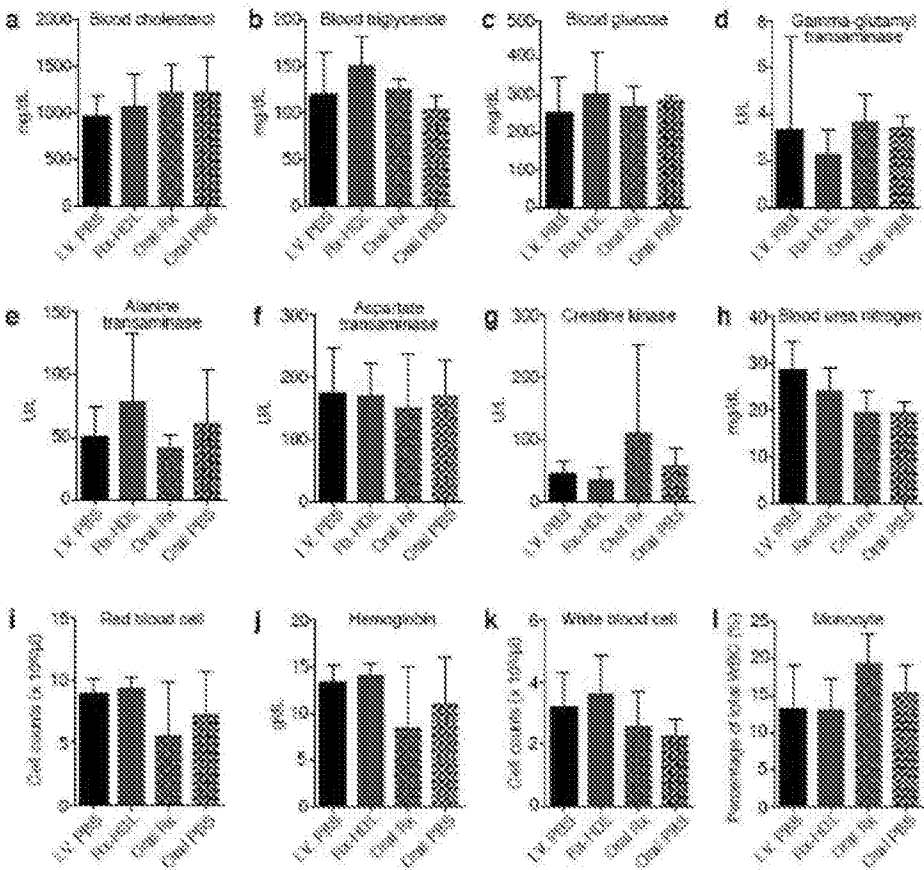
Supplementary Figure 5A-E



Supplementary Figure 6A-H



Supplementary Figure 7A-L



16/17

Supplementary Figure 8

Supplementary table 1. The chemical composition of the nanoparticle library

Nanoparticle	1	2 ^a	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Schematic cartoon																	
Composition																	
Phospholipids (weight ratio)	DMPC MHPC (9:1)	DMPC MHPC (9:1)	DMPC MHPC (9:1)	DMPC	DMPC	DMPC	DMPC	POPC PIIPC (9:1)	POPC PIIPC (9:1)	POPC PIIPC (9:1)	POPC PIIPC (9:1)	DMPC MHPC (2:1)	DMPC MHPC (2:1)	DMPC MHPC (2:1)	DMPC MHPC (2:1)	N/A	DTPC Chol
PL-to-APOA1 weight ratio	2.5	2.5	1.25	2.5	1.25	2.5	1.25	2.5	1.25	2.5	1.25	3.2	3.2	3.2	3.2	N/A	N/A
PL-to-TG weight ratio	N/A	N/A	N/A	N/A	N/A	2	2	N/A	N/A	2	2	N/A	N/A	N/A	N/A	N/A	N/A
Polymer	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	PLA	PLA	PLGA	PLGA	N/A	N/A
PL-to-polymer weight ratio	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1	1	1	1	N/A	N/A
PEGylated PL	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	DSPE- PEG- 2000	DSPE- PEG- 2000
PEGylated PL percentage (%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	100	25
PL-to-cholesterol weight ratio	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	4.5

Abbreviations: N/A, not available; PL, phospholipid; TG, triglyceride; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; MHPC, 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PHPC, 1-palmitoyl-2-hexadecyl-*sn*-glycero-3-phosphocholine; Chol, cholesterol; PLA, polylactic acid; PLGA, poly(lactic-co-glycolic acid); DSPE-PEG2000, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000].

^aAPOA1 of nanoparticle 2 were oxidized by a chemical process.

Supplementary Figure 9

Supplementary table 2. Blood half-lives of nanoparticle library.

Nanoparticle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Blood half-life (hr)	5.01	2.99	2.49	2.53	3.47	0.97	0.92	1.73	1.70	6.26	3.99	0.71	5.00	0.67	0.98	3.24	0.78

Blood half-life values were determined from 5 mice per nanoparticle.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/56149

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 9/14; A61K 9/51; A61K 31/195; A61K 31/395 (2017.01)

CPC - A61K 9/145; A61K 9/146; A61K 9/5153; A61K 9/5192; Y10T 428/2982; A61K 31/195; A61K 31/395

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 -- Y -- A	US 2015/0182461 A1 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 02 July 2015 (02.07.2015) para [0094]-[0096], [0117]-[0118], [0233], [0290]-[0292], Table 4; para [0308]-[0309]	17, 18/17 ----- 20-24 ----- 1-16, 18/(1-16) ----- 20-24 ----- 1-16, 18/(1-16)
Y --- A	WO 2016/106328 A1 (UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC.) 30 June 2016 (30.06.2016) para [0005], [0070], [0157], [0170], Fig 1	1-16, 18/(1-16)
A	US 2014/0328759 A1 (THE UNIVERSITY OF BRITISH COLUMBIA) 06 November 2014 (06.11.2014) para [0006], [0099], [0109]	1-16, 18/(1-16)
A	DUCLOS, 'Synthesis of 1-palmitoyl-2-hexadecyl-sn-glycero-3-phosphocholine (PHPC)', Chemistry and Physics of Lipids, 1993, Vol.66, pp 161-170. abstract; pg 162, Fig 1.	1-16, 18/(1-16)
A	US 8,252,775 B2 (KANTER et al.) 28 August 2012 (28.08.2012) claim 3	1-16, 18/(1-16)

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

21 November 2017

Date of mailing of the international search report

29 DEC 2017

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/56149

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

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

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

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

3. ☒ Claims Nos.: 19 and 25
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.