Translating lipid-driven inflammation in atherosclerosis

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CHAPTER 10

LIPOSOMAL PREDNISOLONE AGGRAVATES ATHEROSCLEROSIS
BY PROMOTING MACROPHAGE LIPOTOXICITY


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ABSTRACT

Rationale: Atherosclerosis is a lipid-driven inflammatory disease, for which novel anti-inflammatory strategies are under evaluation. Prednisolone is a potent anti-inflammatory compound, yet, potential proatherogenic effects have been observed upon targeting of plaque macrophages using liposomal nanoparticles loaded with prednisolone phosphate (LN-PLP) in patients with atherosclerosis. We aimed to unravel the direct effects of LN-PLP on macrophages residing in the lipid-rich environment of atherosclerosis.

Methods and Results: In low-density lipoprotein receptor knockout (LDLr⁻/⁻) mice fed a high-fat diet, we show that LN-PLP accumulates in plaque macrophages. Biweekly injections of LN-PLP at 10mg/kg induces (i) enhanced monocyte recruitment to plaques after 2 weeks, followed by (ii) increased macrophage content, more advanced plaque stages, and larger necrotic core sizes after 6 weeks of LN-PLP administration. In vitro, we observed that both murine and human macrophages polarize into a lipid-avid phenotype after LN-PLP exposure, leading to increased lipid accumulation, endoplasmatic reticulum (ER) stress and late apoptosis.

Conclusion: These findings indicate that macrophage targeting in plaques with prednisolone may accelerate atherosclerosis by promoting macrophage lipotoxicity, thereby highlighting the challenges of anti-inflammatory therapy in atherosclerosis.
INTRODUCTION

Atherosclerosis is a chronic disease of the arterial wall, characterized by a lipid-rich, low-grade inflammatory milieu. Despite the success of lifestyle changes and statins in cardiovascular prevention and treatment, disability and death from cardiovascular disease (CVD) is still increasing worldwide. Consequently, novel anti-atherosclerotic strategies, amongst which anti-inflammatory compounds, are being assessed. Glucocorticoids are potent anti-inflammatory agents, yet, long-term and systemic administration in CVD patients is considered undesirable due to systemic adverse effects.

Nanotechnology holds a promise by increasing drug accumulation in target tissues, while reducing systemic exposure. In support of this concept, liposomal nanoparticles loaded with prednisolone phosphate (LN-PLP) were shown to accumulate in aortic lesions of rabbits, of which a high degree of LN-PLP co-localized with lesional macrophages. Moreover, LN-PLP rapidly reduced the inflammatory activity of atherosclerotic lesions in rabbits. In humans, the accumulation of LN-PLP in plaque macrophages isolated from patients after intravenous infusion was also found. However, in patients with advanced atherosclerosis, LN-PLP treatment tended to increase the degree of arterial wall inflammation.

To elucidate the impact of LN-PLP on plaque macrophages in advanced atherosclerosis, we first addressed its effect in low-density lipoprotein receptor knockout (LDLr⁻/⁻) mice, characterized by lipid-rich and macrophage-rich plaques. We found an increased number of inflammatory cells in the plaque after LN-PLP infusions for 2 weeks. In line with this initial proinflammatory effect, we next show that prolonged exposure to LN-PLP for 6 weeks induced more advanced plaque stages in this atherosclerotic mouse model. Subsequent in vitro studies in both murine and human macrophages corroborated that LN-PLP induced lipotoxic effects in macrophages residing in a lipid-rich environment.

METHODS

Nanoparticle formulations

The empty liposomal nanoparticles (LN) and liposomal prednisolone phosphate (LN-PLP) were formulated as previously described and detailed in the Supplementary material.

LDLr⁻/⁻ mice studies

In vivo studies, as illustrated in Figure S1, were performed in 8 weeks old LDLr⁻/⁻ mice on a C57BL/6 background purchased from Jackson Laboratories, fed a high fat diet (HFD; Hope Farms) containing 0.15% (w/w) cholesterol, 16% fat (w/w) and no cholate for 6 weeks. All animal experiments were approved by the Committee for Animal Welfare of Amsterdam.
Medical Centre or Mount Sinai New York and were carried out in compliance with guidelines issued by the local governments.

**Biodistribution of LN-PLP: LDLr^-/-** mice were sacrificed 24 hours after tail vein injection of either PBS (n=4) or 10mg/kg cy5.5-labelled liposomal prednisolone (LN-PLP; n=4)\textsuperscript{11}. LN-PLP uptake was assessed with near infrared fluorescence (NIRF) imaging and flow cytometric analysis of blood, spleen and aortic arches, as described in the Supplementary methods.

**LN-PLP efficacy studies:** Phosphate-buffered saline (PBS), free prednisolone (PLP, only in 6 weeks drug study), empty liposomes (LN) or LN-PLP tail vein injections of 10mg/kg were given twice a week for either 2 or 6 weeks (n=66 and n=64, respectively). Efficacy of LN-PLP was assessed using cytochemical and immunohistochemical stainings of aortic roots or arches, flow cytometric assays and mRNA expression in aortic arches.

**In vitro macrophage assays**

Bone marrow cells were isolated from both femurs and tibiae of wild-type mice (C57BL/6; n=2). Cells were cultured in RPMI-1640 with 100U/ml penicillin/streptomycin, 10% fetal bovine serum (FBS; all GIBCO Invitrogen) and 15% L929 conditioned medium (LCM) for 8 days to generate bone marrow-derived macrophages (BMDM) as previously described\textsuperscript{12}. BMDM of two mice were pooled and seeded at a density of 0.15x10\(^6\) cells/cm\(^2\) 24 hours prior to stimulation. THP-1 monocytes (ATCC) were cultured in RPMI-1640 supplemented with 100U/ml penicillin/streptomycin and 10% FBS. Cells were differentiated into macrophages by adding 50ng/ml phorbol-12-myristate-13-acetate (PMA) for 24 hours. RAW264.7 stable cell line containing the 3x-NFκB-luc plasmid\textsuperscript{13} was cultured in DMEM-high glucose (GIBCO Invitrogen) containing 100U/ml penicillin/streptomycin and 10% FBS. For each in vitro experiment, cells were stimulated with a low (20μM) and high (80μM) dose of PLP. LN-PLP was given at a concentration of 10μg/ml (equivalent to 20μM PLP) or 40μg/ml (equivalent to 80μM PLP). The empty liposomes (LN) were added in the same liposomal concentrations as LN-PLP. Macrophage assays comprised of immunostaining, NFκB transcriptional activity, mRNA gene expression, cholesterol efflux, oil-red-O staining and flow cytometric assays.

**Statistical analysis**

All experiments were at least performed in duplicate. Statistical analyses were performed using GraphPad Prism version 5 (San Diego, CA). An unpaired t-test (Welch corrected when necessary) or Mann Whitney test was used to define differences between 2 groups. For differences in plaque stage, the Chi2 test was used. Statistical significance between the 3 or 4 treatment groups was assessed using the 1-way ANOVA. Data are presented as mean ± SEM. The significance level was set at p<0.05.
RESULTS

LN-PLP accumulates in plaque macrophages

Near infrared fluorescence (NIRF) imaging substantiated accumulation of LN-PLP in the atherosclerotic plaques located in the aortas of LDLr<sup>−/−</sup> mice, 24 hours after tail vein injection (Figure 1A). In line with previous experiments<sup>16</sup>, LN-PLP was also found in the liver, lung, spleen and kidney (Figure 1A). Flow cytometry assays showed that LN-PLP was predominantly taken up by myeloid cells, whereas not by non-myeloid cells (Figure 1B). In the circulating and splenic myeloid cells, the highest uptake was found in proinflammatory Ly6C<sup>+</sup> monocytes and macrophages (Figure 1B). Also in the aortic arch, uptake of LN-PLP in macrophages and monocytes was demonstrated (Figure 1C).

![A] NIRF imaging

![B] Flow cytometry spleen and blood

**FIGURE 1.** LN-PLP uptake in LDLr<sup>−/−</sup> mice

(A) NIRF images showing accumulation and distribution of Cy5.5-labelled LN-PLP (n=4) or PBS (n=4) 24 hours after tail vein injection (10mg/kg) in 8-week old LDLr<sup>−/−</sup> mice, 6 weeks on HFD. (B,C) Cellular distribution of LN-PLP in spleen, blood and aorta was assessed by flow cytometry. Data are presented as mean ± SEM. HFD indicates high fat diet; LN-PLP, liposomal prednisolone; NIRF, near infrared fluorescence; PBS, phosphate-buffered saline.
**FIGURE 1.** Continued

(B,C) Cellular distribution of LN-PLP in spleen, blood and aorta was assessed by flow cytometry. Data are presented as mean ± SEM. HFD indicates high fat diet; LN-PLP, liposomal prednisolone; NIRF, near infrared fluorescence; PBS, phosphate-buffered saline.

## Two weeks LN-PLP induces monocyte recruitment

To evaluate drug effects in lipid-rich atherosclerotic plaques, we fed 8-week old LDLr⁻/⁻ mice a high fat diet (HFD) for 6 weeks inducing substantial atherosclerosis in both the arches and roots (baseline; Figure 2A,B). Followed by 2 weeks administration of LN-PLP, empty liposomes (LN) or PBS (10 mg/kg, biweekly), no difference in plaque size was observed between the treatment groups (Figure 2A-B). However, the percentage of proinflammatory monocytes was significantly higher in aortic arches of the LN-PLP mice (11±6%) compared with LN (5±3%, p<0.05) and PBS (6±3%, p<0.01; Figure 2C). To analyse the recruitment of monocytes to the lesions, we assessed the expression of ER-MP58; a characteristic of circulating immature myeloid cells, which is lost upon differentiation into macrophages. Immunostaining of the roots showed a significantly higher positivity for ER-MP58 after LN-PLP (24±11%) compared with LN (13±4%, p<0.001) and PBS (17±8%, p<0.05; Figure 2D), which indicates an increased presence of freshly influxed monocytes. No increase in the number of blood monocytes was observed after LN-PLP (Figure 2E). Of note, the body weight in LN-PLP treated mice was lower, whereas plasma cholesterol levels among the main lipoprotein classes were not different between groups (Figure S2A,B). Also, RBCs, platelets and haematocrit were similar between groups (Figure S2C).
FIGURE 2. LN-PLP induces monocyte recruitment

(A,B) Plaque size was quantified in (A) the aortic arches (original magnification x25; scale bars represent 1mm) and (B) the roots using Hematoxylin-Eosin (HE) staining, 6 weeks after HFD (baseline, n=6) and an additional 2 weeks with PBS, LN or LN-PLP iv administration (n=20/group, 10mg/kg, biweekly). (C) Pro-inflammatory monocytes were assessed by flow cytometry of the arches, and (D) % of the lesion positive for ER-MPS8 staining, indicative of freshly recruited monocytes in the roots, (E) the number of blood monocytes. Data are presented as mean ± SEM; *P<0.05, **P<0.01. LN indicates empty liposomes; LN-PLP, liposomal prednisolone; PBS, phosphate-buffered saline.
Six weeks LN-PLP aggravates atherosclerotic plaques

To assess whether increased monocyte influx after a 2-week LN-PLP administration translated into accelerated atherogenesis, we performed an additional experiment using a similar approach in 8-week old LDLr−/− mice fed a HFD for 6 weeks, yet, now receiving 6 weeks of intravenous LN-PLP, LN, PLP or PBS (10 mg/kg, biweekly). After the start of LN-PLP body weight decreased, whereas plasma cholesterol levels and circulating blood cells were not different between groups (Figure S3A-C). Plaque size was not different between groups (Figure 3A), yet, plaque stage was more advanced 6 weeks after LN-PLP (40% of plaques in stage V) compared with PLP, LN and PBS (for all <10% of plaques in stage V, p<0.001; Figure 3B). Immunohistochemical stainings revealed an increased macrophage content after LN-PLP (25±13%) compared with PLP (14±8%, p<0.05), LN (18±7%, p<0.01) or PBS (14±5%, p<0.01; Figure 3C). In addition, plaques had lower smooth muscle cell content in the LN-PLP mice (SMCs; 7±2%) compared with PLP (10±3%), LN (11±5%) and PBS (11±4%, for all p<0.05; Figure 3D). In agreement, less collagen was present in the LN-PLP group (43±11) compared with PLP (52±20%, p<0.05), LN (62±12%, p<0.01) and PBS (61±12%, p<0.01; Figure S3D). Finally, enlarged necrotic core areas were found after LN-PLP (68±4%) compared with PLP and LN (both 61±1%, both p<0.05), and PBS (52±3%, p<0.01; Figure 3E). In addition, the mRNA expression of chemokines Mcp-1 and Sdf-1α in the aortic arches was higher after LN-PLP compared with control groups. In addition, the expression of Chop, a gene activated upon endoplasmic reticulum (ER) stress, was upregulated after LN-PLP in comparison to control groups (Figure 4).
FIGURE 3. LN-PLP aggravates atherosclerotic plaques
After 6 weeks HFD alone, followed by 6 weeks PBS, PLP, LN or LN-PLP administration (n=16/group, 10mg/kg iv, biweekly), (A) plaque size and (B) plaque stage were determined in HE stained sections of the roots. (C-E) Representative photomicrographs and quantifications (C) of macrophage content (MAC-3), (D) smooth muscle cell content (1A4) and (E) necrotic core size are shown. A,C,D original magnification x25; E, x100; scale bars represent 1mm. Data are presented as mean±SEM; *P<0.05, **P<0.01, ***P<0.001. LN indicates empty liposomes; PLP, free prednisolone; LN-PLP, liposomal prednisolone; PBS, phosphate-buffered saline.
FIGURE 4. Gene expression in aortic arches

mRNA was isolated from aortic arches of LDLr⁻/⁻ mice after 6 weeks administration of either PBS, PLP, LN or LN-PLP (n=16, 10mg/kg iv, biweekly) to analyse gene expression of monocyte migration factors, Mcp-1 and Sdf-1α, and the endoplasmatic reticulum stress marker, Chop. Gene expression was normalized to 36B4 housekeeping gene. Data are presented as mean ± SEM; *P<0.05, **P<0.01. Chop indicates C/EBP homologous protein; LN, empty liposomes; LN-PLP, liposomal prednisolone; Mcp-1, monocyte chemotactic protein-1; PBS, phosphate-buffered saline; PLP, free prednisolone; Sdf-1α, stromal cell-derived factor-1α.

LN-PLP induces macrophage lipotoxicity in vitro

In vitro, RAW264.7 NFκB-luc cells were used to assess prednisolone’s classical anti-inflammatory effect.(13) As expected, pretreatment of cells with either LN-PLP or PLP significantly decreased NFκB activity upon LPS challenge (8.6±0.8 and 9.8±0.4 for the highest dose, respectively) compared with control (23.8±3.8; for both p<0.001), whereas its activity was not influenced by empty LN (23.3±3.5; Figure 5A).

In view of the lipid-rich environment in atherosclerotic plaques, we addressed the effect of LN-PLP on lipid-handling pathways in BMDM. The expression of the major cholesterol efflux protein Abca1 was significantly diminished after LN-PLP (0.007±0.001) or PLP (0.035±0.002) compared with control (0.013±0.002; p<0.001 and p<0.01, respectively; Figure 5B). In line, the cholesterol efflux capacity of macrophages reduced after LN-PLP and PLP (0.73±0.18% and 1.34±0.26%, respectively) compared with LN (1.65±0.45) or control (2.00±0.27, p<0.01; Figure 5B). Also, BMDM lipid content was higher after LN-PLP and free PLP compared with empty LN and control (Figure 5C).
Lipsomal prednisolone promotes macrophage lipotoxicity

**FIGURE 5.** LN-PLP promotes macrophage lipotoxicity

- **A** NFκB activity was determined in RAW267.4 NFκB-luc cells as the relative fold change to control without 100ng/ml LPS stimulation.
- **B** BMDM were isolated from C57BL/6 mice to study gene expression levels of ATP-binding cassette transporter A1 (Abca1) and cholesterol efflux towards the acceptor apo-A1.
- **C** BMDM lipid content after 48 hours oxLDL (50 ug/ml) using oil-red-O stain.
- **D** Gene expressions of the intracellular lipid transporter Fabp4, and (E) ER-stress markers Perk and Chop in BMDM exposed to oxLDL (50 ug/ml) for 24 hours.
The expression of the intracellular lipid transporter *Fabp4* was also increased after both LN-PLP (0.81±0.07) and PLP (0.79±0.08), compared with control (0.47±0.05, both p<0.01), whereas after LN the *Fabp4* expression was decreased (0.09±0.01, p<0.001; Figure 5D). Increased levels of intracellular lipid trafficking by *Fabp4* have been shown to promote ER-stress and induce an unfolded protein response (UPR) \(^{16}\). Indeed, we observed that in lipid-rich macrophages LN-PLP markedly increased the mRNA expression of the ER-stress markers *Perk* (p<0.05 compared with either LN or PBS; Figure 4D) and *Chop* (p<0.05 compared with both control groups; Figure 5E). Activation of *Perk* and *Chop* are elementary in the switch from pro-survival to pro-death signalling; in line, 23% of the macrophages underwent late apoptosis and 7% necrosis after LN-PLP, as compared with <15% and <3% of the macrophages, respectively, in control conditions (Figure 5F-G).

Comparable results were observed using the human THP-1 cell line; a reduced cholesterol efflux towards apo-A1, increased *Fabp4* gene expression, and attenuated apoptosis in response to both PLP and LN-PLP (Figure S4).
Lipsomal prednisolone promotes macrophage lipotoxicity

DISCUSSION

In the present study, we show that LN-PLP administration for 2 weeks increases monocyte influx into the plaques of LDLr−/− mice. Following these early proinflammatory changes, administration of LN-PLP for 6 weeks results in an increased plaque macrophage content. In addition, prolonged LN-PLP administration aggravates plaque stage, characterized by a decreased collagen and smooth muscle cell content, as well as increased necrotic core area. Using an in vitro approach, we show that in a lipid-rich environment, LN-PLP decreases the macrophage cholesterol efflux capacity, driving lipid-induced ER-stress and subsequent apoptosis/necrosis. These findings indicate that local exposure to prednisolone elicits a proatherogenic, lipotoxic effect in plaque macrophages in LDLr−/− mice.

The early proinflammatory effect of LN-PLP in LDLr−/− mice is discordant with the previously observed reduction in inflammatory cell content in rabbit's atherosclerotic lesions only days after a single LN-PLP administration8. Explanations for this discrepancy may include differences in timing (2 days versus 2 weeks treatment), dosing (single versus multiple injections of LN-PLP) and differences in plaque composition between rabbits and mice17. The latter may be of particular relevance, since the acute inflammatory response following the double-balloon injury in the rabbit model more closely represents ‘classical’ inflammation18, explaining the beneficial impact of glucocorticoids. Conversely, the lipid-driven inflammation in LDLr−/− mice, and for that matter also in patients, reflects a chronic inflammatory disease state19.

The relevance of the early proinflammatory effect of LN-PLP is substantiated by the observation that after 6 weeks of LN-PLP administration, the number of macrophages, tissue descendants of monocytes, are higher in plaques compared with PLP, LN or PBS administration. In addition, mRNA expression of chemoattractants20, Mcp-1 and Sdf-1α, was upregulated in the aortic arches of the LN-PLP treated mice and may be an explanation for the observed increased monocyte influx and macrophage plaque content. This higher macrophage number after LN-PLP corresponds to our previous observation in patients treated with LN-PLP who exhibited a 7% increase in carotid 18F-fluorodeoxyglucose (18F-FDG) uptake9, which is a marker of plaque macrophage content21.

Since we have shown that LN-PLP is taken up by plaque macrophages in the atherosclerotic plaques, we focused on the direct effects of LN-PLP on plaque macrophages. It has been previously reported that glucocorticoids induce the repolarization of inflammatory (M1-like) macrophages towards reparative (M2-like) macrophages22. In support, we show that NFκB activity is decreased after (LN-)PLP. Interestingly, this reparative phenotype has also been reported to bear an increased vulnerability for lipid stress23, as suggested by increased expression of the intracellular lipid chaperone Fabp424 and decreased expression of the major cholesterol efflux transporter Abca125. Here, we report the consequences of these phenotypical changes in a lipid-rich environment, by showing that (LN-)PLP decreases the efflux capacity of lipid-laden macrophages, leading to accelerated foam cell formation.
Increased lipid burden in macrophages is known to promote cellular stress responses. We show that exposure of lipid-rich macrophages to (LN-)PLP augments ER-stress and increases the number of macrophages undergoing late apoptosis and necrosis.

The finding of a direct proatherogenic effect adds to previous suggestions that prednisolone predominantly has indirect adverse effects on known cardiovascular risk factors such as glucose homeostasis, blood pressure and lipids. Several observations strengthen the concept of an adverse effect of prednisolone itself on macrophages: (i) in vitro macrophages respond in a similar fashion to liposomal encapsulated PLP (LN-PLP) as to free PLP, and (ii) the lipotoxic effects of (LN-)PLP in vitro correspond to the observed proatherogenic changes in vivo, both in mice (higher number of monocytes/macrophages and more advanced plaque stages) as well as in patients with atherosclerotic plaques (increased 18F-FDG uptake).

Several limitations merit consideration. First, although we show local accumulation of LN-PLP in plaque macrophages in LDLr-/- mice, we cannot provide an absolute quantification of the drug concentration in the atherosclerotic plaques. With the advent of novel nanoparticle therapies for atherosclerosis, future studies need to address drug delivery efficiency. Second, whereas we have focused on the effects of LN-PLP on macrophages in a lipid-rich environment, we cannot exclude that other mechanisms of glucocorticoids may also have contributed to the observed proatherogenic effects. For instance, glucocorticoids have been shown to induce an upregulation of 11β-hydroxysteroid dehydrogenase type 1 (11β-Hsd1), which converts inactive glucocorticoids into active glucocorticoids. In line, 11β-Hsd1 inhibitors decrease the active intracellular glucocorticoids and attenuate atherosclerosis progression in ApoE-/- mice.

The present findings highlight the challenges of applying anti-inflammatory strategies in atherosclerosis. Thus, favourable effects of an anti-inflammatory compound in a classical inflammatory disease cannot be easily extrapolated to a comparable efficacy in the lipid-rich environment of an atherosclerotic plaque. Hence, future drug candidates need to undergo a multifaceted screening with careful consideration of the lipid-rich, atherosclerotic microenvironment.

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Society (ENDO) as well as from the “3E”: Exchange in Endocrinology Expertise program by the European Union of Medical Specialists (U.E.M.S.). All other authors declare that they have no conflict of interest and no relationships with industry relevant to this study.

REFERENCES


SUPPLEMENT

LN-PLP formulation. The liposomal nanoparticles (LN) were composed of a hydrophilic core, either empty or encapsulating prednisolone phosphate (PLP), surrounded by a lipid bilayer of phospholipids and cholesterol, which was coated with polyethylene glycol (PEG) to increase plasma half-life and reduce rapid plasma clearance of the LN through the mononuclear phagocyte system.

In vivo mice studies:

Assays for LN-PLP uptake. Hearts, aortas, livers, lungs, spleens, kidneys, and femoral muscle were collected and imaged by near infrared fluorescence imaging (NIRF) using the IN VivoSpectrum optical imaging system (PerkinElmer). Overview of the whole aortic roots was taken with 20x magnification using digital stitching of Zeiss Axioplan2IE microscope. Spleens were homogenized using a 70μM cell strainer. Before flow cytometric analysis, blood and splenic erythrocytes were lysed. Aortas were diced into small pieces and digested with an enzyme cocktail containing 4U/ml liberalase TH (Roche), 0.1mg/ml DNase I (Sigma-Aldrich), and 60U/ml hyaluronidase (Sigma-Aldrich) in PBS at 37°C for 60 minutes. The following antibodies were used: 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), Ly-6C (clone AL21), CD11b (clone M1/70), CD11c (clone N418) and F4/80 (clone BM8). DAPI stain was used to exclude dead cells from analysis. All antibodies were either purchased from eBioscience, BD Biosciences, or Biolegend. Fluorescence was detected by flow cytometry (BD Biosciences LSR II), and the data were analyzed using FlowJo software (Tree Star).

Assays for LN-PLP efficacy. After sacrifice, blood was drawn from the mice after 4 hours fasting; and the hearts and aortic arches were taken out. The hearts were cut perpendicular to the heart axis just below the atrial tips. Tissue was embedded in paraffin and cut into sections, or cryosections were made (both 7μm). The lesions were visualized using hematoxylin-eosin (H&E) stain. The aortic arches were either embedded in paraffin, snap frozen, or processed for flow cytometry. Plaque size and necrotic core were quantified using Adobe Photoshop CS5 software. Plaque stage was determined as previously described (de Waard et al, Current Protocols in Mouse Biology 2012).

Aorta flow cytometry. The aortic arches were dissected, put in 20mM HEPES in RPMI (GIBCO Invitrogen) and cut into small pieces. Digestion was performed by 30 minutes incubation at 37°C with a Collagenase I, XI, DnaseI and Hyaluronidase enzyme mix (all from Sigma). The lysate was transferred into a FACS tube with cell strainer (BD Biosciences) and centrifuged for 5 minutes at 1600 rpm. A live/dead stain was performed according to the manufacturer’s protocol (Fixable Aqua Dead Cell Stain Kit; Life Technologies), Fc receptors were blocked.
with an antibody against CD32/CD16 (eBioscience) and cells were labeled for analysis on the LSRFortessa (BD Biosciences) with the antibodies recognizing: CD45, Ly6C and CD11b (eBiosciences).

**Mouse blood parameters.** Red blood cells (RBC), platelets and hematocrit (Hct) were measured using a Coulter Counter. The total cholesterol distribution among the main lipoprotein classes of the plasma samples was measured using fast performance liquid chromatography (FPLC) analysis as described previously (Levels JHM et al. Crit Care Med. 2003). In brief, the system contained a PU-980 ternary pump with an LG-980-02 linear degasser and an UV-975 UV/VIS detector (Jasco, Tokyo, Japan). An extra PU 2080i-plus pump (Jasco, Tokyo, Japan) was used for in-line cholesterol RTU enzymatic reagent (Biomerieux, Marcy l’Etoile, France) addition at a flow rate of 0.1 mL/min respectively. EDTA plasma was diluted 1:1 with Tris buffered saline and 30 μL sample/buffer mixture was loaded on a Superose 6 HR 10/30 column (GE Health care, Life sciences division, Diegem, Belgium) for lipoprotein separation at a flow rate of 0.31 mL/min. Chromatographic profiles of commercially available plasma lipid standards (SKZL, Nijmegen, The Netherlands) served as a reference.

**Cytochemical and immunohistological stainings.** Cryosections (2 weeks study) were dried and subsequently fixed with dry acetone (Sigma). Newly influxed monocytes were shown using an antibody against ER-MP58 (Bioconnect) followed by detection with a biotin-labeled rabbit anti-rat antibody and staining with the ABC kit (Vector Labs). After counterstaining with hematoxylin sections were embedded with Vectamount (Vectashield). ER-MP58 positive cells were counted manually. Paraffin sections (6 weeks study) were deparaffinized and rehydrated. Aortic roots were stained for 60 minutes with a 0.2% Picro Sirius Red solution and incubated for 2 minutes in acidified water (0.01M HCl). The sections were dehydrated, embedded in pertex (Histolab) and collagen content was quantified using Adobe photoshop CS5 software. In addition, sections were incubated with antibodies detecting macrophages (MAC-3; Pharmingen) and smooth muscle cells (SMC; 1A4; Dako) followed by a horseradish peroxidase (HRP)-conjugated secondary antibody. DAB substrate (ImmunoLogic) was used for detection. After counterstaining with hematoxylin sections were embedded in pertex. Macrophage and smooth muscle cell area was quantified using Adobe photoshop CS5 software.

**RNA isolation, cDNA synthesis and RT-qPCR.** From snap frozen aortic arches, total RNA was extracted after crushing (under liquid nitrogen) using Trizol (Life technologies) and cDNA was synthesized from 300ng of total RNA using iScript cDNA Synthesis kit (BioRad). Semi-quantitative real-time PCR was performed using SensiFAST™ SYBR® (BC Biotech) and measured with the CFX384 system (Bio-Rad). Specific primers for monocyte chemotactic protein-1 (Mcp-1), stromal cell-derived factor-1α (Sdf-1α) and C/EBP homologous protein (Chop), and ribosomal protein 36B4 (to correct for cDNA content) were designed using Primer 3 software (see Table S1).
In vitro macrophage assays:

In vitro uptake of LN. Macrophages were seeded on Lab Tek chamber slides (Nunc), stimulated and incubated overnight. Cells were washed twice with PBS before a 20 minute fixation step with 100% methanol (Sigma) followed by a permeabilization/blocking step with 0.15% Triton 10% FBS in PBS for 1 hour at room temperature. Liposomes were detected with an antibody against polyethylene glycol (PEG; Epitomics) followed by an anti-Rabbit-FITC labelled secondary (Jackson Immuno Research). Slides were mounted using DAPI mounting media (Vectashield) and pictures were taken.

NFκB transcriptional activity. RAW267.4 NFκB-luc cells were cultured in a 96-well plate at a density of 70,000 cells/well. Cells were treated with compounds, stimulated with 100ng/ml LPS (Sigma) after 2 hours and luciferase activity was determined 22 hours later using the One-Glo reporter assay system (Promega) according to the manufacturer’s instructions.

RNA isolation, cDNA synthesis and RT-qPCR. From cultured BMDM, total RNA was extracted using a similar approach, and cDNA made from 500ng RNA. Semi-quantitative real-time PCR was performed using SensiFAST™ SYBR® (BC Biotech) and measured with the CFX384 system (Bio-Rad). Specific primers for fatty acid binding protein 4 (Fabp4), ATP-binding cassette transporter A1 (Abca1), C/EBP homologous protein (Chop), pancreatic endoplasmic reticulum kinase (Perk), and ribosomal protein 36B4 and 18S (to correct for cDNA content) were designed using Primer 3 software (see Table S1).

Cholesterol efflux (3H cholesterol). BMDM or THP-1 macrophages were loaded with cholesterol for 24 hours using 30μg/ml cholesterol (Sigma) mixed with 0.5μCi/ml Tritium-labeled cholesterol (Amersham) and the compounds in RPMI supplemented with 100U/ml penicillin/streptomycin and 0.2% free fatty acid (FFA)-free bovine serum albumin (BSA; Sigma). After washing the cells with PBS containing 0.2% FFA-free BSA, they were incubated for 24 hours with 20μg/ml apolipoprotein A1 (apo-A1; Calbiochem) in RPMI containing 100U/ml penicillin/streptomycin and 0.2% FFA-free BSA. Both cell culture medium and the cells were harvested, and Tritium was measured using a Scintillation counter.

Oil-red-O. BMDM were cultured on a Lab Tek chamber slide, incubated with the compounds overnight and thereafter stimulated with 50μg/ml oxLDL (Alfa Aesar) for 48 hours. Cells were fixed with 3.5% formalfix and incubated with isopropanol for 10 minutes. Cellular lipids were stained with Oil-red-O (0.3g/100ml 60% isopropanol) for 30 minutes and briefly counterstained with hematoxylin. Slides were embedded in glycergel (Dako) and photomicrographs were taken with a 400x magnification.
Late apoptosis and necrosis (Annexin-V / propidium iodine). BMDM or THP-1 macrophages were stimulated with 50μg/ml oxLDL and the compounds for 72 hours. To determine late apoptosis and necrosis by Flow cytometry 5x10⁵ cells were suspended in 50μl Annexin-V binding buffer, and stained with 1:50 propidium iodine and 1:100 FITC-labelled Annexin-V (Life Technologies). Samples were measured on the FACS Canto II (BD Biosciences) and the data analysis was performed using FlowJo software (Tree Star).

**TABLE S1.** Primer sequences used for semi-quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence (5’-3’)</th>
<th>Reverse primer sequence (5’-3’)</th>
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<tr>
<td>36B4</td>
<td>ACGGGTACAAACGAGGTCTCTG</td>
<td>GCCTTGACCTTTCTCAGCAAG</td>
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†indicates human primers.
I. Biodistribution

II. 2 week treatment

II. 6 week treatment

*biweekly dosing

FIGURE S1. Schematic of in vivo studies in LDLr−/− mice

Eight-week old LDLr−/− mice were fed a HFD for 6 weeks, followed by (i) a single dose of LN-PLP 10mg/kg or PBS and sacrifice after 24 hours, (ii) 2 weeks administration of LN-PLP, LN or PBS (10mg/kg, biweekly) or (iii) 6 weeks of LN-PLP, LN, PLP or PBS (10mg/kg, biweekly). LN indicates empty liposomes; LN-PLP, liposomal prednisolone; PBS, phosphate-buffered saline; PLP, free prednisolone.
FIGURE S2. Safety parameters of LDLr<sup>+</sup> mice after 2 weeks treatment

(A) Body weight of the mice was monitored weekly. (B) After 6 weeks of HFD, followed by 2 weeks of drug administration, plasma cholesterol among the main lipoprotein classes were measured. (C) Red blood cells (RBC), platelets and hematocrit (Hct) was determined by Coulter Counter upon harvest. Data are presented as mean ± SEM; **p<0.01, ***p<0.001. LN indicates empty liposomes; LN-PLP, liposomal prednisolone; PBS, phosphate-buffered saline.
FIGURE S3. Safety parameters and plaque collagen content in LDLr<sup>−/−</sup> mice after 6 weeks treatment

(A) Body weight of the mice was monitored weekly. (B) After 6 weeks of HFD, followed by 6 weeks of drug administration, total cholesterol was measured in the main lipoprotein classes (C) Upon harvest red blood cells (RBC), platelets and hematocrit (Hct) was determined by Coulter Counter, (D) collagen content in the roots was assessed with sirius red staining (original magnification x25; scale bars represent 1mm). Data are presented as mean±SEM; *P<0.05, ***P<0.001. LN indicates empty liposomes; LN-PLP, liposomal prednisolone; PBS, phosphate-buffered saline; PLP, free prednisolone.
FIGURE S4. LN-PLP effects in human macrophages

THP-1 cells were differentiated into macrophages by PMA treatment and (A) cholesterol efflux towards apo-A1, (B) gene expression of intracellular lipid transporter Fabp4 after 24 hours oxLDL (50 μg/ml), and (C) oxLDL-induced late apoptosis (50 μg/ml, 72 hours) were determined. Data are presented as mean±SEM; *P<0.05, **P<0.01, ***P<0.001. LN indicates empty liposomes; LN-PLP, liposomal prednisolone; PBS, phosphate-buffered saline; PLP, free prednisolone.