BASIC SCIENCE

Nanomedicine: Nanotechnology, Biology, and Medicine
12 (2016) 1463–1470

Original Article

nanomedjournal.com

Liposomal prednisolone promotes macrophage lipotoxicity in experimental atherosclerosis

Fleur M. van der Valk, PhD, MD[a,1], Dominik M. Schulte, PhD, MD[a,b,1], Svenja Meiler, PhD[c], Jun Tang, PhD[d], Kang He Zheng, PhD, MD[a], Jan Van den Bossche, PhD, MSc[c], Tom Seijkens, PhD, MD[c], Matthias Laudes, PhD, MD[b], Menno de Winther, PhD, MSc[c], Esther Lutgens, PhD, MD[c], Amr Alaarg, PhD[e], Josbert M. Metselaar, PhD[e], Geesje M. Dallinga-Thie, PhD[a], Willem J.M. Mulder, PhD, MSc[a,d], Erik S.G. Stroes, PhD, MD[a,*], Anouk A.J. Hamers, PhD, MSc[a]

[a]*Department of Vascular Medicine, Academic Medical Center, Meibergdreef 9, Amsterdam, The Netherlands*
[b]*Department of Internal Medicine I, University Schleswig-Holstein, Arnold-Heller-Straße 3, Kiel, Germany*
[c]*Experimental Vascular Biology, Department of Medical Biochemistry, Academic Medical Center, Meibergdreef 9, Amsterdam, The Netherlands*
[d]*Translational and Molecular Imaging Institute, Icahn School of Medicine at Mount Sinai, 1 Gustave, NY, USA*
[e]*Department of Biomaterials Science and Technology, Targeted Therapeutics section, MIRA Institute, University of Twente, Drienerlolaan 5, NB, Enschede, The Netherlands*

Received 2 December 2015; accepted 25 February 2016

Abstract

Atherosclerosis is a lipid-driven inflammatory disease, for which nanomedicinal interventions are under evaluation. Previously, we showed that liposomal nanoparticles loaded with prednisolone (LN-PLP) accumulated in plaque macrophages, however, induced proatherogenic effects in patients. Here, we confirmed in low-density lipoprotein receptor knockout (*LDLr* $^{-/-}$) mice that LN-PLP accumulates in plaque macrophages. Next, we found that LN-PLP infusions at 10 mg/kg for 2 weeks enhanced monocyte recruitment to plaques. In follow up, after 6 weeks of LN-PLP exposure we observed (i) increased macrophage content, (ii) more advanced plaque stages, and (iii) larger necrotic core sizes. Finally, *in vitro* studies showed that macrophages become lipotoxic after LN-PLP exposure, exemplified by enhanced lipid loading, ER stress and apoptosis. These findings indicate that liposomal prednisolone may paradoxically accelerate atherosclerosis by promoting macrophage lipotoxicity. Hence, future (nanomedicinal) drug development studies are challenged by the multifactorial nature of atherosclerotic inflammation.

Key words: Atherosclerosis; Macrophages; Lipotoxicity; Prednisolone; Liposomal nanoparticles

Abbreviations: CVD, cardiovascular disease; DPPC, dipalmitoylphosphatidylcholine; DSPE, distearoylphosphatidylethanola-mine; GC, glucocorticoids; LDLr−/−, low-density lipoprotein receptor knockout; LN, empty liposomal nanoparticles; LN-PLP, liposomal nanoparticle encapsulating prednisolone phosphate; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PLP, free prednisolone phosphate

Author disclosures and funding: This work was supported by a European Framework Program 7 grant (ESS: FP7-Health 309820: Nano-Athero); the Dutch network for Nanotechnology NanoNext NL, in the subprogram "Drug Delivery" and a NWO Vidi (W.J.M.M.). J.M.M. is affiliated with the company Enceladus Pharmaceuticals (Amsterdam, The Netherlands). These other authors declare that they have no competing interests. D.M.S. received support from the International Endocrine Scholars Program (IESP) by the European Society of Endocrinology (ESE) and the Endocrine 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.

Previous abstract: American Heart Association 2015, USA.

*Corresponding author at: Academic Medical Center, Department of Vascular Medicine, Room F4-211, 1100 DD, Amsterdam, the Netherlands.
E-mail addresses: f.m.valkvander@amc.nl (F.M. van der Valk), dominik.schulte@uksh.de (D.M. Schulte), s.u.meiler@amc.uva.nl (S. Meiler), jun.tang.mssm@gmail.com (J. Tang), k.h.zheng@amc.uva.nl (K.H. Zheng), j.vandenbossche@amc.uva.nl (J. Van den Bossche), t.t.seijkens@amc.uva.nl (T. Seijkens), matthias.laudes@uk-sh.de (M. Laudes), m.dewinther@amc.uva.nl (M. de Winther), e.lutgens@amc.uva.nl (E. Lutgens), A.M.S.A.Alaarg@uu.nl (A. Alaarg), bart@enceladus.nl (J.M. Metselaar), g.m.dallinga@amc.uva.nl (G.M. Dallinga-Thie), wjmmulder@gmail.com (W.J.M. Mulder), e.s.stroes@amc.uva.nl (E.S.G. Stroes), a.a.hamers@amc.uva.nl (A.A.J. Hamers).
[1] Authors contributed equally.

http://dx.doi.org/10.1016/j.nano.2016.02.022
1549-9634/

Atherosclerosis is a chronic disease of the arterial wall, characterized by a lipid-rich, low-grade inflammatory milieu.[1] Despite the success of lifestyle changes and statins in cardiovascular prevention and treatment,[2] disability and death from cardiovascular disease (CVD) is still increasing worldwide.[3] Consequently, anti-inflammatory drugs, amongst which nanomedicinal approaches, are being assessed.[4]

Nanotechnology holds a promise by increasing drug accumulation in target tissues, while reducing systemic exposure.[5,6] In support of this concept, liposomal nanoparticles loaded with anti-inflammatory 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.[7] Moreover, LN-PLP rapidly reduced the inflammatory activity of atherosclerotic lesions in rabbits.[7] In humans, the accumulation of LN-PLP in plaque macrophages isolated from patients after intravenous infusion was also found.[8] However, in patients with advanced atherosclerosis, LN-PLP treatment tended to increase the degree of arterial wall inflammation.[8]

Unfortunately, discrepant drug effects between the preclinical stage and first-in-human are no exception.[1] Whereas previous studies focused on the inflammatory metrics, here, we addressed LN-PLP's effect on atherosclerosis 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[9] and detailed in the Supplementary Material.

LDLr $^{-/-}$ *mice studies*

In vivo studies, as illustrated in Supplementary Figure 1, 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 10 mg/kg cy5.5-labelled liposomal prednisolone (LN-PLP; $n = 4$).[10] 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 10 mg/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.[11] BMDM of two mice were pooled and seeded at a density of 0.15×10^6 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 50 ng/ml phorbol-12-myristate-13-acetate (PMA) for 24 hours. The RAW264.7 stable cell line containing the 3x-NFκB-*luc* plasmid[12] 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 (20 μM) and high (80 μM) dose of PLP. LN-PLP was given at a concentration of 10 μg/ml (equivalent to 20 μM PLP) or 40 μg/ml (equivalent to 80 μM PLP). The empty liposomes (LN) were added in the same liposomal concentrations as LN-PLP. Macrophage assays comprised of immunostaining, NFκB transcriptional activity (RLU were normalized to untreated cells), mRNA gene expression, cholesterol efflux, oil-red-O staining and flow cytometric assays.

Statistical analysis

In vivo mice studies were performed in at least $n = 4$ (see Supplementary Figure 1). *In vitro* experiments were performed at least three times in triplicate. Statistical analyses were performed using GraphPad Prism version 5 (San Diego, CA). A Shapiro–Wilk normality test was performed before either 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 and 4 treatment groups was assessed using the 1-way ANOVA. Data in the text are presented as mean ± SD. The significance level was set at $P < 0.05$.

Results

LN-PLP accumulates in plaque macrophages of LDLr $^{-/-}$ *mice*

Near infrared fluorescence (NIRF) imaging substantiated accumulation of LN-PLP in the atherosclerotic plaques located

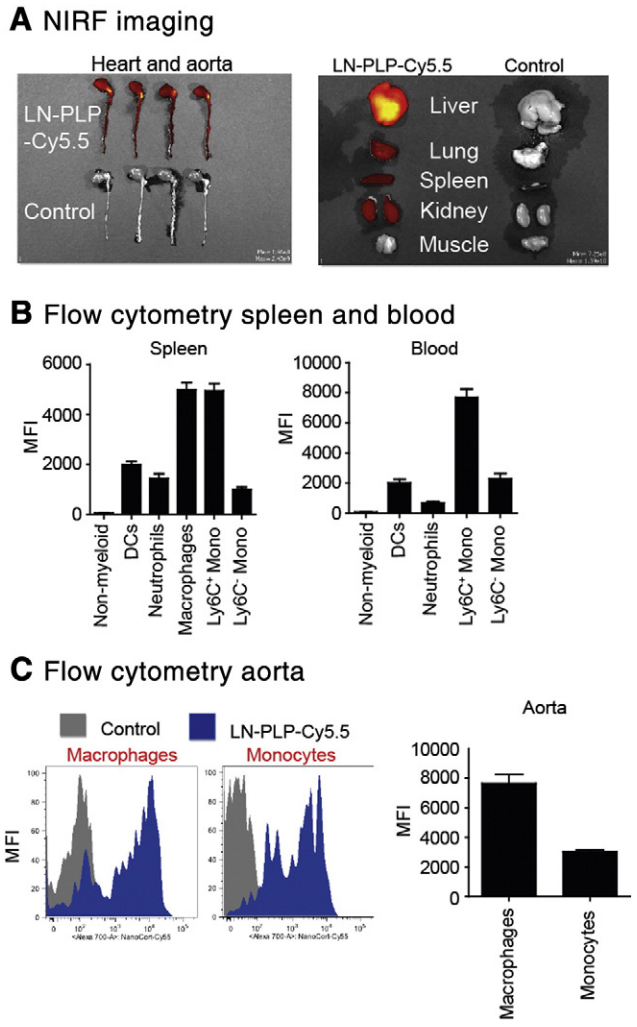

Figure 1. LN-PLP uptake in *LDLr* $^{-/-}$ mice. (**A**) NIRF images showing accumulation and distribution of Cy5.5-labelled LN-PLP ($n = 4$) or PBS ($n = 4$) 24 hours after tail vein injection (10 mg/kg) in 8-week old *LDLr* $^{-/-}$ mice, 6 weeks on HFD. (**B,C**) Cellular distribution of LN-PLP in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

in the aortas of *LDLr* $^{-/-}$ mice, 24 hours after tail vein injection (Figure 1, *A*). In line with previous experiments,[13] LN-PLP was also found in the liver, lung, spleen and kidney (Figure 1, *A*). Flow cytometry assays showed that predominantly myeloid cells take up LN-PLP (Figure 1, *B*). In the circulating and splenic myeloid cells, the highest uptake was found in proinflammatory Ly6C^{+} monocytes and macrophages (Figure 1, *B*). Also in the aortic arch, uptake of LN-PLP by macrophages and monocytes was demonstrated (Figure 1, *C*).

Two weeks LN-PLP induces monocyte recruitment into atherosclerotic plaques

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 2, *A,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 2, *A-B*). However, the percentage of proinflammatory monocytes was significantly higher in aortic arches of the LN-PLP mice (11% ± 6; mean ± SD) compared with LN (5% ± 3, $P < 0.05$) and PBS (6% ± 3, $P < 0.01$; Figure 2, *C*). 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.[14] Immunostaining of the roots showed a significantly higher number of ER-MP58 positive cells after LN-PLP (24 ± 11; mean ± SD total ER-MP58+ cells per mouse) compared with LN (13 ± 4, $P < 0.0001$) and PBS (17 ± 8, $P < 0.05$; Figure 2, *D*), which indicates an increased presence of freshly influxed monocytes. No increase in the number of blood monocytes was observed after LN-PLP (Figure 2, *E*). 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 (Supplementary Figure 2, *A,B*). Also, RBCs, platelets and haematocrit were similar between groups (Supplementary Figure 2, *C*).

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, now receiving 6 weeks of intravenous LN-PLP, LN, PLP or PBS infusion (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 (Supplementary Figure 3, *A-C*). Plaque size was not different between groups (Figure 3, *A*), 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 3, *B*). 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 3, *C*). 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 3, *D*). In agreement, less collagen was present in the LN-PLP group (43% ± 11) compared with PLP (52% ± 20, $P < 0.05$), LN (62% ± 12, $P < 0.0001$) and PBS (61% ± 12, $P < 0.001$; Supplementary Figure 3, *D*). 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 3, *E*). In addition, the mRNA expression of chemokines *Mcp-1* and *Sdf-1α* (Figure 4, *A-B*) in the aortic arches was higher after LN-PLP compared with control groups. In addition, the expression of *Chop* (Figure 4, *C*), a gene activated upon endoplasmic reticulum (ER) stress, was upregulated after LN-PLP in comparison to control groups.

LN-PLP induces macrophage lipotoxicity in vitro

In vitro, RAW264.7 NFκB-*luc* cells were used to assess prednisolone's classical anti-inflammatory effect.[12] As expected,

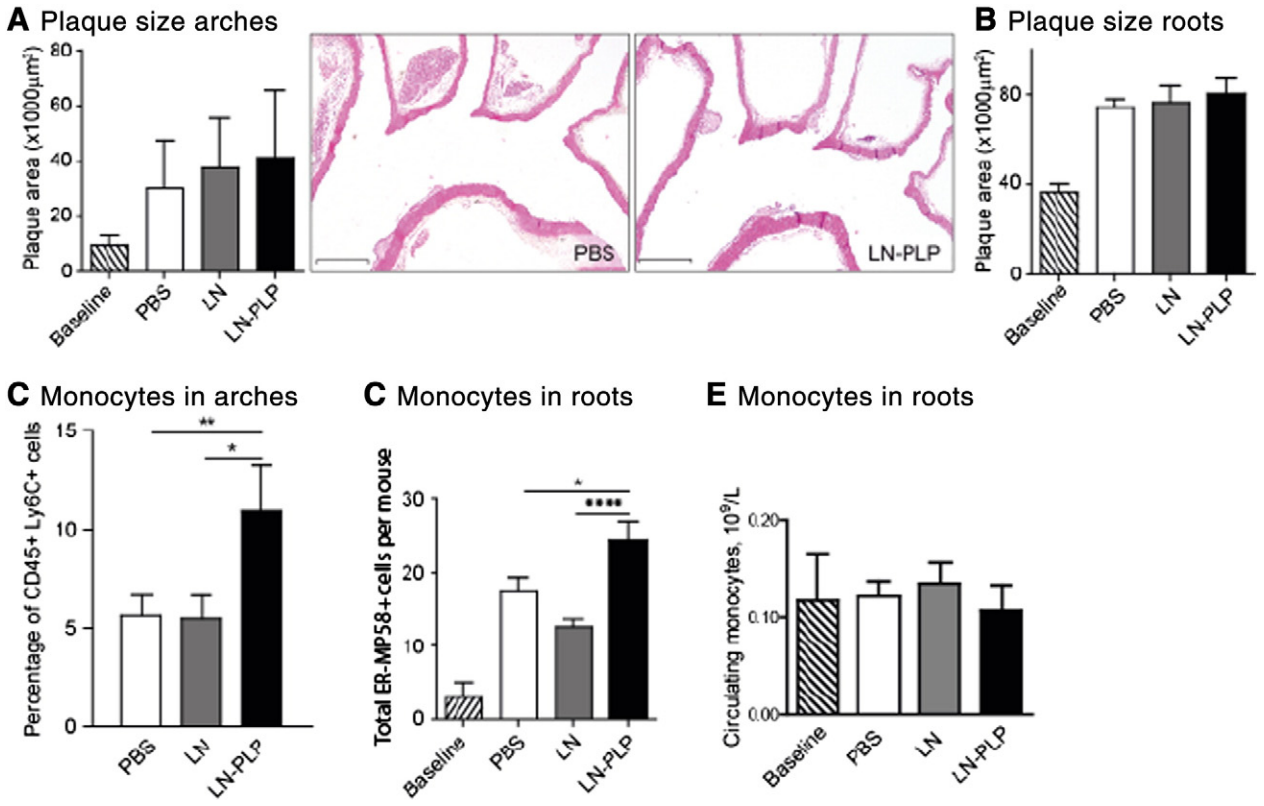

Figure 2. LN-PLP induces monocyte recruitment in plaques of *LDLr* $^{-/-}$ mice. (**A,B**) Plaque size was quantified in (**A**) the aortic arches (original magnification ×25; scale bars represent 1 mm) and (**B**) the roots using Hematoxylin-Eosin (HE) staining, 6 weeks after HFD (baseline, $n = 6$) and an additional 2 weeks with PBS, LN or LN-PLP iv administration ($n = 20$/group, 10 mg/kg, biweekly). (**C**) Pro-inflammatory monocytes were assessed by flow cytometry of the arches, and (**D**) total number of cells positive for ER-MP58 staining, indicative of freshly recruited monocytes in the roots, (**E**) the number of blood monocytes. Data are presented as mean ± SEM; *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. LN = empty liposomes, LN-PLP = liposomal prednisolone, PBS = phosphate-buffered saline.

pretreatment of cells with either LN-PLP or PLP significantly decreased NFκB activity (RLU were normalized to untreated cells) 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 5, *A*).

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 5, *B*). In line with the decreased expression of *Abca1,* 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 5, *B*). Also, BMDM lipid content was higher after LN-PLP and free PLP compared with empty LN and control (Figure 5, *C*).

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 5, *D*). Increased levels of intracellular lipid trafficking by *Fabp4* have been shown to promote ER-stress and induce an unfolded protein response (UPR).[15] 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 4, *D*) and *Chop* ($P < 0.05$ compared with both control groups; Figure 5, *E*). Activation of *Perk* and *Chop* are elementary in the switch from pro-survival to pro-death signalling; in line with this, 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 5, *F-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 (Supplementary Figure 4).

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

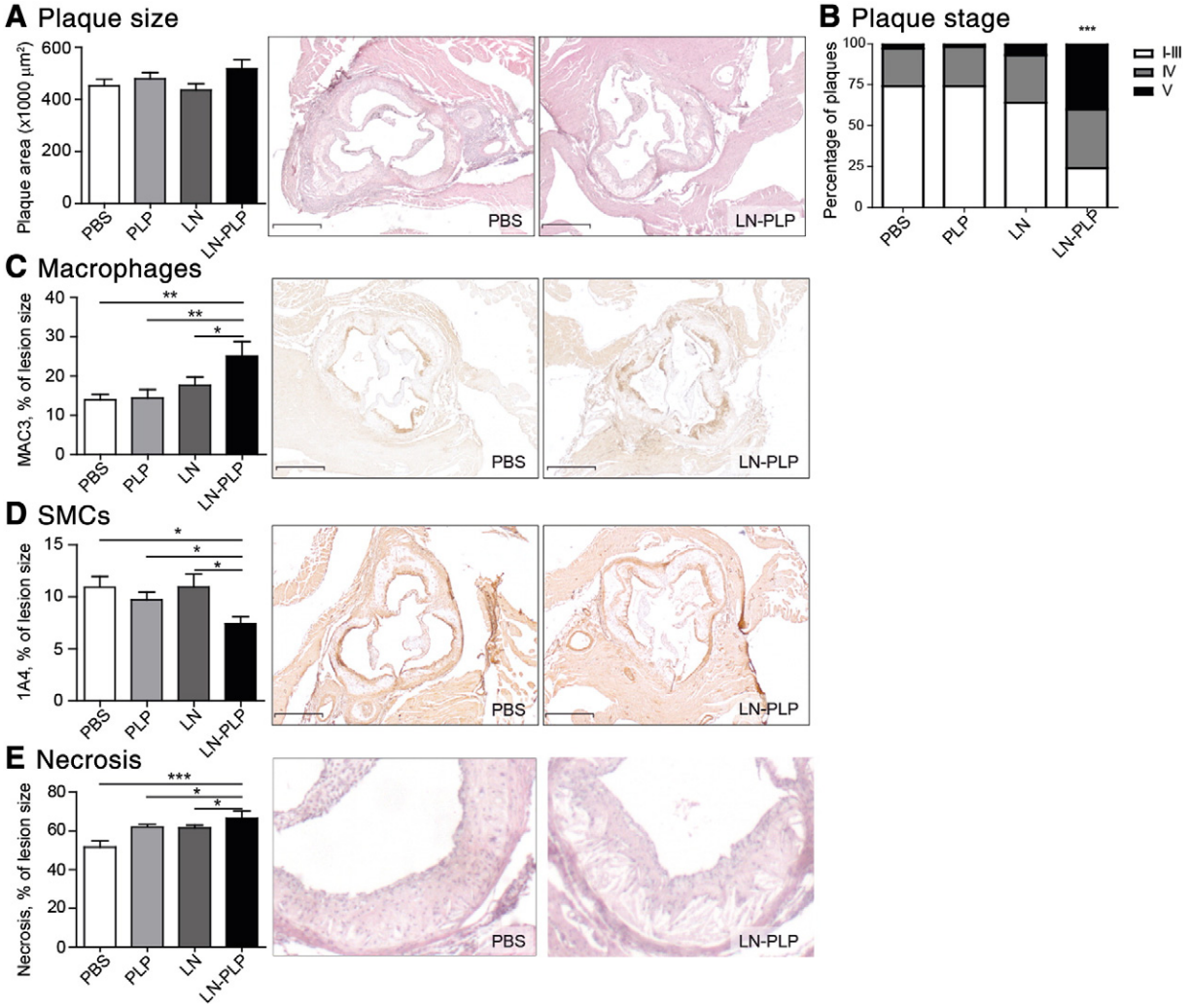

Figure 3. LN-PLP aggravates atherosclerotic plaques in *LDLr* $^{-/-}$ mice. (**A**) After 6 weeks HFD alone, followed by 6 weeks PBS, PLP, LN or LN-PLP administration (n = 16/group, 10 mg/kg iv, biweekly), (**A**) plaque size and (**B**) plaque stage were determined in HE stained sections of the roots. (**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 ×25; **E**, ×100; scale bars represent 1 mm. Data are presented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001. LN = empty liposomes, PLP = free prednisolone, LN-PLP = liposomal prednisolone, PBS = phosphate-buffered saline.

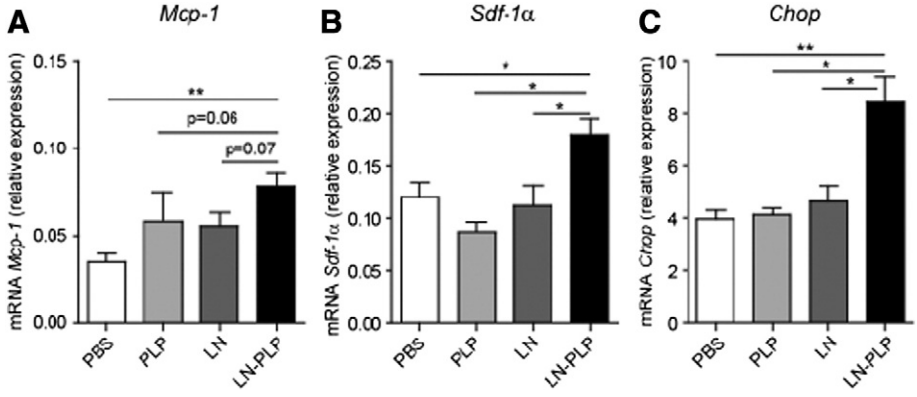

Figure 4. Gene expression in aortic arches from *LDLr* $^{-/-}$ mice. (**A-C**) mRNA was isolated from aortic arches of *LDLr* $^{-/-}$ mice after 6 weeks administration of either PBS, PLP, LN or LN-PLP (n = 16, 10 mg/kg iv, biweekly) to analyse gene expression of monocyte migration factors, *Mcp-1* (**A**) and *Sdf-1α* (**B**), and the endoplasmatic reticulum stress marker, *Chop* (**C**). Gene expression was normalized to *36B4* housekeeping gene. Data are presented as mean ± SEM; *P < 0.05, **P < 0.01.*Chop* = 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.

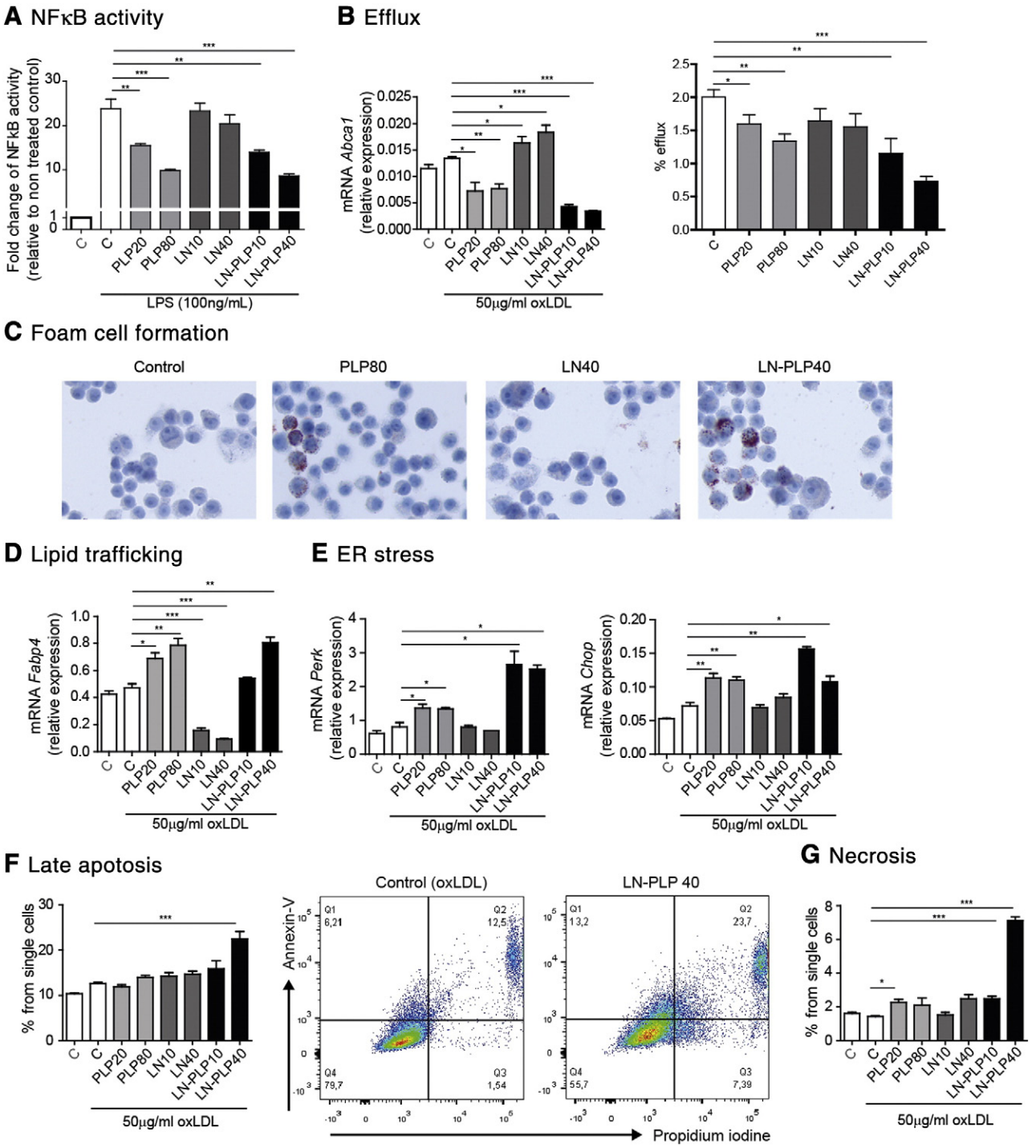

Figure 5. LN-PLP promotes macrophage lipotoxicity. (**A**) NFκB activity was determined in RAW267.4 NFκB-*luc* cells as the relative fold change to control without 100 ng/ml LPS stimulation. (**B**) BMDM were isolated from C57BL/6 mice to study gene expression levels of ATP-binding cassette transporter 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. (**F-G**) Late apoptosis and necrosis following a 72-hour oxLDL (50 ug/ml) incubation were assessed by Annexin-V / propidium iodine flow cytometry. Gene expression was normalized to *36B4* and *18S* housekeeping genes. Oil-red-O photomicrographs were taken with a 400× magnification. Data are presented as mean ± SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.BMDM = bone marrow derived macrophages, *Chop* = C/EBP homologous protein, *Fabp4* = fatty acid binding protein 4, LN = empty liposomes, LN-PLP = liposomal prednisolone, PBS = phosphate-buffered saline, *Perk* = pancreatic endoplasmic reticulum kinase, PLP = free prednisolone.

subsequent apoptosis/necrosis. These findings indicate that local exposure to prednisolone elicits a proatherogenic, lipotoxic effect in plaque macrophages in *LDLr* $^{-/-}$ mice.

The finding of enhanced monocyte influx in plaques of *LDLr* $^{-/-}$ mice after 2 weeks of LN-PLP is discordant with the previously described reduction in inflammatory cell

content in rabbit's atherosclerotic lesions only days after a single LN-PLP administration.[7] 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 mice.[16] 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' inflammation,[17] 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 state.[18]

The relevance of the enhanced monocyte response after 2 weeks is substantiated by the observation that after 6 weeks of LN-PLP administration, the number of macrophages, tissue descendants of monocytes, is higher in plaques compared with PLP, LN or PBS administration. In addition, mRNA expression of chemoattractants,[19] *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 ^{18}F-fluorodeoxyglucose (^{18}F-FDG) uptake,[8] which is a marker of plaque macrophage content.[20]

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) macrophages.[21] 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 stress,[22] as suggested by increased expression of the intracellular lipid chaperone *Fabp4*[23] and decreased expression of the major cholesterol efflux transporter *Abca1*.[24] 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.[15] 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.[25] 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 ^{18}F-FDG uptake).[8]

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,[26,27] 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.[28,29]

Here, we show in an atherosclerosis mouse model, that LN-PLP infusions induce early monocyte recruitment to plaques, followed by increased macrophage content, more advanced plaque stages, and larger necrotic core sizes. These *in vivo* findings were recapitulated *in vitro*, since we observed that both murine and human macrophages polarize into a lipid-avid phenotype after LN-PLP exposure. In summary, these findings indicate that macrophage targeting in plaques with prednisolone may accelerate atherosclerosis by promoting macrophage lipotoxicity. This also highlights the challenges of applying anti-inflammatory strategies in atherosclerosis; 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.

Acknowledgements

We thank Alinda Schimmel, Stefan Havik, Miranda Versloot and Linda Becker for their crucial support in experimental and lab work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2016.02.022.

References

1. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature* 2011;**473**:317-25.
2. Ford I, Murray H, Packard CJ, Shepherd J, Macfarlane PW, Cobbe SM, et al. Long-term follow-up of the west of scotland coronary prevention study. *N Engl J Med* 2007;**357**:2373-83.
3. Roth GA, Forouzanfar MH, Moran AE, Barber R, Nguyen G, Feigin VL, et al. Demographic and epidemiologic drivers of global cardiovascular mortality. *N Engl J Med* 2015;**372**:1333-41.
4. van der Valk FM, van Wijk DF, Stroes ESG. Novel anti-inflammatory strategies in atherosclerosis. *Curr Opin Lipidol* 2012;**23**:532-9.
5. Lobatto ME, Fuster V, Fayad ZA, Mulder WJM. Perspectives and opportunities for nanomedicine in the management of atherosclerosis. *Nat Rev Drug Discov* 2011;**10**:835-52.

6. Schiener M, Hossann M, Viola JR, Ortega-Gomez A, Weber C, Lauber K, et al. Nanomedicine-based strategies for treatment of atherosclerosis. *Trends Mol Med* 2014;**20**:271-81.
7. Lobatto ME, Fayad ZA, Silvera S, Vucic E, Calcagno C, Mani V, et al. Multimodal clinical imaging to longitudinally assess a nanomedical anti-inflammatory treatment in experimental atherosclerosis. *Mol Pharm* 2010;**7**:2020-9.
8. van der Valk FM, van Wijk DF, Lobatto ME, Verberne HJ, Storm G, Willems MCM, et al. Prednisolone-containing liposomes accumulate in human atherosclerotic macrophages upon intravenous administration. *Nanomedicine* 2015;**11**:1039-46.
9. Lobatto ME, Calcagno C, Otten MJ, Millon A, Ramachandran S, Paridaans MPM, et al. Pharmaceutical development and preclinical evaluation of a GMP-grade anti-inflammatory nanotherapy. *Nanomedicine* 2015;**11**:1133-40.
10. Tang J, Lobatto ME, Hassing L, Van Der Staay S, Van Rijs SM, Calcagno C, et al. Inhibiting macrophage proliferation suppresses atherosclerotic plaque inflammation. *Sci Adv* 2015;**1**:e1400223.
11. Hamers AAJ, Vos M, Rassam F, Marinković G, Marincovic G, Kurakula K, et al. Bone marrow-specific deficiency of nuclear receptor Nur77 enhances atherosclerosis. *Circ Res* 2012;**110**:428-38.
12. Carlsen H, Moskaug JO, Fromm SH, Blomhoff R. In Vivo Imaging of NF- B Activity. *J Immunol* 2002;**168**:1441-6.
13. Metselaar JM, Storm G. Liposomes in the treatment of inflammatory disorders. *Expert Opin Drug Deliv* 2005;**2**:465-76.
14. Goossens P, Vergouwe MN, Gijbels MJJ, Curfs DMJ, van Woezik JHG, Hoeksema MA, et al. Myeloid IκBα deficiency promotes atherogenesis by enhancing leukocyte recruitment to the plaques. *PLoS One* 2011;**6**:e22327.
15. Erbay E, Babaev VR, Mayers JR, Makowski L, Charles KN, Snitow ME, et al. Reducing endoplasmic reticulum stress through a macrophage lipid chaperone alleviates atherosclerosis. *Nat Med* 2009;**15**:1383-91.
16. Getz GS, Reardon CA. Animal models of atherosclerosis. *Arterioscler Thromb Vasc Biol* 2012;**32**:1104-15.
17. Lobatto ME, Calcagno C, Metselaar JM, Storm G, Stroes ESG, Fayad ZA, et al. *Imaging the efficacy of anti-inflammatory liposomes in a rabbit model of atherosclerosis by non-invasive imaging*. Methods in enzymology. Elsevier Inc.; 2012211-28.
18. Viola J, Soehnlein O. Atherosclerosis – A matter of unresolved inflammation. *Semin Immunol* 2015:1-10.
19. Bleul CC. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med* 1996;**184**:1101-9.
20. Tarkin JM, Joshi FR, Rudd JHF. PET imaging of inflammation in atherosclerosis. *Nat Rev Cardiol* 2014;**11**:443-57.
21. Liu G, Yang H. Modulation of macrophage activation and programming in immunity. *J Cell Physiol* 2013;**228**:502-12.
22. Isa SA, Ruffino JS, Ahluwalia M, Thomas AW, Morris K, Webb R. M2 macrophages exhibit higher sensitivity to oxLDL-induced lipotoxicity than other monocyte/macrophage subtypes. *Lipids Health Dis* 2011;**10**:229.
23. Hu W, Zhou X, Jiang M, Duan Y, Chen Y, Li X, et al. Statins synergize dexamethasone-induced adipocyte fatty acid binding protein expression in macrophages. *Atherosclerosis* 2012;**222**:434-43.
24. Liew YY, Docter E, Ray DW, Hutchinson IV, Brogan IJ. Effect of rapamycin and prednisolone in differentiated THP-1 and U937 cells. *Transplant Proc* 2002;**34**:2872-3.
25. Czock D, Keller F, Rasche FM, Häussler U. Pharmacokinetics and pharmacodynamics of systemically administered glucocorticoids. *Clin Pharmacokinet* 2005;**44**:61-98.
26. Fredman G, Kamaly N, Spolitu S, Milton J, Ghorpade D, Chiasson R, et al. Targeted nanoparticles containing the proresolving peptide Ac2-26 protect against advanced atherosclerosis in hypercholesterolemic mice. *Sci Transl Med* 2015;**7**:275ra20.
27. Zhang X-Q, Even-Or O, Xu X, van Rosmalen M, Lim L, Gadde S, et al. Nanoparticles containing a liver X receptor agonist inhibit inflammation and atherosclerosis. *Adv Healthc Mater* 2015;**4**:228-36.
28. Luo MJ, Thieringer R, Springer MS, Wright SD, Hermanowski-Vosatka A, Plump A, et al. 11 β-HSD1 inhibition reduces atherosclerosis in mice by altering pro-inflammatory gene expression in the vasculature. *Physiol Genomics* 2012;**7**:47-57.
29. Hadoke PWF, Kipari T, Seckl JR, Chapman KE. Modulation of 11β-hydroxysteroid dehydrogenase as a strategy to reduce vascular inflammation. *Curr Atheroscler Rep* 2013;**15**:320.