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Liposomal targeting of glucocorticoids to the inflamed synovium inhibits cartilage matrix destruction during murine antigen-induced arthritis

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ABSTRACT

Encapsulation of glucocorticoids into long-circulating liposomes provides targeting of these drugs to the inflamed synovium in experimental arthritis and thereby strongly improves their therapeutic index. The goal of this study was to evaluate the effect and mechanisms of intravenous liposomal delivery of prednisolone phosphate (Lip-PLP) on protease mediated cartilage destruction during murine antigen-induced arthritis (AIA). Mice treated with a single injection of Lip-PLP showed a pronounced suppression of synovial immune cell infiltration compared to control, PBS-treated mice. Liposomal PLP also significantly suppressed interleukin 1β (3.6 fold) in the synovium, but not in the blood serum. Furthermore, expression of the proteases MMP-3, -9, -13 and -14 and ADAMTS-4 and -5 was suppressed by Lip-PLP in the synovium, but not within the articular cartilage of the femur and tibia, demonstrating the specific action of Lip-PLP on the synovium. Lip-PLP is phagocytosed by macrophages *in vitro* and suppresses their expression of IL-1β and MMPs after LPS activation. Moreover, Lip-PLP suppresses destruction of the cartilage matrix during AIA mediated by active MMPs and ADAMTS, as assessed by immunostaining of their respective neoepitopes VDIPEN and NITEGE in various cartilage layers of the knee joint. *Conclusion*: liposomal delivery of glucocorticoids protects against cartilage matrix destruction during experimental arthritis by inhibiting protease expression and activity in the inflamed synovium.

1. Introduction

Rheumatoid arthritis (RA) is an auto-immune disease characterized by inflammation of the joint with a gradual destruction of cartilage and bone. Glucocorticoids, are still widely used in the treatment of RA for their potent anti-inflammatory effects (Hoes et al., 2007). However, chronic use of glucocorticoids is disadvantageous because of its widespread side effects, such as bone demineralisation, metabolic syndrome and suppression of the hypothalamic–pituitary–adrenal axis (McDonough et al., 2008). Characteristically, glucocorticoids have a relatively large volume of distribution and short half life, which requires relatively high and frequent dosing of the drug. A way to improve on these characteristics is the delivery of glucocorticoids within long-circulating liposomes, which increases its circulation time and therapeutic index, which has been verified in several experimental arthritis models (Metselaar et al., 2003, 2004; Rauchhaus et al., 2009; Anderson et al., 2010).

Long-circulating PEGylated liposomes are also called 'stealth' for their ability to evade uptake by the mononuclear phagocyte system. When given intravenously, stealth liposomes specifically accumulate within inflamed tissues of the joint, where they are engulfed by resident phagocytes within the synovial lining layer. This layer, which contains macrophages and fibroblast-like cells, plays a pivotal role in the onset and progression of arthritis. The synovial lining cells produce cytokines and enzymes which activate and stimulate inflammation and joint destruction. The importance of macrophages in this process was highlighted by selective depletion of lining macrophages prior to arthritis induction, which completely inhibited immune cell influx and development of joint destruction (van Lent et al., 1998a).

In murine models of arthritis, pro-inflammatory cytokines, particularly interleukin 1beta (IL-1β) and tumour necrosis factor-alpha (TNF-α) play an important role in driving joint destruction and can activate chondrocytes towards a catabolic phenotype (Page et al., 2010). In addition, activated synovial lining macrophages, which are the dominant producers of pro-inflammatory cytokines, also produce mediators like oxygen radicals which contribute to the activation of latent pro-enzymes produced within the inflamed knee joint (van Meurs et al., 1999a).

Cartilage destruction starts with breakdown and release of proteoglycans which eventually leads to destruction of collagen type

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Table 1
Liposome characteristics. S.D., standard deviation. Encapsulation efficiency was calculated as [PLP concentration after extrusion and dialysis]/[PLP concentration before extrusion (100 mg/ml)] × 100.

	Empty Lip.	Lip-PLP	Rh-PE Lip-PLP
Mean diameter (S.D.)	114.3 (3.0) nm	108.5 (1.0) nm	105.5 (2.5) nm
Polydispersity index (S.D.)	0.040 (0.014)	0.025 (0.007)	0.045 (0.007)
Encapsulation efficiency	–	6.5%	6.8%
End concentration of Lip-PLP	–	6.5 mg/ml	6.8 mg/ml

II and complete loss of the matrix. The destruction of the cartilage matrix is induced primarily by elevated production of matrix metalloproteases (MMPs) and aggrecanases (ADAMTS) which are produced by chondrocytes as well as by macrophages (van Lent et al., 2008).

In the present study, we investigated whether liposomal targeting of glucocorticoid to synovial macrophages during experimental arthritis offers an effective treatment strategy against cartilage destruction during RA. To this end we chose long-circulating liposomes encapsulating the water soluble prednisolone phosphate (PLP) as they are efficient in silencing inflammation in experimental arthritis (Metselaar et al., 2003), are known to target the inflamed synovium (Metselaar et al., 2004) and have clinical relevance (Barrera et al., 2008). We studied the effect of a single injection of liposomal PLP (Lip-PLP) on MMPs and ADAMTS mediated cartilage destruction in murine antigen-induced arthritis (AIA) and compared this to the effects of free PLP. As glucocorticoids are often administered as a pulse-dose treatment to RA patients, we also compared the single injection of Lip-PLP with 4 repeated, once-daily injections of free PLP.

The AIA a is model that develops a gradual destruction of the cartilage matrix at the femur, tibia and patella which can be measured by the presence of neo-epitopes VDIPEN and NITEGE-indicative for MMP and ADAMTS activity respectively after their visualisation with immunohistochemistry against these markers (van Meurs et al., 1998, 1999b). Furthermore, we also measured the expression of MMPs and ADAMTS directly within the inflamed synovium and levels of activating cytokines present within the inflamed knee joint and in blood serum. To determine whether Lip-PLP can be taken up directly by macrophages and suppress their expression of IL-1β and MMPs we studied these parameters on macrophages *in vitro*.

2. Materials and methods

2.1. Preparation of liposomal prednisolone phosphate

Liposomes were prepared as described by Woodle (Woodle et al., 1994), using a lipid composition of dipalmitoyl phosphatidylcholine (DPPC) (Lipoid GmbH, Ludwigshave, Germany), PEG 2000-distearoyl phosphatidyl-ethanolamine (DSPE) and cholesterol (Sigma–Aldrich, Poole, UK) in a molar ratio of 1.85:0.15:1.0. For the fluorescently labelled liposomes, rhodamine-PE was added as 0.1% of the total molar weight of the lipid formulation. The lipids were dissolved together in ethanol which was then evaporated from a round-bottom flask to create a lipid film. The lipid film was hydrated in water to create empty liposomes or in a solution of 100 mg/ml prednisolone disodium phosphate (PLP) (Bufa, Uitgeest, the Netherlands) in water to create liposomal PLP. Nanosized vesicles were obtained by filtering the liposomal dispersion multiple times through polycarbonate filter membranes decreasing in pore diameter until the liposomes had a mean diameter in the range of 90–120 nm with a polydispersity of <0.2. Mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd., Malvern, UK). Unencapsulated PLP was removed by dialysis against 0.9% phosphate buffered saline using Slide-A-Lyzer dialysis cassettes with a molecular weight cut-off of 10,000 (Pierce, Rockford, USA). Encapsulation efficiency of PLP was determined using ultra performance liquid chromatography (UPLC) from the aqueous phase from liposomal preparations after extraction with chloroform. The UPLC was performed using a mobile phase acetonitril–water with pH of 2, connected to an UV-detector, which was set at 254 nm. Both prednisolone and its phosphate ester could be measured in one single run. The final characteristics of the liposomes are listed in Table 1.

2.2. In vitro culture and treatment

The murine macrophage-like cell line RAW 264.7 was cultured in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen, Paisley, UK) containing 10% fetal calf serum with added pyruvate and antibiotics. Before stimulation, cells were seeded into 6-well culture plates at a concentration of 1×10^6 cell/well. The following day, the RAW cells were incubated with liposomal PLP for 20 h (doses of 1, 10 or 100 μg/ml) in culture medium. All incubations were performed in triplicates. Subsequently, the medium was replaced with medium containing 100 ng/ml lippopolysaccharide (LPS, Sigma–Aldrich) for 4 h. After this time point, culture supernatant was stored at −80 °C until measurement of IL-1β by Luminex. After washing, cells were lysed with Trizol reagent (Sigma–Aldrich) and stored at −20 °C until RNA isolation.

For uptake of liposomes, RAW macrophages were seeded into 0.45 cm^3 tissue chambers at a concentration of 6×10^5 cells/well. The following day, the cells were incubated with fresh medium containing rhodamine-PE labelled PLP liposomes (10 μg/ml) for indicated durations (15 min, 2, 4 and 6 h). Subsequently the cells were washed, dried and sealed with Prolong antifade reagent (Invitrogen). Microscopic images were obtained with a Leica DMR microscope (Leica, Heerbrugg, Austria), mounted with a digital camera and images were processed with QuantiMed software (Leica).

2.3. Animals

Male C57Bl/6 mice were purchased from Elevage–Janvier (Le Genest Saint Isle, France) and were housed in filter-top cages and fed a standard diet and water ad libitum. Animal studies were approved by the Institutional Review Board (RU-DEC 2006-182) and were performed according to the related codes of practice.

2.3.1. Antigen-induced arthritis

Antigen-induced arthritis was performed as described previously (van den Berg et al., 1981). Briefly, mice at an age of 8–12 weeks were immunized with 100 μg methylated bovine serum albumin (mBSA, Sigma–Aldrich), emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, USA) which was injected into the flanks and the footpath of the forelegs. Heat-killed *Bordetella pertussis* (RIVM, Bilthoven, the Netherlands) was administered intraperitoneally as an additional adjuvant. Two subcutaneous booster injections with in total 50 μg mBSA/Freund's complete adjuvant were given in the neck region 1 week after the initial immunization. Two weeks after these injections, AIA was induced by intra-articular injection of 60 μg of mBSA in 6 μl of phosphate-buffered saline into both knee joints.

Table 2
Primer sequences.

Name	Forward primer (5′ > 3′)	Reverse primer (5′ > 3′)
GAPDH	GGCAAATTCAACGGCACA	GTTAGTGGGGTCTCGCTCCTG
MMP-3	TGAAGCACCAACATCAGGA	TGGAGCTGATGCATAAGCCC
MMP-9	GGAACTCACACGACATCTTCCA	GAACTCACACGCCAGAAGAATTT
MMP-13	AGACCTTGTGTTTGCAGAGCACTAC	CTTCAGGATTCCCGCAAGAG
MMP-14	GCCTGCATCCATCAATACT	CAGTGCTTATCTCCTTTGAAGAAG
ADAMTS-4	CACTGACTTCCTGGACAATGGTTAT	GGAAAAGTCGCTGGTAGATGGA
ADAMTS-5	GATGATCACGAAGAGCACTACGA	TCACATGAATGATGCCCACAT

2.3.2. Treatment, sacrifice and tissue isolation

At day 3, when severe arthritis had developed, groups consisting of 5 mice each (n = *5*), were treated with either a single intravenous injection of PBS (control group), empty liposomes (control group), 10 mg/kg liposomal PLP, 10 mg/kg free PLP or 4 repeated once-daily injections of 10 mg/kg free PLP. At day 8 after AIA-induction, whole blood was obtained by retro-orbital bleeding of mice anaesthetized with isoflurane (5%), into MiniCollect tubes (Greiner bio-one, Kremsmuenster, Austria). Hereafter, mice were sacrificed by cervical dislocation and the whole knee joints (right side) were stored in formalin for histology. Synovial biopsies of 3 mm diameter were taken on either side of the left knee joint and were stored in liquid nitrogen until RNA isolation. Synovial wash-outs were obtained by incubating the synovium, surrounding the patella of the left knee joint, in 200 μl RPMI culture medium containing anti-biotics for 2 h and were subsequently stored at −80 °C. Serum samples were obtained after coagulation and centrifugation of whole blood samples and were stored at −80 °C.

2.4. Histology

Whole knee joints of mice were isolated at day 8 after arthritis induction and fixed in 10% formalin. After decalcification for 2 weeks in 5% formic acid, the specimens were processed for paraffin embedding. Standard frontal sections of 7 μm were mounted on superfrost slides (Menzel–Gläser, Braunschweig, Germany) for histology and immunostaining. Histology was performed on sections stained with hematoxylin and eosin (HE). The severity of joint inflammation was determined as described previously (van Lent et al., 2003), by scoring the amount of cellular infiltration into the synovium using an arbitrary scale (0–3), for three representative knee joint sections for each mouse (5 mice for each treatment group). Scoring was performed in a blinded manner by two independent observers: 0, no cells; 1, mild cellularity; 2, moderate cellularity and 3, maximal cellularity.

2.5. Immunohistochemistry

Prior to immunostaining, sections were deparaffinised. The immunostaining of VDIPEN was based on the method by Singer et al. (1995) and performed as described previously (van Meurs et al., 1998). Briefly, sections were digested after rehydration with chondroitinase ABC (0.25 U/ml, 0.1 M Tris–HCl, pH 8.0; Sigma) for 1 h at 37 °C, to remove chondroitin sulphate from the proteoglycans. Sections were then treated with 1% H_2O_2 in methanol for 20 min and subsequently 5 min with 0.1% (v/v) Triton X-100 in PBS. After incubation with 1.5% normal goat serum for 20 min, sections were incubated with affinity-purified anti-VDIPEN IgG overnight at 4 °C. Hereafter, sections were incubated with biotinylated goat anti-rabbit *i*Gg and binding was detected using avidin–peroxidase staining (Elite kit; Vector Laboratories Inc., Burlingame, USA). Peroxidase activity was detected using nickel enhancement and counterstaining was performed with orange G (2%) for 5 min. Sections were embedded and preserved in Permount (Fisher chemicals, Fair Lawn, USA).

The immunostaining of NITEGE was performed as described previously (van Meurs et al., 1999b). Briefly, sections were incubated with rabbit-anti-murine NITEGE or with normal rabbit IgG (X0936; DAKO, Glostrup, Denmark) in phosphate-buffered saline containing 5% milk powder, 3% fetal calf serum and 2% BSA. Subsequently, the sections were incubated with biotinylated swine anti-rabbit IgG (E0431; DAKO) followed by labelling with streptavidin–horseradish peroxidase (Po397; DAKO). Peroxidase was developed with diaminobenzidine as substrate. Sections were counterstained with hematoxylin for 1 min and embedded in Permount (Fisher Chemicals).

The amount of staining was quantified using QuantiMed software (Leica), as a percentage per articular cartilage surface on the medial and lateral side of the femur and tibia, the frontal side of the femur and of the patella. The mean amount of staining was determined for three representative sections for each mouse (5 mice for each treatment group).

2.6. Measurement of cytokine levels

Cytokine levels of TNF-α, IL-1β, IL-6 and IL-10 were measured in synovial wash-outs and in blood serum samples and levels of IL-1β in culture supernatant using Luminex multianalyte technology (Bio-Rad Laboratories, Hercules, USA). Protein levels were calculated from a standard curve of known cytokine concentrations. Data analysis was performed with Bio-Plex Manager software (Bio-Rad Laboratories).

2.7. Quantitative reverse transcriptase polymerase chain reaction (Q-PCR)

To quantify mRNA levels in the synovium, RNA was extracted from 2 synovial biopsies (3 mm diameter) per knee joint: one from the medial side and one from the lateral side, which were stored in liquid nitrogen. The samples were thawed and submersed in Trizol reagent (Sigma–Aldrich) and RNA was isolated with a RNeasy kit (Qiagen, Venlo, The Netherlands). Isolated nucleic acids were treated with DNAse before being reverse transcribed into complementary DNA using oligo (dT) primers and MMLV reverse transcriptase. The quantitative real-time PCR was performed using the ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, USA) for quantification with SYBR Green (Applied Biosystems) and melting curve analysis. Primers were cDNA specific and designed with Primer Express Version 2.0 (Applied Biosystems). Primer sequences are listed in Table 2. PCR conditions were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Primer concentrations were 300 nM. All PCR's were performed in a total volume of 20 μl. Relative quantification of the PCR signals was performed by subtracting the threshold cycle (Ct) value, of the gene of interest of each sample with the Ct values of the reference gene GAPDH of the sample (dCt). Fold change was calculated as 2 to the power of the dCt value.

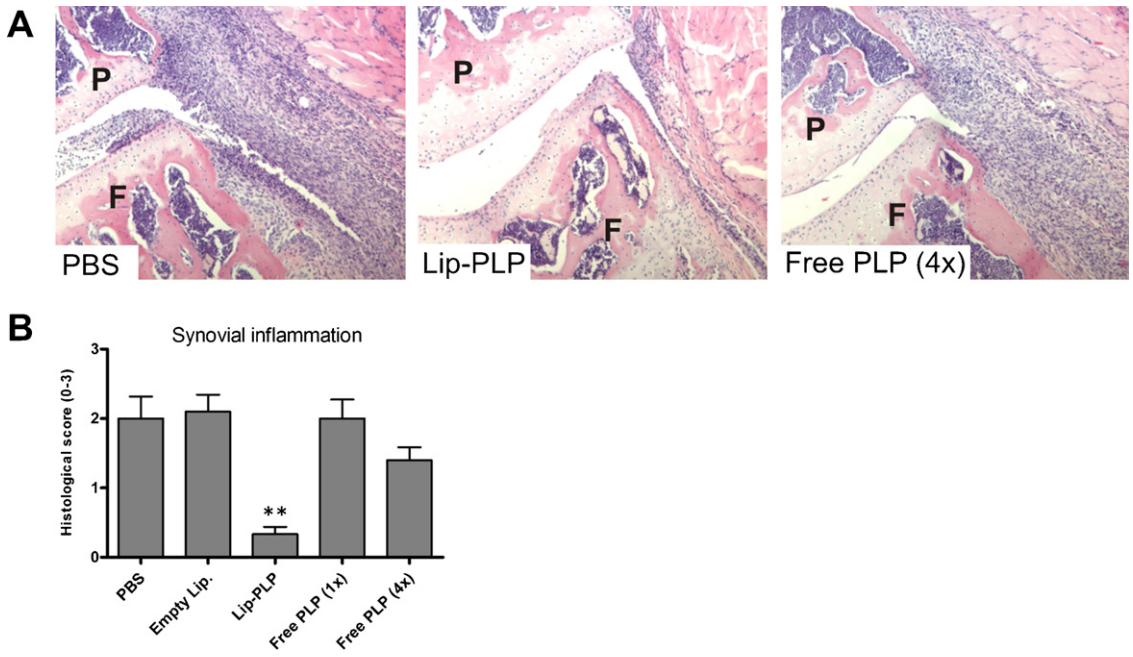

Fig. 1. Histology of the knee joints. Frontal HE stained knee joint sections show synovial infiltration which is significantly reduced by Lip-PLP. (A) Representative microphotograpghs (original magnification ×100). P, patella and F, femur. (B) Histological score of the inflammatory infiltrate in the synovium. Values are mean ± S.D. (n = 5), $^{**}p < 0.01$ by Mann–Whitney *U* test versus all other treatment groups.

2.8. Statistical analysis

Differences between experimental groups were tested for statistical significance by Student's *t*-test or Mann–Whitney U as non-parametric test, using Graphpad Prism 5.0 software. Data are expressed as mean ± standard deviation (S.D.). *p*-Values less than 0.05 were considered as statistically significant.

3. Results

3.1. Liposomal delivery of PLP strongly suppresses knee joint inflammation during antigen-induced arthritis (AIA)

After induction of AIA, arthritis was monitored using histology of frontal sections of the whole knee joint. Mice were treated at day 3 and sacrificed at day 8 after induction of AIA (5 days after treatment). At that time point, there was a massive inflammatory cell infiltrate present within the synovium of control treatment groups (PBS and empty liposomes) as well as in mice treated with a single injection of 10 mg/kg free PLP (Fig. 1). Although joint inflammation was somewhat lower in mice treated with 4 × 10 mg/kg free PLP (mean suppression of 33%, as compared to PBS treatment), the degree of inflammation was still florid. In sharp contrast, synovial infiltration was largely inhibited in mice that were treated with Lip-PLP (by 73%, as compared to PBS treatment).

3.2. Liposomal PLP suppresses synovial expression of IL-1β

Since joint inflammation is largely driven by cytokines, that can have either local or systemic effects, we additionally measured pro- and anti-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-10) in blood serum and in synovial wash-outs of mice sacrificed at day 8 after AIA.

Suppression of serum levels of TNF-α was statistically significant when compared to empty liposomal control treatment, but not when compared to PBS control treatment or free PLP (Fig. 2A). The differences between the levels of pro-inflammatory cytokines IL-1β and IL-6 and the anti-inflammatory cytokine IL-10 with those

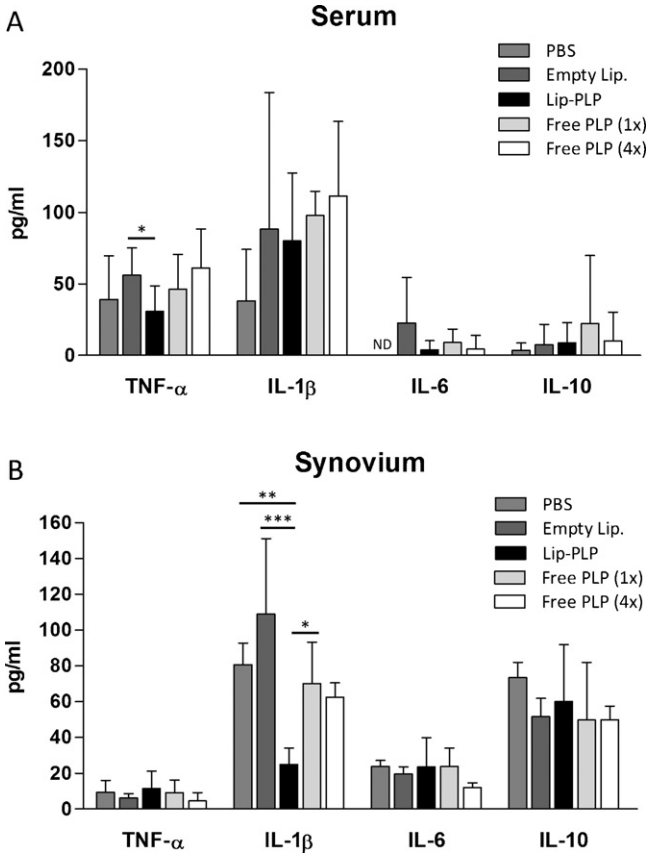

Fig. 2. Cytokine levels as measured by Luminex in blood serum (A) and synovial wash-outs (B). Note that Lip-PLP significantly suppressed TNF-α in serum and IL-1β in the synovium. Values are mean ± S.D. (n = 5), $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ by Student's *t*-test compared to indicated treatments. ND, not detected.

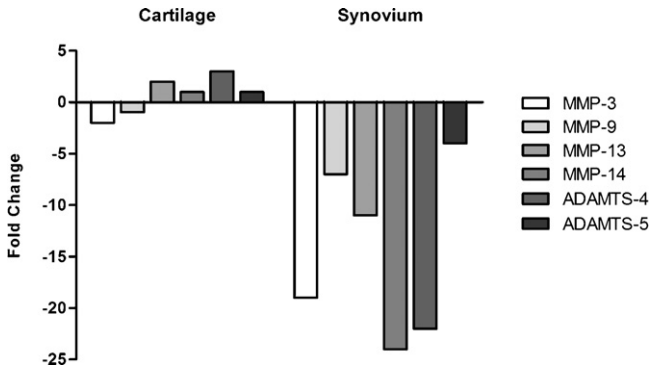

Fig. 3. Effect of Lip-PLP on expression of MMPs and aggrecanases in the synovium and in articular cartilage of the inflamed knee joint. Data are expressed as the fold change of mean expression values of the Lip-PLP treated group compared to the control, PBS-treated group Note that Lip-PLP gives a strong suppression of synovial expression compared to expression in cartilage. Statistical significance was determined from dCt levels of Lip-PLP treated mice compared to PBS treated mice (n = 5).

from controls or free PLP were not statistically significant (Fig. 3A). In contrast, in synovial wash-outs, levels of IL-1β, but not those of TNF-α, IL-6 and IL-10 were significantly suppressed by Lip-PLP when compared to control treatments or free PLP (Fig. 2B).

3.3. Liposomal PLP suppresses MMP and ADAMTS expression in the synovium

To study the effect of Lip-PLP treatment on protease mediated destruction within the joint, we measured gene expression of several MMPs and ADAMTS not only in the synovium, but also directly in the articular cartilage. The latter was isolated from femur and tibia head at day 8 after AIA induction. Interestingly, liposomal PLP strongly suppressed synovial gene expression of MMPs (MMP-3 19-fold, MMP-9 7-fold, MMP-13 11-fold and MMP-14 24-fold inhibition) and ADAMTS (ADAMTS-4 22-fold and ADAMTS-5 4-fold inhibition). However, Lip-PLP did not exert a statistically significant effect on MMP and ADAMTS expression within the articular cartilage (Fig. 3). This shows that Lip-PLP inhibits protease activity primarily in the synovium, rather than in articular cartilage.

3.4. Lip-PLP suppresses IL-1β and MMP expression by macrophages in vitro

As liposomes are selectively taken up by macrophages in the inflamed knee joint when administered systemically, the effect of Lip-PLP on MMP expression by this cell type was studied. We therefore incubated LPS-activated macrophages *in vitro* with Lip-PLP. When macrophages were cultured with rhodamine-PE labelled Lip-PLP, a rapid uptake was observed and was already detected inside macrophages within 15 min and maximal uptake was found after 4 h (Fig. 4A). When incubated for 6 h, no further uptake was observed (data not shown).

Macrophages that were stimulated with LPS for 24 h produced high levels of IL-1β. Lip-PLP treated macrophages showed a dose-dependent suppression of IL-1β production (Fig. 4B). Moreover, LPS induced activation of macrophages elevated expression of mRNA levels of various MMPs as measured by quantitative RT-PCR. MMPs 3, 9, 13 and -14 were raised by 3-, 16-, 379- and 3-fold respectively (Fig. 4C). When LPS-activated macrophages were incubated with Lip-PLP for 24 h, a significant reduction in mRNA levels of MMPs -3 (100%), 9 (76%), -13 (73%) and 14 (67%) was found, indicating that liposomal targeting of PLP to macrophages offers an effective strategy to inhibit the factors that contribute directly and indirectly to destruction of cartilage matrix during arthritis.

3.5. Liposomal PLP inhibits protease mediated cartilage matrix destruction

We further investigated the effect of Lip-PLP treatment on cartilage destruction by MMPs and ADAMTS *in vivo*. During AIA, aggrecan, an important component of the cartilage matrix, is cleaved by MMPs and ADAMTS within its interglobular domain leaving neo-epitopes VDIPEN and NITEGE respectively (van Meurs et al., 1999b; Nagase and Kashiwagi, 2003). In our study, we detected both neo-epitopes in cartilage layers of the femur,

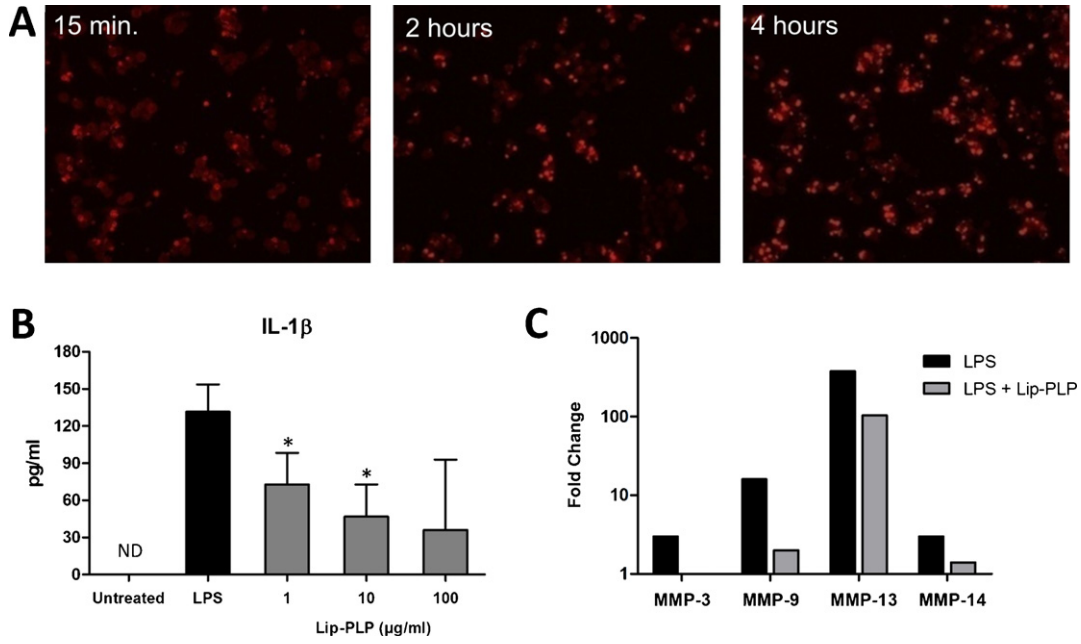

Fig. 4. Effect of Lip-PLP on RAW macrophages. (A) Microphotographs of RAW cells treated with rhodamine-PE labelled liposomes (original magnification ×200). Note an increase in liposome phagocytosis over time. (B) Dose dependent suppression of IL-1β by Lip-PLP during LPS activation. (C) Expression of MMPs by activated RAW macrophages is suppressed by Lip-PLP. Values are mean ± S.D. of triplicates, $^{*}p < 0.05$ by Student's *t*-test compared to control (LPS activation). ND, not detected.

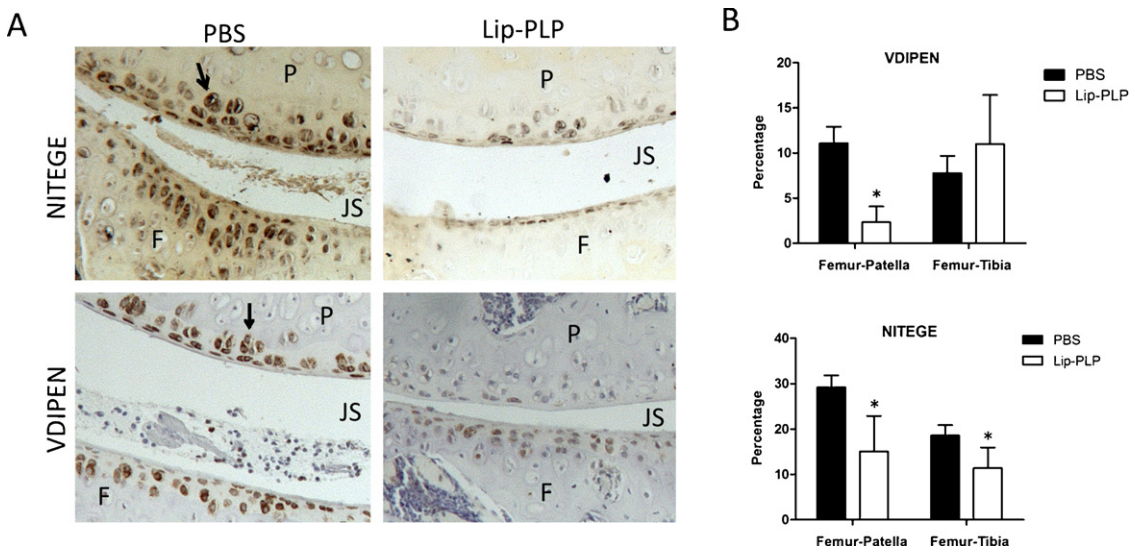

Fig. 5. Expression of the neo-epitopes NITEGE and VDIPEN. (A) Microphotographs of articular cartilage layers of patella (P) and femur (F) stained with immunolocalisation against VDIPEN or NITEGE (original magnification ×200) treated with PBS or Lip-PLP. (B) Quantification of VDIPEN and NITEGE staining in frontal knee joint sections. Note that Lip-PLP significantly suppresses VDIPEN and NITEGE expression. Values are mean ± S.D. (n = 5), $^*p < 0.05$ by Student's *t*-test compared to PBS control. JS, joint space. Arrows point to positive staining of the pericellular matrix around chondrocytes.

tibia and patella by immunohistochemistry (Fig. 5). Interestingly, VDIPEN expression in the femur–patella region in Lip-PLP-treated mice was reduced by 79% when compared to PBS treated mice (Fig. 5B). Expression of NITEGE was reduced in both the femur–tibia regions (35%) as well as in the femur–patella region (49%) when compared to PBS-treated mice (Fig. 5B). These data indicate that suppression of IL-1β and MMPs/ADAMTS by Lip-PLP has functional consequences resulting in less destruction of the cartilage matrix.

4. Discussion

In the present study we demonstrate that a single systemic injection of liposomal PLP (Lip-PLP) significantly inhibits cartilage destruction during antigen-induced arthritis (AIA). Cartilage destruction in AIA develops gradually and is largely mediated by cytokines and proteinases. In the onset of AIA, ADAMTS-4 and ADAMTS-5 cleave proteoglycans, thereby leaving neoepitopes NITEGE behind in the cartilage. Previous studies performed by Stanton et al. have shown that in ADAMTS-5 knockout mice, the cartilage destruction occurring after induction of AIA, was completely abrogated (Stanton et al., 2005). At a later time-point starting at day 5 after induction of AIA, MMPs become activated which not only further degrade proteoglycans, thereby leaving neoepitopes behind which end at the amino acid sequence VDIPEN, but more importantly also break down the collagen type II network which eventually causes irreversible matrix destruction (van Meurs et al., 1999a; Sondergaard et al., 2006). Cartilage destruction is thought to be mediated by MMPs produced by both chondrocytes in the cartilage as well as by inflamed synovium (Young et al., 2005; Goldbach-Mansky et al., 2000). Our study shows that a single intravenous injection of Lip-PLP strongly decreases MMP expression in the synovium but not in the cartilage. This suggests that the effect of liposomal PLP treatment is largely due to targeting the inflamed synovium. MMPs and ADAMTS are produced in large amounts by the synovial lining layer and synovial fibroblasts are the major cell type involved in MMP production (Pap et al., 2000). Macrophages which lie adjacent to these fibroblasts become activated and form the dominant producers of cytokines like TNF-α and IL-1β. These master cytokines stimulate fibroblast and chondrocytes to produce MMPs.

In the present study, a single injection of Lip-PLP inhibited joint inflammation which correlated with strong inhibition of local but not systemic IL-1β production. In AIA, IL-1β is the major cytokine driving severe cartilage destruction. Earlier studies showed that blocking IL-1β by specific antibodies or by the inhibitory IL-1β receptor antagonist (IL-1RA) released by osmotic pumps completely inhibited severe cartilage destruction (Joosten et al., 1996). Local production of IL-1β is not only important directly by promoting cartilage destruction, but also indirectly by regulating joint inflammation. Overexpression of IL-1RA using an adenovial vector significantly inhibited joint inflammation during experimental arthritis (Smeets et al., 2005). Liposomal PLP treatment may thus particularly act by down-regulating local IL-1β production by synovial macrophages which was substantiated by our finding that systemic Lip-PLP treatment causes significant inhibition of IL-1β production only locally in the inflamed joint but not in the serum. Furthermore, our *in vitro* studies show that macrophages which were activated by LPS and subsequently treated with liposomal PLP also show significantly reduced IL-1β production. This suppression of IL-1β is likely to be mediated by activation of the glucocorticoid receptor (GR) which has a strong suppressive effect on NFκB mediated gene expression (de Bosscher et al., 2003).

Apart from blocking cytokines like IL-1β, liposomally targeted corticosteroids may also downregulate crucial factors which are involved in the activation of MMPs. These proteases are secreted in a latent form and need a cleavage step in order to become active. Antigen-induced arthritis is a disease-model mediated by IgG immune complexes (IgG-IC). These IgG-IC bind to Fcγ receptors (FcγRs) present on the surface of macrophages which then become activated and thereby produce factors like oxygen radicals and enzymes which have been shown to activate MMPs (Blom et al., 2000). The FcγR's are crucial in mediating MMP activation during AIA, as was shown by us in earlier studies using FcγR knockout mice (van Lent et al., 2006). Liposomal PLP may be a potent inhibitor of FcγR expression on macrophages which may further contribute to protection of cartilage (data not shown).

The PLP-liposomes used within this study had a diameter around 100 nm and this size allows them to travel passively through the endothelial cell layer of the dilated blood vessels present within the inflamed synovium during arthritis. This effect is known as the

'enhanced permeability and retention (EPR) effect' and together with the sustained circulation of long-circulating liposomes is responsible for their strong effects in the inflamed joint (Torchilin, 2000). Moreover, most of the blood vessels lie just beneath the lining layer predominantly consisting of macrophages. Once the PLP-liposomes leave the blood vessels they are directly phagocytosed by these lining macrophages which have been shown to regulate the onset and propagation of joint inflammation during antigen-induced arthritis (van Lent et al., 1998b). Selective depletion of lining macrophages using clodronate-containing liposomes completely abrogated joint inflammation during experimental arthritis (van Lent et al., 1998a). Therefore, macrophage targeting by liposomal PLP explains their efficacy in inhibiting cartilage destruction during experimental arthritis.

In a recent phase I study it was shown that a single treatment with liposomal PLP was well tolerated by RA patients and proved more effective than a depot injection of methyl-prednisolone (Depo-Medrol) in reducing disease activity scores (DAS) (Barrera et al., 2008). Future studies should determine the specific advantages of the cellular targeting of liposomal glucocorticoid and its effect on the disease course of both experimental arthritis and RA.

5. Conclusion

Liposomal PLP inhibits cartilage matrix destruction in antigen-induced arthritis by suppressing synovial inflammation and the expression and activity of MMPs and ADAMTS. The inhibitory effect on cartilage destruction is likely to be mediated via targeting of macrophages in the inflamed synovium as these cells contribute largely to MMP/ADAMTS-mediated cartilage matrix destruction and as they are also suppressed in their activity by Lip-PLP *in vitro*.

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