Journal of Controlled Release xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Review

Liposomal corticosteroids for the treatment of inflammatory disorders and cancer

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ARTICLE INFO

Article history:
Received 9 April 2014
Accepted 20 May 2014
Available online xxxx

Keywords:
Liposomes
Corticosteroids
Nanomedicine
Inflammation
Cancer

ABSTRACT

Glucocorticoids (GC) are known for their potent immunosuppressive and anti-inflammatory properties. As a result, they are extensively used for the treatment of many different diseases. Prolonged and/or high-dose GC therapy, however, generally comes with severe side effects, resulting not only from their very diverse mechanism(s) of action, but also from their relatively poor biodistribution. Drug delivery systems, and in particular liposomes, have been extensively used to enhance the biodistribution and the target site accumulation of GC, and to thereby improve the balance between their efficacy and their toxicity. Many different types of liposomes have been employed, and both local and systemic treatments have been evaluated. We here summarize the progress made in the use of liposomal GC formulations for the treatment of asthma, rheumatoid arthritis, multiple sclerosis and cancer, and we show that the targeted delivery of GC to pathological sites holds significant clinical potential.

1. Introduction

1.1. Liposomes

Ever since their first description by Alec Bangham more than half a century ago, liposomes have been extensively used for drug delivery applications [1–3]. Because of their relatively straightforward preparation, as well as their excellent biodegradability and biocompatibility, liposomal systems have progressed into one of the most extensively used and clinically most advanced drug delivery platforms [4].

Liposomes are composed of phospholipids, which, due to their amphiphilic nature, spontaneously self-assemble into vesicular structures when dispersed in aqueous media. In these lipid vesicles, the hydrophilic head groups line up and face the outer aqueous environment, while another layer of polar heads face the aqueous interior, segregating the hydrophobic tail groups of both layers from the aqueous environment (Fig. 1). The vesicular membrane [2], which in fact may consist of a number of bilayers, provides the liposome with structural stability, and enables the encapsulation of pharmacologically active agents, either in the layer itself for lipophilic compounds, or – more commonly – in the aqueous core for hydrophilic compounds [5]. When administered locally, the liposomal formulation allows for prolonged retention of the encapsulated drug at the injected site by limiting its diffusion and degradation ('depot' function). By limiting renal excretion and hepatic degradation, some liposome formulations, especially those with high transition-temperature saturated phospholipids and high cholesterol content, optionally containing a small percentage of PEGylated lipids (so-called 'long circulating liposomes') improve the pharmacokinetics of encapsulated drugs when administered systemically, allowing them to circulate for prolonged periods of time.

In addition, the 'Enhanced Permeability and Retention' (EPR) effect [7] promotes the accumulation of liposomes in tissues characterized by enhanced vascular leakiness, such as tumors and sites of inflammation, while at the same time attenuating their localization in healthy non-target tissues. In addition, liposomes can also be administered locally, such as through inhalation, and can increase the delivery and accumulation of drug molecules in the target tissue. As a consequence, liposomal drugs tend to be more effective and less toxic than standard (low-molecular-weight) drugs, they can be administered less frequently, and can improve both time- and cost-effectiveness.

1.2. Glucocorticoids

Glucocorticoids (GC) are a class of steroid hormones that possess strong immunosuppressive and anti-inflammatory activity. Ever since their introduction in the 1950s, GC have therefore been extensively used in diseases caused by an excessively active immune system, such as allergies, asthma, autoimmune diseases and sepsis [8]. GC exert

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http://dx.doi.org/10.1016/j.jconrel.2014.05.039
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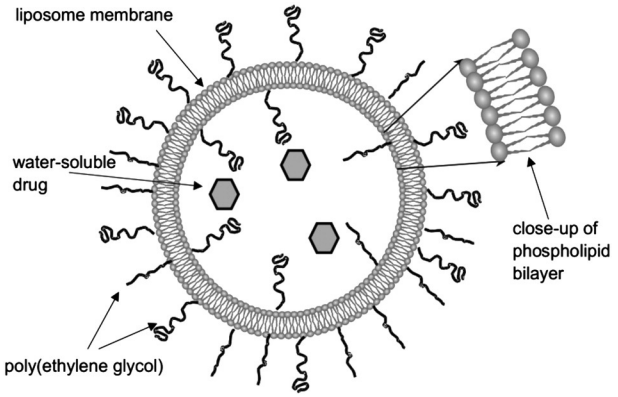

Q2 **Fig. 1.** Schematic depiction of a long-circulating liposome.
Image reproduced, with permission, from [6].

their effects by binding to the glucocorticoid receptor (GR) [9], which, once inside the nucleus, modulates several DNA transcription factors. This leads to the up-regulation of anti-inflammatory protein production and to a concomitant down-regulation of pro-inflammatory protein production (Fig. 2) [10].

In addition to such relatively slow genomic effects, GC also display more rapid non-genomic effects, including e.g. inhibition of arachidonic acid release and alterations in cation transport across the plasma membrane [12]. The genomic and non-genomic effects together, change the metabolism of lipids, carbohydrates, proteins and have been shown to affect bones, neurons, glial cells, and the electrolyte and water balance [7]. Although the glucocorticoid receptor (GR) is involved, the exact molecular mechanism driving the non-genomic activity of GC still remains unclear [7,12,13].

Because of their broad pharmacologic activity, GC are notorious for their side effects. These include immunosuppression (and the increased risk of infection), musculoskeletal complications (such as osteoporosis, osteonecrosis, myopathy), growth suppressive effects (in children), hypertension, rapid weight gain, diabetes, hypertriglyceridemia, hypercholesterolemia, dermatological effects (fat redistribution, thinning of the skin, allergic reactions), glaucoma, peptic ulcer disease, decelerated wound healing, and electrolyte imbalance [7,14].

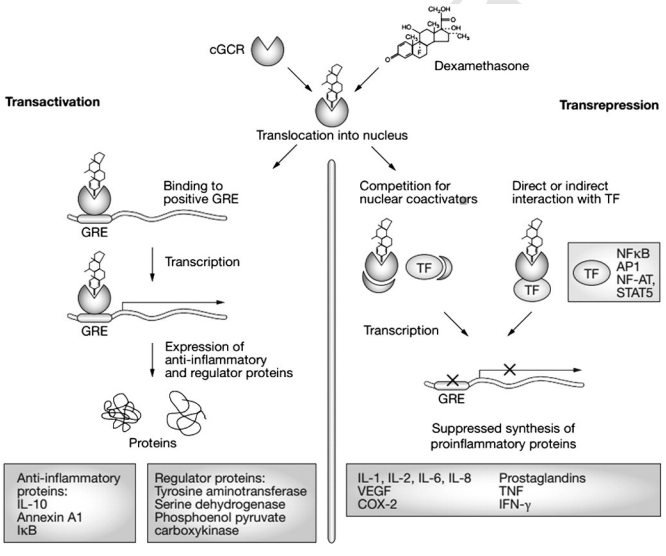

Fig. 2. Mechanism of action of glucocorticoids (GC). Upon binding to the cytosolic glucocorticoid receptor (cGCR), GC activate and repress a large number of important (anti-) inflammatory mediators. Only genomic effects, at the transcriptional level, are shown.
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The encapsulation of GC in liposomes has been extensively evaluated over the past 2–3 decades. This is done to reduce the volume of distribution and the off-target accumulation of GC, thereby lowering their toxicity, as well as to increase and prolong drug levels at the pathological site, to improve their therapeutic efficacy. We here summarize several key advances in this area of research, and provide an overview of studies showing the potential usefulness of liposomal GC for improving the treatment of asthma, multiple sclerosis, rheumatoid arthritis and cancer.

2. Liposomal glucocorticoids for inflammatory disorders

2.1. Asthma

2.1.1. Pathophysiology of asthma and therapeutic role of glucocorticoids

Asthma is a chronic respiratory disorder with a strong allergic component, which is characterized by an obstruction of the pulmonary airways, causing shortness of breath, wheezing, coughing and chest tightness or pain [15]. The disease initially develops with bronchial provocation and hyper-responsiveness, followed by bronchial inflammation and swelling of the inner walls of the airways (lamina reticularis). In addition, increased growth of mucus cells leads to mucus hypersecretion and a thicker mucus structure. This results in an increased tendency to lung hyperinflation, smooth muscle hypertrophy, edema and cilia cell disruption [15]. Although the symptoms of asthma are mostly reversible, the associated inflammation of the pulmonary tract may lead to permanent structural changes, also known as airway remodeling [16].

Asthma therapy generally aims to reduce symptoms, maintain pulmonary function, prevent recurrent exacerbations, and minimize hospitalization [17]. In addition to non-steroidal therapeutics, such as bronchodilators, inhaled glucocorticoids (IGC) are prescribed frequently in asthma therapy, because of their effective anti-inflammatory properties [18]. However, IGC have some limitations due to long-term side effects, especially in older patients [19]. Additionally, with the need of daily dosing, these effects may lead to patient noncompliance and treatment failure. Several studies have demonstrated that the employment of a drug delivery platform for inhaled GC therapy in asthma may have a direct and distinct pulmonary effect with reduced side effects [20–22]. This review focuses on local delivery of liposomes to the lungs for therapy of asthma, which provide an optimized pulmonary residence time of the drug by increasing lung deposition and decreasing upper respiratory tract retention, while drug redistribution to non-target tissues is attenuated [23,24].

2.1.2. Liposomal glucocorticoids for pulmonary therapy of asthma

One of the first clinical studies involving liposomal GC in asthma evaluated the use of nebulizers to administer dilauroyl phosphatidylcholine (DLPC) liposomes containing beclomethasone dipropionate (Bec-DP) [25]. Using 18 different types of nebulizers, the local lung deposition efficiency of liposomes with a diameter of 1–3 μm was evaluated. While the majority of nebulizers were able to provide acceptable performance for delivering Bec-DP liposomes, only two of them, i.e. Aerotech II and Spira, achieved high localization in alveolar airways, and relatively low deposition in mouth and throat.

The lung deposition and clearance of ^{99m}Technetium-labeled Bec-DLPC liposomes was visualized and quantified in a follow-up study [26]. These experiments showed that ~75% of the inhaled liposomes were in the pulmonary tract, ~12% in the nasopharynx, and ~13% in the stomach and intestine (Fig. 3A). Although free ^{99m}Tc was cleared within minutes, ~50% of the liposome-associated radioactivity was still found to be present in the lungs 24 h after inhalation, indicating a substantially prolonged retention of radiolabeled liposomes in the lungs (Fig. 3B).

Also in healthy human volunteers, a strong deposition in the lungs and oropharynx was observed upon using the Aerotech II and Spira

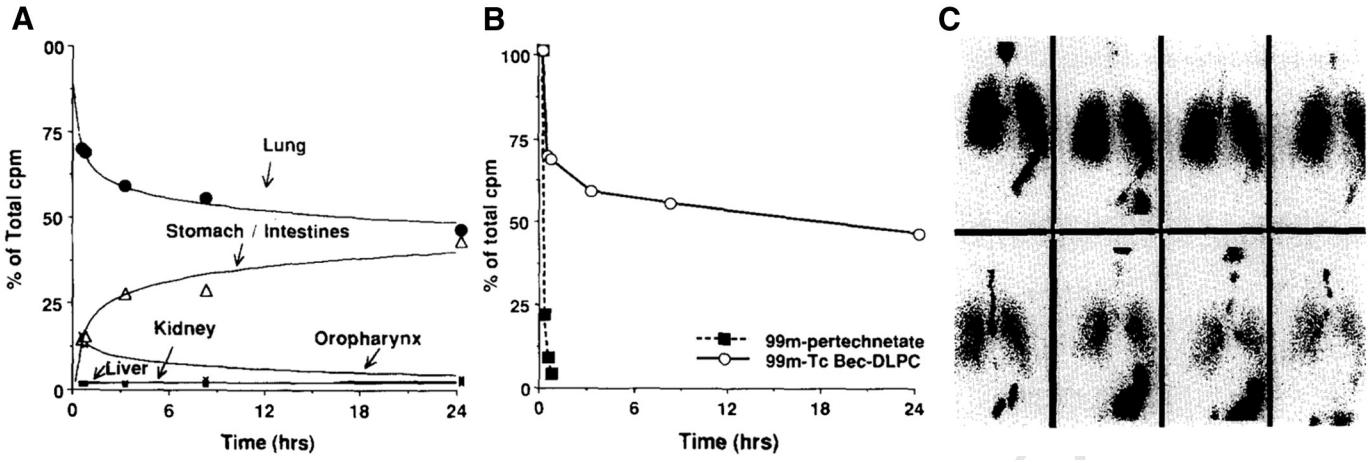

Fig. 3. Pharmacokinetics of ^{99m}Tc-labeled Bec-DLPC liposomes in mice after inhalation. A. Distribution of liposomes in lung, liver, kidney, oropharynx and stomach/intestine, showing 50% of the liposome-related radioactivity still present in lungs after 24 h. B. The different lung clearance rates observed for free ^{99m}Tc and ^{99m}Tc-labeled Bec-DLPC liposomes exemplify significantly prolonged retention in the lung upon using liposomes. C. Scintigraphic scans showing deposition of ^{99m}Tc-labeled Bec-DLPC liposome in the lungs of healthy volunteers upon using the Aerotech II (top) and the Spira nebulizer (bottom), at 0,1, 2 and 3 h after inhalation (left to right).
Image reproduced, with permission, from [26].

nebulizer. Once deposited, a large proportion of inhaled radiolabeled liposomes remained in the lung for >3 h [26]. The clearance levels differed between the two nebulizers, likely because of aerosol particle size (much larger in the case of the Spira; resulting in less deep and less homogenous deposition, and faster mucociliary clearance; Fig. 3C). No significant side effects, either local or systemic, were observed upon assessing the tolerability of Bec-DLPC liposomal aerosol formulations in healthy volunteers, in spite of efficient deposition and distribution [27]. Also in patients, the inhalation of Bec-DLPC liposomes resulted in a beneficial distributional pattern: high overall localization in the lungs, but moderate to low deposition in the upper respiratory tract (i.e. the oropharynx, mouth, and throat) and in the gastrointestinal (GI) tract [28]. In the case of severe asthma, as compared to mild asthma, there was some increased clearance and inhomogeneous deposition [29], but in both groups, more than half of the dose was still present in the lung 1 day after administration. A similar study using DPPC liposomes showed 88% lung deposition at 6 h after aerosol inhalation, suggesting that a single daily dose of inhaled liposomal GC might suffice for proper therapeutic efficacy [30].

In some cases, when moderate IGC administration is ineffective, its combination with a long-lasting β2-agonist, such as formoterol, provides better therapeutic outcome than just using higher IGC doses [31]. Similarly, the co-administration of formoterol with ^{99m}Tc-labeled Bec-DLPC liposomes significantly improved the liposomal localization and therapeutic activity, as measured by spirometry, of pulmonary administered GC liposomes in asthma. Although formoterol could potentially stimulate liposomal clearance (since β2 agonists are known to improve mucociliary clearance both *in vitro* and *in vivo* [32]; particularly in patients with bronchitis [33]), the pulmonary retention of the ^{99m}Tc-labeled liposomes was unaffected by formoterol therapy.

Finally, it is worth noting that PEGylated liposomal aerosols have also been evaluated, e.g. containing budesonide. As shown in Fig. 4, in a mouse model for asthma, weekly administration of budesonide-loaded PEGylated liposomes resulted in a similar efficacy as equal daily doses of free budesonide [34]. Interestingly, the therapeutic efficacy of weekly-administered budesonide-encapsulating conventional liposomes was much lower. Moreover, the budesonide-loaded PEG-liposomes induced an effective decrease in serum eosinophil peroxidase activity (EPO, an eosinophilic activation marker in asthma), while the other treatments tested, including non-PEGylated liposomal budesonide, failed to demonstrate an effect. This positive contribution of PEGylation on the efficacy of pulmonary administered liposomal aerosol formulation seems to be related to their improved physicochemical stability (i.e. less aggregation, less opsonization, etc.) as compared to unPEGylated liposomes.

2.2. Rheumatoid arthritis

2.2.1. Pathophysiology of rheumatoid arthritis and therapeutic role of glucocorticoids

Rheumatoid arthritis (RA) is a chronic, systemic and progressive autoimmune disease, characterized by inflammation of the joints [35].

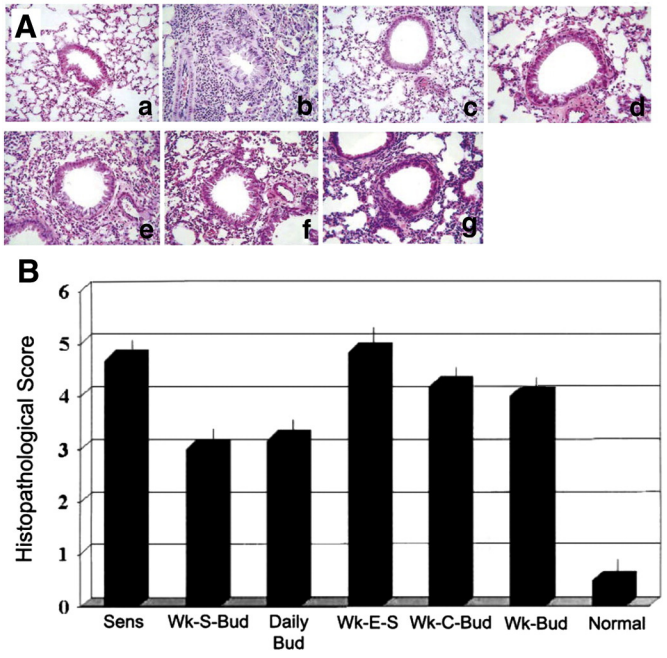

Fig. 4. Histopathology of lung tissue in asthmatic mice, showing the therapeutic benefit of entrapping budesonide in PEGylated liposomes. A. Immunohistochemical staining of: a Healthy lung tissue. b–g. Diseased lung tissue; b. untreated control (sens). c. After weekly administration of budesonide in stealth liposomes (Wk-S-Bud). d. After daily free budesonide administration (Daily Bud). e. After weekly administration of budesonide in conventional liposomes (WK-C-Bud). f. After weekly administration of empty stealth liposomes (Wk-E-S). g. After weekly administration of free budesonide (Wk-Bud). B. Scores of inflammation in lung tissues, calculated based on histopathology (none = 0, mild = 1–2, moderate = 3–4 and severe = 5–6).
Image reproduced, with permission, from [34].

Although a genetic basis has been suggested, and although certain environmental factors, such as viruses, bacteria and fungi may trigger RA, the exact pathophysiologic mechanisms have not yet been elucidated [36]. The inflammation generally occurs in the lining tissue (synovium) of the joint, which normally consists of layers of mostly fibroblast- and macrophage-like synoviocytes. Especially macrophages play an important role in the onset and progression of RA [37,38]. Among other pathologic phenomena, the inflammation leads to large-scale infiltration of macrophages, which causes expansion of the synovial lining, resulting in thickening and excessive synovial fluid production [39]. This leads to inflammation of the joints, with symptoms such as swelling, stiffness, pain and loss of function. In severe cases, there may be destruction of articular cartilage and bone, which leads to joint erosion and eventually deformation, resulting in chronic pain and progressive non-reversible joint damage. When activated, macrophages produce pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1), triggering the production of other inflammatory mediators, such as cyclooxygenase type 2 (COX2), prostaglandin-E2 (PGE2) and nitric oxide (NO). These mediators stimulate and amplify the inflammation. IL-1 plasma levels have been correlated with RA disease severity, and although IL-1 and TNFα have overlapping effects, studies have shown that IL-1 knockout rats did not develop RA, whereas TNFα knockout animals could still develop the disease [36].

RA therapy focuses on reducing joint inflammation, maximizing joint function, limiting damage and preventing deformation. GC were introduced for the clinical management of RA during the 1950s. As they are capable of inducing a dramatic decrease in joint inflammation, they have become extensively used for the treatment of arthritis [40]. Unfortunately, as alluded to above, GC tend to be fairly unspecific, and since they have an effect on many different organs and tissues, their use generally comes with numerous side effects [41]. To exploit the efficient anti-arthritic effects of GC, and to at the same time attenuate their off-target effects, various liposome formulations have been designed and evaluated over the years for RA-targeted GC delivery.

2.2.2. Liposomal glucocorticoids for local rheumatoid arthritis therapy

Liposomes have been studied as delivery vehicles for local GC therapy (intra-articular administration) as well as for inflamed joint targeting after systemic (intravenous) injection. Local liposomal GC delivery was already studied in 1976 by Shaw and coworkers who explored whether a nanomedicine formulation would enhance the interaction between the drug and the target cell, enabling more effective therapy using a lower dose and with less systemic toxicity [42]. Cortisol palmitate or cortisol octanoate was loaded into the lipid bilayer of different liposomal formulations, based on DMPC, DPPC or DSPC. More efficient incorporation was achieved with cortisol octanoate, which has a longer lipid chain. The release rate of cortisol octanoate was significantly higher for DMPC liposomes as compared to the other formulations, and particularly DPPC liposomes showed very slow release. The lower encapsulation efficiency of cortisol palmitate and the fast release of DMPC liposomes likely result from the lower lipid chain melting temperature of the steroid-derivative and of the phospholipid, respectively. Shorter chains have lower melting temperatures, and the bilayers that contain short-chained compounds are less organized and less stable at body temperature compared to the formulations composed of longer lipid chains [43]. In a subsequent study, liposomal cortisol palmitate was evaluated in a rabbit model of acute arthritis, in which it demonstrated an efficient anti-inflammatory effect in vivo [44]. In addition, a similar study showed a high proportion of liposomes localizing in the synovium, which indicated successful drug targeting [45]. This was further investigated in a study of Phillips et al., where the effect of various doses of liposomal cortisol palmitate were examined in rabbit knee joints with experimental arthritis [46]. The administration of DPPC/EPC liposomes loaded with cortisol palmitate into the inflamed knee joint of rabbits resulted in a sustained improvement of the temperature and diameter of the inflamed joints.

2.2.3. Liposomal glucocorticoids for systemic rheumatoid arthritis therapy

Even more interesting from a drug targeting perspective is a series of the studies regarding the use of intravenously injected liposomal formulations of GC in preclinical arthritis models of RA. Several methods have been developed to improve *in vitro* and *in vivo* stability, such as the inclusion of cholesterol, which improves molecular packaging within the bilayers, resulting in increased stability [47]. The stability of systemically administered liposomes can be further improved by incorporating PEG into the liposomal bilayer, delaying opsonization and recognition by macrophages of the mononuclear phagocytic system (MPS) [48,49], enabling the liposomes to stay in the circulation for a prolonged period of time, and thereby increasing their passive target site accumulation and their therapeutic efficacy [49].

The principle of passive joint targeting using liposomes has been demonstrated via comparative pharmacokinetic analyses, as well as via radiolabeling and scintigraphic imaging of biodistribution and target site accumulation [50]. As shown in Fig. 5, liposomal prednisolone phosphate (PLP) circulated for much longer than free PLP, and the liposomes efficiently accumulated in arthritic joints, resulting in a dramatic increase in the therapeutic efficacy of the (targeted) GC. Repeated daily dosing of 10 mg/kg free PLP (7×; pulse therapy) was only able to halt the disease progression, whereas a single 10 mg/kg dose of PLP liposomes resulted in a complete resolution of joint inflammation (Fig. 5). Moreover, when free PLP treatment was stopped, inflammation reappeared within a day, while with liposomal PLP the effect was also significantly more prolonged.

To optimize joint targeting, the pharmacokinetics and the biodistribution of different liposome formulations were also compared [50]. Small PEGylated liposomes (100 nm) resulted in slow plasma clearance and in a significant increase in PLP half-life, as compared to larger PEG liposomes (500 nm) or small (100 nm) liposomes without PEG coating (Fig. 6A). These findings correlated with their biodistribution, showing the highest levels of arthritic joint targeting for small PEGylated liposomes, and the lowest levels in MPS organs such as spleen (Fig. 6B). Arthritic rats furthermore showed a 7-fold higher accumulation of 100 nm-sized PEGylated in their hind paws as compared to healthy rats, providing proof-of-principle for efficient passive targeting.

In a subsequent study, the effect of a single dose of PLP liposomes on joint inflammation and cartilage degradation was evaluated in another arthritis model (collagen-induced arthritis (CIA)) [51]. As in rats with AIA, these studies showed that a single 10 mg/kg administration of liposomal PLP resulted in a complete reversal of joint inflammation (Fig. 7A). A single 10 mg/kg dose of free PLP did not result in significant disease inhibition. When administered daily, free PLP did display anti-inflammatory activity, however this effect was similar to that of single administration of liposomal PLP at a 10 times lower dose (Fig. 7B). At the histopathological level, cartilage damage was much lower upon treatment with PLP liposomes, indicating efficient and long-lasting inhibition of inflammation. These promising preclinical results have provided the basis for clinical studies in which liposomal PLP are evaluated in RA patients [52].

Avnir and colleagues [53] evaluated PEGylated liposomes that were remotely loaded (using the transmembrane calcium acetate gradient method) with betamethasone-hemisuccinate and methylprednisolone-hemisuccinate in arthritic rats. When compared to similar, passively loaded liposomes, these remotely loaded liposomes demonstrated enhanced encapsulation efficiency, therefore allowing the administration of reduced lipid doses and thus limiting potential side effects related to the liposomal lipids or vehicle [54]. Also these studies revealed an increased anti-arthritic activity of liposomal GC versus free GC. Moreover, the efficacy of liposomal methylprednisolone and betamethasone, either administered subcutaneously or intravenously, was superior to

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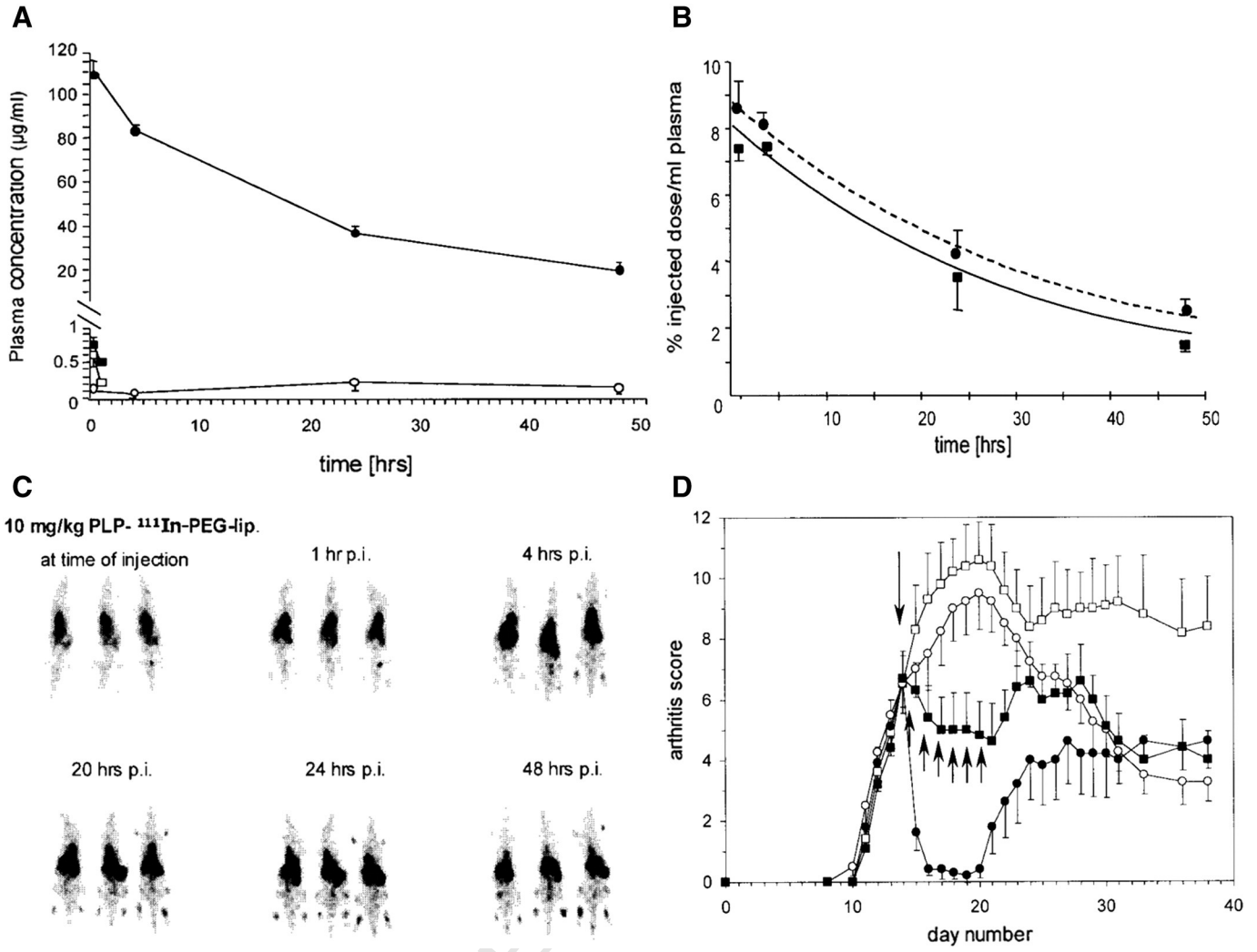

Fig. 5. Superiority of liposomal PLP as compared to free PLP for the treatment of RA. A. Levels of prednisolone phosphate (PLP) and prednisolone (PL) in plasma upon the i.v. injection of liposomal PLP and free PLP. (PLP after liposomal PLP = solid circles, PL after liposomal PLP = open circles, PLP after free PLP = solid squares, PL after free PLP = open squares). B. Plasma concentration (as % injected dose) of PLP liposomes (solid circles) and drug-free ^{111}In-labeled liposomes (solid squares). C. Scintigraphic imaging of the biodistribution and arthritic joint accumulation of ^{111}In-labeled PEGylated PLP liposomes, showing efficient targeting to the pathological site. D Arthritis scores after a single injection PEGylated PLP liposomes (10 mg/kg; solid circles), as compared to seven doses of free PLP (7 × 10 mg/kg; solid squares), empty PEG liposomes (open circles) and saline (open squares). A clear therapeutic benefit of liposome GC targeting can be observed.
Image reproduced, with permission, from [50].

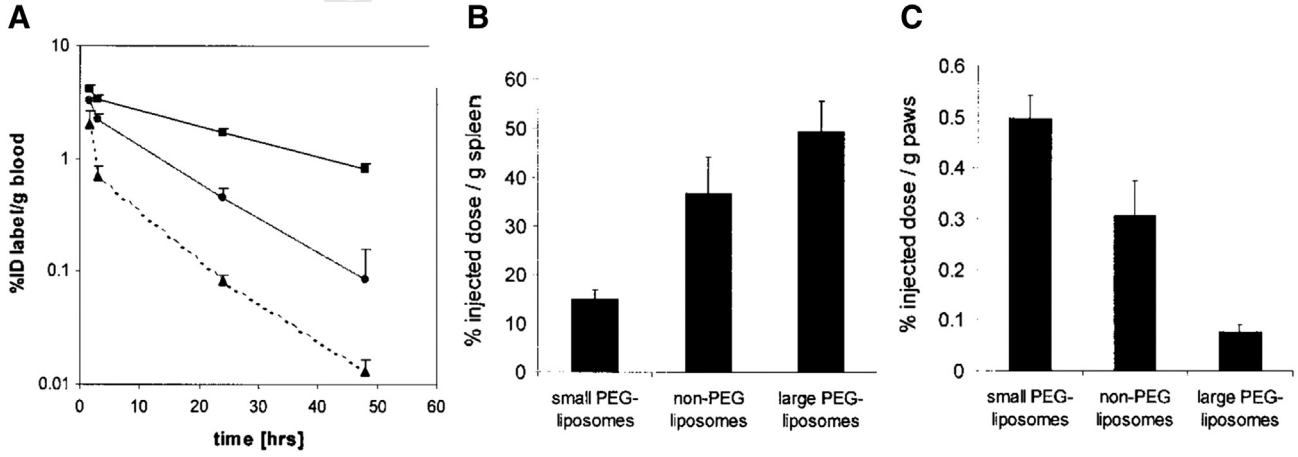

Fig. 6. Pharmacokinetics and localization of liposomes depend on liposome size and PEGylation. A. Blood levels of small PEGylated liposomes (solid squares), non-PEGylated liposomes (solid circles), and large PEGylated liposomes (solid triangles). B. Localization of small PEGylated liposomes, large PEGylated liposomes and non-PEGylated liposomes in spleen and in arthritic paws.
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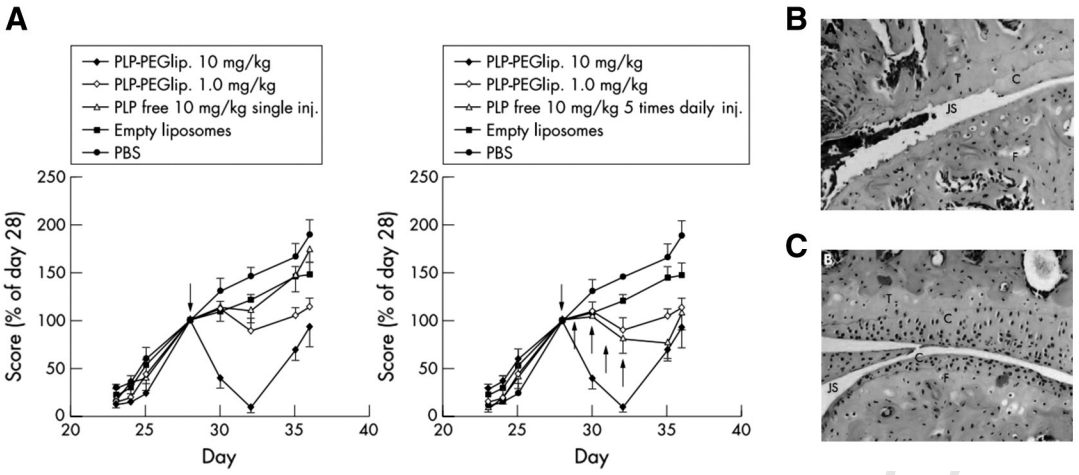

Fig. 7. Therapeutic efficacy of PEGylated PLP in collagen-induced arthritis (CAI). A. Comparison of the efficacy of PEGylated PLP liposomes (single dose, 10 and 1 mg/kg), free PLP (single dose and 5×, at 10 mg/kg), empty liposomes and PBS in rats with CAI. B–C. Cartilage loss in arthritic knees following saline (B) and 10 mg/kg PEGylated liposomal PLP (C) treatment, showing good conservation in the case of the latter.
Image reproduced, with permission, from [51].

that of the TNF-α inhibitors Infliximab and Etanercept, indicating that liposomal GC should be considered a promising option for the future management of RA [55].

Another group studied liposomal formulations of the potent GC derivative budesonide phosphate (BUP) and compared this formulation to PLP liposomes in mice with AIA [56]. The effect on joint swelling suppression after a single injection of liposomal BUP was superior to that of liposomal PLP, in spite of the fact that the latter was given at a 10 times higher dose. At the same time, the suppressive effect of GC treatment on the hypothalamic–pituitary–adrenal (HPA) axis, which can be used as a measure of systemic GC adverse effects [57], was more pronounced for PLP liposomes. This indicates that BUP-loaded liposomes are promising candidates for future (pre-) clinical evaluation.

Finally, since some authors have reported immunogenicity of PEG after repeated injections of PEG-containing materials [58–61], several studies have also looked at non-PEGylated GC formulations [62,63]. Rauchhaus et al. designed a non-PEGylated liposome formulation containing dexamethasone phosphate (DXP). In their study on rats with AIA, free DXP only demonstrated a partial remission while PEG-free liposomal DXP showed significant and long-lasting suppression on joint swelling and joint destruction. Interestingly, in contrast to the above studies (Fig. 6), there was no clear localization of the non-PEGylated formulation in the inflamed joints. There was considerable uptake of the non-PEGylated liposomes in the spleen, though, which indicates that systemic processes, such as macrophage polarization to the more anti-inflammatory phenotype of M2 and non-specific non-genomic effects that occur following the administration of high dose GC, may be partially responsible for its efficacy [62].

2.3. Multiple sclerosis

2.3.1. Pathophysiology of multiple sclerosis and therapeutic role of glucocorticoids

Multiple sclerosis (MS) is a chronic autoimmune disease of unknown etiology. It is associated with demyelination of nerve cells in the brain and spinal cord, and with deceleration or complete blockage of messenger transport to and from the brain [64]. Although some MS patients are mildly affected by disease symptoms, MS ultimately leads to severe disability, e.g. inability to write, speak and walk, and as often results in mortality. Symptoms usually consist of episodes of fast deterioration (i.e. relapses and exacerbations) interspersed with periods of low disease activity, however, often with slow progressive worsening. Four subtypes of MS have been described: (1) relapsing–remitting, (2) secondary progressive, (3) primary progressive and (4) progressive relapsing [65]. Patients with relapsing–remitting MS suffer from unpredictable attacks and long periods of remission, which corresponds to the initial symptoms of 80% of patients [66]. The second type, secondary progressive MS, is characterized by progressive acute attacks with a minor but indefinite remission period; 65% of patients with relapsing–remitting MS evolve into secondary progressive MS within 20 years from disease onset [67]. For 10–20% of patients, the initial symptoms of the disease comprise progressive neurological disability and no (or very few) remission periods. This refers to the primary progressive subtype [68]. The last subtype of MS, progressive relapsing MS, is characterized by neurological deterioration immediately from the onset of the disease, with superimposed exacerbations. Although classic symptoms are not observed during a remission, neurological deterioration continues also in these phases [69]. MS lesions are commonly observed in the white matter of the brain, as autoimmune inflammatory processes cause a loss of myelin, which is important for neuronal signaling [70]. Remyelination is limited, due to the loss of oligodendrocytes, which promote the production and restoration of myelin sheaths [71].

MS is currently incurable, and medical management therefore aims at slowing down disease progression, controlling the symptoms, and preventing exacerbations and disabilities. GC therapy addresses the exacerbations of MS, and often involves a course of 3–5 days of high-dose (1 g per day; pulse therapy) intravenous administration of methylprednisolone (MP) [72]. Higher doses (2 g per day) have been shown to provide even better results with regard to inhibiting inflammation and infiltration, likely due to additional non-genomic mechanisms [73,74]. An important drawback of this type of intervention is the toxicity caused by high dose GC treatment. Since MS therapy benefits from high tissue concentrations of GC in the affected area and low systemic concentrations, significant efforts have been invested in the development of (liposomal) drug delivery systems for GC.

2.3.2. Liposomal glucocorticoids for targeted multiple sclerosis therapy

The inflammatory processes in MS are assumed to result in the partial disturbance of the otherwise impermeable blood–brain barrier (BBB) in the central nervous system (CNS), allowing liposomes and other nanomedicines to pass through and access the MS lesion [75]. This principle has been validated in a routinely used animal model for MS, i.e. experimental autoimmune encephalomyelitis (EAE), which has similar inflammatory properties as MS, but does not display the characteristic demyelination of neurons [76].

As shown in Fig. 8A, upon the i.v. injection of ^{99m}Tc-labeled liposomes, increased liposomal accumulation was observed in the

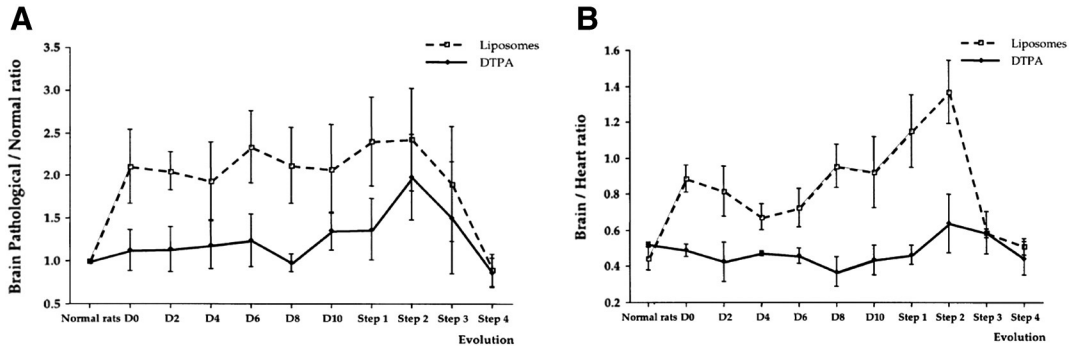

Fig. 8. Accumulation of ^{99m}Tc-DTPA and ^{99m}Tc-DTPA-labeled liposomes in the brain of EAE rats. A. Ratio of localization in pathological to normal brain tissue upon i.v. injection of liposomes vs. free ^{99m}Tc-DTPA. B. Ratio of localization in brain vs. heart for liposomes or free ^{99m}Tc-DTPA.
Image reproduced, with permission, from [76].

brains of EAE rats as compared to the brains in healthy rats. To correlate BBB permeability with liposome localization, ^{99m}Tc-DTPA (a low-molecular-weight control that can also cross the BBB in case of injury/inflammation-associated permeability) was evaluated in a similar setup and, interestingly, a much lower relative localization to pathological brains was observed. In line with this, the brain-vs.-heart ratio was very different for ^{99m}Tc-DTPA-labeled liposomes as compared to free ^{99m}Tc-DTPA (Fig. 8B). These findings demonstrate that liposomes can pass through permeable BBB lesions in MS, but they also hint at an alternative mode of liposome uptake by pathological brain lesions (since the kinetics of accumulation are different from those of free ^{99m}Tc-DTPA). A potential explanation for this could be related to the activation of inflammatory macrophages: in the early phase of induction, before obvious BBB disruption has occurred, liposomes could be transported into the diseased tissue by recruited monocytes of the MPS, which possess a phagocytosing disposition for nanosized particulate matter [77,78].

Schmidt and colleagues [79] investigated a formulation consisting of PEGylated long-circulating loaded prednisolone phosphate (PLP), to achieve high dose delivery of GC to the CNS in a myelin basic protein (MBP)-induced adoptive transfer EAE rat model of MS. When comparing the effect of single dose free and liposomal PLP (10 mg/kg), as well as of free methylprednisolone-hemisuccinate (MP; 50 mg/kg) on BBB permeability and brain inflammation, they showed that the efficacy of PLP liposomes was superior to that of free MP or PLP, with improved BBB integrity and with a reduced infiltration of TNFα-positive T cells. This effect is likely due to the selective targeting of PLP liposomes to the MS lesions, as suggested by 3–5 fold higher accumulation of ^{3}H-labeled liposomes in the CNS of EAE-induced rats as compared to healthy rats, whereas the accumulation in tissues other than the CNS did not differ between both groups (Fig. 9A). Detailed histological studies using colloidal gold-loaded liposomes demonstrated that the majority of these vesicles were taken up by phagocytic cells, in particular by macrophages in the spinal cord, spleen and liver. Upon

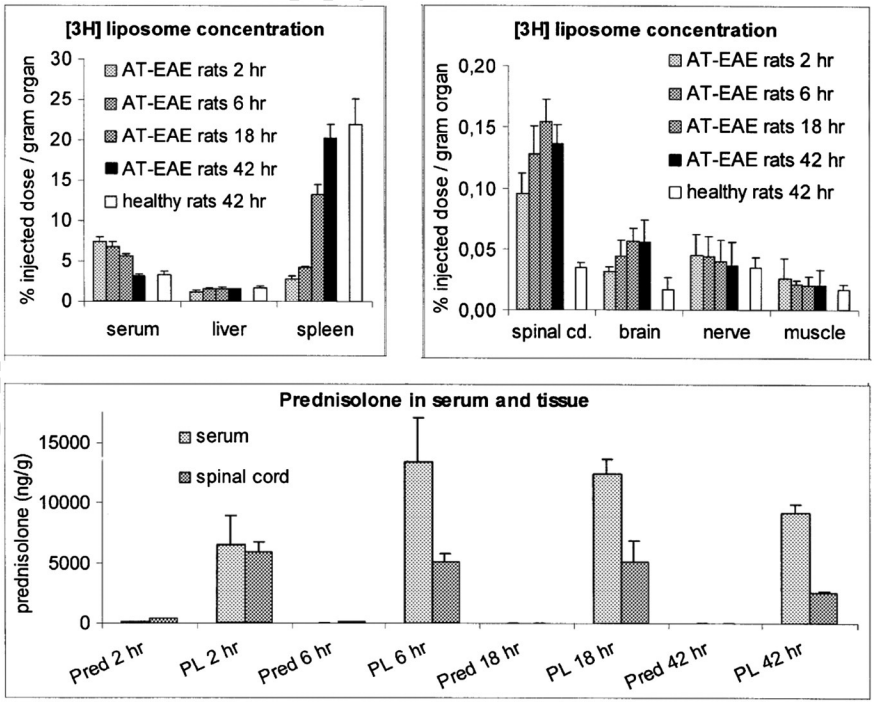

Fig. 9. Localization of ^{3}H-labeled PLP liposomes shows efficient delivery of PLP to target tissues, and is higher than localization of PLP after free drug injection. A. Concentration of ^{3}H-labeled PLP liposomes in serum, liver and spleen, spinal cord, brain, nerve and muscle. B. Comparison of prednisolone concentrations in serum and spinal cord following a single injection of prednisolone liposomal (PL) or free drug (Pred).
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quantification, target tissue levels of prednisolone were markedly higher upon administration of PLP-loaded liposomes than upon administration of the free drug. In addition, liposomal PLP prolonged GC exposure, as concentrations decreased very slowly, with significant levels still present at 42 h after injection, whereas free prednisolone displayed fast elimination and was undetectable after 6 h (Fig. 9B).

The therapeutic efficacy of PLP-containing liposomes was compared to that of free MP and MP-containing liposomes in two different myelin oligodendrocyte glycoprotein (MOG)-induced EAE models [80]. The relapsing MOG-EAE induction model is considered more realistic in reflecting the histopathological changes of MS, displaying not only dominant macrophage infiltration, but also demyelination and axonal damage, as well as T-cell infiltration [81]. As opposed to free MP, a single 10 mg/kg dose of liposomal PLP during the first relapse provided rapid remission of MS activity, displaying a reduced amount of periventricular lesions, and prevented further relapses over the course of the disease, resulting in a significantly reduced overall mortality rate. The effect of liposomal PLP was more prolonged than that of free MP and liposomal MP, as rats treated with the former were almost completely protected during the third relapse. This is confirmed by the lower levels of microglia and T-cell infiltration at the onset of the second relapse (Fig. 10). Furthermore, PLP liposomes preserved integrity of the BBB and prevented demyelination, leading to higher axon densities (Fig. 11), providing an effect superior to that of other treatments.

Several formulations, including liposomal dexamethasone, have been used to investigate the (cellular) mechanism of action of liposomal GC in mice suffering from EAE [82]. While free dexamethasone acted primarily on T-cells, liposomal GC had only limited effect on T-cells, but primarily drove the polarization of macrophages to a more anti-inflammatory phenotype, M2 [83]. This indicates that liposomal encapsulation of GC may not only modulate the disease by enhanced *in vivo* target site accumulation, but also by targeting a different cell population as compared to free GC [83–85].

The impact of the loading method of GC on therapeutic efficacy was evaluated by Avnir and colleagues [86]. They remotely (actively) loaded methylprednisolone via a transmembrane calcium acetate gradient into the aqueous core of PEGylated liposomes, where the drug then precipitates as a salt. When compared to passively loaded MP liposomes, remote loading provided an almost 50 times higher encapsulation efficiency. Likely as a direct result of this, remotely loaded liposomal MP provided very strong inhibition of disease activity in EAE mice. A direct comparison of actively and passively loaded liposomes was unfortunately not performed. However, the authors conclude that their remote-loaded liposomal GC formulation is not stable *in vivo* as the pharmacokinetic and biodistribution studies demonstrate zero-order slow-release kinetics from these liposomal systems.

Attempts have also been made to actively target liposomes to MS lesions and across the BBB. To this end, Gaillard and colleagues [87] developed the same remotely loaded methylprednisolone hemisuccinate liposomes as Avnir et al., and functionalized their surface with glutathione to facilitate transport across the BBB, and to increase the accumulation of GC in the CNS. As compared to long-circulating MP liposomes without targeting ligands (passive targeted), the glutathione-targeted (active targeted) were more efficient in suppressing signs of EAE in mice, leading the authors to conclude specific targeting across the BBB might be advantageous in case of MS.

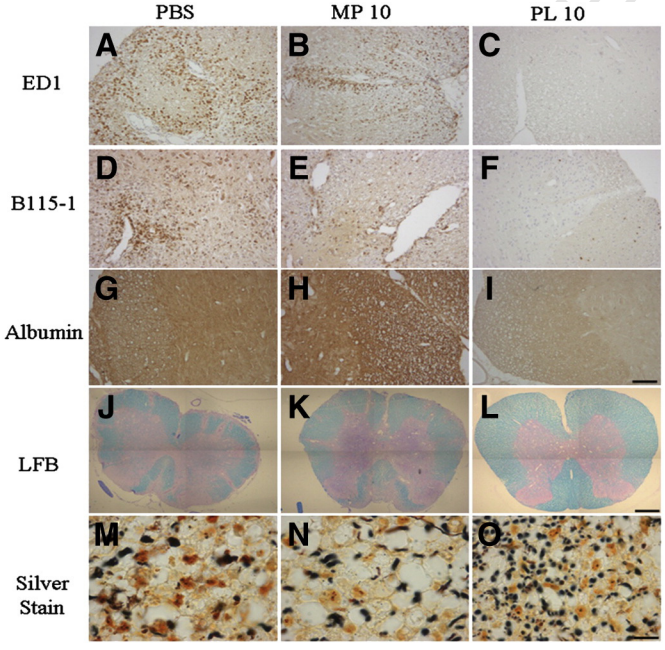

Fig. 10. Histopathological analysis of MS disease severity upon treatment with PBS, free MP (3 × 10 mg/kg), and PLP liposomes (1 × 10 mg/kg). Assessment of macrophage/microglia infiltration (ED1 staining), T cell infiltration (B115-1 staining), BBB integrity (albumin staining), demyelination (Luxol Fast Blue staining) and axon preservation (silver staining) demonstrated higher efficacy of PLP liposomes.
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3. Liposomal glucocorticoids for cancer

3.1. Pathophysiology of cancer and therapeutic role of glucocorticoids

Cancer is a diverse class of diseases characterized by (epi-) genetic abnormalities causing uncontrolled cell growth. Cancer cells can invade adjacent tissues and spread to other body parts by metastasis, making cancer a progressive disease with a high mortality rate. Symptoms of cancer can be classified into 3 groups: i) local symptoms, such as unusual lumps/swelling (tumor), hemorrhage, pain and ulceration; ii) symptoms of metastasis (spreading), such as enlarged lymph nodes, cough and hemoptysis, hepatomegaly, bone pain; and iii) systemic symptoms, such as weight loss, fatigue and cachexia, excessive sweating and anemia [88].

Although the human body is capable of protecting itself against cancer using several mechanisms (e.g. immunological protection, apoptosis, etc.), this protection may fail for several reasons, resulting in chromosomal aberrations and malignant transformation. This may occur due to environmental factors (carcinogens) or may be randomly acquired or inherited by errors in DNA replication. Environmental factors include tobacco smoke, radiation, chemicals, particles (such as asbestos) and infectious pathogens (such as HIV, human papillomavirus, hepatitis B, hepatitis C, Epstein–Barr and human T-lymphotropic virus) [89–92]. These genetic changes form the basis of the seven hallmarks of cancer: i) uncontrolled growth; ii) loss of capacity for apoptosis; iii) loss of capacity for senescence (unlimited replication); iv) sustained angiogenesis (unlimited tumor growth); v) ability to invade other tissues; and vi) ability to metastasize to distant sites [93, 94]. In addition to this, inflammation has recently been recognized as the 7th hallmark of cancer [95].

The main goal of cancer therapy is to eradicate the disease from the body, by removing the tumor and/or killing the tumor cells. There are several types of therapeutic options to achieve this, including surgery, radiotherapy and chemotherapy. The treatment of choice and its success rate depend on the type, location and stage of the malignancy. In many cases, the pharmacological reduction of symptoms, such as pain, nausea, vomiting and infusion-related reactions, is as important as the (chemo-) therapeutic treatment itself. Glucocorticoids play an important role in the secondary management of cancer, e.g. to reduce edema in gliomas, or to suppress infusion-related inflammatory reactions upon the administration of Taxol and Taxotere. Via their anti-angiogenic and general anti-inflammatory properties [96], GC might also have some direct/intrinsic antitumor potential. In general, efficacious GC anticancer treatment requires high and frequent dosing, and this generally comes with severe side effects. To overcome this

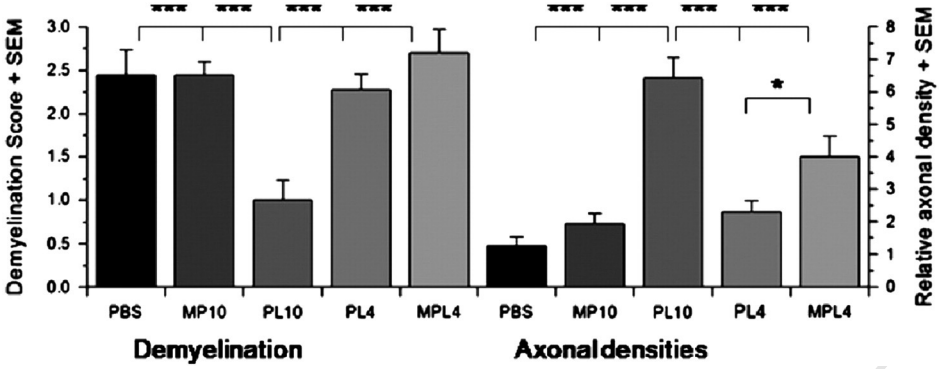

Fig. 11. Injection of 10 mg/kg PLP liposomes (PL) prevents demyelination and increases axonal density as compared to free (MP) and liposomal methylprednisolone (MPL). Image reproduced, with permission, from [80].

limitation, several different long-circulating liposomal GC formulations have been designed and evaluated. In general, long-circulating liposomes exploit vascular abnormalities and high endothelial permeability in the tumor to enable tumor-selective drug delivery via the EPR effect. Similar to inflammatory diseases, upon extravasation from systemic circulation into the tumor interstitium, liposomes tend to be phagocytized by tumor-associated macrophages (TAM). As TAM are important cells in promoting inflammation, angiogenesis and tumor growth [97], the accumulation of liposomal GC in TAM may partially explain the observed therapeutic effect, inhibiting angiogenesis and attenuating tumor growth [97,98]. Below, selected studies exemplifying the therapeutic potential of liposomal GC will be described and discussed.

3.2. Liposomal glucocorticoids for targeted cancer therapy

A significant portion of the studies on long-circulating liposomes for selective GC delivery to tumors have been performed by Schiffelers and colleagues [7,99–105]. They for instance evaluated the antitumor

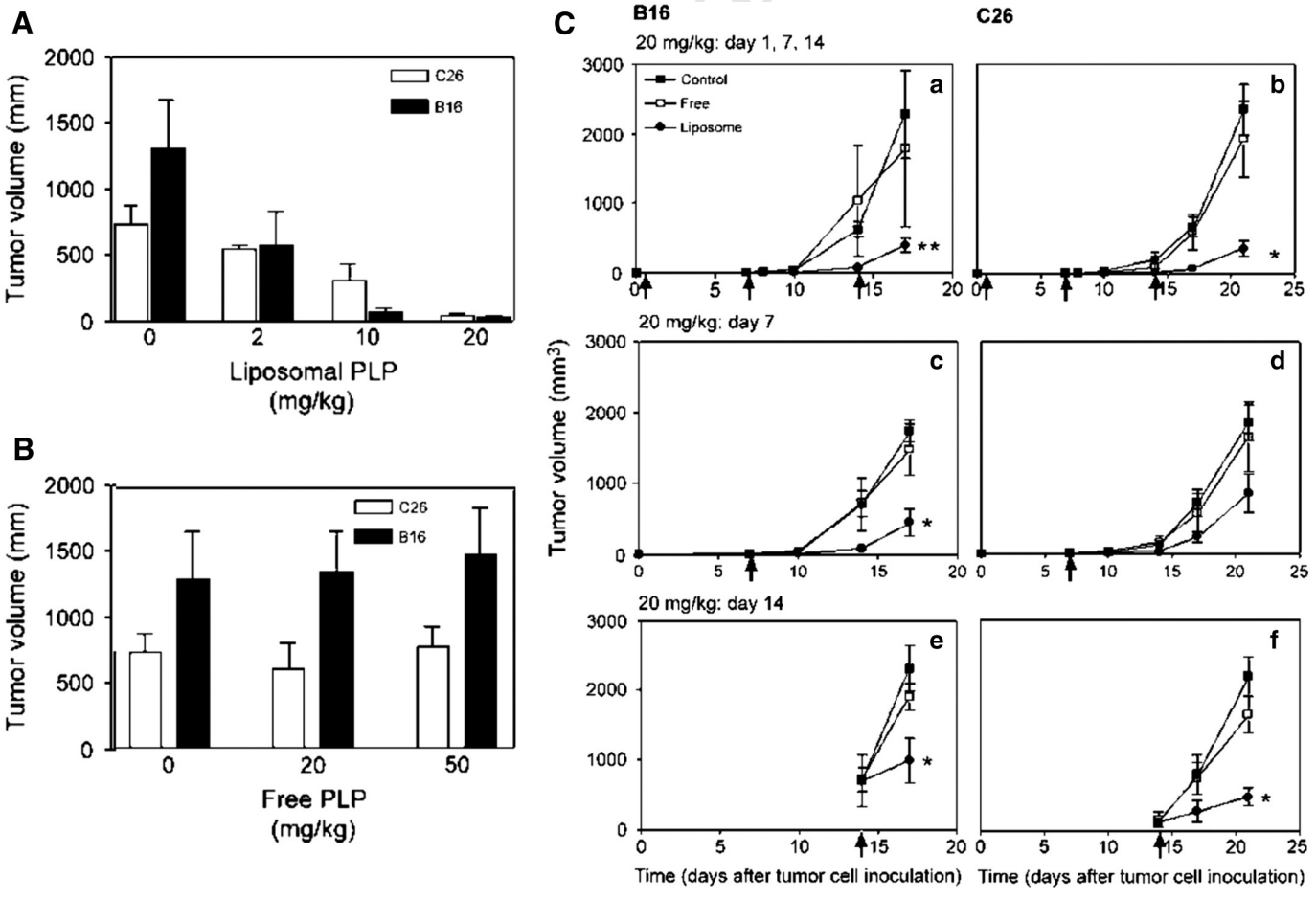

Fig. 12. Liposomal PLP has a higher therapeutic efficacy in B16.F10 or C26 tumor-bearing mice than free PLP. A. Tumor volumes at the end of the study after the single i.v. administration of liposomal PLP at different doses. B. Tumor volumes after injection of free PLP at different doses. C. Tumor growth after different treatment regimens of 20 mg/kg liposomal PLP versus 20 mg/kg free PLP (A–B = drug administration on days 1, 7 and 14; C–D = administration on day 7; E–F = administration on day 14). Image reproduced, with permission, from [99].

Please cite this article as: B. Ozbakir, et al., Liposomal corticosteroids for the treatment of inflammatory disorders and cancer, J. Control. Release (2014), http://dx.doi.org/10.1016/j.jconrel.2014.05.039

activity of PLP encapsulated in PEGylated liposomes, in both C26 colon carcinoma and B16.F10 melanoma mouse models, and compared it to the antitumor activity of free PLP [99]. A single administration of long-circulating liposomes loaded with PLP significantly reduced tumor growth in both models, in a dose-dependent manner (Fig. 12). The free drug, on the other hand, had no effect, independent of dose. Interestingly, although there was a clear growth inhibiting effect of liposomal PLP when tumors were established, no delay was observed if treatment was administered immediately after tumor engraftment (i.e. 1 day after inoculation), indicating that some (leaky) tumor blood vessels are required for efficient passive drug targeting. To investigate the importance of the long-circulating property of PEGylated liposomes with regard to tumor growth inhibition, a short-circulating PEG-free liposomal PLP formulation was also evaluated. Although there was some tumor growth inhibition as compared to control animals, the observed effect was much less prominent than upon using standard PEGylated liposomes, demonstrating that the long-circulating properties are highly important for conveying the antitumor activity of liposomal GC (Fig. 13).

Follow-up studies by the same group of authors aimed at identifying the mechanism of action of liposomal GC [106]. Using an *in vitro* assay to evaluate the direct cytotoxic effects of liposomal and free PLP on tumor and endothelial cells, a moderate anti-proliferative effect was observed for both formulations in both cell lines. When encapsulated in liposomes, the anti-proliferative activity of PLP was somewhat stronger, but not to a level explaining the differences in *in vivo* tumor growth. When the mechanism of action was evaluated *in vivo*, using protein expression arrays, a potent inhibition of pro-inflammatory and pro-angiogenic protein production was observed upon treatment with liposomal PLP, which was significantly stronger as compared to free PLP. As a matter of fact, as exemplified by Fig. 14, liposomal PLP reduced the expression levels of 14 out of the 17 pro-angiogenic proteins analyzed. Follow-up studies using different GC formulations confirmed this notion, strongly suggesting that the antitumor efficacy of liposomal GC is related, at least in part, to the inhibition of angiogenesis [12,107,108].

Not surprisingly, the effect of liposomal GC on *in vitro* proliferation and survival, as well as on *in vivo* tumor growth, is strongly dependent on the type of GC used [104]. When comparing different GC with different potencies in terms of anti-proliferative and cytotoxic effects, the more potent GC dexamethasone phosphate (DXP) and in particular budesonide phosphate (BUP) were much more effective than methylprednisolone phosphate (MPLP) and prednisolone phosphate (PLP), both in liposomal and in free form. As exemplified by Fig. 14A, this

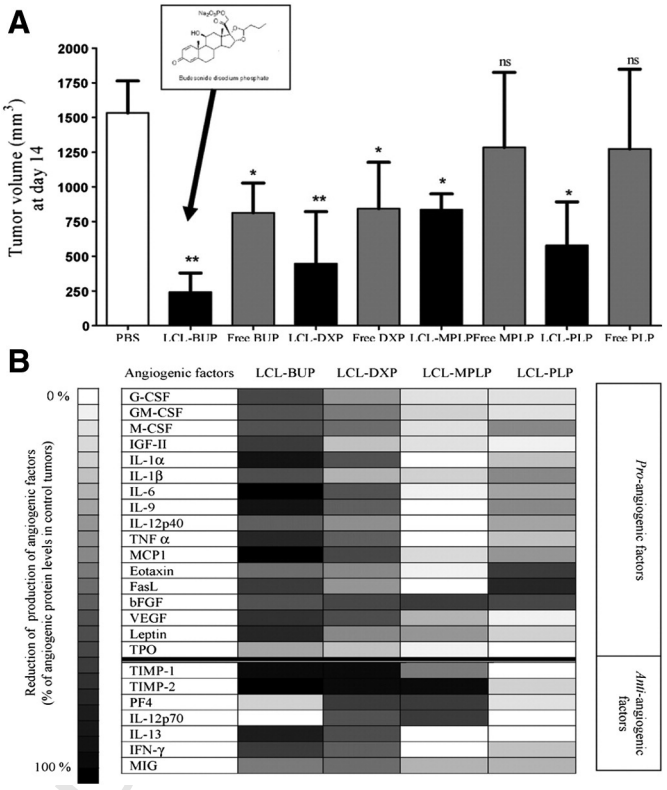

Fig. 14. Antitumor and anti-angiogenic effects of different liposomal GC. A. Tumor volumes after treatment with four different free and liposomal GC. B. The production of angiogenic factors after LCL-GC injections.
Image reproduced, with permission, from [104].

trend was also evident *in vivo*. In mice bearing B16.F10 tumors, tumor growth inhibition corresponded very well with GC potency (BUP > DXP > PLP > MPLP), with the strongest effects detectable for liposomal BUP, and the weakest for liposomal MPLP. In line with this, the reduction in pro-angiogenic and pro-inflammatory protein production also corresponded very well with the potency of the different GC (Fig. 14B). Liposomal BUP reduced the expression levels of all proteins evaluated, and it did so in a very strong manner, in particular also in the case of VEGF, which is undoubtedly one of the key factors in inducing/sustaining angiogenesis [109,110]. Upon encapsulating

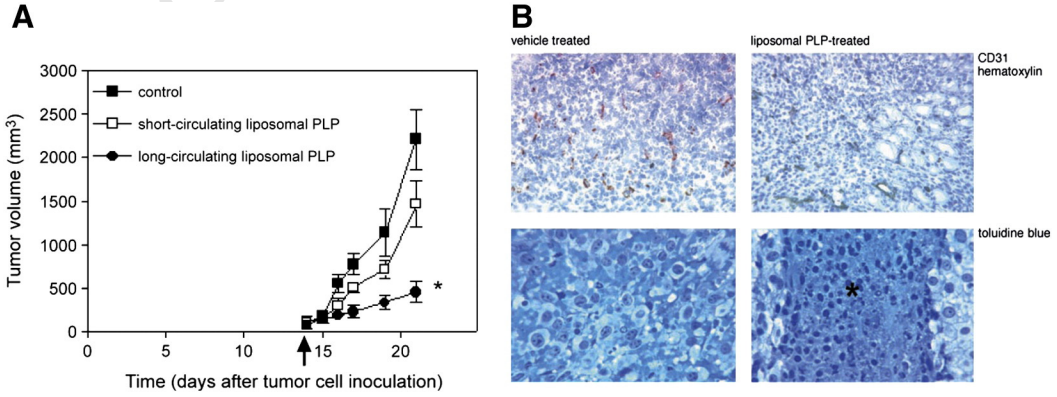

Fig. 13. Long-circulating PEGylated liposomal PLP has a higher therapeutic efficacy compared to short-circulating non-PEGylated liposomal PLP. A. Effect of long- and short-circulating PLP liposomes on C26 tumor volume, B. C26 tumor tissues and blood vessel density after a single liposomal PLP injection (control tumors = upper panels, liposomal PLP treated tumors = lower panels).
Image reproduced, with permission, from [99].

MPLP, the least potent GC, into liposomes, only a couple of proteins were affected, and the levels of suppression were much lower. Liposomal PLP and DXP nicely confirmed the GC potency-dependent inhibition of inflammation and angiogenesis in tumors.

Extending these efforts, Banciu and colleagues showed that the tumor localization of the four different liposomal GC was similar (3–4% of the injected dose; at 24 h post), thereby excluding the possibility that the observed differences in antitumor and anti-angiogenic activity result from differences in target site accumulation [105]. In the same study, extensive dose–response analyses were performed, showing that in all cases, higher dose treatment resulted in stronger tumor growth inhibition (Fig. 15). However, in between the different liposomal GC, significant differences in dose–response were observed, correlating very well with their intrinsic potency. As shown in Fig. 15, liposomal BUP clearly was the most potent formulation, with already at 3 mg/kg, tumor growth inhibition in the order of 75% (as compared to PBS-treated controls). For comparable efficiency, liposomal DXP had to be administered at a dose of 10 mg/kg, liposomal PLP at 20 mg/kg and liposomal MPLP at 30 mg/kg. With regard to toxicity, when administered at the highest dose, all liposomal GC except liposomal MPLP resulted in some body weight loss, as well as in a significant reduction of spleen size (by 40–50%).

Tumor-associated macrophages (TAM), subsets of which are known to be actively involved in promoting inflammation and angiogenesis, are important for tumor growth [111]. Therefore, clodronate-containing liposomes, which deplete macrophage pools [112,113], were employed to study the role of TAM in the antitumor activity of liposomal GC [102]. In athymic Foxn1$^{nu-/nu-}$ mice (to exclude T-cell-mediated antitumor effects) bearing B16.F10 tumors, a strong effect of liposomal clodronate was observed on the amount of TAM present in tumor tissue. Treatment with liposomal PLP also reduced the TAM pool in tumors, but to a lesser extent. Tumors of animals treated with liposomal clodronate were 55% smaller than control tumors on day 14 after i.v. administration, and levels of pro-angiogenic proteins were significantly lower (35%) than those in control mice, highlighting the pivotal role of TAM in tumor growth (Fig. 16). Upon pretreatment with liposomal clodronate, no additive tumor growth inhibition could be achieved by subsequent liposomal PLP treatment. In line with this, no added effect on the reduction of angiogenic protein production was observed for liposomal PLP after liposomal clodronate pretreatment. In good agreement with their proposed mechanism of action, i.e. the inhibition of the pro-inflammatory and pro-angiogenic activity of TAM, these findings show that TAM indeed are important effector cells for conferring the antitumor efficacy of liposomal GC.

4. Conclusions and perspectives

Although GC are highly potent drugs, and although they have been proven to be useful for the treatment of many different diseases, the severe side effects associated with their prolonged and/or high-dose use have somewhat limited their broad clinical applicability. Consequently, in order to improve drug efficacy and at the same time reduce toxicity, significant research efforts have focused on the development of drug delivery systems for GC.

Particularly liposomes have been used for targeted GC delivery, and they have been extensively employed for this purpose for over 30 years. Early studies have illustrated a positive impact of liposomal encapsulation for local applications in asthma and RA, increasing their anti-inflammatory activity and/or prolonging the duration of effective treatment. In subsequent studies, upon the development of PEGylated liposome systems with prolonged circulation kinetics, liposomal GC have also shown great promise for systemic anti-inflammatory therapies. Such systemic anti-inflammatory interventions have attracted considerable attention in the last couple of years, not only with regard to liposomal drug delivery systems, but also with other nanomedicine formulations (such as polymers and micelles) [114]. An important advantage of liposomes, as compared to other drug delivery platforms, is the relative ease of varying the type of encapsulated GC, allowing for the selection of the appropriate GC for a specific therapeutic intervention. Conversely, other drug delivery systems might be more suitable for applications in which carefully controlled (and sustained) drug release kinetics are required.

Taken together, as comprehensively discussed in the current manuscript, long-circulating liposomal GC have demonstrated impressive preclinical potential in RA and MS. As a result of this, they are currently being evaluated for these applications in clinical trials. Given their

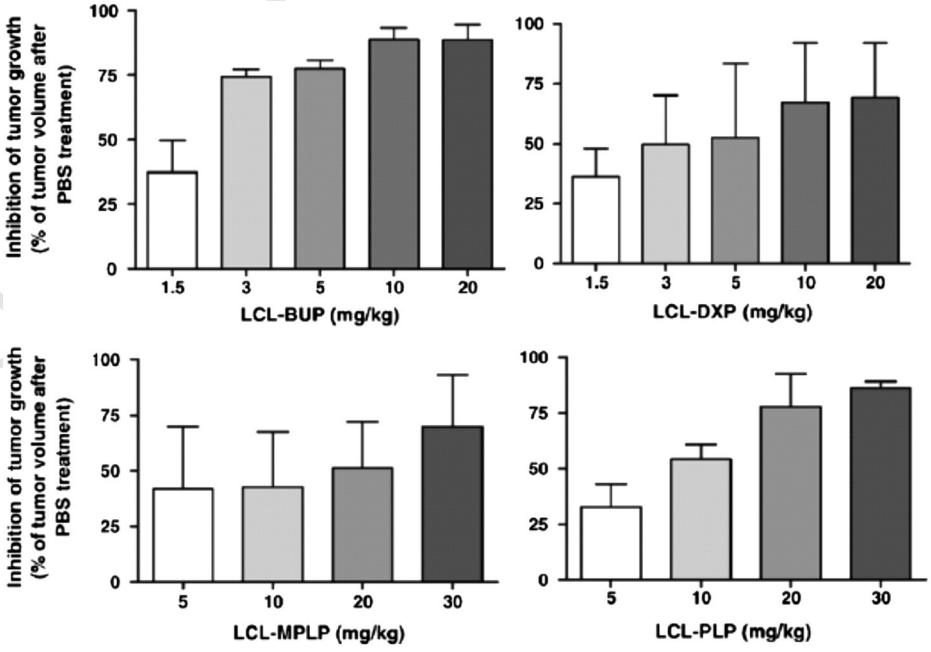

Fig. 15. Dose-dependent inhibition of tumor growth by liposomal GC.
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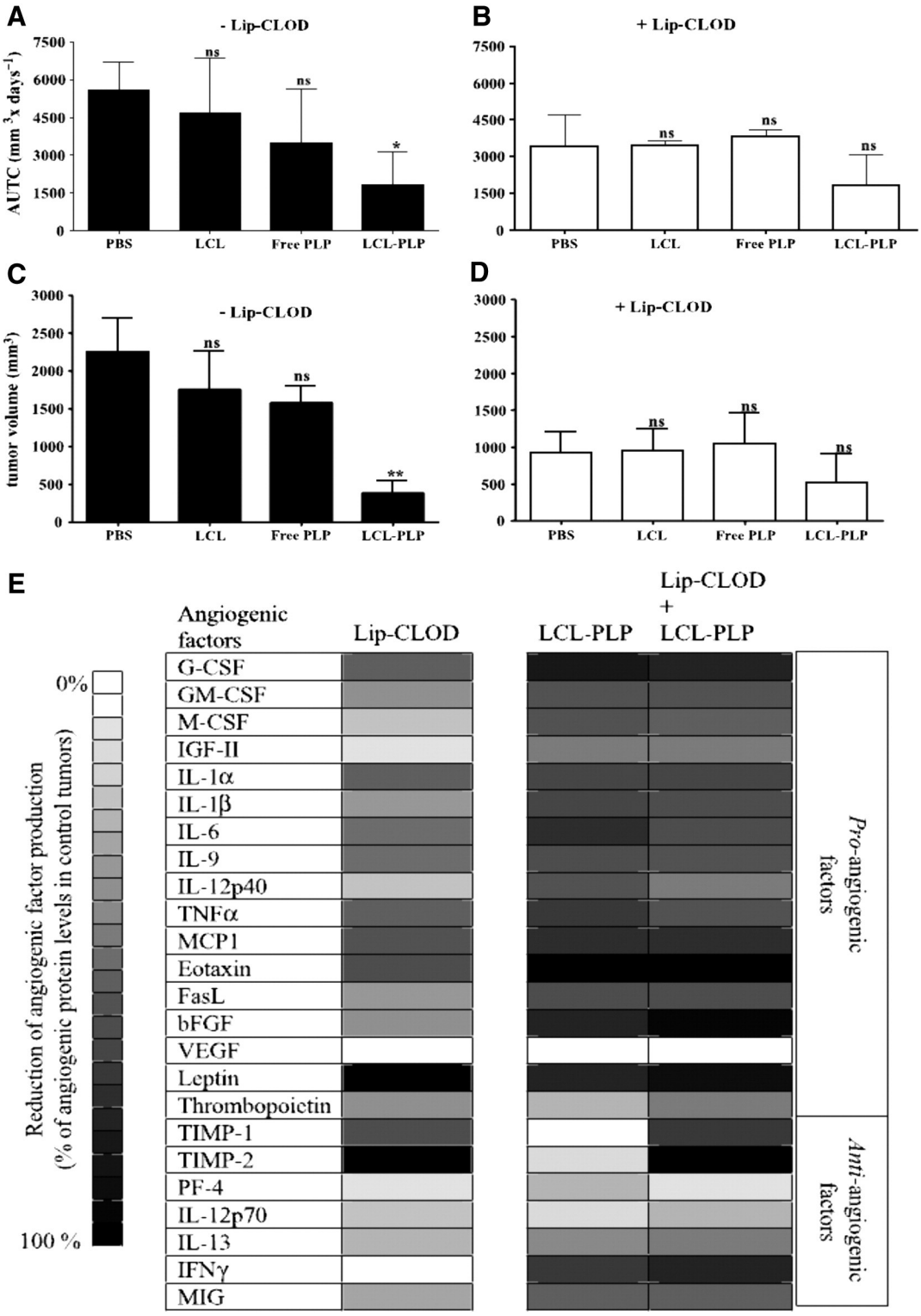

Fig. 16. Impact of macrophage depletion (via pre-treatment with clodronate-containing liposomes) on the antitumor efficacy of long-circulating liposomal prednisolone phosphate (LCL-PLP). A. Area under tumor growth curve (AUTC) after free and liposomal PLP treatment. B. AUTC after free and liposomal PLP treatment upon pretreatment with liposomal clodronate (Lip-CLOD). C. Tumor volume at the end of the experiment upon free and liposomal PLP treatment. D. Tumor volume at the end of the experiment upon free and liposomal PLP treatment preceded by Lip-CLOD pretreatment. E. Effect of Lip-CLOD pretreatment on the LCL-PLP-induced downregulation of antiangiogenic proteins.
Image reproduced, with permission, from [102].

ability to inhibit tumor growth, studies in cancer patients also seem to be warranted, in particular in case of malignancies which are currently being treated with high-dose GC therapy, such as malignant myeloma. Furthermore, liposomal GC have recently also demonstrated promising preclinical efficacy in other inflammatory disorders and infectious diseases, such as atherosclerosis, stroke, inflammatory bowel disease

and malaria [115–118]. Consequently, liposomal glucocorticoids are considered to be useful and broadly applicable tools for the treatment of inflammatory disorders and cancer, and a bright clinical future appears to be ahead of them.

5. Uncited reference

[63]

Acknowledgments

The authors gratefully acknowledge financial support by the European Research Council (ERC-StG-309495: NeoNaNo), by the European Union (COST-Action TD1004), by NWO (HIFUCHEM, Rubicon) and by the DFG (LA 2937/1-2).

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Please cite this article as: B. Ozbakir, et al., Liposomal corticosteroids for the treatment of inflammatory disorders and cancer, J. Control. Release (2014), http://dx.doi.org/10.1016/j.jconrel.2014.05.039