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Macrophages and liposomes in inflammatory disease: Friends or foes?

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ABSTRACT

Liposome-encapsulated corticosteroids have shown to exert strong beneficial effects in inflammatory diseases, such as arthritis and cancer. To extend the clinical applicability of these potent nanomedicines, the therapeutic effect of dexamethasone phosphate loaded long-circulating liposomes (LCL-DXP) was evaluated in animal models of multiple sclerosis (MS) and Crohn's disease (CD).

In mice with experimental autoimmune encephalitis (EAE), a model for MS, treatment with LCL-DXP, but not free DXP, resulted in a decrease in disease activity when compared to PBS treated mice. In contrast, in mice with chronic DSS-induced colitis, a model for CD, treatment with LCL-DXP did not induce an improvement, but in fact worsened the fecal blood loss after treatment, indicating an aggravation of the disease. It is hypothesized that modulation of macrophage polarization towards a M2 phenotype underlies the efficacy of corticosteroid-based drug delivery systems, which is supported by the presented data. On the one hand, M1 polarized macrophages are part of the pathogenesis of MS; the modulation to M2-polarization by LCL-DXP is therefore beneficial. On the other hand, M1-polarized intestinal macrophages fulfill a protective and inflammation-suppressing role in intestinal homeostasis; changing their phenotype to M2 causes reduced protection to invading microorganisms, leading to a more severe intestinal inflammation. These findings therefore indicate that the interplay between the specific phenotype of macrophages and the specific inflammatory context of the inflammatory disease in question may be an important determining factor in the therapeutic applicability of liposomal corticosteroids in inflammatory disease.

1. Introduction

In the last decade, targeting strategies using liposome-encapsulated corticosteroids have shown to have a distinct therapeutic effect in several animal models of inflammatory diseases, such as rheumatoid arthritis (RA), multiple sclerosis (MS) and cancer (Metselaar et al., 2003; Schiffelers et al., 2005; Schmidt et al., 2003). The increased leakiness of blood vessels and the reduced lymphatic drainage occurring in inflamed tissues and tumors, referred to as the Enhanced Permeability and Retention (EPR) effect (Matsumura and Maeda, 1986), enables enhanced accumulation of long-circulating nanosized carrier systems in these target tissues (Sandanaraj et al., 2010). In this regard, liposomes with a poly(ethylene glycol) coating are most advanced, with a long circulation time and favorable biodistribution, and therefore frequently used as drug carriers (Fenske and Cullis, 2008). Although the administration of such long-circulating liposomes leads to higher concentrations of the encapsulated drug at the inflamed site, the precise mechanism of how these encapsulated drugs interact with the target inflammatory tissue is still quite unclear.

There is compelling evidence that macrophages play a crucial role in this mechanism. Macrophages and other phagocytic cells of the reticuloendothelial system (RES) are essential in the elimination of liposomes from the circulation (Allen et al., 1995; Hsu and Juliano, 1982). Although these cells are mainly residing in the liver and spleen, they are also responsible for the phagocytosis of liposomes in other (target) tissues (Huang et al., 1992). After degradation of the liposomal carrier by these target tissue macrophages, the drug that had been encapsulated can have various fates: (1) the liberated drug molecules are degraded within the lysosomal vesicles, (2) liposome-phagocytosing macrophages act as a 'reservoir' for the liberated drugs and can slowly release the drug in time

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(Storm et al., 1988), or (3) the liberated drug has an effect on the macrophage itself and inhibits macrophage activity (Banciu et al., 2008). In the case of liposomal glucocorticoids, there is strong evidence that the latter mechanism is the most important; in similar models for inflammatory diseases, therapy with bisphosphonate clodronate liposomes, which induce apoptosis of phagocytosing macrophages, leads to equivalent therapeutic results as therapy with liposomal corticosteroids (Banciu et al., 2008; van Rooijen and van Kesteren-Hendrikx, 2002). Macrophages, like other cells of the immune system, show a strong intracellular expression of the glucocorticoid receptor (GR), which, after activation by the liberated corticosteroids, leads to suppression of NF-κB activity, downregulation of macrophage activity, and thus reduction of secretion of pro-inflammatory cytokines, such as TNF-α, IFN-γ, IL-2 and IL-12 (Auphan et al., 1995; Elenkov, 2004; Glass and Saijo, 2010).

Even though therapy with liposomal corticosteroids proved successful in a number of preclinical models of inflammatory disease, additional research is still ongoing. In some cases more evidence is needed to confirm previously obtained preclinical results, to justify a next step to clinical assessment. For example, both liposomal prednisolone phosphate and liposomal 6α-methylprednisolone phosphate have successfully been employed to attenuate the inflammatory response in experimental autoimmune encephalitis (EAE) induced in rats (Linker et al., 2008; Schmidt et al., 2003). The rat EAE model is a well-accepted model for MS, however, there are several methods in use to induce this condition in species other than the rat, each with its own similarities to the human pathophysiology (Gold et al., 2006). For this reason, SJL mice with myelin proteolipid protein-induced EAE, which have a disease profile similar to human MS, were used in the present study (Tuohy et al., 1988).

The therapeutic indication of liposome-encapsulated corticosteroids could be expanded to other inflammatory diseases, including Crohn's disease (CD). CD is a chronic inflammatory disease of the gastrointestinal tract, which together with Colitis Ulcerosa is commonly referred to as inflammatory bowel disease (IBD). The disease activity in CD is characterized by an intermittent course with exacerbations and remissions; the latter most commonly being induced by corticosteroid treatment (Benchimol et al., 2008; Cummings et al., 2008). In light of this intermittent disease activity, which shows resemblance with that of MS and RA, it can be envisaged that liposomal corticosteroids will be beneficial in the treatment of CD, as indicated already in preclinical models of RA and MS.

In the current study, the therapeutic efficacy of liposomal dexamethasone phosphate (DXP) was assessed in mouse models of MS and CD. DXP is a phosphate derivative of dexamethasone, which is a corticosteroid with strong anti-inflammatory potency. Dexamethasone is found to be 6–10 times more potent than prednisolone and 5 times more potent than 6α-methylprednisolone with respect to their glucocorticoid activities (Gilman and Goodman, 2006; Grossmann et al., 2004). It was anticipated that the employment of long-circulating DXP-liposomes would reduce the disease activity in both models of inflammatory disease, which would aid in the development of liposomal corticosteroid formulations for clinical anti-inflammatory therapy.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) and dipalmitoylphosphatidylcholine (DPPC) were provided by Lipoid (Germany). Cholesterol was purchased from Sigma–Aldrich (Germany). Dexamethasone phosphate (DXP) was obtained from BUFA (The Netherlands). Posphate buffered saline (PBS) with pH 7.4 was purchased from B. Braun (The Netherlands). Dextran sodium sulfate (DSS) with molecular weight of 36–50 kDa was supplied by MP Biomedicals (France). Beckman Coulter Hemoccult® SENSA® tests were obtained from Bipharma Diagnostics B.V. (The Netherlands).

2.2. Preparation of long-circulating liposomes

DPPC, DSPE-PEG_{2000} and cholesterol were dissolved in a 1.85:0.15:1 molar ratio in a roundbottom flask in 5–10 mL ethanol. A lipid film was formed by rotary evaporation (Buchi, Switzerland), which was dried further under a nitrogen flow. The lipid film was hydrated with a 100 mg/mL solution of DXP in reversed osmosis water to form liposomes. The size and polydispersity of the liposomes were decreased by extruding the dispersion through two polycarbonate filters (Whatman, USA) mounted in an LIPEX extruder (Northern Lipids Inc, Canada). Starting with two extrusions through a double 200 nm filter and two extrusions through a 200 and 100 nm filter, the liposomes were extruded ten times through two 100 nm filters. DXP not encapsulated into the liposomes was removed by means of dialysis in PBS at 4 °C for 48 h, while replacing the PBS regularly in order to remove all free corticosteroid. Particle size and polydispersity of extruded dispersions was determined by dynamic light scattering using a Malvern ALV CGS-3 (Malvern Instruments). DLS results are given as a *z*-average particle size diameter and a polydispersity index (PDI), which is expressed on a scale of 0–1; 0 meaning complete monodispersity and 1 meaning complete polydispersity. All used liposomes had an average diameter of around 100 nm and a PDI smaller than 0.1. The DXP concentration in the liposomal dispersion was measured using ultra performance liquid chromatography (UPLC) (Waters) equipped with a Acquity UPLC BEH C18 column, using 1:3 acetonitrile in water (pH 2) as eluent. Prior to analysis the DXP was recovered from the liposomes after extraction of the aqueous phase (Bligh and Dyer, 1959).

2.3. Animal experiments

Mice were housed in groups of 8–10 per cage and supplied ad libitum with water and standard diet pellets for rodents (801730 CRM (E) Expanded, Special Diets Services, England). All animal experiments were conducted in agreement with local law.

2.4. EAE model

Female SJL mice (Charles River) were immunized by subcutaneous injection in each flank of proteolipid protein in Complete Freund's Adjuvant containing Mycobacterium tuberculosis. Immediately after, animals received a solution of pertussis toxin by intraperitoneal injection. Starting from day 7 post-immunization, clinical disease activity was assessed daily and scored on a range from 0 to 5: 0 = normal, 0.5 = partial tail paralysis, 1 = tail paralysis, 1.5 = tail paralysis and partial unilateral hindlimb paralysis, 2 = tail paralysis and hindlimb weakness or partial hindlimb paralysis, 2.5 = tail paralysis and unilateral hindlimb paralysis or severe hindlimb paresis (lowered pelvis), 3 = tail paralysis and complete hindlimb paralysis, 3.5 = tail paralysis and complete hindlimb paralysis and incontinence, 4 = tail paralysis and hindlimb paralysis and weakness or partial paralysis of forelimbs, 5 = moribund or dead. Clinical signs were monitored daily in each group of treatment in a blind fashion. At the onset of disease (score ≥ 1, day 0), each animal was randomly assigned to one of four experimental groups. Groups of 9–10 mice were treated intravenously with a single dose of PBS, DXP (10 mg/kg), or long-circulating DXP-liposomes

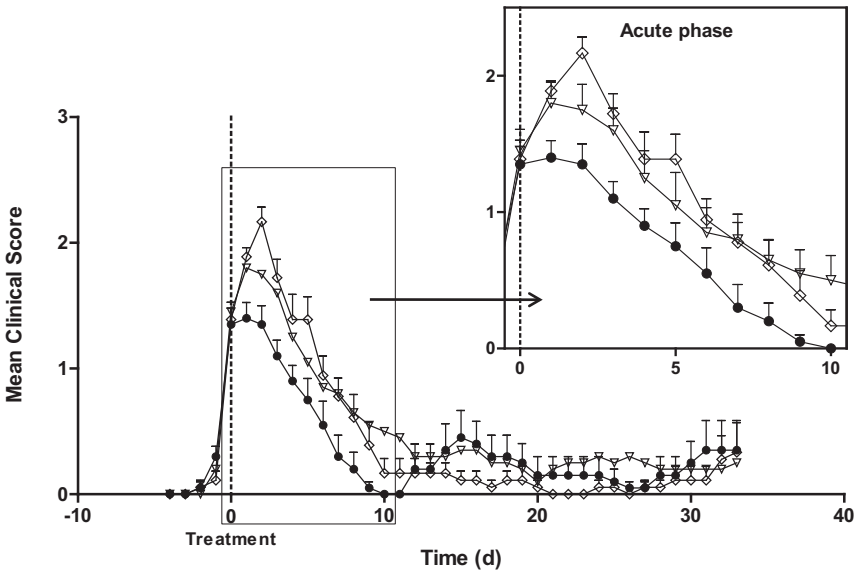

Fig. 1. Clinical EAE score of mice with EAE after treatment with (liposomal) DXP. Experimental autoimmune encephalomyelitis (EAE) was induced in female SJL mice by immunization with proteolipid protein in Complete Freund's Adjuvant containing mycobacterium tuberculosis, followed by a boost of pertussis toxin. The clinical score was monitored daily. At the onset of disease (clinical score ≥ 1, day 0) mice were treated with PBS (◊), 10 mg/kg DXP (▽) or 10 mg/kg long-circulating DXP-liposomes (LCL-DXP, ●). Depicted are the mean clinical score and SEM of each experimental group (n = 9–10) until 49 days post-immunization. Clinical score: 0 = normal, 0.5 = partial tail paralysis, 1 = tail paralysis, 1.5 = tail paralysis and partial unilateral hindlimb paralysis, 2 = tail paralysis and hindlimb weakness or partial hindlimb paralysis, 2.5 = tail paralysis and unilateral hindlimb paralysis or severe hindlimb paresis (lowered pelvis), 3 = tail paralysis and complete hindlimb paralysis, 3.5 = tail paralysis and complete hindlimb paralysis and incontinence, 4 = tail paralysis and hindlimb paralysis and weakness or partial paralysis of forelimbs, 5 = moribund or dead.

(LCL-DXP, 10 mg/kg). All animals were sacrificed 7 weeks after immunization. All statistical analyses were performed using the PBS treated mice as reference.

2.5. DSS-induced colitis model

Murine chronic colitis was induced using a protocol for dextran sodium sulfate (DSS) induced colitis (Okayasu et al., 1990; Wirtz et al., 2007). Female, 12-week-old C57BL/6 mice (Charles River) were given 1.5% DSS in their drinking water during 5 consecutive days, followed by 10 days of normal drinking water. This cycle was repeated two more times to induce a chronic inflammatory response. Before each DSS interval, a fresh DSS solution was prepared by dissolving 1.5% (w/v) in standard tap water. Bottles were wrapped in foil to protect the solution from light, and were washed after each interval.

All mice were assessed 4 times a week for clinical signs of colitis, i.e. stool consistency and fecal blood loss. Fresh droppings of each animal, which were collected by temporarily separating mice in individual cages, were smeared on the detection window of a Hemoccult® SENSA® test card to determine the consistency of the stool and the presence of any visible blood. The stool consistency was recorded using a stool score ranging from 0 to 4: 0 = normal stool, 1 = smearable stool, 2 = loose stool, 3 = very loose/shapeless stool and or slime, 4 = diarrhea. Within one week after stool collection, the tests cards were developed and the animals were scored for the degree of fecal blood loss: 0 = no fecal blood loss, 2 = occult fecal blood loss (no visible blood, positive test), 4 = macroscopic fecal blood loss. Test cards were stored for at least 48 h at room temperature before development, to reduce the chance on false positive results due to dietary peroxidases, as specified in the product instructions.

Mice were randomly assigned to an experimental group, and evenly distributed among the cages to exclude any cage related effects. Mice were treated after the third interval of DSS administration (day 36). Three groups of 8 mice were treated intravenously with a single dose of DXP (5 mg/kg), long-circulating liposomes containing PBS (LCL-PBS) or LCL-DXP (5 mg/kg). Two groups of 4 mice received LCL-DXP at doses 10 mg/kg or 20 mg/kg. Three groups of 5 mice, which did not receive DSS in their drinking water, served as healthy controls and were injected with DXP (5 mg/kg), LCL-PBS or LCL-DXP (5 mg/kg). After treatment, all mice were assessed on a daily basis and scored for stool consistency and hematochezia by an experimenter who was blinded for the treatment they received. All animals were sacrificed 9 days after treatment. All statistical analyses were performed using the LCL-PBS treated mice as reference.

3. Results

3.1. EAE model

To assess the effect of long-circulating DXP-liposomes in a chronic relapsing murine EAE, female SJL mice were immunized with proteolipid protein and scored on a daily basis until 7 weeks (49 days) after immunization (Tuohy et al., 1988). Between 11 and 16 days after immunization all mice developed clinical EAE (score ≥ 1), at which time they were treated with a single dose of 10 mg/kg DXP, 10 mg/kg LCL-DXP or PBS (day 0). After onset, the clinical course of the disease could be separated into a severe acute phase from day 0 to day 10, and a much more moderate disease activity in the following chronic phase (Fig. 1). Treatment with free DXP or PBS, did not show an effect on the disease activity in the acute or chronic phase. Lack of effect was also the case with LCL-DXP treatment in the chronic phase of EAE (data not shown). In the acute phase, however, the maximal clinical score that LCL-DXP treated mice received throughout the experiment was significantly lower than that of mice treated with PBS ($p < 0.01$, nonparametric one-way ANOVA) (Fig. 2). Finally, the total disease load, as represented by the area-under-the-curve (AUC), was in the acute phase significantly lower for animals treated with LCL-DXP compared to the animals that received PBS ($p < 0.05$, one-way ANOVA) (Fig. 3).

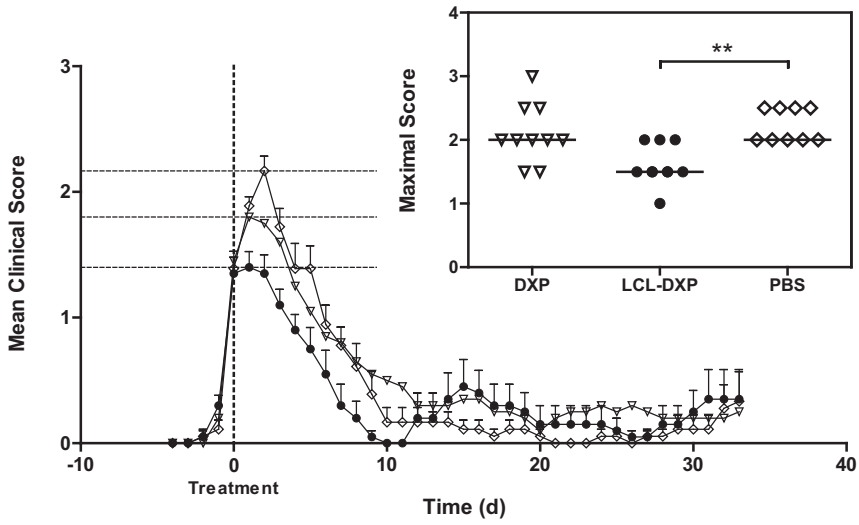

Fig. 2. Maximal clinical score of mice with EAE after treatment with (liposomal) DXP. The maximal clinical score of each animal was determined by the largest clinical score they received throughout the experiment, which in all cases corresponded to the height of the peak on day 1, 2 or 3. Depicted is the averaged clinical score curve of each experimental group with the maximal mean clinical score (dotted lines) of the group (main graph), and the maximal score of each individual mouse treated with PBS (◊), DXP (▽) or LCL-DXP (●) and the median of the maximal scores within the group (insert). There was a significant reduction of median maximal score after treatment with LCL-DXP (**$p < 0.01$), but not after treatment with DXP (compared with PBS, one-way ANOVA (nonparametric Kruskal–Willis test, followed by Dunn's multiple comparison test)).

3.2. DSS-induced colitis model

To assess the effect of long-circulating DXP-liposomes in a chronic colitis model of CD, DSS-induced colitis was established in female C57BL/6 mice (Okayasu et al., 1990). By periodically giving 1.5% DSS in their drinking water, in three cycles of 5 days of DSS followed by 10 days of tap water, a chronic inflammation of the large intestine was induced. On the day the mice switched for the third time to standard tap water (day 36), they were treated with a single i.v. injection of DXP (5 mg/kg), 'empty' long-circulating liposomes (LCL-PBS), or LCL-DXP (5 mg/kg, 10 mg/kg or 20 mg/kg). Before treatment, all mice were scored 4 times a week for stool consistency and fecal blood loss. After treatment, scoring was done on a daily basis until the end of the study, nine days after treatment (day 44). The control groups of mice, that did not receive DSS but only the treatments, did not show any signs of colitis throughout the study (data not shown). In the groups with DSS-induced colitis, all mice developed an acute colitis immediately after starting the first cycle of DSS, as indicated by their stool consistency (Fig. 4) and fecal blood loss (Fig. 5). The disease severity dropped in intensity after returning to normal tap water, and subsequently increased, although less severe, during the second and third cycle of DSS. Treatment of the mice with DXP, LCL-PBS or LCL-DXP after the third interval of DSS administration did not lead to any significant differences in their stool consistency score (Fig. 4). Interestingly, there was a clear effect of treatment on the fecal blood loss of the mice which received LCL-DXP in any dosage. However, unexpectedly, LCL-DXP treatment did not result in an improvement but in a worsening of the disease (Fig. 5). Moreover, the total fecal blood score after treatment was significantly larger for the mice treated with 5, 10 or 20 mg/kg LCL-DXP compared with LCL-PBS treated mice ($p < 0.05$, $p < 0.01$, $p < 0.001$ respectively, one-way ANOVA) (Fig. 6). Although a higher average total blood score was found in mice receiving a higher dose of LCL-DXP, this relation was not significant.

4. Discussion

So far, long-circulating liposome-encapsulated prednisolone and 6α-methylprednisolone have been studied for their efficacy in models of RA (rat), MS (rat) and cancer (mouse) (Metselaar et al., 2003; Schiffelers et al., 2005; Schmidt et al., 2003). This study aimed to broaden the applicability of liposome-encapsulated corticos-

Mean disease load of each treatment group during the acute phase

Treatment	Mean Disease load
PBS	12.7
DXP	11.8
LCL-DXP	8.0

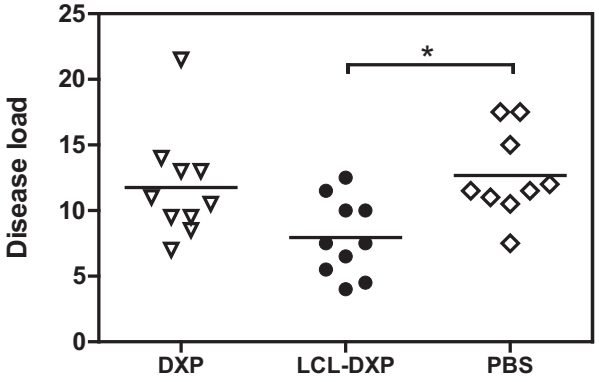

Fig. 3. Disease load during acute phase of mice with EAE after treatment with (liposomal) DXP. The disease load of each animal during the acute phase was defined as the area under the clinical score curve during the first 10 days after treatment. Depicted is the mean disease load each experimental group (table) and the disease load of each mouse treated with PBS, DXP or LCL-DXP, with the average of the experimental group as a straight line (graph). There was a significant reduction of mean disease load after treatment with LCL-DXP (*$p < 0.05$), but not after treatment with DXP (compared with PBS, one-way ANOVA followed by Bonferroni's multiple comparison test).

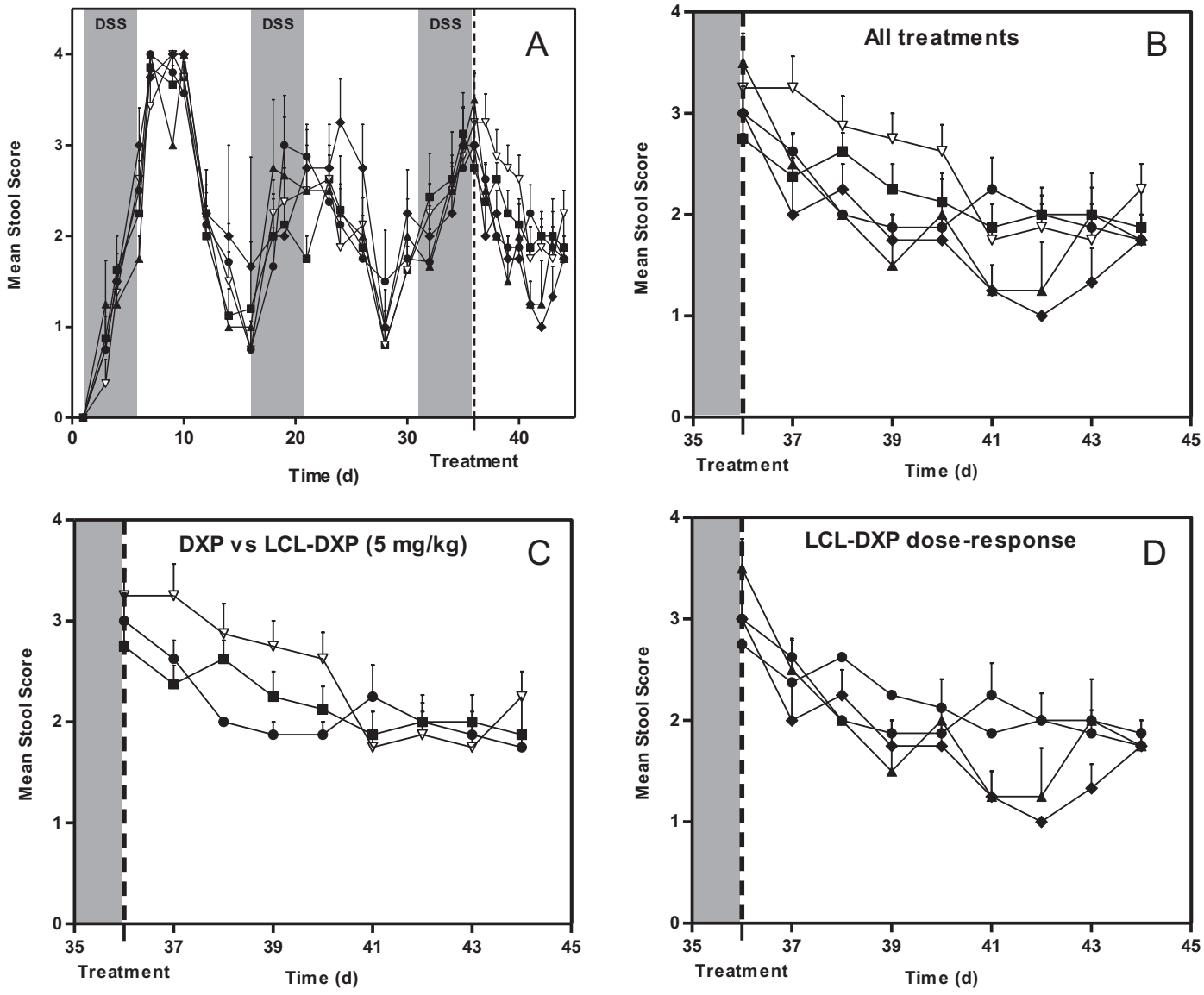

Fig. 4. Stool consistency of mice with DSS-induced colitis after treatment with (liposomal) DXP. Chronic colitis was induced in female C57BL/6 mice by changing their drinking water during three cycles of 5 days of 1.5% DSS and 10 days of normal tap water. After the last interval of DSS (day 36), all mice were treated with 'empty' long-circulating liposomes (LCL-PBS ■), DXP (5 mg/kg ▽), or liposomal DXP (LCL-DXP, 5 mg/kg ●, 10 mg/kg ▲, or 20 mg/kg ♦). (A) The average stool score and SEM of all groups from disease induction (day 1) until 9 days after treatment (day 44). (B) Stool score of all groups after treatment. (C) Stool score after treatment of mice treated with DXP (5 mg/kg) or liposomal DXP (5 mg/kg). (D) Stool score after treatment of mice treated with different dose of LCL-DXP (5, 10 and 20 mg/kg). Depicted are mean of stool score and SEM of the experimental group ($n = 8$). No significant differences in stool score after treatment was observed (versus LCL-PBS, two-way ANOVA). Stool score for consistency: 0 = normal stool, 1 = smearable stool, 2 = loose stool, 3 = very loose/shapeless stool and or slime, 4 = diarrhea.

teroids by evaluating another corticosteroid in mouse models for MS and CD. Both liposomal prednisolone phosphate and liposomal 6α-methylprednisolone phosphate have shown therapeutic efficacy in rat models for MS (Linker et al., 2008; Schmidt et al., 2003), but to date this has never been confirmed in other species. CD forms a completely new indication in the research on the therapeutic applicability of liposomal corticosteroids. In both preclinical models it was expected that treatment with liposome-encapsulated DXP, which has a higher glucocorticoid potency than prednisolone, would lead to a clinical improvement. In the EAE model there was indeed a significant improvement after single-dose treatment with 10 mg/kg LCL-DXP, with a reduced disease intensity and disease load. However, surprisingly, in the DSS-induced IBD model, there was a worsening of the disease after treatment with 5, 10 or 20 mg/kg LCL-DXP, indicated by a significant increase in fecal blood loss. The question arises why long-circulating DXP-liposomes are favorable in case of EAE and not in DSS-induced colitis. Since previous data showed that the effect of liposomal glucocorticoids is – at least partly – mediated by local tissue macrophages (Banciu et al., 2008; Storm et al., 1988; van Rooijen and van Kesteren-Hendrikx, 2002), these results suggest a contrasting role of these macrophages in MS and CD. Our hypothesis is that the dissimilar effects of LCL-DXP relate to differences in the polarization status of the target tissue macrophages in both diseases.

The concept that macrophage phenotype and function is dependent on the environment where it resides, is not new (Gordon and Taylor, 2005). Populations of macrophages have previously been classified as M1- versus M2-polarized, making a distinction between a cytotoxic and a regulatory macrophage activity, respectively. This is similar to the Th1 and Th2 T-helper cell classification (Mantovani et al., 2004; Martinez et al., 2009). This classification has been developed further into a model where macrophages are categorized into 'classical activated', 'wound-healing', and 'regulatory' macrophages (Edwards et al., 2006; Mosser and Edwards, 2008).

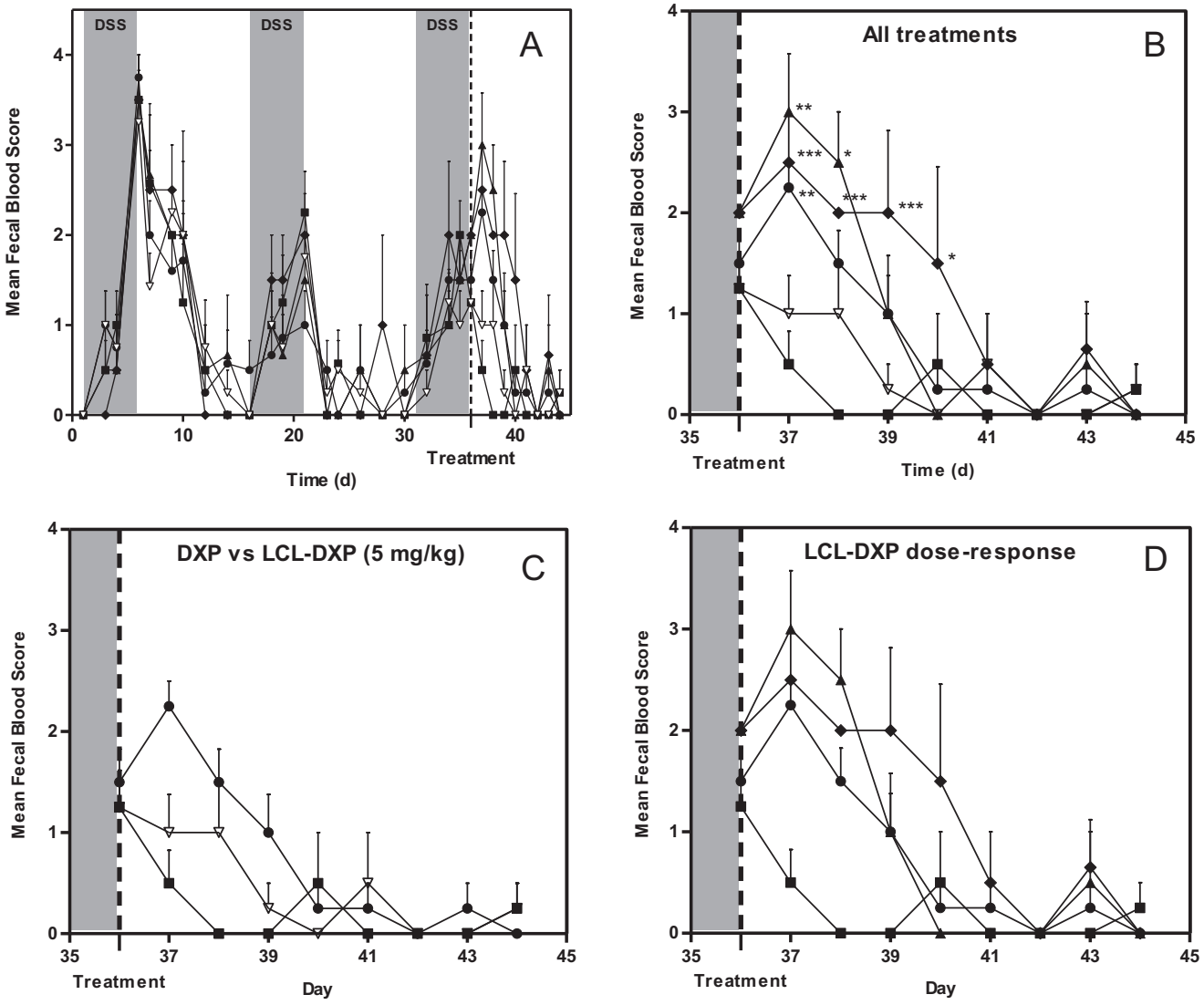

Fig. 5. Fecal blood score of mice with DSS-induced colitis after treatment with (liposomal) DXP. Chronic colitis was induced in female C57BL/6 mice by changing their drinking water during three cycles of 5 days of 1.5% DSS and 10 days of normal tap water. After the last interval of DSS (day 36), all mice were treated with 'empty' long-circulating liposomes (LCL-PBS ■), DXP (5 mg/kg ▽), or liposomal DXP (LCL-DXP, 5 mg/kg ●, 10 mg/kg ▲, or 20 mg/kg ♦). (A) Average fecal blood score and SEM of all groups from disease induction (day 1) until 9 days after treatment (day 44). (B) Fecal blood score of all groups after treatment. (C) Fecal blood score after treatment of mice treated with DXP (5 mg/kg) or liposomal DXP (5 mg/kg). (D) Fecal blood score after treatment of mice treated with different doses of LCL-DXP (5, 10 and 20 mg/kg). Depicted are the average fecal blood score and SEM of the experimental group ($n = 8$). Treatment was a significant parameter in the observed variance in fecal blood loss ($p < 0.0001$, two-way ANOVA). $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ compared to LCL-PBS (two-way ANOVA, followed by Bonferroni's post-test). Fecal blood score: 0 = no fecal blood loss, 2 = occult fecal blood loss (no visible blood, positive test), 4 = macroscopic fecal blood loss.

Classical activated macrophages are generally formed in response to TNF-α and IFN-γ, and are strongly associated with Th1- and Th17-mediated cellular immunity. When stimulated, they release pro-inflammatory cytokines such as IL-1, IL-6 and IL-23. Wound-healing macrophages arise after exposure to IL-4 and IL-13, which are important regulatory cytokines in Th2-associated humoral immunity. As the name implies, this type of macrophage is involved in the process of tissue regeneration, by producing collagen for the extracellular matrix and releasing tissue-repair promoting factors (Martinez et al., 2009). Finally, regulatory macrophages are formed following exposure to IL-10 produced by regulatory T-cells. Regulatory macrophages, together with regulatory T-cells, dampen the inflammatory response and stimulate tissue repair by inducing Th2- and M2-polarization, which result in high levels of IL-4 and consequently formation of wound-healing macrophages (Mosser and Edwards, 2008). The polarization of macrophages is not definite: macrophages retain the capability to change their phenotype according to the local environment (Stout et al., 2005; Stout and Suttles, 2004). In particular, glucocorticoids have been shown to interfere with the macrophage phenotype: they drive the macrophage population towards a more anti-inflammatory, regulatory M2 polarization (Ehrchen et al., 2007). This means that, in light of the natural tropism of liposomes for macrophages, DXP encapsulated in liposomes is even more potent in doing so. This is supported by the observed results in the EAE model, and previously in RA, which are primarily M1 macrophage-mediated diseases (Brennan et al., 1998; Schönrock et al., 2000). In EAE, shifting the macrophage polarization towards a M2/Th2 polarization, results in amelioration of the clinical disease activity (Imler Jr and Petro, 2009; Mikita et al., 2010). The finding that liposomal dexamethasone reduces the clinical score in mice with EAE, can therefore be explained by a similar modulation of the macrophages in the nervous system. Intestinal macrophages, however, respond differently to their surroundings than other tissue macrophages; a property

Mean total fecal blood score of each treatment group after treatment

Treatment	Mean total fecal blood score
LCL-PBS	2.5
DXP (5 mg/kg)	4.0
LCL-DXP (5 mg/kg)	7.0
LCL-DXP (10mg/kg)	9.0
LCL-DXP (20 mg/kg)	11.0

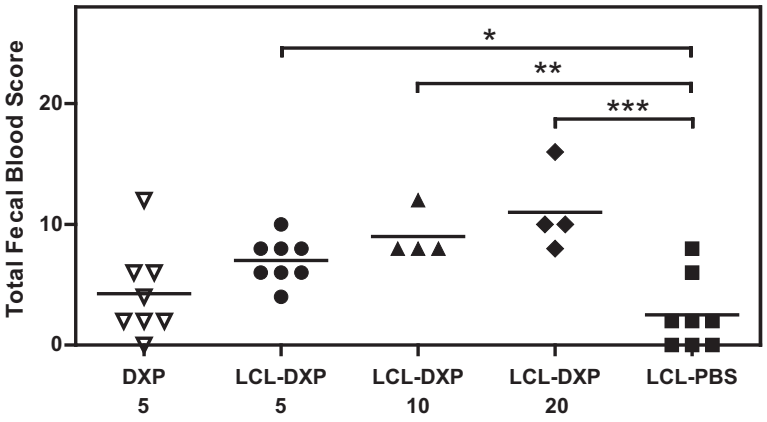

Fig. 6. Total fecal blood score of mice with DSS-induced colitis after treatment with (liposomal) DXP. The total fecal blood score of each mouse was defined by the sum of fecal blood scores after treatment, corresponding to the area under the fecal blood score curve. Depicted is the mean total fecal blood score curve of each experimental group (table), and the total fecal blood score of each mouse treated with LCL-PBS, DXP or LCL-DXP (5, 10 or 20 mg/kg) and the average of the experimental group (graph). There was a significant increase of total fecal blood score of mice treated with LCL-DXP at all doses ($^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$), but not of mice treated with DXP (compared with LCL-PBS, one-way ANOVA followed by Bonferroni's multiple comparison test).

that can be related to the constant threat of commensal microorganisms from the intestinal lumen (Heinsbroek and Gordon, 2009). Intestinal macrophages therefore possess the capability to phagocytose and eradicate microorganisms and act in a M1 polarized manner, without showing a pro-inflammatory phenotype. In fact, there is strong evidence that intestinal macrophages are key regulators in maintaining an immunological tolerance towards these invading microorganisms (Platt and Mowat, 2008). In the absence of such control mechanism for intestinal homeostasis, a permanent inflammation of the whole gastrointestinal tract would occur. Also in CD, several reports indicate that M1 activated macrophages indeed are essential in the preservation of mucosal integrity and defense, and fulfill a protective role in the development of the disease. First of all, DSS-induced colitis in intestinal macrophage-depleted mice has a more severe course compared to mice with an intact intestinal macrophage function (Qualls et al., 2006). Secondly, therapy with granulocyte macrophage colony-stimulating factor (GM-CSF), which stimulates innate immunity by inducing a shift in macrophage polarization towards M1, leads to improvement of DSS colitis (Bernasconi et al., 2010; Sainathan et al., 2008). In fact, Sargramostim, a recombinant GM-CSF, shows beneficial effects in CD patients, and is currently in phase II clinical trials (Valentine et al., 2009). Therefore, changing the phenotype of intestinal macrophages from M1 to M2 by treatment with DXP-liposomes may indeed lead to a worsening of clinical symptoms in colitis, making CD a notable exception in the field of inflammatory diseases, which does not respond favorably to liposomal corticosteroid treatment.

In conclusion, the current study shows contrasting disease outcomes after treatment with liposomal DXP in models of CD and MS. In EAE, a model for MS, treatment with liposomal DXP leads to a clinical improvement of the disease, resulting in a reduction of maximal disease intensity and disease load. In contrast, in the DSS-induced colitis model for CD treatment with liposomal DXP leads to a clinical worsening, marked by an increase in fecal blood loss. This remarkable difference could be due to the different inflammatory context of both diseases, which makes that modulation of macrophage polarization to M2 in each of these diseases leads to these opposing effects.

Since most, if not all, nanomedicines generally accumulate in cells of the RES, including those at inflammatory sites, this could prove to be a valuable lesson for future efforts in the development of anti-inflammatory targeted drug formulations.

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