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Liposomal encapsulation of dexamethasone modulates cytotoxicity, inflammatory cytokine response, and migratory properties of primary human macrophages

Matthias Bartneck, PhD[a,*,1], Franziska Marie Peters[a,1], Klaudia Theresa Warzecha, MSc[a], Michaela Bienert, MSc[b], Louis van Bloois[c], Christian Trautwein[a], Twan Lammers[c,d,e], Frank Tacke[a]

[a]*Department of Medicine III, Medical Faculty, RWTH University, Aachen, Germany*
[b]*Helmholtz-Institute for Biomedical Engineering, Biointerface Laboratory, RWTH Aachen, Germany*
[c]*Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands*
[d]*Department of Controlled Drug Delivery, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands*
[e]*Department of Experimental Molecular Imaging, Helmholtz Institute for Biomedical Engineering, Medical Faculty, RWTH University, Aachen, Germany*

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Abstract

The encapsulation of drugs into liposomes aims to enhance their efficacy and reduce their toxicity. Corticosteroid-loaded liposomes are currently being evaluated in patients suffering from rheumatoid arthritis, atherosclerosis, colitis, and cancer. Here, using several different fluorophore-labeled formulations, we comprehensively studied the impact of liposome encapsulation of the prototypic corticosteroid dexamethasone on various primary human cells *in vitro*. Liposomal dexamethasone targeted several primary cell types in a dose and time-dependent manner, but specifically reduced cytotoxicity against human fibroblasts and macrophages in comparison to the solute drug. Furthermore, macrophage maturation and polarization markers were altered. Interestingly, liposomal dexamethasone induced proinflammatory cytokine secretion (specifically TNF, IL1β, IL6) in unstimulated cells, but reduced this response under inflammatory conditions. Monocyte and macrophage migration was significantly inhibited by dexamethasone-loaded liposomes. The findings indicate that the encapsulation of dexamethasone into liposomes modulates their cellular mechanism of action, and provides important indications for follow-up *in vivo* investigations.

From the Clinical Editor: This study investigates mechanism of action of liposomal dexamethason in the treatment of inflammatory conditions. It is concluded that liposomal dexamethasone actually induces proinflammatory cytokine secretion in unstimulated cells, but reduces the same response under inflammatory conditions. Monocyte and macrophage migration was also inhibited. The findings indicate that liposomal dexamethasone may have different mechanisms of action than its native counterpart.

Key words: Liposomes; Corticosteroids; Glucocorticoids; Dexamethasone; Primary human macrophages; Immune cells; Cell migration; Cell activation; Cytokine release; Inflammation

Liposomes are in clinical use as nanocarriers of glucocorticoids (GC) that are intended to suppress inflammatory diseases such as arthritis, colitis, and atherosclerosis.[1,2] GC act via the GC receptor and lead to the downregulation of proinflammatory mediators.[3] Liposomes are approved for autologous stem cell transplantation induction regimes,[4] autoimmune encephalomyelitis,[3] and cancer treatment.[5] Liposomal encapsulation of vaccines has also been done to increase vaccination efficiency,[6] a process that strongly relies on antigen-presenting cells such as monocytes and macrophages. The pharmaceutical advantages of encapsulated glucocorticoids are based on the antiinflammatory activities of GC. The decoration of nanoparticle surfaces with polyethylene glycol (PEG) is known to increase the circulation time of nanoparticles,[2,7]

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*Corresponding author. Department of Medicine III Medical Faculty RWTH Aachen Pauwelsstraße 30, Aachen, Germany.

E-mail address: mbartneck@ukaachen.de (M. Bartneck).

[1] These authors contributed equally.

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and we could correlate this increased circulation with a decreased clearance of PEGylated particles by macrophages.[8,9]

There are two major subpopulations of macrophages that can be classified as either classically (proinflammatory) or alternatively (antiinflammatory) activated. Classical activation of macrophages is induced by bacterial products such as lipopolysaccharides or immune cell-derived interferon γ and the corresponding cells are referred to as M1 macrophages. In contrast, alternative activation is a response to stimulation with glucocorticoids such as dexamethasone (Dex)[10] and the designated cells are defined as M2 macrophages. The M2 subtype is known to express typical markers of alternative activation like CD163.[11] A recent study has shown that liposomal encapsulation of prednisolone increases M2 marker expression by macrophages.[3] However, the effects of encapsulation of the more frequently used GC dexamethasone specifically on immune cell activation would be of great interest for clinicians.

Furthermore, it is known that Dex can have cytotoxic effects.[12] This might be especially relevant to cell compartments in the liver, where GC as well as nanocarriers are typically cleared from the circulation.[9] For experimental purposes, fluorescent labeling of nanocarriers might assist in verifying their cell targeting capacities and facilitates their detection in fluorescence-based applications such as immunofluorescence microscopy or flow cytometry.

In order to address these unknown aspects of liposome-based GC targeting, we generated several different formulations: we equipped liposomes with a diameter of 100 or 400 nm with a fluorophore resulting in a green fluorescent signal, additionally PEGylated them and loaded them with Dex. We studied the cytotoxicity of solute and encapsulated Dex and of liposomes using four different primary cell types. The uptake of different liposomal formulations by the most relevant scavenging cells was studied using flow cytometry, and cellular morphology changes were studied in macrophage cell cultures. Changes in macrophage phenotype were monitored using flow cytometric detection of surface markers. Inflammatory cytokine expression was studied using quantitative real-time-PCR and cytokine multiplex assays. Using these sophisticated methods on primary cell populations, our study revealed distinct effects of liposomal versus free Dex on cell-specific targeting, cytotoxicity and interactions with immune cell function and migration.

Methods

Liposome preparation and characterization

Liposomes were prepared according to the film-method.[13] In brief, Dipalmitoyl phosphatidylcholine (DPPC) and PEG-(2000)-distearoyl phosphatidylethanolamine (PEG-(2000)-DSPE) were obtained from Lipoid (Ludwigshafen, Germany), cholesterol was obtained from Sigma (St. Louis, MO, USA) and (*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-snglycero-3-phosphoethanolamine, triethyl-ammonium salt) (NBD-PE) was obtained from Molecular Probes (Grand Island, New York, USA). All other chemicals were of reagent grade. A mixture of chloroform/methanol (10:1 volumetric ratio) containing DPPC, PEG-(2000)-DSPE, NBD-PE and cholesterol was prepared at a molar ratio of 1.85:0.15:0:1. As a fluorescent marker 1 mol % of NBD-PE was added to the organic phase in relation to the total amount of lipid, including cholesterol. The organic phase was evaporated with a rotavapor (BUCHI Labortechnik AG, Flawil, Switzerland), followed by nitrogen flushing for removal of residual organic solvent. The lipid film was hydrated at 50 °C in an aqueous solution of dexamethasone phosphate in a concentration of 100 mg/mL at a phospholipid concentration of 100 mM. Empty liposomes without dexamethasone phosphate were dispersed in phosphate buffered saline (PBS). The liposomes were sequentially extruded through two stacked polycarbonate filters with pore sizes of 600, 200, and 100 nm (Nuclepore, Pleaston, USA) under nitrogen pressure, using a Lipex high pressure extruder (Lipex, Nortern Lipids, Vancouver, Canada). Unentrapped dexamethasone phosphate was removed by dialysis at 4 °C against PBS using Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, USA) with a molecular cut-off of 10 kD.

The mean particle size was determined by dynamic light scattering with an ALVCGS-3 system (Malvern Instruments, Worcestershire, United Kingdom). Zeta potential was determined using a Zetasizer Nano Z (Malvern Instruments Ltd., Worcs, UK). The phospholipid content was determined with a phosphate assay[14,15] on the organic phase after extraction of the liposomal preparation with chloroform. The aqueous phase after extraction was used to determine the liposomal dexamethasone phosphate concentration of the liposomes by high performance liquid chromatography using a mobile phase of acetonitrile/water of pH 2 in a ratio of 25/75 and monitoring the eluents with a UV-detector at 254 nm. The batch of 100 nm PEGylated liposomes that was used in this study had a zeta potential of 5.1 +/− 1.4 mV, and its loading capacity was 0.13 mg dexamethasone phosphate per μmol of phospholipid. Earlier studies have shown that these liposomes are physically and chemically stable for up to 1 year at temperatures up to 40 °C, with no loss of the encapsulated corticosteroid, either upon dilution, or upon interaction with body fluids.[2,16] The formulations were extensively dialyzed, according to previously established procedures,[17] to remove all free and surface-associated corticosteroid. A limulus amebocyte lysate (LAL) assay QCL-1000 was obtained from Lonza (Walkersville, MD, USA) to test for possible endotoxin contaminations of the liposomes. The kit was used according to the instructions of the manufacturer.

Cell isolation and culture

Human primary blood leukocytes were isolated using dextran sedimentation and purification of peripheral blood mononuclear cells (PBMC) was done using Ficoll-based density gradient centrifugation as reported earlier.[8] To isolate monocytes for macrophage culture, PBMC were incubated at 37 °C on bacterial grade Petri dishes at a density of three million cells per mL in RPMI1640 (Sigma-Aldrich, St. Louis, MA, USA) containing 5% human autologous serum for 35 min in a humidified incubator with 5% CO_2. During this period monocytes become adherent

and lymphocytes were removed with the supernatant. To obtain macrophages, monocytes were cultured for seven days in RPMI1640 medium supplemented with 5% autologous serum. To detach adherent macrophages from culture plates, the plates were put on ice for 20 min and the cells were then detached using a rubber-based cell scraper.

Serum was recovered from commercial collection tubes (Sarstedt, Nümbrecht, Germany). Human primary dermal fibroblasts were isolated from human preputium skin. Human umbilical vein endothelial cells were isolated as described earlier in detail[18] and primary murine hepatocytes were isolated from liver of C57BL6/J wildtype mice (for details, see supporting information).

In the cell culture experiments, the amount of liposomes to be used for stimulation was normalized to the Dex content, and 10 μg/mL of Dex were used for the stimulation of cells. For comparison, the identical volume of unloaded liposomes, normalized to their phospholipid content of 77 nmol/mL (77 μM), was used. This Dex concentration was chosen as it is considered to reflect an *in vivo* dose of 1 mg/kg body weight, based on earlier data on nanoparticle uptake by human macrophages *in vitro* and murine macrophages *in vivo*.[8,9]

Fluorescence microscopy

For nuclear staining, cells were incubated in a humidified 5% CO_2 incubator in RPMI1640 with 5% FCS and liposomes for 24 h. Afterwards, cells were rinsed two times with PBS with calcium and magnesium. Fixation was done using 2% paraformaldehyde, followed by three times of rinsing using PBS. For permeabilization, PBS was removed and cells were treated with 0.1% Triton X-100 for ten minutes. Cells were rinsed with PBS three times. Staining with 4',6-diamidino-2-phenylindole (DAPI) was done at 1 μg/mL of DAPI in PBS and excess DAPI was removed by washing for three times with PBS. The liposome fluorescence was recorded at the green channel. We used a Zeiss Axio Observer Z1 for the generation of micrographs (Carl Zeiss, Oberkochen, Germany).

Flow Cytometry

Cell surface antigens were stained with directly conjugated mouse anti-human antibodies anti-CD14, anti-CD16, anti-HLA-DR (all Becton Dickinson, Franklin Lakes, NJ), anti-CD163 (R&D systems, Minneapolis, USA) for 30 min at a dilution of 1:400 in a PBS-based blocking buffer containing 3% bovine serum albumin, 1% human, 1% rat, 1% mouse, and 1% rabbit serum at 4 °C. After staining, the cells were washed with PBS to remove excess antibodies. Cells were then stained with Hoechst 33258 (Sigma-Aldrich, St. Louis, MA, USA) in PBS 1:2000 for dead cell exclusion. All results are given relative to the untreated control.

Uptake and migration studies

To study the uptake of liposomes by the different cell types, cells were incubated with the formulations in RPMI1640 with 5% FCS and at 37 °C under continuous shaking conditions (500 rpm) in 1.5 mL tubes on a Thermomixer comfort (Eppendorf, Hamburg, Germany).

The experiments for spontaneous migration were done using 5 μm pores sizing transwell insert inlets for 24-well plates (Millipore, Billerica, Massachusetts, USA). Cells were incubated with liposomes in RPMI1640 with 5% FCS for one hour at 37 °C to assure uptake of liposomes interaction of substances with cells. Two million blood leukocytes or one million macrophages were then resolved in 200 μL RPMI1640 with 1% FCS and added on top of the transwell insets containing 800 μL RPMI1640 with 1% FCS, in 24-well plates. Migration of blood leukocytes was done for one hour at 37 °C due to their enhanced migration *in vitro* shown before[19] whereas macrophages were left to migrate for 16 h at 37 °C in a humidified incubator. For the evaluation of the test, the insets were removed after the designated time-points and the cells that had migrated were counted using a FACS Canto II (Becton Dickinson, Franklin Lakes, NJ). In some chemotaxis experiments, monocyte migration was tested towards 100 ng/mL recombinant human CCL2 (Peprotech, Rocky Hill, NJ, USA).

Real-time PCR

Cells were washed with medium after incubation with the different formulations. RNA was purified using the peqGold kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany), and complementary DNA was generated from RNA using the First Strand cDNA synthesis kit (Roche, Penzberg, Germany). Quantitative real-time polymerase chain reaction was done based on SYBR Green Reagent (Roche, Penzberg, Germany). Reactions were done as triplicates, and β-actin was used to normalize gene expression. Primer sequences are available upon request.

Cytokine detection

The release of six different cytokines (IL1β, TNFα, IL6, CCL2, TGFβ (LAP), IL10) by macrophages into the cell culture medium was quantified using the Flow Cytomix system, according to the instructions of the manufacturer (Ebioscience, San Diego, USA). All results on differential cytokine release are based on the comparison to the untreated control.

Statistical analysis

Statistical analysis of the biological data was performed using Graph Pad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). One way ANOVA with Bonferroni's post test was done to test significance of data versus untreated control cells. $P < 0.05$ was considered statistically significant.

Results

To generate liposomes that can simultaneously be traced based on a fluorescent signal and loaded with drugs, we synthesized liposomes sizing 100 or 400 nm. We PEGylated a batch of them to reduce unspecific binding with protein and cellular uptake,[20] and subsets of liposomes were either equipped with the fluorescent dye NBD-PE or additionally loaded with dexamethasone (Figure 1, *A*). The size distribution of the liposomes was controlled using dynamic light scattering analysis (Figure 1, *B*). Importantly, the endotoxin content of all liposomes

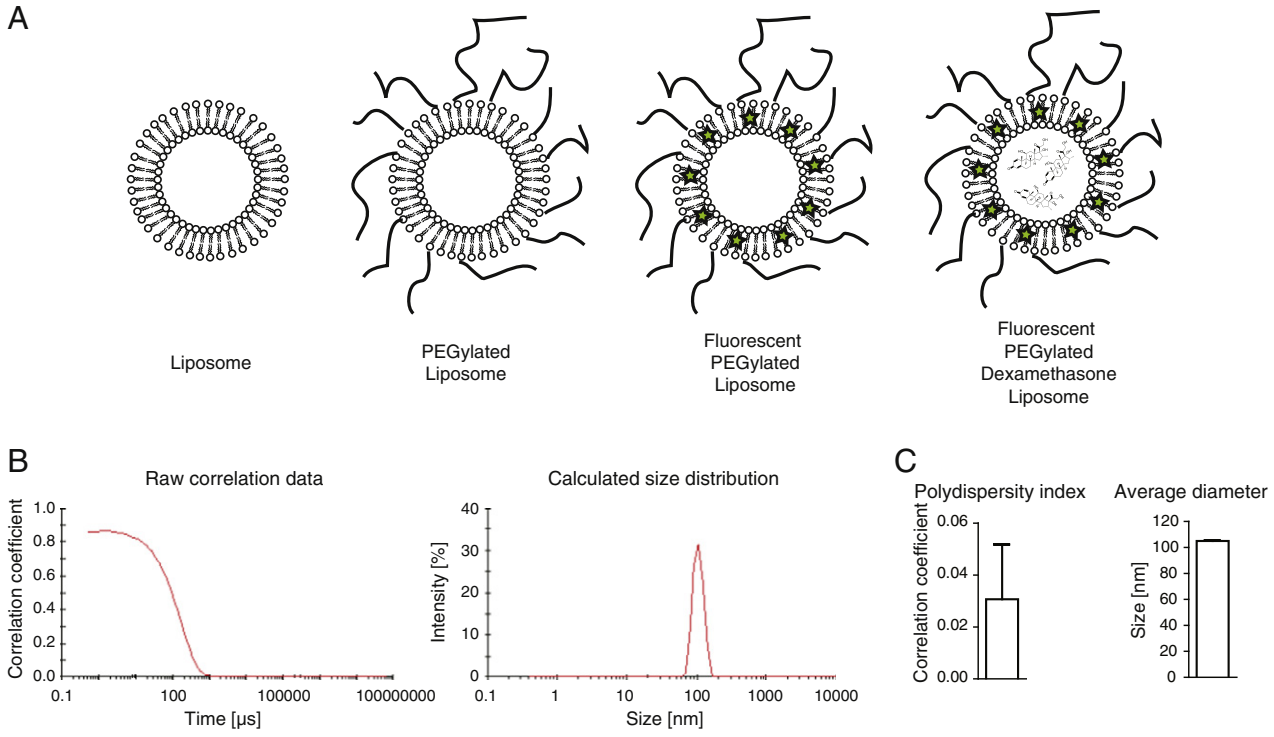


Figure 1. Liposome formulations used in this study. Schematic depiction: liposomes were either unmodified, PEGylated, additionally equipped with a fluorescent dye incorporated into the membrane, and loaded with dexamethasone within the core **(A)**. Liposomes were characterized using dynamic light scattering based assessment of the polydispersity index, and their size distribution was calculated based on the raw correlation data **(B)**, and average polydispersity index and average diameter **(C)**. Representative analyses for 100 nm PEGylated liposomes are depicted.

used in this study was below the detection limit of a commercial LAL assay, thus effects of bacterial contaminations on cell activation can be excluded.

Fluorescent liposomes can optionally be loaded with the drug Dex, which enables a combined analysis of cellular uptake and viability in fluorescence microscopy. We studied three different concentrations of PEGylated 100 nm liposomes, and identical liposomes additionally loaded with Dex, in comparison to free Dex. Notably, the uptake of liposomes by macrophages was similar both for FCS or human autologous serum (Figure S2). We found that after incubation with macrophages, liposomes were located in defined regions in the cells. In contrast, the particles were distributed throughout the cell in hepatocytes. The liposome uptake by both cell types was concentration dependent (Figure 2, *A*). We analyzed the effects of the different liposomes on the viability of four different primary cell types, human monocyte-derived macrophages, endothelial cells, fibroblasts, and hepatocytes after 48 h of incubation, based on a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based assay. Macrophage and fibroblast viability was significantly reduced at 10 µg Dex/mL (77 nmol/mL phospholipid content), while the viability of hepatocytes was unaffected and that of endothelial cells even increased (Figure 2, *B*).

To study the effects of the different liposomal formulations on macrophage morphology in cell culture, we generated macrophages using human monocytes from healthy volunteers and cultured them with either Dex, PEGylated 100 nm and Dex-loaded liposomes, 100 nm PEGylated liposomes, or lipopolysaccharides (LPS) for 24 h (Figure S1). While untreated control macrophages exhibited different cellular shapes (Figure S1, *A*), Dex led to cell shrinkage and the appearance of big round cells (Figure S1, *B*), whereas Dex liposomes reduced the number of adherent cells (Figure S1, *C*). Liposomes alone did not change macrophage morphology compared to the control (Figure S1, *D*), and LPS induced a characteristic stretched morphology (Figure S1, *E*).

We next compared the uptake of different liposomes by blood circulating immune cells granulocytes, monocytes, and lymphocytes (Figure 3, *A*), and compared them to tissue resident macrophages after seven days of culture (Figure 3, *B*). The different cell populations were differentiated by their forward scattering (FSC, reflecting cell size) and sideward scattering (SSC, cell granularity) profile in flow cytometry. The number of cells that internalized liposomes was quantified based on their emission of green fluorescence (excitation at 488 nm and emission at 420 nm wavelength). The fluorescent signal was determined either based on gating on the green fluorescent cells or by assessing the mean fluorescent intensity (MFI) of the fluorescent signal. Interestingly, macrophages internalized all types of liposomes, irrespective of size or PEGylation (Figure 3, *B*). Considering autofluorescence is an important issue in studies focused on the uptake of fluorescent particles, one should note that even macrophages that received no liposomes exhibited a significantly enhanced fluorescent signal compared to the other cell types (Figure 3, *C*).

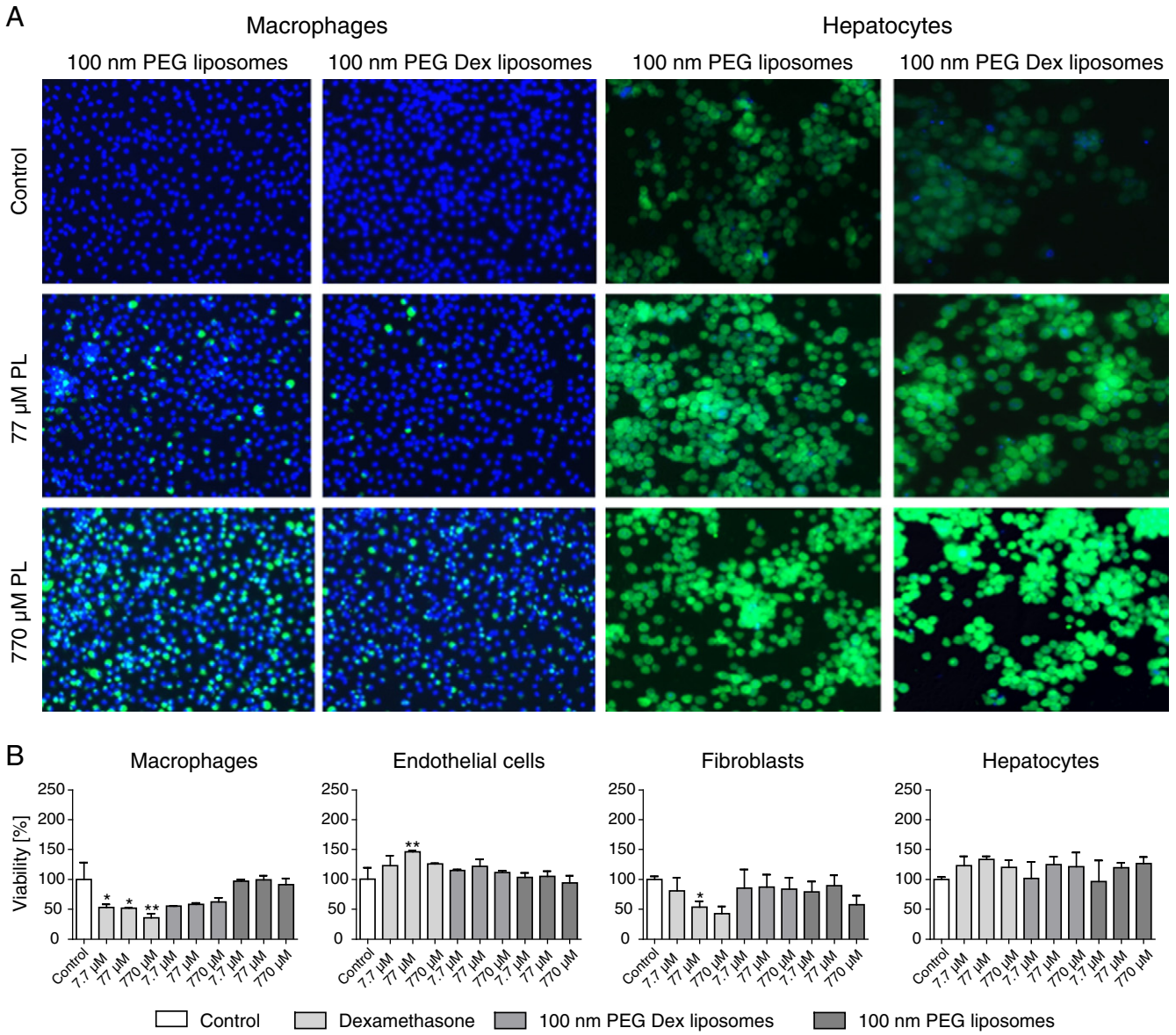
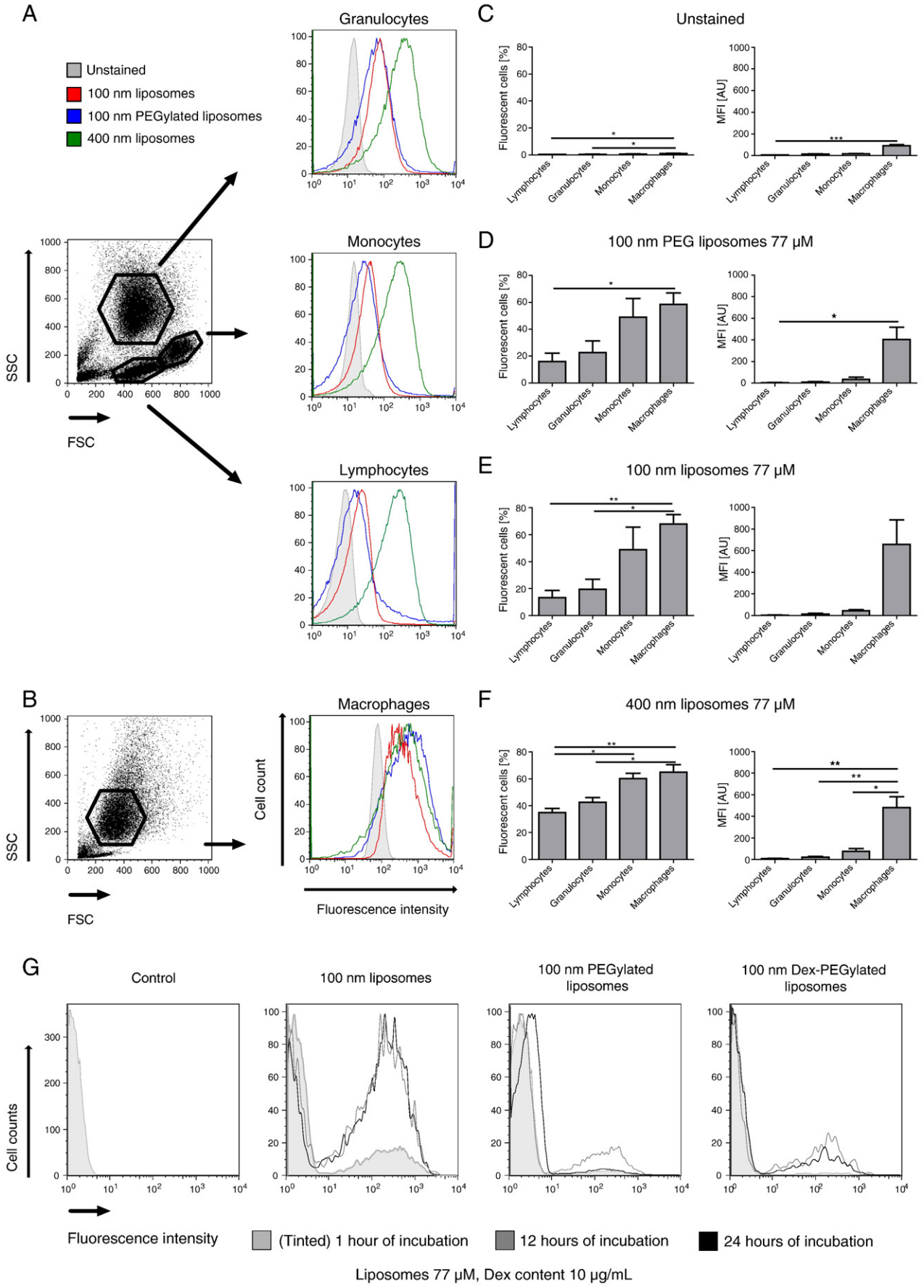


Figure 2. Cell type specific cytotoxicity of liposome encapsulated dexamethasone. Human primary macrophages and primary murine hepatocytes were incubated with 100 nm PEGylated liposomes with and without dexamethasone (Dex) for 24 h in RPMI1640 with 5% fetal calf serum. Green fluorescence reflects liposome internalization and nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI, blue), normalized on the phospholipid (PL) concentration of liposomes **(A)**. Viability screening of human primary macrophages, endothelial cells, fibroblasts, and murine primary hepatocytes with dexamethasone (Dex), 100 nm PEGylated Dex liposomes, and 100 nm PEGylated liposomes based on a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 48 h of incubation. A liposome concentration of 77 µM corresponds to a Dex content of 10 µg/mL **(B)**. Mean data of $n = 4$. *$P < 0.05$, **$P < 0.005$ (one way ANOVA, compared to control).

PEGylated liposomes 100 nm in diameter were mostly taken up by macrophages, followed by monocytes, granulocytes and lymphocytes (Figure 3, *D*). PEGylation appeared to have a low impact on the short-term uptake by the different blood cells but strongly reduced their internalization by macrophages considering especially the MFI data (Figure 3, *E*). Liposomes up to 400 nm were increasingly found also in other immune cells, yet still they were mostly localized in macrophages (Figure 3, *F*). Due to the increased location of the 400 nm liposomes in blood cells and the associated reduction in macrophage uptake which in turn would mean a decreased delivery to an organ such as the liver based on the high presence of hepatic macrophages (Kupffer cells) in liver,[21] we focused all follow-up experiments on the 100 nm liposomes. On the basis of the cellular uptake studies, an additional loading of liposomes with dexamethasone enables a cell-specific targeting of macrophages with the drug (Figure 3, *A-F*). The uptake of the liposomes by macrophages was also time dependent as shown in representative flow cytometry derived signal overlays: after one hour, comparatively few liposomes were internalized by macrophages, whereas the maximum uptake was


A
Unstained
100 nm liposomes
100 nm PEGylated liposomes
400 nm liposomes
SSC
FSC
Granulocytes
Monocytes
Lymphocytes
B
Macrophages
Cell count
Fluorescence intensity
C
Unstained
D
100 nm PEG liposomes 77 µM
E
100 nm liposomes 77 µM
F
400 nm liposomes 77 µM
Fluorescent cells [%]
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Lymphocytes
Granulocytes
Monocytes
Macrophages
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Control
100 nm liposomes
100 nm PEGylated liposomes
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Cell counts
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(Tinted) 1 hour of incubation
12 hours of incubation
24 hours of incubation
Liposomes 77 µM, Dex content 10 µg/mL

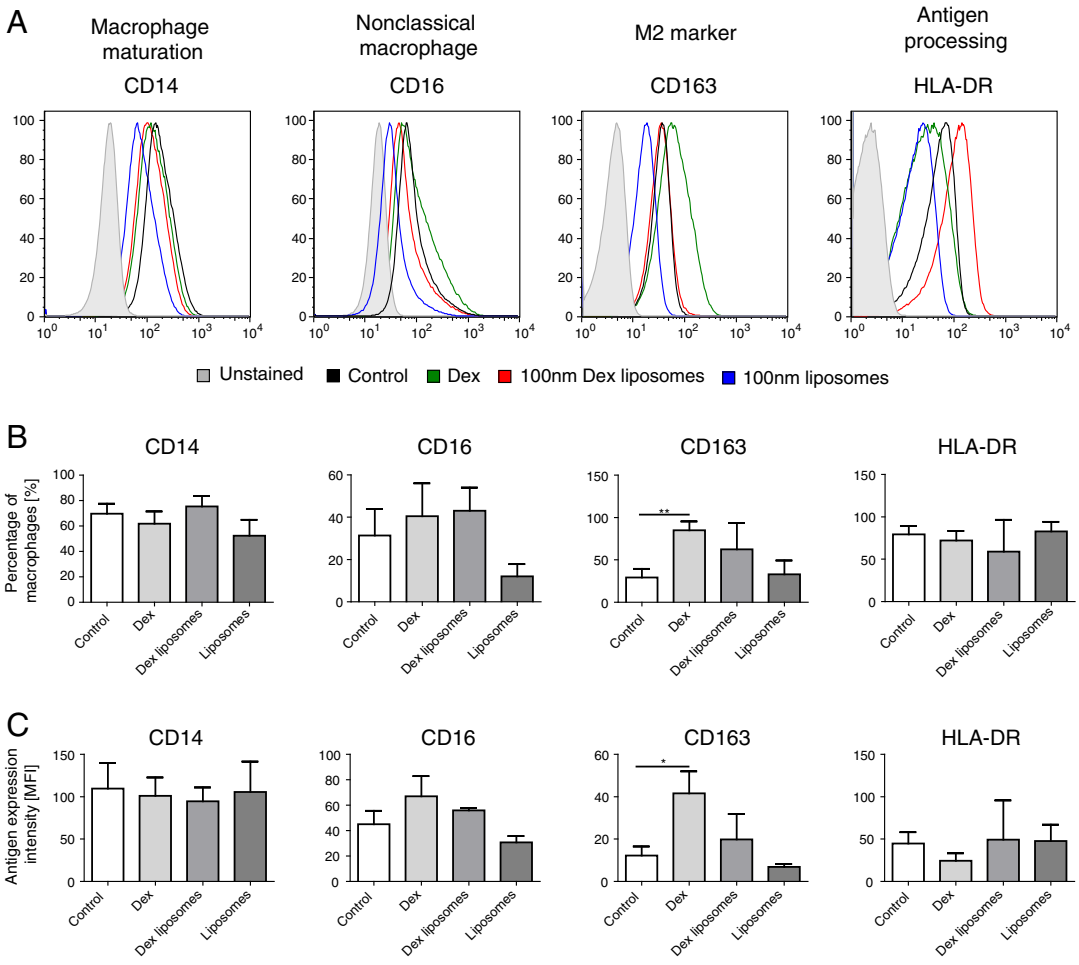

Figure 4. Macrophage polarization by dexamethasone loaded liposomes. Human primary macrophages were generated by seven days of monocyte culture in RPMI1640 supplemented with 5% autologous serum and left untreated, or were incubated with 10 μg/mL dexamethasone (Dex), 100 nm PEGylated Dex liposomes with the same amount of Dex and a phospholipid (PL) content of 77 μM (77 nM/mL), or 100 nm PEGylated liposomes with the same amount of PL, for 24 h. Flow cytometric analysis of the expression of the macrophage differentiation markers CD14, CD16, CD163, and HLA-DR of one representative donor **(A)**. Statistical summary of the expression of the designated surface markers based on gating as percentage of the total macrophage pool **(B)**, or on the mean fluorescent intensity (MFI) of the markers **(C)**. Mean data of $n = 4$. *$P < 0.05$, **$P < 0.005$ (one way ANOVA).

reached after 12 h. PEGylation of liposomes reduced their uptake by macrophages what becomes most clear after 12 h of incubation, similar to the uptake of PEGylated Dex liposomes (Figure 3, *G*).

Due to the pronounced effects of solute and encapsulated glucocorticoids, we hypothesized that there might be also consequences for macrophage differentiation after 24 h of incubation with the formulations. We therefore performed flow cytometry for the functional macrophage markers CD14, the receptor for the LPS binding protein that is known to be expressed by classical monocytes and macrophages, for CD16, the Fc receptor γRIII, for CD163, the haptoglobin scavenging receptor that is expressed by alternatively activated macrophages, and HLA-DR which is involved in antigen processing (Figure 4, *A*). We found, both using individual gating (Figure 4, *B*) and quantifying the MFI (Figure 4, *C*), that Dex induced a significant up-regulation of CD163 expression, confirming literature,[10] whereas CD163 expression was not significantly induced by the Dex liposomes (Figure 4, *B* and *C*).

It should be noted that macrophage marker expression after corresponding stimuli changes rather slowly whereas inflammatory

Figure 3. Immune cell specific uptake of fluorescent liposomes. Human primary granulocytes, monocytes, and lymphocytes were isolated from human blood using dextran sedimentation **(A)**. Blood cells were distinguished using flow cytometry, and their liposome uptake was studied after 60 min of incubation in RPMI1640 medium with 5% fetal calf serum (FCS) based on the green fluorescent signal of the liposomes. Human primary macrophages were generated by seven days of monocyte culture in RPMI1640 supplemented with 5% autologous serum **(B)**. Flow cytometry-based quantification of liposome uptake after 60 min based on their emission of fluorescence or mean fluorescent intensity (MFI, right column). Signals without fluorescent liposomes **(C)**, and after incubation with 77 μM (77 nM/mL) of 100 nm PEG liposomes **(D)**, 100 nm liposomes **(E)**, or 400 nm liposomes **(F)**. Flow cytometry-based quantification of liposome uptake by macrophages after different intervals of incubation with fluorescent 100 nm liposomes, 100 nm PEGylated liposomes, and 100 nm PEGylated Dex liposomes **(G)**. Mean data of $n = 4$. *$P < 0.05$, **$P < 0.005$ (one way ANOVA).

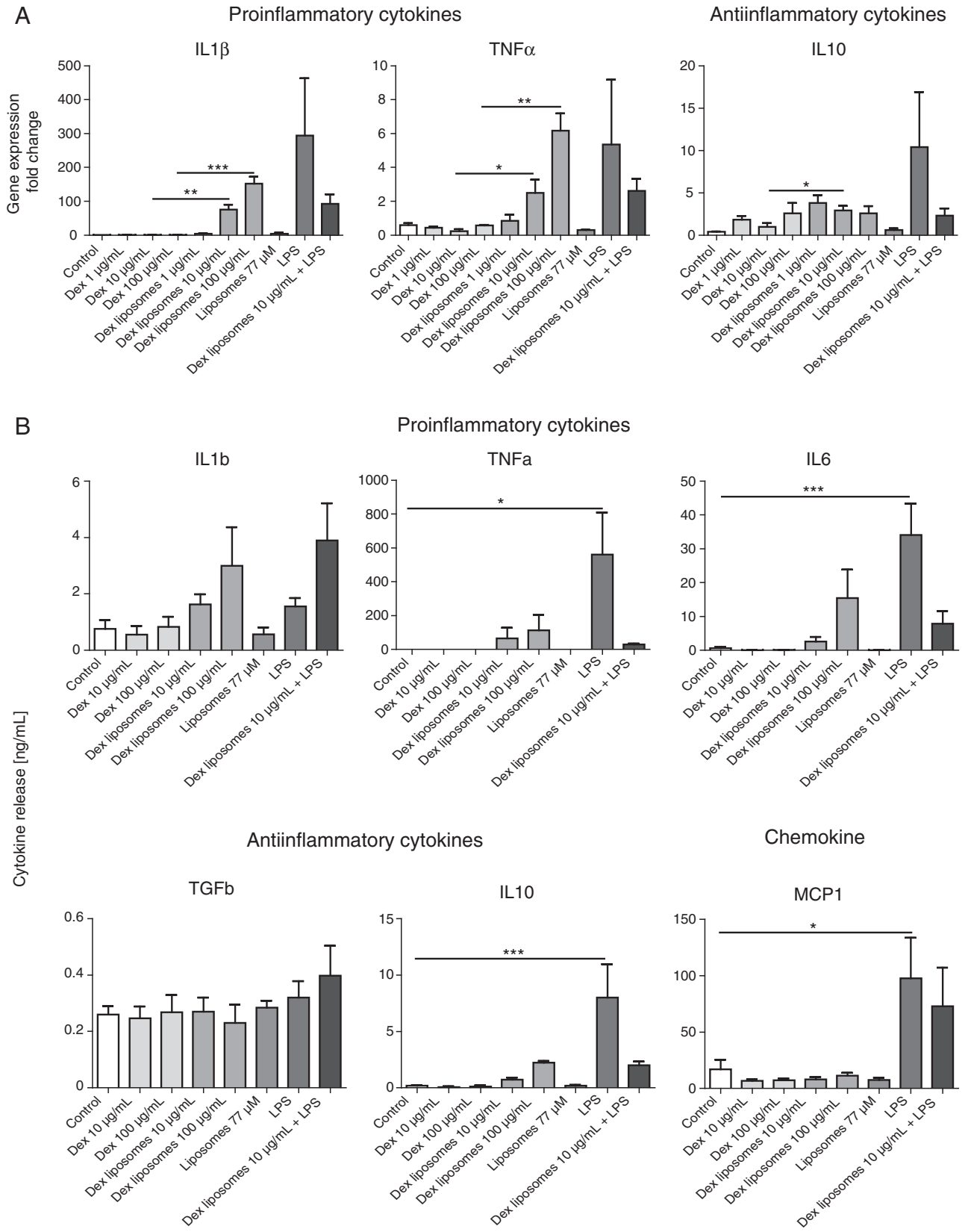

Figure 5. Alterations of macrophage functionality by dexamethasone loaded liposomes. Human primary monocyte-derived macrophages were generated by seven days of monocyte culture on bacterial-grade petri dishes in RPMI1640 with 5% autologous serum. Cells were incubated for 24 h with dexamethasone (Dex), PEGylated 100nm liposomes loaded with Dex, 100nm liposomes, lipopolysaccharides (LPS), or with LPS and Dex liposomes. Gene expression levels of inflammatory and antiinflammatory cytokines **(A)**. Cytokine secretion into the supernatant by macrophages **(B)**. Mean data of $n = 4$. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$ (one way ANOVA).

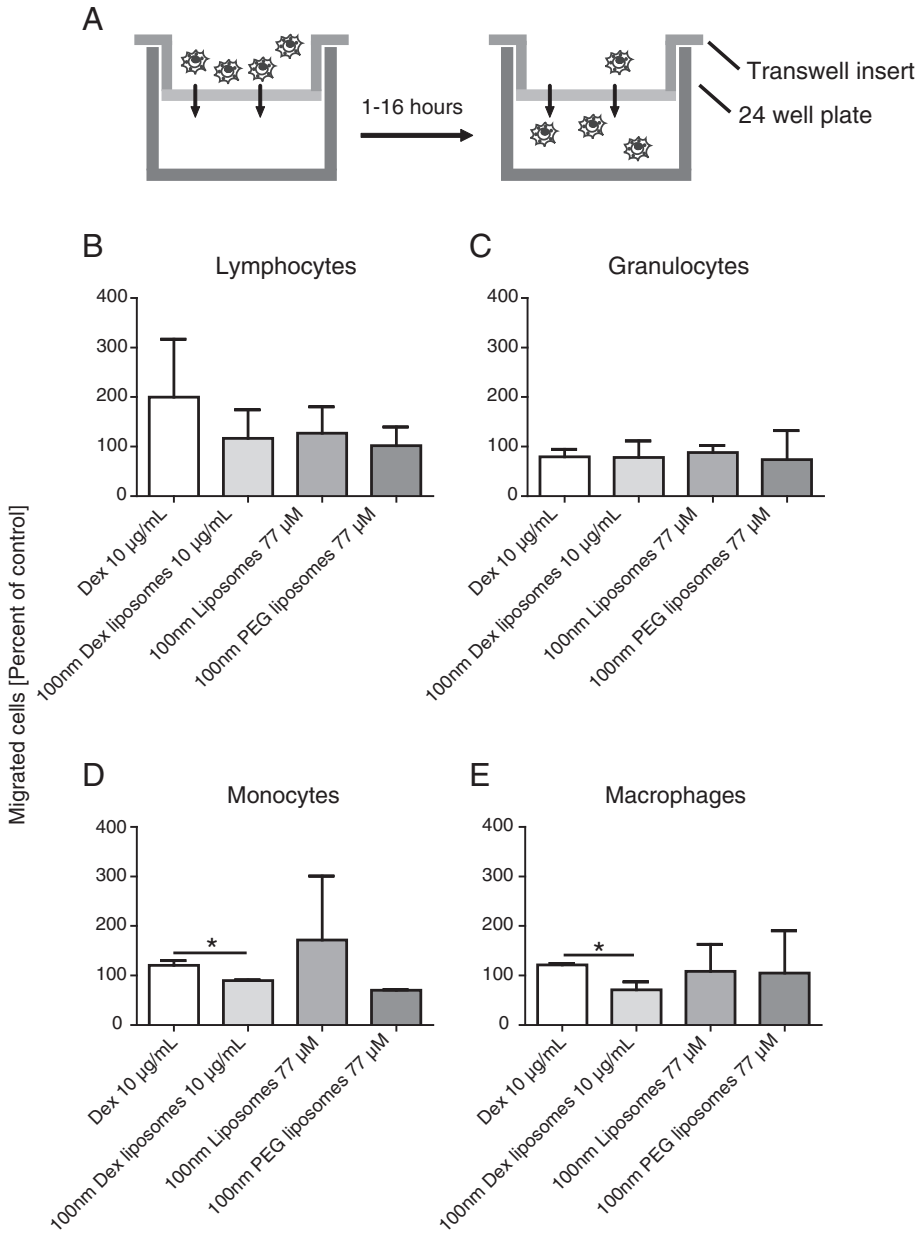

Figure 6. Influence of dexamethasone loaded liposomes on immune cell migration. Schematic depiction of the migration assay principle before **(A)** and after the experiment **(B)**. Human primary blood leukocytes represented by lymphocytes **(C)**, granulocytes **(D)**, and monocytes **(E)** were isolated from heparinized blood of healthy volunteers and purified using dextran sedimentation. Human primary macrophages were generated by seven days of monocyte culture in RPMI1640 with 5% of autologous serum **(F)**. Prior to the migration experiment, cells were incubated for one hour with the formulations in RPMI1640 medium with 5% fetal bovine serum (FCS). Blood cells were left to migrate for one hour, whereas macrophages were left to migrate for 16 h in RPMI1640 medium with 1% FCS. Mean data of $n = 4$. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$ (one way ANOVA).

gene expression was shown to change within only one day.[8] We therefore performed analyses of inflammatory gene expression and found that expression of the mRNA of proinflammatory cytokines interleukin 1 β (IL1β) and of the tumor necrosis factor (TNFα) was significantly enhanced by encapsulation of the GC compared to their solute form. Similarly, IL10 was significantly increased after 24 h of incubation with encapsulated Dex. Interestingly, costimulation with LPS and 100 nm PEGylated Dex liposomes, mimicking inflammatory conditions in which macrophages are activated, led to IL1β and TNFα mRNA levels comparable to those of Dex liposomes. Liposomes alone had no effect on inflammatory gene expression (Figure 5, *A*).

To verify whether the pronounced changes in gene expression might also lead to changes in protein release, we performed a multiplex analysis of six different cytokines into the cell culture medium. We found that also on the level of protein release, encapsulated Dex led to increased levels especially of IL1β, TNFα, and IL6, as well as of the antiinflammatory IL10. The

transforming growth factor β (TGFβ) was unaffected by the different GC formulations, and levels of the monocyte chemoattractant chemokine 1 (MCP1) were slightly decreased by all formulations except for LPS stimulation. Time courses of proinflammatory cytokine release over 48 h after the addition of Dex liposomes showed that the levels of IL1β were constantly increased compared to controls, TNFα was released within 4 h and declined to control levels after 48 h, whereas IL6 was released during the first 8 h after stimulation and remained at similar elevated levels (Figure S3). Interestingly, costimulation of encapsulated Dex and LPS resulted in an accumulated level of IL1β whereas the amounts of TNFα and IL6 were strongly reduced by encapsulated Dex (Figure 5, *B*).

Leukocytes are migratory cells that, upon attraction, translocate into tissues to encounter pathogens and body foreign material. Any influence on immune cell migration might therefore render their protective abilities. In order to study the potential effects of glucocorticoids and their encapsulation on macrophage activation, we incubated blood leukocytes and macrophages with Dex, PEGylated 100 nm Dex liposomes, 100 nm PEGylated liposomes, or 100 nm liposomes for one hour and then subjected the cells to migration in 24-well plates through a membrane with a pore size of 5 μm. Earlier studies have shown that the migratory activity of blood phagocytes (monocytes) is significantly higher than that of macrophages[19] and therefore, blood cells were incubated for one hour whereas macrophages were left to migrate for 16 h (Figure 6, *A*). In this assay, cells migrate through a membrane consisting of 5 μm pores. We found that glucocorticoid encapsulation did not affect the spontaneous migration of lymphocytes (Figure 6, *B*) or granulocytes (Figure 6, *C*), but the migration of monocytes (Figure 6, *D*) and macrophages (Figure 6, *E*) was significantly reduced by encapsulation of Dex.

We further performed a chemotaxis assay using recombinant CCL2 (MCP1) to simulate inflammatory conditions (Figure S4) in which high levels of CCL2 are found in injured tissue.[22] The cytokine was added into the bottom well, towards which the cells migrated. Under the influence of CCL2, monocyte migration increased by 49%. Treatment of cells with Dex resulted in a similar induction of 33% increased migration towards CCL2. Compared to the free Dex, the CCL2-dependent monocyte migration was moderately reduced in cells pretreated with Dex liposomes (+13%), PEGylated liposomes (+26%), and plain liposomes (+18%) (Table S1), further corroborating that liposomal uptake might inhibit migratory properties of monocytes and macrophages.

Discussion

We generated Dex-loaded liposomes that enable a combined approach of fluorescent detection and drug loading for cell culture and *in vivo* studies on the effects of the drugs. Interestingly, and in contrast to studies using prednisolone which after encapsulation was shown to enhance the antiinflammatory properties of the GC,[3] we found that encapsulation of Dex reduces its cytotoxicity for cells that are otherwise sensitive to free Dex, such as fibroblasts and macrophages. *In vivo*, this might translate into the situation that hepatocytes and endothelial cells are protected from Dex-induced cytotoxicity whereas hepatic Kupffer cells and hepatic stellate cells, activated fibroblasts that trigger liver fibrosis, decrease in their viability. This means that a systemic administration of Dex might be more efficient in targeting macrophages and fibroblasts, cell types that critically promote liver fibrosis,[22] compared to liposomal administration of Dex.

The differences in macrophage morphology and attachment that were observed in the macrophage cultures might be explained by the toxic effects of free compared to encapsulated Dex on macrophages observed in the MTT test. In contrast, in its encapsulated form, it likely accumulates inside the cells and reduces their attachment, which is in well agreement with the migration assays, in which encapsulated Dex inhibited monocyte and macrophage migration compared to solute Dex.

Similar to earlier studies facilitating gold and polymer-based nanoparticles,[7,8] also PEGylation of liposomes reduced their uptake by macrophages. However, the reduction in liposome uptake by macrophages after PEGylation is less pronounced compared to PEGylation of gold nanoparticles.[8] This might be related to the fact that the PEGylation of liposomes was done via the incorporation of PEG into the liposome shell with only a small portion of PEG being exposed to the exterior. In contrast, gold nanoparticles have a dense PEG brush on their surface that shields them from interactions with macrophages.[8]

Interestingly, Dex encapsulation reduced the effects of GC in terms of the expression of the M2 marker CD163, which was unexpected from earlier studies that have shown that encapsulated prednisolone, another GC, efficiently induces CD163 expression.[3] However, an activated cytokine signature of macrophages occurs on the level of inflammatory marker expression. This suggests that Dex-containing liposomes might exhibit antitumoral activity due to their M1-converting properties; it is known that M1 polarized macrophages exhibit antitumoral activity.[23] The *in vivo* depletion of macrophages using clodronate-containing liposomes is based on the specific intracellular accumulation of clodronate following the phagocytic uptake of liposomes and an induction of apoptosis due to intracellular clodronate.[24] A similar mechanism might lead to the intracellular accumulation of Dex in the macrophages that activates their danger sensing and thereby IL1β release[25] as the Dex concentration probably reaches toxic levels.[12] We hypothesize that, in contrast to its encapsulated form, free Dex might not accumulate in the macrophages as it passively diffuses through the cell membrane, but liposomes are internalized via phagocytosis[24] and their content accumulates inside the cell whereas the phospholipids fuse with the cell membrane.

Dex-containing liposomes not only influenced cellular viability and activation marker expression of macrophages, but they also inhibited the migratory competence of monocytes, the circulating precursor cells for tissue macrophages.[26] The migratory pattern of monocytes is known to be crucial in many different diseases,[27] and therefore, an inhibition of monocyte trafficking might influence the progression of inflammatory diseases. One might speculate that systemic administration of Dex-containing liposomes would target circulating blood

monocytes, which would be less responsive to migratory signals, such as chemokines released from injured tissue, and in turn might alter the course of inflammatory disorders.

The observation that macrophage costimulation with LPS and Dex liposomes reduced IL1β and TNFα mRNA levels, but in case of protein release clearly reduced TNFα and IL6 production, suggests that they inhibit proinflammatory activation dependent on TNFα or IL6, but not IL1β-driven inflammation. TNFα-overexpression has been associated with a variety of diseases including rheumatoid arthritis, Crohn's disease, HIV and cancer.[28] TNFα is known to be a key regulator of inflammatory cytokines[29] and TNFα-directed therapies represent an established clinical concept in the treatment of chronic inflammatory diseases, such as inflammatory bowel disease (IBD).[30] However, IBD patients are at risk for liver failure due to hepatotoxicity of TNF antagonists.[31] Therefore, Dex liposomes might represent a promising therapeutic alternative to current anti-TNF directed agents used in the treatment of inflammatory disorders. Their inhibition of TNF and IL6 might represent the molecular mechanism of action of corticoid containing liposomes as shown for prednisolone liposomes in arthritis.[17] Nevertheless, it needs to be considered that also antiinflammatory cytokines like IL10 can be affected by Dex liposomes, which could possibly limit its therapeutic applications in distinct inflammatory conditions.

In contrast to TNFα, IL1β is part of the inflammasome, a central cellular system of danger sensing[25] that can be activated by many different stimuli, and probably also by increased concentrations of Dex after liposome uptake. Due to their enhancing effects on IL1β production, Dex liposomes should not be coadministered during therapies that intend to inhibit IL1β such as type 2 diabetes therapies.[32] The beneficial effects of liposomes on vaccination[6] might be related to an accumulation of the encapsulated substances in phagocytes upon phagocytosis[24] and a subsequent activation of the cells due to profound intracellular accumulation.

However, it should also be considered that the macrophage activating properties of Dex liposomes might also partially be related to the fact that there is no metabolization of the liposomes *in vitro* that exist *in vivo*. Nevertheless, our data might assist in understanding the mechanism of action of Dex liposomes and other encapsulated corticosteroids.

In conclusion, our findings demonstrate that the mode of action of dexamethasone is cell type-specific and fundamentally changes upon encapsulation into liposomes, especially considering the effects of this model corticosteroid on macrophages. Any drug may, upon encapsulation, accumulate in macrophages, altering their state of activation or even deactivating them while being non-cytotoxic for other cell types. *In vivo* studies should incorporate these findings into comprehensive analyses of the mode of action of encapsulated compounds.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2014.02.011.

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