In Vitro Study of a Liposomal Curcumin Formulation (Lipocurc™): Toxicity and Biological Activity in Synovial Fibroblasts and Macrophages

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Abstract. *Background/Aim: The polyphenol curcumin is produced in the rhizome of Curcuma longa and exhibits potent anti-inflammatory, antioxidant, and chemopreventive activities. Due to the fact that curcumin is poorly soluble in water, many delivery systems have been developed to improve its solubility and bioavailability achieving optimum therapeutic application. In this study, we evaluated the biological effects of a liposomal curcumin formulation (Lipocurc™) on human synovial fibroblasts (SW982) and mouse macrophages (RAW264). Material and Methods: Cellular uptake of liposomes was studied using calcein-loaded liposomes. Effects of Lipocurc™ on cell viability and proliferation were determined with Celltox green cytotoxicity assay and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay, respectively. To induce cytokine/chemokine expression, the cells were stimulated with interleukin (IL)1β or lipopolysaccharide (LPS). The release of IL6, IL8, and tumor necrosis factor-alpha (TNFα) was quantified by enzyme-linked immunosorbent assay (ELISA). Results: Data showed that the liposomal curcumin formulation Lipocurc™ was significantly less toxic to synovial fibroblasts and macrophages compared to non-encapsulated, free curcumin. Furthermore, Lipocurc™ effectively reduced pro-inflammatory cytokine/chemokine expression in synovial fibroblasts as well as in macrophages without affecting cell viability, suggesting that this curcumin nanoformulation might be a promising tool for the treatment of inflammatory diseases.*

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Curcuminoids are yellow, lipid-soluble polyphenols, extracted from the rhizome of turmeric (*Curcuma longa*), consisting of three active components: curcumin, demethoxycurcumin, and bisdemethoxycurcumin (1). Curcuminoids have been shown to suppress inflammatory processes and induce apoptotic cell death of a wide variety of tumor cells (2-6). In 2009, Funk *et al.* (7) and Bharti *et al.* (8) reported that curcumin may be a promising agent for the treatment of chronic inflammatory diseases such as neurodegenerative and cardiovascular diseases, as well as different forms of arthritis. Recently, we showed that curcumin had potent anti-inflammatory and pro-apoptotic effects on synovial fibroblasts from patients with rheumatoid arthritis (9). However, sensitivity to light, extremely low water solubility and poor oral availability restrict the therapeutic application of curcumin. In order to overcome these problems, many nanocarrier systems have been developed, including liposomes, polymeric nanoparticles, biodegradable microspheres, cyclodextrin, and hydrogels (10-12). A cyclodextrin-based curcuminoid formulation (Curcumin Extract 45) has been synthesized by Wacker Chemie AG (Munich, Germany) and is manufactured by Dr. Wolz Zell GmbH (Geisenheim, Germany). Administration of this formulation to humans led to a 45-fold higher amount of total curcuminoids in blood plasma, expressed as the sum of free curcumin and its metabolites (curcumin sulfates and curcumin glucuronides), than that achieved by use of pure curcumin powder.

Liposomes are artificially prepared vesicles made of phospholipid bilayers (12). Previous studies demonstrated that curcumin encapsulation in liposomes significantly improved the stability and bioactivity of curcumin (13, 14). A novel liposomal curcumin formulation, named Lipocurc™ was developed by Polymun Scientific GmbH (Klosterneuburg, Austria) and has recently been tested in a clinical phase I study (15). In the present work, we evaluated the cytotoxic, anti-proliferative and

anti-inflammatory effects of Lipocurc™ on human synovial fibroblasts (SW982) and mouse macrophages (RAW264) and compared them with those of uncapsulated, free curcumin.

Materials and Methods

Chemicals and compounds. All chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) and Carl Roth (Karlsruhe, Germany) at analytical grade and used without further purification. Curcumin powder was from Sami Labs Limited (Bangalore, India), calcein-loaded liposomes, liposomal curcumin (*Lipocurc™*) and empty liposomes were obtained from Polymun Scientific GmbH (Klosterneuburg, Austria). Curcumin powder was dissolved in dimethylsulfoxide (DMSO; purity ≥99.5%) and diluted into cell culture medium. Stock solutions of calcein-loaded liposomes, Lipocurc™ and empty liposomes were diluted in phosphate-buffered saline (PBS; pH 7.4) before being added to cell culture medium.

Characteristics of liposomes. Calcein-loaded liposomes: Lipid content: 1-Palmitoyl,2-oleoyl-*sn*-glycero-3-phosphocholine (POPC; 90 mg/ml); 1,2-dimyristoyl-*sn*-glycero-3-phosphorylglycerol (DMPG; 10 mg/ml); total content of lipids: 7.5 mM; mean particle diameter: 121.8 nm; Zeta potential: –15.8 mV (pH 7.32). Curcumin-loaded liposomes (batch CUR0214-A): Lipid content: 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC; 72 mg/ml); DMPG (8 mg/ml); content curcumin: 6.0 mg/ml; mean particle diameter: 117 nm; Zeta potential: –36 mV (pH 5.0). Empty liposomes: Same lipid characteristics as curcumin liposomes but without curcumin.

Cell culture. Human synovial fibroblasts (SW982 cell line) and mouse macrophages (RAW264 cell line) were purchased from the European Collection of Authenticated Cell Cultures (Public Health England, Salisbury, UK) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA) at 37°C with 5% CO_2 in a humidified incubator.

Fluorescence microscopy. To study the cellular uptake of liposomes, synovial fibroblasts and macrophages were plated at a density of 5×10^4/well in 24-well plates and incubated with calcein-loaded liposomes (6.4 μM of total lipids) for 48 and 72 h. At each time point, plates were washed twice with PBS and imaged using fluorescence microscopy (40× magnification).

Cell viability assay. Synovial fibroblasts and macrophages were seeded in 96-well plates (1×10^4 cells/well) and incubated for 24 h with or without different concentrations (1-20 μM) of Lipocurc™ or free curcumin. Cytotoxicity was evaluated using the Celltox green cytotoxicity assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Fluorescence ($485 – 500_{Ex}/520 – 530_{Em}$) was measured in a fluorometer (GloMax Multi Detection System; Promega). All experiments were performed in triplicates, and data are presented relative to vehicle-treated control cells.

Cell proliferation assay. Synovial fibroblasts and macrophages were seeded in 96-well plates (1×10^4 cells/well) and treated for 48 h with or without different concentrations (1-20 μM) of Lipocurc™ or free curcumin. Cell growth was determined using the Cell Proliferation Kit II (Roche Diagnostics GmbH, Vienna, Austria), containing XTT

reagent. Absorbance at 490/655 nm was measured using a microplate reader (iMark™; Bio-Rad Laboratories, Hercules, CA, USA). All experiments were performed in triplicates, and the relative cell proliferation (%) was expressed as a percentage relative to that of vehicle-treated control cells.

Cytokine/chemokine expression assay. Synovial fibroblasts and macrophages (5×10^4 cells/well) were seeded in 24-well plates and pre-incubated for 24 h with or without different concentrations (1-20 μM) of Lipocurc™, empty liposomes or free curcumin before being stimulated for 24 h with IL1β (5 ng/ml) or LPS (100 ng/ml). The concentrations of IL6, IL8, and TNFα in cell culture supernatants were quantified by ELISA obtained from ebioscience (San Diego, CA, USA) and used according to the manufacturer's instructions. The absorbance of the resulting yellow-colored product was measured by a microplate reader (Bio-Rad) at 405/450 nm. All experiments were performed in duplicates, and expression levels are presented relative to those of IL1β- or LPS-stimulated cells.

Statistical analysis. The results are expressed as the mean±standard error of the mean (SEM). Statistical differences within groups were evaluated by one-factor analysis of variance (ANOVA) followed by *post-hoc* analysis using Bonferroni test as appropriate. A value of $p<0.05$ was considered statistically significant.

Results

Cellular uptake of liposomes. In order to study the cellular uptake of liposomes by synovial fibroblasts and macrophages, the cells were incubated for different time intervals (48 and 72 h) with calcein-loaded liposomes and imaged by fluorescence microscopy. The data show that liposomes were not absorbed by fibroblasts but only attached to the outer cell membrane (Figure 1A and B). In contrast to fibroblasts, liposomes were fully encapsulated by macrophages in a time-dependent manner, reaching a maximum of entrapment after 72 h (Figure 1C and D).

Effects of liposomal curcumin on cell viability. In order to investigate possible toxic effects of Lipocurc™ on synovial fibroblasts and macrophages, the cells were treated for 24 h with or without different concentrations (1-20 μM) of curcumin either encapsulated in liposomes (Lipocurc™) or as free drug. DNA release from damaged cells served as an indicator of cytotoxicity. The data show that free curcumin, even at low concentrations (5 μM), had toxic effects on both fibroblasts and macrophages (Figure 2A and B), whereas treatment with liposomal formulation (Lipocurc™) significantly reduced the toxicity of the free drug (Figure 2A and B). However, when the curcumin concentration in liposomes was increased to 20 μM, a significant loss of viability was observed in both cell types (Figure 2A and B).

Effects of liposomal curcumin on cell proliferation. In order to examine the effects of Lipocurc™ on proliferatiion of synovial fibroblasts and macrophages, the cells were cultured
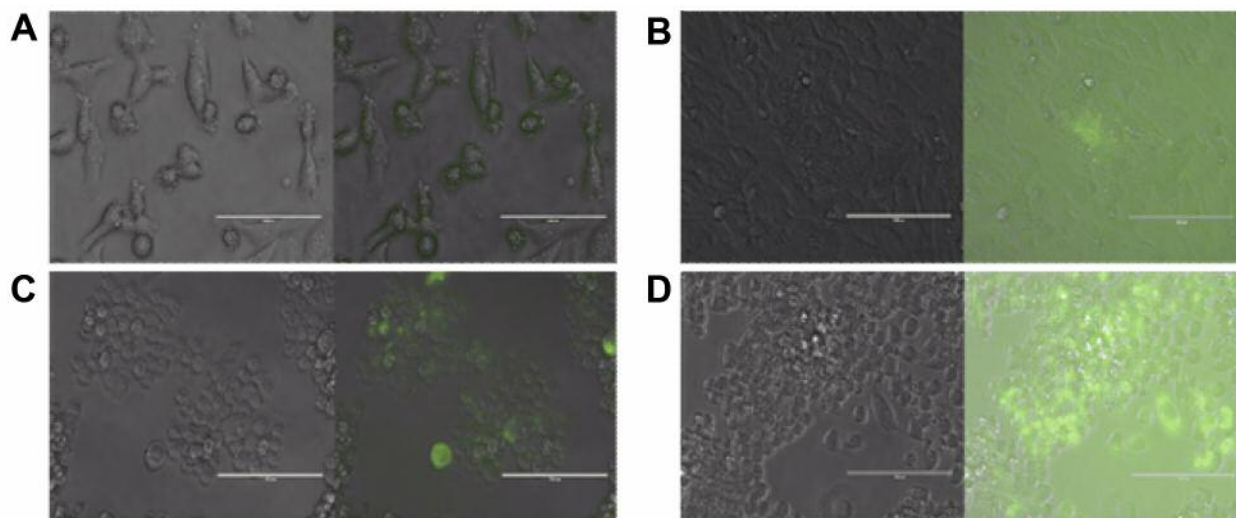
Figure 1. *Cellular uptake of liposomes. Synovial fibroblasts and macrophages were incubated for different time intervals (48 and 72 h) with calcein-loaded liposomes. At each time point, cellular uptake was assessed by fluorescence microscopy. Images of SW982 fibroblasts (A, B) and RAW264 macrophages (C, D) after 48 h (A, C) and 72 h (B, D).*

for 48 h with or without different concentrations (1-20 μM) of Lipocurc™ or free curcumin. As shown in Figure 3A, 10 μM curcumin encapsulated in liposomes had a minor effect on cell proliferation (~10% inhibition), whereas free curcumin reduced cell growth of fibroblasts by about 50%. Similar effects were observed for macrophages (Figure 3B).

Effects of liposomal curcumin on pro-inflammatory cytokine/ chemokine expression. Next, we investigated the anti-inflammatory potential of Lipocurc™ in synovial fibroblasts and macrophages. In these experiments, the cells were pre-incubated for 24 h with or without different concentrations (1-20 μM) of Lipocurc™, empty liposomes or free curcumin before being stimulated for 24 h with IL1β or LPS. In fibroblasts, incubation with 10 μM Lipocurc™ reduced IL6 and IL8 levels by about 30%, and 10%, respectively (Figure 4A and B), whereas the same concentration of free curcumin blocked IL6 and IL8 expression by about 50%, indicating that the cellular uptake of liposomes by fibroblasts was limited. At 20 μM Lipocurc™, the inhibitory effect on IL6 and IL8 synthesis was most effective but this was accompanied with increased cytotoxicity. Empty liposomes did not affect IL6 and IL8 expression and had no effect on cell viability (data not shown). In macrophages, the efficacy of Lipocurc™ in inhibiting cytokine expression was significantly greater compared to that synovial fibroblasts, for indicating that liposomes were indeed encapsulated by macrophages. Treatment with 5 μM Lipocurc™ resulted in reduction of IL6 and TNFα levels by about 80% and 20%, respectively (Figure 4C and D), whereas free curcumin blocked IL6 and TNFα expression by about 70% and 50%, respectively. Data demonstrated that treatment with

10 μM Lipocurc™ was most effective: at this concentraion, IL6 synthesis was almost completely blocked (Figure 4C) and TNFα level was reduced by about 80% (Figure 4D), demonstrating the great anti-inflammatory potential of Lipocurc™ in LPS-activated macrophages.

Empty liposomes diminish cytokine expression in macrophages. Interestingly, we observed that IL6 and TNFα expression in macrophages was also significantly blocked by empty liposomes (Figure 5A and B) without affecting cell viability (data not shown). At 10 μM empty liposomes, IL6 synthesis was reduced to a similar extent as under treatment with liposomal curcumin (Figure 4C), whereas inhibition of TNFα synthesis was less pronounced: 40% *versus* 80%. We conclude that phospholipids themselves may have anti-inflammatory effects or may interact with LPS or LPS-binding protein, thereby preventing the binding of LPS to the surface marker CD14 or toll-like receptors (TLR)-2 or -4.

Discussion

Curcumin has been characterized as an excellent molecule among many naturally occuring compounds for cancer therapeutics (16). Curcumin is involved in the modulation of transcription factors such as nuclear factor-kappa B (NFκB) and transcription factor activator protein-1, as well as in mitogen-activated protein kinase and protein kinase B signaling pathways (17). However, a major limiting factor of therapeutic application of curcumin is its extremely low solubility in water (namely 0.0004 mg/ml at pH 7.3). Furthermore, curcumin molecules are extremely unstable at
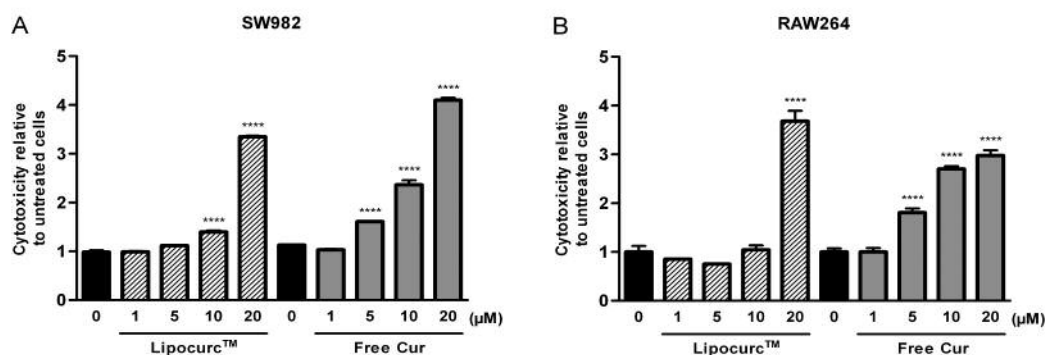
Figure 2. *Effects of Lipocurc™ and free curcumin (Cur) on cell viability. Synovial fibroblasts and macrophages were incubated for 24 h with or without the indicated concentrations of Lipocurc™ or free curcumin. Cytotoxicity was determined with CellTox green cytotoxicity assay. A: SW982 fibroblasts; B: RAW264 macrophages. Data are presented as the mean values±SEM of two independent experiments. ****p<0.0001 compared to vehicle-treated control.*
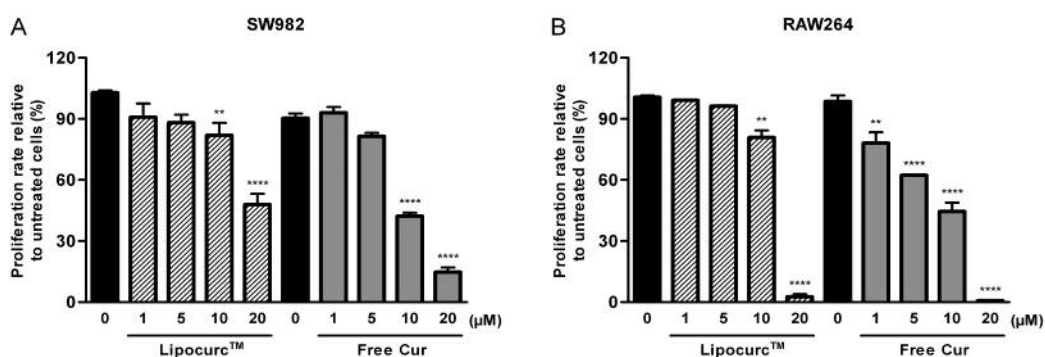

Figure 3. *Effects of Lipocurc™ and free curcumin (Cur) on cell proliferation. Synovial fibroblasts and macrophages were treated for 48 h with or without the ndicated concentrations of Lipocurc™ or free curcumin. After the addition of XTT reagent, the cells were incubated for another 24 h. A: SW982 fibroblasts; B: RAW264 macrophages. Data are expressed as the mean values±SEM of two independent experiments **p<0.01 and ****p<0.0001 compared to vehicle-treated control.*

physiological pH (18-20). Many pre-clinical and clinical studies in mice, rats and humans revealed a low bioavailability of curcumin (16, 21).

In recent years, numerous delivery systems for curcumin have been developed to improve drug bioavailability. Various types of nanoparticles (NPs), such as polymer NPs, polymeric micelles, liposome/phospholipid, nano-/microemulsions, nanogels, solid lipid NPs etc. are suitable systems for the delivery of curcumin (22). One of the most studied delivery systems for curcumin are liposomes. Liposomes are well established systems able to incorporate poorly soluble drugs and enable their aqueous-based administration (23). Liposomal curcumin was reported to have higher stability than free curcumin in PBS, human blood, plasma and cell culture medium (24). Based on these studies, it is evident that liposomes enhance the stability, bioavailability and cellular uptake of curcumin.

In the present study, we evaluated the toxicity and anti-inflammatory potential of a liposomal curcumin formulation (Lipocurc™) *in vitro*. Cellular uptake of liposomes by synovial fibroblasts and macrophages was studied by the encapsulation of calcein-loaded liposomes. Fluorescence images show that liposomes were not absorbed by synovial fibroblasts but only attached to the cell membrane. Whether the attachment of liposomes to the cell membrane is sufficient for the cellular uptake of curcumin is not clear and needs to be investigated in further experiments. In contrast to fibroblasts, liposomes were encapsulated by macrophages in a time-dependent manner. After 72 h, a maximum of entrapment was observed. Generally, the cytotoxicity of free curcumin was significantly lowered by treatment of cells with liposomal curcumin (Lipocurc™), suggesting that both cellular uptake of liposomes and intracellular release of curcumin take place relatively slow
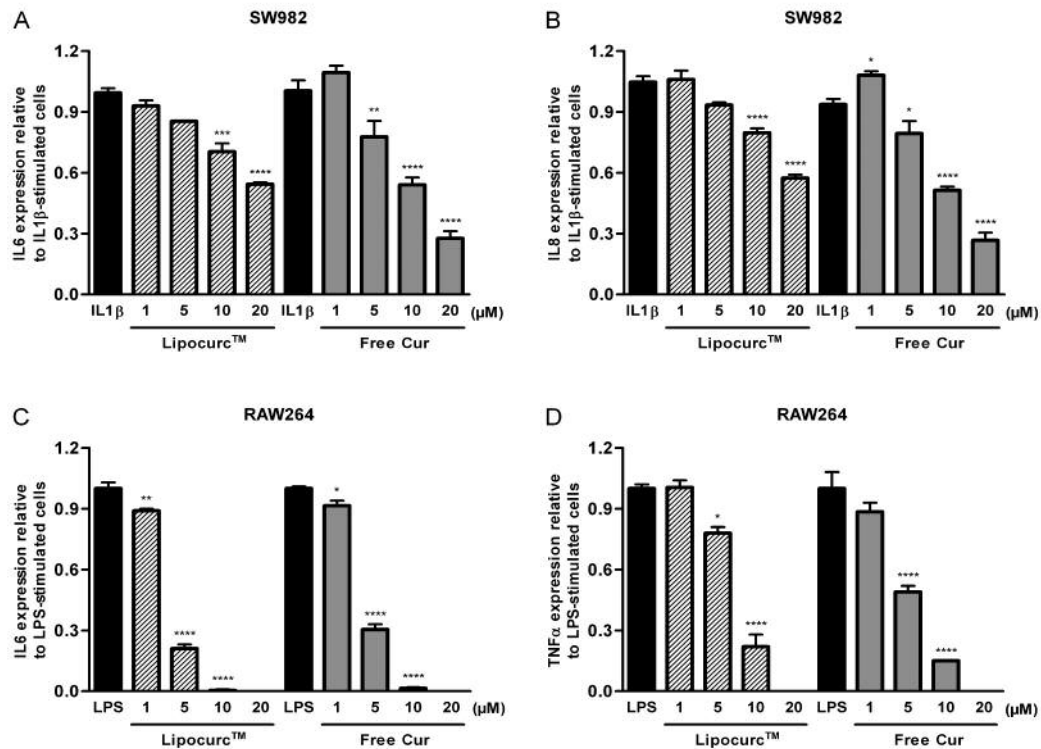
Figure 4. *Effects of Lipocurc™ and free curcumin (Cur) on cytokine/chemokine expression. Synovial fibroblasts and macrophages were pre-incubated for 24 h with indicated concentrations of Lipocurc™ or free curcumin before being stimulated for 24 h with interleukin (IL)1β or lipopolysaccharide (LPS). IL6, IL8 and tumor necrosis factor α (TNFα) release was quantified by ELISA. Relative expression of IL6 and IL8 in IL1β-stimulated SW982 fibroblasts (A,B) and relative IL-6 and TNFα expression in LPS-stimulated RAW264 macrophages (C,D). Data are presented as the mean±SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared to IL1β- or LPS-stimulated cells.*
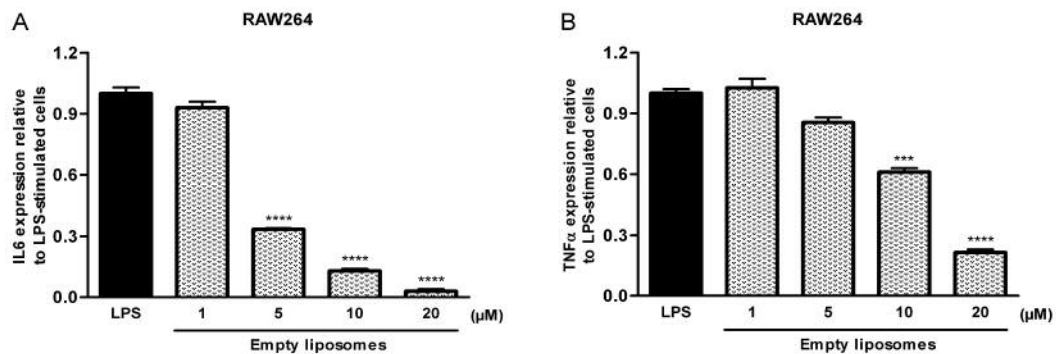

Figure 5. *Empty liposomes diminish cytokine synthesis in macrophages. The cells were pre-incubated for 24 h with indicated concentrations of empty liposomes before being stimulated for 24 h with lipopolysaccharide (LPS). Relative interleukin 6 (IL6) (A) and tumor necrosis factor α (TNFα) (B) expression in LPS-stimulated RAW264 macrophages. Data are presented as the mean±SEM of three independent experiments. ***p<0.001 and ****p<0.0001 compared to LPS-stimulated cells.*

without affecting cell viability. Notably, our data show that higher concentrations of curcumin-loaded liposomes (20 μM) had cytotoxic effects. We do not know whether curcumin from liposomes was released into cell culture medium *via* a diffusion process. If this was the case, this could explain the increased toxicity of Lipocurc™ at higher concentrations. In synovial fibroblasts, Lipocurc™ had limited anti-inflammatory potential, suggesting that

fibroblasts were not able to incorporate liposomes. In macrophages, however, the anti-inflammatory effects of Lipocurc™ were more pronounced. At 10 μM, both Lipocurc™ and free curcumin reduced IL6 and TNFα expression to a similar extent, indicating that liposomes were absorbed by macrophages. Notably, we observed that empty liposomes also diminished LPS-induced pro-inflammatory cytokine expression in macrophages but not in IL1β-stimulated synovial fibroblasts. IL6 expression was blocked by Lipocurc™ and empty liposomes to a similar extent. However, inhibition of TNFα synthesis was more effective in cells treated with Lipocurc™ (about two-fold), indicating that both phospholipids and curcumin may act in a synergistic manner.

It is well known that the membrane-bound receptors CD14 and TLR4 are involved in the activation of mononuclear cells by LPS and that activation may be enhanced by soluble LPS-binding protein. Mueller *et al.* reported that phospholipids (cardiolipin) strongly inhibited LPS-induced TNFα release when added to the cells before stimulation (25). Activation of cells by LPS was dependent on the presence of cell-associated LPS-binding protein, thus making LPS-binding protein a possible target for the antagonistic action of phospholipids (25). Yeh *et al.* showed that both empty and curcuminoid-loaded liposomes down-regulated IL1β-induced cyclo-oxygenase-2 and matrix metalloproteinase-3 expression in 7F2 osteoblasts (26). Our results are also in agreement with those of Treede *et al.*, who demonstrated that phosphatidylcholine inhibited TNFα-induced pro-inflammatory gene expression and NFκB activation in Caco-2 cells (27). Lipocurc™ formulation contains two types of phospholipids: DMPC and DMPG, suggesting that LPS-induced IL6 and TNFα synthesis in macrophages may be blocked by two components, phospholipids and curcumin.

Conclusion

To summarize, our data clearly demonstrate that liposomal curcumin (Lipocurc™) has lower cytotoxicity compared to non-encapsulated, free curcumin. Lipocurc™ was effectively absorbed by macrophages but only attached to synovial fibroblasts. Lipocurc™ reduced pro-inflammatory cytokine/ chemokine expression in both cell types, but its efficacy was significantly pronounced in macrophages. Furthermore, we observed that empty liposomes also inhibited LPS-induced cytokine expression. Thus, these data allow us to suggest that the liposomal curcumin formulation combines the anti-inflammatory potential of two compounds (phospholipids and curcumin), which may act in a synergistic manner by: i) inhibition of the interaction between LPS and LPS-binding protein outside of the cell by phospholipids; and ii) blocking the NF-κB signaling pathway inside the cell by curcumin.

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