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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/102717 since 2016-11-04T15:54:57Z

Published version:

DOI:10.1016/j.ejps.2010.01.013

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17 July 2024

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Solid lipid nanoparticles as anti-inflammatory drug delivery system in a human inflammatory bowel disease whole blood model

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Abstract

Standard treatment for inflammatory bowel diseases (IBD) necessitates frequent intake of anti-inflammatory and/or immunosuppressive drugs, leading to significant adverse events.
To evaluate the role solid lipid nanoparticles (SLN) play as drug delivery system in enhancing anti-inflammatory activity for drugs such as dexamethasone and butyrate in a human inflammatory bowel diseases whole blood model. ELISA assay and the peripheral blood mononuclear cell (PBMC) cytokine mRNA expression levels were evaluated by quantitative SYBR Green real-time RT-PCR to determine the IL-1β, TNF-α, IFN-γ and IL-10 secretion in inflammatory bowel diseases patients' PBMC culture supernatants. There was a significant decrease in IL-1β (p<0.01) and TNF-α (p<0.001) secretion, while IL-10 (p<0.05) secretion significantly increased after cholesteryl butyrate administration, compared to that of butyrate alone at the highest concentration tested (100 μM), at 24 h exposure. There was a significant decrease in IL-1β (p<0.01), TNF-α (p<0.001) and IL-10 (p<0.001) secretion after dexamethasone loaded SLN administration, compared to dexamethasone alone at the highest concentration tested (250 nM) at 24 h exposure. No IFN-γ was detected under any conditions and no cytotoxic effects observed even at the highest concentration tested.
The incorporation of butyrate and dexamethasone into SLN has a significant positive anti-inflammatory effect in the human inflammatory bowel disease whole-blood model.

Keywords

Solid lipid nanoparticles, Inflammatory bowel diseases, Butyrate, Dexamethasone, Cytokines

1. Introduction

Ulcerative colitis, limited to the colon and Crohn's disease, which, although may involve any part of the gastrointestinal tract from the mouth to the anus, most commonly affects the small intestine and/or the colon, are major forms of inflammatory bowel disease (IBD), characterized by chronic uncontrolled inflammation of intestinal mucosa. As a rule, there is a waxing and waning course in intensity and severity, affecting millions of people worldwide. The inflammatory responses are triggered by an infiltration of neutrophils and macrophages, where the activated macrophages produce a potent mixture of broadly active inflammatory cytokines, including the tumor necrosis factor (TNF)-α and interleukin (IL)-1β (Hanuer, 2006; Podolski, 2002).

Standard treatment for IBD necessitates the frequent intake of anti-inflammatory drugs, leading to significant adverse events. Therefore, a carrier system able to deliver the drug specifically and exclusively to the inflamed regions for prolonged periods of time after oral administration, would be a distinct improvement on the existing delivery systems and would result in significantly fewer side effects compared to conventional chemical anti-inflammatory compounds (Hanuer, 2006). Nowadays, it is known that, in the presence of IBD, there is a strong cellular immunoresponse from the inflamed regions. That is to say there is usually an increased amount of neutrophils, natural killer cells, mast cells and regulatory T cells. All of which have been demonstrated to play an important role in IBD pathophysiology (Podolski, 2002). As it has been reported that nanoparticles and microspheres can be efficiently taken up by immunocompetent cells, it is reasonable to presume that this cell uptake would allow for the accumulation of the particular carrier in a determinate area (Lamprecht et al., 2001; Probert et al., 1996). Nowadays, solid lipid nanoparticles (SLN) are under extensive worldwide study as promising alternative carriers for drugs and diagnostics.

Bioavailability can be increased by SLN, prepared from a warm microemulsion and carrying a drug (either hydrophilic or lipophilic), with modification of the pharmacokinetic parameters and tissue distribution of the incorporated drug. Indeed, SLN have been already tested *in vivo* on laboratory animals via duodenal, intravenous and ocular administration, with promising results (Gasco, 2007; Zara et al., 2002, Manjunath et al., 2006; Cavalli et al., 2002; Manjunath et al., 2005).

Short-chain fatty acids (SCFA: acetate, propionate and *n*-butyrate) derived from the enteric bacterial fermentation of non-digested dietary carbohydrates provide an essential energy substrate for the colonic epithelium: an SCFA deficiency may trigger the development of diversion colitis. Butyrate, a naturally occurring monocarboxylate produced in the lumen of the colon by microbial fermentation of complex carbohydrates that escapes digestion in the small intestine, is the principal metabolic fuel for colonic epithelial cells and exerts a variety of effects essential for intestinal health and function. It is of particular interest due to its anti-inflammatory properties and has been studied in various *in vitro* systems (Dyer et al., 2005; Scheppach W et al., 2004; Segain et al., 2000; Klampfer et al., 2003; Menzel et al., 2004; Zapolska-Downar et al., 2004). Butyrate enemas were found to be cytoprotective in experimental rat colitis (D'Argenio et al., 1996; Venkatraman et al., 2003). Moreover, a double-blind multicenter clinical trial demonstrated that combined treatment with topical butyrate and 5-aminosalicylic acid (5-ASA) is significantly more effective than is 5-ASA alone in the management of refractory distal colitis (Vernia et al., 2003). An *in vitro* study, which evaluated the whole-blood model of healthy volunteers, demonstrated that butyrate possessed a wide inhibitory effect on cytokine production (Lichtenstein et al., 2006). However, unfortunately, the clinical use of sodium butyrate is limited by its short half-life, due to its rapid metabolism and excretion (Pellizzaro et al., 1999). Our group used cholesteryl butyrate (chol-but) as an SLN lipid matrix (Salomone et al., 2001) and evaluated its function

as a pro-drug of butyric acid. Chol-but SLN displayed a higher antineoplastic effect than butyrate alone in several tumor cell lines and fluorescent SLN showed chol-but SLN was taken up by the cells within a few minutes (Pellizzaro et al., 1999; Salomone et al., 2001; Serpe et al., 2004).

Recently, we have shown that both sodium butyrate and chol-but SLN exert rapid anti-inflammatory effects on peripheral blood mononuclear cell (PBMC), by assessing their capacity to modulate adhesion to vascular endothelial cells, superoxide anion (O_2^-) production and myeloperoxidase release *in vitro*. The latter is much more potent than the former since it acts at concentrations of three logs lower and displays a broader inhibitory effect on PBMC activation than does superoxide anion. The stronger effect of chol-but SLN might be ascribed to their rapid uptake into the cytoplasmic compartment, which is maximal within a few minutes in both PBMC and human umbilical vein endothelial cells (HUVEC) (Dianzani et al., 2006).

Corticosteroids, which include parental, oral and topical agents, have been used for decades to treat active IBD. Their use in IBD patients is intended to reduce inflammatory mediators through the binding of the glucocorticoid receptor expressed by the immune cells. The binding of glucocorticoids to their receptors leads to the trans-repression of pro-inflammatory transcription factors, such as nuclear factor B (NF-kB) and apoptosis of target inflammatory cells and activated lymphocytes. Several controlled clinical studies have evaluated how systemic corticosteroids induce remission in IBD (Hanuer et al., 2006; Podolski et al., 2002; Lichtenstein et al., 2006).

Although glucocorticoids are effective in treating IBD flares, their considerable side effects have raised significant concerns among both physicians and patients and has, consequently, limited their use. Thus, a formulation that maintains intestinal therapeutic effects, whilst, at the same time, minimizes the systemic side effects would provide a major

therapeutic advance. Recently, we have prepared a new formulation of dexamethasone incorporated into SLN, aimed at improving the targeting and penetration of the drug into the inflammatory cells. This study reports data on the influence of butyrate alone, chol-but SLN, dexamethasone alone and dexamethasone loaded SLN, on cytokines with pro-inflammatory actions (TNF-α, IL-1β) and with implied functions in the Th_1/Th_2 balance [interferon (IFN)-γ, IL-5, IL-10, IL-12, IL-13]. We used a whole-blood model in which cytokine release in patients with IBD was measured after stimulation by lipopolysaccharide (LPS) alone and/or in the presence of the various drug formulations.

2. Materials and Methods

2.1. Solid Lipid Nanoparticles

2.1.1. *Preparation of dexamethasone solid lipid nanoparticles*

Dexamethasone loaded SLN (dexa SLN) were prepared from a warm microemulsion made up of stearic acid (Sigma-Aldrich, Milano, Italy) as lipidic matrix, sodium taurocholate (PCA, Basaluzzo, Italy) as cosurfactant, Epikuron 200® (containing about 95% of soy phosphatidylcholine; Lucas Meyer, Hamburg, Germany) as surfactant, dexamethasone (Sigma-Aldrich) and water. The warm microemulsion was dispersed in cold water (2-3°C) and the dexa SLN dispersion was washed by tangential filtration, using the Vivaflow 50 Sartorius system (100,000 RC membrane) (Sartorius, Goettingen, Germany) and then sterilized by autoclaving (30 min, 121°C).

2.1.2. *Preparation of cholesteryl butyrate solid lipid nanoparticles*

Epikuron 200® (15%) as surfactant and cholesteryl butyrate (Sigma-Aldrich) (12%) as lipidic matrix were melted at 85°C and a warm water solution (59%) of sodium taurocholate (3%) and butanol (Sigma-Aldrich) (11%) as cosurfactants were added to obtain a clear system. The microemulsion was immediately dispersed (1:10, v/v) in cold water (2-3°C) and the dispersion was washed by ultrafiltration and then sterilized by autoclaving (30 min, 121°C).

2.1.3. *Chemico-physical characterization of solid lipid nanoparticles*

After sterilization, the average diameter, polydispersity index and zeta potential were measured by photon correlation spectroscopy, using a Zetasizer 3000HS_A (Malvern

Instruments, Malvern, U.K.) at a fixed 90 degree angle. The measurements were performed in triplicate at 25°C.

2.1.4. *Determination of dexamethasone concentration in solid lipid nanoparticle dispersion*

The amount of dexamethasone in the sterilized dexa SLN dispersion was determined by high pressure liquid chromatography (HPLC). The flow-rate delivered by a PU-2080 plus pump (Jasco, Tokyo, Japan) was 1.0 ml/min and the eluate was monitored at 246 nm by a UV-2075 plus ultraviolet (UV) detector (Jasco). The analytical column was a Tracer Extrasil 5 μm ODS-1 (15 x 460 mm) (Teknokroma, Barcellona, Spain). The mobile phase consisted of 2 mM acetate buffer at pH 4.8 and acetonitrile (65:35, v/v) . The flow rate was 1 ml/min and the UV detector was set at 246 nm.

2.1.5. *Determination of cholesteryl butyrate concentration in solid lipid nanoparticle dispersion*

The cholesteryl butyrate concentration in the sterilized chol-but SLN dispersion was determined by HPLC- UV (Jasco), according to the Duncan et al. method (Duncan et al., 1979). Briefly, the analytical column was a reverse-phase column Water Spherisorb 5 μm ODS2 (4.6 x 250 mm), the mobile phase consisted of isopropanol and acetonitrile (40:60, v/v), the flow rate was 1.2 ml/min and the UV detector was set at 210 nm.

2.2. PBMC isolation and culture

PBMC were isolated from the heparinized peripheral blood of five IBD patients (age range: 30-78 years; median age: 52 years; 80% female and 20% male) with active disease (60% Crohn's disease and 40% ulcerative colitis) and no anti-inflammatory drug intake, by density-

gradient centrifugation over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) assay in two independent experiments. The study was approved by the Local Ethic Committee, Piemonte Region, Italy and written informed consent was obtained.

PBMC viability was assayed by trypan blue dye exclusion and 1 x 10^6/ml cells were cultured in 24-well culture plates in a RPMI 1640 medium (Sigma-Aldrich) containing 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100 units of streptomycin/ml, 200 units of penicillin/ml and 1 μg/ml of LPS (Sigma-Aldrich) for 24 h at 37°C, 5% CO_2. Where required, the PBMC were incubated with increasing concentrations of Na-but/chol-but SLN (1-100 μM), or dexa/dexa SLN (2.5-250 nM) at the start of the culture period. In order to exclude the possibility of the drugs affecting cell viability, a trypan blue dye exclusion assay was performed for each condition at a 24 h incubation.

2.3. RNA isolation and SYBR Green real time RT-PCR for cytokines

The expression level of the mRNA cytokines was measured at 4 and 24 h incubation by quantitative SYBR Green real-time RT-PCR. Total RNA was obtained from PBMC using RNeasy Plus Mini Kit (Qiagen, Milano, Italy) according to the manufacturer's instructions. The RNA concentrations were measured at spectophotometry at 260 and 280 nm with correction for background at 300 nm. The integrity of the RNA was confirmed by assessing the clarity of the ribosomal bands on ethidium-stained agarose. QuantiTect Primer Assay (Qiagen) was used as the gene-specific primer pair for *IL-1β* (Cat. No. QT00021385), *TNF-α* (Cat. No. QT00067277), *IFN-γ* (Cat. No. QT00000525) and *IL-10* (Cat. No. QT00041685). Real-time RT-PCR analysis was carried out using 1 μg of total RNA, which was reverse transcribed in a 20 μl complementary deoxyribonucleic acid (cDNA) reaction using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions,

100 ng of cDNA was used for each 25 μl real-time RT-PCR reaction. Quantitative RT-PCR was performed using the QuantiTect SYBR Green RT-PCR Kit (Qiagen).

To normalise mRNA data, the housekeeping *Act-β* (Cat. No. QT00095431) gene transcript and real-time PCR was performed by an iQ5 real-time PCR detection system (Bio-Rad). The PCR protocol conditions were as follows: HotStarTaq DNA polymerase activation step at 95°C for 15 min, followed by 40 cycles at various temperatures/times: i.e. 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. All samples were run in duplicate. At least two non-template controls were included in all PCR runs.

At the end of the PCR runs, baseline and threshold values (C_T) for *IL-1β*, *TNF-α*, *IFN-γ, IL-10* and *Act-β* were set using the iQ5 real-time PCR Software and the calculated C_T values were exported to Microsoft Excel for analysis. The relative expression of the mRNA cytokine was calculated using the comparative C_T method, according to the manufacturer's literature. The relative amount of the mRNA cytokine, standardised against the amount of *Act-β* mRNA, was expressed as $-\Delta C_T = [C_{Tcytokines} - C_{TAct\text{-}\beta}]$. The cytokine mRNA/*Act-β* mRNA ratio i.e. the relative cytokine expression, was then calculated as $2^{-\Delta C_T}$.

2.4. Immunoassays for cytokines

The IL-1β, TNF-α, IL-10 and IFN-γ protein concentrations in culture supernatants of PBMC were determined at 4 and 24 h incubation by specific sandwich enzyme immunoassay (Inalco, Milano, Italy) according to the manufacturer's instructions. The lower detection limits were: IL-1β, 7 pg/ml; TNF-α, 8 pg/ml; IFN-γ, 5 pg/ml; IL-10 5 pg/ml.

2.5. Statistical analyses

Data are expressed throughout as mean values ± SD of two separate experiments. Statistical comparisons between treatment groups were performed by the analysis of variance (two-way ANOVA) and the threshold of significance was calculated by Bonferroni's test. Statistical significance was set at $p<0.05$.

3. Results

3.1. Solid Lipid Nanoparticles

Table 1 shows the parameter values of SLN characterization and the amount of loaded drugs in the SLN dispersions.

3.2. Cholesteryl butyrate solid lipid nanoparticles and cytokine production

3.2.1. *Il-1β*

There were no statistically significant changes in the basal IL-1β mRNA expression in PBMC at 4 and 24 h (Figs. 1a and 1b), at any of the Na-but concentrations investigated, whereas a significantly increased IL-1β mRNA expression was observed at the highest chol-but SLN concentration (100 μM) only at 4 h (Figs. 1a and 1b).

A statistically significant dose-independent decrease in the production of the IL-1β in the PBMC culture supernatant was observed at a protein level, with Na-but at 4 h and only the highest concentration (100 μM) still showed a decreased secretion at 24 h (Figs. 1c and 1d). Conversely, there was a statistically significant chol-but SLN decrease in the production of the IL-1β cytokine in the PBMC culture supernatant, at 100 μM at 4 h and at all the concentrations tested at 24 h (Figs. 1c and 1d). Noteworthy was the fact that the IL-1β PBMC secretion decrease was significantly higher at all the concentrations tested compared to Na-but at 24 h (Fig. 1d).

3.2.2. *TNF-α*

Na-but determined a significant reduction in the basal TNF-α mRNA expression in PBMC at the highest concentration (100 μM) at 4 h, but lower concentrations (1 and 10 μM) led to an increased TNF- α mRNA expression at 24 h (Figs. 2a and 2b). On the contrary, chol-but SLN significantly increased TNF-α mRNA expression at 4 h, whilst there was a significant decrease at all the chol-but SLN concentrations at 24 h (Figs. 2a and 2b).

TNF-α secretion in PBMC supernatant was unaffected by Na-but at 4 h as it was at 24 h at all but the highest concentration, which determined a 50% TNF-α secretion reduction (Figs. 2c and 2d). Chol-but SLN only at the highest concentration exerted a significant decrease in TNF-α secretion, both at 4 and 24 h (Figs. 2c and 2d). When the two butyrate formulations were compared, it was observed that chol-but SLN determined a significantly higher reduction in TNF-α secretion than did Na-but (Figs. 2c and 2d).

3.2.3. *IFN–γ*

Neither IFN–γ mRNA, nor protein level of expression was detectable.

3.2.4. *Il-10*

Although Na-but significantly increased the IL-10 mRNA production at 4 h, at all the concentrations, this increase was maintained at 24 h at only 100 μM (Figs. 3a and 3b). Similarly, chol-but SLN led to a significant dose-independent IL-10 mRNA production increase at 4 h, but not at 24 h (Figs. 3a and 3b).

No IL-10 was detected in the PBMC supernatants at 4 h, either with Na-but, or chol-but SLN. At 24 h, Na-but had no significant effect on the IL-10 secretion level, while chol-but SLN significantly decreased the IL-10 production at 1 and 10 μM (Fig. 3c).

3.3. Dexamethasone solid lipid nanoparticles and cytokine production

3.3.1. *Il-1β*

Dexamethasone (dexa) exerted a significant increase in the basal IL-1β mRNA expression at 2.5 and 25 nM, at 24 h, whilst the SLN formulation had a similar effect only at the lower concentration with a five-fold IL-1β mRNA expression decrease at the highest concentration (Fig. 4b).

Dexa alone induced a 50% dose-independent reduction in IL-1β secretion compared to control cells at 4 h, while the decrease was dose-dependent at 24 h, reaching a 50% reduction of the cytokine production at the highest concentration (Figs. 4c and 4d). Moreover, dexa SLN determined a 40-50% dose-independent decrease in the IL-1β production at 4 h, while the decrease was dose-dependent at 24 h, reaching as high as 10% of the control cell production (Figs. 4c and 4d). Noteworthy, was the statistically higher decrease in IL-1β PBMC secretion with the drug SLN formulation at all concentrations than that observed with the drug alone at 24 h (Fig. 4d).

3.3.2. *TNF-α*

Dexa modified the TNF-α mRNA expression basal level with a slight dose-independent increase in the cytokine mRNA expression at 24 h (Figs. 5a and 5b). Conversely, dexa SLN determined a statistically significant increase in the mRNA cytokine expression at the highest concentration at 4 h, which dropped at 24 h to roughly two-fold that of the control cells (Figs. 5a and 5b).

Dexa affected the TNF-α secretion in PBMC supernatants (Fig. 5c), only at 24 h with a dose-dependent decrease in cytokine secretion, reaching 45% of the control cell production (Fig. 5d). Conversely, dexa SLN decreased the cytokine secretion earlier i.e. at 4 h, to less

than 10% of the control cell production at the higher concentrations (Fig. 5c). The same trend was detectable at 24 h with a 50% decrease compared to control cell production even at the lowest concentration (Fig. 5d). When the two drug formulations were compared, the reduction in the TNF-α production was significantly higher both at 4 and 24 h with the SLN formulation, which was more pronounced for the highest concentration (Figs. 5c and 5d).

3.3.3. *IFN–γ*

Neither IFN–γ mRNA, nor protein level of expression was detectable.

3.3.4. *Il-10*

Dexa determined a statistically significant increase in the IL-10 mRNA expression at the two highest concentrations, at 24 h (Figs. 6a and 6b). Conversely, dexa SLN determined a ten-fold increase in the IL-10 mRNA expression even at 4 h at the lowest concentration and led to a twenty-five fold increase at the highest concentration (Fig. 6a). The IL-10 mRNA expression increase was maintained only for the highest concentration at 24 h, with about a seven-fold increase over that of the control cells (Fig. 6b).

No IL-10 concentration was detectable in PBMC supernatants at 4 h, either with dexa alone, or dexa SLN. Both dexa formulations induced a dose-dependent IL-10 secretion decrease in the PBMC supernatants at 24 h, which was more pronounced with the SLN formulation (Fig. 6c).

4. Discussion

Although IBD remain a challenge for physicians, the last few decades have witnessed advances in this chronic disease treatment, also enhancing drug delivery to the gastrointestinal tract, leading to more effective and safer medications. Most of these delivery systems rely on temporal control, changes in pH along the gastrointestinal tract and changes in intraluminal pressure (Friend, 2005).

IBD are characterised by chronic inflammation of the gut mucosa where aberrant T lymphocyte responses underlie inflammation, as in Crohn's disease where there seems to be a deregulation of the T helper cell phenotype in favour of T helper-type 1 (Th_1) cells (Fiocchi, 1999). Indeed, one of the major regulatory mechanisms of the mucosal immune system is the control of cytokine production. Cytokine secretion is a self restricted phenomenon of short duration and cytokine synthesis begins with the "de novo" transcription of the respective genes. Mediators of natural immunity, produced by mononuclear phagocytes in response to infective agents (IFN-α, IFN-β, TNF, IL-1, IL-6) and regulators of immuno-mediated inflammation, trigger non-specific inflammatory cells raised through the antigenic specific recognition by T lymphocytes (IFN-γ, IL-10, IL-12). IL-10, secreted by monocytes, activated macrophages and lymphocytes, also acquire anti-inflammatory properties, including the inhibition of nuclear transcription and metallo-proteinase factors, the inhibition of macrophage and monocyte pro-inflammatory cytokine production and the promotion of phenotypic changes in the lymphocyte Th_2 phenotype (Hanuer, 2006; Podolski, 2002). Noteworthy, is the fact that the Th_1-type cytokine IFN-γ, was not produced under the cell culture conditions tested. After LPS stimulation *in vitro*, butyrate has a wide spectrum of activity on cytokine release and has been reported to decrease LPS-induced IL-1β and TNF-α secretion in healthy human PBMC (Segain et al., 2000).

It is believed that the effects of butyrate on decreased LPS-induced TNF-α secretion might be modulated by a gene transcriptional regulation of NF-κB, a key transcription factor in the regulation of immune and inflammatory response, in combination with cyclooxygenase and lipoxygenase activities, via histone acetylation (Segain et al., 2000; Usami et al., 2008). Segain et al. (2000), not only demonstrated that butyrate (2 mM) reduced LPS induced TNF-α production by PBMC in both control subjects and patients with Crohn's disease, but also that butyrate (2 and 10 mM) decreased TNF-α production and pro-inflammatory cytokine (IL-1β, IL-6) mRNA expression, in intestinal biopsies and lamina propria mononuclear cells from subjects with Crohn's disease. Noteworthy, chol-but SLN promoted a statistically significant reduction in the LPS stimulated release of IL-1β and TNF-α at concentrations significantly lower than butyrate alone (Figs. 1 and 2). There was a 50% decrease in LPS induced TNF-α secretion with 100 μM butyrate and a 90% decrease with 100 μM chol-but SLN, at 24 h. Whilst only with chol-but SLN was there a decrease in the level of the TNF-α mRNA expression, at 24 h (Fig. 2). The fact that chol-but SLN was responsible for a greater inhibition of the stimulated release of the pro-inflammatory cytokine is likely related to an increase in both the uptake and lifetime of butyrate effects with this formulation.

In contrast to the potent effect of butyrate on Th_1 cytokine production, there was little effect on the production of the anti-inflammatory cytokine IL-10. Moreover, it seems to be an association between the IL-10 genotypes and IBD in humans, implying that a genetic predisposition to produce less IL-10, favours the tendency to develop IBD (Tagore et al., 1999**).** *In vitro* studies have further demonstrated that IL-10 down-regulates the activation of T cells from IBD patients in the active phases of the disease (Saemann et al., 2000).

Thus overall, butyrate led to a marked shift away from a Th_1-dominated response, indicating that the efficacy of butyrate may stem from an alteration in the T cell phenotype i.e. towards an anti-inflammatory phenotype, as reported by Nancey et al. (2002).

We observed that chol-but SLN determined a two-fold dose-independent increase in the IL-10 mRNA expression, over and above that of butyrate alone, at 4 h. Whilst, IL-10 was detectable in PBMC supernatants only at 24 h (Fig. 3). In agreement with the results obtained by Nancey et al (2002), although there was a significant increase in IL-10 mRNA expression, as early as 4 h, butyrate alone did not increase the stimulated release of IL-10. It has been reported that this wide inhibiting effect of butyrate on pro-inflammatory cytokines, is not so much linked to cell toxicity, as it is to the ability of butyrate to inhibit NF-κB activation (Usami et al., 2008). We observed an early mRNA expression induced pro- and anti-inflammatory cytokine stimulation, that failed at 24 h, where, at a protein level, the cytokine secretion decreased and the chol-but SLN formulation was more powerful at the highest concentration tested on IL-1β ($p<0.01$) and TNF-α secretion ($p<0.001$), compared to butyrate alone (Figs. 1 and 3).

The glucocorticoid effect on cytokine modulation in IBD is achieved by a combination of genomic mechanisms. The translocated and activated glucocorticoid–receptor complex can both bind to, and inactivate, key pro-inflammatory transcription factors, such as NF-kB. It can bind to glucocorticoid responsive elements (GRE), thereby up-regulating the cytokine-inhibitory protein expression, like IkB, which inactivates the NF-kB transcription factor. Moreover, it reduces the half-life and utility of cytokine mRNA (Cavaglieri et al., 2003). Glucocorticoids suppress the production of various inflammation mediators, like cytokines (TNF-α, IL-1, GM-CSF, IL-6, IL-8), but do not decrease IL-10 production. They also decrease the stability and life time of IL-1, IL-2, IL-6, TNF-α, GM-CSF and IL-8 mRNA (Lucas et al., 2007; Rogler et al., 1999).

An *in vivo* study conducted by Nakase et al. (2001), oral administration of dexamethasone microspheres appeared to ameliorate mucosal injury in a rat model of colitis, more than a conventional formulation. To the best of our knowledge this is the first report as to how SLN vehiculated drugs affect human pro- and anti-inflammatory cytokine production.

Our data showed that dexamethasone loaded SLN determined a more than 90% decrease in the IL-1β and TNF-α mRNA expression compared to control cells, at the highest concentration, at 24 h (Figs. 4 and 5). Whilst, the SLN formulation at the highest concentration stimulated TNF-α mRNA expression at 4 and 24 h, but inhibited protein secretion (Fig. 6). When the two dexamethasone formulations were compared, the IL-1β and TNF-α production was significantly reduced, both at 4 and 24 h, with the SLN formulation, which was more evident for the highest concentration (Figs. 4 and 5). Moreover, free dexamethasone led to a dose-dependent inhibition of TNF-α production, reaching its maximum at 250 nM with a 25% reduction with the free formulation and a 90% reduction with the SLN formulation, compared to the control cells (Fig. 5).

In conclusion, the incorporation of butyrate and dexamethasone into SLN is able to enhance the anti-inflammatory efficacy of the drugs on the PBMC of IBD patients stimulated by LPS and that this effect may well be related to the favoured uptake of the SLN formulation by the PBMC.

Acknowledgments

This work was supported by the Fondazione IBD Onlus, with a grant by the "Fondazione Compagnia di San Paolo", Italy (grant number 2002.2003).

We also wish to thank Mrs. Barbara Wade for her linguistic advice.

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Figure Legends

Fig. 1 - IL-1β mRNA expression in PBMC analyzed by real time RT-PCR at 4 h (panel a) and 24 h (panel b). IL-1β secretion in PBMC culture supernatants analyzed by ELISA at 4 h (panel c) and 24 h (panel d). Cells were treated with 1, 10 and 100 μM sodium butyrate (Na-but) and cholesteryl-butyrate (chol-but SLN) for 4 and 24 h. * $p< 0.05$, ** $p<0.01$ and $p<0.001$ (Na-but *vs* chol-but SLN).

Fig. 2 - TNF-α mRNA expression in PBMC analyzed by real time RT PCR at 4 h (panel a) and 24 h (panel b). TNF-α secretion in PBMC culture supernatants analyzed by ELISA ELISA at 4 h (panel c) and 24 h (panel d). Cells were treated with 1, 10 and 100 μM sodium butyrate (Na-but) and cholesteryl-butyrate (chol-but SLN). * $p< 0.05$, ** $p<0.01$ and $p<0.001$ (Na-but *vs* chol-but SLN).

Fig. 3 - IL-10 mRNA expression in PBMC analyzed by real time RT PCR at 4 h (panel a) and 24 h (panel b). IL-10 secretion in PBMC culture supernatants analyzed by ELISA at 24 h (panel c). Cells were treated with 1, 10 and 100 μM sodium butyrate (Na-but) and cholesteryl-butyrate (chol-but SLN). * $p< 0.05$, ** $p<0.01$ and $p<0.001$ (Na-but *vs* chol-but SLN).

Fig. 4 - IL-1β mRNA expression in PBMC analyzed by real time RT PCR at 4 h (panel a) and 24 h (panel b). IL-1β secretion in PBMC culture supernatants analyzed by ELISA at 4 h (panel c) and 24 h (panel d). Cells were treated with 2.5, 25 and 250 nM dexamethasone (Dexa) and dexamethasone loaded SLN (Dexa SLN). * $p< 0.05$, ** $p<0.01$ and *** $p<0.001$ (Dexa *vs* Dexa SLN).

Fig. 5 - TNF-α mRNA expression in PBMC analyzed by real time RT PCR at 4 h (panel a) and 24 h (panel b). TNF-α secretion in PBMC culture supernatants analyzed by ELISA at 24 h (panel c). Cells were treated with 2.5, 25 and 250 nM dexamethasone (Dexa) and dexamethasone loaded SLN and (Dexa SLN). * $p< 0.05$, ** $p<0.01$ and *** $p<0.001$ (Dexa *vs* Dexa SLN).

Fig. 6 - IL-10 mRNA expression in PBMC analyzed by real time RT PCR at 4 h (panel a) and 24 h (panel b). IL-10 secretion in PBMC culture supernatants analyzed by ELISA at 4 h (panel c) and 24 h (panel d). Cells were treated with 2.5, 25 and 250 nM dexamethasone (Dexa) and dexamethasone loaded SLN (Dexa SLN). * $p< 0.05$, ** $p<0.01$ and *** $p<0.001$ (Dexa *vs* Dexa SLN).

Figure 1

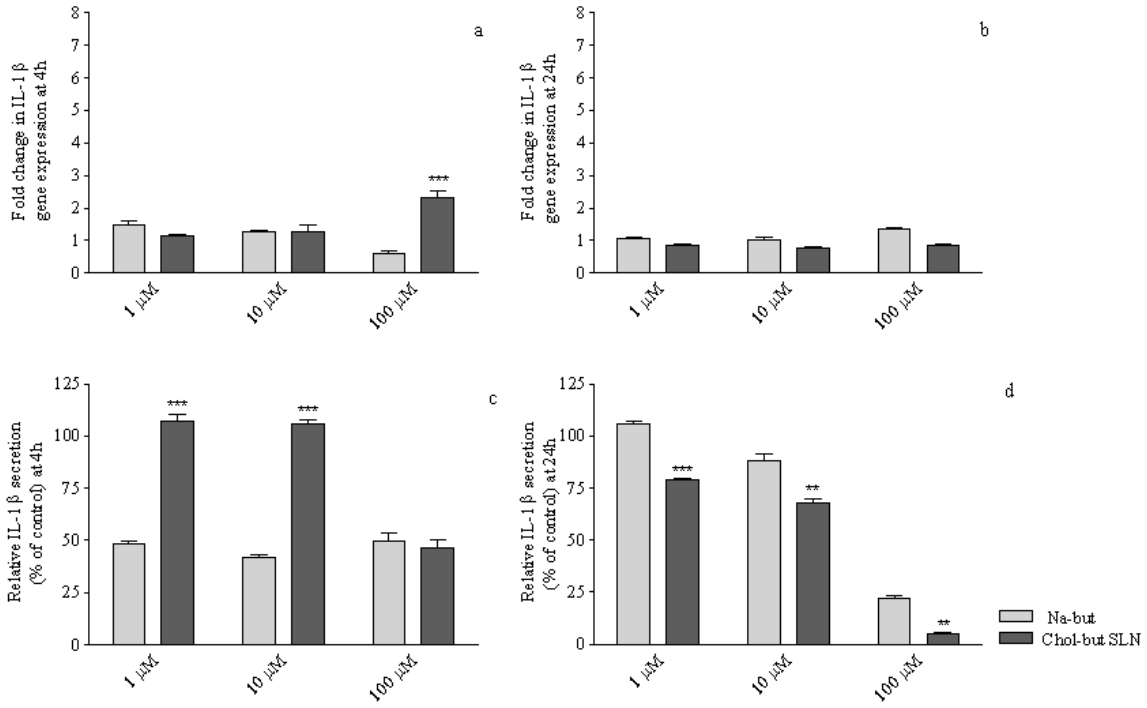

a
b
c
d
Fold change in IL-1 β gene expression at 4h
Fold change in IL-1 β gene expression at 24h
Relative IL-1 β secretion (% of control) at 4h
Relative IL-1 β secretion (% of control) at 24h
1 µM
10 µM
100 µM
***
**
Na-but
Chol-but SLN

Figure 2

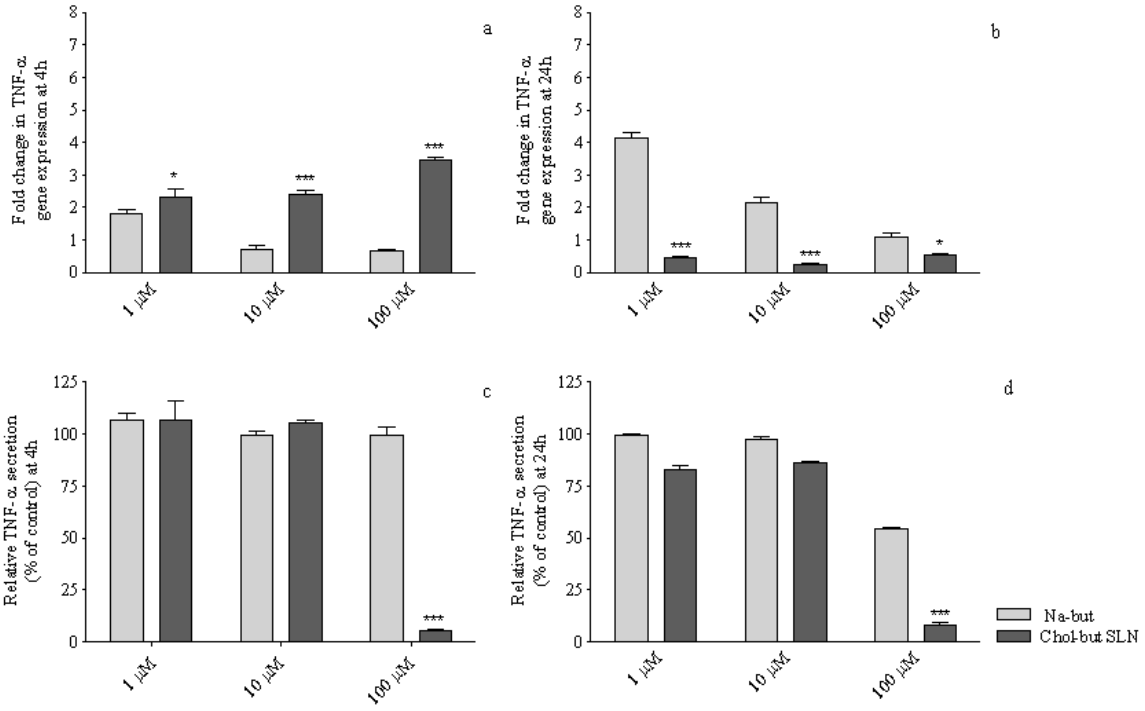

a
b
c
d
Fold change in TNF-α gene expression at 4h
Fold change in TNF-α gene expression at 24h
Relative TNF-α secretion (% of control) at 4h
Relative TNF-α secretion (% of control) at 24h
1 µM
10 µM
100 µM
Na-but
Chol-but SLN

Figure 3

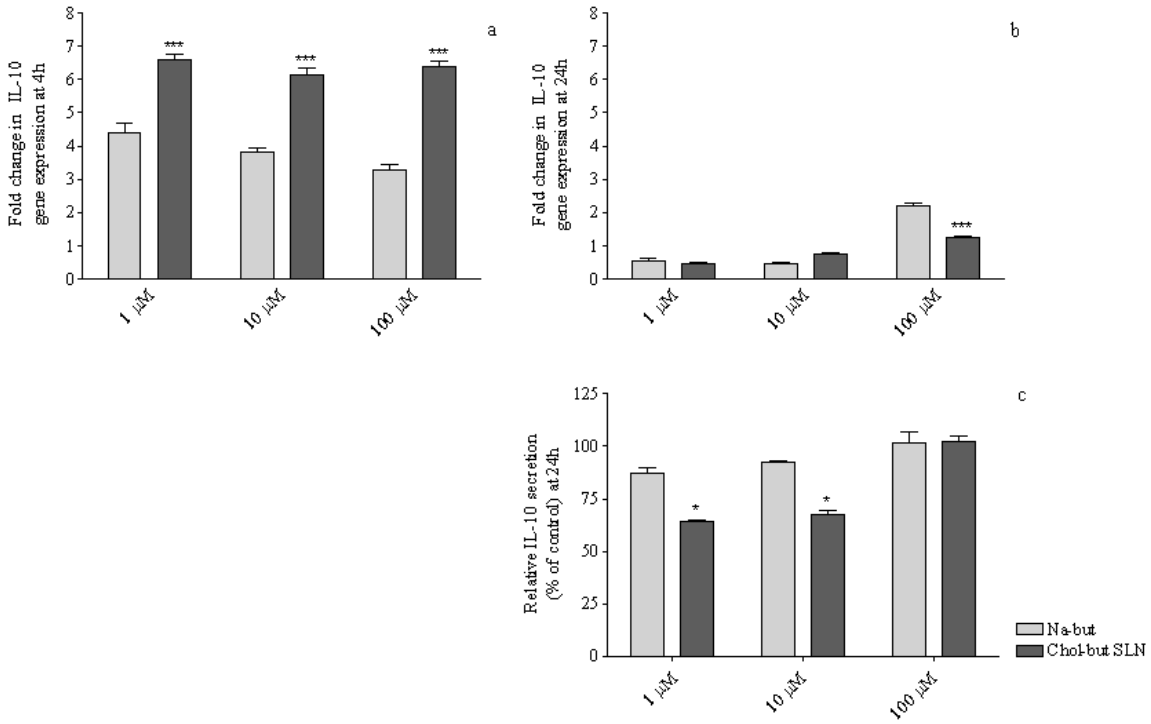

a
Fold change in IL-10 gene expression at 4h
***
***
***
1 µM
10 µM
100 µM
b
Fold change in IL-10 gene expression at 24h
***
1 µM
10 µM
100 µM
c
Relative IL-10 secretion (% of control) at 24h
*
*
1 µM
10 µM
100 µM
Na-but
Chol-but SLN

Figure 4

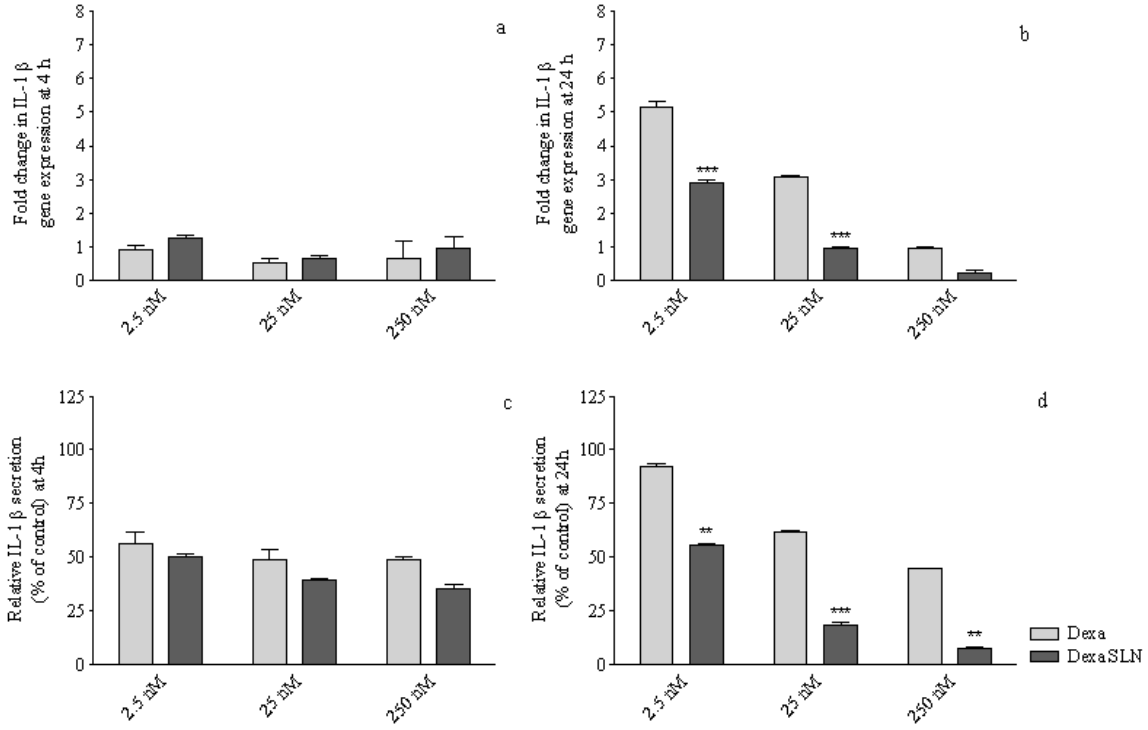

a
b
c
d
Fold change in IL-1 β gene expression at 4 h
Fold change in IL-1 β gene expression at 24 h
Relative IL-1 β secretion (% of control) at 4h
Relative IL-1 β secretion (% of control) at 24h
2.5 nM
25 nM
250 nM
***
**
Dexa
DexaSLN

Figure 5

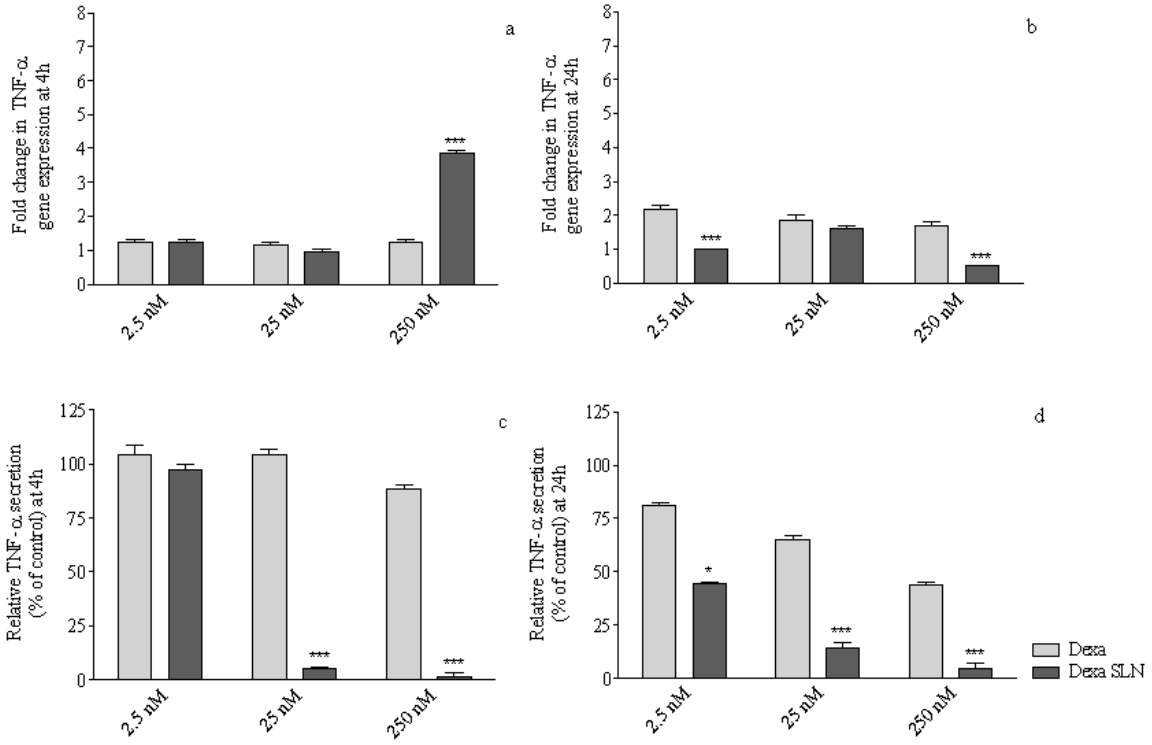
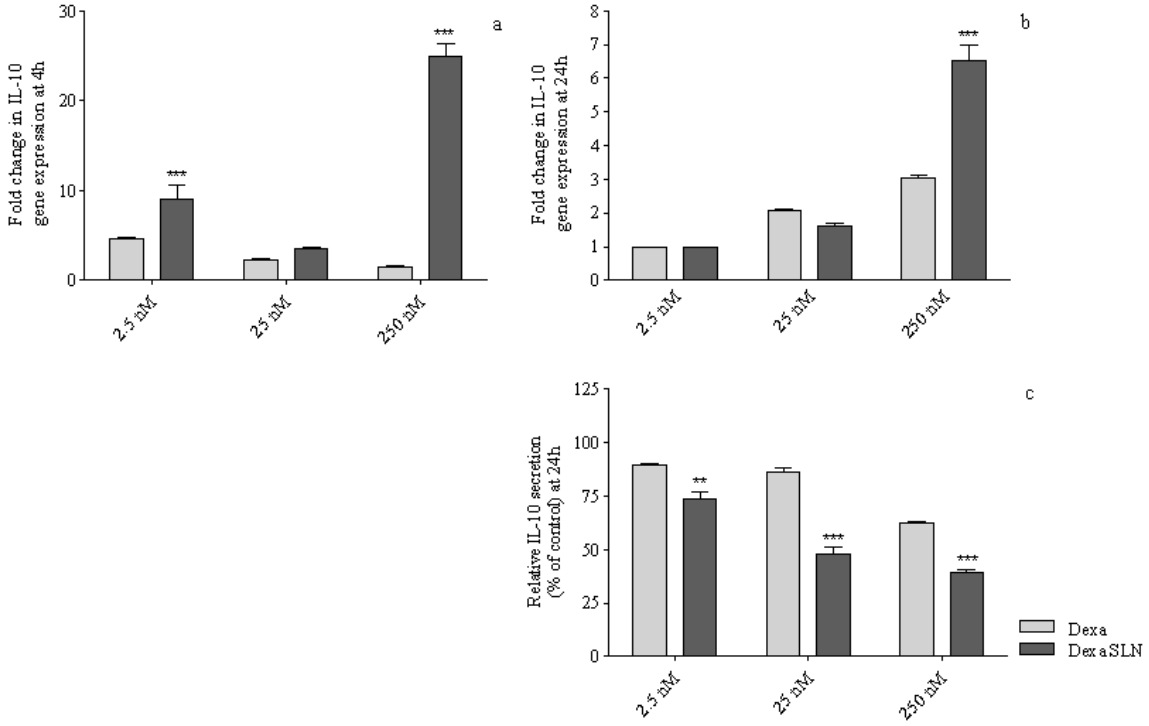

a
Fold change in TNF-α gene expression at 4h
8
7
6
5
4
3
2
1
0
***
2.5 nM
25 nM
250 nM
b
Fold change in TNF-α gene expression at 24h
***
***
c
Relative TNF-α secretion (% of control) at 4h
125
100
75
50
25
0
***
***
d
Relative TNF-α secretion (% of control) at 24h
*
***
***
Dexa
Dexa SLN

Figure 6


a
Fold change in IL-10 gene expression at 4h
30
20
10
0
***
***
2.5 nM
25 nM
250 nM
b
Fold change in IL-10 gene expression at 24h
8
7
6
5
4
3
2
1
0
***
2.5 nM
25 nM
250 nM
c
Relative IL-10 secretion (% of control) at 24h
125
100
75
50
25
0
**
***
***
2.5 nM
25 nM
250 nM
Dexa
DexaSLN