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Anti-inflammatory action of lipid nanocarrier-delivered Myriocin: therapeutic potential in Cystic Fibrosis

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Abstract

Background—Sphingolipids take part in immune response and can initiate and/or sustain inflammation. Various inflammatory diseases have been associated with increased ceramide content, and pharmacological reduction of ceramide diminishes inflammation damage *in vivo*. Inflammation and susceptibility to microbial infection are two elements in a vicious circle. Recently, sphingolipid metabolism inhibitors were used to reduce infection. Cystic Fibrosis (CF) is characterized by a hyper-inflammation and an excessive innate immune response, which fails to evolve into adaptive immunity and to eradicate acute infection. This results in chronic infections, lung damage, and patient morbidity. Indeed, ceramide content in mucosa airways is higher in CF mouse models and in patients than in control mice or healthy subjects.

Methods—The potential of the *de novo* ceramide synthesis inhibitor myriocin in CF therapy was investigated in cells and mice models.

Results—We treated CF human respiratory epithelial cells with myriocin, an inhibitor of the rate limiting enzyme involved in *de novo* ceramide synthesis Serine Palmitoyl Transferase (SPT). This treatment resulted in reduced basal, as well as TNF α -stimulated, inflammation. In turn, TNF α induced an increase in SPT in these cells, linking *de novo* synthesis of ceramide and inflammation in a noxious loop. Furthermore, myriocin-loaded nanocarrier, injected intratrachea prior to *P.aeruginosa* challenge, allowed a significant reduction of lung infection and reduced inflammation.

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Conclusion—The presented data suggest that *de novo* sphingolipid synthesis is constitutively enhanced in CF mucosa and that it can be envisaged as pharmacological target for modulating inflammation and restoring effective innate immunity against acute infection.

General significance—Myriocin stands as a powerful immunomodulatory agent for inflammatory and infectious diseases.

Introduction

Sphingolipids (SPL) are a broad class of membrane components and signaling mediators involved in cell survival and function. It is becoming increasingly apparent that SPL take part to inflammation and to host innate response upon infection (1-3). The sphingolipid ceramide is highly effective in the activation of inflammation related transcription factors, such as NF- κ B (4) and AP1(5) and in receptors clustering /signaling upon inflammatory stimuli (6). Patients suffering from chronic inflammation such as irritable bowel syndrome (7), emphysema lung injury and chronic obstructive pulmonary disease (8-10), exhibit increased levels of mucosal ceramide as compared to controls. A vicious loop relates the excessive inflammation to the susceptibility to microbial infection. Hyper-inflammation associates with the inability to clear infections at their early stage, as well as to mature the adaptive immune response, thus allowing the chronic establishment of pathogens communities. Ceramide accumulation may derive from increased synthesis *de novo* or from altered metabolism of complex sphingolipids such as sphingomyelin or glycosphingolipids. The role of sphingomyelinases, neutral and acidic, in inflammation has been extensively investigated (11; 12). The hydrolysis of plasma-membrane sphingomyelin is responsible for ceramide-rich membrane platforms formation and required for signal transduction of inflammatory stimuli, such as TNF α , IL β , INF γ (6; 8; 13; 14), increased vascular permeability (15; 16) as well as for the internalization of microorganisms (13; 17). On the other side, only a few reports underscore the involvement of *de novo* synthesis of ceramide in the inflammatory responses (10; 18-20). Thus, emphysema lung damage (10) and immune-reactivity in murine dorsal horn of the lumbar spinal cord were reduced by Fumonisin B1, an inhibitor of ceramide synthase (18). Furthermore, mice feeding with Myriocin (Myr), an inhibitor of Serine Palmitoyl Transferase (SPT), decreased radiation-induced inflammation and fibrosis (21). A recent finding demonstrated that reduced *de novo* ceramide synthesis by fenretinide, associated with the increase of its precursor dihydroceramide, impairs bacterial infection in macrophages (19).

Cystic Fibrosis (CF) is an inherited autosomal recessive disease caused by CF transmembrane conductance regulator (CFTR) mutations. CF patients develop mucus viscosity, impaired mucociliary transport, hyper- inflammation and severe alteration of all mucosal functions with lung disease (22; 23) and chronic opportunistic infections (mainly *Pseudomonas aeruginosa*, *Burkholderia cepacia complex*, and *Staphylococcus aureus*) remaining the main cause of morbidity and mortality (24; 25). Inflammation is an independent risk factor for CF disease progression. Even the uninfected CF lungs of foetuses or two years old infants, develop a pathological inflammatory condition (26-29), confirming the severe immune alteration of mucosa in the respiratory tract.

In mouse models of CF, an age-related accumulation of ceramide in respiratory epithelium was associated to the pathological inflammatory state and infection susceptibility. Sphingomyelinase (9; 30; 31), or ceramide synthase (9) inhibition, or fenretinide, an inhibitor of ceramide formation from its precursor dihydro-ceramide (32), reduced inflammation and infection in CF mice.

Similarly, in CF patients, increased ceramide content was found in nasal respiratory epithelium and lungs (30; 33; 34). Amitriptyline, an inhibitor of acid sphingomyelinase used in a phase II study, reduced ceramide levels in the respiratory epithelial cells of treated patients and this was accompanied by a significant increase in lung function (30; 31).

In spite of the increased ceramide mass in CF mucosa, there is no evidence on what is the rate of ceramide synthesis *versus* its release from membrane sphingomyelin. We hypothesized that preventing *de novo* sphingolipid synthesis with Myr could reduce excessive lung inflammation in CF and allow an efficient innate response to acute infection. In this article we demonstrated that Myr reduces IL-8 and IL6 release in human CF respiratory epithelium. Given the hydrophobicity of the compound, we sought to deliver Myr *in vivo*, in murine airways, by means of Solid Lipid Nanoparticles (SLN) (35). We demonstrated that Myr- loaded SLN are able to reduce inflammation and infection in CF mice lung.

Materials and Methods

Reagents and Antibodies

Myr was purchased from Fermentek LTD (Israel), MTT and bovine serum albumin (BSA) were from Sigma-Aldrich (US). LHC Basal, LHC-8 w/o gentamicin culture media (Gibco, US), Penicillin and streptomycin (Invitrogen) were purchased from Life Technologies Italia (Italy). Fetal bovine serum (FBS) and the chemiluminescence system LiteAbLot were purchased from EuroClone Life Science Division (Italy). Human Fibronectin and Bovine Collagen were from Becton-Dickinson Italia (Italy). Human and mouse IL-8, IL-6 mini EDK and Human TNF- α were from Peprotech (UK). The synthetic oligonucleotides used in this study were purchased from M-Medical (Italy). All reagents were of the maximal available purity degree.

Myriocin stock solution preparation

Myr powder was weighted and dissolved in DMSO by warming up at 37°C, to a final concentration of 2 mM. Solution was sterile filtered (0.22 μ m pore diameter, Nalgene) and stored at 4°C until used. This stock solution was diluted in medium for cell treatment (final treatment concentration: 10 μ M) and in sterile saline for animal treatments (final treatment concentration: 420 μ M, equal to 11,95 μ g of Myr *per* mouse).

Myriocin loaded-Solid Lipid Nanoparticles (SLN) used for mice treatment

Treatment of mice with Myr was achieved by using Myr-loaded SLNs (Nanovector srl, Italy) prepared as previously described (35). SLN loaded with drug were measured for Myr content and a 1 mM Myr-SLN stock solution was prepared. This solution was diluted 1:12

in sterile saline and 75 μ l (1.7 μ g of Myr and 8% SLN) were used for each mouse administration in the airways.

Cell lines and treatments

IB3-1 cells, an adeno-associated virus-transformed human bronchial epithelial cell line derived from a CF patient (F508/W1282X) and its isogenic C38 cells, corrected by insertion of CFTR, have been both obtained from LGC Promochem (US) and kindly provided by the Cystic Fibrosis animal Core Facility (CFaCore, San Raffaele Hospital, Milan, Italy). Cells were grown in LHC-8 media supplemented with 5% FBS. Both culture flasks and plates were coated with a solution of LHC-basal medium containing 35 μ g/ml bovine collagen, 1 μ g/ml bovine serum albumin and 10 μ g/ml human fibronectin as described (36). For experiments, cells were seeded in 6 multi-wells plate or 100 mm petri dishes at 3×10^5 and 2×10^6 cells/plate respectively. Twenty four h after seeding, when cells reached about 60% confluence, medium was replaced with fresh one, containing either Myr (10 μ M) or vehicle (DMSO). Eight h after Myr treatment, human TNF α (20ng/ml) was added to both treated and untreated cells. Incubation proceeded for further 18 h and then samples were collected for analyses.

MTT assay

Cell proliferation was examined in triplicate samples by the MTT assay as previously described (37). Proliferation index represents fold increase proliferation over vehicle-treated cells at time zero.

ELISA

Released IL-8 and IL-6 were determined in supernatants collected from the cell cultures using ELISA kits according to the manufacturer's instructions (Vinci Biochem, Italy). Values were normalized to 10^6 cells. KC and IL-6 concentration were determined in lung homogenates by ELISA, according to manufacturer's instructions.

LC-MS analysis

Sphingolipid extracts from both mice lungs and treated cells, fortified with internal standards (*N*-dodecanoylsphingosine, *N*-dodecanoylglucosylsphingosine, *N*-dodecanoylsphingosyl phosphorylcholine, C17-sphinganine (0.2 nmol each) and C17-sphinganine-1-phosphate (0.1 nmol), were prepared and analysed as reported (38).

Western blotting

Cells were scraped in ice-cold phosphate buffered saline (PBS) containing proteases inhibitors (Roche Italia, Milan, Italy) and spun at $1,200 \times g$ for 5 min at 4°C. An aliquot was used for protein quantification; the remaining cells were resuspended in Laemmli buffer, boiled for 8 min and stored at -20°C. Cytosolic and nuclear extracts were obtained by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, US), according to manufacturer protocols. Equal amount of proteins (20 μ g for SPT and 15 μ g for NF-kB 40 μ g for IK-B α) were separated on 10% acrylamide gels by SDS-electrophoresis and transferred onto nitrocellulose membranes. After blocking unspecific binding sites with 5% dry skimmed

milk in PBS-Tween 0.1% (PBST), the membranes were incubated (47°C/overnight) with primary antibodies (Anti-Serine Palmitoyltransferase, anti-SPTLC 1 and 2, antibodies were kindly provided by Dr. T. Hornemann University Hospital Zürich, Switzerland; anti IK-B α and anti NF- κ B p65 were from Calbiochem, US) diluted 1:1,000 in PBST-3% BSA, followed by incubation (room temperature/2 h) with the appropriate HRP-secondary antibodies (Jackson Laboratories US) diluted 1:10,000 in PBST-3% BSA. The same membranes were immunoblotted against β -actin (dilution 1:5,000) for data normalization. Proteins were detected by chemiluminescence and bands intensity was quantified by Gel Doc 2000, using Quantity One Software (BioRad Life Science, US).

RNA extraction and quantitative RT-PCR

RNA extraction and quantitative RT-PCR for murine KC and IL-6 and human IL-8 and IL-6 were performed as previously reported (39). Human SPTLC 1 and 2 transcripts (mRNA) were evaluated by RT-PCR as previously reported (40).

Mice treatment and infection

Fourteen weeks old gut-corrected CFTR deficient mice B6.129P2-Cftr^{tm1UNC}TgN(FABPCFTR) (group KO) and congenic wild-type (Case Western Reserve University, Cleveland, Ohio, USA) (group WT) male and female mice were used (41; 42).

Mice were housed in filtered cages under specific-pathogen conditions and permitted unlimited access to food and water. Once a deep stage of anesthesia with 2,2,2-tribromoethanol (Avertin, Sigma-Aldrich, US) was reached, Myr either dissolved in DMSO or uploaded in SLN, was intra-trachea instilled by means of MicroSprayer® Aerosolizer – Model IA1C, attached to “FMJ-250 High Pressure Syringe” (Penn-Century Inc., US). Control animals were treated with the corresponding empty vehicle. The total volume introduced in lungs was 75 μ l, in mice of approximately 30 gr each. This volume contained either 11.95 μ g of compound/mouse in 10%DMSO-saline solution or 1.75 μ g of compound/mouse in 8%SLN-saline solution. 24 h after treatment, animals (both KO and WT) were infected with $3-10 \times 10^5$ CFU of *P. aeruginosa* strain PAO1 planktonic cells, as previously described (43). After 18 h post-infection, mice were euthanized and lungs were perfused with PBS and homogenized in 1 ml of PBS containing protease inhibitors (Roche Italia, Italy). Part of the lung homogenates was centrifuged at 13,000 rpm for 30 minutes at 4°C and the supernatants were stored at –20°C for cytokine analysis. The remaining lung homogenate was divided in two aliquots: one part was lyophilized and lipids were extracted for LC–MS analysis; the other aliquot was serially diluted 1:10 in PBS and plated onto TSA plates to determine lung bacterial load. All experiments were performed with a minimum of five animals per group.

Ethics Statement

Animal studies were conducted according to protocols approved by the San Raffaele Scientific Institute (Milan, Italy) Institutional Animal Care and Use Committee (IACUC) and adhered strictly to the Italian Ministry of Health guidelines for the use and care of experimental animals.

Statistical analysis

Data significance was evaluated by unpaired two-tailed Student t-test ($P < 0.05$) or by one-way ANOVA followed by the Bonferroni multiple comparisons test when significant ($P < 0.05$). Data are expressed as mean \pm SEM.

Results

1. Myriocin differently affects proliferation of IB3 vs C38 cells

To assess whether IB3 cells (a cystic fibrosis cell line, 508/W128X) and C38 cells (a cell line derived from IB3, but stably expressing the wild-type CFTR) respond to sphingolipid *de novo* synthesis inhibitor, we evaluated the proliferation rate of cells treated with Myr vs untreated control cells (DMSO vehicle only) for different times. Myr inhibitory effect on proliferation was significantly lower (50%) on IB3 with respect to C38 cells, at 48 and at 72 h (Figure 1A and B). In order to exclude that sphingolipid synthesis inhibition was affecting cell survival, we performed a Trypan blue exclusion test in IB3 and C38 cells treated with Myr. As shown in Figure 1C, percentage of alive cells was not affected by Myr treatment at any time point (24, 48, 72 h).

2. Inflammation increases ceramide *de novo* synthesis and Myriocin reduces the inflammatory response in IB3 cells

To understand the above reported different behaviour of the two cell lines upon sphingolipid *de novo* synthesis inhibition, we measured ceramide in Myr pre-treated (8 h) and/or TNF α treated (24 h) cells. First of all, we observed a higher content of ceramide in IB3 than in C38 unstimulated cells (Figure 2A). The higher ceramide levels found in IB3 may account for their reduced sensitivity to the anti-proliferative effect of sphingolipid synthesis inhibition (see Figure 1 A). Second, we observed that ceramide was elevated by two folds in TNF α treated IB3 but not in C38 cells (Figure 2A), indicating that *de novo* synthesis of ceramide is hyper-stimulated in the CFTR mutant cells upon inflammatory stimulation. As expected, Myr treatment drastically reduced ceramide levels in both IB3 and C38 cell lines. Notably, in TNF α stimulated IB3 cells, Myr reduced ceramide levels from 900 ± 68.3 to 187 ± 62.7 pmoles/mg protein. To further assess the correlation between sphingolipid *de novo* synthesis and inflammatory signalling, we stimulated IB3 and C38 cells with TNF α and measured SPT (the sphingolipid synthesis rate-limiting enzyme, SPT1 and 2 subunits) transcription by Real Time PCR. TNF α induced an increase in SPT 1 and 2 mRNA levels. Such an increase was significantly higher in IB3 than in C38 cells (Figure 2B). The transcript increase was confirmed by an augmented protein expression of both subunits of the enzyme (Figure 2 C and 2 D). We then evaluated if sphingolipid synthesis inhibition was able to modulate cytokines release upon TNF α stimulation. IB3 and C38 cells were treated with TNF α (24 h), either alone or in combination with a pre-treatment with Myr (8 h). Myr significantly reduced TNF α stimulated IL-8 mRNA expression and protein release in IB3 cells, whereas cytokines expression and release was slightly affected in C38 treated cells (Figure 3 A and C). IL-6 mRNA expression was down-regulated by Myr alone both in C38 and IB3 cells. When cells were stimulated with TNF α , Myr was able to reduce (about 2 folds) the IL-6 mRNA in IB3 but no significant reduction was observed in C38 cell line (Figure 3 B). IL-6 protein release was not affected by either TNF α or by Myr stimulation in C38 cells.

Whereas Myr alone did not alter the levels of secreted IL-6 protein in IB3 cells, it was effective in decreasing cytokines release induced by TNF α in IB3 (Figure 3 D).

3. Myriocin downregulates NF-kB activation in IB3 cells

Human and murine CF epithelium was demonstrated to sustain inflammatory signaling via NF-kB activation (44-46). Therefore we investigated on Myr ability to alter NF-kB activation in human CF epithelial cell line. IB3 cells were treated with TNF α (24 h) either alone or in combination with Myr pre-treatment (8 h). Cytosolic fractions were separated from nuclear fractions. We observed that TNF α -induced degradation of the cytosolic I κ -B α (the NF-kB inhibitor, Figure 4 A and B) and nuclear accumulation of NF-kB p65 (Figure 4 C and D), were both reversed by Myr.

4. Myriocin modulates *P. aeruginosa* airways inflammation in Cfr^{tm1UNC}TgN(FABPCFTR) mice

At the aim of validating the results obtained on human cell lines in an *in vivo* model, Cfr^{tm1UNC}TgN(FABPCFTR) mice (KO) and congenic control (WT) mice were treated with Myr dissolved in 10% DMSO, by intra-tracheal micro-sprayer nebulisation for 24 h prior to infection with *P. aeruginosa* (PAO1) by intra-tracheal injection. Animals were sacrificed 18 h after infection. Myr treatment was significantly effective in reducing lungs Cer level in infected KO mice (from 722 \pm 9 to 313.3 \pm 6.9 pmoles/mg protein) and small reduction was detected in infected WT mice lungs (from 784.5 \pm 60.5 to 612.5 \pm 129.5 pmoles/mg protein) (Figure 5).

On the contrary, sphingolipid synthesis inhibition was associated to a reduced KC mRNA content (Figure 6 A) as well as in KC protein level (Figure 6 C), both in KO and in WT mice lungs homogenate. IL-6 mRNA was down-modulated by Myr in both KO and WT animals and IL-6 protein release was reduced in both animal groups (Figure 6 B and D, respectively). All animal experiments have been repeated three times.

5. SLN delivery of Myriocin enhances its effects on infection and inflammation in murine airways

Myr is highly lipophilic and poorly soluble, even in a 10% DMSO solution. At the aim of optimizing lungs delivery and prolonging the effect of the inhibitor by increasing its half-life in the lungs, Myr was loaded into SLN and injected as aerosol solution, by intra-tracheal microsprayer nebulisation, 24 h prior to infection with *P. aeruginosa*. Animals were sacrificed at 18 h post infection. SLN- mediated Myr delivery ensured a high efficacy on sphingolipid synthesis inhibition as demonstrated by 37% or 60% of lungs ceramide reduction in WT and KO infected animals respectively (Figure 7 A). The inhibition of *de novo* ceramide synthesis in CF mouse airways was associated to a marked reduction of glucosyl-galactosyl ceramide pool. No changes were detected in the overall content of sphingomyelins (supplementary Figure 1). This suggests that in CF models, *de novo* generated ceramide can be preferentially metabolized by glycosylation. This hypothesis is in line with the anti-inflammatory effect of miglustat, an inhibitor of ceramide glycosylation, obtained by Dechecchi and coworkers (39).

Notably, SLN- mediated Myr delivery reduced *P.aeruginosa* lung colonies 6 times in treated KO treated compared to untreated KO mice, while no differences were found between treated and untreated WT mice (Figure 7 B). Sphingolipid synthesis inhibition *via* Myr-loaded SLN was associated to a significant reduction in lungs KC and IL-6 mRNA expression (Figure 8 A and B). Lung KC and IL-6 protein levels were similarly down-regulated by Myr both in KO and WT infected mice. All animal experiments have been repeated three times.

Discussion

The role of sphingolipid *de novo* synthesis pathway in inflammation is still largely unexplored. The data presented in this article demonstrate for the first time that *de novo* sphingolipid synthesis is enhanced during inflammation and, in turn, it promotes the production of inflammatory mediators (Figure 2 and 4). In CF mice model, we proved that blocking SPL *de novo* synthesis can reduce hyper-inflammation favouring the recovery of an effective response to *P.aeruginosa* infection in the respiratory tract. It is important to consider that CF is a chronic inflammatory disease, prior to become an infective chronic disease: noteworthy even CF uninfected infants exhibit high levels of pulmonary inflammation (27). Hence, it is mandatory to cure innate immunity in CF patients and immunomodulatory agents are required to reduce the persistent inflammation that favours infection stabilization. From literature evidences it is clear that acute and chronic inflammation modulate sphingolipids and are, in turn, tuned by sphingolipid metabolites (2; 31; 39; 47). Accordingly, in CF human cells we showed: i) a basal higher amount of endogenously synthesized ceramide, that provides the cells with the ability to partially overcome the antiproliferative effect of sphingolipid *de novo* synthesis inhibition; ii) an increased expression of the rate limiting enzyme of sphingolipid *de novo* synthesis upon TNF α stimulation (Figure 2 B). We also proved that *de novo* sphingolipid synthesis inhibition in this cells reduced NF-kB activation (Figure 4) and IL8 and IL6 release (Figure 3) upon TNF α stimulation. These evidences demonstrate the existence of a noxious loop between ceramide *de novo* synthesis and inflammation in CF. Gulbins and colleagues demonstrated first in CF mice and then in CF human patients, that inhalation of an acid sphingomyelinase inhibitor reduces ceramide accumulation and lung burden of inflammation and infection (31). On the other side, in pulmonary infections, rapid acid sphingomyelinase activation and formation of ceramide-enriched membrane platforms was shown to be required for the internalization/clearance of different bacteria, including *P.aeruginosa* (17; 48). It was also found that infection of acid sphingomyelinase-deficient mice with *P. aeruginosa* caused increased bacterial load, cytokine storm (49) and finally death of the mice (50). Thus the activity of acid sphingomyelinase seems to be required for a proper inflammatory response and for pathogens clearance (48; 51), casting doubt on a long term use of acid sphingomyelinase inhibitor, which may negatively impact on the immunity system of the respiratory tract. Petrache and co-workers demonstrated that increased rate of sphingolipid *de novo* synthesis is associated with inflammation and, importantly, with an increase in the secretion of acid sphingomyelinase, which is responsible for a high release of ceramide in respiratory mucosa of COPD mice. This ceramide contributes to mucus thickness and inflammation (10). Our hypothesis is that CFTR mutation, possibly by

inducing endogenous stress, associates with an enhanced rate of *de novo* sphingolipid synthesis and with an increase in the content of ceramide within the respiratory mucosa. In addition to the block of ceramide release from secreted sphingomyelinase activity by amitriptyline, the correction of the hyper-stimulated *de novo* synthesis, which may be responsible of increased sphingomyelinase activity (10) by Myr, is able to counteract the excessive inflammatory reaction upon infection and to favour bacteria clearance.

Sphingolipid targeting for human therapy has the problem of hydrophobicity of most of the known metabolism inhibitors. Lipophilic ceramide was previously delivered *in vivo* by means of liposomes carriers (52). Recently, the use of solid lipid nanocarriers was successfully experienced in mouse eye, by external administration of eye drops containing Myr-loaded SLN (35). We here demonstrate that surfactant like containing nanoparticles allow a good uptake and delivery of Myr. Comparing the efficacy of the compound in terms of ceramide content reduction, we observed approximately a 7 fold decrease in the effective drug concentration when using SLN delivery in respect to the concentration required when Myr was dissolved in DMSO and directly diluted into saline. It is feasible that aqueous suspensions and perhaps dry powder formulations of SLN can be used for pulmonary inhalation. We think that SLN may be a very important tool for any drug targeting sphingolipid metabolism *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

De novo synthesis of ceramide is increased in CF respiratory tract

Myriocin reduces ceramide in CF respiratory tract

Myriocin reduces inflammation in CF respiratory tract

Nanocarriers delivery of myriocin in vivo reduces lung infection in CF mice

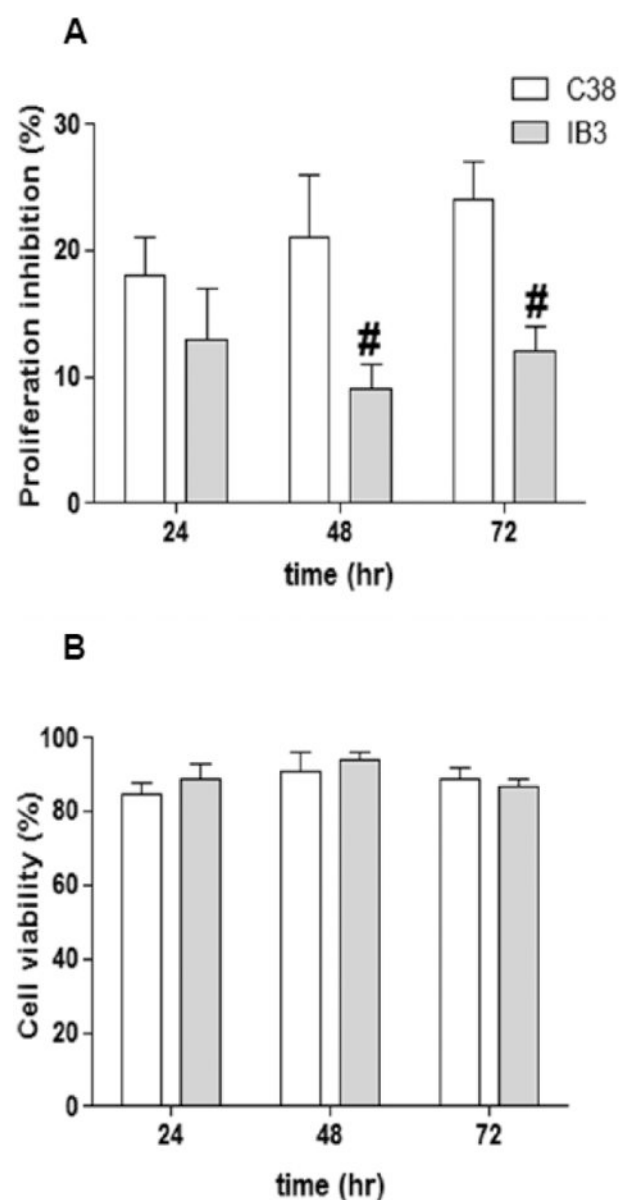


Figure 1. Myriocin differentially affects proliferation of C38 vs IB3 cells

Inhibitory effect of Myr (10 μ M) on IB3 and C38 cells proliferation at 24, 48, 72 h, expressed as percentage of proliferation inhibition in treated vs control (A). Significance was evaluated by one-way ANOVA as reported in Table 1. #, vs C38 cells. Cell viability, calculated as percentage of alive vs total cells in IB3 and C38 cells treated with Myr (10 μ M) for 24, 48, 72 h (B).

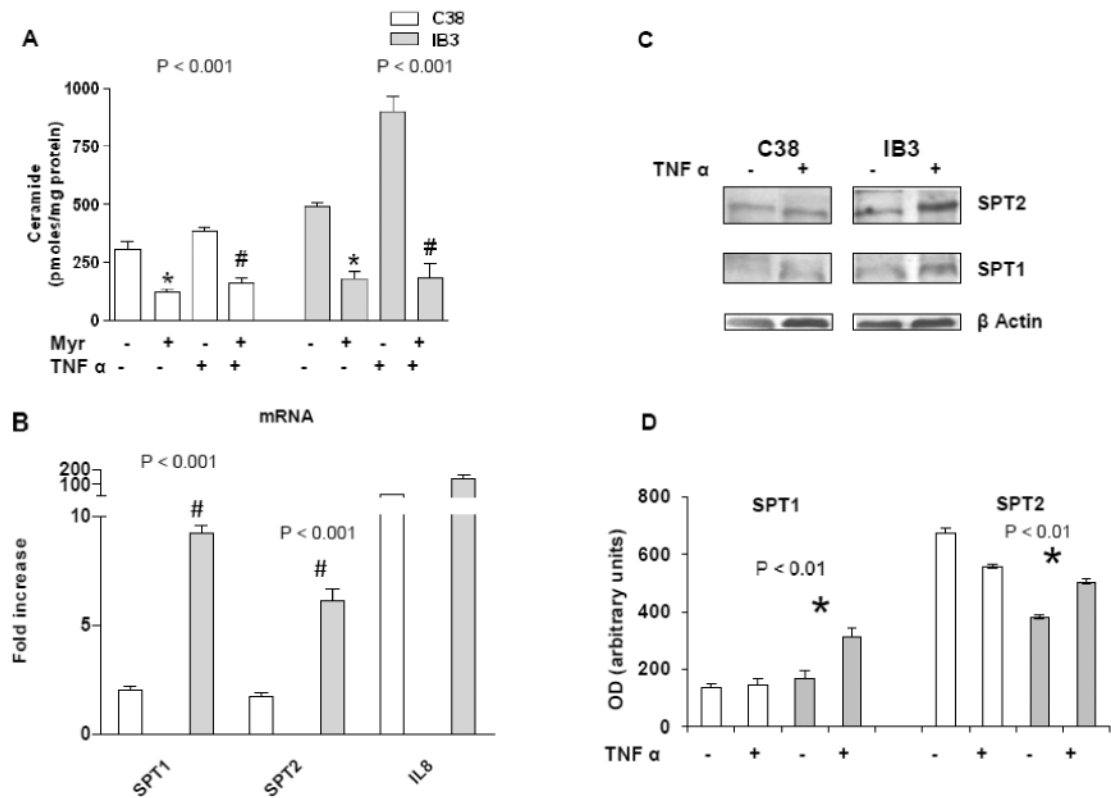


Figure 2. TNF α differently induces *de novo* ceramide synthesis in IB3 vs C38 cells

Cer quantitation, by LC–MS analysis, in IB3 and C38 cells stimulated with TNF α (20 ng/ml) either alone or in combination with Myr (10 μ M, 8 h pre-treatment) for 24 h. Significance was evaluated by one-way ANOVA (value reported on the graphs). #, *, vs TNF α -treated and untreated cells (Bonferroni post-test: P < 0.01 and P < 0.001 in C38 and IB3, respectively) (**A**). Real time PCR of SPT 1 and 2 transcripts in C38 and IB3 cells cells stimulated with TNF α (20 ng/ml) for 24 hours. IL8 was evaluated as positive control of TNF α -induced inflammation. Data are expressed as fold increase of treated cells vs. untreated cells (**B**). SPT protein expression (SPT1, SPT2) in IB3 and C38 cells TNF α -treated and untreated for 24 h. β actin was used as loading control (**C**). Densitometric analysis of the SPT protein bands normalized on the corresponding β actin value. Medium value of three independent experiments. Significance was evaluated by one-way ANOVA (value reported on the graphs). SPT2: *, vs TNF α -treated IB3 and untreated C38 cells. SPT1: *, vs TNF α -treated C38 cells (Bonferroni post-test: P < 0.05) (**D**).

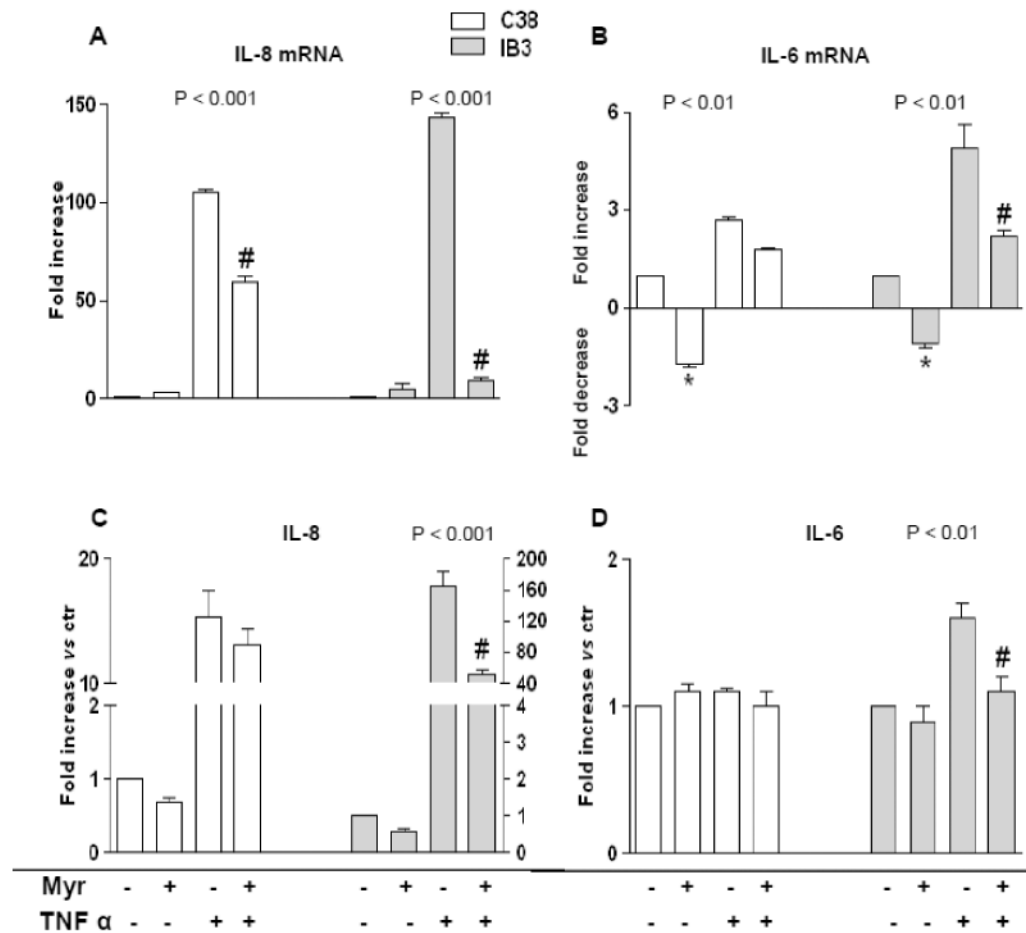


Figure 3. Myriocin effect on IL-8 and IL-6 transcription and release in TNF α -stimulated IB3 and C38 cells

IL-8 and IL-6 mRNA expression (A and B, respectively) and protein release (C and D, respectively) in IB3 and C38 cells stimulated with TNF α (20 ng/ml) either alone or in combination with Myr (10 μ M, 8 h pre-treatment) for 24 h. Significance was evaluated by one-way ANOVA (value reported on the graphs). Panel A, C: #, vs TNF α -treated cells ($P < 0.001$, Bonferroni post-test). Panel B, D: #, *, vs TNF α -treated and untreated cells, respectively ($P < 0.05$, Bonferroni post-test).

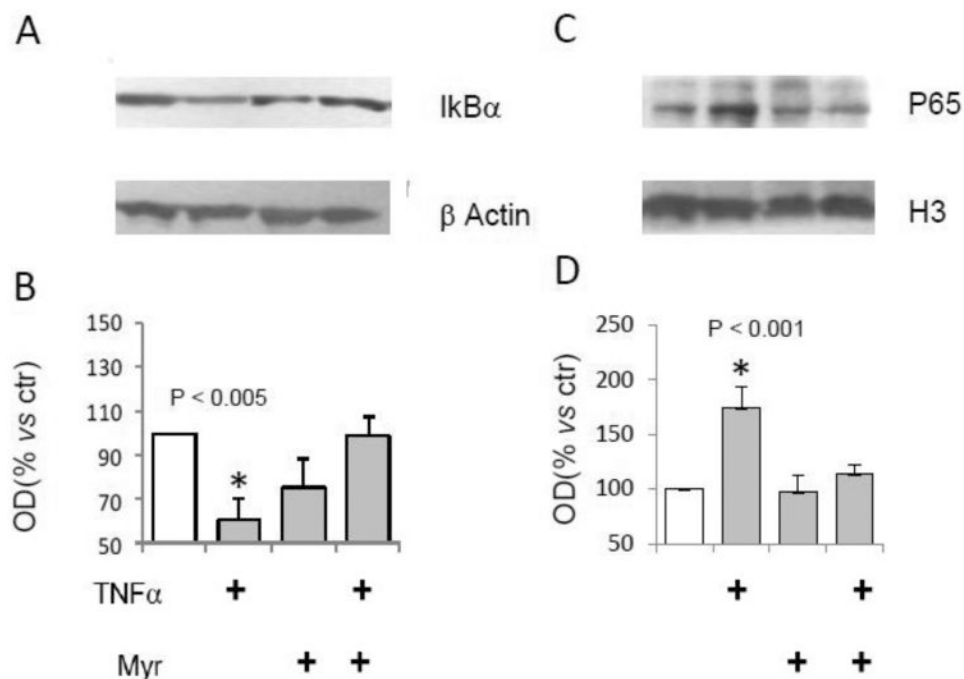


Figure 4. Myriocin effect on NF- κ B activation in TNF α -stimulated IB3

IB3 cells stimulated with TNF α (20 ng/ml) for 24 h. Western blotting evaluation of I- κ B from cytosolic extracts, compared to β -actin expression (A). OD from optical densitometry of 3 independent experiments was reported (B). Western blotting evaluation of NF- κ B p65 from nuclear extracts, compared to histone H3. Significance was evaluated by one-way ANOVA (value reported on the graphs, $P < 0.001$, Bonferroni post-test).

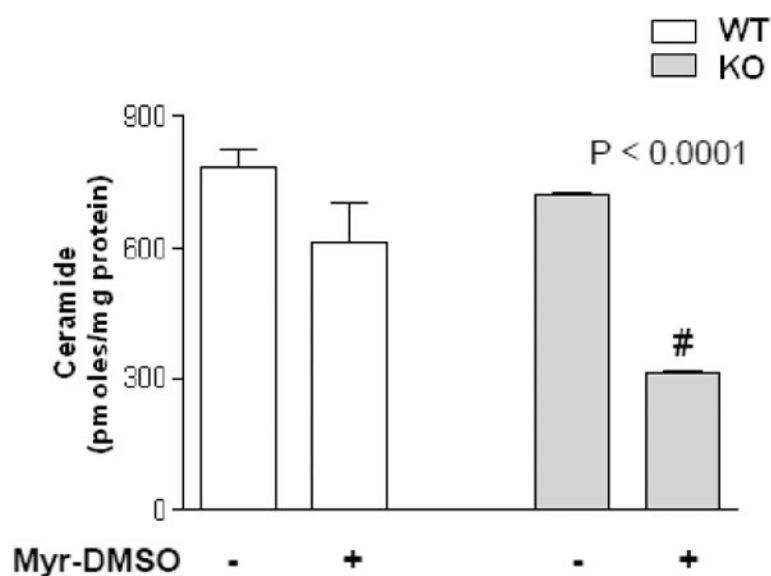


Figure 5. Myriocin effect on ceramide lung content and infection in KO and WT mice
Cer quantitation, by LC-MS analysis, in KO and WT mice treated (or untreated, DMSO vehicle only) with DMSO-solved Myr (11.95 μ g of Myr/mouse lungs) 24 h prior to infection with *P. aeruginosa*. Animals were sacrificed 18 h post infection. Significance was evaluated by unpaired two-tailed Student t-test. #, vs untreated mice.

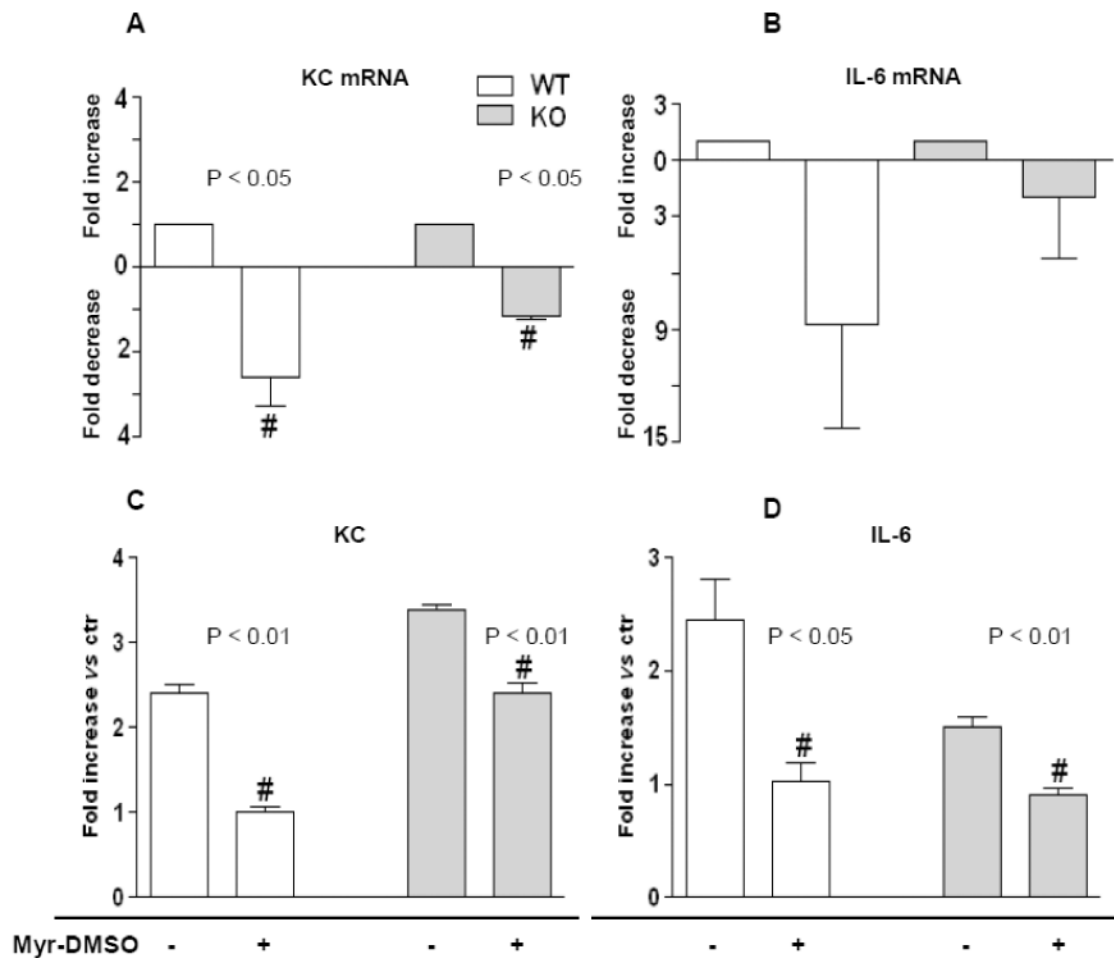


Figure 6. Myriocin effect on KC and IL-6 transcription and release in PAO1-infected lung of KO and WT mice

KC and IL-6 mRNA expression (A and B, respectively) and lung protein content (C and D, respectively) in KO and WT mice treated with DMSO-solved Myr (11.95 μ g of Myr/mouse lungs) 24 h prior to infection with *P. aeruginosa* (PAO1). Animals were sacrificed 18 h post infection. mRNA values are expressed as fold change vs ctr (untreated mice). Protein values are expressed as fold increase vs ctr. Significance was evaluated by unpaired two-tailed Student t-test. #, vs ctr mice.

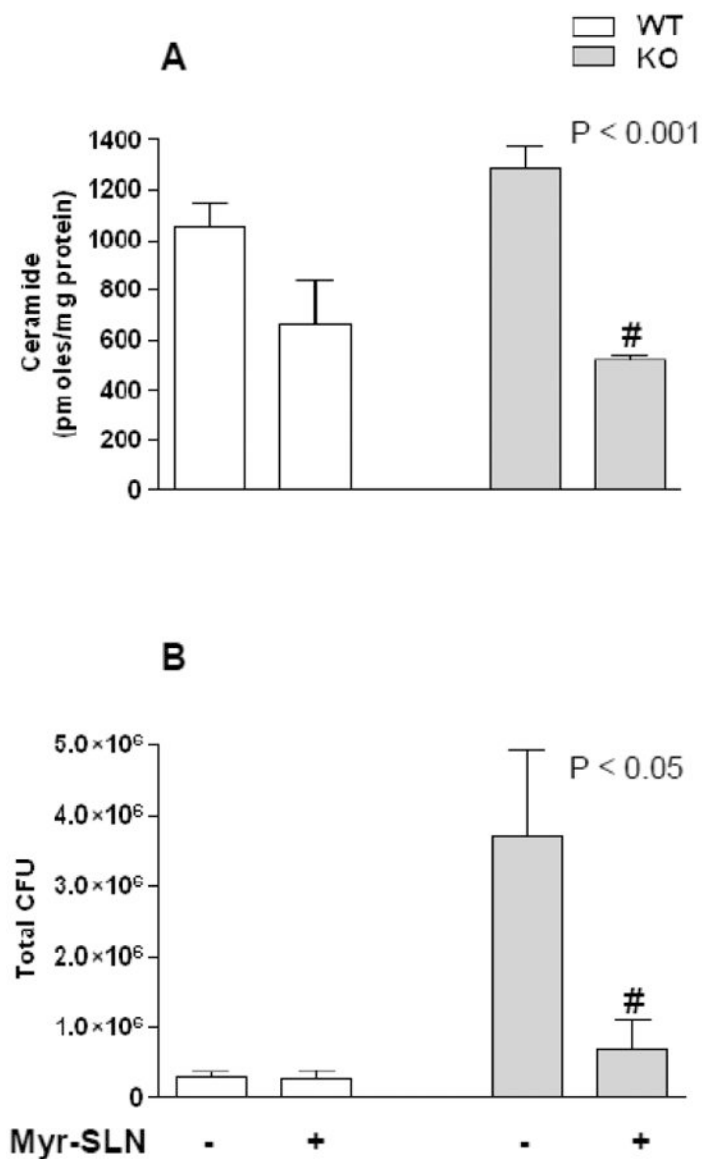


Figure 7. Myriocin-SLN effect on ceramide and infection in KO and WT mice
 KO and WT mice treated with Myr-SLN (1.7 μ g of Myr/mouse lungs) or untreated (empty SLN), 24 h prior to infection with *P. aeruginosa* (PAO1). Animals were sacrificed 18 h post infection. Cer quantitation, by LC-MS analysis (**A**). PAO1 colonies formation assay from lungs homogenate of infected KO and WT mice treated or untreated (**B**). Significance was evaluated by unpaired two-tailed Student t-test. #, vs untreated mice.

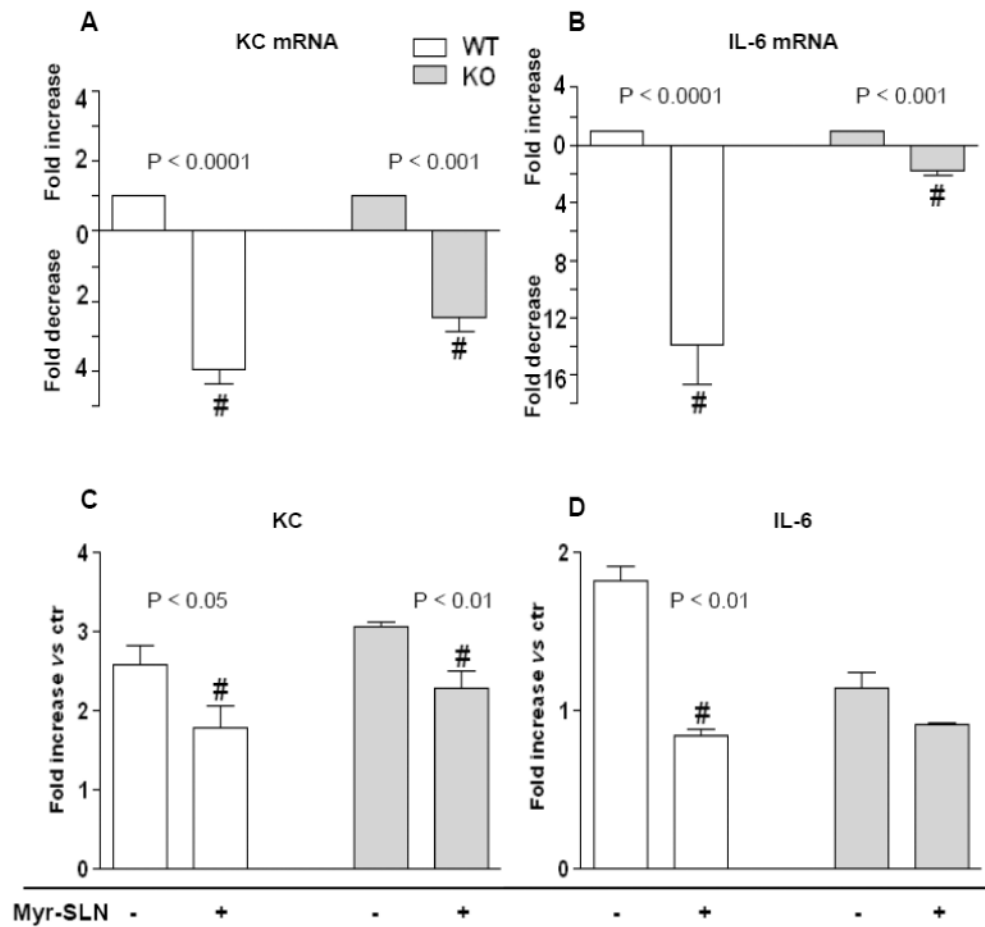


Figure 8. Myriocin-SLN effect on KC and IL-6 transcription and release in PAO1-infected lung of KO and WT mice

KO and WT mice treated with Myr-SLN (1.7 μ g of Myr/mouse lungs) or untreated (empty SLN), 24 h prior to infection with *P. aeruginosa* (PAO1). Animals were sacrificed 18 h post infection. KC and IL-6 mRNA expression (**A** and **B**, respectively) and lungs protein content (**C** and **D**, respectively) in KO and WT mice. mRNA values are expressed as fold change vs ctr (untreated mice). Protein values are expressed as fold increase vs ctr. Significance was evaluated by unpaired two-tailed Student t-test. #, vs ctr.