

The anti-inflammatory fungal compound (*S*)-Curvularin reduces pro-inflammatory gene expression in an in-vivo model of rheumatoid arthritis

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The abbreviations used are: AIRE, autoimmune regulator; Anks1, ankyrin repeat and SAM domain containing 1; AP-1, activator protein-1; Camp, cathelicidin-related anti-microbial peptide; Cfp, Factor P; CIA, collagen induced arthritis; CII, chicken collagen II; C5a, complement component 5a; CCL, chemokine (C-C motive) ligand; Coro1a, Coronin 1A; Defb6, Defensin B6; Dex, dexamethasone; Dsc2, Desmocollin-2; GC, glucocorticoid; GR, glucocorticoid receptor; Gpr34, Probable G-protein coupled receptor 34; Gpsm3, G-protein signaling modulator 3; IFN- γ , interferon- γ ; IL, interleukin; IL-1 β , interleukin-1 β ; JAK, janus kinase; LPS, Lipopolysaccharide; Magi3, Membrane-associated guanylate kinase 3; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; MMP,

matrixmetalloproteases; MPO, myeloperoxidase; NF- κ B, nuclear factor κ B; Nr2c1, nuclear receptor subfamily 2 group C member 1; iNOS, inducible NO synthase; Ogn, Osteoglycin; Pbp, peptidylprolyl cis-trans isomerase B; PBMC, peripheral blood monocytes; qRT-PCR, quantitative real time reverse transcription polymerase chain reaction; RA, rheumatoid arthritis; Rab27, Ras-related protein Rab-27B; RANKL, receptor activator of nuclear factor- κ B ligand; ROS, reactive oxygen species; Sart1, Squamous cell carcinoma antigen recognized by T cells; SC, (S)-Curvularin; STAT-1 α , signal transducer and activator of transcription-1 α ; TCR, T-cell receptor; TNF- α , tumor necrosis factor- α ; WB, Western Blot

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Abstract

In previous studies we identified the fungal macrocyclic lactone (*S*)-Curvularin (SC) as an anti-inflammatory agent using a screening system detecting inhibitors of the JAK/STAT-pathway. The objective of the present study was to investigate whether SC is able to decrease pro-inflammatory gene expression in an *in-vivo* model of a chronic inflammatory disease. Therefore, the effects of SC and dexamethasone were compared in the model of collagen induced arthritis (CIA) in mice. Total genomic microarray analyses were performed to identify SC target genes. Also in human C28/I2 chondrocytes and MonoMac6 monocytes the effect of SC on pro-inflammatory gene expression was tested at the mRNA and protein level. In the CIA model SC markedly reduced the expression of a number of pro-inflammatory cytokines and chemokines involved in the pathogenesis of CIA as well as human RA. In almost all cases the effects of SC were comparable to those of dexamethasone. In microarray analyses we identified additional new therapeutic targets of SC. Some of them, such as S100A8, myeloperoxidase or cathelicidin, an antimicrobial peptide, are known to be implicated in pathophysiological processes in RA. Similar anti-inflammatory effects of SC were also observed in human C28/I2 chondrocyte cells, which are resistant to glucocorticoid treatment. These data indicate that SC and glucocorticoid effects are mediated via independent signal transduction pathways. In summary, we demonstrate that SC is a new effective anti-inflammatory compound, which may serve as a lead compound for the development of new drugs for the therapy of chronic inflammatory diseases.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune and destructive joint disease affecting 1% of the population. RA is characterized by abnormal accumulation of immune cells in joints. These immune cells together with endothelial cells, fibroblasts and chondrocytes express and export a complex mixture of pro-inflammatory mediators like cytokines (TNF- α , IL-1, IL-6 etc.), chemokines (MCP-1, MCP-4 etc.), lipids, growth factors, transcription factors (like NF- κ B, STATs, AP-1) and destructive enzymes (e.g. matrix metalloproteinases) which are critically involved in local tissue destruction and fibrotic processes (McInnes and Schett, 2007).

Glucocorticoids (GC) at high doses are used to control acute RA disease attacks (Kirwan and Power, 2007) and are prescribed at low concentrations as long-term medication. The anti-inflammatory effects of GCs are mediated by their ability to modulate expression of pro-inflammatory genes, such as IL-1 β or TNF- α , as well as T-cell proliferation (Chikanza, 2002). Although GCs are very potent anti-inflammatory drugs, their long-term usage is limited by a number of severe side effects such as osteoporosis, insulin resistance, skin thinning, hypertension, obesity and inhibition of wound repair. Moreover, about 30% of patients with RA fail to respond to steroid therapy (Barnes and Adcock, 2009). Therefore the development of new anti-inflammatory drugs with the therapeutic potency of GCs but without their adverse effects is essential.

In our previous work we identified the fungal macrocyclic lactone (*S*)-Curvularin (SC) as a potent inhibitor of pro-inflammatory gene expression in human alveolar epithelial A549/8 cells. SC reduced the expression of the human inducible nitric oxide synthase (iNOS), an enzyme critically involved in pro-inflammatory immune processes, by inhibition of the cytokine-induced promoter activity. Studies on the mode of action revealed that SC inhibited the phosphorylation and thereby the activation of the tyrosine kinase (janus kinase) JAK2

and, consecutively, of the transcription factor signal transducer and activator of transcription-1 (STAT-1) resulting in the inhibition of STAT-1-dependent gene expression (Yao et al., 2003). Inhibition of STAT-1-mediated signal transduction seems to be an attractive concept in RA therapy because it has been demonstrated that genes transcriptionally regulated by the JAK/STAT-1 pathway were upregulated in RA patients with severe joint inflammation (van der Pouw Kraan et al., 2003).

Chondrocytes play an important role in the cartilage degradation observed in human joint diseases (Otero and Goldring, 2007). Under stress conditions they produce a variety of cytokines such as TNF- α or IL-1 β , metalloproteases, NO, prostaglandins and other mediators all associated with the inflammatory process. We formerly demonstrated that human C28/I2 chondrocytes did not respond to GC-treatment due to the lack of glucocorticoid receptor α (GR α) expression. In contrast to GC, treatment of C28/I2 cells with SC inhibited cytokine induced iNOS expression (Schmidt et al., 2010). These findings suggest that SC may be useful as anti-inflammatory agent to treat glucocorticoid-resistant inflammatory diseases.

In the current study we used the mouse model of collagen-induced arthritis (CIA), which represents a wide accepted animal model of rheumatoid arthritis. In this model immunization with heterologous collagen-type-II (CII) induces severe synovitis with redness and swelling of the joints. CII seems to be also a relevant autoantigen in humans and there is evidence that both, in human and mice, the disease is driven by CII-specific T- and B-cells and their secreted cytokines (Kim et al., 2000).

In this chronic inflammation model SC-treatment decreased the expression of important pro-inflammatory cytokines and chemokines, involved in the onset and progression of RA, in a similar manner as the glucocorticoid dexamethasone (Dex). In microarray-analyses we identified several immuno-modulatory genes regulated during the course of CIA which are significantly affected by the therapeutic intervention with SC. Additionally, SC reduced the

expression of pro-inflammatory genes in human C28/I2 chondrocytes and human MonoMac6 monocytes at mRNA- and protein level.

In conclusion we demonstrate that SC possesses a similar therapeutic power as Dex in regulating pro-inflammatory gene expression in cell culture as well as in in-vivo models.

Methods

Animals

DBA/1 mice, expressing a transgenic TCR β -chain (V β 12) obtained from a CII-specific T cell clone (Mori et al., 1992) were used. Mice were housed under specified pathogen free conditions in accordance with standard animal care requirements and maintained on a 12 / 12 hour light-dark cycle. Water and food were given *ad libitum*. The animal studies were approved by the ethical board and they were performed with the current regulation on animal handling.

Induction and treatment of CIA

CIA induction was performed as described (Hess et al., 1996) with little modifications. Chicken collagen type II (CII) (Sigma Deisenhofen, Germany) was dissolved in 0.01 M acetic acid at 4 mg/ml. Animals were injected intradermally with 200 μ g of CII emulsified in complete Freund's adjuvant (CFA) Becton Dickinson, Heidelberg, Germany) in both ears (50 μ g each) and the base of the tail (100 μ g). Two booster injections of 100 μ g CII in CFA were given intradermally 11 and 21 days later. Control animals were injected intradermally with PBS. From day 21 to day 37 CII-immunized mice were injected intraperitoneally every second day with 10 mg/kg (S)-Curvularin, 5 mg/kg dexamethasone or PBS/ETOH as solvent control. At day 21, 25, 29, 33 and 37 eight mice of each treatment group were euthanized by inhalation of CO₂ for subsequent analyses.

Assessment of arthritis

Mice were visually checked for the appearance of arthritis in peripheral joints every two days and grades of an established scoring system (Brand et al., 2007) ranging from 0 to 4 were allotted to each limb. A maximum score of 16 could be achieved for each mouse. The arthritis score was checked by a minimum of two people but not in a fully blinded manner.

(S)-Curvularin

(S)-Curvularin has been obtained by fermentation of the producer strain *Penicillium* spec. IBWF3-93 and isolation from the culture fluid by chromatographic methods as previously described (Yao et al., 2003). The purity of S-Curvularin as estimated by HPLC-DAD/MS analysis was greater than 99 % (Supplemental Figure 1).

RNA Isolation

Total RNA of paws was prepared by homogenizing the sample in GIT-buffer (Chomczynski and Sacchi, 1987) and RNA was isolated as described (Rodriguez-Pascual et al., 2000). Total RNA from C28/I2 and MonoMac6 cells was prepared using the RNeasy mini kit (Qiagen, Hilden, Germany).

Microarray-analysis

For microarray-analysis, total RNA was prepared from all paws of each animal with the RNeasy Midi Kit (Qiagen, Hilden, Germany). Then the RNA of all paws of one mouse was pooled. RNA quality analysis, generation of fluorescent labeled cDNAs, microarray hybridization and microarray data analysis were performed as described using mouse total genome OpArrays (Eurofins MWG Operon, Ebersberg, Germany) (Pautz et al., 2009). The study was conducted according to standards developed by the Microarray Gene Expression

Data Society (MGED), and data complying with the "minimum information about microarray experiments" (MIAME) have been deposited in the EMBL-EBI ArrayExpress repository (<http://www.ebi.ac.uk/arrayexpress>; experiment name: HK_CIA_experiment).

Real-time reverse transcription polymerase chain reaction analysis

Gene expression in mouse paws was quantified in a two-step real-time RT-PCR as described (Schmidt et al., 2010). In the RT step, cDNA was reverse transcribed from 0.5 - 1 µg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's recommendations. Real-time PCR analysis was performed in a total volume of 25 µl in a 96-well spectrofluorometric thermal cycler (iCycler, Bio-Rad Laboratories, München, Germany). For real-time PCR (40 cycles of 15 s 94°C, 60 s 60°C), the following oligonucleotides served as sense and antisense primers and Taqman hybridization probe:

MMP3

sense	TGGAGATGCTCACTTTGACG
antisense	ATGGAAACGGGACAAGTCTG
probe	CACTCAGCCAAGGCTGAAGCTCTGA

RANKL

sense	G TTCCTGTACTTTTCGAGCGC
antisense	TGTGTTGCAGTTCCTTCTGC
probe	CATCGGGTTCCATAAAGTCACTCTGTCT

Pol2A

sense	ACCACGTCCAATGATATTGTGGAG
antisense	ATGTCATAGTGTCACACAGGAGCG
probe	CTGGGCATTGAGGCTGTGCGGAA

Notch2

sense	TACCTGTCACATGCTCAGCC
antisense	TCACACTTCTGCCCTGTGAG
probe	TGCCTGTCTATCTCATCCCTGCGAA

The mRNA expression in human C28/I2 chondrocytes and MonoMac6 cells was analyzed by two-step real-time RT-PCR as described (Jung et al., 2009; Schmidt et al., 2010). cDNA was prepared from 0.5 - 1 µg total cellular RNA. The reverse transcription was performed with the “RevertAid™ H Minus First Strand cDNA Synthesis kit” (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. PCR products were synthesized from cDNA (100 or 300 ng) using functionally validated gene expression assays, as the ABsolute qPCR SYBRm Green fluorescein mix obtained from ABgene. A two-step amplification protocol was chosen: initial denaturation step at 95 °C for 10 min followed by 45 cycles with 15 s denaturation at 94 °C, 30 s annealing at 56 °C and 30 s extension at 72 °C. To analyze the mRNA expression of investigated genes, qRT-PCR was carried out using the following gene-specific primers:

MCP-1

sense	ATCAATGCCCCAGTCACC
antisense	AGTCTTCGGAGTTTGGG

COX2

sense TTCAAATGAGATTGTGGGAAAATTGCT

antisense AGATCATCTCTGCCTGAGTATCTT

GAPDH

sense CCTCCGGGAAACTGTGG

antisense AGTGGGGACACGGAAG

IL-8

sense TGCCAAGGAGTGCTAAAG

antisense CTCCACAACCCTCTGCAC

TNF- α

sense TCTTCTGCCTGCTGCACTTTGG

antisense ATCTCTCAGCTCCACGCCATTG

S100A8

sense CCATGCCGTCTACAGGGATG

antisense TATCCAACCTCTTTGAACCAGACGTC

To calculate the relative mRNA expression the $2^{(-\Delta\Delta C(T))}$ method (Livak and Schmittgen, 2001) was used.

Western blot experiments

To study protein-expression in mouse tissue 50-100 μ g protein was separated on SDS polyacrylamide gels and transferred to nitrocellulose membranes by semi-dry electroblotting.

Total cell extracts of MonoMac6 cells or mouse tissue were prepared using RIPA detergent buffer (137mM NaCl, 2.7mM KCl, 8.1mM Na₂HPO₄ 2 H₂O, 1.5mM KH₂PO₄, pH 7.4, 1.0% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 1.0 mM Na₃VO₄, complete protease inhibitor cocktail 1 : 50 (Roche Diagnostics, Mannheim, Germany)). All further steps were performed as described (Rodriguez-Pascual et al., 2000). For murine protein detection a monoclonal anti-S100A8-antibody (SantaCruz, Heidelberg, Germany), polyclonal anti-PpiB-, anti-MPO- (both R&D Systems, Wiesbaden, Germany) and anti-p38MAPK-antibodies (NEB, Frankfurt a.M., Germany) all in an 1 to 1000 dilution were used. Human proteins were detected with anti-S100A8-antibody and anti-GAPDH-antibody (1/1000 dilution, both SantaCruz, Heidelberg, Germany).

Cell culture and stimulation protocols

Human immortalized C28/I2 chondrocyte cells (Goldring et al., 1994) were cultured as a superconfluent monolayer as described (Schmidt et al., 2010). For qRT-PCR and proteome profiling, cells were starved for 16 h in DMEM with 0.5% fetal calf serum. Afterwards cells were pretreated with SC in serum-reduced medium for 1 h. Stimulation with a cytokine mixture (CM) consisting of 10 ng/mL TNF- α , 5 ng/mL IL-1 β and 10 ng/mL IFN- γ was performed for 6 h (qRT-PCR) and 16 h (proteome profiling), respectively. Human immortalized MonoMac6 monocyte cells were cultured as described (Erkel et al., 2007). For qRT-PCR and western blot analysis cells were starved for 16 h in RPMI 1640 medium with 0.5% fetal calf serum, pretreated with SC in serum-reduced medium for 1 h and then stimulated with a mixture of 1 μ g/ml LPS, 10 ng/ml IFN- γ , 10 ng/mL TNF- α and 5 ng/mL IL-1 β for 24 h.

Giemsa staining

The cytotoxicity of (*S*)-Curvularin against C28/I2 cells was determined in a Giemsa staining assay according to Mirabelli et al. (Mirabelli et al., 1985). Absorbances were read by a Lambda Spectral 340 Microplate reader (MWG Biotech Ebersberg, Germany) at a wavelength of 600 nm and a reference wavelength of 405 nm. Data were displayed as percent of the control absorbances (relative to untreated cells).

Proteome ProfilerTM (R&D Systems)

To analyze pro-inflammatory protein expression in murine samples or in culture supernatants of C28/I2 cells we used the proteome profilerTM "Mouse Cytokine Array Panel A" and the "Human Cytokine Array Panel A" according to the protocol of the manufacturer. The QuantityOne software (BioRad, Munich; Germany) was used for quantification.

Statistics - Data represent means + SEM. Statistical differences were determined by factorial analysis of variance followed by Fisher's protected least-significant-difference (PLSD) test for comparison of multiple means.

Results

The expression of different pro-inflammatory mediators was decreased by SC

Our previous analyses showed that SC inhibits the JAK/STAT-pathway (STAT-1 α -phosphorylation) and thereby pro-inflammatory gene expression in different human and murine cell systems ((Yao et al., 2003; Elzner et al., 2008; Schmidt et al., 2010) and data not shown). Also SC was able to reduce pro-inflammatory gene expression in LPS-treated mice in vivo (data not shown). Therefore we evaluated the anti-inflammatory properties of SC in the CIA mouse model. CIA was induced in transgenic V β 12 DBA/1 mice as described in Materials and Methods. In comparison to PBS-injected control mice, mice immunized with CII displayed severe symptoms of an inflammation, visible as redness and swelling of the

digits or other parts of the paws. First signs of inflammation were seen between day 21 and 25 after the first immunization with collagen type II and the maximum was reached around day 33 with an average arthritis score of 8.2 ± 2.22 (data not shown).

To investigate the effect of (*S*)-Curvularin in the setting of a chronic inflammation in the CIA-model the CII-immunized mice were injected intraperitoneally every second day from day 21 on with (*S*)-Curvularin dissolved in PBS/10%ETOH (**CII+SC**) (10 mg/kg), dexamethasone (**CII+Dex**) (5 mg/kg) dissolved in PBS/10%ETOH or PBS/10%ETOH (**CII**) as a solvent control. To analyze the effects of SC and Dex on the expression of different CIA-related cytokines and chemokines we performed a proteome profile analysis with a mouse cytokine/chemokine array using cell lysates isolated from paws on day 33 after immunization with CII (figure 1A). CII-treatment (**CII**) significantly elevated the expression (between 1.6 to 32 fold), which is presented as log 2 ratios, of chemokines like C5a, MCP-1, CCL17, Mip-1 α , MIP-1 β , MIP-2 or CXCL12. These chemokines are responsible for the recruitment of immune cells to the inflamed tissue (figure 1B). The largest effect of Dex and SC was detected on CCL17 expression which was nearly reduced to level of control animals by both substances. CII induced protein expression of MCP-1 and Mip-1 α was reduced to 30 % respectively 60 % by Dex and to 40 % respectively 80 % by SC. So in almost all cases the SC-effects were similar to those of Dex. The expression of different pro-inflammatory RA-relevant cytokines, like IFN- γ , IL-1 β , IL-12p70, IL-6 or TNF- α was also increased (1.8 to 10 fold) upon CII treatment (figure 1C). Dex and SC markedly inhibited CII induced IL12p70 expression (reduction to 18 % respectively 22 %). The effect of both substances on TNF- α - or IL-10 production was less pronounced. Dex treatment resulted in a 70 % inhibition of CII induced TNF- α and 50 % inhibition of IL-10 production whereas SC therapy led to a 60 % decrease of TNF- α - and a 40 % inhibition of IL-10 production. Surprisingly, Dex and SC had opposite effects on IFN- γ and IL-1 β expression. Only SC-treatment led to a significant reduction of IL-1 β production (reduction to 30 %), whereas Dex

further increased CII-induced IL-1 β expression (2 fold). Similar results were obtained for IFN- γ . SC did not affect IFN- γ -levels while Dex enhanced IFN- γ -production (6 fold) in the CIA setting.

In summary, SC-treatment of CIA was nearly as effective as the therapy with Dex. SC significantly reduced the expression of many pro-inflammatory genes critically involved in the pathogenesis of CIA and RA.

The expression of markers for synovial joint destruction was decreased by SC

As markers for destruction of cartilage and bone loss in the synovial joint we measured the mRNA levels of the matrix metalloproteinase 3 (MMP3) and the receptor activator of nuclear factor- κ B ligand (RANKL) by quantitative real time RT-PCR (qRT-PCR). On day 33 after the first immunization with CII (**CII**), we detected a 20 fold increase in MMP3 mRNA levels and a 6 fold enhanced RANKL mRNA expression. In CII-immunized mice treated with SC (**CII+SC**) or Dex (**CII+Dex**) the elevated mRNA levels of MMP3 and RANKL were reduced to the level of PBS-treated control mice (**PBS**) (figure 2 A+B).

Microarray-analyses to identify CIA- and SC regulated genes

To identify genes which are regulated by Dex and SC during CIA development we performed microarray experiments using whole genome mouse arrays allowing analysis of the expression of 25.000 genes in parallel. RNA was isolated from the paws of PBS-, CII-, CII+Dex- or CII+SC-treated mice on day 33 and used for labeled cDNA probe preparation.

Immunization with CII significantly altered the expression of 2228 genes at least two fold (≤ 0.05 *P* value threshold) compared to control PBS mice. The analysis of the microarray data obtained after Dex and SC treatment revealed that 100 genes were at least two fold up- or downregulated (≤ 0.05 *P* value threshold) by the treatment with Dex as well as SC compared to CII-immunized mice (data not shown, the array data have been deposited in the

EMBL-EBI ArrayExpress repository - <http://www.ebi.ac.uk/arrayexpress>; experiment name: HK_CIA_experiment). We next focused our microarray data analysis on genes differentially regulated by SC treatment in CII-immunized mice. *In silico* biological process analysis by PANTHER database (Thomas et al., 2003) revealed that 20 genes (figure 3A+B) involved in signal transduction- and immune defense mechanisms were significantly up- or downregulated (log₂ scale) by SC treatment. Amongst others the mRNA expression of cathelicidin antimicrobial peptide (Camp), peptidylprolyl isomerase B (Pbip), defensin beta (Defb6), myeloperoxidase (Mpo) and the calcium binding protein S100A8 (S100A8) was significantly increased (induction 10 to 100 fold) in the paws of the animals upon CII-immunization. In mice injected with SC we detected an approximately 50% reduced expression of those genes whereas Dex treatment seems to be less effective (figure 3A). Furthermore administration of SC reversed the down-regulation of genes like the notch homologue 2 (Notch2), the forkhead box 1 (Foxc1) transcription factor or the autoimmune regulator inhibitor (AIRE) during arthritis development (figure 3B).

In order to confirm the data obtained from our microarray analyses Western blot experiments for the protein expression of selected genes were performed. We detected a marked increase in the protein expression of MPO, Pbip and S100A8 in the paws of CII-treated mice (**CII**), which was significantly reduced by the administration of SC (**CII+SC**) or Dex (**CII+Dex**) (figure 4A). In qRT-PCR experiments we measured a 4 fold upregulation of Notch2 mRNA-levels in SC treated CII-mice. These results confirmed our microarray data. On the other hand treatment of CII-mice with Dex not only showed no reversion of the reduction of Notch2 mRNA-levels but also resulted in an additional repression of this gene (figure 4B).

SC decreased pro-inflammatory gene expression in C28/I2 chondrocytes

To extend our studies to the human system we tested the effect of SC on the mRNA expression of relevant pro-inflammatory genes in human C28/I2 chondrocytes by qRT-PCR. The cells were stimulated with a mixture of pro-inflammatory cytokines (**CM**) and treated with 30 µg/ml SC (**CM+30µg/ml SC**) as described. Compared to control cells the mRNA expression of IL-8, MCP-1, TNF- α and COX-2 was significantly enhanced (minimal 5 - / maximal 100 fold) upon cytokine stimulation and reduced by SC-treatment (reduction to 30 - 60 %; figure 5A).

To study the effect of SC on pro-inflammatory protein expression in C28/I2 cells we used a human proteome profiler. We detected a 15 fold up-regulation of MCP-1 upon cytokine stimulation that was reversed by SC to a level of 10% of CM incubated C28/I2 cells. The cytokine-induced expression of IL-8 (2 fold) and CXCL10 (7 fold) was slightly reduced by SC-treatment (reduction to 90%), whereas the effect on cytokine induced IL-28 (2 fold) and CXCL11 (6 fold) expression was more pronounced. Here we detected a 40% reduction compared to cytokine stimulated control cells (figure 5B). The data are presented as log 2 ratios in comparison to untreated control cells or CM treated cells to evaluate the SC effects. Comparable to the data obtained in the CIA mouse model C5a expression was also significantly reduced upon SC-treatment in CM-stimulated C28/I2 cells. Giemsa staining of C28/I2 cells demonstrated that SC had no pronounced cytotoxic effects in the concentrations used in our experiments (figure 5C). These data showed that SC reduced the expression of different pro-inflammatory cytokines and chemokines also in human C28/I2 chondrocytes.

SC decreased the expression of S100A8 in human MonoMac6 cells

Monocytes and macrophages are critically involved in the pathogenesis of RA and are the major source of the pro-inflammatory marker S100A8. In the human monocyte cell line MonoMac6 pre-treatment with 20 µg/ml SC (**CM+SC**) prevented the 3 fold LPS/CM-induced increase of S100A8 (**CM**) mRNA and protein expression (figure 6A-C). These data

highlighted that SC effects detected in the mouse *in-vivo* model were reproducible in the human system.

In summary, our data demonstrate that SC is a potent inhibitor of pro-inflammatory gene expression in a murine *in-vivo* model of RA as well as in different human cells important for RA disease pathogenesis.

Discussion

We previously demonstrated that the fungal macrocyclic lactone *S*-Curvularin (SC) reduced the expression of the pro-inflammatory enzyme iNOS, by inhibition of the JAK-STAT pathway (Yao et al., 2003). In addition we observed inhibition of NF- κ B dependent promoter activity by SC in human A549/8 cells (data not shown). As the JAK-STAT - and the NF- κ B signal pathways play a pivotal role in the initiation and progression of inflammation and recruitment of immune cells, we tested the effect of SC in the murine CIA model.

Chemokines promote CIA and RA by recruitment of immune cells to the synovial tissue. For example, the complement component 5a (C5a) attracts neutrophils and macrophages to the synovium (Wang et al., 1995; Grant et al., 2002). Beside C5a the expression of other important chemokines like MCP-1, Mip-1 α , Mip-1 β , Mip-2, CXCL12 or CCL17 was inhibited by SC-treatment. These effects were similar to those observed in the Dex-treated control group (figure 1B). These data imply that SC may improve CIA or RA by diminishing the recruitment of immune cells to the inflamed joint.

The imbalance of pro- and anti-inflammatory cytokines promotes chronic inflammation and destruction of the joints in the CIA model and in RA. In consistence, we detected a significant increase of pro-inflammatory cytokines upon immunization with CII. Our data demonstrated that SC decreased the expression of these cytokines (figure 1C). Beside IFN- γ and IL-1 β , the effects observed with SC were similar to the Dex-mediated effects.

Surprisingly, Dex-intervention elevated the protein amount of IFN- γ and IL-1 β in the paws. Increase of IL-1 β expression after treatment with high doses of glucocorticoids has also been described in human PBMC (Markova et al., 2007).

TNF- α is a key mediator of inflammation in the joints by stimulating the production of further pro-inflammatory cytokines like IL-1, IL-8, IL-6 or GM-CSF or different chemokines. The significant down-regulation of TNF- α expression by SC might account for the reduction of the expression of a number of cytokines and chemokines seen in our experiments (figure 1C). The development of drugs, that block the activity of TNF- α , IL-1 and IL-6 has been a major clinical advance in the treatment of RA indicating the benefit of therapeutic strategies to modulate pro-inflammatory signal pathways. (Brennan and McInnes, 2008). An important hallmark of RA is the destruction of cartilage and bone. SC inhibited the expression of RANKL, which regulates osteoclast differentiation, maturation, and induction of resorptive activity. The expression of RANKL is regulated by inflammatory cytokines and mediators such as TNF- α (Karmakar et al., 2010). In addition we found that SC inhibited the expression of MMP9 (data not shown) and MMP 3, enzymes that degrade cartilage and non-collagen matrix components of the joint (figure 2 A+B). These results indicate that SC may improve the disease by reducing the expression of proteins involved in degenerative processes in the joint.

In total genome microarray experiments we detected considerable differences in the effects of SC and Dex on CII-modulated gene expression indicating that SC- and Dex-induced effects are mediated by different pathways. A more detailed analysis revealed that SC-treatment primarily affected the expression of genes involved in signal- and immune-processes and therefore we focused on those genes whose expression was regulated by CII and SC in an opposite manner (figure 3A+B).

Peptidylprolyl cis-trans isomerase B (Ppib, Cyclophilin B) is upregulated by CII in V β -12 mice and was down-regulated by SC-treatment (figure 3A+4A). In the articular cartilage

Ppib is secreted by chondrocytes and its release is mediated by matrix metalloproteinases (De Ceuninck et al., 2003). Ppib has been shown to induce efficiently chemotaxis of human neutrophils and T-cells (Pakula et al., 2007) and down-regulation of Ppib expression by SC could ameliorate CIA or RA symptoms by inhibiting the recruitment of immune cells.

Myeloperoxidase (MPO) has pro-inflammatory and pro-oxidative properties and is expressed in different immune cells. In inflamed tissues the enzyme can be released to the extracellular space resulting in enhanced production of reactive oxygen species (ROS) and increased oxidative stress. ROS, as a second messenger, can activate NF- κ B and thus promotes the onset and progression of arthritis (Miesel et al., 1996). In chronic inflammatory joint diseases elevated levels of MPO were detected (Maki-Petaja et al., 2008; Feijoo et al., 2009). In the study of Maki-Petaja et al. MPO levels correlated positively with the amount of the inflammation marker C-reactive protein and iNOS activity and negatively with the endothelial function (Maki-Petaja et al., 2008). This data indicate that the inflammatory and oxidative processes in RA trigger the development of endothelial dysfunction and thereby may promote the cardiovascular risk of RA patients. Our data showed that SC- and Dex-treatment significantly reduced MPO mRNA and protein expression in the CIA model (figure 3A+4A). Thus SC-mediated decrease of MPO expression may result in reduced oxidative stress during RA.

The highly conserved Notch signaling pathway plays a critical role in cell proliferation, differentiation and apoptosis (Baron, 2003). Notch2 expression in the synovium of RA patients has been demonstrated, but the role of this protein in the context of RA is poorly understood (Ishii et al., 2001). One report linked Notch2 signaling with the induction of apoptosis in chondrocytes (Hattori et al., 2005). Moreover, an altered expression of Notch2 was detected in T-helper cells of RA patients with an active disease (Jiao et al., 2010). So modification of Notch2 expression by SC may have beneficial effects in RA therapy.

Unexpectedly, in case of dexamethasone the qRT-PCR analyses do not reflect the microarray data on Notch2 mRNA expression (figure 3B + 4B).

The S100 calcium binding proteins (S100A8, S100A9, S100A12) are primarily expressed by neutrophils, monocytes and activated macrophages and mediate pro-inflammatory and chemotactic effects partly by amplifying NF- κ B-dependent gene expression (Ryckman et al., 2003; Sunahori et al., 2006). S100A8 and S100A9 form heterodimers and elevated levels of these complexes were detected in the synovial fluid and serum of RA patients (Frosch et al., 2000; Bovin et al., 2004). In addition, a positive correlation of S100A8 expression with the severity of arthritis symptoms was described in RA patients as well as in the mouse model of antigen induced arthritis (Sunahori et al., 2006); (van Lent et al., 2008). Deletion of the gene coding for S100A9 also abrogates the expression of S100A8. In S100A9^{-/-} mice, a significant reduction of the swelling of the paws as well as an inhibition of MMP-mediated degradation of articular cartilage was detected (van Lent et al., 2008). As S100A8 seems to be important for the pathogenesis of CIA and RA, downregulation of the protein by SC-treatment (figure 4A) in combination with reduced expression of other mediators (see above) may have a significant effect on the course of the disease.

We could translate our data from the CIA-mouse model to human immortalized C28/I2 chondrocytes, where SC treatment efficiently reduced the cytokine-stimulated expression of various pro-inflammatory mediators. This demonstrates the anti-inflammatory efficacy of SC also in a RA-relevant human cell model (figure 5A). We found that SC inhibited the synthesis of the chemotactic factors C5a and MCP-1 in CM-induced C28/I2 cells (figure 5B) corroborating the results of the murine proteome profile analyses. These data indicate that SC-treatment in the human disease may also efficiently inhibit the recruitment of immune cells to the inflamed tissue. We previously described that C28/I2 cells are resistant to glucocorticoid-treatment, due to lack of GR α expression (Schmidt et al., 2010). In contrast, SC markedly reduced pro-inflammatory gene expression in these cells and this clearly

indicates that SC-mediated effects are not mediated via glucocorticoid signal transduction pathways.

Recent studies have shown that S100A8 expression is strongly enhanced in the synovial fluid of RA patients and could serve as an early biomarker for the onset of the disease (Liao et al., 2004; Baillet et al., 2010). It has been described that glucocorticoids increases S100A8 expression in human macrophages (Hsu et al., 2005). In contrast, SC inhibited the expression of this important marker in the human monocyte cell line MonoMac6 (figure 6A-C).

Furthermore, we have no evidence for major adverse effects of SC. Mice treated with SC neither show a loss of weight nor abnormalities in cytochrome P450 expression (data not shown). Also Giemsa staining of C28/I2 cells demonstrated that the reduced cytokine and chemokine expression was not due to cytotoxic effects of SC (figure 5C). According to data in the literature myelosuppressive side effects could be expected through inhibition of the JAK2-STAT1 signal transduction pathway (Purandare et al., 2012). Whether this occurs in SC treated mice must be investigated in further experiments.

In summary our study demonstrated that in the CIA mouse model the fungal compound SC reduced the expression of pro-inflammatory genes nearly as effective as the established glucocorticoid Dex. Moreover, we provide evidence that SC is also effective in a glucocorticoid-resistant human cellular model. Altogether, SC may serve as a promising lead-structure for the development of new therapeutics for the treatment of chronic inflammatory diseases, such as RA or inflammatory bowel disease. However it will be important to describe efficacy of SC using a therapeutic dosing regime in future experiments.

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Authorship Contributions

Participated in research design: Erkel, Kleinert, Pautz

Conducted experiments: Schmidt, Art, Forsch, Werner, Jung

Contributed new reagents or analytic tools: Erkel

Performed data analysis: Schmidt, Pautz, Kleinert, Erkel

Wrote or contributed to the writing of the manuscript: Pautz, Kleinert, Erkel, Horke

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Footnotes

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Figure legends

Figure 1: Analysis of the expression of pro-inflammatory mediators in the CIA model. TCR- β tg-mice were immunized with 200 μ g collagen II (CII) in complete CFA. A booster injection of 100 μ g CII was given on day 11 and 21. PBS-injected mice served as control group. From day 21 on CII-immunized mice were treated with 5 mg/kg dexamethasone (CII+Dex) or with 10 mg/kg (*S*)-Curvularin (CII+SC) or PBS/10% ETOH as solvent control (CII) each second day by intraperitoneal (i.p.) injection. For the “mouse cytokine array” proteome profile analyses paws of PBS, CII, CII+Dex or CII+SC treated animals (eight animals per group) were taken on day 33 after immunization for protein isolation. The results are expressed as log 2 ratios of relative protein levels of CII treated mice versus PBS injected control mice (black bars) or CII+Dex vs. CII treated (grey bars) or CII+SC vs CII treated mice (white bars). **A:** The illustration shows a representative part of the membranes used for the proteome profiler analysis. **B:** The data show quantitative protein analyses of significantly regulated chemokines. The data (means + SEM) are presented as log 2 ratios in comparison to PBS- or CII-injected mice. (***: $p < 0.001$ vs. PBS-treated mice; ###: $p < 0.001$ vs. CII-induced mice). **C:** The data show protein analyses of significantly regulated cytokines. The data (means + SEM) are presented as log 2 ratios in comparison to PBS- or CII-injected mice. (*: $p < 0.05$; ***: $p < 0.001$ vs. PBS-treated mice; #: $p < 0.05$; ##: $p < 0.01$; ###: $p < 0.001$; ns#: not significant vs. CII-induced mice).

Figure 2: The mRNA expression of RANKL and MMP3, both markers for joint destruction, was measured in paws of PBS, collagen II (CII), CII + dexamethasone (CII+Dex), or CII + (*S*)-Curvularin (CII+SC) treated mice on day 33 after the first immunization. Each treatment group contained eight mice. **A:** The data show the relative RANKL mRNA expression in

paws of PBS, CII, CII+Dex and CII+SC treated mice. Data represent the relative RANKL mRNA expression (+ SEM) compared to CII-treated mice (**: $p < 0.01$; vs. CII-treated mice). **B:** qRT-PCR analyses were performed using the same RNAs as described above. The data (means + SEM) represent relative MMP3 mRNA expression (***: $p < 0.001$ vs. CII-induced mice).

Figure 3: To analyze the effects of collagen II (CII) treatment as well as the CII + dexamethasone (CII+Dex) or CII + (*S*)-Curvularin (CII+SC) treatment on total genomic gene expression DNA microarray analyses were performed. RNA of all four paws of an animal (four animals for each treatment group, day 33) was isolated and pooled. This RNA was used for the labeling reaction. **A:** The data show the relative RNA-expression of genes involved in signal transduction and immunity response, which are significantly upregulated by CII treatment (CII vs. PBS, black bars) and downregulated after SC-treatment (CII+SC vs. CII, white bars). Also the effect of Dex (CII+Dex vs CII, grey bars) treatment is indicated. The values are presented as log 2 ratios in comparison to untreated PBS or CII immunized mice. **B:** Data show the relative RNA-expression of genes involved in signal transduction and immunity response, which are significantly upregulated after SC-treatment (CII+SC vs. CII, white bars) in comparison to CII-immunized mice (CII vs PBS, black bars). Also the effect of Dex (CII+Dex vs. CII, grey bars) treatment is indicated. The values are presented as log 2 ratios in comparison to untreated PBS or CII immunized mice.

Figure 4: Western Blot and qRT-PCR experiments were performed to verify the results of the microarray analyses. To investigate the expression of genes regulated by (*S*)-Curvularin (SC) in paws of CIA mice, RNA or protein was isolated on day 33 after immunization. Each treatment group contained eight mice. **A:** Western blots were performed using specific anti-Ppib-, anti-MPO-, anti-S100A8- and anti-p38 MAPK antibodies and protein extracts from

paws of PBS, collagen II (CII), CII + dexamethasone (CII+Dex)- and CII+SC treated TCR- β tg-mice. The blots are representative of two other blots showing similar results. **B:** qRT-PCR using RNA of paws of PBS, CII, CII+Dex and CII+SC treated TCR- β tg-mice are shown. Data represent the relative Notch2 mRNA expression (+ SEM) compared to CII-treated mice (***: $p < 0.001$; *: $p < 0.05$; ns: not significant vs. CII-treated mice).

Figure 5 The data demonstrate the effect of (*S*)-Curvularin (SC) on the expression of selected pro-inflammatory genes in CM (IL-1 β + IFN- γ + TNF- α) stimulated C28/I2 cells. **A:** For qRT-PCR experiments C28/I2 cells were pretreated with 30 μ g/ml SC before cytokine stimulation. A summary of three independent experiments is shown using RNA of C28/I2 cells. Data represent the relative mRNA expression (+ SEM) compared to CM-stimulated cells (***: $p < 0.001$; **: $p < 0.01$; vs. CM-induced cells). **B:** To examine the effect of SC (50 μ g/ml) on cytokine- and chemokine-production in C-28/I2 cells stimulated for 16 h with CM, human proteome profile analyses were performed. Values are expressed as log 2 ratios of relative protein levels of CM cells versus non-stimulated cells (black bars) and CM versus CM+SC treated C-28/I2 cells (grey bars). The data (means + SEM, n=3) represent relative protein amounts of significantly regulated cytokines and chemokines (***: $p < 0.001$ vs. non-stimulated C28/I2 cells; ###: $p < 0.001$ vs. CM stimulated C28/I2 cells). **C:** Giemsa staining of C28/I2 cells incubated with 0, 10, 30, 50, 70 or 100 μ g/ml SC for 24 h. The data (means +/-SEM) represent relative cell viability compared to non-treated control cells (***: $p < 0.001$; ns: not significant vs. non-treated C28/I2 cells).

Figure 6: The data show the effect of 20 μ g/ml (*S*)-Curvularin (CM+SC) on the expression of the pro-inflammatory mediator S100A8 in MonoMac6 cells stimulated with a mixture of LPS, TNF- α , IL-1 β and IFN- γ (CM). Non-stimulated cells were used as control (CO). **A:** A summary of three independent experiments demonstrated the relative S100A8 mRNA

expression (+ SEM) compared to CM-stimulated MonoMac6 cells (*: $p < 0.05$; vs. CM-induced cells). **B:** For western blot analyses MonoMac6 cells were treated as described above and total cell extracts (200 μ g protein) were blotted with a specific anti-human S100A8- and an anti-GAPDH antibody. The blot is representative for two other blots. **C:** The data show quantitative analyses of S100A8 protein expression in MonoMac6 cells. The data (means + SEM) represent relative protein amounts (**: $p < 0.01$ vs. CM induced MonoMac6 cells).

Figure 1

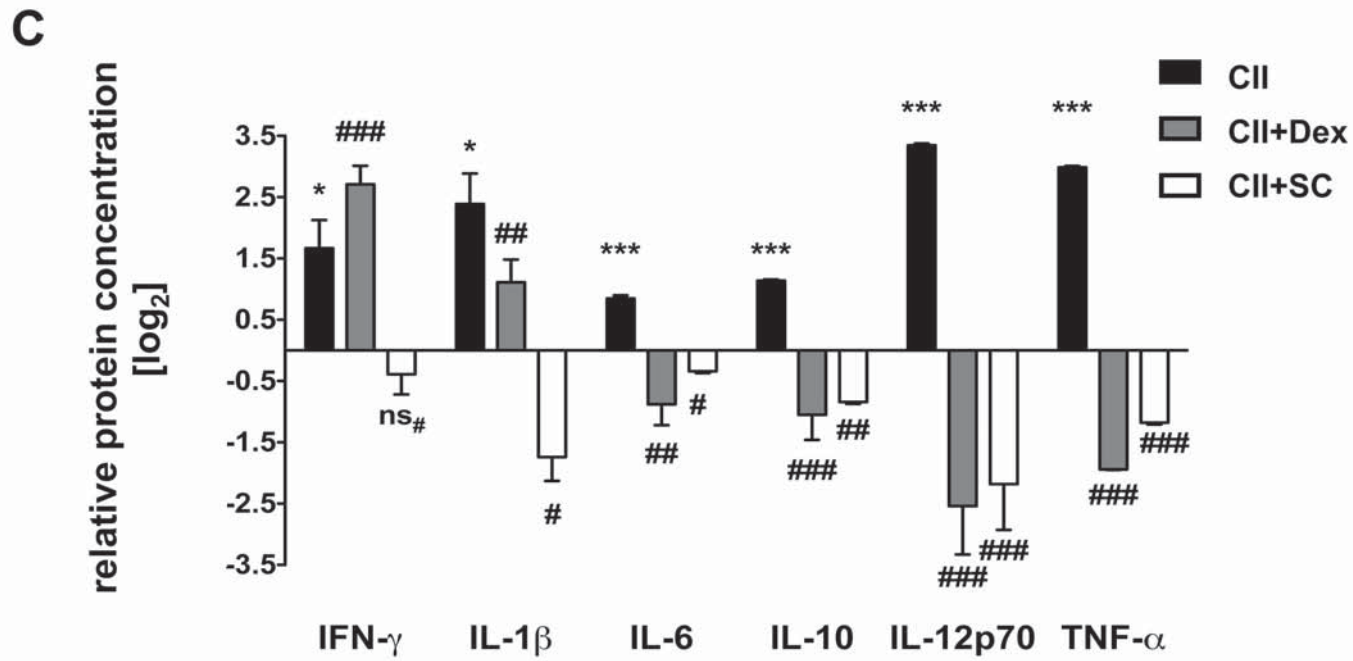
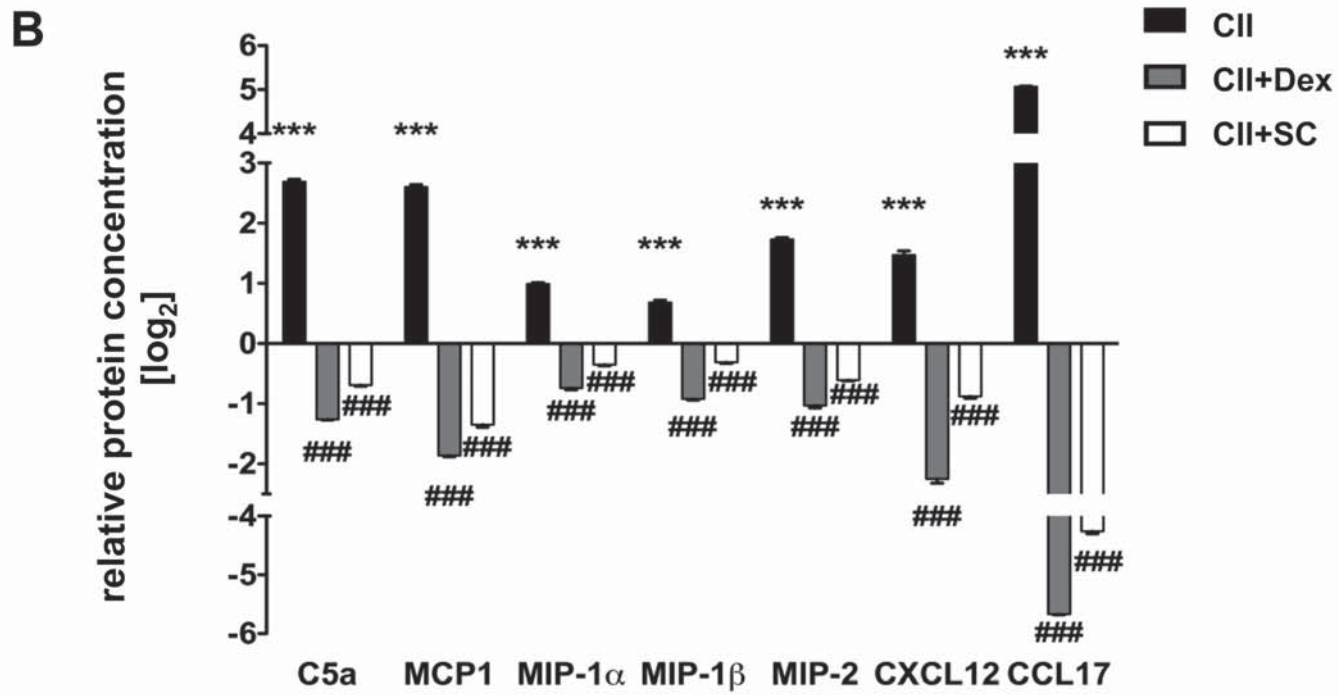
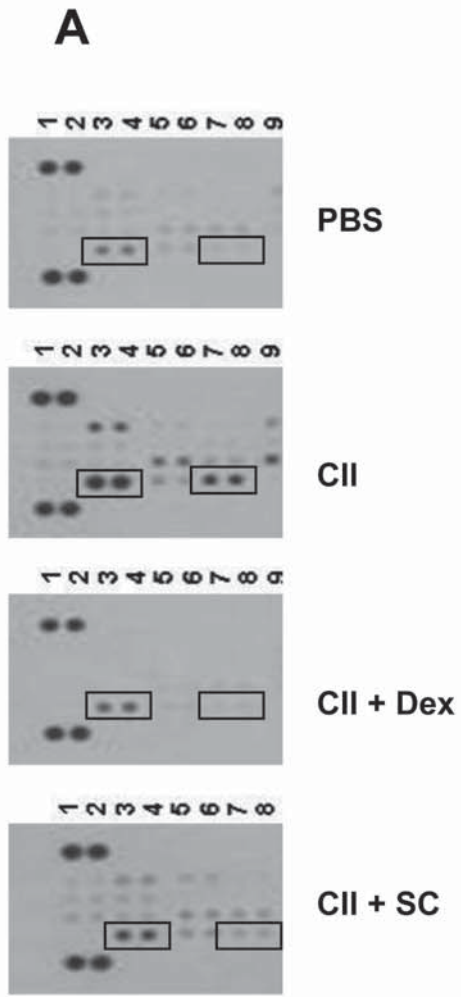
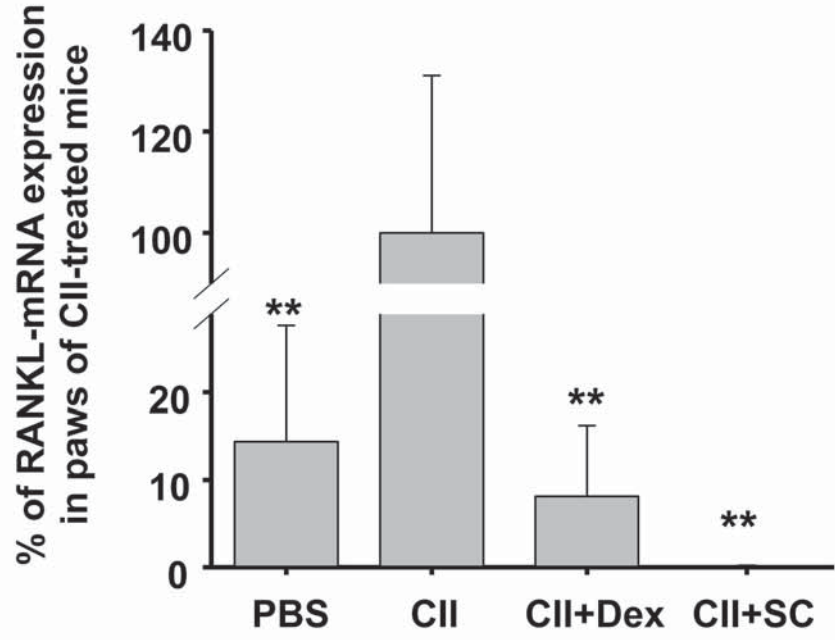


Figure 2

A



B

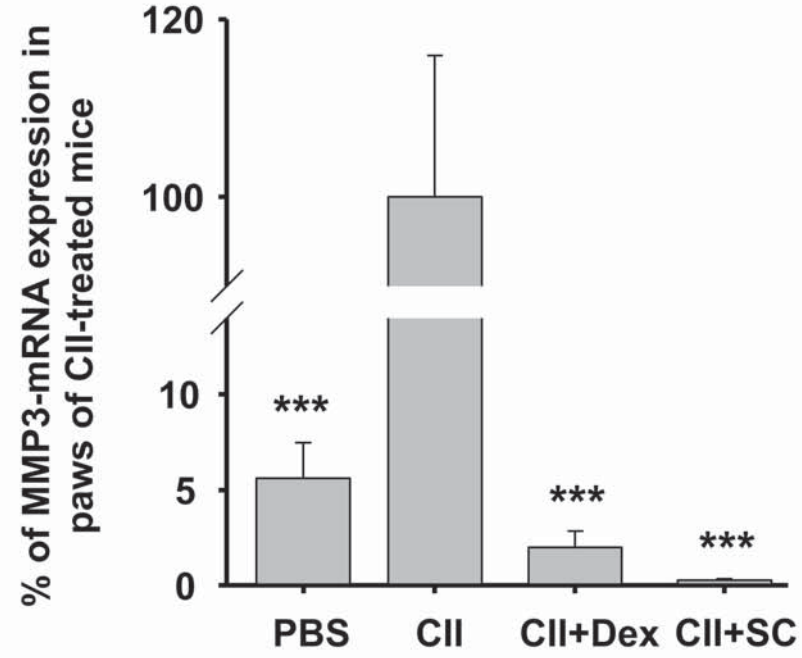


Figure 3

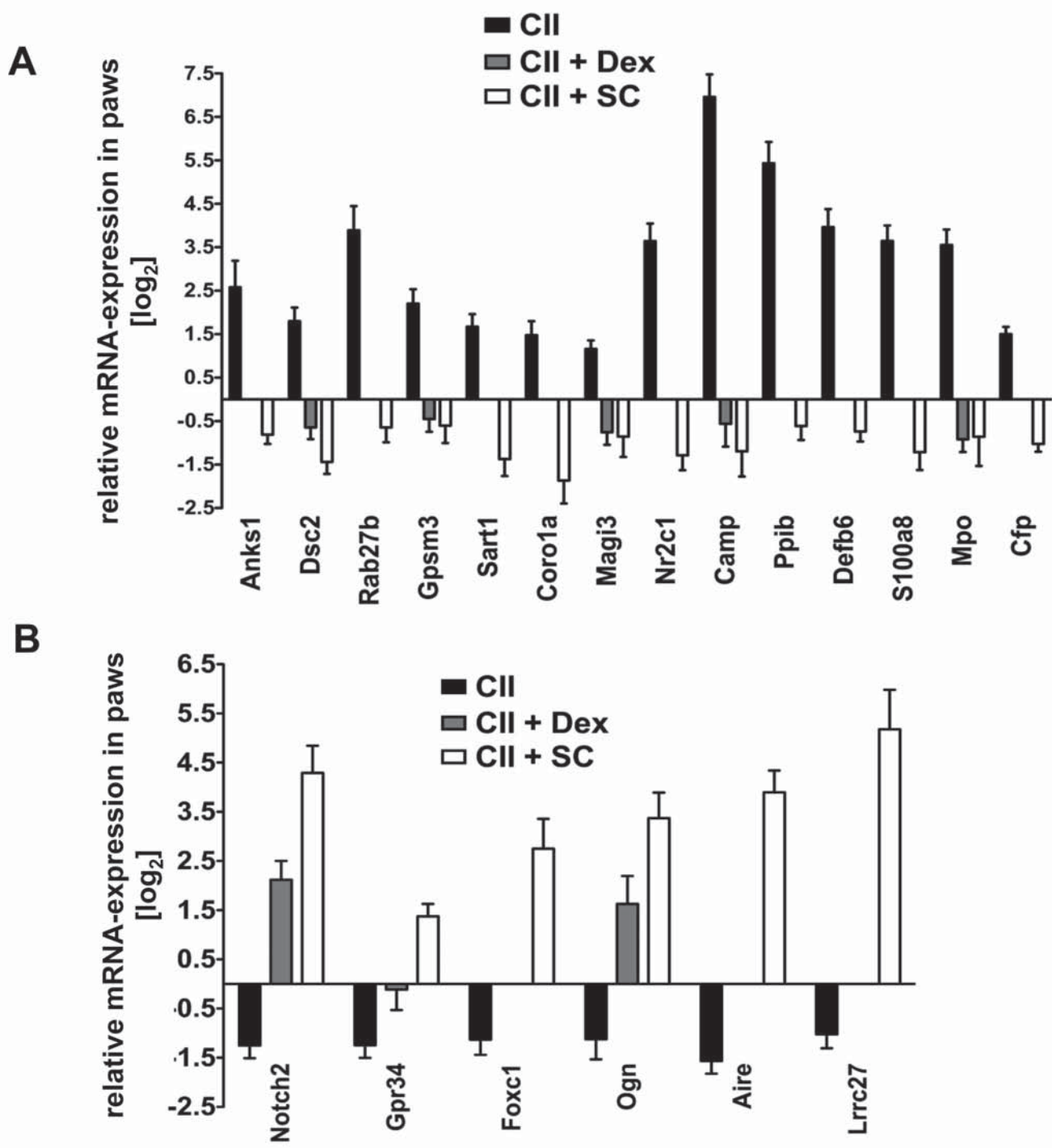


Figure 4

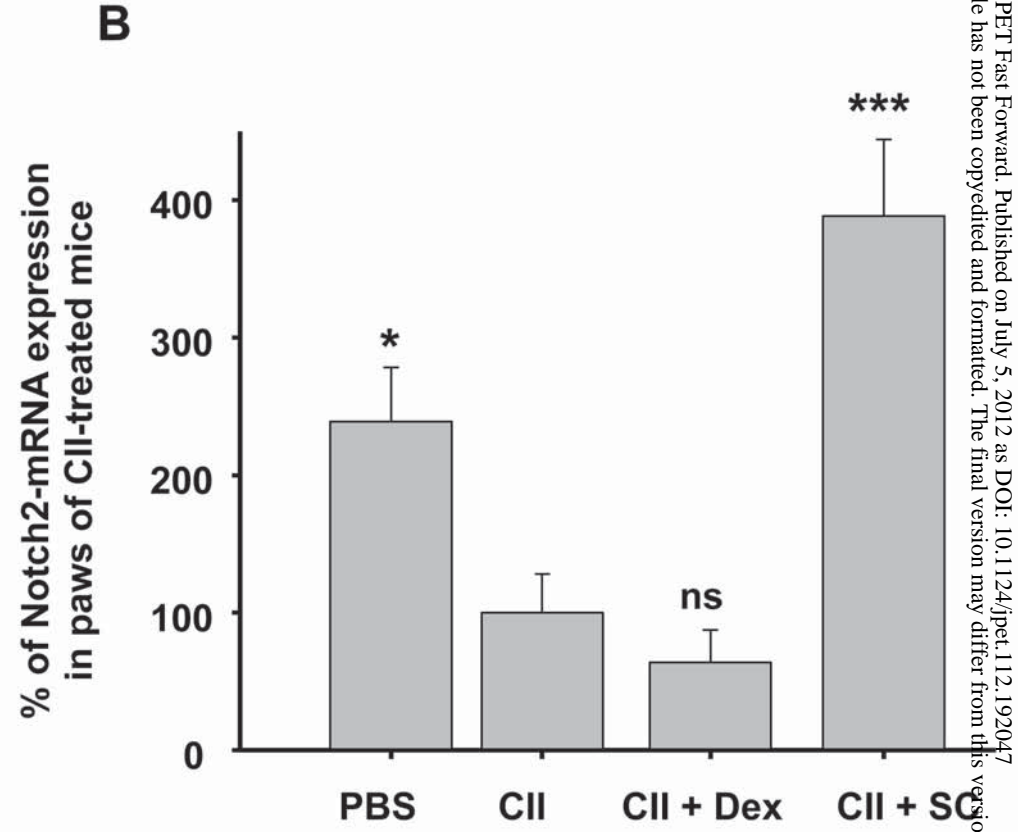
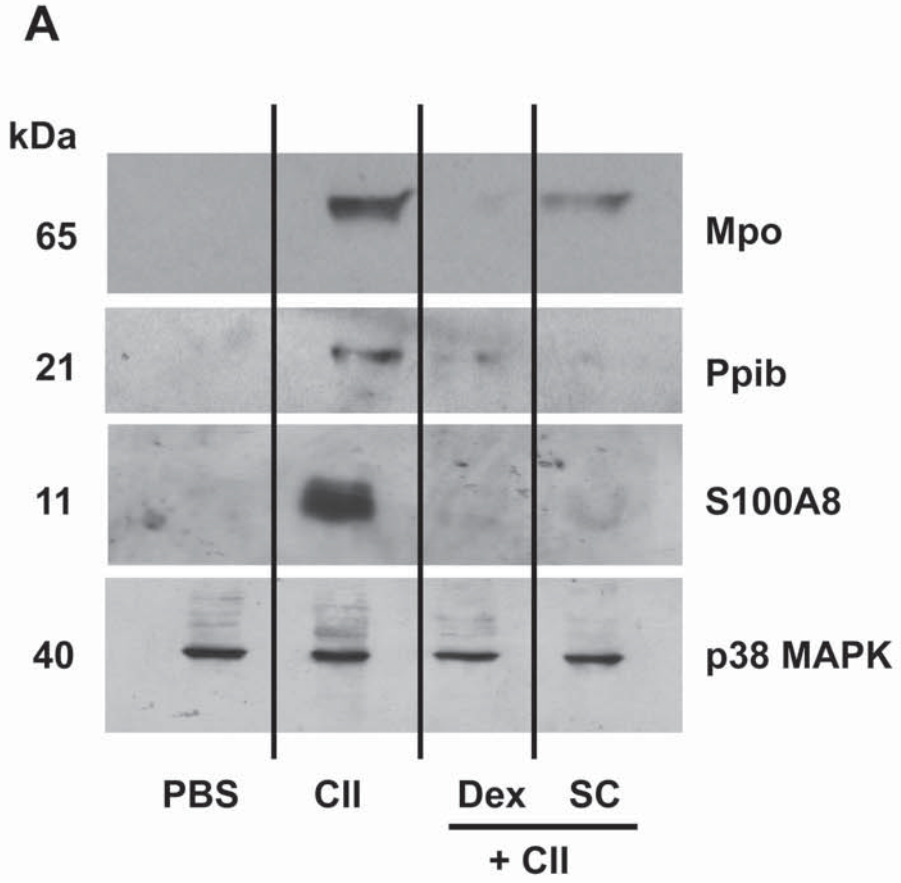


Figure 5

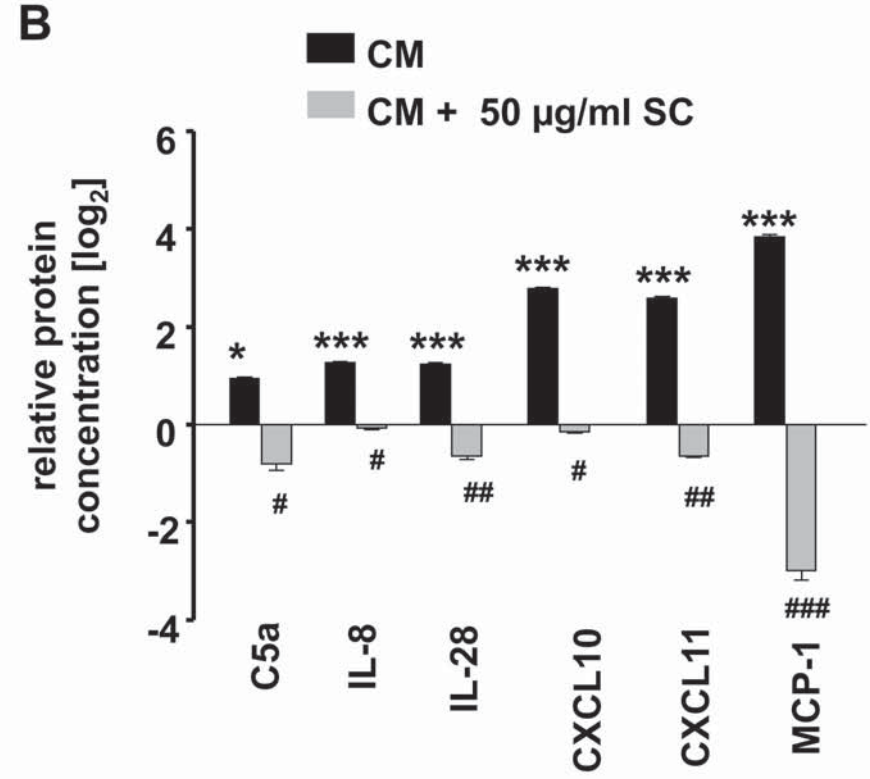
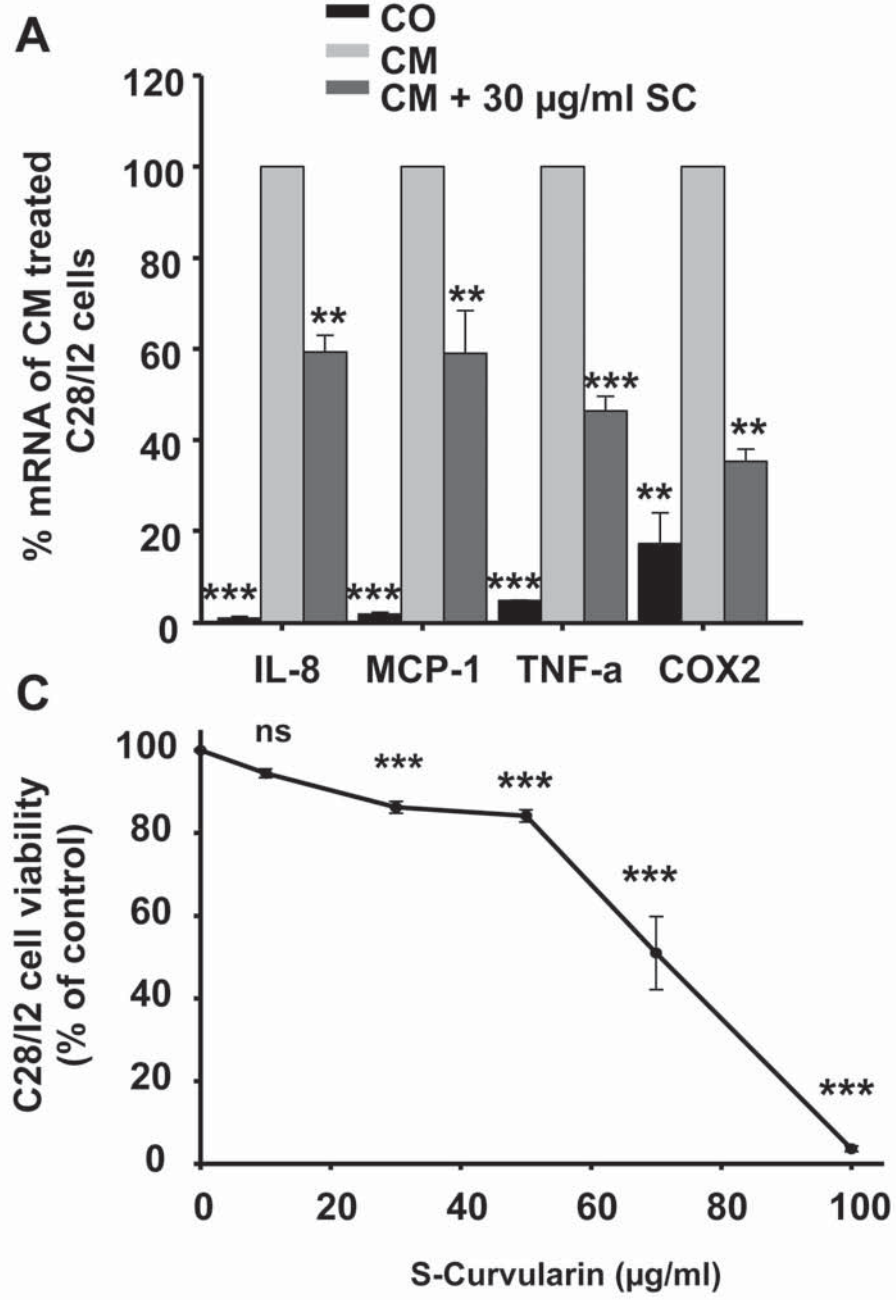
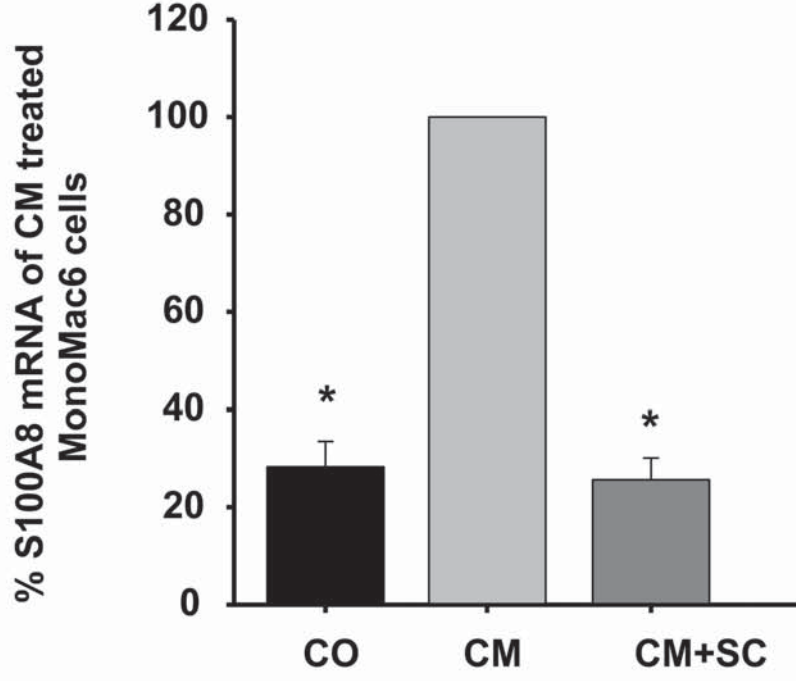
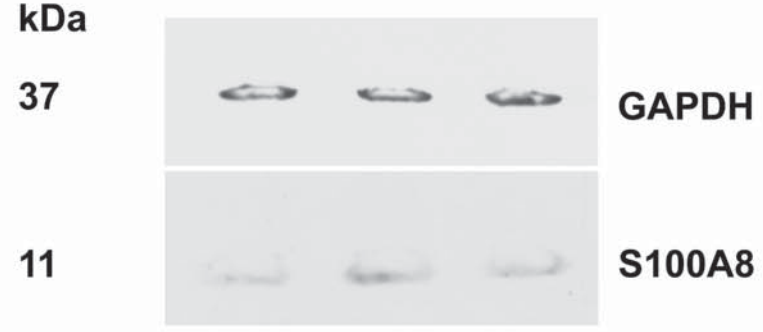


Figure 6

A



B



C

