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Transcriptional and post-transcriptional regulation of iNOS expression in human chondrocytes

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Category: Inflammation and Immunopharmacology

Abstract

1
2 Chondrocytes are important for the development and maintenance of articular cartilage.
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4 However, both in osteoarthritis (OA) and rheumatoid arthritis (RA) chondrocytes are
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6 involved in the process of cartilage degradation and synthesize important immunomodulatory
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8 mediators, including nitric oxide (NO) generated by the inducible NO synthase (iNOS). To
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10 uncover the role of iNOS in the pathomechanisms of OA and RA, we analyzed the regulation
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12 of iNOS expression using immortalized human chondrocytes as a reproducible model.
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16 In C-28/I2 chondrocytes, iNOS expression was associated with the expression of the
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18 chondrocyte phenotype. Peak induction by a cytokine cocktail occurred between 6 and 8 h
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20 and declined by 24 h. Inhibition of p38MAPK, NF- κ B and the JAK2-STAT-1 α pathways
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22 resulted in a reduction of iNOS expression. In contrast to other cell types, the cytokine-
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24 mediated induction of the human iNOS promoter paralleled the induction rate of the iNOS
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26 mRNA expression in C-28/I2 chondrocytes. However, in addition post-transcriptional
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28 regulation of iNOS expression by the RNA binding protein KSRP seems to operate in these
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30 cells. As seen in other chondrocyte models, glucocorticoids were not able to inhibit cytokine-
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32 induced iNOS expression in C-28/I2 cells, due to the lack of the glucocorticoid receptor
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34 mRNA expression. In this model of glucocorticoid-resistance, the new fungal anti-
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36 inflammatory compound S-curvularin was able to inhibit cytokine-induced iNOS expression
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38 and iNOS-dependent NO production.
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46 In summary, we demonstrate for the first time that differentiated human immortalized C-28/I2
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48 chondrocytes are a representative cell culture model to investigate iNOS gene expression in
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50 human joint diseases.
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55 **Key words:** chondrocytes, iNOS, glucocorticoids, NF- κ B, JAK-STAT, KSRP
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Abbreviations: AG490, N-Benzyl-3,4-dihydroxybenzylidenecyanoacetamide; ARE, AU-rich element; Bay11-7082, E-3-(4-Methylphenylsulfonyl)-2-propenenitrile; CM, cytokine mixture; CO, control/untreated cells; COL2A1, collagen, type II-alpha 1; C(T), cycle threshold; Dex, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular regulated kinase; FCS, fetal calf serum; GC, glucocorticoid, GCR, glucocorticoid receptor; IL-1 β , interleukin-1 β ; IFN- γ , interferon- γ ; iNOS, inducible nitric oxide synthase; JAK, janus kinase; JNK, Jun N-terminal kinase; KSRP, KH-type splicing regulatory protein; MAPK, mitogen activated protein kinase; NF- κ B, nuclear factor- κ B; NO, nitric oxide; NOS, NO synthase; OA, osteoarthritis; PD98059, 2'-amino-3'-methoxyflavone; Pol2a, large subunit of RNA polymerase II; Pred, prednisolone; qRT-PCR, quantitative reverse transcription polymerase chain reactions; RA, rheumatoid arthritis; RNA-BP, RNA binding protein; SB203580, 4-(4'-fluorophenyl)-2-(4'-methylsulfinylphenyl)-5-(4'-pyridyl) imidazole; SOX9, SRY (sex determining region Y)-box 9; SP600125, (Antra[1,9-*cd*]pyrazol-6(2*H*)-one1,9-pyrazoloanthrone); STAT-1 α , signal transducer and activator of transcription-1 α ; TNF- α , tumor necrosis factor- α ; UTR, untranslated region; WB, Western blot

1. Introduction

Nitric oxide (NO), generated by the inducible nitric oxide synthase (iNOS), fulfills a great variety of physiological and pathophysiological functions. Aberrant iNOS expression plays an important role in human inflammatory diseases such as asthma, multiple sclerosis, colitis, psoriasis, neurodegenerative diseases, transplant rejection, septic shock and tumor development [1-3]. Also in osteoarthritis (OA) and rheumatoid arthritis (RA), the induction of iNOS expression and iNOS-generated NO seems to be crucially involved in the pathomechanisms of these diseases [4, 5].

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In human cells, the induction of iNOS expression needs a complex cytokine mixture including interferon- γ (IFN- γ), interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) [6]. The signaling pathways responsible for induction of iNOS expression vary in different cells and species [6]. In human cells, the activation of the mitogen activated protein kinase (MAPK) pathways (p42/44 MAPK, p38MAPK, JNK), the janus kinase 2-signal transducer and activator of transcription-1 α (JAK2-STAT-1 α) pathway and the nuclear factor- κ B (NF- κ B) pathway is important for increasing iNOS expression. Consecutively, the induction of iNOS can be inhibited by a wide variety of immunomodulatory compounds acting at different levels of the signaling pathways involved [6]. Further, there are reports describing the regulation of iNOS expression by NO itself. The mechanisms involve, for example, NO-dependent inhibition of NF- κ B activity, STAT-1 α activity, or upregulation of p53 activity and thereby inhibition of the iNOS promoter [6].

Transcriptional as well as post-transcriptional mechanisms are critically involved in the regulation of human iNOS expression [6]. In most human cells analyzed there is a great discrepancy between human iNOS promoter inducibility (maximally up to 10-fold) and the induction rate of iNOS mRNA expression (more than 100-fold) [6]. Also basal iNOS promoter activity without appreciable iNOS mRNA expression has been described [7, 8]. In addition, a much larger 5'-flanking sequence of the human iNOS gene (more than 3.6 kb) [7] is necessary to obtain a measurable cytokine-mediated induction of the promoter activity than needed for full functional promoter activity in the murine system (around 1 kb) [9]. In most human cells, binding of the transcription factors NF- κ B and STAT-1 α to their cognate sequences in the human iNOS promoter (located around -5.5 kb)[10, 11] is central for induction of iNOS promoter activity. Moreover in human coloncarcinoma DLD-1 cells, the regulation of iNOS expression markedly depends on the activity of RNA binding proteins (RNA-BP) modulating iNOS mRNA stability. A complex network of RNA-BP with a central

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role of the KH-type splicing regulatory protein (KSRP) has been shown to be essential for the post-transcriptional regulation of iNOS expression [8, 12-14].

In the literature, the role of iNOS in joint diseases is controversially discussed [4, 5]. Analyses in iNOS-deficient mice have shown that the iNOS pathway plays an essential role in IL-1 β -induced bone destruction in RA [15]. In experimental OA model in dogs, the selective inhibition of iNOS activity by the specific inhibitor N-NIL attenuated OA disease progression [16]. However, in a murine OA-model, the deletion of the iNOS gene enhanced OA severity rather than reducing it [17]. Therefore, the exact role of iNOS-generated NO in joint diseases is not clear.

Chondrocytes are crucially involved in the development, growth, and maintenance of articular cartilage [18], but they also play an important role in the process of cartilage degradation seen in human joint diseases like OA or RA [5, 19]. In adult human articular cartilage, the chondrocytes display low metabolic activity with low turnover of matrix components [5]. However, under stress conditions present in OA or RA, chondrocytes are able to produce and release a variety of cytokines such as TNF- α or IL-1 β , metalloproteases, NO, prostaglandins and other mediators, all of which are associated with inflammation and can accumulate at high local concentrations in the joint fluids and tissues. In addition, chondrocytes express receptors for some of these mediators, which therefore may act in an autocrine/paracrine way to regulate the metabolic activity in these cells [5, 19].

Since chondrocytes from different donors may exhibit variable responses in the regulation of iNOS, we decided to analyze iNOS expression in a reproducible model of human immortalized C-28/I2 chondrocytes [20, 21]. These cells were derived from primary human chondrocytes derived from juvenile costal cartilage, by retroviral transduction with the SV40 large T antigen. In contrast to primary chondrocytes, C-28/I2 cells proliferate continuously without loss of chondrocytic morphology [20, 21].

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C-28/I2 chondrocytes were grown as monolayer and alginate cultures for different time periods. To induce iNOS expression, the cells were incubated with a cytokine mixture known to induce iNOS expression in other human cell types. To examine the signaling pathways responsible for iNOS induction in C-28/I2 chondrocytes, selective inhibitors were used. In addition, transcriptional and post-transcriptional mechanisms regulating iNOS expression were analyzed. As glucocorticoids have been described to have no effects on gene expression in human primary or hMSC-derived chondrocytes [22], the effects of dexamethasone and prednisolone on iNOS expression in C-28/I2 cells were analyzed.

The current study aimed to analyze the regulation of iNOS expression in stabilized human C-28/I2-chondrocytes and to elucidate the effects of the new anti-inflammatory compound S-Curvularin (SC) in this cell model.

2. Material and Methods

2.1. Materials

Trypsin-, glutamine-, and pyruvate-solutions, BSA, horseradish-peroxidase-coupled anti-mouse IgG, sodium alginate, anti-tubulin antibodies, prednisolone and dexamethasone were purchased from Sigma (Deisenhofen, Germany). All oligonucleotides and dual labeled probes were from MWG Biotech (Ebersberg, Germany). Human IFN- γ , IL-1 β and TNF- α were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). FCS and DMEM were purchased from PAN-Systems (Nürnberg, Germany). SB203580, AG490, SP600125, PD98059, Bay11-7082 were purchased from Calbiochem (Darmstadt, Germany). The Bradford reagent mix for determination of protein concentration was obtained from BioRad (Munich, Germany). The FuGENE transfection reagent was obtained from Roche Diagnostics, Mannheim, Germany. Zeocin and psiRNAhH1-GFPzeo were purchased from InvivoGen, San Diego, U.S.A. pcDNA3 and the Flp-In system were purchased from

Invitrogen, Groningen, The Netherlands. Restriction enzymes, Klenow DNA polymerase, dNTPs, and NTPs were from New England Biolabs, Frankfurt a.M., Germany. The monoclonal anti-human iNOS antibody was purchased from R&D systems (Wiesbaden, Germany) and the monoclonal anti-tubulin antibodies were from Sigma. The eucaryotic expression vector pEGFP-C1 coding for an enhanced green fluorescent protein was purchased from Takara/Clontech, St-Germain-en-Laye, France. The monoclonal anti-KSRP antibody and the eucaryotic expression vector coding for a His-tagged KSRP protein (pcDNA3.1-His-KSRP) were a kind gift of Dr. Douglas L. Black (Howard Hughes Medical Institute at UCLA, Los Angeles, USA). S-curvularin was isolated from the culture fluids of *Penicillium* strain 48-93, by bioactivity-guided fractionation using standard chromatographic methods as described before [23].

2.2. Cell culture, cytokine treatment, RNA isolation and nitrite measurement

Human epithelial A549/8 carcinoma and EA.hy 926 endothelial cells were cultured as described [24, 25]. C-28/I2 cells are a human immortalized chondrocyte cell line obtained after clonal expansion of the T/C-28 cells [20, 21]. These cells were grown in monolayer or alginate cultures at 37°C in a humidified gas mixture containing 5% CO₂. For monolayer cultures C-28/I2 chondrocytes were grown in DMEM with 2 mM L-glutamine, penicillin and streptomycin, 1 mM sodium pyruvate and 10 % heat-inactivated fetal bovine serum for the time points indicated. For culturing in alginate beads, confluent C-28/I2 monolayer cultures were treated with trypsin-EDTA, washed with phosphate buffered saline (PBS), and resuspended in a filter-sterilized solution of 1.2% (weight/volume) sodium alginate in 150 mM NaCl (4 x 10⁶ cells/ml alginate solution). The cell suspension was passed drop-wise through a 22-gauge needle into a 102 mM CaCl₂ solution. Following polymerization for 10

1 min, beads were washed with PBS and cultured in the same medium as used for the
2 monolayer cultures.
3

4 Eighteen hours before cytokine induction, cells were incubated with DMEM containing
5 2 mM L-glutamine in the absence of serum and phenol red. iNOS expression was induced
6 with a triple cytokine mixture (CM) containing IFN- γ (100 U/ml), IL-1 β (50 U/ml) and
7 TNF- α (10 ng/ml) for the corresponding time periods depending on the experiment. In some
8 experiments, cells were treated with AG490 (30 μ M), SB203580 (10 μ M), SP600125
9 (10 μ M), PD98059 (50 μ M), Bay11-7082 (10 μ M), S-curvularin (1 to 100 μ M),
10 dexamethasone (5 μ M) or prednisolone (15 μ M) 1 h before and during cytokine incubation.
11 Afterwards, supernatant of the cells was used to measure NO₂⁻ with the Sievers Nitric Oxide
12 Analyzer (ADIInstruments, Spechbach, Germany). For RNA isolation, cells in monolayer
13 culture or in alginate beads were lysed by adding GIT-buffer (4 M
14 guanidiniumisothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% laurylsarcosine, 0.1 M
15 β -mercaptoethanol), and RNA was isolated by acid phenol/chloroform extraction as
16 described previously [8].
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39 **2.3. Establishment of C-28/I2 cells stably transfected with a 16kb human iNOS** 40 **promoter luciferase reporter gene construct** 41

42 C-28/I2 cells were transfected by lipofection with FuGENE according to the
43 manufacturer's recommendations. To generate C-28/I2 cells stably transfected with a
44 construct containing a 16 kb fragment of the human iNOS promoter cloned in front of a
45 luciferase reporter gene (C-28/I2-16kb cells), cells were plated onto 6-well plates and
46 transfected with 3.2 μ g of pNOS2(16)Luc [7] and 0.8 μ g of psiRNAhH1-GFPzeo (containing
47 a zeocin resistance gene) per well. The transfected cells were selected by Zeocin treatment
48 (0.2 mg/ml). Different cell clones were analyzed for luciferase activity and checked for
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integration of the transfected DNA by PCR.

2.4. Establishment of C-28/I2 cells overexpressing an EGFP-KSRP fusion protein

For the generation of C-28/I2 cells overexpressing an EGFP-KSRP fusion protein (C-28/I2-EGFP-KSRP) or EGFP alone (C-28/I2-EGFP) the Flp-In (Invitrogen) system was used. According to the manufacturer's recommendations C-28/I2 cells were transfected with pFRT/lacZeo to generate cell lines containing a FRT integration site. Resulting cell clones were selected by Zeocin incubation and clones with high β -galactosidase expression were isolated (C-28/I2-FRT). The cDNA coding for KSRP was isolated from pcDNA3.1-His-KSRP [13] and cloned into pEGFP-C1 (Clontech) generating pEGFP-KSRP. The cDNA coding for the EGFP-KSRP fusion protein as well as for EGFP alone were isolated from pEGFP-KSRP or pEGFP-C1 and inserted into pcDNA5/FRT/TO (Invitrogen) generating pcDNA5-FRT/TO/EGFP-KSRP and pcDNA5-FRT/TO-EGFP. These plasmids were used to transfect the C-28/I2-FRT cell clone to generate C-28/I2-EGFP-KSRP and C-28/I2-EGFP cells. Cells were selected for Hygromycin resistance. The Hygromycin-resistant cell pools were also selected for EGFP expression by fluorescence-activated cell sorting. In addition, Western blots using a monoclonal KSRP-antibody were used for testing the overexpression of EGFP-KSRP fusion protein.

2.5. Analysis of the expression of the glucocorticoid receptor

To test C-28/I2 chondrocytes for the expression of the human glucocorticoid receptor analytical reverse transcription polymerase chain reactions (RT-PCR) were performed. In the RT step, cDNA was reverse transcribed from total RNA samples from C-28/I2, A549/8 or EA.hy 926 cells using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's recommendations. PCR

was performed in 25 μ l reactions in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). The final reaction mix contained: 1.5 U Taq-DNA polymerase, Taq-DNA polymerase buffer and dNTPs (final concentration: 2.5 μ M each; PEQLab Biotechnology, Erlangen, Germany), forward and reverse primers at final concentrations of 0.4 μ M for each primer and 2 μ l cDNA or 10 ng of pHG0, an expression plasmid for the human glucocorticoid receptor (kindly provided by Dr. P Chambon). For the PCR reaction (40 cycles of 60 s 94°C, 30 s 55°C, 60 s 72°C), following oligonucleotides were used: hGCR primer set 1, hGCR_f1 5'-CCTAAGGACGGTCTGAAGAGC-3' and hGCR_r1 fl 5'-ATAGAGGGCCAAGACTTGGC-3' (length of the expected fragment: 478 bp); hGCR primer set 2, hGCR_f2 5'-TTTGCTCCTGATCTGATTATTAATGAGC-3' and hGCR_r2 5'-GTATGTTTCCTCTGAGTTACACAGGCT-3' (length of the expected fragment: 102 bp).

2.6. Real-time reverse transcription polymerase chain reaction analysis

Gene expression was quantified in a two-step real-time RT-PCR. The RT step was performed as described above. Real-time PCR was performed in a total volume of 25 μ l in a 96-well spectrofluorometric thermal cycler (iCycler, Bio-Rad Laboratories, München, Germany). For all genes the final reaction mix contained: 2.5 U Taq-DNA polymerase, Taq-DNA polymerase buffer and dNTPs (final concentration: 2.5 μ M each), forward and reverse primers at final concentrations of 0.4 μ M for each primer, the corresponding probe at the final concentration of 0.2 μ M and 2 μ l cDNA.

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 probes: iNOS, sense 5'-TGCAGACACGTGCGTTACTCC-3', antisense 5'-GGTAGCCAGCATAGCGGATG-3', probe 5'-TGGCAAGCACGACTTCCGGGTG-3'; Pol2a, sense 5'-GCACCACGTCCAATGACAT-3', antisense 5'-GTGCGGCTGCTTCCATAA-3', probe

1 5'-TACCACGTCATCTCCTTTGATGGCTCCTAT-3'; COL2A1, sense 5'-
 2 AGATTGAGAGCATCCGCAGC-3', antisense 5'-GCAGAAAACCTTCATGGCGTC-3',
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 4 probe 5'-AGAGTGGAGACTACTGGATTGACCCCAACCA-3'; SOX9, sense 5'-
 5 CAGTACCCGCACTTGCACAAC-3', antisense 5'-GCTGGTACTTGTAATCCGGGTG-3',
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 7 probe 5'-CTGGGCAAGCTCTGGAGACTTCTGAACG-3'; luciferase, sense 5'-
 8 CACAGGTCTCTTCCTGGTTTG-3' , antisense 5'-TCTTCCAGCGGATAGAATGG-3',
 9
 10 probe 5'-CAGCTGCAAGCCCCACAGTG3'.

11 Taqman hybridization probes were double labeled with 6-carboxyfluorescein (FAM) as
 12 reporter fluorophore and carboxytetramethyl-rhodamine (TAMRA) as quencher.
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 14 Fluorescence was monitored at each 60°C step. Each experimental reaction was performed in
 15
 16 triplicate. All primer/probes sets had efficiencies of 100% ($\pm 10\%$).

17 To calculate the relative expression of Col2A, iNOS, luciferase or SOX9 mRNA in
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 19 C-28/I2 cells the $2^{(-\Delta\Delta C(T))}$ method [26] was used. According to this method the C(T) values
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 21 for Col2A, iNOS, luciferase or SOX9 mRNA expression in each sample were normalized to
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 23 the C(T) values of Pol2a mRNA in the same sample.

2.7. Western blot experiments

24 To study the expression of iNOS- or KSRP protein in C-28/I2 cells total cellular protein
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 26 (10-50 μg protein) was separated on SDS polyacrylamide gels and transferred to
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 28 nitrocellulose membranes by semi-dry electroblotting. All further steps were performed as
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 30 described previously [8]. For detection of iNOS a monoclonal anti-iNOS-antibody (R&D
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 32 systems, Wiesbaden, Germany) was used. To study the KSRP protein expression a
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 34 monoclonal anti-KSRP-antibody [27] was used. The immunoreactive proteins on the blots
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 36 were visualized by the enhanced chemiluminescence detection system (ECL, Amersham).

2.8 Statistics

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2 Data represent means + SEM. Statistical differences were determined by factorial
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4 analysis of variance (One-way ANOVA) followed by Tukey's multiple comparison test for
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6 comparison of multiple means using GraphPad Prism 5.0. Detailed results of these analyses
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8 are shown in the supplemental data (Supplemental information to statistical analyses).
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3. Results

3.1 Induction of iNOS expression in human chondrocytes is associated with differentiation.

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21 In the literature conflicting results concerning the role of human iNOS in the onset and
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23 progression of OA and RA exist. Therefore, the aim of the study was to investigate the
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25 regulation of human iNOS expression in a reproducible chondrocyte cell culture system to
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27 elucidate the role of the enzyme in inflammatory joint diseases. In C-28/I2 cells, chosen as a
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29 chondrocyte cell culture model, we first analyzed iNOS expression upon cytokine
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31 stimulation.
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36 C-28/I2 chondrocytes were cultured for 1 to 6 days in alginate beads or in monolayer.
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38 Then cells were incubated for 16 h in medium without FCS and subsequently treated for 6 h
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40 with a triple cytokine mixture (CM) containing TNF- α , IFN- γ and IL-1 β . As seen in **Fig. 1A**
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42 **to 1C**, significant cytokine-mediated induction of iNOS expression (mRNA and protein) was
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44 seen after 6 h of treatment of 6-day alginate or monolayer cultures, but not 1- or 2-day
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46 cultures. As no difference in iNOS mRNA expression between alginate and monolayer
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48 culture after 6 days could be detected, we used C-28/I2 chondrocytes cultured for 6 days in
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50 monolayer ("superconfluent") for further experiments.
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55 To elucidate the time dependence of iNOS induction, C-28/I2-cells were incubated with
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57 CM for different periods (2 to 24 h; **Fig. 1D** and **1E**). These analyses showed maximal iNOS
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1 mRNA expression (**Fig. 1D**) after 6 h CM incubation and maximal iNOS protein expression
2 after 6 to 8 h (**Fig. 1E**), both of which declined after 24 h.
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4 To explore if iNOS expression depends on chondrogenic differentiation we analyzed in
5 the same RNA samples as used above (**Fig. 1B**) the mRNA expression of the differentiated
6 chondrocyte marker genes COL2A1 (**Fig. 2A**) and SOX9 (**Fig. 2B**)[18]. As shown in **Fig.**
7 **2A&B**, the expression of both genes was markedly enhanced on day 6 of the monolayer
8 culture, indicating a metabolically active phenotype at this timepoint. Our results indicate that
9 maximal iNOS expression in C-28/I2 cells is associated with the differentiated chondrocyte
10 phenotype.
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24 **3.2 Inhibition of iNOS activity does not modulate cytokine-induced iNOS expression** 25 **in C-28/I2 chondrocytes.** 26 27

28 It has been shown that NO modulates gene expression in chondrocytes [28]. In addition,
29 several reports demonstrate that NO regulates iNOS expression in several cell types [6].
30 Therefore, we examined whether NO affects iNOS expression in C-28/I2 chondrocytes.
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34 Coincubation of cytokine-induced superconfluent C-28/I2 cells with aminoguanidine,
35 an inhibitor of iNOS activity, resulted in marked inhibition of cytokine-induced NO
36 production (**suppl. Fig. 1A**). However, (**suppl. Fig. 1B**), aminoguanidine had no effect on
37 cytokine-induced iNOS mRNA expression. These data clearly demonstrate that NO by itself
38 is not able to regulate iNOS expression in C-28/I2 chondrocytes.
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50 **3.3 Cytokine-induced iNOS expression and iNOS-related NO production is reduced** 51 **after inhibition of p38MAPK, JAK2-STAT-1 α and the NF- κ B signal transduction** 52 **pathways.** 53 54 55 56 57 58 59 60 61 62 63 64 65

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The induction of iNOS expression in human cells depends on the activation of several signaling pathways in a cell type-specific manner [6]. Therefore, we analyzed the effects of the inhibition of major signaling pathways on cytokine-mediated induction of iNOS expression in C-28/I2 chondrocytes. As shown in **Fig. 3A**, inhibition of JNK by SP600125 (direct JNK inhibitor) or inhibition of ERK activation by PD98059 (MEK inhibitor, blocks activation of ERK1/2) did not affect the cytokine-induced iNOS mRNA expression. In contrast, blockade of p38MAPK by SB203590 (direct p38MAPK α and - β inhibitor), the NF- κ B pathway by Bay11-7082 (IKK inhibitor, inhibits I- κ B phosphorylation and thereby NF- κ B activation) and the JAK2-STAT-1 α pathway by AG490 (JAK2/3 inhibitor, blocks JAK2-dependent activation of STAT-1 α) resulted in a significant reduction of cytokine induced iNOS mRNA (**Fig. 3A**) and protein (**Fig. 3B**) expression as well as iNOS-dependent NO production (**Fig. 3C**).

In summary, iNOS induction in C-28/I2 chondrocytes depends on the same signal transduction pathways described to be involved in iNOS expression in most other human cells [6].

3.4 Cytokine-induced iNOS promoter activity parallels cytokine-induced iNOS mRNA expression in C-28/I2 chondrocytes.

In several cell lines, marked differences between the inducibility of the human iNOS promoter and the iNOS mRNA expression have been described [6]. In A549/8-, AKN- and DLD-1 cells (run-on analyses, transient and stable transfections) a low basal human iNOS promoter activity was detected, which did not result in appreciable iNOS mRNA expression. Cytokine incubation increased human iNOS promoter activity only 10-fold, whereas iNOS mRNA expression was enhanced to a much greater extent (> 100 fold)[7, 8]. To test the inducibility of the human iNOS promoter in C-28/I2 chondrocytes, cells stably transfected

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with a construct containing a 16 kb fragment of the human iNOS promoter in front of the luciferase reporter gene were generated. These cells enable the parallel analysis of human iNOS promoter activity (measurement of the luciferase mRNA, **Luc**) and endogenous iNOS mRNA expression by qRT-PCR. As shown in **Fig. 4** no significant iNOS- or luciferase mRNA expression was seen in C-28/I2-chondrocytes after 1 day of monolayer culture and treatment with **CM** for 6h. As shown above CM-treatment resulted in a marked induction of iNOS mRNA expression after 6 days in monolayer culture. The same rate of inducibility was seen for the luciferase (**Luc**) mRNA. These data show, that in contrast to most other human cell lines, in C-28/I2 chondrocytes the cytokine-induced iNOS promoter activity directly reflects iNOS mRNA expression.

3.5 KSRP regulates cytokine-induced iNOS expression in human C-28/I2 chondrocytes

Beside transcriptional regulation, human iNOS expression has been shown to depend on post-transcriptional mechanisms [6]. In DLD-1 cells we demonstrated that the RNA-BP KSRP is a major post-transcriptional regulator of human iNOS expression mediating degradation of the iNOS mRNA [13]. As shown above in differentiated C-28/I2 chondrocytes, the cytokine-induced iNOS promoter activity nearly parallels the iNOS mRNA expression. Therefore, we wanted to elucidate whether KSRP plays an important role in the regulation of iNOS expression in this cell system. We generated cell lines, which overexpress an EGFP-KSRP fusion protein (C-28/I2-EGFP-KSRP) and as control C-28/I2-EGFP cells expressing EGFP alone (see **Fig. 5A**). As shown in **Fig. 5B** and **C**, overexpression of KSRP markedly inhibited cytokine-induced iNOS expression. These data indicate that, although in C-28/I2 cells the iNOS promoter activity seems to directly reflect the iNOS mRNA

1 expression, the major negative regulating RNA-BP KSRP still modulates iNOS expression
2 post-transcriptionally.
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7 **3.6 Glucocorticoids do not inhibit cytokine-induced iNOS mRNA and protein** 8 **expression or iNOS-dependent NO production in C-28/I2 chondrocytes.** 9

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11 Glucocorticoids (GC) are used as a therapeutic agent to treat human inflammatory
12 autoimmune diseases such as bowel disease and asthma as well as RA [29]. Moreover, they
13 have been described to inhibit iNOS induction in several cell systems and animal models [6].
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19 Interestingly, coincubation of cytokine-induced superconfluent C-28/I2 cells with
20 dexamethasone or prednisolone did not result in any inhibition of cytokine-induced iNOS
21 mRNA- or protein expression (**Fig. 6A and B**) as well as iNOS-dependent NO-production
22 (**Fig. 6C**).
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31 **3.7 C-28/I2 chondrocytes do not express the glucocorticoid receptor mRNA** 32

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34 As most effects of GC are mediated by binding and activation of the glucocorticoid
35 receptor (GCR), we analyzed the expression of the GCR mRNA in C-28/I2 chondrocytes
36 using two different primer pairs. Set 1 is specific for the human GCR α mRNA. Set 2 detects
37 the mRNAs of both GCR α and - β . As shown in **Fig. 6D**, RT-PCR of total RNA from C-28/I2
38 cells showed no expression of the human GCR mRNA. In contrast, in total RNA from human
39 epithelial A549/8- and endothelial EA.hy 926 cells expression of the human GCR was
40 detected. In both cell lines, effects of GC on gene expression have been described [24, 30].
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51 Our results clearly demonstrate that C-28/I2 cells do not express the GCR mRNA and
52 that this may account for the lack of efficacy of GC treatment.
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3.8 The anti-inflammatory fungal compound S-curvularin inhibits cytokine-induced iNOS mRNA and protein expression as well as iNOS-dependent NO production in C-28/I2 chondrocytes

Novel strategies for the therapy of chronic inflammatory diseases especially in cases of glucocorticoid resistance are based on the development of compounds that suppress pro-inflammatory gene expression, either by inhibition of the corresponding signal transfer pathways or, directly, by inhibition of the transcription factors involved. S-curvularin isolated from different imperfect fungi [23] is able to inhibit 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 (STAT-1 α) resulting in the inhibition of STAT-1 α -dependent gene expression [23]. Therefore, we tested the effects of S-curvularin on iNOS expression in C-28/I2 chondrocytes. As shown in **Fig. 7** cocubation of cytokine-induced superconfluent C-28/I2 cells with S-curvularin (SC) resulted in inhibition of cytokine-induced iNOS mRNA or protein expression (**Fig. 7A and B**), as well as iNOS-dependent NO-production (**Fig. 7C**).

4. Discussion

The opportunities for the molecular analysis of physiologic or pathophysiologic gene expression in primary cultures of human chondrocytes are limited. Primary chondrocytes are difficult to obtain, vary in their behavior according to the conditions and the ages of the patients [5], and exhibit loss of differentiated phenotype and significant changes in gene expression patterns [31]. Therefore, we have used a reproducible cell culture system of immortalized human chondrocytes established by retroviral transduction of primary human chondrocytes with the SV40 large T antigen [20]. These cells, which retain chondrocytic morphology and maintain continuous proliferation in monolayer culture, have proven useful

in molecular gene expression analyses [21].

NO generated by iNOS seems to be involved in the pathophysiologic mechanisms leading to OA [19] or RA [32] by regulation of chondrocyte functions [28] and gene expression [33]. Therefore, we wanted to establish a reliable model for analyzing the regulation of iNOS expression in human immortalized C-28/I2 chondrocytes.

Our results (**Fig. 1**) demonstrate that culture of C-28/I2 chondrocytes in alginate or monolayer for 1 or 2 days is not sufficient to permit induction of iNOS expression. In contrast culturing the cells for 6 days before cytokine treatment resulted in a marked increase of iNOS expression in both culture types. This implies that iNOS induction in chondrocytes needs some “superconfluency-derived” stress signals to enable the cytokines to activate iNOS expression. As iNOS-expression is regulated by Rho-mediated changes in the structure of the actin cytoskeleton [34], it is tempting to speculate that the above-mentioned stress signals may relate to superconfluency-induced changes in the structure of the actin cytoskeleton. In addition, there is evidence that the Rho-mediated modulation of the actin cytoskeleton is involved in the expressional changes essential for chondrogenesis [35]. Also the high density of cells in the superconfluent cultures may result in local hypoxia. It has been shown that hypoxia via hypoxia-inducible factor (HIF)-1 α is able to modulate iNOS expression [36, 37]. Indeed, hypoxia-induced activation of HIF-1 α has been related to chondrogenesis [38].

In contrast to data published for human primary [39] and chondrocytes derived from human mesenchymal stem cells (hMSC) [22], IL-1 β , TNF- α or IFN- γ alone or in double combinations were not able to induce iNOS expression in these cells (data not shown). Thus, iNOS induction in superconfluent C-28/I2 cells depends on a triple cytokine mixture, as known for most other human cells [6]. In addition, induction of iNOS expression in superconfluent C-28/I2 chondrocytes was paralleled by a marked increase of the mRNA expression of COL2A1 and SOX9 (**Fig. 2**), both marker genes for chondrogenic

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differentiation [18]. Therefore, we speculate that the induction of iNOS expression depends on chondrocyte differentiation, as shown in human primary [40] or mesenchymal stem cell-derived chondrocytes [22]. In summary, these data reveal superconfluent C-28/I2 cells as an appropriate cell culture model to study the role of iNOS in physiological and pathophysiological processes in articular cartilage.

NO has been described to auto-regulate cytokine-induced iNOS expression either in a positive or negative manner [6]. However, our results indicate that NO does not modulate iNOS expression in C-28/I2 chondrocytes (**suppl. Fig. 1**). Signaling pathways resulting in the induction of iNOS expression vary in different cells or species. Accordingly, diverse results have been described regarding the effects of specific pharmacological or molecular inhibitors of these pathways on iNOS induction [6]. We observed that inhibition of the JNK pathway by SP600125 or the ERK-pathway by PD98059 did not result in a change in cytokine-induced iNOS mRNA expression in superconfluent C-28/I2 chondrocytes (**Fig. 3**). The expression and inducibility of both JNK and ERK in C-28/I2 cells have been described [41]. In line with our data, no influence of ERK or JNK inhibition on AGE-induced iNOS expression has been seen in human primary chondrocytes [42].

Inhibition of p38MAPK by SB203590 reduced cytokine-induced iNOS expression, as well as iNOS-mediated NO production in superconfluent C-28/I2 chondrocytes, suggests the essential involvement of p38MAPK in iNOS induction. Similar results have been published for human primary chondrocytes [42]. Activation of p38MAPK has been described to be essential for iNOS induction in most mammalian (including human) cells [6]. Despite the finding of p38MAPK activation via regulation of NF- κ B activity on iNOS promoter activity [43], it is generally believed, that p38MAPK affects pro-inflammatory gene expression mainly on the post-transcriptional level [44]. We have recently demonstrated that inhibition of p38MAPK by incubation with SB203590 or by overexpressing a dominant negative

1 p38MAPK in human DLD-1 cells markedly reduces cytokine-induced iNOS expression.
2 Detailed analyses revealed a post-transcriptional regulation of iNOS expression in DLD-1
3 cells by the cytokine-mediated p38MAPK-dependent induction of the RNA binding protein
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10 Cytokine-mediated induction of NF- κ B activity and the JAK2-STAT-1 α pathway are
11 essential for the induction of iNOS expression in most mammalian cells [6]. In agreement, we
12 found that inhibition of JAK2 activation by AG490 or inhibition of NF- κ B activation by
13 Bay11-7082 decreases cytokine-induced iNOS expression in C-28/I2 chondrocytes (**Fig. 3**).
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31 Similar data regarding NF- κ B-dependent iNOS expression were published for human primary
32 chondrocytes [45]. In the murine chondrogenic cell line ADTC5, critical dependence of iNOS
33 induction from the JAK2 activity has been described [46]. Therefore, cytokine-induced iNOS
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32 Regulation of iNOS transcription is considered to be one of the important control
33 mechanisms for iNOS expression [6]. However, in most human cell lines analyzed there is a
34 marked difference between the inducibility of the human iNOS promoter activity (maximal
35 10-fold) and the induction rate seen for iNOS mRNA expression (more than 100-fold). Also,
36 basal iNOS promoter activity without an appreciable iNOS mRNA expression was described
37 [7, 8]. In contrast to the murine system where a 1 kb fragment of the 5'-flanking sequence of
38 the iNOS gene displays full inducibility [9], much longer fragments of the 5'-flanking
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iNOS promoter activity closely parallels the induction rate of the iNOS mRNA expression. This indicates a much more direct dependence of the iNOS expression for the promoter activity in C-28/I2 cells than seen in other human cell types. In addition, the cytokine-dependent induction rate of the human iNOS promoter in C-28/I2 chondrocytes depends on their differentiation status. Interestingly, also Du et al. described cell type-specific inducibility of a 7.2 kb fragment of the human iNOS promoter in transient transfection experiments [47]. Therefore, it seems that the cytokine-inducibility of the human iNOS promoter activity depends on the genetic background and the differentiation status of the cell type analyzed.

The complex mechanisms regulating human iNOS expression involve also post-transcriptional regulation [6]. Our studies in DLD-1 cells have shown that the RNA-BP KSRP is a major negative regulator of human iNOS expression [12, 13]. KSRP binds to the AU-rich elements (ARE) in the 3'-untranslated region (3'-UTR) of the human iNOS mRNA and recruits the exosome (an exonuclease complex) to the iNOS mRNA, thus enabling 3' to 5' mRNA degradation. As seen in DLD-1 cells, overexpression of KSRP in C-28/I2 chondrocytes resulted in marked inhibition of cytokine-induced iNOS expression (see **Fig. 5**). Therefore, it is very likely that although iNOS promoter activity seems to reflect the iNOS mRNA expression in C-28/I2 cells directly, the major negative regulating RNA-BP KSRP still modulates iNOS expression on the post-transcriptional level.

Glucocorticoids (GC), often used for the treatment of pro-inflammatory diseases [29], are also known to inhibit iNOS expression [48]. GC-mediated inhibition of iNOS expression has been implicated in the anti-inflammatory effects of GC in asthma [49]. Surprisingly, the potent glucocorticoid dexamethasone was not able to inhibit cytokine-induced iNOS expression in superconfluent C-28/I2 chondrocytes (**Fig. 6A - C**). Supporting our data, Mais et al. [22] demonstrate that dexamethasone has also no effect on cytokine-induced chondrogenic differentiation-dependent NO-production in human primary and hMSC-derived

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chondrocytes. These authors even postulate glucocorticoid-insensitive iNOS expression as a marker of chondrogenic differentiation. However, in contrast to the data described above, IL-17 induced iNOS expression and iNOS-dependent NO production in human primary chondrocytes has been described to be inhibited by dexamethasone [45]. The glucocorticoid-resistance of C-28/I2 chondrocytes may be due to the absence of GCR α and- β mRNAs (**Fig. 6D**). In contrast, in A549/8 or EA.hy 926 cells, which display GC-dependent changes in gene expression [24, 30], the expression of GCR mRNA was detected. Resistance to glucocorticoids has been described as a major problem for the treatment of several common inflammatory diseases and up to 30% of the RA patients do not respond to glucocorticoids [50]. Therefore, C-28/I2 chondrocytes may constitute an interesting tool for the development of new anti-inflammatory compounds with effects on a glucocorticoid-resistant background.

As C-28/I2 chondrocytes seem to represent a reliable molecular model of glucocorticoid-resistant RA, we investigate the effects of S-curvularin (SC), a new anti-inflammatory compound isolated from fungi shown to inhibit the JAK/STAT-1 α pathway [23], on iNOS expression. As seen in **Fig. 7**, SC was able to inhibit the cytokine-induced iNOS expression (**Fig. 7A and B**) and iNOS-dependent NO-production (**Fig. 7C**) in superconfluent C-28/I2 chondrocytes most likely by inhibition of cytokine induced JAK/STAT activation. Thus, as shown in this glucocorticoid-resistant chondrocyte culture model, drugs derived from the JAK/STAT-1 α inhibitor SC may open new ways to treat glucocorticoid-resistant courses of pro-inflammatory diseases.

In summary, as shown for human primary chondrocytes, iNOS induction in C-28/I2 cells is related to their state of chondrocyte differentiation and depends on the activation of the p38MAPK, JAK2-STAT-1 α , and NF- κ B pathways. In contrast to most human cells analyzed, cytokine-induced iNOS promoter activity in C-28/I2 chondrocytes directly reflects

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iNOS mRNA expression. However, post-transcriptional regulation by the RNA-BP KSRP also plays a role in modulating cytokine-induced iNOS expression in these cells. Similar to human primary and hMSC-derived chondrocytes, iNOS induction in C-28/I2 chondrocytes is not inhibited by glucocorticoids. This lack of GC-mediated inhibition is correlated to the absence of the expression of the GCR. In contrast to glucocorticoids, the JAK/STAT-1 α pathway inhibitor SC is able to inhibit cytokine-induced iNOS expression indicating the possible efficacy of SC-derived drugs for the treatment of glucocorticoid-resistant chronic inflammatory diseases.

Conflict of interest

The authors have no conflict of interest.

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Footnotes

both authors contributed equally to this work

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Figure legends

Figure 1. Induction of iNOS expression in human immortalized C-28/I2 chondrocytes

C-28/I2 chondrocytes were cultured for 1 (**d1**), 2 (**d2**) or 6 (**d6**) days in monolayer or alginate culture. Afterwards, the cells were treated without (**CO**) or with **CM** for 6 h (mRNA and protein analyses) or 24 h (NO production). **(A)** A summary of 6 qRT-PCR analyses to determine iNOS mRNA expression is shown using RNAs from C-28/I2 chondrocytes after alginate culture. Data (means + SEM) represent relative iNOS mRNA levels (100% = CM 1d; * $p < 0.05$; ns = non-significant vs. CM-treated C-28/I2 cells incubated for 1 day in alginate culture). **(B)** A summary of 6 qRT-PCR analyses to determine iNOS mRNA expression is shown using RNAs from C-28/I2 chondrocytes after monolayer culture. Data (means + SEM) represent relative iNOS mRNA levels (100% = CM 1d; *** $p < 0.001$; ns = non-significant vs. CM-treated C-28/I2 cells incubated for 1 day in monolayer). **(C)** iNOS and α -tubulin protein expression were analyzed in Western blot experiments. This blot is representative of three other blots showing similar results. **(D)** A summary of 4 qRT-PCR analyses to determine iNOS mRNA expression is shown using RNAs from superconfluent C-28/I2 chondrocytes incubated without (**CO**) or with **CM** for 2 to 24 h. Data (means + SEM) represent relative iNOS mRNA levels (100% = CM 6h; *** $p < 0.001$; ns = not significant vs. cells treated with CM for 6 h).

(E) iNOS and β -tubulin protein expression were analyzed in Western blot experiments using whole cell extracts from superconfluent C-28/I2 cells incubated for 2 to 24 h with **CM**. This blot is representative of two other blots showing similar results.

Figure 2. Expression of differentiated chondrocyte marker genes in C-28/I2 chondrocytes

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(A) A summary of 4 qRT-PCR analyses to determine COL2A1 mRNA expression is shown using the same RNAs as in (Fig. 1B) from C-28/I2 chondrocytes after monolayer culture for 1 (d1), 2 (d2) or 6 (d6) days without CM-treatment. Data (means + SEM) represent relative COL2A1 mRNA levels (100 % = d1; *** p < 0.001; ns = non-significant vs. C-28/I2 cells incubated for 1 day in monolayer). (B) A summary of 4 qRT-PCR analyses to determine SOX9 mRNA expression is shown using the same RNAs as in (Fig. 1B) from C-28/I2 chondrocytes after monolayer culture for 1 (d1), 2 (d2) or 6 (d6) days without CM-treatment. Data (means + SEM) represent relative SOX9 mRNA levels (100 % = d1; * p < 0.05; ns = non-significant vs. C-28/I2 cells incubated for 1 day in monolayer).

Figure 3. Inhibition of the p38MAPK, the JAK/STAT-1 α and the NF- κ B pathway reduces cytokine-induced iNOS expression in superconfluent C-28/I2 chondrocytes

(A) A summary of 6 qRT-PCR analyses to determine iNOS mRNA expression is shown using RNAs from superconfluent C-28/I2 chondrocytes after treatment without (CO) or with CM for 6 h in the presence or absence of 10 μ M SP600125 (SP), 50 μ M PD98059 (PD), 10 μ M SB203590 (SB) 30 μ M Bay11-7082 (Bay) or 30 μ M AG490 (AG). Data (means + SEM) represent relative iNOS mRNA levels (100% = CM; *** p < 0.001; ns = not significant vs. CM-treated C-28/I2 cells in the absence of inhibitors). (B) iNOS and β -tubulin protein expression in superconfluent C-28/I2 cells after treatment without (CO) or with CM for 6 h in the presence or absence of 30 μ M AG, 10 μ M SB or 30 Bay were analyzed in Western blot experiments. This blot is representative of three other blots showing similar results. (C) A summary of 3 nitrite analyses using supernatants from superconfluent C-28/I2 cells treated without (CO) or with CM for 24 h in the presence or absence of 30 μ M AG, 10 μ M SB or 30

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μM Bay is shown. Data (means + SEM) represent relative nitrite levels (100% = CM; *** $p < 0.001$ vs. CM-treated C-28/I2 cells in the absence of inhibitors).

Figure 4. Induction of iNOS promoter activity parallels the induction of iNOS mRNA expression in C-28/I2 chondrocytes

C-28/I2-16kb cells were cultured for 1 (d1) or 6 (d6) days in monolayer culture. Afterwards cells were treated without (CO) or with CM for 6 h and total cellular RNA was purified. A summary of 6 qRT-PCR analyses to determine iNOS- and luciferase mRNA expression in C-28/I2-16 kb cells is shown. Data (means + SEM) represent relative iNOS- or luciferase mRNA levels. The iNOS- or luciferase mRNA levels in cells incubated for 6 days in monolayer and treated with CM for 6h were set to 100 % (*** $p < 0.001$, iNOS-mRNA; ### $p < 0.001$, luciferase mRNA, each vs. CM-treated C-28/I2-16kb cells after 6 days in monolayer; ns = not significant vs. CM-treated cells after one day in monolayer).

Figure 5. KSRP reduces cytokine-induced iNOS expression in C-28/I2 chondrocytes

C-28/I2-EGFP or -EGFP-KSRP cells were cultured for six days (d6) in monolayer culture. Afterwards cells were treated without (CO) or with CM for 6 h and total cellular RNA or protein was purified. (A) Western blots were performed using specific anti-KSRP- and anti- β -tubulin antibodies and extracts from C-28/I2-EGFP or -EGFP-KSRP cell pools. The blots are representative of two other blots showing similar results. The positions of EGFP-KSRP, KSRP and β -tubulin are indicated. (B) A summary of 8 qRT-PCR analyses to determine iNOS mRNA expression in C-28/I2-EGFP or -EGFP-KSRP cells is shown. Data (means + SEM) represent relative iNOS mRNA levels. The iNOS mRNA level in C-28/I2-EGFP cells incubated for 6 days in monolayer and treated with CM for 6h were set to 100 % (*** $p < 0.001$ vs. CM-treated C-28/I2-EGFP cells). (C) iNOS and β -tubulin protein expression were

analyzed in Western blot experiments using whole cell extracts from superconfluent C-28/I2-EGFP or -EGFP-KSRP cells incubated for 6h with **CM**. This blot is representative of three other blots showing similar results. The positions of iNOS and β -tubulin are indicated.

Figure 6. Glucocorticoids do not inhibit iNOS expression in C-28/I2 chondrocytes – lack of the expression of the glucocorticoid receptor

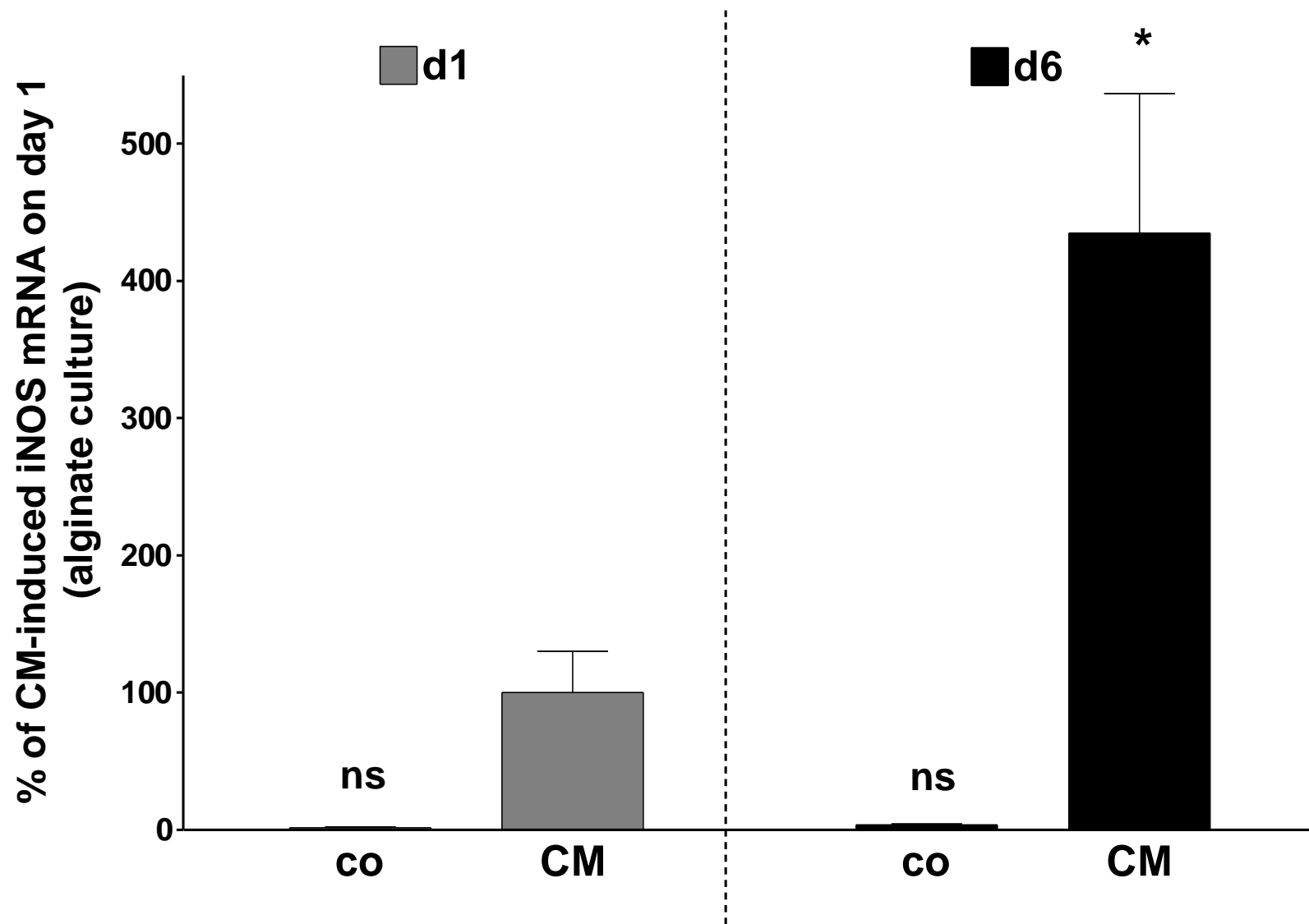
(A) A summary of 4 qRT-PCR analyses to determine iNOS mRNA expression is shown using RNAs from superconfluent C-28/I2 chondrocytes and **CM**-treatment for 6 h in the presence or absence of 5 μ M dexamethasone (**Dex**) or 15 μ M prednisolone (**Pred**). Data (means + SEM) represent relative iNOS mRNA levels (100% = **CM**; *** $p < 0.001$; ns non-significant vs. **CM**-treated C-28/I2 cells in the absence of glucocorticoids). (B) iNOS and β -tubulin protein expression in superconfluent C-28/I2 cells treated without (**CO**) or with **CM** for 6 h in the presence or absence of 5 μ M **Dex** were analyzed in Western blot experiments. This blot is representative of two other blots showing similar results. (C) A summary of 4 nitrite analyses using supernatants from superconfluent C-28/I2 cells treated without (**CO**) or with **CM** for 24 h in the presence or absence of 5 μ M **Dex** is shown. Data (means + SEM) represent relative nitrite levels (100% = **CM**; *** $p < 0.001$; ns = not significant vs. **CM**-treated C-28/I2 cells in the absence of dexamethasone). (D) The expression of the human glucocorticoid receptor mRNA was analyzed performing RT-PCR reactions using total RNA isolated from **C-28/I2** chondrocytes cultured for 6 days in monolayer or total RNA isolated from confluent human epithelial **A549/8** and human endothelial **EA.hy 926** monolayer cells. RT-PCR reactions were performed with the oligonucleotides hGCR_f1 and hGCR-r1 (**Set 1**; detecting the α -isoform of the human GCR; 478 bp PCR fragment expected) and hGCR_f2 and hGCR_r2 (**Set 2**; detecting both the α and β -isoform of the human GCR; 102 bp PCR fragment expected). As positive control **pHG0** plasmid DNA (eucaryotic expression vector

for the human GCR α) was used instead of RNA. The positions of the specific GCR PCR fragments are indicated (**M**: molecular weight marker).

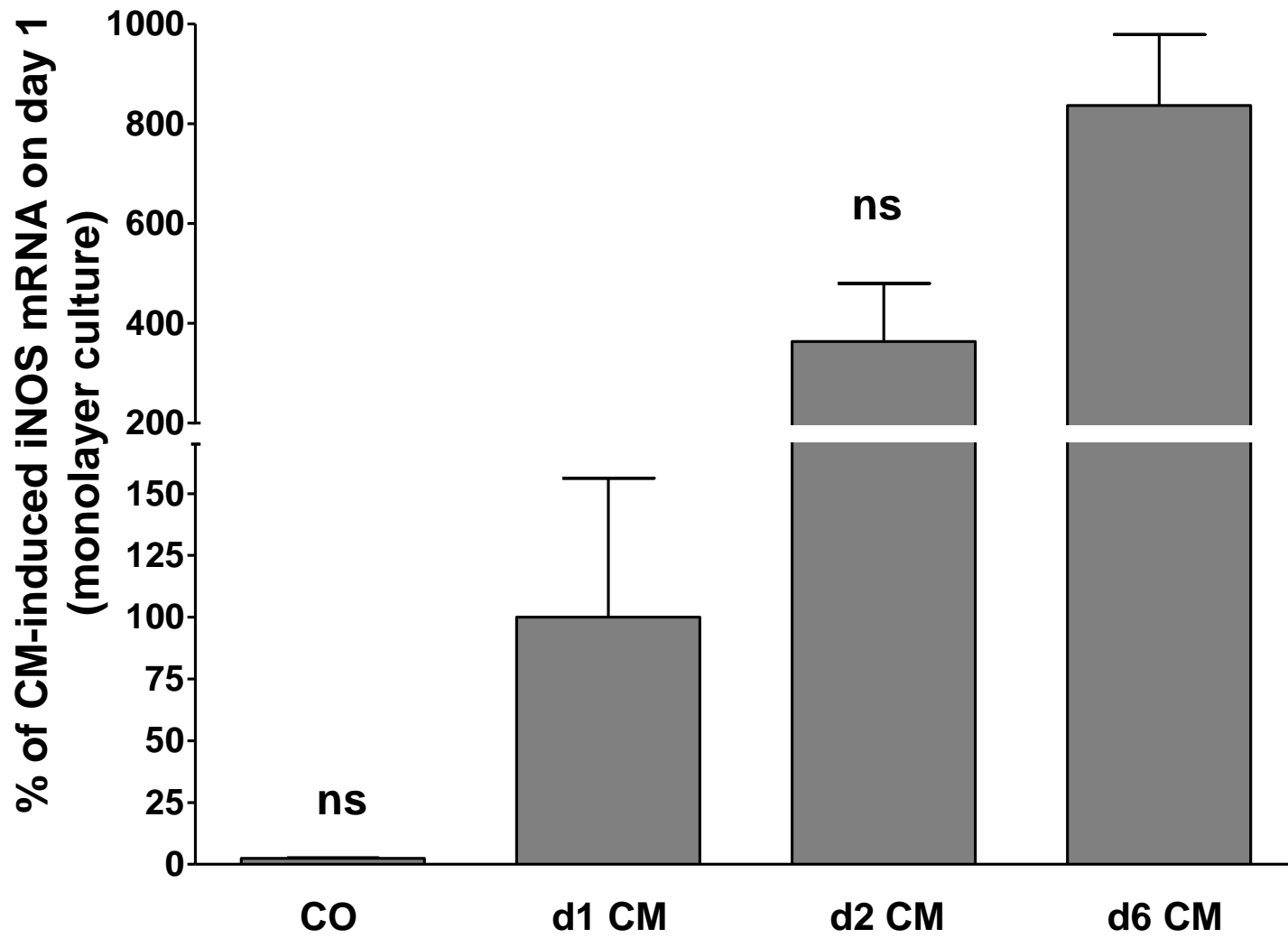
Figure 7. Inhibition of cytokine-induced iNOS expression and iNOS-dependent NO-production by the fungal metabolite S-curvularin

C-28/I2 chondrocytes were cultured for 6 days in monolayer culture. Afterwards cells were preincubated for 1 h with 5 μ M dexamethasone (**Dex**) or S-curvularin (**SC**; 1 to 100 μ M) and then treated without (**CO**) or with **CM** for 6 h (mRNA and protein analyses) or 24 h (NO production). **(A)** A summary of 7 qRT-PCR analyses to determine iNOS mRNA expression is shown using RNAs from superconfluent C-28/I2 chondrocytes after treatment without (**CO**) or with **CM** for 6 h in the presence or absence of 5 μ M dexamethasone (**Dex**) or 100 μ M S-curvularin (**SC**). Data (means + SEM) represent relative iNOS mRNA levels (100% = **CM**; *** $p < 0.001$; ns = not significant vs. **CM**-treated C-28/I2 cells in the absence of inhibitors). **(B)** iNOS and β -tubulin protein expression in superconfluent C-28/I2 cells after treatment without (**CO**) or with **CM** for 6 h in the presence or absence of 5 μ M **Dex**, or 1 to 100 μ M **SC** were analyzed in Western blot experiments. This blot is representative of three other blots showing similar results. **(C)** A summary of 5 nitrite analyses using supernatants from superconfluent C-28/I2 cells treated without (**CO**) or with **CM** for 24 h in the presence or absence of 5 μ M **Dex** or 100 μ M **SC** is shown. Data (means + SEM) represent relative nitrite levels (100% = **CM**; *** $p < 0.001$ vs. **CM**-treated C-28/I2 cells in the absence of inhibitors).

1A



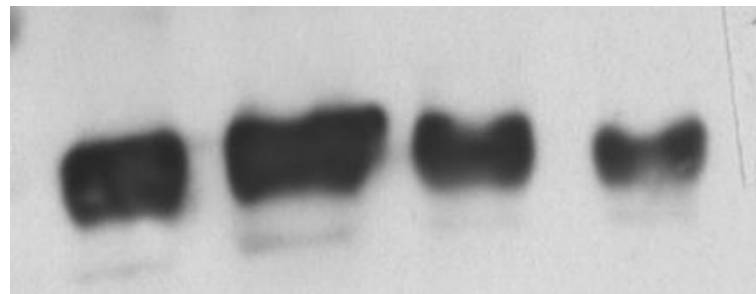
1B



1C



iNOS



α -tubulin

CO

CM

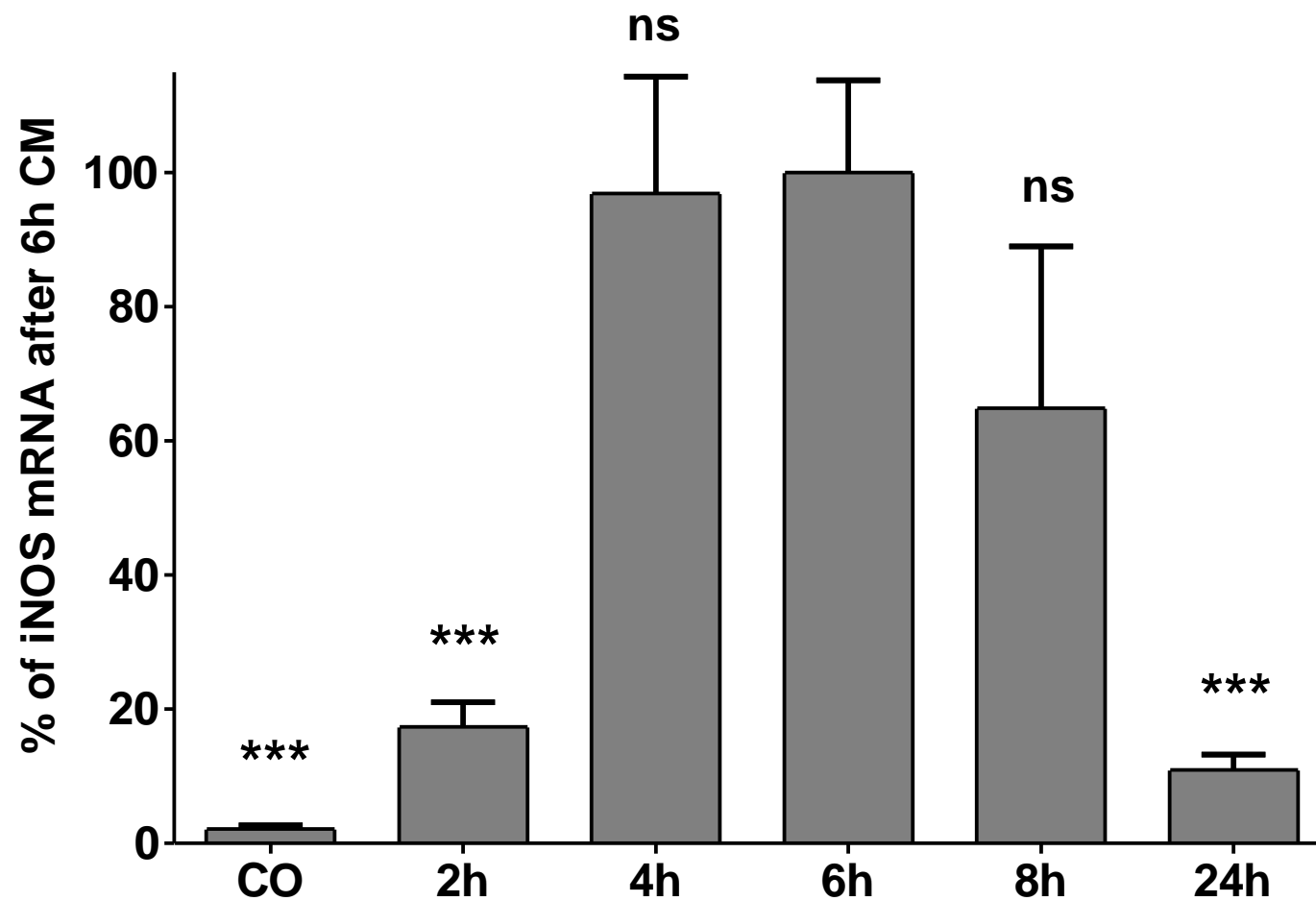
CO

CM

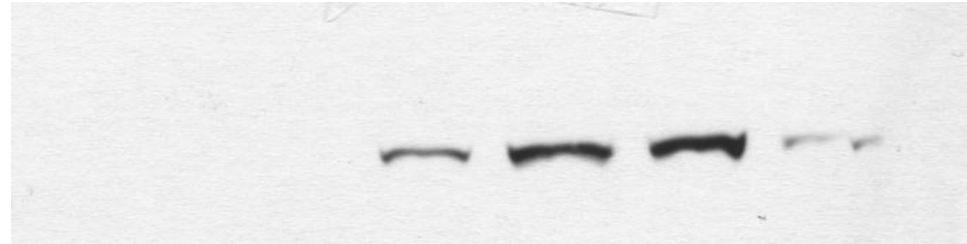
d1

d6

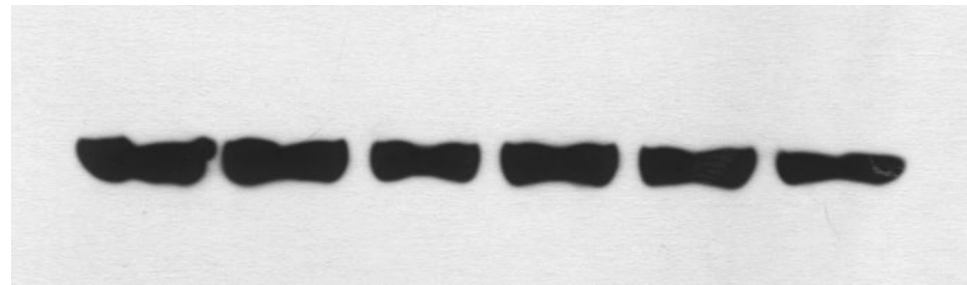
1D



1E



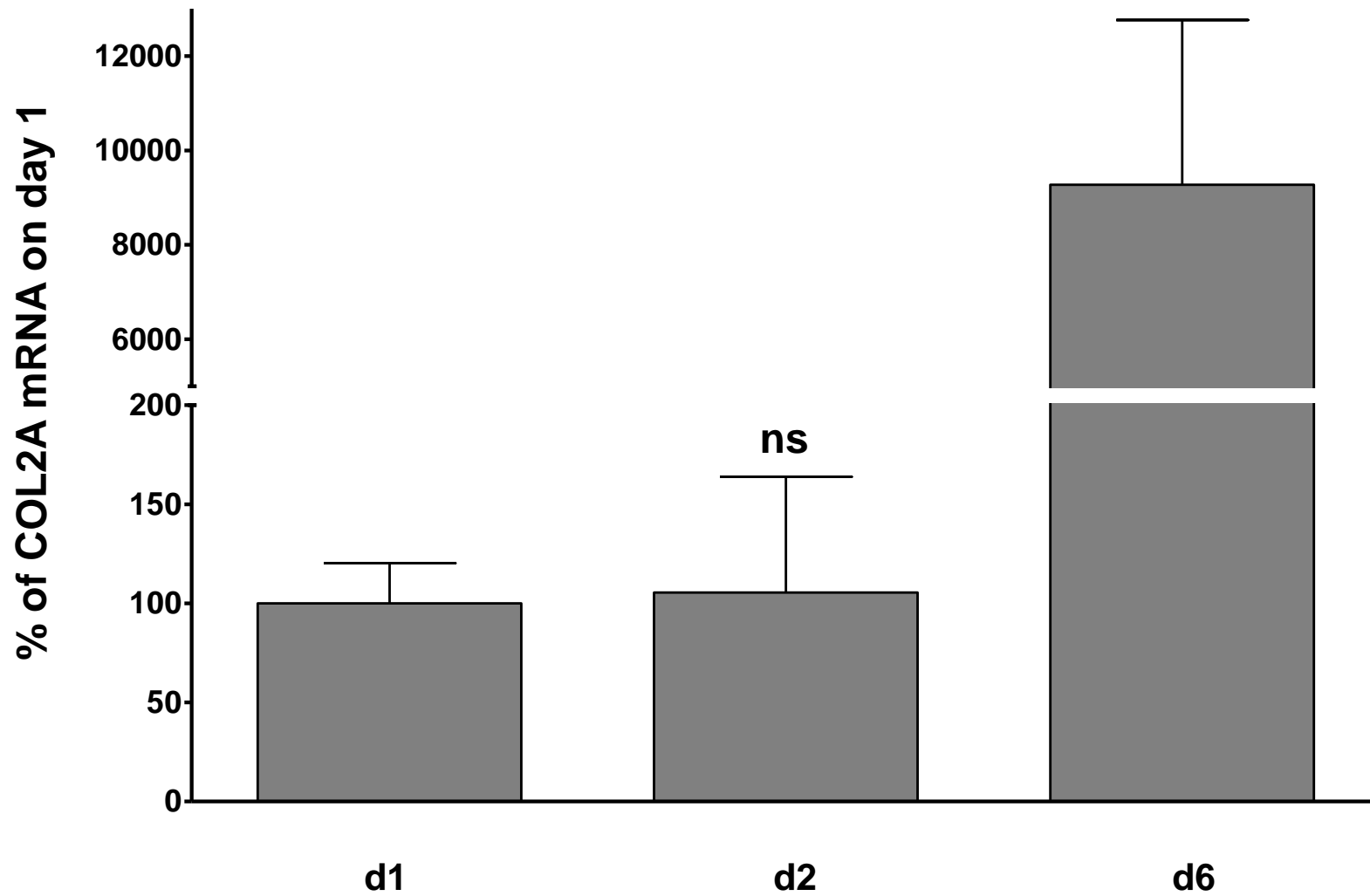
iNOS



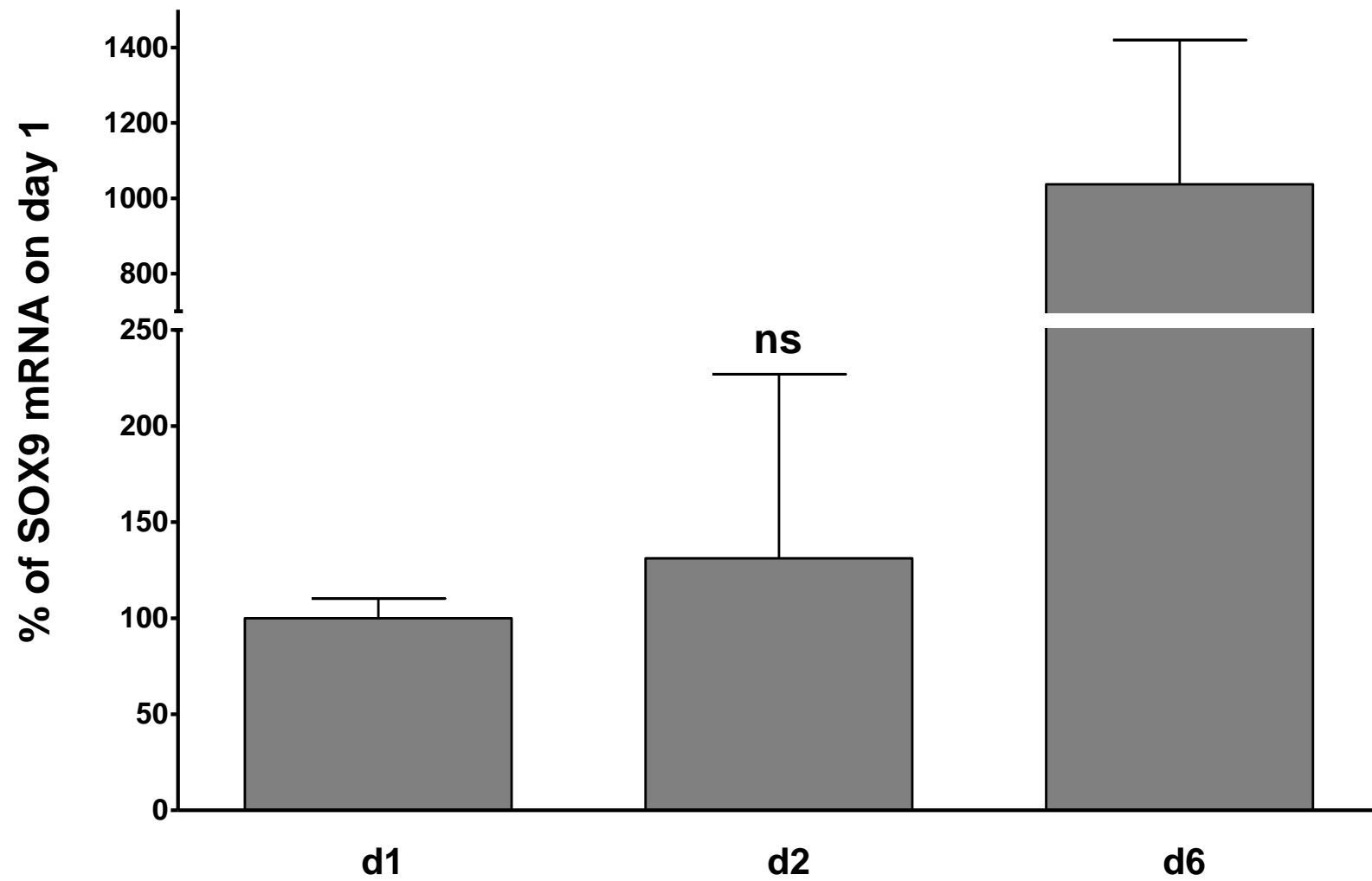
β -tubulin

CM - 2h 4h 6h 8h 24h

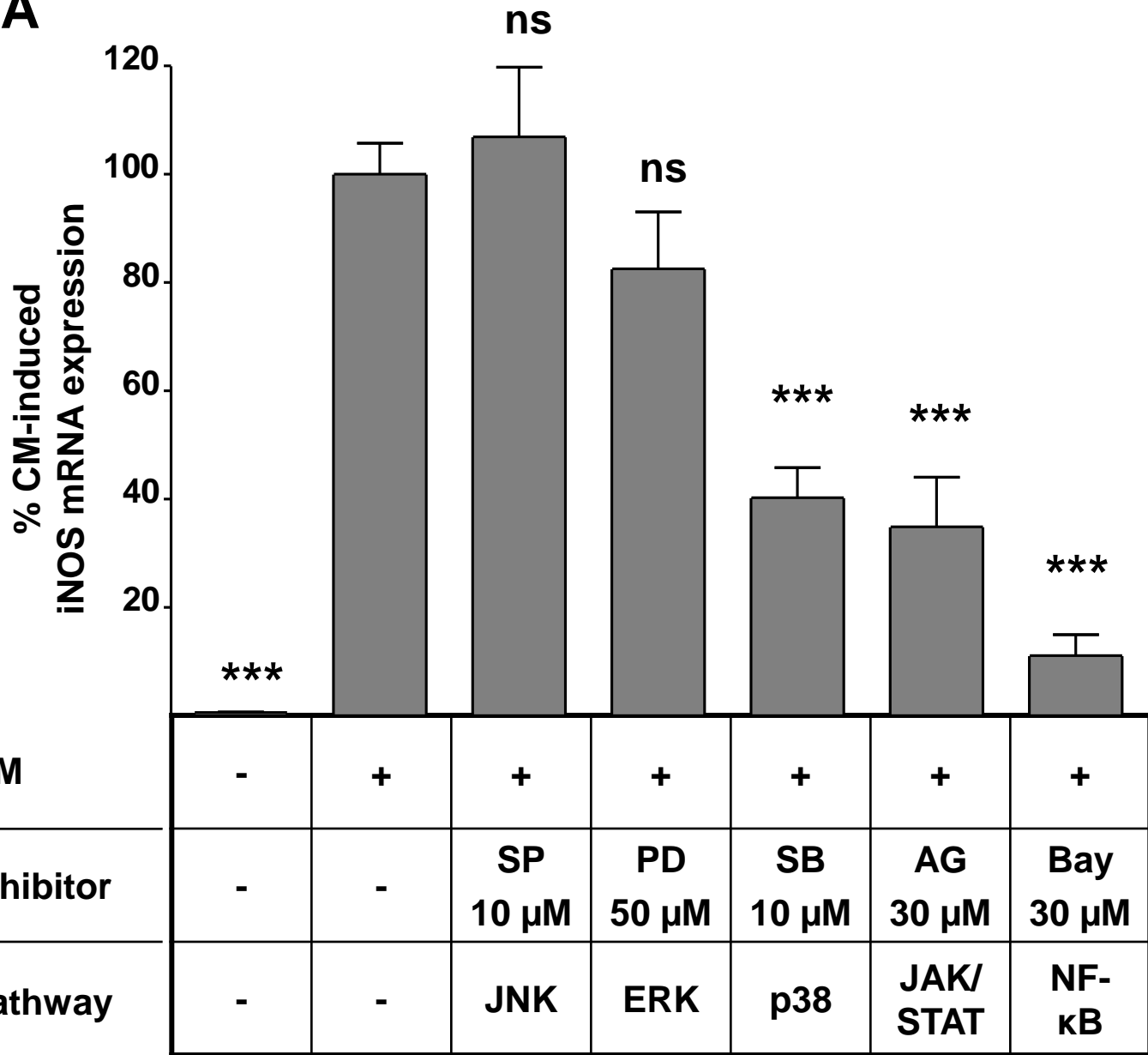
2A



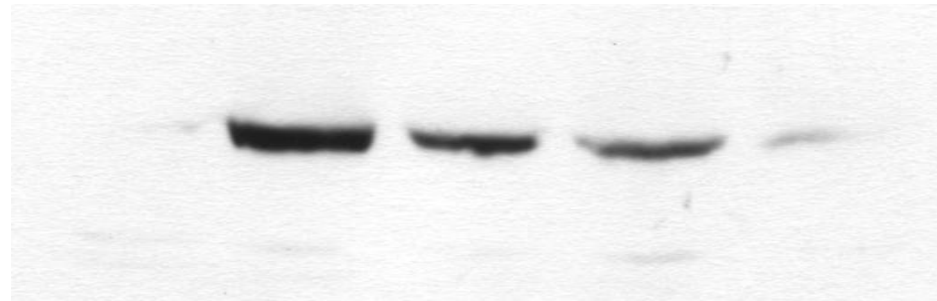
2B



3A



3B



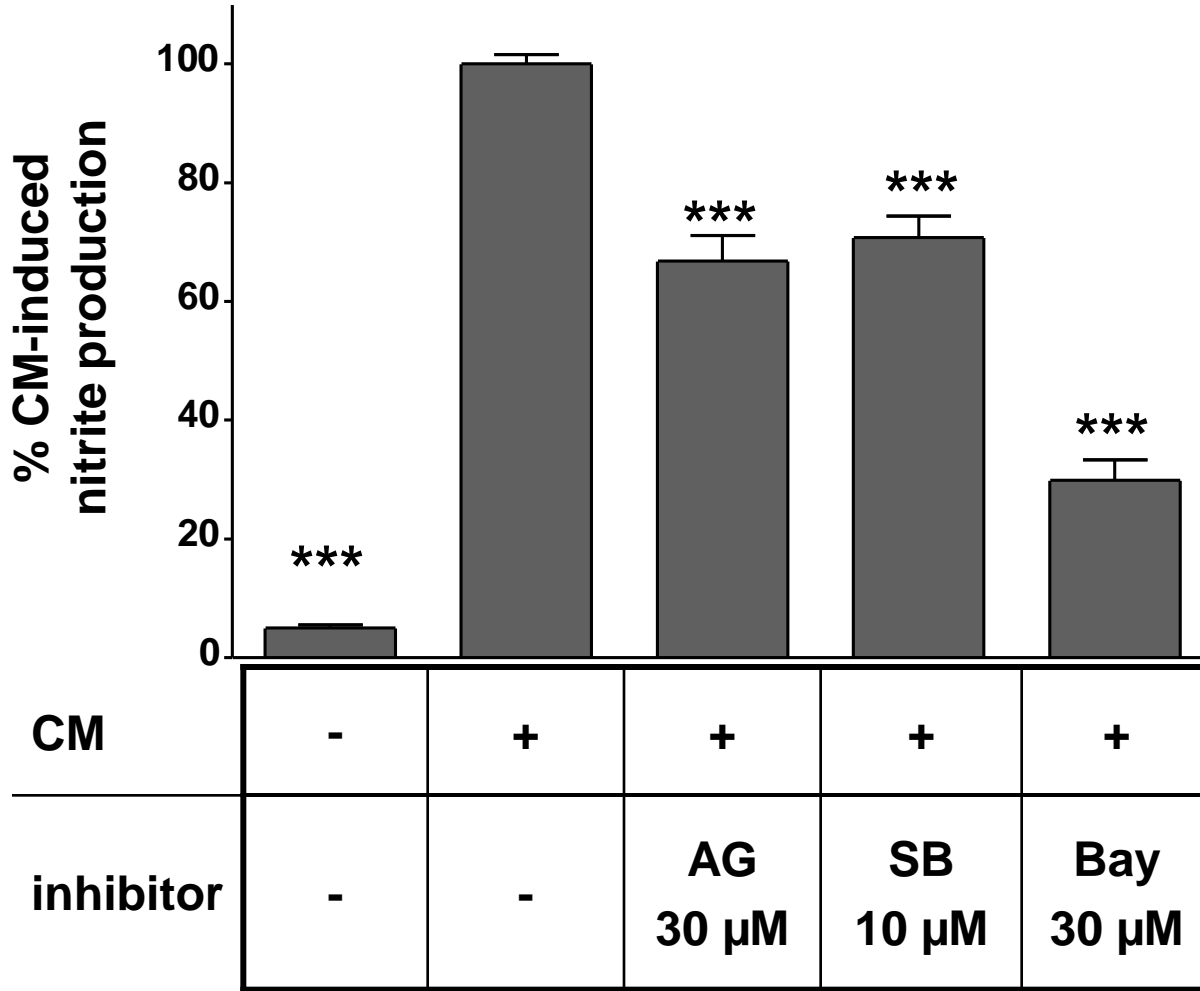
iNOS

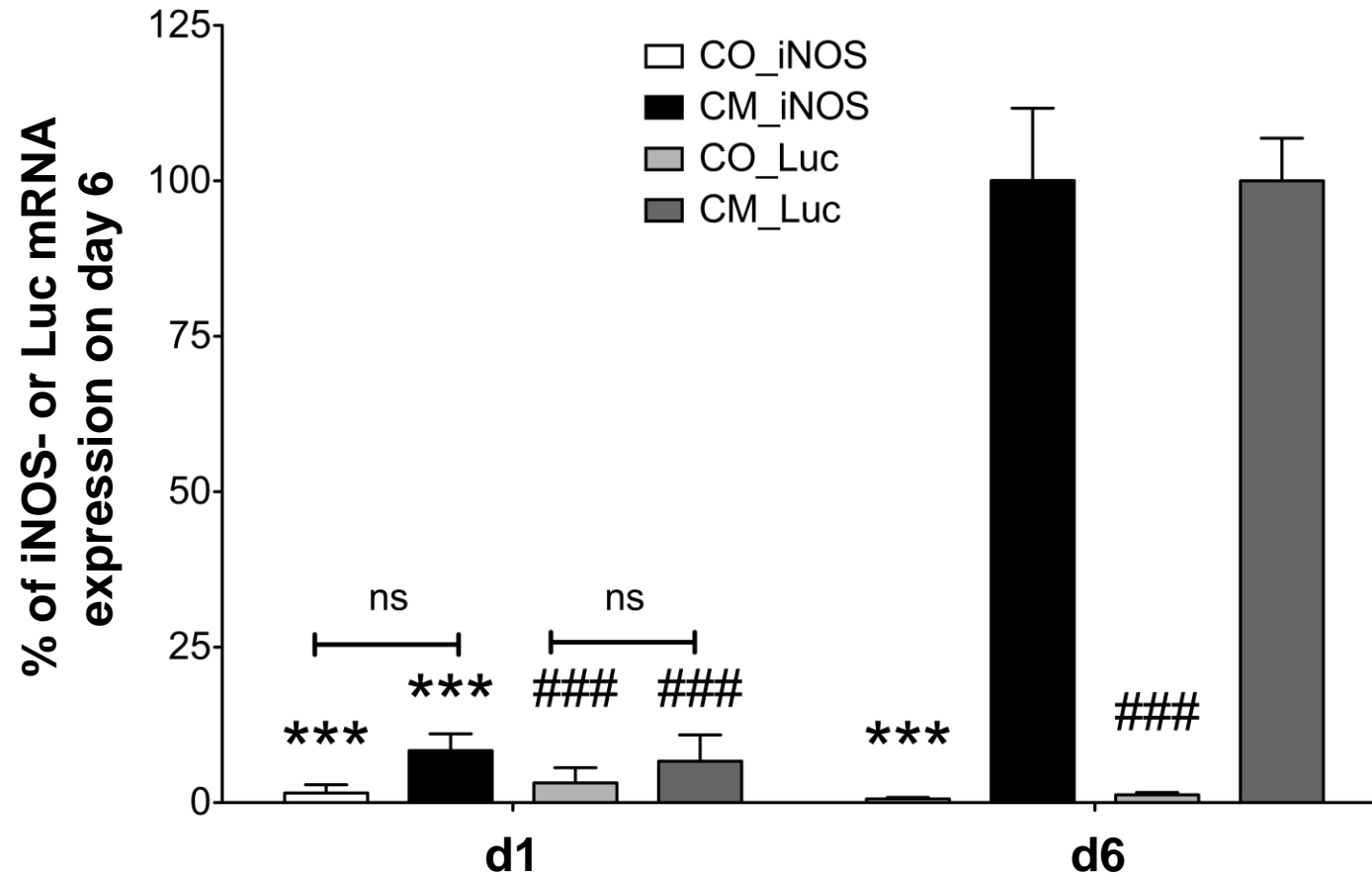


β-tubulin

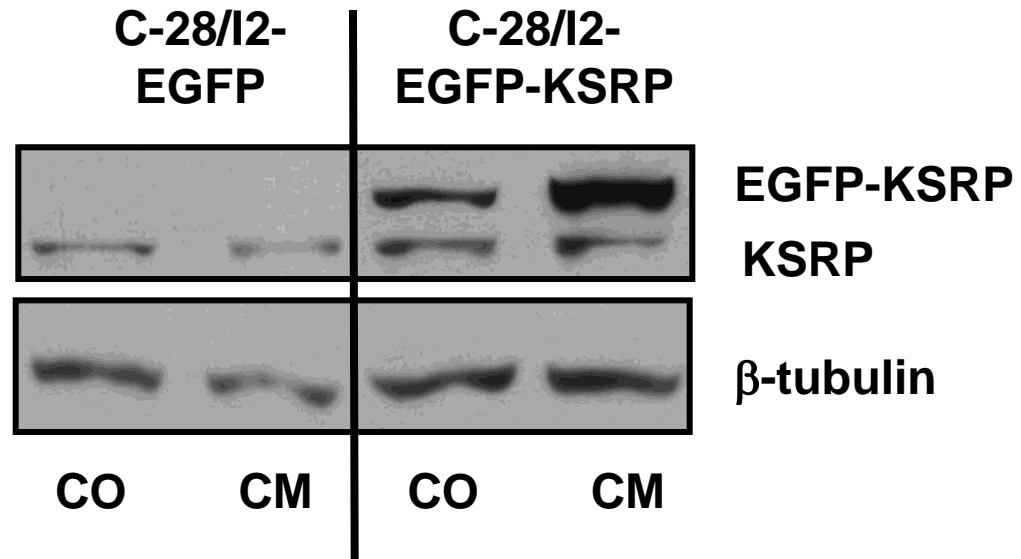
CM	-	+	+	+	+
inhibitor	-	-	AG 30 μM	SB 10 μM	Bay 30 μM

3C

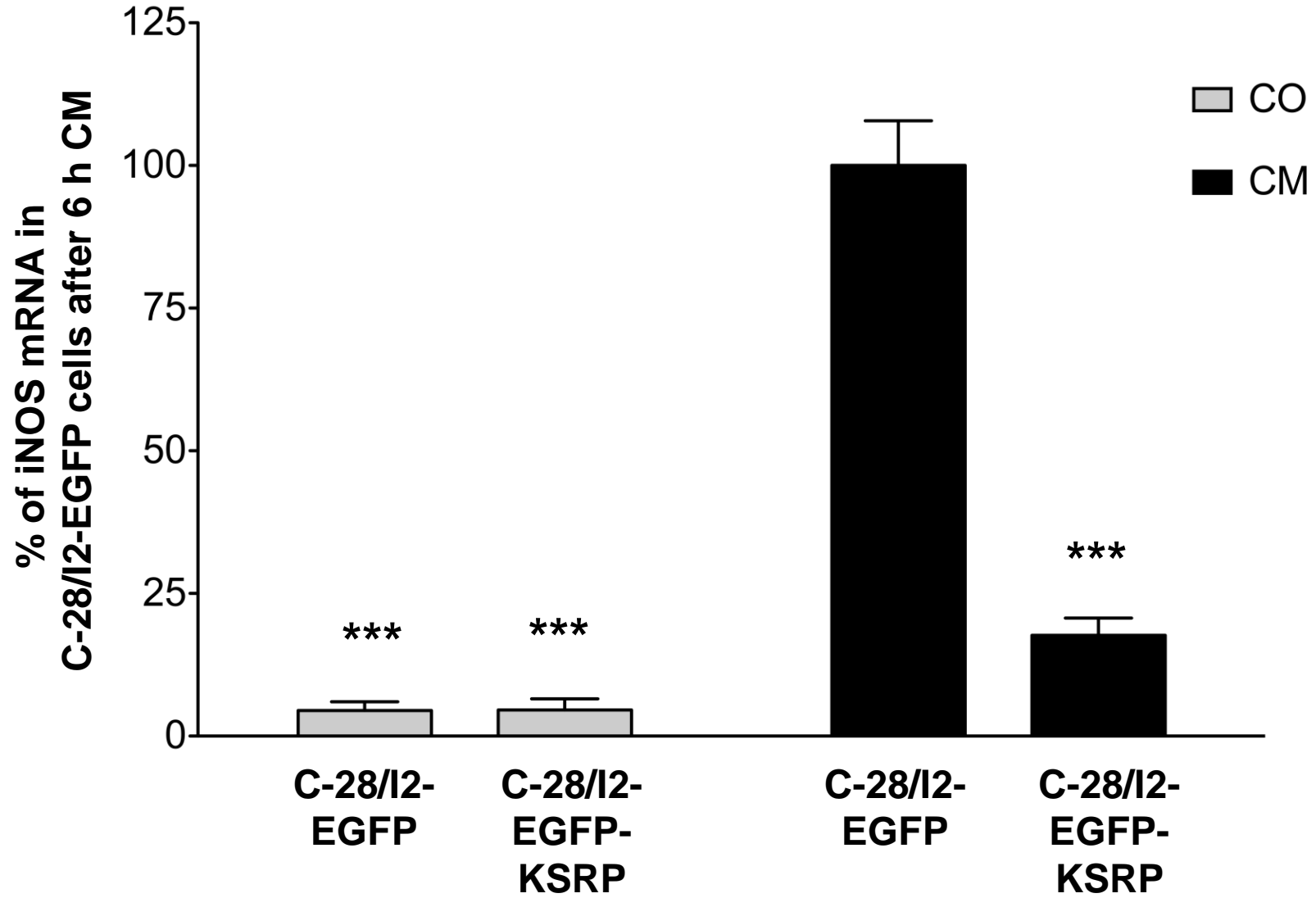




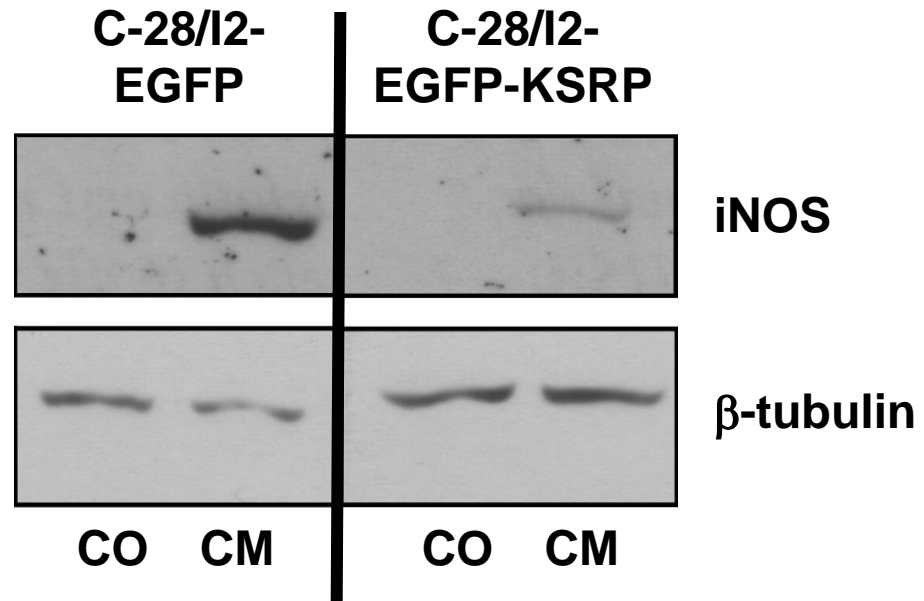
5A



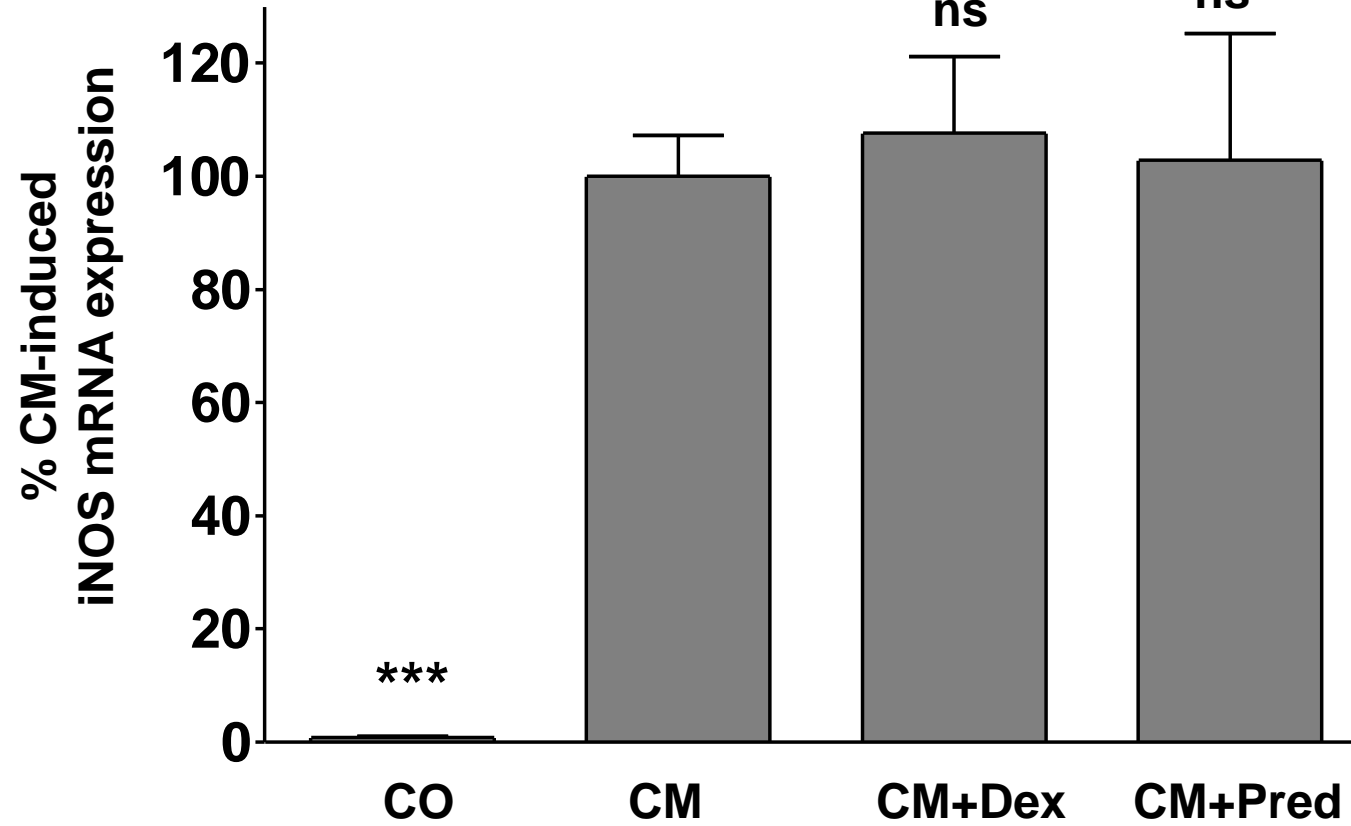
5B



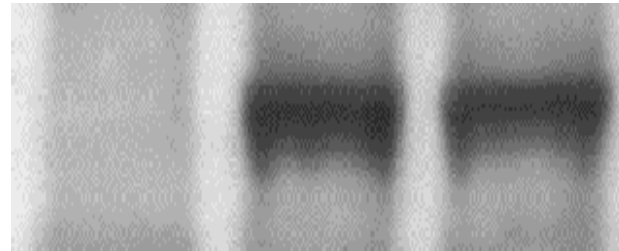
5C



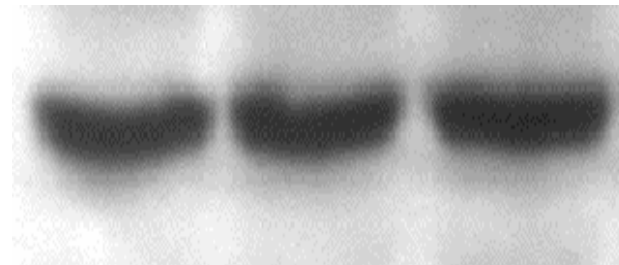
6A



6B



iNOS



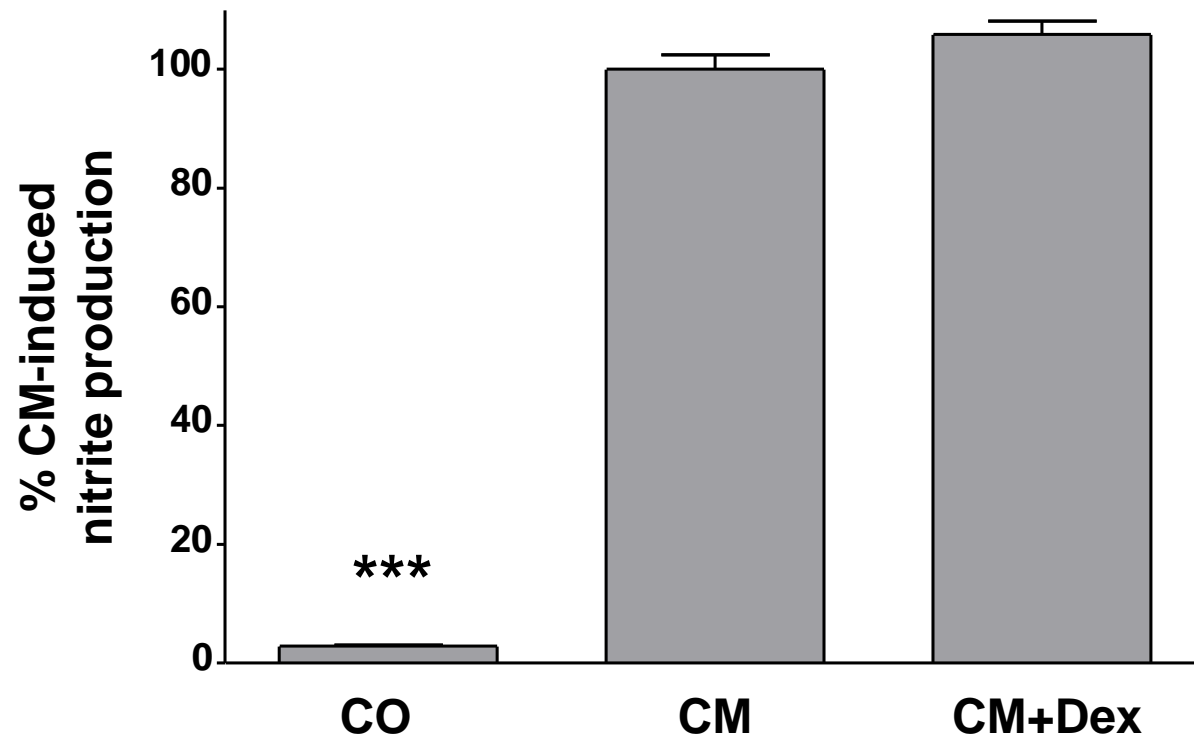
β-tubulin

CO

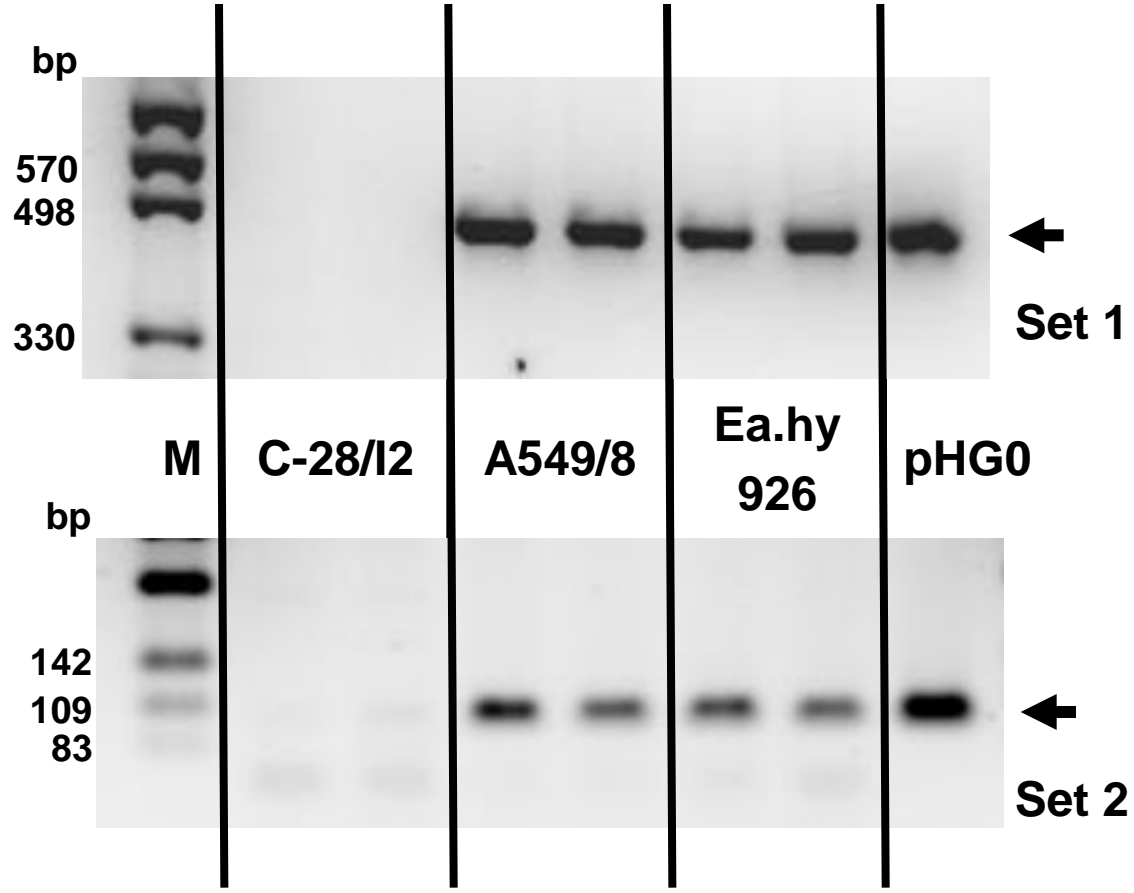
CM

**CM +
5µM Dex**

6C

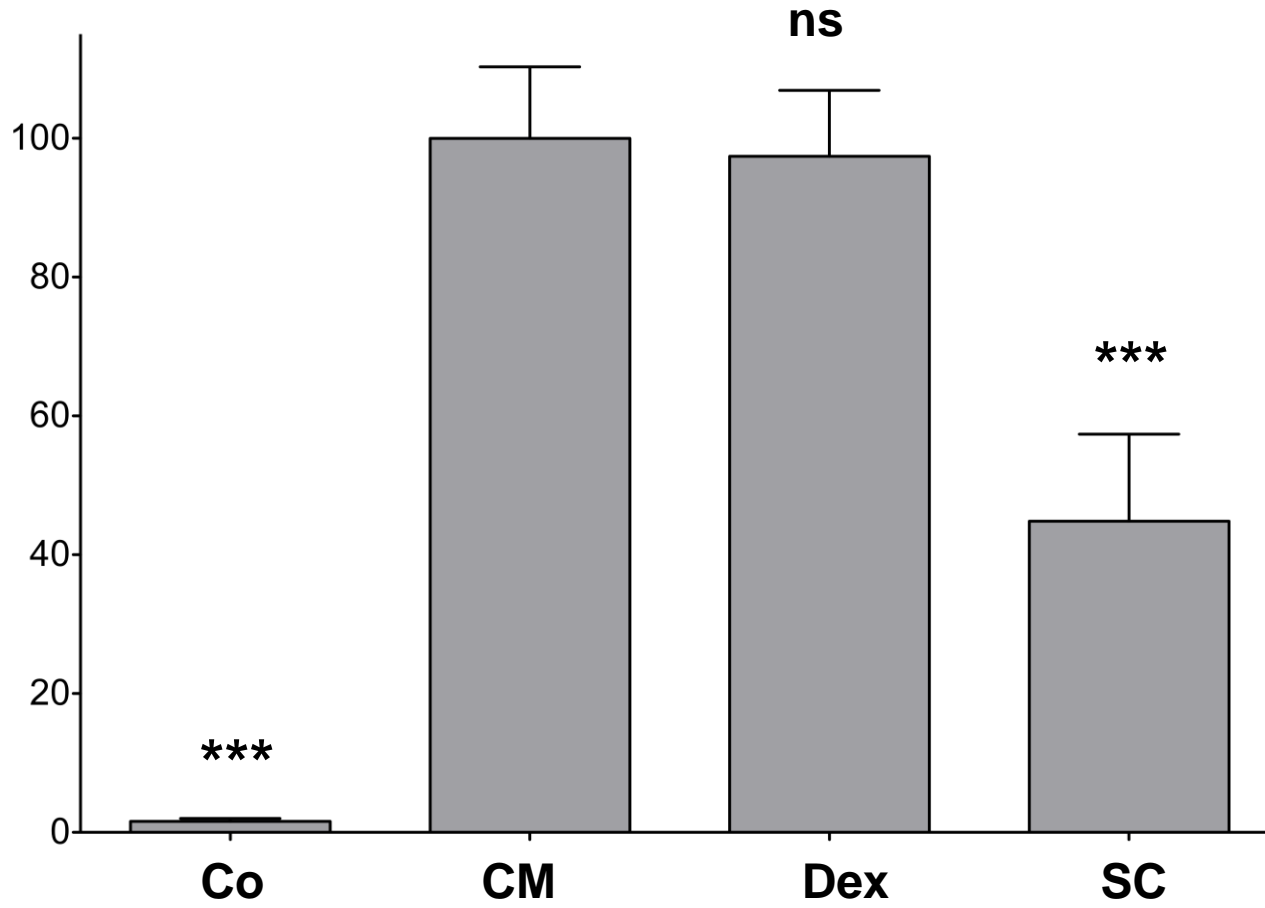


6D

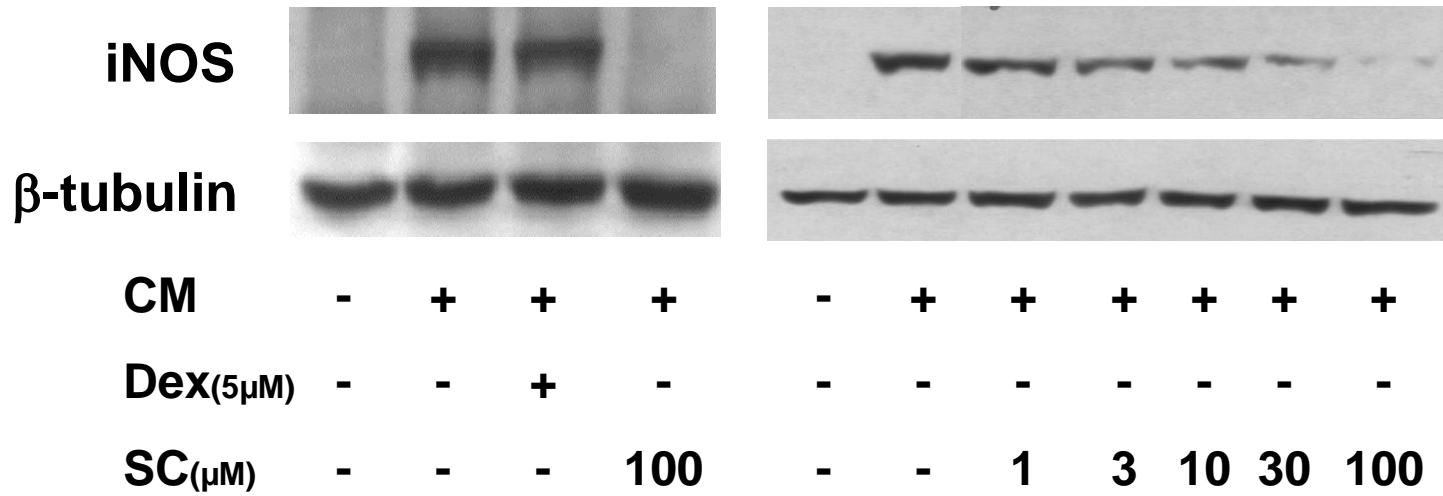


7A

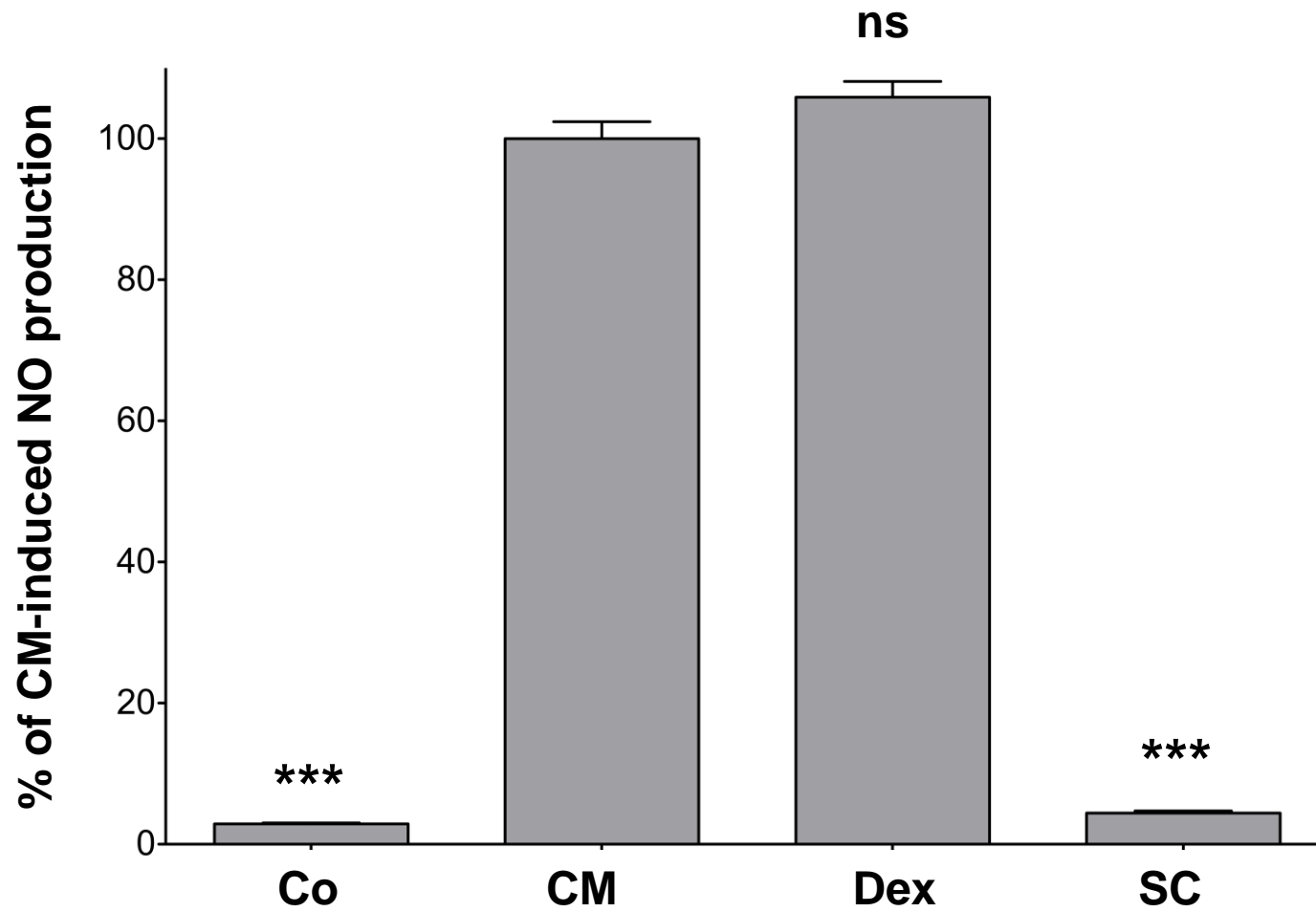
% of CM-induced iNOS mRNA expression



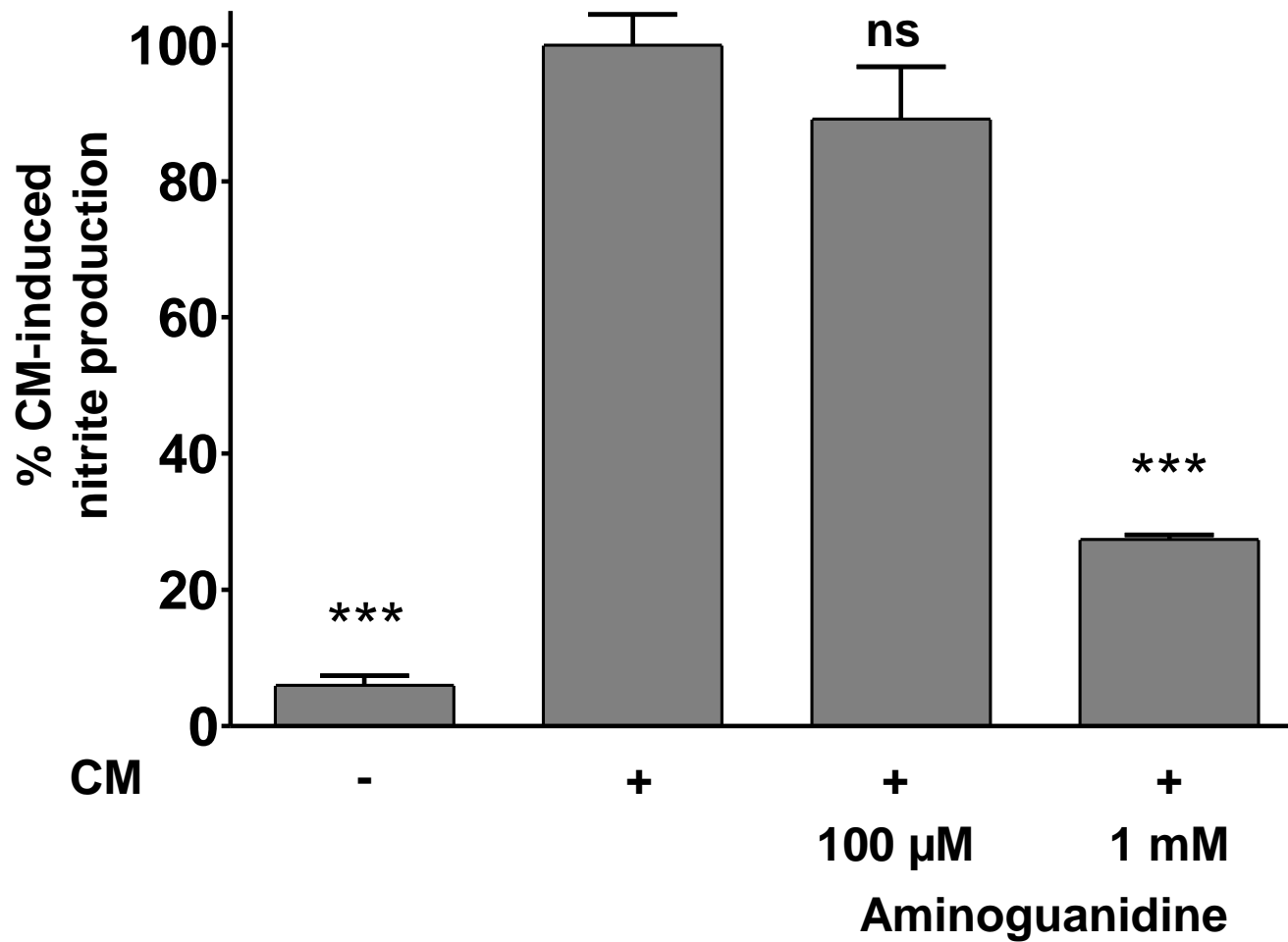
7B



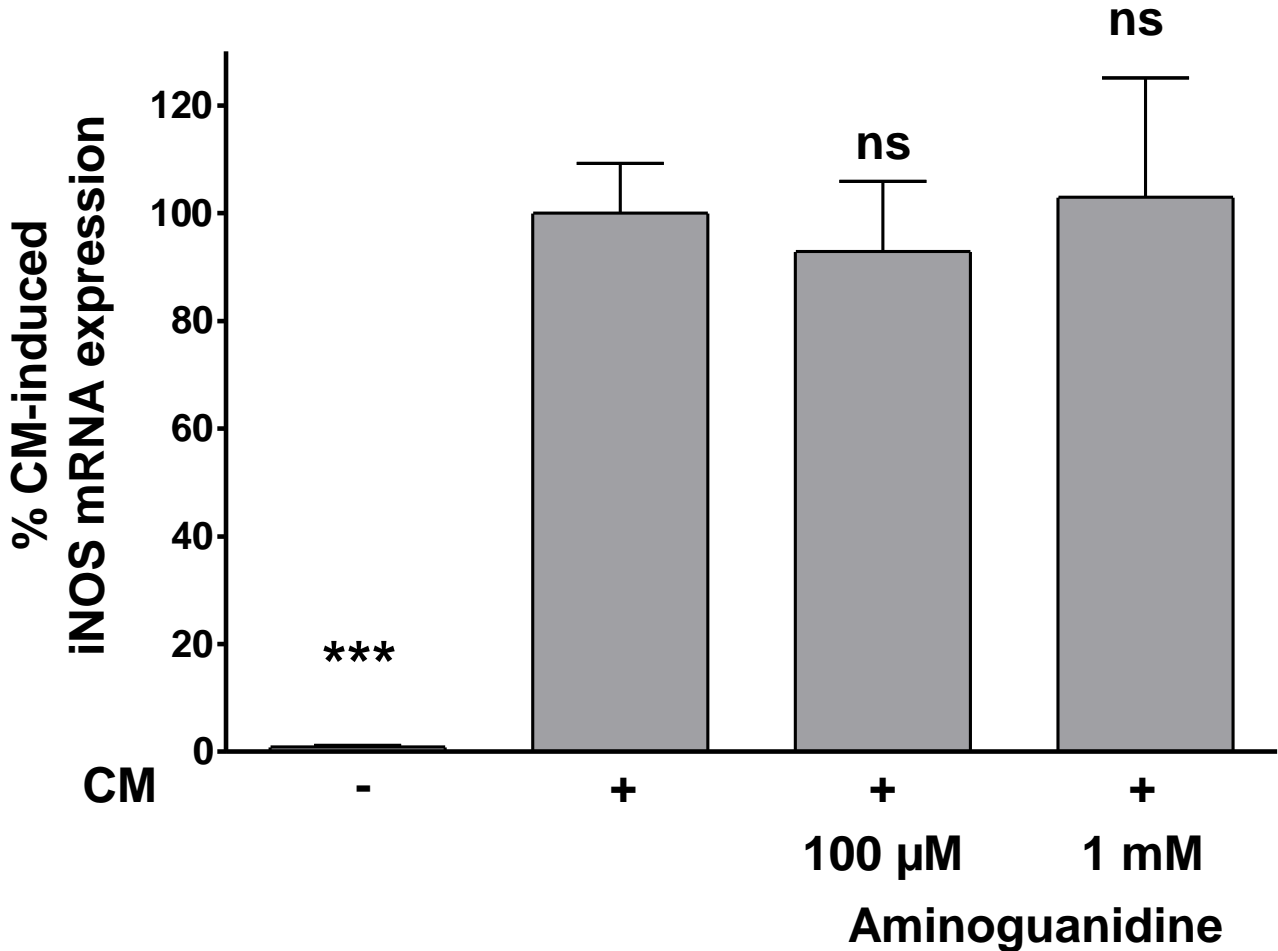
7C



suppl. 1A



suppl. 1B



Legend to supplementary Figure 1.**Inhibition of iNOS-dependent NO-production does not modulate iNOS mRNA expression**

(A) A summary of 5 nitrite analyses using supernatants from superconfluent C-28/I2 treated with (CM) or without (CO) the cytokine mixture for 24 h in the presence or absence of 100 μ M and 1 mM aminoguanidine is shown. Data (means + SEM) represent relative nitrite levels (100% = CM; *** $p < 0.001$; ns not significant vs. CM-treated C-28/I2 cells in the absence of aminoguanidine).

One-way analysis of variance compared to CM	
P value	< 0.0001
F	104,3
total degrees of freedom	23
Tukey's Multiple Comparison Test	
Co vs. CM	***
CM vs. 100 μ M AG	ns
CM vs. 1 mM AG	***

(B) A summary of 5 qRT-PCR analyses to determine iNOS mRNA expression is shown using RNAs from C-28/I2 chondrocytes after monolayer culture for 6 days and CM-treatment for 6 h in the presence or absence of 100 μ M or 1 mM aminoguanidine. Data (means + SEM) represent relative iNOS mRNA levels (100% CM; *** $p < 0.001$; ns = non-significant vs. CM-treated C-28/I2 cells in the absence of aminoguanidine).

One-way analysis of variance compared to CM	
P value	< 0.0001
F	19,05
total degrees of freedom	33
Tukey's Multiple Comparison Test	
Co vs. CM	***
CM vs. 100 μ M AG	ns
CM vs. 1 mM AG	ns

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Supplemental information to statistical analyses

*** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$; ns = not significant

Fig. 1A:

One-way analysis of variance compared to CM_1d	
P value	< 0.0001
F	12,73
total degrees of freedom	29
Tukey's Multiple Comparison Test	
Co_1d vs. CM_1d	ns
Co_6d vs. CM_1d	ns
CM_6d vs. CM_1d	*

Fig. 1B:

One-way analysis of variance compared to CM_1d	
P value	< 0.0001
F	19,98
total degrees of freedom	36
Tukey's Multiple Comparison Test	
Co vs. d1 CM	ns
d1 CM vs. d2 CM	ns
d1 CM vs. d6 CM	***

Fig. 1D

One-way analysis of variance compared to CM 6h	
P value	< 0.0001
F	27,08
total degrees of freedom	46
Tukey's Multiple Comparison Test	
Co vs. 6h	***
2h vs. 6h	***
4h vs. 6h	ns
8h vs. 6h	ns
24h vs. 6h	***

Fig. 2A:

One-way analysis of variance compared to d1	
P value	< 0.0001
F	12,79
total degrees of freedom	40
Tukey's Multiple Comparison Test	
d1 vs. d2	ns
d1 vs. d6	***

Fig. 2B:

One-way analysis of variance compared to d1	
P value	0,0056
F	5,841
total degrees of freedom	46
Tukey's Multiple Comparison Test	
d1 vs. d2	ns
d1 vs. d6	*

Fig 3A:

One-way analysis of variance compared to CM	
P value	< 0.0001
F	72,00
total degrees of freedom	113
Tukey's Multiple Comparison Test	
Co vs. CM	***
CM vs. SP	ns
CM vs. PD	ns
CM vs. SB	***
CM vs. AG	***
CM vs. Bay	***

Fig 3B:

One-way analysis of variance compared to CM	
P value	< 0.0001
F	524,5
total degrees of freedom	108
Tukey's Multiple Comparison Test	
Co vs. CM	***
CM vs. AG	***
CM vs. SB	***
CM vs. Bay	***

Fig. 4:

One-way analysis of variance compared to d6 (iNOS)	
P value	< 0.0001
F	33,77
total degrees of freedom	80
Tukey's Multiple Comparison Test	
Co_iNOS_d1 vs. CM_iNOS_d1	ns
Co_iNOS_d1 vs. CM_iNOS_d6	***
CM_iNOS_d1 vs. CM_iNOS_d6	***
Co_iNOS_d6 vs. CM_iNOS_d6	***

One-way analysis of variance compared to d6 (Luc)	
P value	< 0.0001
F	94,15
total degrees of freedom	80
Tukey's Multiple Comparison Test	
Co_Luc_d1 vs. CM_Luc_d1	ns
Co_Luc_d1 vs. CM_Luc_d6	***
CM_Luc_d1 vs. CM_Luc_d6	***
Co_Luc_d6 vs. CM_Luc_d6	***

Fig. 5B:

One-way analysis of variance compared to C-28/I2-EGFP_CM	
P value	< 0.0001
F	106,2
total degrees of freedom	138
Tukey's Multiple Comparison Test	
Co_EGFP vs. CM_EGFP	***
CM_EGFP vs. CO_KSRP	***
CM_EGFP vs. CM_KSRP	***

Fig. 6A:

One-way analysis of variance compared to CM	
P value	< 0.0001
F	42,62
total degrees of freedom	62
Tukey's Multiple Comparison Test	
Co vs. CM	***
CM vs. Dex	ns
CM vs. Pred	ns

Fig. 6C:

One-way analysis of variance compared to CM	
P value	< 0.0001
F	930,8
total degrees of freedom	26
Tukey's Multiple Comparison Test	
Co vs. CM	***
CM vs. Dex	ns

Fig. 7A:

One-way analysis of variance compared to CM	
P value	< 0.0001
F	34,27
total degrees of freedom	77
Tukey's Multiple Comparison Test	
Co vs. CM	***
CM vs. Dex	ns
CM vs. SC	***

Fig. 7C:

One-way analysis of variance compared to CM	
P value	< 0.0001
F	1211
total degrees of freedom	35
Tukey's Multiple Comparison Test	Significance
Co vs. CM	***
CM vs. Dex	ns
CM vs. SC	***

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Regulation of human iNOS expression in C-28/I2-chondrocytes

