

## **Anti-inflammatory actions of St John's wort: inhibition of human iNOS expression by down regulating STAT-1 $\alpha$ activation**

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**Abbreviations:** CM, cytokine mixture; GAS, gamma-activated site; EMSA, electrophoretic mobility shift assay; IFN- $\gamma$ , interferon- $\gamma$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; IP, immunoprecipitation; JAK, janus kinase; NF- $\kappa$ B, nuclear factor  $\kappa$ B; NO, nitric oxide; NOS, NO synthase; iNOS, inducible NO-synthase; RPA, RNase protection assay; SJW, St John's wort; STAT-1 $\alpha$ , signal transducer and activator of transcription-1 $\alpha$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

## Abstract

St John's wort (SJW) has been described to show anti-inflammatory properties due to its inhibitory effects on the expression of pro-inflammatory genes like cyclooxygenase-2, interleukin 6 and inducible nitric oxide synthase (iNOS). Since iNOS plays a critical role in chronic inflammatory diseases, we have focused our attention on the regulation of iNOS expression by SJW in two different human epithelial cell lines, alveolar A549/8- and colon DLD-1 cells. SJW extract concentration-dependently inhibited human iNOS expression evaluated by measuring the amounts of iNOS mRNA, iNOS protein and NO production in both cell lines. This inhibitory effect resulted from transcriptional inhibition as shown in reporter gene experiments. With electrophoretic mobility shift experiments, we found a SJW-mediated down regulation of the DNA binding activity of the transcription factor "signal transducer and activator of transcription-1 $\alpha$ " (STAT-1 $\alpha$ ), but not of "nuclear factor- $\kappa$ B" (NF- $\kappa$ B). This down regulation of the STAT-1 $\alpha$  DNA binding was shown to result from reduced tyrosine-phosphorylation of the STAT-1 $\alpha$  protein. The diminished STAT-1 $\alpha$  tyrosine-phosphorylation resulted from SJW-mediated reduction of janus kinase 2 activity. These data suggest that extracts from SJW may be a promising anti-inflammatory principle in chronic inflammatory diseases.

Extract from *Hypericum perforatum*, commonly called St John's wort (SJW), is widely used in traditional medicine as anti-depressive, anti-hypertensive (Schwarz and Cupp, 2000) and mild anti-inflammatory drugs (Barnes et al., 2001). SJW contains a number of compounds such as quercetin, hypericin, hyperforin and myricetin, etc (Barnes et al., 2001). In contrast to the significant number of reports on the anti-depressive effects of SJW, little is known about the molecular basis of its anti-inflammatory action. There are some reports attributing the anti-inflammatory effects of SJW or its ingredient quercetin to inhibition of nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation (Bork et al., 1999), inhibition of protein kinase C (PKC) (Agostinis et al., 1996) and reduction of the lipopolysaccharide-, cytokine- or substance P-induced expression of cyclooxygenase-2 (COX-2) (Raso et al., 2001; Raso et al., 2002), inducible nitric oxide synthase (iNOS) (Raso et al., 2001; Raso et al., 2002; Wang and Mazza, 2002) or interleukin 6 (Fiebich et al., 2001). Also its anti-depressive effect has been attributed to the inhibition of interleukin 6 synthesis (Calapai et al., 2001).

Nitric oxide (NO) synthase is a widely distributed enzyme catalyzing the synthesis of NO from L-arginine. There are three isoforms of NO synthase, encoded by distinct genes, including the constitutively expressed neuronal and endothelial NO synthase and the inducible expressed iNOS. iNOS is expressed in most cell types analyzed after induction by various compounds (e.g. by cytokines in inflammatory conditions (Kleinert et al., 2000)). NO produced by iNOS may reach high concentrations (up to mM) around the site of inflammation. These massive amounts of NO are believed to be involved in the elimination of intruding organisms such as bacteria, parasites and viruses (MacMicking et al., 1997). However, these large amounts of NO are also potentially toxic to host tissues. This may explain the involvement of iNOS in the pathobiology of several human diseases (Kröncke et al., 1998). In addition to its immunological role, iNOS-derived NO seems to be important for wound repair (Stallmeyer et al., 1999) and may exert protective functions especially in stress situations of the liver (Kim et al., 1997). iNOS-derived NO seems to be important for human cancer development, either as a weapon of the immune system against the

tumor (Umansky and Schirmacher, 2001) or as an anti-apoptotic (Payne et al., 1999) and pro-angiogenetic (Jaiswal et al., 2001) factor supporting tumor growth. Therefore it is reasonable to expect, that the expression of iNOS mRNA is finely controlled by a concerted action of two categories of compounds: inducers and suppressors (Kleinert et al., 2000). Pro-inflammatory cytokines such as interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL1- $\beta$ ) belong to the former. The latter includes transforming growth factor- $\beta$  (TGF- $\beta$ ), basic fibroblast growth factor (bFGF), estrogen, morphine and dexamethasone (Kleinert et al., 2000). However, in recent years it has been shown that regulation of iNOS expression is cell- and species specific (Kleinert et al., 2000).

Induction of human iNOS gene expression has been shown to depend at least on two major transcription factors, nuclear factor- $\kappa$ B (NF- $\kappa$ B) and the signal-transducer and activator of transcription-1 $\alpha$  (STAT-1 $\alpha$ ) (Geller and Billiar, 1998; Kleinert et al., 2000). The relative importance of these two transcription factors varies with the cell type analyzed (Kleinert et al., 2000). NF- $\kappa$ B is rapidly activated by lipopolysaccharide (LPS), TNF- $\alpha$  and IL-1 $\beta$  (Adcock, 1997), whereas the activation of STAT-1 $\alpha$  is triggered by IFN- $\gamma$  (Darnell et al., 1994). Activated NF- $\kappa$ B and STAT-1 $\alpha$  stimulate the activity of the promoter region of iNOS, thereby inducing expression of the iNOS gene (Kleinert et al., 1998; Ganster et al., 2001). Regulation of the activation of NF- $\kappa$ B and STAT-1 $\alpha$  involves both tyrosine- and serine-phosphorylation. Therefore, the modulation of this process should control the time-spatial expression of iNOS and the successive NO production.

In the current study we tested the hypothesis that the anti-inflammatory action of SJW could depend on an inhibition of the pro-inflammatory transcription factors STAT-1 $\alpha$  and/or NF- $\kappa$ B. Our data indicate, that SJW efficiently inhibits cytokine-induced STAT-1 $\alpha$  activation (but not NF- $\kappa$ B activation), thereby reducing iNOS expression.

## Methods

### *Reagents*

Trypsin-, glutamine-, and pyruvate-solutions, agarose, tRNA and bovine serum albumin (BSA) were purchased from Sigma, Deisenhofen, Germany. Isotopes were obtained from ICN Biomedicals, Eschwege, Germany. T3 and T7 RNA polymerase, RNase A, RNase T1 and DNase I were obtained from Roche Diagnostics, Mannheim, Germany. Human interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were obtained from Strahtmann, Hannover, Germany. Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from PAN-Systems, Nürnberg, Germany. The Dual-Luciferase Reporter Assay System and Passive Lysis Buffer were purchased from Promega, Heidelberg, Germany. Restriction enzymes, Klenow DNA polymerase, dNTPs, NTPs and the PhosphoPlus<sup>®</sup> STAT-1 $\alpha$  (Tyr701) Antibody Kit were from New England Biolabs, Frankfurt a.M., Germany. The monoclonal anti-JAK2 antibody and the monoclonal anti-phospho-tyrosine antibody were obtained from Upstate, Milton Keynes, United Kingdom. The monoclonal anti-IFN- $\gamma$ -receptor- $\alpha$ -subunit antibody and the protein A agarose were obtained from Santa Cruz, Heidelberg, Germany. The St John's wort (SJW) extract was obtained from Indena, Milan, Italy.

### *Cell culture, cytokine treatment, RNA isolation and nitrite measurement*

The human alveolar epithelial A549/8 cells, human colon carcinoma DLD-1 cells and human ECV304 cells were grown in DMEM with 5 to 10 % fetal bovine serum, 2 mM L-glutamine, penicillin and streptomycin. For RNA isolation, they were plated onto 10 cm-diameter (58 cm<sup>2</sup>/well) dishes, whereas those experiments involving luciferase activity determinations or NO production studies were performed with cells plated onto 6-well plates (9.6 cm<sup>2</sup>/well) or 24-well plates (1.75 cm<sup>2</sup>/well). Eighteen hours prior to cytokine induction, cells were washed with PBS solution and incubated with DMEM containing 2 mM L-glutamine in the absence of serum and phenol red. After this preincubation time cells were pretreated with different concentrations of

SJW extract for 1 h. A549/8- and DLD-1 cells were induced with a cytokine mixture (CM) composed of IFN- $\gamma$  (100 U/ml), IL-1 $\beta$  (50 U/ml) and TNF- $\alpha$  (10 ng/ml) for the time periods indicated. Afterwards, the supernatant of the cells (300  $\mu$ l) was used to measure NO $_2^-$  by the Griess reaction and cells were processed for RNA isolation by guanidinium thiocyanate/phenol/chloroform extraction as described (Kleinert et al., 1998).

### ***RNase protection analysis***

For the generation of radio labeled human iNOS- and human GAPDH-antisense probes for RNase protection assays 0.5  $\mu$ g of the linearized plasmids pCR\_iNOS\_human (Kleinert et al., 1996) or pXcm\_GAPDH\_human (Witteck et al., 2003) were *in vitro* transcribed using T3 or T7 RNA polymerase and  $\alpha$ - $^{32}$ P-UTP. To quantify human iNOS mRNA levels, RNase protection experiments were performed as described (Kleinert et al., 1998). In all experiments, GAPDH mRNA expression was determined for normalization purposes. Densitometric analyses were performed using a PhosphorImager (BioRad, Munich, Germany). The protected fragments of human iNOS- and human GAPDH-mRNA were 386 nt or 195 nt and 105 nt, respectively.

### ***Analysis of the human iNOS promoter activity and human eNOS promoter activity***

In order to investigate the effect of SJW extract on cytokine-induced iNOS promoter activity, pools of stably transfected A549/8 or DLD-1 cells (containing a 16 kb fragment of the human iNOS promoter cloned in front of a luciferase reporter gene; (Yao et al., 2003)) were incubated for 18h with DMEM without FCS and without phenol red. Before cytokine induction the cells were pretreated with SJW extract in the concentrations indicated. After CM incubation for 4.5 h in the presence or absence of SJW extract cells were lysed in 1x Passive Lysis Buffer.

In order to investigate the effect of SJW extract on the constitutive human eNOS promoter activity, pools of stably transfected ECV 304 cells (containing a 3.5 kb fragment of the human eNOS promoter cloned in front of a luciferase reporter gene; (Yao et al., 2003)) were incubated for 24h with DMEM without FCS and without phenol red. Then cell were incubated with or without different concentrations of SJW extract for 4 to 5h. Then the cells were lysed in 1x Passive Lysis Buffer.

Firefly luciferase activity was determined using the Dual-Luciferase Assay Kit. Protein concentrations of the extracts were determined by Bradford reagent using BSA as standard. Protein content of the extracts was used for normalization of the luciferase activity.

### ***Western blot experiments***

#### ***Detection of STAT-1 $\alpha$ phosphorylation***

A549/8 and DLD-1 cells incubated with or without CM in the presence or absence of SJW extract for 0.5 h were lysed on ice with 20 mM Hepes pH 7.4, 420 mM NaCl, 1% NP40, 1 mM EGTA, 1mM EDTA for 15 min. After centrifugation for 15 min at 12,000 rpm, proteins (50  $\mu$ g/lane) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 7.5% gel, electroblotted onto PVDF membrane (Millipore S.p.A. Rome, Italy), and reacted with anti-STAT-1 $\alpha$  phosphotyrosine 701 antibody diluted 1:700 (New England Biolabs, Hetchin, England) and after stripping, with anti-STAT-1 $\alpha$  antibody diluted 1:1000 according to standard procedures. Immune complexes were detected by using anti-rabbit horseradish peroxidase-conjugated immunoglobulin for detection of the primary antibody. The immunoreactive proteins on the blot were detected by enhanced chemiluminescence detection system (ECL, Amersham).

#### ***Detection of iNOS protein***

DLD-1 cells incubated with or without CM in the presence or absence of SJW extract for 24h were lysed on ice with RIPA buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 1%



NP40, 1 mM EGTA, 1mM EDTA) for 20 min. After centrifugation for 20 min at 12,000 rpm, proteins (80 µg/lane) were fractionated by SDS-PAGE in a 7.5% gel, electroblotted onto PVDF membrane (Millipore S.p.A. Rome, Italy), and reacted with a polyclonal anti-iNOS antibody (Transduction Laboratories, Lexington, KY) diluted 1:200 according to standard procedures. Immune complexes were detected by using anti-rabbit horseradish peroxidase-conjugated immunoglobulin for detection of the primary antibody. The immunoreactive proteins on the blot were detected by enhanced chemiluminescence detection system (ECL, Amersham).

***Immunoprecipitation analyses of tyrosine-phosphorylated JAK2 or IFN- $\gamma$  receptor subunit  $\alpha$***

A549/8 and DLD-1 cells incubated with or without CM in the presence or absence of SJW extract for 0.5 h were lysed on ice with RIPA buffer for 15 min. After centrifugation for 15 min at 12,000 rpm, proteins (500 µg/sample) were incubated overnight at 4 °C with a monoclonal anti-JAK2- or a monoclonal anti-IFN- $\gamma$ -receptor-subunit- $\alpha$  antibody at the concentration recommended by the supplier. Then the immunocomplexes were incubated with protein A-agarose and precipitated by centrifugation. After 3 washing steps with RIPA buffer (2x) and PBS (1x) the immunoprecipitated material was resuspended in Laemmli sample buffer and separated on SDS-PAGE in a 5 or 7.5% gel, electroblotted onto PVDF membrane (Millipore S.p.A. Rome, Italy), and reacted with an anti-phosphotyrosine antibody (Upstate) diluted 1:1000 according to standard procedures. Immune complexes were detected by using anti-rabbit horseradish peroxidase-conjugated immunoglobulin for detection of the primary antibody. The immunoreactive proteins on the blot were detected by enhanced chemiluminescence detection system (ECL, Amersham). After detection of tyrosine-phosphorylated JAK2 or IFN- $\gamma$ -receptor-subunit- $\alpha$  the blots were stripped and reprobed with a monoclonal anti-JAK2- or anti-IFN- $\gamma$ -receptor-subunit- $\alpha$  antibody, respectively (for normalization).

### ***Electrophoretic mobility shift assay***

Nuclear extracts were prepared from cells treated with or without cytokines in the presence or absence of SJW extract according to Osborn et al. (Osborne et al., 1997) in the presence of 10 µg/ml leupeptin, 5 µg/ml antipain and pepstain, and 1 mM PMSF (Sigma). Protein concentration in the nuclear extracts was determined by using the Bradford reagent. Nuclear extracts (10 µg) were incubated at room temperature for 20 min with a <sup>32</sup>P-labeled double-stranded oligonucleotide, containing the STAT-1α binding site (sis-inducible factor-binding recognition element, SIE/m67) from the c-fos promoter (5'-gtcgaCATTTCCCGTAAATCg-3'), or the NF-κB binding sequence from the IL-6 promoter (5'-gatcCAGAGGGGACTTTCCGAGt-3') (Promega, Milan, Italy), in a 15 µl reaction mixture containing 20 mM HEPES, pH 7.9, 50 mM KCl, 10% glycerol, 0.5 mM DTT, 0.1 mM EDTA, 2 µg poly(dI-dC), 1 µg salmon sperm DNA. Products were fractionated on a nondenaturing 5% polyacrylamide gel in TBE (Tris-Borate-EDTA buffer; 1.08 % Tris, pH 8.3, 0.55 % boric acid, and 20 mM EDTA).

### ***Calculations***

All data are presented as mean ± SEM. Differences between means were tested for statistical significance using factorial ANOVA followed by Fisher's PLSD test as the posthoc test (StatView software, SAS Institute).

## Results

### ***St John's wort (SJW) extract inhibited human iNOS mRNA- and protein expression and nitrite production in A549/8 and DLD-1 cells***

As shown in figure 1, SJW extract (**SJW**) inhibited concentration-dependently CM-elicited iNOS mRNA expression in A549/8 and DLD-1 cells. Also the cytokine-induced expression of iNOS protein was inhibited by SJW extract (figure 2). Inhibition of cytokine-induced NO production was also observed (figure 3).

### ***SJW extract inhibited human iNOS promoter activity in stably transfected A549/8- and DLD-1 cells***

In order to analyze the effect of SJW extract on human iNOS promoter activity, we incubated A549/8 and DLD-1 cells, stably transfected with pNOSII(16)Luc (Yao et al., 2003), with different concentrations of SJW extract. The plasmid pNOSII(16)Luc contains a 16 kb fragment of the human iNOS promoter, cloned in front of a luciferase reporter gene. In line with previous reports (Hausding et al., 2000; Witteck et al., 2003; Yao et al., 2003), CM incubation of these pools of stably transfected A549/8- and DLD-1 cells resulted in a up to 5-fold enhancement of the luciferase expression (figure 4). SJW extract inhibited concentration-dependently the CM-induced iNOS promoter activity in these cells (figure 4).

### ***Only moderate inhibition by SJW extract of a human 3.5 kb eNOS promoter.***

To determine whether the inhibition of iNOS expression was a result of the inhibition of general transcription factors, the effect of SJW extract on a constitutively active promoter was analyzed. For this purpose ECV-pNOS III-Hu-3500-Luc-neo cells (Yao et al., 2003) were used. These cells derive from human ECV304 cells transfected with the plasmid pNOS III-Hu-3500-Luc-neo. This plasmid contains containing a 3.5 kb fragment of the human eNOS promoter cloned in front of a luciferase reporter gene. This promoter displayed a significant constitutive activity in the stably transfected cells (see figure 5). Incubation of ECV-pNOS III-Hu-3500-Luc-

neo cells with the highest concentrations of SJW extract only resulted in a moderate reduction of eNOS promoter activity (control cells 100%; SJW extract, 50 µg/ml, 65.1% ± 4.3%, n = 14).

***SJW extract inhibited STAT-1 $\alpha$  DNA binding, but did not effect NF- $\kappa$ B DNA binding***

In order to study the effect of SJW extract on the DNA binding activity of transcription factors in A549/8- and DLD-1 cells we performed an EMSA (electrophoretic mobility shift assay) for STAT-1 $\alpha$  and NF- $\kappa$ B, known to play a critical role in human iNOS expression. As shown in figures 6 and 7, cytokine treatment rapidly induced both STAT-1 $\alpha$  and NF- $\kappa$ B DNA binding in both cell lines. However, only STAT-1 $\alpha$  DNA binding was reduced concentration-dependently by co-incubation of cytokine-treated cells with SJW extract (figure 6). In contrast, cytokine-induced NF- $\kappa$ B DNA binding remained unchanged (figure 7).

***SJW extract inhibited the activation of STAT-1 $\alpha$  in A549/8 cells and DLD-1 cells***

To further analyze the mechanism SJW-mediated inhibition of STAT-1 $\alpha$  activity in DLD-1 and A548/9 cells, we studied STAT-1 $\alpha$  activation by immunoblot analyses using an antibody, which specifically detects the tyrosine-(Tyr701)-phosphorylated form of STAT-1 $\alpha$  (Ihle, 1995). This phosphorylation is critical for the activation of the STAT-1 $\alpha$  protein and enables it to dimerize, to migrate into the nucleus and to bind to specific STAT-1 $\alpha$  binding sites on the DNA (the GAS elements). Incubation of A549/8 and DLD-1 cells for 30 min with CM resulted in a marked enhancement of STAT-1 $\alpha$  tyrosine phosphorylation (figure 8). Co-incubation of CM-treated A549/8 and DLD-1 cells with SJW extract (50 µg/ml) completely inhibited the Tyr701 phosphorylation, in line with the inhibition of DNA binding activity seen upon treatment with SJW (figure 6).

### **SJW extract inhibited the activity of JAK2 in DLD-1 cells**

Finally, we analyzed the mechanism of inhibition of STAT-1 $\alpha$  activation by testing the effect of SJW extract on janus kinase 2 (JAK2) activity. JAK2 activation has been described to be an essential step for STAT-1 $\alpha$  activation in several cell types (Darnell et al., 1994). Specific inhibition of JAK2 activation by tyrphostin AG490 has been described to inhibit cytokine-induced iNOS expression in human cells (Kleinert et al., 1998). Therefore, we performed immunoprecipitation analyses for JAK2 tyrosine-autophosphorylation (figure 9A) and for tyrosine-phosphorylation of the IFN- $\gamma$  receptor subunit- $\alpha$  (figure 9B). Both assays indicated that SJW extract (50  $\mu$ g/ml) inhibited cytokine-mediated JAK2 activation in A549/8 and DLD-1 cells.

## Discussion

Induction of iNOS expression is a hallmark of inflammatory diseases, especially at the early phase of inflammation. Under certain conditions, time-spatially well-controlled production of NO by iNOS are beneficial for the body, because of their involvement in the elimination of intruding organisms causing inflammation (MacMicking et al., 1997). However, deregulation of iNOS expression, leading to either prolonged or spatially abnormal production of massive amounts of NO, can trigger severe tissue damage, and is often observed in chronic inflammation (Kröncke et al., 1998; Kleinert et al., 2000). Studies in which cells were treated with (i)NOS enzyme inhibitors, in an attempt to decrease overproduction of NO, often produced conflicting results. This may be due in part to toxic secondary effect of these inhibitors or other nonspecific effects (Alderton et al., 2001). Also the use (i)NOS enzyme inhibitors can cause unwanted effects, such as hypertension (Husain, 2002).

An alternative approach to block iNOS-dependent NO production is the suppression of iNOS induction (Yao et al., 2003). In contrast to eNOS and nNOS, which are constitutively expressed, expression of iNOS has to be induced by cytokines and other compounds (Kleinert et al., 2000). Also the transcription machinery involved in the expression of the three NOS isoforms differs markedly (Kleinert et al., 2000). Therefore, there is a good probability, that specific inhibitors of iNOS expression can be generated.

At least two distinct transcription factors, NF- $\kappa$ B and (probably more important) STAT-1 $\alpha$ , are necessary for the induction of human iNOS expression (Kleinert et al., 2000). The transcription factor NF- $\kappa$ B, activated by LPS, TNF- $\alpha$  and IL-1 $\beta$ , has been considered a promising target of drugs that reduce the expression of pro-inflammatory genes (Makarov, 2000). However, in some cases inhibition of NF- $\kappa$ B failed to reduce iNOS-derived NO production (Kleinert et al., 1998; Leonard et al., 1998). Glucocorticoids, which are widely used as anti-inflammatory drugs are

effective NF- $\kappa$ B inhibitors. However their (prolonged) clinical use is associated with serious side effects (Schacke et al., 2002).

More recently, several authors have described the dependence of iNOS induction on the IFN- $\gamma$ -JAK-STAT pathway (Kleinert et al., 1998; Kleinert et al., 2000; Dell'Albani et al., 2001; Ganster et al., 2001; Ohmori and Hamilton, 2001). Therefore, the IFN- $\gamma$ -JAK-STAT pathway seems to be a reasonable target for the development of inhibitors of iNOS expression.

However, most inhibitors of STAT-1 $\alpha$  activation are associated with severe cell toxicity. Recently some compounds like the green tea polyphenol epigallocatechin-3-gallate (Menegazzi et al., 2001), statins (Sadeghi et al., 2001) and the fungal metabolites S-curvularin, S14-95 and sporogen (Yao et al., 2003) have been reported as inhibitors of STAT-1 $\alpha$  activation with low toxicity.

SJW, a plant extract widely used in phyto-medicine, has been reported to exert some anti-inflammatory effects. Since the molecular mechanism of this anti-inflammatory actions are unknown we tested the hypothesis that SJW could interfere with pro-inflammatory transcription factors thereby inhibiting iNOS expression. As cellular models we chose two epithelial derived human cell lines: DLD-1 colon carcinoma and A548/9 alveolar carcinoma cells, because several chronic inflammatory diseases of the colon and the lung correlate with an aberrant expression of iNOS (Guslandi, 1998; Kröncke et al., 1998).

Data presented in this work clearly show that SJW extract inhibited concentration-dependently iNOS synthesis (mRNA and protein, figures 1 and 2) and NO production (figure 3) in these cell lines. This inhibition of iNOS expression seemed to result from SJW-mediated inhibition of human iNOS promoter activity (figure 4). In all cases, the concentrations required for half maximal inhibition were below the concentrations showing an effect on an unrelated promoter (eNOS) used for control purposes (figure 5). Thus the inhibitory effect of SJW extract on iNOS expression is unlikely to result from the inhibition of general transcription factors or cell viability.

As both NF- $\kappa$ B and STAT-1 $\alpha$  have been reported as regulators of human iNOS promoter activity, we analyzed the effect of SJW extract on these two transcription factors. As shown in figures 6 and 7 SJW extract inhibited concentration-dependently STAT-1 $\alpha$ -, but not NF- $\kappa$ B DNA binding activity in human epithelial A549/8- and DLD-1 cells. Therefore, the SJW-mediated inhibition of iNOS expression is likely to result from SJW-related inhibition of STAT-1 $\alpha$  activation. To analyze the mechanism of SJW-related inhibition of STAT-1 $\alpha$  activation, we determined the effect of SJW on the tyrosine phosphorylation of STAT-1 $\alpha$  and on the tyrosine kinase JAK2, described to be essential for IFN- $\gamma$ -mediated STAT-1 $\alpha$  activation (Briscoe et al., 1996). As shown in figure 8, SJW extract inhibited STAT-1 $\alpha$  tyrosine phosphorylation, which had been described to be essential for STAT-1 $\alpha$  DNA binding and activation of promoter activity of STAT-1 $\alpha$ -dependent genes. This down regulation of STAT-1 $\alpha$  tyrosine phosphorylation resulted from SJW extract-mediated inhibition of cytokine-induced JAK2 activity (figure 9).

Our data show that inhibition of the IFN- $\gamma$ -JAK-STAT-1 $\alpha$  pathway by SJW extract may be an efficient way to down-regulate iNOS-related NO production. Therefore, in treating inflammatory diseases, the use of SJW may be an alternative or adjuvant to the use of anti-inflammatory steroids or may help to reduce the steroid dose. This can be clinically relevant, because the long term use of anti-inflammatory steroids is associated with significant side effects (Shanley et al., 2002), and glucocorticoids failed to exert a substantial effect on intestinal NO synthesis in inflammatory bowel disease (Leonard et al., 1998).

Interestingly in contrast the murine system (Raso et al., 2002) we did not observe any inhibitory effect of SJW extract on the LPS/cytokine-induced NF- $\kappa$ B binding activity in human epithelial A549/8- or DLD-1 cells. Therefore in human, unlike murine cells, inhibition of NF- $\kappa$ B is unlikely to be involved in the reduction of iNOS expression by SJW. This is in agreement with our previous report, showing that induction of iNOS depends only partially on NF- $\kappa$ B activation in human cells (Kleinert et al., 1998).



In conclusion, SJW extract represents an effective inhibitory principle of human iNOS induction. The mayor mechanism of action seems to be suppression of IFN- $\gamma$ -elicited STAT-1 $\alpha$  activation.

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## **Footnotes**

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## Legends to Figures

### Figure 1. Effect of St John's wort (SJW) extract on cytokine-induced human iNOS mRNA expression in epithelial A549/8 and DLD-1 cells

**Panel A:** Representative RNase protection assay for iNOS mRNA performed with total RNA from human A549/8 cells. Cells were preincubated for 18h in serum-free medium and for an additional 1h with different concentrations of SJW extract (**SJW**; 1 - 100  $\mu$ g/ml). Then a cytokine mixture (**CM**, 100 U/ml interferon- $\gamma$ , 100 U/ml interleukin-1 $\beta$  and 10 ng/ml tumor necrosis factor- $\alpha$ ) was added to some of the cultures and the cells were incubated for an additional 8h with or without the above concentrations of SJW. Experiments were performed using antisense RNA probes for human iNOS and GAPDH (used for normalization). The positions of the protected iNOS- and GAPDH fragments are indicated. (**M**: molecular weight standard,  $\Phi$ X174-restricted with *Hinf* I; **I**: iNOS antisense probe; **G**: GAPDH antisense probe).

**Panel B:** Densitometric analyses of six different gels using RNA isolated from A549/8 cells. Columns (means  $\pm$  SEM) represent relative iNOS mRNA levels at the different concentrations of SJW extract (\*  $p < 0.05$ ; \*\*\*  $p < 0.001$ ; ns = not significant vs. CM).

**Panel C:** Data obtained using RNA isolated from human DLD-1 cells. Columns (means  $\pm$  SEM,  $n = 5$ ) represent relative iNOS mRNA levels at the different concentrations of SJW extract (\*\*\*  $p < 0.001$ ; ns = not significant vs. CM).

### Figure 2. Effect of SJW extract on cytokine-induced iNOS protein expression in human DLD-1 cells

DLD-1 cells were preincubated for 18h with serum-free DMEM medium. Then cells were incubated for 24 h with or without a cytokine mixture (**CM**) in the presence or absence of SJW extract (**SJW**, 50  $\mu$ g/ml). Total cell extracts (80  $\mu$ g total proteins) were analyzed for iNOS- and (for normalization) for STAT-1 $\alpha$  protein expression by



Western blotting using a polyclonal anti-iNOS antibody and a monoclonal anti-STAT-1 $\alpha$  antibody, respectively. The blot shown is representative for three experiments showing the same results.

**Figure 3. Effect of SJW extract on cytokine-induced nitrite production in human epithelial A549/8 and DLD-1 cells**

**Panel A:** Statistical analysis of 9 different Griess assays performed with supernatants from untreated A549 cells or cells stimulated for 24h with a cytokine mixture (**CM**). Cells were preincubated for 18h in serum-free medium, some cultures then received SJW extract (**SJW**, 10 - 50  $\mu$ g/ml) for an additional 1h. The extract remained present for the subsequent 24h incubation (see above). Columns (means  $\pm$  SEM) represent the relative nitrite levels determined (100% corresponds to 400 pmol/ml/24h; \*\*\*  $p < 0.001$  vs. CM).

**Panel B:** Data generated with DLD-1 cells using 10 to 100  $\mu$ g/ml SJW extract. Columns (means  $\pm$  SEM) represent the relative nitrite levels determined under the conditions indicated (100% corresponds to 400 pmol/ml/24h; \*\*\*  $p < 0.001$  vs. CM).

**Figure 4. Effect of SJW extract cytokine-induced human iNOS promoter activity in stably transfected A549/8 and DLD-1 cells**

A549/8- and DLD-1 cells, stably transfected with pNOSII(16)Luc a construct containing a 16 kb fragment of the human iNOS promoter in front of a luciferase reporter gene (Yao et al., 2003), were preincubated in serum-free medium for 18h and then with SJW extracts for an additionally 1h. Then cells were incubated with a cytokine mixture (**CM**) for another 4h in the presence or absence of the SJW extract. Luciferase activity and protein content were determined in cell extracts.

**Panel A:** Columns (means  $\pm$  SEM;  $n = 18$ ) represent the relative luciferase activities (% of the CM effect) in extracts of stably transfected A549/8 cells, incubated with SJW extract (**SJW**, 1 to 50  $\mu$ g/ml; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; ns = not significant vs. CM).

**Panel B:** Columns (means  $\pm$  SEM; n = 16) represent the relative luciferase activities (% of the CM effect) in extracts of stably transfected DLD-1 cells, incubated with SJW extract (**SJW**, 1 to 100  $\mu$ g/ml; \*\* p < 0.01; \*\*\* p < 0.001; ns = not significant vs. CM).

**Figure 5. Effect of SJW extract on the activity of the constitutive human eNOS promoter in stably transfected ECV-pNOS III-Hu-3500-Luc-neo cells**

ECV-pNOS III-Hu-3500-Luc-neo cells (Yao et al., 2003) stably transfected with pNOS III-Hu-3500-Luc-neo, a construct containing a 3.5 kb human eNOS promoter fragment in front of a luciferase reporter gene were preincubated with serum-free medium for 18h. Then cells were incubated with or without SJW extract (**SJW**; 1 to 50  $\mu$ g/ml) for additional 6h in serum-free medium. Cell extracts were prepared and luciferase activity and protein content of the extracts were determined. Columns (means  $\pm$  SEM; n = 12) represent relative luciferase activities (% of control cells) in the presence of the different concentrations of the SJW extract (\* p < 0.05; ns = not significant vs. control).

**Figure. 6: Effect on SJW extracts on cytokine-induced STAT-1 $\alpha$  DNA binding activity in human A549/8 and DLD-1 cells.**

Gel shift experiments using a radiolabeled oligonucleotide containing a consensus STAT-1 $\alpha$  binding site and nuclear extracts from untreated A549/8 and DLD-1 cells, or A549/8- and DLD-1 cells stimulated with a cytokine mixture (**CM**) in the presence or absence of SJW extract (**SJW**, 1 to 50  $\mu$ g/ml). The position of the DNA-protein complex (**STAT**) and the free oligonucleotide is indicated. The gels are representative of four gels each showing similar results.

**Figure 7: Effect on SJW extracts on cytokine-induced NF- $\kappa$ B DNA binding activity in human A549/8 and DLD-1 cells.**

Gel shift experiments using a radiolabeled oligonucleotide containing a consensus NF- $\kappa$ B binding site and nuclear extracts from untreated A549/8 and DLD-1 cells, or A549/8- and DLD-1 cells stimulated with a cytokine mixture (**CM**) in the presence or absence of SJW extract (**SJW**, 1 to 50  $\mu$ g/ml). The position of the DNA-protein complex (**NF- $\kappa$ B**) and the free oligonucleotide is indicated. The gels are representative of three gels each showing similar results.

**Figure 8. Effect of SJW extract on cytokine-induced activation of STAT-1 $\alpha$  in human A549/8 and DLD-1 cells**

**Panel A:** A549/8 cells were preincubated for 18h with serum-free and then for 1h with SJW extract (**SJW**, 50  $\mu$ g/ml). Subsequently, cells were incubated for 30 min with or without a cytokine mixture (**CM**) in the presence or absence of SJW extract. Nuclear extracts were prepared and 50  $\mu$ g nuclear proteins were analyzed for STAT-1 $\alpha$  tyrosine-(Tyr701) phosphorylation by Western blotting using the PhosphoPlus<sup>®</sup> STAT-1 $\alpha$  (Tyr701) antibody kit. The blots shown are representative for four experiments yielding the same results. The upper parts of each panel show a Western blots using a STAT-1 $\alpha$ -tyrosine-(Tyr701)-phosphate-specific antibody. The lower parts show the same blot (after stripping) using an anti-STAT-1 $\alpha$  antibody, which detects phosphorylated and non-phosphorylated STAT-1 $\alpha$ .

**Panel B:** Experiments similar to panel A generated with nuclear extracts from DLD-1 cells.

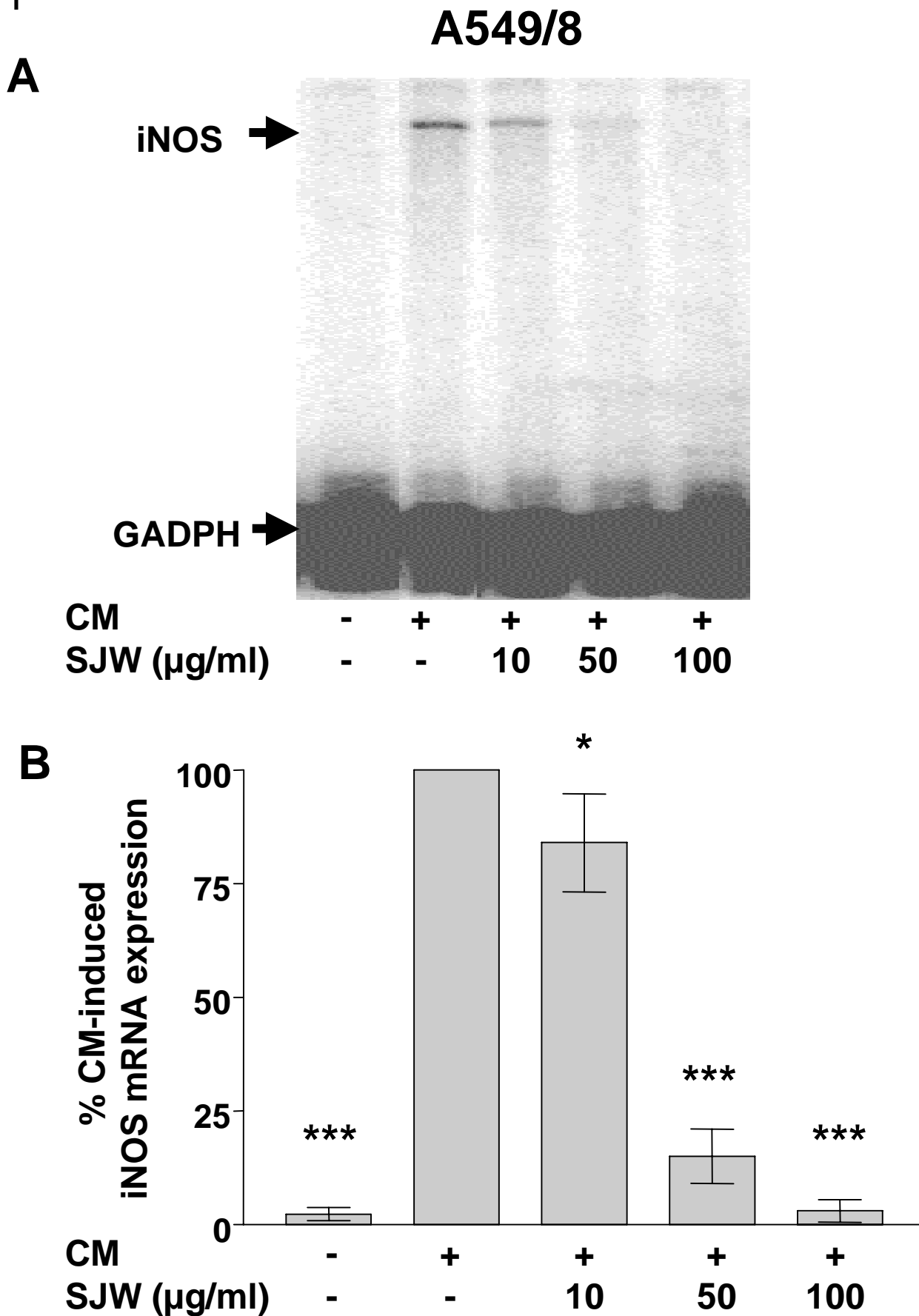
**Figure 9. Effect of SJW extract on cytokine-induced JAK2 activity in human A549/8 and DLD-1 cells**

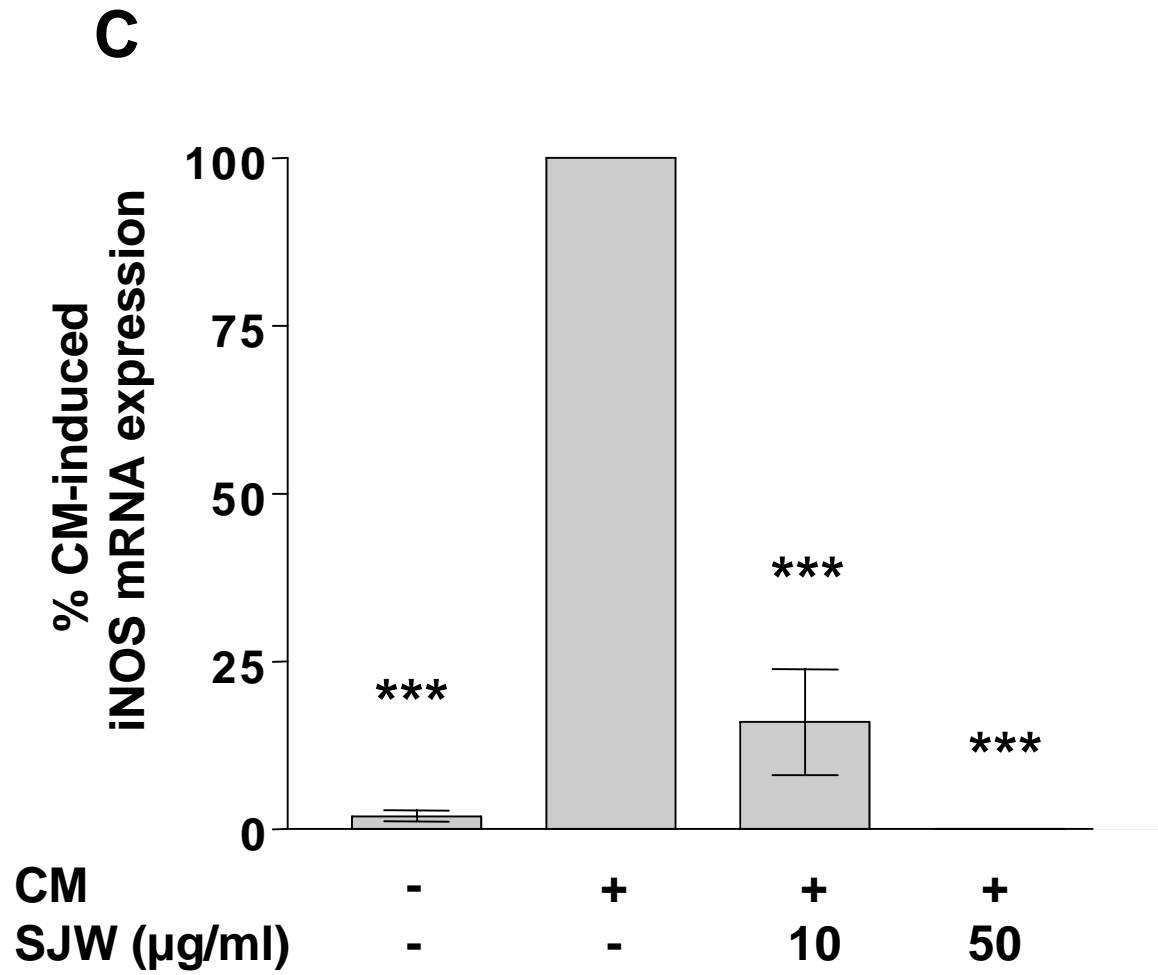
**Panel A:** A549/8 cells were preincubated for 18h with serum-free DMEM medium and then for 1h with SJW extract (**SJW**, 50  $\mu$ g/ml). Then cells were incubated for 30 min with or without a cytokine mixture (CM) in the presence or absence of SJW

extract. Total protein extracts were prepared and immunoprecipitated with a monoclonal anti-JAK2 antibody using protein A agarose. The immunoprecipitated protein was loaded on a SDS PAGE, and after blotting, tyrosine phosphorylation of JAK2 was analyzed using an anti-phospho-tyrosine antibody. The blots shown are representative for four experiments showing the same results. The upper part shows a Western blot using the anti-phospho-tyrosine specific antibody. The lower part shows the same blot (after stripping) using an anti-JAK2 antibody, which detects phosphorylated and non-phosphorylated JAK2.

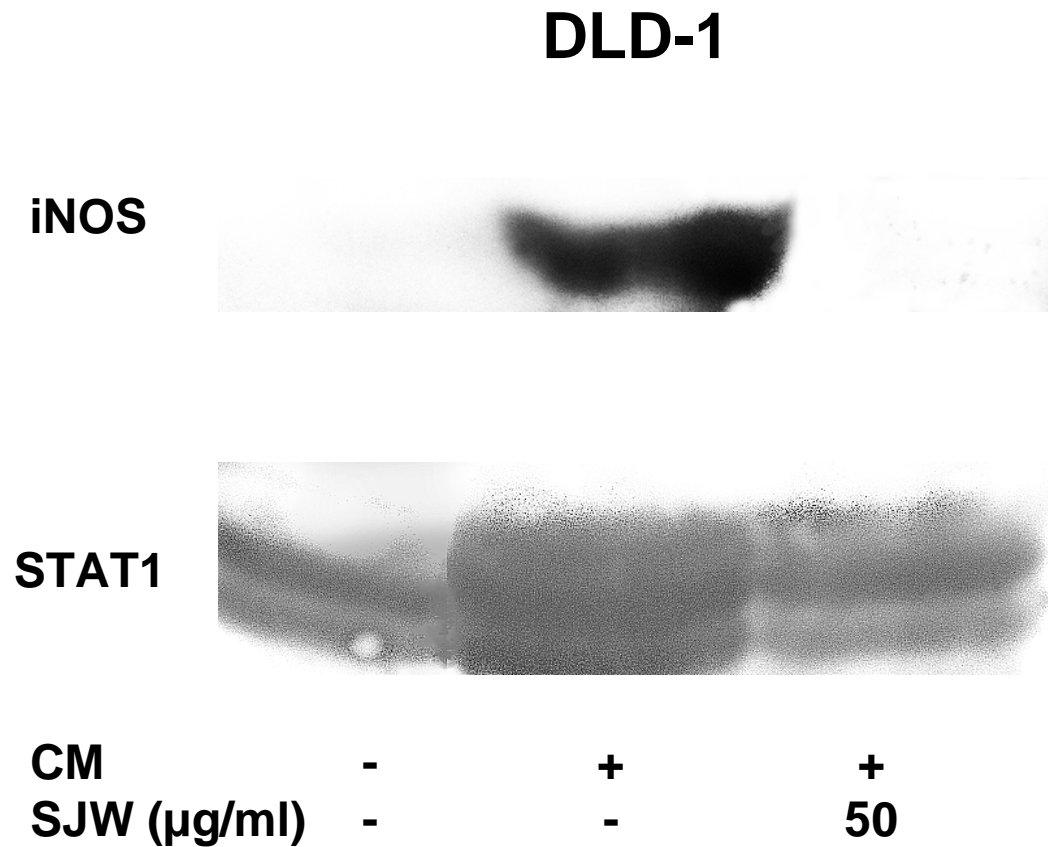
**Panel B:** DLD1 cells were preincubated for 18h with serum-free DMEM medium and then for 1h with SJW extract (**SJW**, 50  $\mu$ g/ml). Then cells were incubated for 30 min with or without a cytokine mixture (CM) in the presence or absence of SJW extract. Total protein extracts were prepared and immunoprecipitated with a monoclonal anti-IFN- $\gamma$ -receptor-subunit- $\alpha$  antibody using protein A agarose. The immunoprecipitated protein was loaded on a SDS page, and after blotting, tyrosine phosphorylation of IFN- $\gamma$ -receptor-subunit- $\alpha$  was analyzed using an anti-phospho-tyrosine antibody. The blots shown are representative for three experiments showing the same results. The upper part shows a Western blot using the anti-phospho-tyrosine specific antibody. The lower part shows the same blot (after stripping) using an anti-IFN- $\gamma$ -receptor-subunit- $\alpha$  antibody, which detects phosphorylated and non-phosphorylated IFN- $\gamma$ -receptor-subunit- $\alpha$ .

**Fig 1**



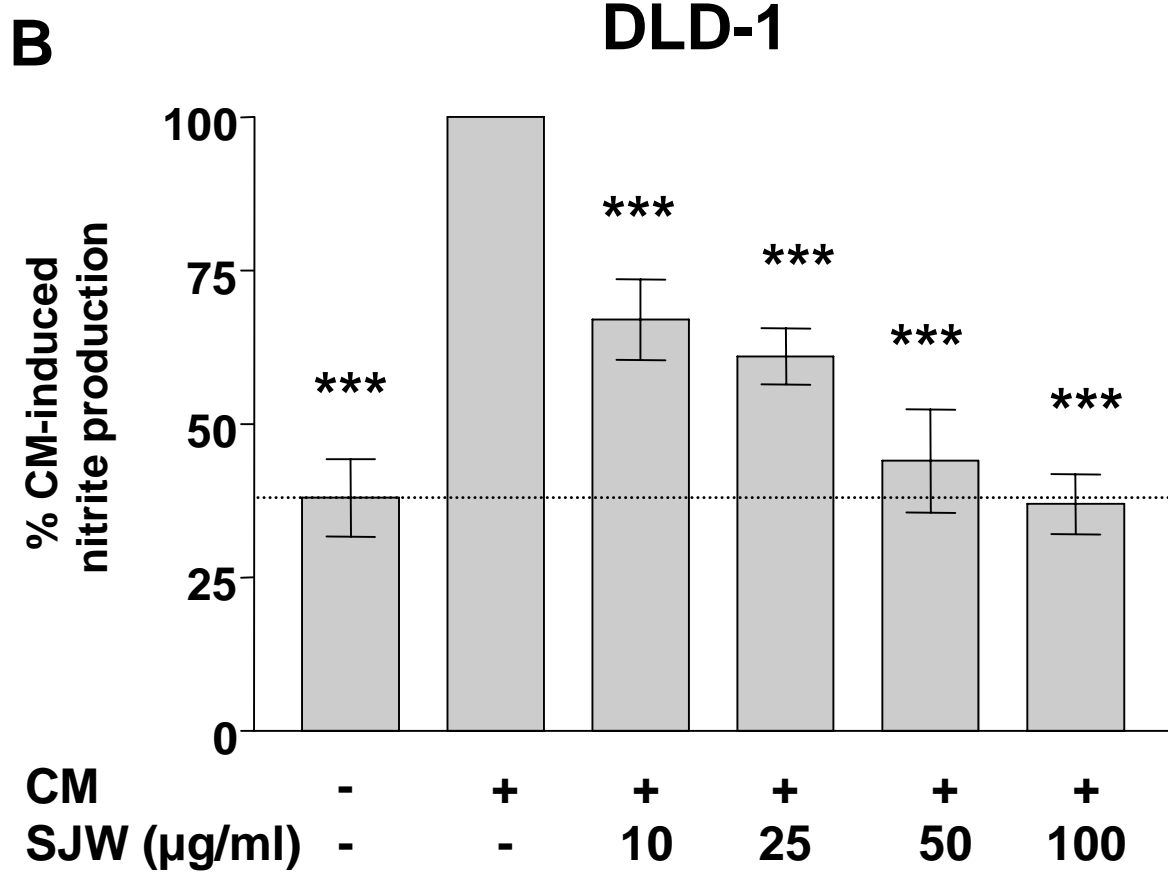
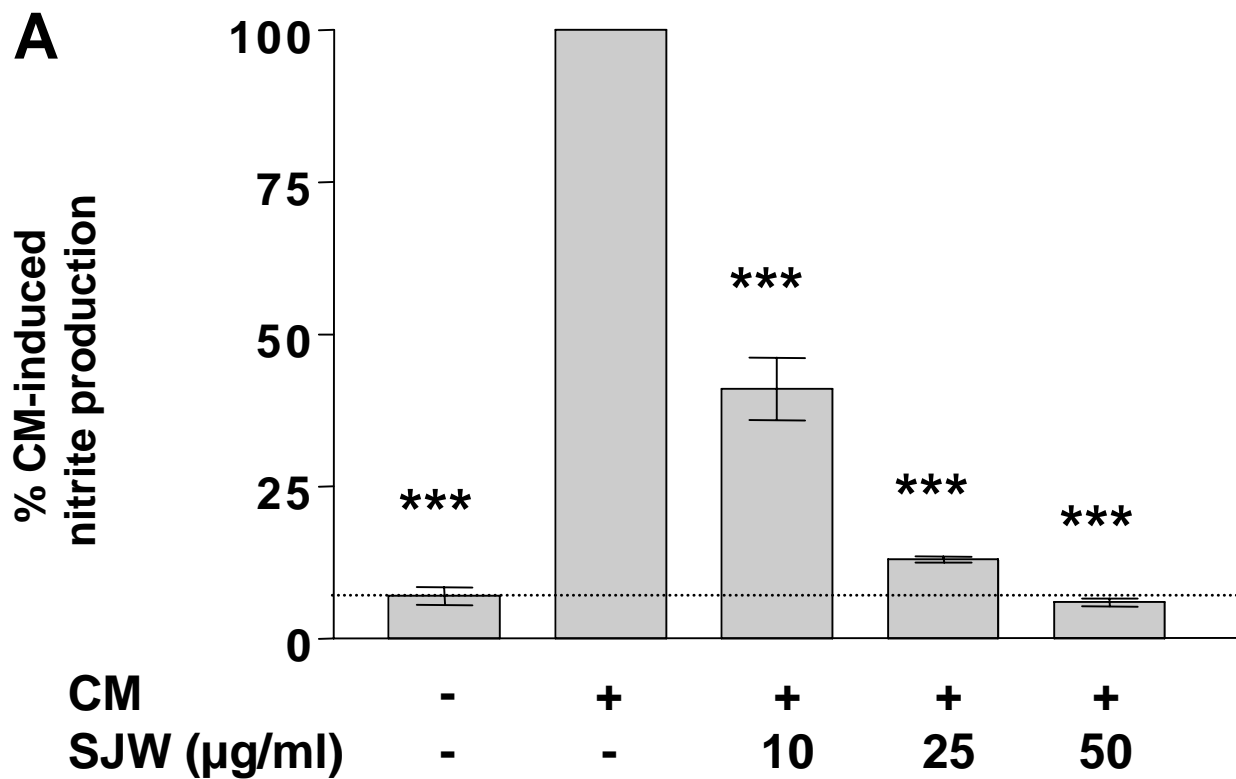


**Fig 2**



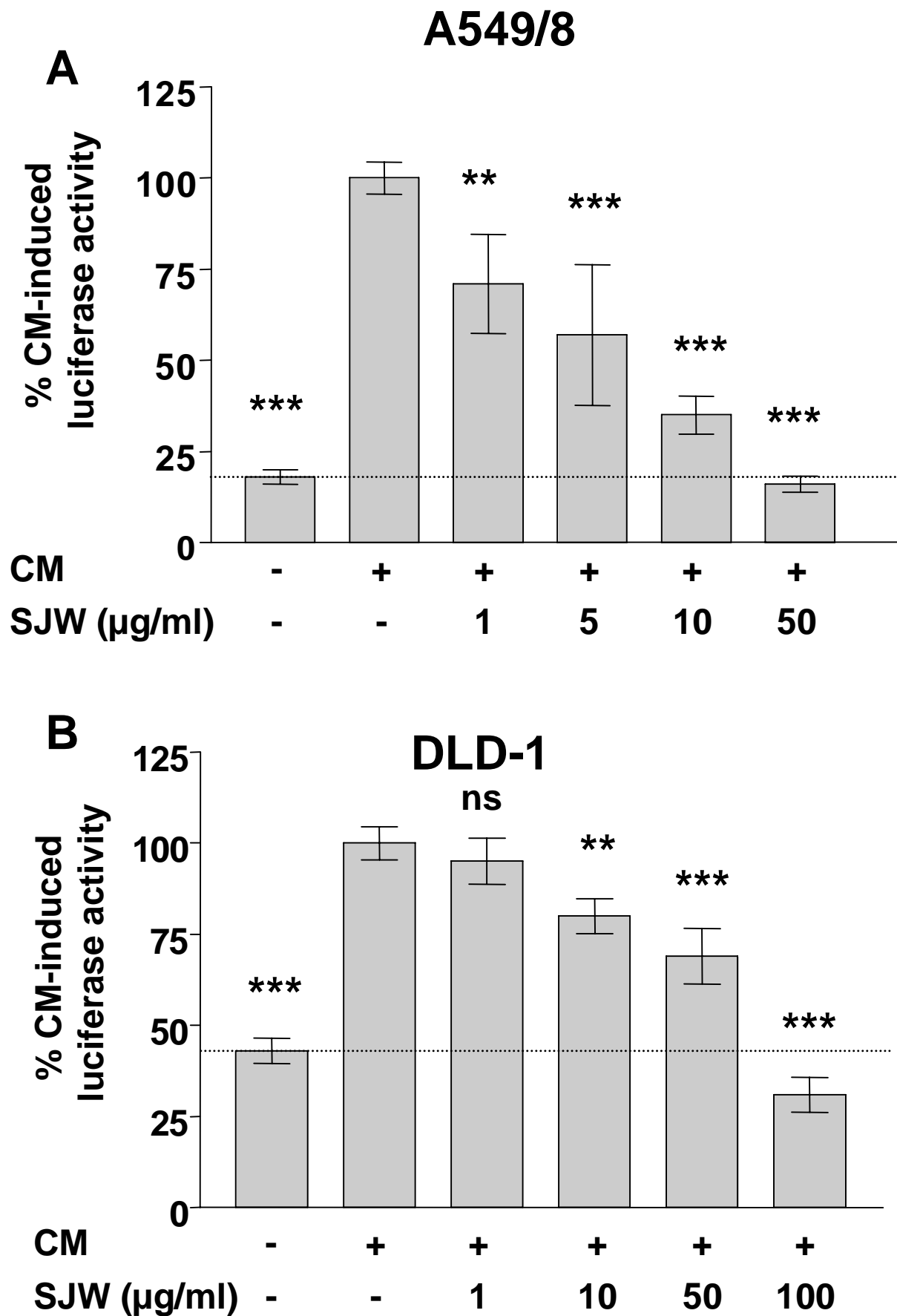
**Fig 3**

## A549/8





**Fig 4**



**Fig 5**

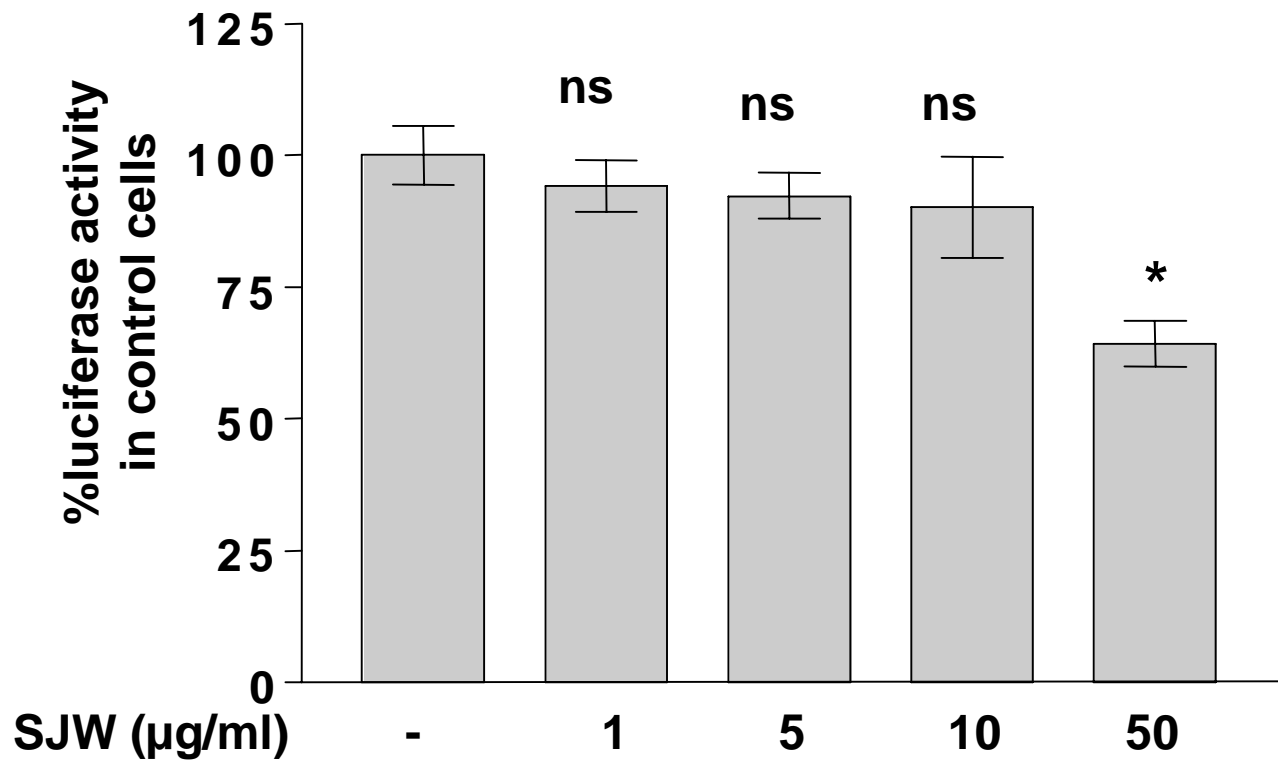


Fig 6

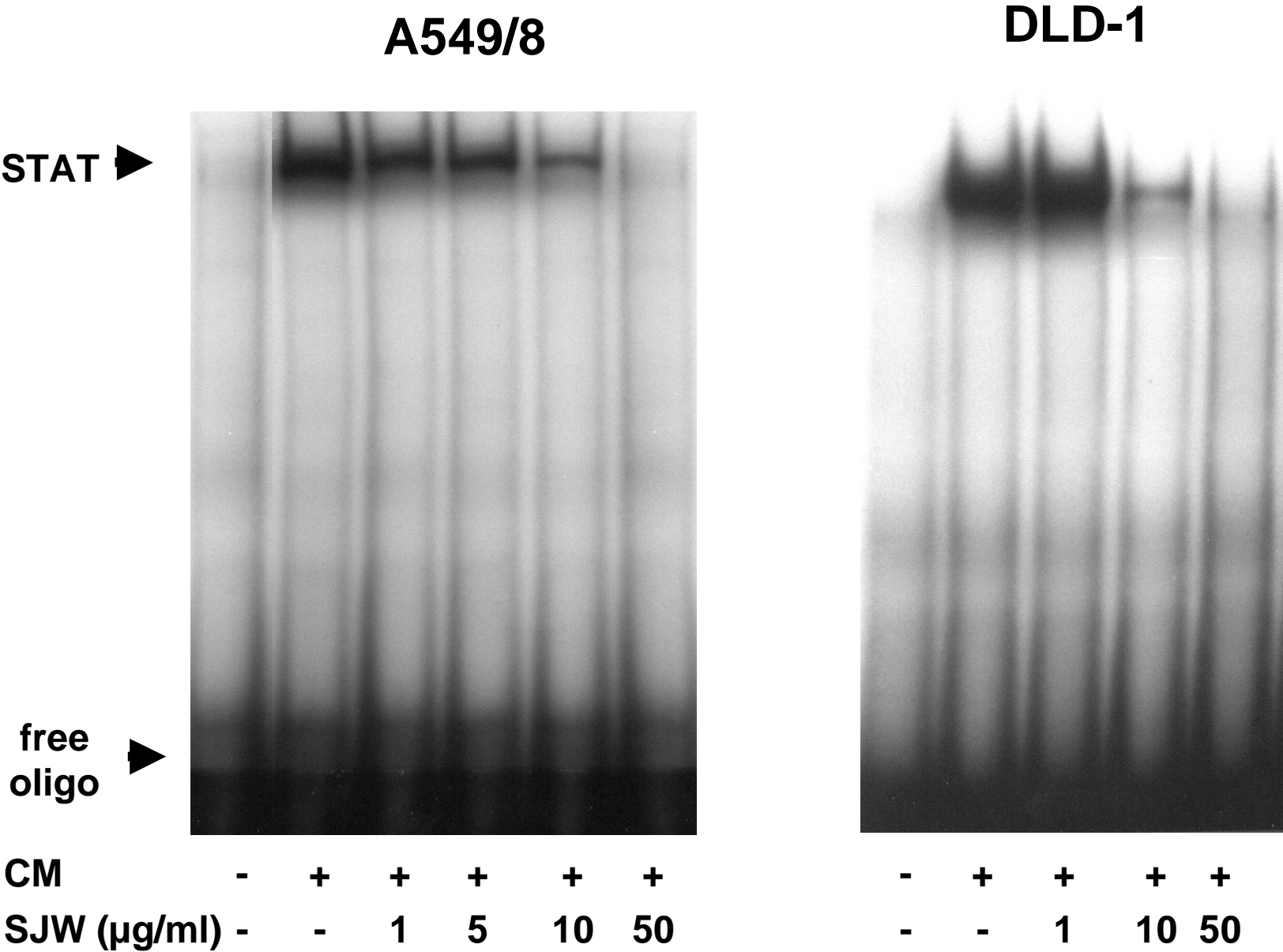
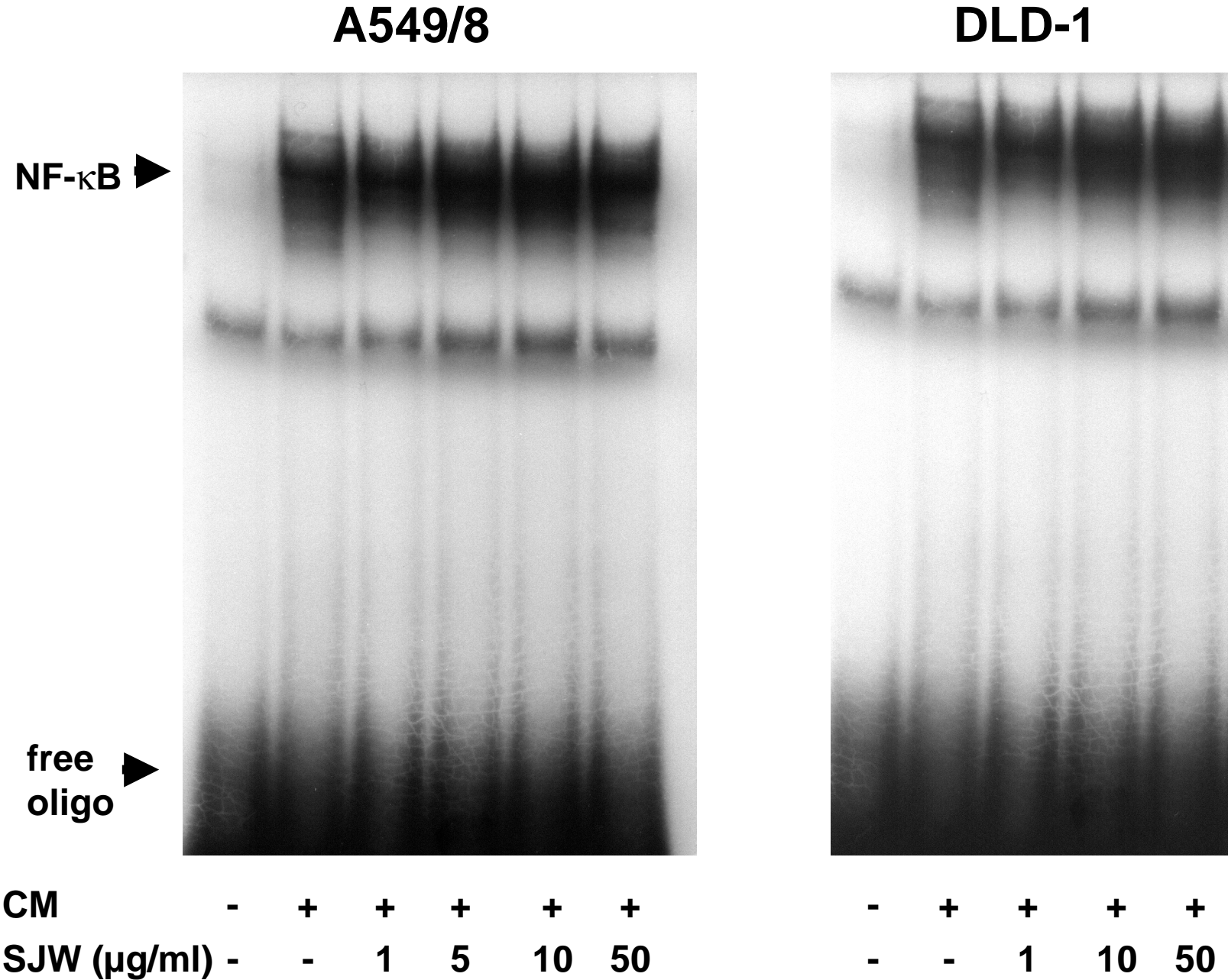
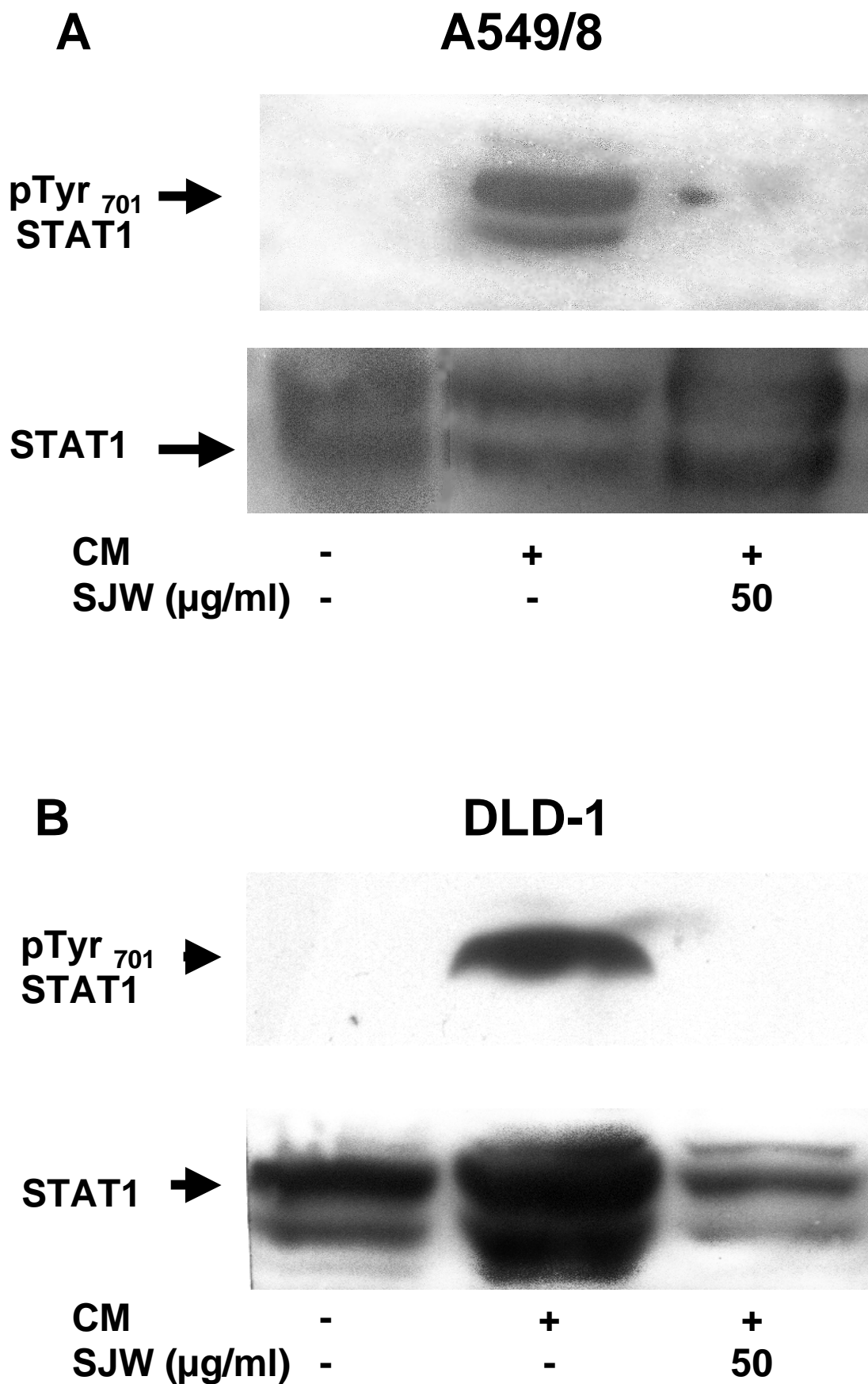


Fig 7



**Fig 8**



**Fig 9**

