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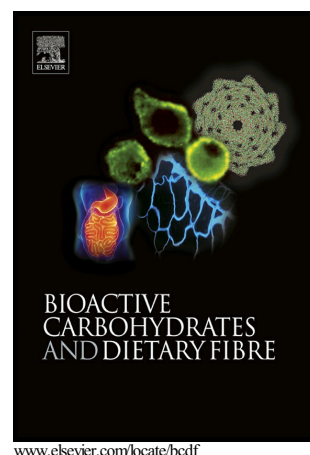
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# Cordyceps Sinensis: *Anti-fibrotic and inflammatory effects of a cultured polysaccharide extract*

Danfei Huang<sup>1</sup>, Soma Meran<sup>2</sup>, Shao-Ping Nie<sup>1</sup>, Adam Midgley<sup>2</sup>, Junqiao Wang<sup>1</sup>, Steve W. Cui<sup>1,3</sup>, Mingyong Xie<sup>1</sup>, Glyn O Phillips<sup>4\*</sup>, Aled O Phillips<sup>2</sup>

<sup>1</sup>State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, Jiangxi, China 330047

<sup>2</sup>Institute of Nephrology, School of Medicine, Cardiff University, Heath Park, Cardiff, CF14 4XN, Wales, UK

<sup>3</sup>Guelph Food Research Centre, Agriculture and Agri-Food Canada, 93 Stone Road West, Guelph, Ont., Canada N1G 5C9

<sup>4</sup>Phillips Hydrocolloids Research Centre, Glyndwr University, Wrexham, LL11 2AW, Wales UK; Phillips Hydrocolloids Research Ltd, 45 Old Bond Street, London, W1S 4AQ UK.

\*Correspondence to: Institute of Nephrology, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN. Tel.: 44 2920 748411; fax: 44 2920 748470. PhillipsAO@cf.ac.uk

## Abstract

It has been suggested that the traditional Chinese herbal preparation *Cordyceps Sinensis* (CS) may have a beneficial effect in renal disease. To satisfy increasing demand, CS derivatives have been produced by aseptic mycelia cultivation. We have demonstrated antifibrotic activity of cultured CS previously. The aim of this study was to examine bioactivity of a polysaccharide isolated from cultured CS with a complicated monosaccharide composition, mainly consisting of Gal, Glc and Man.

This polysaccharide antagonised the effect of TGF- $\beta$ 1 in stimulating the expression of collagen in the HK2 renal cell line. This was associated with down regulation of the TGF- $\beta$  receptor Alk5. In addition the polysaccharide antagonised IL-1 $\beta$  stimulated sICAM-1 dependent adherence of monocytes to a monolayer of HK2 cell. This was associated with increased expression of the primary receptor for hyaluronan CD44, and was abrogated by removal of the cell surface hyaluronan pericellular coat.

In summary we describe both anti-fibrotic and anti-inflammatory activity in a polysaccharide isolated from cultured CS.

## Key Words

*Cordyceps sinensis*, bioactivity, polysaccharide, fibrosis, inflammation, IL-1 $\beta$ , TGF- $\beta$ 1

## Introduction

Mycelial extracts have a wide range of biological activities that modulate functions of mammalian cells, and many effective pharmaceuticals have been developed from plants and fungi. The traditional medicine known as Chinese caterpillar fungus (Dongchong Xiacao) is composed of the parasitic fungus *Cordyceps sinensis* (CS) (Berk.) Sacc. (Clavicipitaceae) growing on the larva of the ghost moth *Hepialus armericanus* Oberthur (Hepialidae) (G. G.-L. Yue, Lau, Fung, Leung, & Ko, 2008). *Cordyceps sinensis* (CS) has a long history of medicinal use in China, and is commonly currently used as a herbal medicine and health supplement. Its history of medicinal use in China, dates back to the Qing dynasty in A.D.1694 (A. Wang, 1914). According to Chinese tradition and the Chinese Pharmacopoeia (Commission, 2005), *Cordyceps sinensis* can “tonify the lung, replenish the kidneys, arrest bleeding, dissolve phlegm, treat chronic coughs, treat spontaneous sweating and restore strength after an illness” (Commission, 2005; S.P. Li & Tsim, 2004; X. Liu, 1998; Zhu, Halpern, & Jones, 1998a, 1998b). More recent studies suggest that *Cordyceps* may have immunomodulatory (Jordan, Hirsch, & Lee, 2008), anti-proliferative (L.-Y. Yang, Huang, Hsieh, & Lin, 2003), pro-apoptotic (Q. X. Zhang & Wu, 2007) and anti-fibrotic effects (F. H. Li, Liu, Xiong, & Xu, 2006; S. J. Wang, Bai, Wang, & Dai, 2007)

As *C. sinensis* can only grows slowly in high-altitude habitats, its supplies are generally insufficient. During the past two decades, aseptic mycelia cultivation have been developed to

meet the increasing demand of *C. sinensis*. Previously we have demonstrated that both natural and cultured CS have anti-fibrotic properties, antagonizing the effects of the pro-fibrotic cytokine Transforming Growth Factor Beta1 (TGF- $\beta$ 1) (Yao et al., 2013). Furthermore, our data suggested that in the natural form of CS this anti-fibrotic activity is associated with a soluble hydrophilic polysaccharide with a low protein content (0.09%) and molecular weight 11 million (X. L. Zhang, Bi-Cheng, Al-Assaf, Phillips, & Phillips, 2012). Methylation analysis and 2D NMR spectroscopy suggested that the polysaccharide backbone is composed of Glcp joined by 1 $\rightarrow$ 4 linkages and 1 $\rightarrow$ 3 linkages; the branching points are located at O-2 or O-6 of Glcp with - terminal-d-Glcp as side chain (Nie et al., 2011). In this paper we describe both anti-fibrotic and anti-inflammatory effects for a polysaccharide isolated from cultured cordyceps mycelium (CSP).

## Methods

### *Extraction and characterization of Cordyceps polysaccharide (Figure 1A):*

Cultured *Cordyceps Sinensis* was purchased from Jiangxi Chinese Medicine Co., Ltd. Jiangxi Province, China. After soaking by 80% ethanol overnight, the cultured mycelium were dried and extracted three times with hot water (95-100°C), each for 2 h. The combined supernatant was concentrated to a small volume at 55°C under reduced pressure. The crude polysaccharide was obtained by precipitation with four volumes of anhydrate ethanol, designate crude polysaccharide with a yield of 14.32% by weight. The crude polysaccharide were further treated with Sevag reagent (chloroform/1-butanol, v/v = 4:1) for three times to remove proteins. Then it was dialyzed against tap water for 48 h and distilled water for 24 h, and finally lyophilized to give a polysaccharide fraction, namely CSP, with a subsequent

yield of 49.36% based on the crude polysaccharide. Furthermore, CSP contained a total phenolic level of  $7.05 \pm 0.46$  mg GAE/g as evidenced by Folin-Ciocalteu colorimetric method.

#### ***Cell culture:***

All reagents were from Sigma-Aldridge (Poole, Dorset, UK) unless otherwise stated. PCR and Quantitative PCR (QPCR) reagents and primers were purchased from Invitrogen (Paisley, UK) and Applied Biosystems (Cheshire, UK).

Anti-fibrotic and inflammatory effects of cordyceps extract CSP were assessed in HK2 cells (American Type Culture Collection Number CRL-2190) as previously described (X. L. Zhang et al., 2012). HK-2 cells, are human proximal tubular epithelial cells immortalised by transduction with human papilloma virus 16 E6/E7 genes (Ryan et al., 1994). Cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% fetal calf serum (FCS) (Sijiqing, China),  $2 \mu\text{mol/l}$  L-glutamine,  $5 \mu\text{g/ml}$  insulin,  $5 \mu\text{g/ml}$  transferrin,  $5 \text{mg/ml}$  sodium selenite,  $400 \text{ng/ml}$  hydrocortisone and  $20 \text{mmol/l}$  HEPES (Sigma-Aldrich, Shanghai, China),  $100 \text{U/ml}$  penicillin and  $100 \mu\text{g/ml}$  streptomycin. Cells were grown at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  and used at passage 20 or below. Cells were growth arrested in serum-free medium for 24 hours before use in experiments. All experiments were done in serum-free conditions.

#### ***Quantitative RT-PCR:***

Cells were grown to confluence in 35mm dishes and washed with PBS prior to lysis with Tri-reagent and RNA purification according to the manufacturer's protocol. Reverse transcription was performed using high capacity cDNA reverse transcription kits according to the manufacturer's protocol.  $1 \mu\text{g}$  of RNA was added to  $2 \mu\text{l}$  of  $10\times$  RT random primers,  $2 \mu\text{l}$  of  $10\times$  RT buffer and  $0.8 \mu\text{l}$  of  $100 \text{mM}$  dNTP.  $1 \mu\text{l}$  of ( $40 \text{units}/\mu\text{l}$ ) ribonuclease inhibitor RNasin

(Promega) and 1µl of (50units/µl) reverse transcriptase were added to each sample and mixed. The solution was incubated at 25°C for 10 min, 37°C for 120 min and then 85°C for 5 min on a GeneAmp PCR system 9700. As a negative control RT was performed with sterile H<sub>2</sub>O replacing the RNA sample.

PCR for the target gene of interest was performed using the Vii7 Fast Real-Time PCR System from Applied Biosciences. PCR was carried out in a final volume of 20µl/sample. 1µl of RT product, 1µl of target gene primers and probe (commercially designed and purchased from Applied Biosciences), 10µl of Taqman Universal PCR mastermix and 8µl of sterile H<sub>2</sub>O. Amplification was carried out using a cycle of 95°C for 1s and 60°C for 20s for 40 cycles. As a negative control, PCR was performed with sterile H<sub>2</sub>O replacing the cDNA sample. PCR was simultaneously done for ribosomal RNA (primers and probe commercially designed and purchased from Applied Biosciences) as a standard reference gene.

The comparative C<sub>T</sub> method was used for relative quantification of gene expression. The C<sub>T</sub> (Threshold cycle where amplification is in the linear range of the amplification curve) for the standard reference gene (ribosomal RNA) was subtracted from the target gene C<sub>T</sub> to obtain the delta C<sub>T</sub> (dC<sub>T</sub>). The mean dC<sub>T</sub> for similar samples were then calculated. The expression of the target gene in experimental samples relative to expression in control samples was then calculated using the formula:

$$2^{-(dC_T(1) - dC_T(2))}$$

where: dC<sub>T</sub>(1) is the mean dC<sub>T</sub> calculated for the experimental samples and dC<sub>T</sub>(2) is the mean dC<sub>T</sub> calculated for the control samples.

#### ***siRNA Transfection.***

Transient transfection of HK2 was performed with specific siRNA nucleotides to their target genes (Applied Biosystems). Transfection was performed using Lipofectamine 2000

transfection reagent (Invitrogen) in accordance with the manufacturer's protocol. Briefly, cells were grown to 70% confluence in antibiotic free medium in either 35 mm dishes. 5  $\mu$ l of the transfection reagent was diluted in 250  $\mu$ l Opti-MEM reduced growth medium (Gibco) and left to incubate for 5 minutes at room temperature. The specific siRNA oligonucleotides were diluted in Opti-MEM reduced growth medium to achieve a final concentration of 30nM. The transfection agent and siRNA mixtures were then combined and incubated at room temperature for a further 20 minutes. The newly formed transfection complexes were subsequently added to the cells and incubated at 37 °C with 5 % CO<sub>2</sub> for 24 hours in serum-free medium prior to experimentation. As a control, cells were transfected with negative control siRNA (a scrambled sequence that bears no homology to the human genome) (Applied Biosystems).

***Assessment of monocyte binding:***

The monocyte binding assay has been described and validated previously (S. Meran, Martin, Luo, Steadman, & Phillips, 2013). U937 cells, originally derived from a human histiocytic lymphoma, were procured from the American Type Culture Collection (Rockville, MD). The cells were grown in suspension culture in RPMI medium supplemented with L-glutamine and penicillin/streptomycin containing 5% fetal bovine serum until an appropriate cell density of  $1 \times 10^6$  cells/ml was achieved. These cells were then centrifuged and resuspended in fibroblast growth medium (DMEM/F12) and incubated with HK2 cells grown in monolayer to 70% confluence in 35-mm dishes. Before addition of the U937 cells, the HK2 cells were treated as described in individual figure legends. After appropriate periods of incubation (as outlined in the figure legends), the cultures were washed 10 times with 2 ml of PBS to remove any unbound U937 cells. Subsequently, the cells were lysed with Tri-reagent and RT-qPCR was performed for CD45 mRNA expression as described above.



### ***Statistical Analysis.***

All data were expressed as means  $\pm$  standard deviation. Statistical analysis was performed using SPSS v.16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Intergroup differences for continuous variables were assessed by multivariate ANOVA.

## **Results**

### ***Characterisation of CSP:***

CSP was a polysaccharides containing 13% protein. It presented a broad distribution profile with a shoulder peak as shown in Figure. 1B. The molecular weight of the major fraction in CSP were estimated to be approximately 28 KDa through high performance gel permeation chromatography. CSP had a complicated monosaccharide composition (Figure 1C), mainly consisted of Gal, Glc and Man in a percentage of 36.40%, 28.99% and 24.81%, respectively. It also contained small amount of Ara (3.34%) and GalA (7.55%). Methylation and GC-MS results revealed the linkage patterns in CSP were highly complex likely to be a mixture of more than two polysaccharide fractions. with part of the main chain of CSP formed from 1 $\rightarrow$ 4 linked Glc and 1 $\rightarrow$ 4 linked Gal. Man units were found as 1 $\rightarrow$ 2, 1 $\rightarrow$ 6, 1 $\rightarrow$ 2, 6 and 1 $\rightarrow$ 4, 6 linkages. Ara was found as terminal and 1 $\rightarrow$ 5 linked residues. In addition, terminally linked Gal, as well as terminally, 1 $\rightarrow$ 3 and 1 $\rightarrow$ 4, 6 linked Glc was also observed (Figure 1 D).

### ***Anti-fibrotic activity of CSP:***

*Confirmation of anti-fibrotic activity:* Our previous publications have demonstrated antagonism of pro-fibrotic activity of TGF- $\beta$ 1 by CS. In this study, CSP bioactivity was assessed by its ability to antagonize TGF- $\beta$ 1 induction of type 1 collagen mRNA. Significant induction of collagen 1 was seen following stimulation with TGF- $\beta$ 1(Figure 2). At each time point stimulation of cells with TGF- $\beta$ 1 in the presence of CSP led to a significant abrogation of the effect of TGF- $\beta$ 1.

*Potential mechanisms of TGF- $\beta$ 1 antagonism:*

Bone Morphogenic Protein 7 is a member of the TGF- $\beta$  superfamily previously reported to be a natural antagonist of TGF- $\beta$ 1 (Midgley et al., 2015). Whilst stimulation with TGF- $\beta$  led to suppression of the expression of its natural antagonist, stimulation in the presence of CSP had no effect on this inhibitory activity (Figure 3A).

Activation of innate immunity pathways via Toll like receptors (TLRs) is relevant to models of progressive renal disease (Hung, Chang, Chen, et al., 2006; Hung, Chang, Tian, et al., 2006; Ma et al., 2014; Shigeoka et al., 2007). TLRs are also activated by traditional chinese medicines (J. Liu et al., 2014), the most abundant TLR expressed in proximal tubular epithelial cells being TLR-4. In this study however suppression of TLR4 expression by gene silencing had no effect on the bioactivity of CSP (Figure 3C).

Previously we have demonstrated that CS in vivo modulates the expression of the TGF- $\beta$ 1 receptor was attenuated in a model of renal injury (Pan et al., 2013). In the current study TGF- $\beta$ 1 induction of the ALK5 TGF $\beta$  receptor is response to TGF- $\beta$ 1 was attenuated significantly by stimulation in the presence of CSP (Figure 3B).

***Anti-inflammatory activity of CSP:***

*Inhibition of monocyte binding:*

Increased binding of monocytes to non-endothelial, resident tissue cells via cell surface ICAM-is well described (S. Meran et al., 2013; X. L. Zhang, Selbi, De La Motte, Hascall, & Phillips, 2004). Stimulation of HK-2 cells with the pro-inflammatory cytokine IL-1 $\beta$  led to an increase in monocyte binding as quantified by the expression of the monocyte marker CD45 on the cell surface of HK2 monolayers (Figure 4). The increase in cell surface binding of CD45 was abrogated by addition of soluble ICAM confirming the dependence of binding to cell surface ICAM. In addition binding of CD45 to the cell monolayer was significantly inhibited by the addition of CSP suggesting abrogation of IL-1 $\beta$  stimulated ICAM dependent

monocyte binding.

*Mechanism of inhibition of monocyte binding:*

Stimulation of HK-2 monolayers with IL-1 $\beta$  led to a significant increase in the expression of CD44 which is the principle receptor for the cell surface associated matrix polysaccharide hyaluronan (Figure 5A). Addition of IL-1 in the presence of CSP resulted in a further significant stimulation of CD44. Addition of CSP alone did not alter the expression pattern of CD44. Figure 5B shows the effect of prevention of induction of CD44 by gene silencing using CD44 siRNA on monocyte binding. Stimulation with IL-1 in the presence of CSP again attenuated the binding of monocytes. In contrast in cells treated with CD44 siRNA addition of CSP no longer prevented IL-1 induced binding of monocytes.

As described above CD44 is the principle cell surface for the matrix component hyaluronan, and we and others have shown the importance of this receptor in anchoring a peri-cellular hyaluronan coat which regulates many cell responses (S. Meran et al., 2011; Soma Meran et al., 2008; Midgley et al., 2015; W. Selbi et al., 2006; R. Simpson et al., 2009; R. M. L. Simpson et al., 2009; Webber, Jenkins, Meran, Phillips, & Steadman, 2009; Webber, Meran, Steadman, & Phillips, 2009). Our previous data has also suggested that interaction of monocytes with cell surface ICAM may be modulated by this matrix component (W. D. Selbi, De La Motte, Hascall, & Phillips, 2004). The experimental system whereby cell surface hyaluronan is removed by exogenous hyaluronan digestion is well described and validated (R. Simpson et al., 2009). Following removal of cell surface hyaluronan CSP did not inhibit, but rather increased IL-1 dependent binding (Figure 5C).

## **Discussion**

*Cordyceps sinensis* is a well-known traditional Chinese medicinal fungus used both as a health food supplement and also in clinical practice to treat a variety of human conditions including respiratory, renal, hepatic and cardiovascular diseases (reviewed in (K. Yue, Ye,

Zhou, Sun, & Lin, 2013)). The biological and pharmacological activities reside within the mycelial biomass. Growth of CS is limited to the high altitudes on the Qinghai-Tibetan plateau and Southwestern China. Because CS grows in such restricted habitats, the natural product is both rare and expensive. In order to satisfy the increasing demand of this relatively rare resource, with the development of fermentation technology, CS derivatives have been produced by aseptic mycelia cultivation, many of which are commonly sold as health foods (reviewed in (Zhou, Gong, Su, Lin, & Tang, 2009)). Although bio-activity has been demonstrated in cultured strains (Chen, Zhang, Shen, & Wang, 2010; S. P. Li, Li, Dong, & Tsim, 2001), it is clear that culture conditions can influence the profile of the generation of bioactive metabolites (Cui & Zhang, 2011), which therefore raises the question of quality control of CS and its products to ensure their efficacy and safety.

We have previously compared the chemical composition of polysaccharides from natural and cultured *Cordyceps Sinensis* and demonstrated significant differences between polysaccharides obtained from these two sources (J. Wang et al., 2015). In this manuscript we demonstrated that monosaccharides dominating in the polysaccharide isolated from this cultured sample were mainly Gal, Glc and Man in contrast to the natural polysaccharide in which Glc was the dominating monosaccharide. The molecular weight were also significantly different between natural (around 950 kDa) and cultured samples (around 28 kDa). As a result of these significant difference, our aim was to examine the bio-activity of the polysaccharide extracted from cultured CS.

Our primary focus is delineating mechanisms and potential therapeutic targets in chronic kidney disease (CKD), which is an increasingly common condition with limited treatment options placing a major financial and emotional burden on the medical community. Renal interstitial fibrosis is well established as the best predictor of the rate of progression of renal dysfunction (Bader et al., 1980; Bogenschutz et al., 1990; Bohle, Mackensen-Haen, & Gise,

1987; Mackensen-Haen et al., 1992; Wehrmann et al., 1989; Wehrmann et al., 1990). The fibrotic process is at least in part driven by phenotypic activation of proximal tubular epithelial cells by Epithelial-Mesenchymal Transition (EMT) under the influence of TGF- $\beta$ 1 (J. Yang & Liu, 2001), hence our choice of a cell culture model utilizing TGF- $\beta$ 1 stimulation of renal proximal tubular epithelial cell.

Fibrosis can be considered as a form of aberrant wound healing in which there is progression rather than resolution of scarring. It results from excessive accumulation of extra-cellular matrix (ECM) components such as collagen, causing disruption of normal tissue architecture and function. The first key finding of this study is that this polysaccharide isolated from cultured CS has bioactivity, antagonising TGF- $\beta$ , as evidence by inhibition of TGF- $\beta$ 1 stimulated collagen 1 expression. In addition this inhibition was associated with suppression of expression of the ALK5 TGF- $\beta$  receptor and is therefore consistent with previous *in vitro* (Yao et al., 2013; X. L. Zhang et al., 2012) and *in vivo* (Pan et al., 2013; X. L. Zhang et al., 2012) data generated with natural cordyceps and also polysaccharides previously isolated from a different cultured cordyceps preparation. In this study we have also excluded other potential modes of action such as signaling through toll-like receptors and modulation of the natural TGF- $\beta$ 1 antagonist BMP7.

Although TGF- $\beta$ 1 has been highlighted as the final common pathway for fibrosis, inflammatory cell infiltration, particularly monocyte/macrophage infiltration, has been implicated in the pathogenesis of a wide diversity of renal diseases (Lavaud et al., 1996; Sassy-Pringent et al., 2000; Young et al., 1995). We have also demonstrated that an inflammatory infiltrate is a predictor of development of progressive renal disease associated with Diabetes Mellitus (Lewis et al., 2007). Mechanistically we have demonstrated that interaction of infiltrating monocytes with resident cells is a trigger for the generation of TGF- $\beta$ 1 linking inflammation to fibrosis (X. L. Zhang et al., 2004). The second key observation in

this study is the novel finding of an anti-inflammatory effect of Cordyceps polysaccharide as evidenced by the reduction of association of CD45 positive cells with the renal epithelial cell monolayer. Previously we have demonstrated that the hyaluronan pericellular matrix regulates the interaction of inflammatory cells with proximal tubular epithelial cells (W. D. Selbi et al., 2004). The results of this study would support the notion that Cordyceps attenuates monocyte binding through induction of a CD44 dependent hyaluronan matrix which prevents the interaction of monocytes with cell surface ICAM-1.

In summary the data supports the hypothesis that cordyceps carries biological activity that through antagonism of the effects of the pro-fibrotic cytokine TGF- $\beta$ 1 as well as through decreasing the interaction between inflammatory cells and resident cells, which potentially reduces the drive for progressive renal fibrosis. Furthermore although polysaccharide isolated from both natural and cultured Cordyceps had significant differences in monosaccharide composition and structure, they share similar bioactivity profiles.

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### References:

- Bader, R., Bader, H., Grund, K. E., Mackensen-Haen, S., Christ, H., & Bohle, A. (1980). Structure and function of the kidney in diabetic glomerulosclerosis: Correlations between morphological and functional parameters. *Path Res Pract*, 167, 204-216.
- Bogenschutz, O., Bohle, A., Batz, C., Wehrmann, M., Pressler, H., Kendziorra, H., & Gartner, H. V. (1990). IgA Nephritis: On the Importance of Morphological and Clinical

- Parameters in the Long-term Prognosis of 239 Patients. *American Journal of Nephrology*, 10, 137-147.
- Bohle, A., Mackensen-Haen, S., & Gise, H. (1987). Significance of tubulointerstitial changes in the renal cortex for the excretory function and concentration ability of the kidney: A morphometric contribution. *American Journal of Nephrology*, 6, 421-433.
- Chen, W., Zhang, W., Shen, W., & Wang, K. (2010). Effects of the acid polysaccharide fraction isolated from a cultivated *Cordyceps sinensis* on macrophages in vitro. *Cell Immunol*, 262(1), 69-74. doi:S0008-8749(10)00013-4 [pii] 10.1016/j.cellimm.2010.01.001
- Chinese Pharmacopoeia. Chinese Pharmacopoeia Commission Shanghai: Commercial Press.
- Cui, J. D., & Zhang, B. Z. (2011). Comparison of culture methods on exopolysaccharide production in the submerged culture of *Cordyceps militaris* and process optimization. *Lett Appl Microbiol*, 52(2), 123-128. doi:10.1111/j.1472-765X.2010.02987.x
- Hung, C. C., Chang, C. T., Chen, K. H., Tian, Y. C., Wu, M. S., Pan, M. J., . . . Yang, C. W. (2006). Upregulation of chemokine CXCL1/KC by leptospiral membrane lipoprotein preparation in renal tubule epithelial cells. *Kidney Int*, 69(10), 1814-1822. doi:5000362 [pii] 10.1038/sj.ki.5000362
- Hung, C. C., Chang, C. T., Tian, Y. C., Wu, M. S., Yu, C. C., Pan, M. J., . . . Yang, C. W. (2006). Leptospiral membrane proteins stimulate pro-inflammatory chemokines secretion by renal tubule epithelial cells through toll-like receptor 2 and p38 mitogen activated protein kinase. *Nephrol Dial Transplant*, 21(4), 898-910. doi:gfi316 [pii] 10.1093/ndt/gfi316
- Jordan, J. L., Hirsch, G. M., & Lee, T. D. G. (2008). *C. sinensis* ablates allograft vasculopathy when used as an adjuvant therapy with cyclosporin A. *Transplant Immunology*, 19(3-4), 159-166.
- Lavaud, S., Michel, O., Sassy-Prigent, C., Heudes, D., Bariety, J., Chevalier, J., . . . Mandet, C. (1996). Early influx of glomerular macrophages precedes glomerulosclerosis in the Obese Zucker Rat model. *Journal of the American Society of Nephrology*, 7(12), 2604-2615.
- Lewis, A., Steadman, R., Manley, P., Craig, K., de la Motte, C. A., Hascall, V. C., & Phillips, A. O. (2007). Diabetic nephropathy, inflammation, hyaluronan and interstitial fibrosis. *Histology and Histopathology*, In Press.
- Li, F. H., Liu, P., Xiong, W. G., & Xu, G. F. (2006). Effects of corydyceps polysaccharide on liver fibrosis induced by DMN in rats. *Zhongguo Zhong Yao Za Zhi*, 31(23), 1968-1971.

- Li, S. P., Li, P., Dong, T. T., & Tsim, K. W. (2001). Anti-oxidation activity of different types of natural *Cordyceps sinensis* and cultured *Cordyceps mycelia*. *Phytomedicine*, 8(3), 207-212. doi:S0944-7113(04)70030-6 [pii] 10.1078/0944-7113-00030
- Li, S. P., & Tsim, K. W. K. (2004). *The biological and pharmacological properties of Cordyceps sinensis, a traditional Chinese medicine that has broad clinical applications*. New York: Marcel Dekker.
- Liu, J., Peter, K., Shi, D., Zhang, L., Dong, G., Zhang, D., . . . Ma, Y. (2014). Traditional formula, modern application: chinese medicine formula sini tang improves early ventricular remodeling and cardiac function after myocardial infarction in rats. *Evid Based Complement Alternat Med*, 2014, 141938. doi:10.1155/2014/141938
- Liu, X. (1998). *Cordyceps sinensis*. Hong Kong: Hong Kong Medical Publisher 1998.
- Ma, J., Chadban, S. J., Zhao, C. Y., Chen, X., Kwan, T., Panchapakesan, U., . . . Wu, H. (2014). TLR4 activation promotes podocyte injury and interstitial fibrosis in diabetic nephropathy. *PLoS One*, 9(5), e97985. doi:10.1371/journal.pone.0097985 PONE-D-13-16619 [pii]
- Mackensen-Haen, S., Bohle, A., Christensen, J., Wehrmann, M., Kendziorra, H., & Kokot, F. (1992). The consequences for renal function of widening of the interstitium and changes in the tubular epithelium of the renal cortex and outer medulla in various renal diseases. *Clinical Nephrology*, 37(2), 70-77.
- Meran, S., Luo, D. D., Simpson, R., Martin, J., Wells, A., Steadman, R., & Phillips, A. O. (2011). Hyaluronan facilitates transforming growth factor-beta1-dependent proliferation via CD44 and epidermal growth factor receptor interaction. *J Biol Chem*, 286(20), 17618-17630. doi:M111.226563 [pii] 10.1074/jbc.M111.226563
- Meran, S., Martin, J., Luo, D. D., Steadman, R., & Phillips, A. (2013). Interleukin-1beta Induces Hyaluronan and CD44-Dependent Cell Protrusions That Facilitate Fibroblast-Monocyte Binding. *Am J Pathol*. doi:S0002-9440(13)00217-4 [pii] 10.1016/j.ajpath.2013.02.038
- Meran, S., Thomas, D. W., Stephens, P., Enoch, S., Martin, J., Steadman, R., & Phillips, A. O. (2008). Hyaluronan Facilitates Transforming Growth Factor- $\beta$ 1-mediated Fibroblast Proliferation. *J. Biol. Chem.*, 283(10), 6530-6545. doi:10.1074/jbc.M704819200
- Midgley, A. C., Duggal, L., Jenkins, R., Hascall, V., Steadman, R., Phillips, A. O., & Meran, S. (2015). Hyaluronan regulates bone morphogenetic protein-7-dependent prevention and reversal of myofibroblast phenotype. *J Biol Chem*, 290(18), 11218-11234. doi:10.1074/jbc.M114.625939



- Nie, S. P., Cui, S. W., Phillips, A. O., Xie, M. Y., Phillips, G. O., Al-Assaf, S., & Zhang, X. L. (2011). Elucidation of the structure of a bioactive hydrophilic polysaccharide from *Cordyceps sinensis* by methylation analysis and NMR spectroscopy. *Carbohydrate Polymers*, 84, 894-899.
- Pan, M. M., Zhang, M. H., Ni, H. F., Chen, J. F., Xu, M., Phillips, A. O., & Liu, B. C. (2013). Inhibition of TGF-beta1/Smad signal pathway is involved in the effect of *Cordyceps sinensis* against renal fibrosis in 5/6 nephrectomy rats. *Food Chem Toxicol*, 58, 487-494. doi:10.1016/j.fct.2013.04.037
- Ryan, M. J., Johnson, G., Kirk, J., Fuerstenberg, S. M., Zager, R. A., & Torok-Storb, B. (1994). HK-2: An immortalized proximal tubule epithelial cell line from normal adult human kidney. *Kidney International*, 45, 48-57.
- Sassy-Pringent, C., Heudes, D., Mandet, C., Belair, M. F., Michel, O., Perdereau, B., . . . Bruneval, P. (2000). Early glomerular macrophage recruitment in streptozotocin induced diabetic rats. *Diabetes*, 49, 466-475.
- Selbi, W., Day, A. J., Rugg, M. S., Fülöp, C., de la Motte, C. A., Bowen, T., . . . Phillips, A. O. (2006). Over-expression of hyaluronan synthase 2 alters hyaluronan distribution and function in proximal tubular epithelial cells. *The Journal of the American Society of Nephrology*, 17(6), 1553-1567.
- Selbi, W. D., De La Motte, C. A., Hascall, V. C., & Phillips, A. O. (2004). BMP-7 modulates HA mediated proximal tubular cell-monocyte interaction. *Journal of the American Society of Nephrology*, 15(5), 1199-1211.
- Shigeoka, A. A., Holscher, T. D., King, A. J., Hall, F. W., Kiosses, W. B., Tobias, P. S., . . . McKay, D. B. (2007). TLR2 is constitutively expressed within the kidney and participates in ischemic renal injury through both MyD88-dependent and -independent pathways. *J Immunol*, 178(10), 6252-6258. doi:178/10/6252 [pii]
- Simpson, R., Meran, S., Thomas, D., Stephens, P., Bowen, T., Steadman, R., & Phillips, A. (2009). Age related changes in peri-cellular hyaluronan organisation leads to impaired dermal fibroblast to myofibroblast differentiation. *The American Journal of Pathology*, In Press.
- Simpson, R. M. L., Wells, A., Thomas, D., Stephens, P., Steadman, R., & A.O., P. (2009). Ageing Fibroblasts resist phenotypic maturation due to impaired Hyaluronan-dependent CD44/EGF receptor signalling. *The American Journal of Pathology*, In Press.
- Wang, A. (1914). (Vol. 1). Shanghai: Gong He Shu Ju (Republic Book Store).
- Wang, J., Kan, L., Nie, S., Kan, L., Chen, H., Cui, S., . . . Xie, M. (2015). Comparison of structural features and antioxidant activity of polysaccharides from natural and cultured *Cordyceps sinensis*. *Food Science and Biotechnology*, 63, 2-7.

- Wang, S. J., Bai, W., Wang, C. L., & Dai, Z. (2007). Effects of cordyceps sinensi on bleomycin-induced pulmonary fibrosis in mice. *Zhongguo Zhong Yao Za Zhi*, 32(24), 2623-1627.
- Webber, J., Jenkins, R. H., Meran, S., Phillips, A., & Steadman, R. (2009). Modulation of TGF{beta}1-Dependent Myofibroblast Differentiation by Hyaluronan. *Am J Pathol*, 175(1), 148-160. doi:10.2353/ajpath.2009.080837
- Webber, J., Meran, S., Steadman, R., & Phillips, A. O. (2009). Hyaluronan orchestrates TGF-beta 1 dependent maintenance of myofibroblast phenotype. *J. Biol. Chem.*, 284, 9083-9092. doi:10.1074/jbc.M806989200
- Wehrmann, M., Bohle, A., Bogenschutz, O., Eissele, R., Freisleder, A., Ohlschlegel, C., . . . Gartner, H. V. (1989). Long-term prognosis of chronic idiopathic membranous glomerulonephritis. *Clinical Nephrology*, 31(2), 67-76.
- Wehrmann, M., Bohle, A., Held, H., Schumm, G., Kendziorra, H., & Pressler, H. (1990). Long-term prognosis of focal sclerosing glomerulonephritis. An analysis of 250 cases with particular regard to tubointerstitial changes. *Clinical Nephrology*, 33(3), 115-122.
- Yang, J., & Liu, Y. (2001). Dissection of key events in tubular epithelial to myofibroblast transition and its implications in renal interstitial fibrosis. *The American Journal of Pathology*, 159(4), 1465-1475.
- Yang, L.-Y., Huang, W. J., Hsieh, H.-G., & Lin, C.-Y. (2003). H1-A extracted from Cordyceps sinensis suppresses the proliferation of human mesangial cells and promotes apoptosis, probably by inhibiting the tyrosine phosphorylation of Bcl-2 and Bcl-XL. *Journal of Laboratory and Clinical Medicine*, 141(1), 74-83.
- Yao, X., Meran, S., Fang, Y., Martin, J., Midgley, A., Pan, M. M., . . . Phillips, A. O. (2013). Cordyceps sinensis: In vitro anti-fibrotic bioactivity of natural and cultured preparations. *Food Hydrocolloids*, 35, 444-452.
- Young, B. A., Johnson, R. J., Alpers, C. E., Eng, C. E., Gordon, K., Floege, J., & Couser, W. (1995). Cellular events in the evolution of experimental diabetic nephropathy. *Kidney International*, 47, 935-944.
- Yue, G. G.-L., Lau, C. B.-S., Fung, K.-P., Leung, P.-C., & Ko, W.-H. (2008). Effects of Cordyceps sinensis, Cordyceps militaris and their isolated compounds on ion transport in Calu-3 human airway epithelial cells. *Journal of Ethnopharmacology*, 117(1), 92-101.
- Yue, K., Ye, M., Zhou, Z., Sun, W., & Lin, X. (2013). The genus Cordyceps: a chemical and pharmacological review. *J Pharm Pharmacol*, 65(4), 474-493. doi:10.1111/j.2042-7158.2012.01601.x
- Zhang, Q. X., & Wu, J. Y. (2007). Cordyceps sinensis Mycelium Extract Induces Human Premyelocytic Leukemia Cell Apoptosis Through Mitochondrion Pathway. *Experimental Biology and Medicine*, 232(1), 52-57.

- Zhang, X. L., Bi-Cheng, L., Al-Assaf, S., Phillips, G. O., & Phillips, A. O. (2012). Cordyceps sinensis decreases TGF- $\beta$ 1 dependent epithelial to mesenchymal transdifferentiation and attenuates renal fibrosis. *Food Hydrocolloids*, 28, 200-212.
- Zhang, X. L., Selbi, W. D., De La Motte, C. A., Hascall, V. C., & Phillips, A. O. (2004). Renal proximal tubular epithelial cells Transforming Growth Factor  $\beta$ 1 generation and monocyte binding. *The American Journal of Pathology*, 165, 763-773.
- Zhou, X., Gong, Z., Su, Y., Lin, J., & Tang, K. (2009). Cordyceps fungi: natural products, pharmacological functions and developmental products. *J Pharm Pharmacol*, 61(3), 279-291. doi:10.1211/jpp/61.03.0002
- Zhu, J. S., Halpern, G. M., & Jones, K. (1998a). The scientific rediscovery of an ancient Chinese herbal medicine: Cordyceps sinensis: Part . *Journal of Alternative and Complementary Medicine*, 4, 289-303.
- Zhu, J. S., Halpern, G. M., & Jones, K. (1998b). The scientific rediscovery of an ancient Chinese herbal medicine: Cordyceps sinensis: Part II. *Journal of Alternative and Complementary Medicine*, 4, 429-457.

## Figure Legends

Figure1. [A] Extraction process of polysaccharide from cultured *Cordyceps Sinensis* (CSP) (as previously described (J. Wang et al., 2015)). [B] HPGPC profiles of the polysaccharides from cultured mycelium, monitored by RID (straight line) and VWD (dotted line) at 280 nm. [C] monosaccharide composition of extracted polysaccharide. [D] Glycosidic linkages of polysaccharide determined by GC-MS.

Figure 2: Antagonism of TGF- $\beta$ 1 induced mRNA expression of Collagen: Confluent monolayers of HK2 were growth arrested in serum free medium for 48 hours. The medium was then replaced with either serum free medium alone (control), serum free medium containing 5ng/ml TGF- $\beta$ 1 or TGF- $\beta$ 1 together with 750  $\mu$ g/ml CSP, for the time periods indicated. mRNA was extracted and cDNA prepared as described in methods. Collagen 1 mRNA expression was assessed by RT-QPCR. The results are represented as mean  $\pm$ SEM of n=3 experiments, ## and # denote a *p* value of less than 0.01 or 0.05 compared to the control cells treated with serum free medium alone; \*\* and \*denotes a *p* value of less than 0.01 or 0.05 for TGF- $\beta$ 1 stimulation together with polysaccharides compared to stimulation with TGF- $\beta$ 1 alone of each time point.

Figure 3: Mechanism of TGF- $\beta$ 1 Antagonism: Confluent monolayers of HK2 were growth arrested in serum free medium for 48 hours. The medium was then replaced with either serum free medium alone (control), serum free medium containing 5ng/ml TGF- $\beta$ 1 or TGF-

$\beta 1$  together with 750 $\mu$ g/ml CSP. mRNA was extracted and cDNA prepared as described in methods. BMP7 [A] and Alk5 [B] mRNA expression assessed by RT-QPCR. In parallel experiments HK2 cells transfected with TLR4 siRNA [C] were stimulated with serum free medium containing 5ng/ml TGF- $\beta 1$  or TGF- $\beta 1$  together with 750 $\mu$ g/ml CSP. Control cells were exposed to serum free medium alone. After RNA extraction and cDNA preparation collagen 1 mRNA expression was assessed by RT-QPCR. The results are represented as mean  $\pm$ SEM of n=3 experiments; \*\* and \*denotes a *p* value of less than 0.01 or 0.05 for TGF- $\beta 1$  stimulation together with polysaccharides compared to stimulation with TGF- $\beta 1$  alone of each time point.

Figure 4: CSP inhibits IL-1  $\beta$  induced monocyte binding, which is ICAM dependent: Confluent monolayers of HK2 cells were growth arrested in serum-free medium for 48 hours. The medium was then replaced with either serum-free medium alone (control), serum-free medium containing 1 ng/mL IL-1 $\beta$ , serum-free medium containing 1 ng/mL IL-1 $\beta$  together with 750 $\mu$ g/ml CSP, or serum-free medium containing 1 ng/mL IL-1 $\beta$  together with 750 $\mu$ g/ml CSP and soluble ICAM at the concentrations indicated for 72 hours. Subsequently,  $1 \times 10^6$  monocytes (U937 cells) were added per well. These two cells were co-cultured for a further 6 hours. The cultures were then washed 10 times with PBS to remove any unbound U937 cells. The cells were subsequently lysed with Tri-reagent and RNA extracted. RT-qPCR was used to assess CD45 mRNA expression. Results represent mean  $\pm$ SEM of n=3. # denotes a significant increase in CD45 binding compared to the unstimulated controls of each treatment (*p*<0.01). \*\* and \* denotes a *p* value of less than 0.01 or 0.05 between groups.

Figure 5: Association and dependence of monocyte binding with CD44 up-regulation and peri-cellular hyaluronan: [A, B] Confluent monolayers of HK2 cells were growth arrested in serum-free medium for 48 hours. The medium was then replaced with either serum-free medium alone (control), serum-free medium containing 1 ng/mL IL-1 $\beta$ , serum-free medium containing 1 ng/mL IL-1 $\beta$  together with 750 $\mu$ g/ml CSP. Subsequently,  $1 \times 10^6$  monocytes (U937 cells) were added per well. These two cells were co-cultured for a further 6 hours. The cultures were then washed 10 times with PBS to remove any unbound U937 cells. The cells were subsequently lysed with Tri-reagent and RNA extracted. RT-qPCR was used to assess CD44 or HAS 2 mRNA expression. Results represent mean  $\pm$ SEM of n=3. ## and # denote a *p* value of less than 0.01 or 0.05 compared to the control cells treated with serum free medium alone; \*\* and \*denotes a *p* value of less than 0.01 or 0.05 for IL-1 $\beta$  stimulation together with polysaccharides compared to stimulation with IL-1 $\beta$  alone of each time point. [C] The dependence of CD45 binding on expression of CD44 was examined by addition of either serum-free medium containing 1 ng/ml IL-1 $\beta$ , or serum-free medium containing 1

ng/ml IL-1 $\beta$  together with 750 $\mu$ g/ml CSP to HK2 cell transfected with siRNA to CD44. In the control experiment following growth arrested in serum-free medium for 48 hours, the medium was replaced with either serum-free medium containing 1 ng/ml IL-1 $\beta$ , or serum-free medium containing 1 ng/ml IL-1 $\beta$  together with 750 $\mu$ g/ml CSP. Subsequently,  $1 \times 10^6$  monocytes (U937 cells) were added per well. These two cells were co-cultured for a further 6 hours. The cultures were then washed 10 times with PBS to remove any unbound U937 cells. The cells were subsequently lysed with Tri-reagent and RNA extracted. RT-qPCR was used to assess CD45 mRNA expression. Results represent mean  $\pm$ SEM of n=3. \* denotes a significant decrease in CD45 binding compared to stimulation with IL-1 $\beta$  alone (p<0.05).

[D] Inhibitory effect of CSP is dependent on cell surface hyaluronan. Cell surface hyaluronan was removed by incubation of confluent HK2 cell monolayers with 200 mg/ml of bovine testicular hyaluronidase, for 24 hours as indicated. Subsequently either serum-free medium alone (control), serum-free medium containing 1 ng/ml IL-1 $\beta$ , serum-free medium containing 1 ng/ml IL-1 $\beta$  together with 750 $\mu$ g/ml CSP was added to the cell monolayer for 72 hours. Subsequently,  $1 \times 10^6$  monocytes (U937 cells) were added per well. These two cells were co-cultured for a further 6 hours. The cultures were then washed 10 times with PBS to remove any unbound U937 cells. The cells were subsequently lysed with Tri-reagent and RNA extracted. RT-qPCR was used to assess CD45 mRNA expression. Results represent mean  $\pm$ SEM of n=3. ## denote a p value of less than 0.01 compared to the control cells treated with serum free medium containing 200 mg/ml of bovine testicular hyaluronidase; \*\* denotes a significant increase in CD45 binding compared to stimulation with IL-1 $\beta$  alone (p<0.01).

Fig 1A

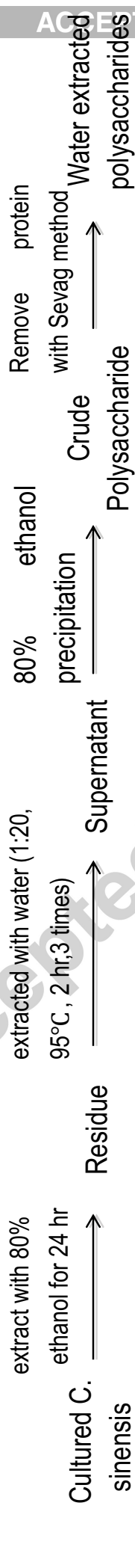


Fig 1B

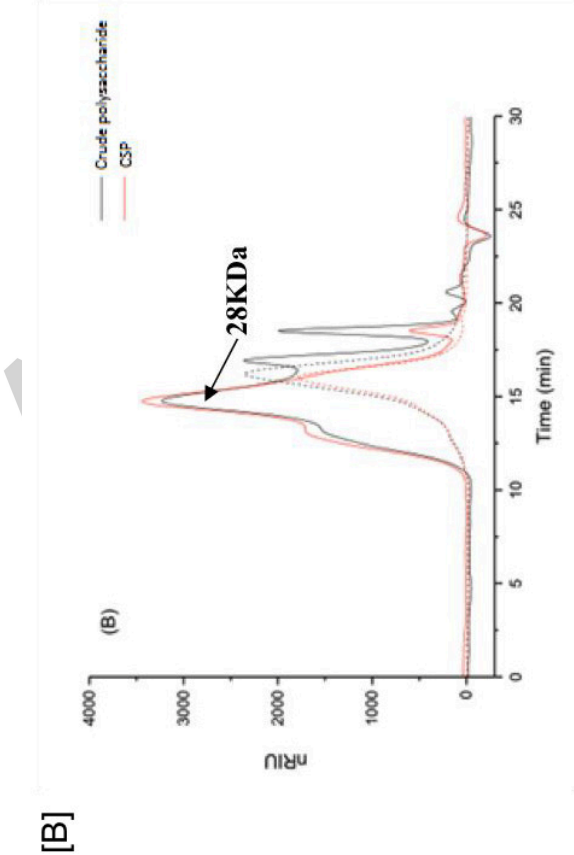


Fig 1C

Sample	Ara,%	Gal,%	Glc,%	Man,%	GalA,%
Crude polysaccharide	trace	38.48	31.99	23.15	6.38
CSP	3.34	36.40	28.99	24.81	7.55

<sup>a</sup> calculated as a molar percentage.

Fig 1D

Glycosyl residues	Linkage pattern	CSP
Ara	t-Araf	1.91
	5-Araf	3.24
Glc	t-Glcp	10.26
	3-Glcp	5.85
	4-Glcp	15.44
	4,6-Glcp	1.42
Gal	t-Galp	6.63
	4-Galp	22.54
Man	2- Manp	5.17
	6- Manp	7.20
	2,6- Manp	3.11
	4,6- Manp	9.20



Fig 2.

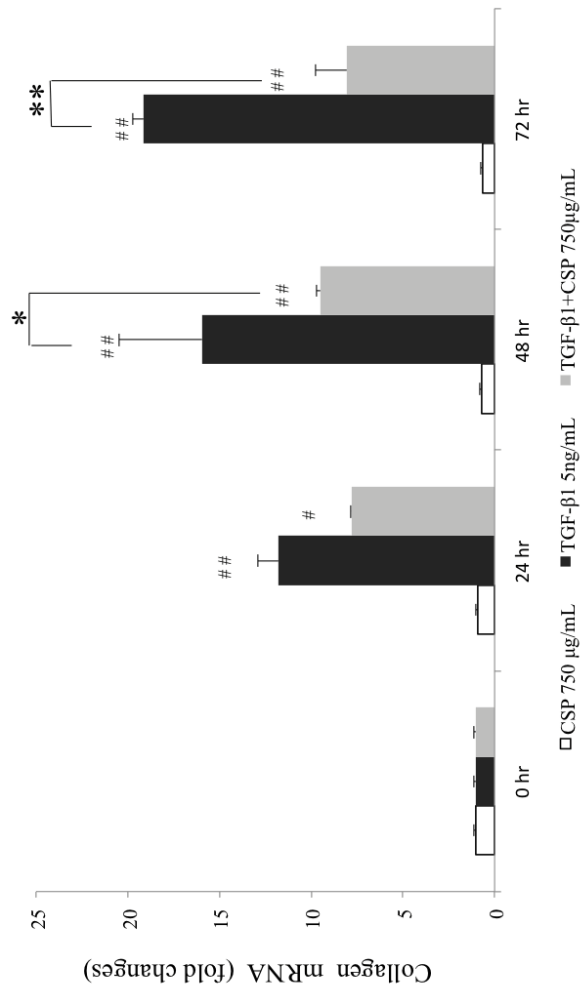


Fig 3 A.

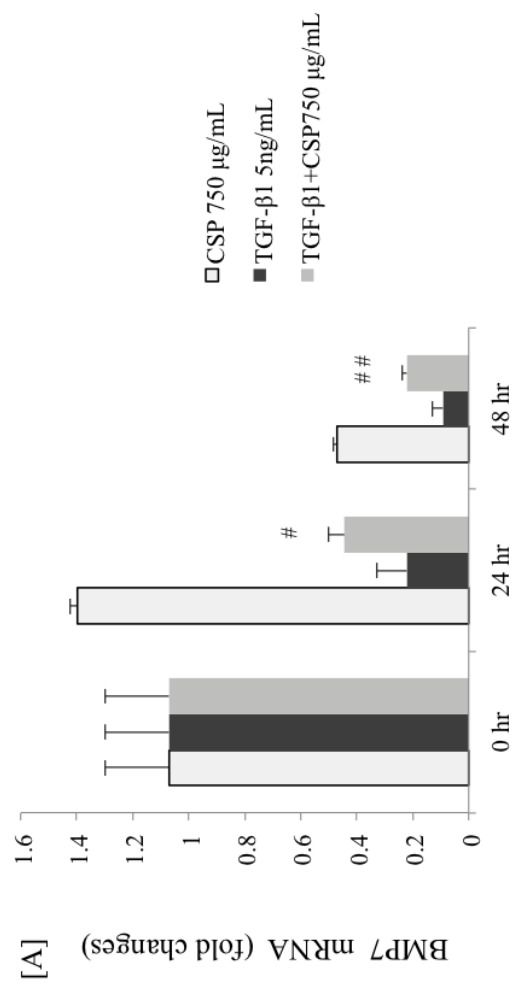


Fig 3B.

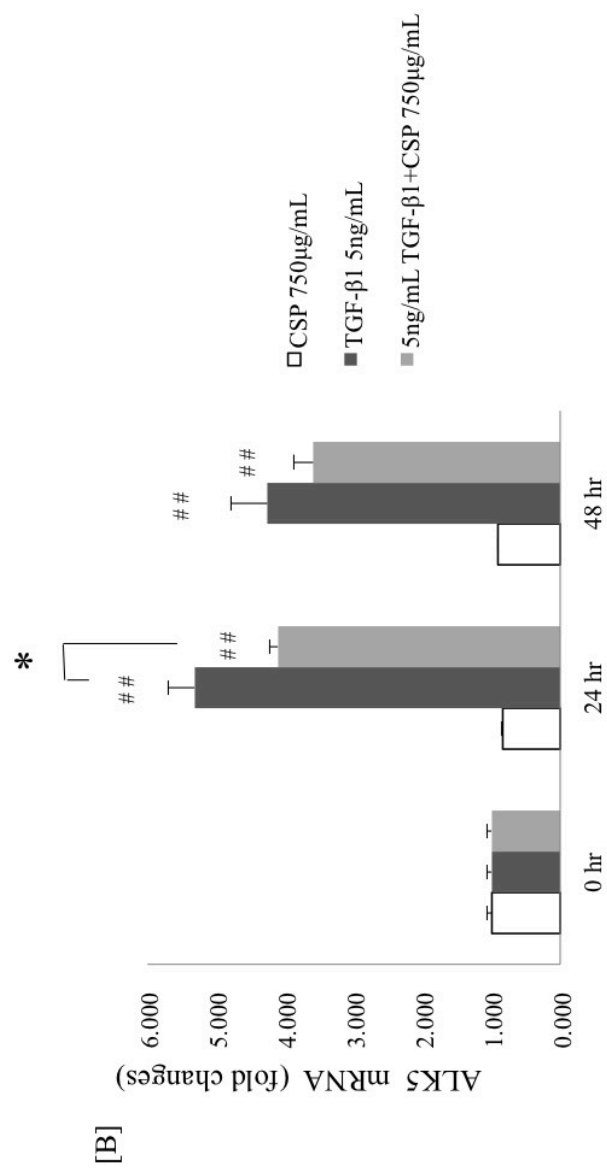


Fig 3C

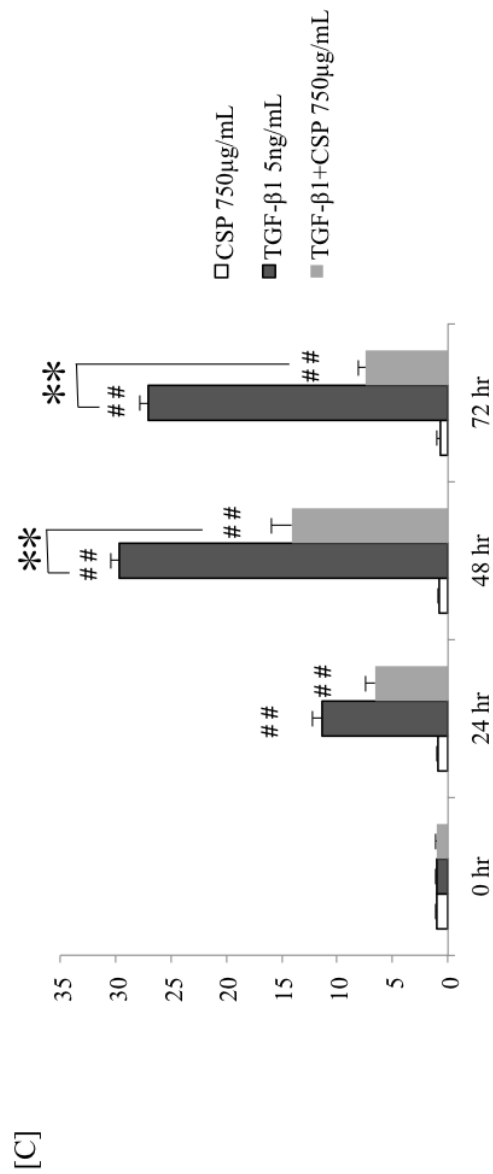


Fig 4.

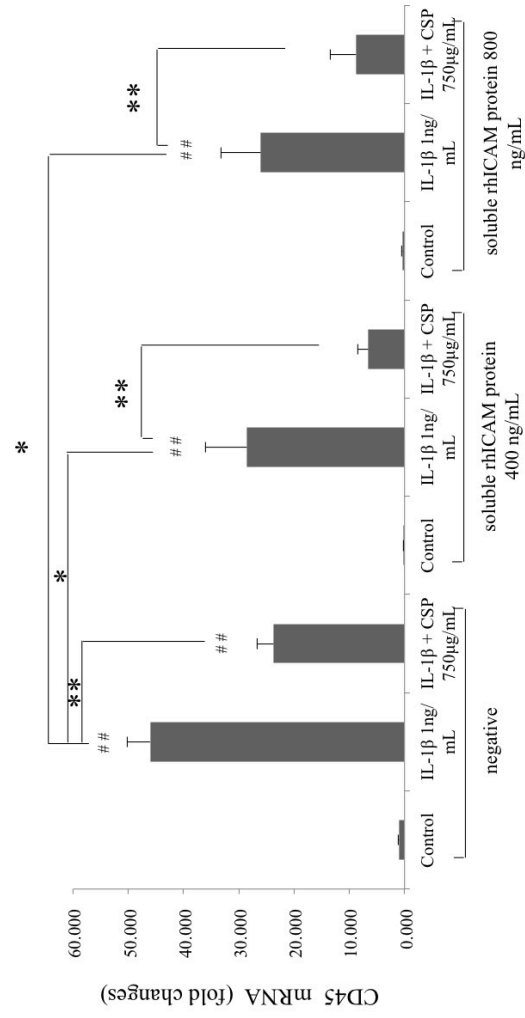


Fig 5 A

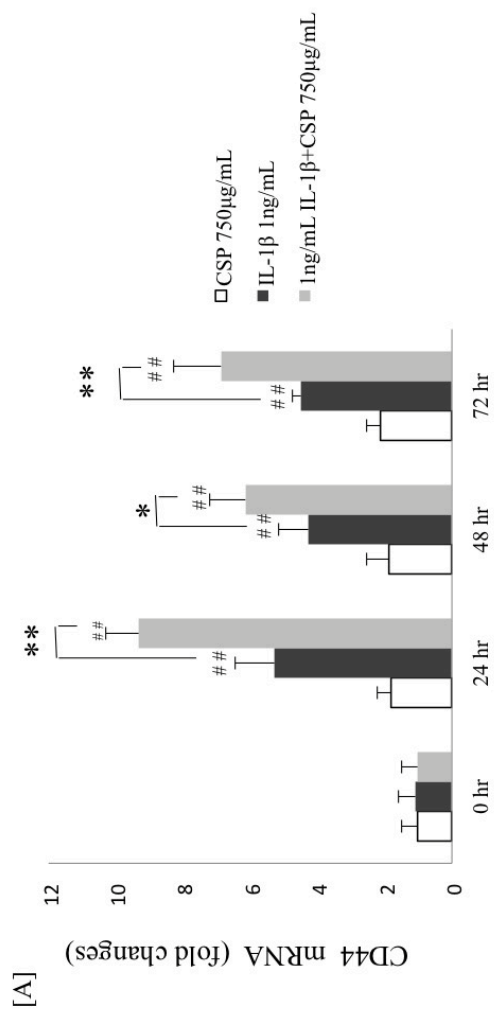


Fig 5B

