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Exposure to cigarette smoke extract induces proliferation and overexpression of CCL2 in A549 cells and migration in lung fibroblasts

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is an interstitial lung disease characterized by an aberrant and deregulated remodeling process, where the immune system plays an important role, with smoking being the main risk factor. Cigarette smoke extract (CSE) induces damage and synthesis of cytokines in the epithelium of the respiratory tract. The CCL2 chemokine is elevated in the bronchoalveolar lavage of patients with IPF. However, it is unknown whether CSE induces its profibrotic effect through CCL2. The objective of the study is to evaluate the role of CCL2 in the migration and expression of profibrotic molecules using an *In vitro* model of A549 lung epithelial cells transfected with the *CCL2* gene and CSE-stimulated CCD25 fibroblasts. Our findings indicate that CSE increases the expression of *CCL2* and modulates the migration and proliferation of A549 cells and the expression and synthesis of TGF- β 1. The conditioned medium of A549 cells that overexpress *CCL2* induces migration and overexpression of *IL6* in fibroblasts. *CCL2* overexpression in CSE-stimulated A549 cells induces a profibrotic effect in CCD25 fibroblasts, serving as an orchestrator in the development of IPF.

Key words: idiopathic pulmonary fibrosis, alveolar epithelial cells, A549 cells, lung fibroblasts, *CCL2*, cigarette smoke extract.

La exposición al extracto del humo del cigarro induce la proliferación y la sobreexpresión de CCL2 en células A549, así como la migración de fibroblastos pulmonares

RESUMEN

La Fibrosis Pulmonar Idiopática (FPI) es una enfermedad en la que el hábito al tabaco es el principal factor de riesgo, presenta un proceso de remodelado complejo y desregulado en la que el sistema inmune tiene un papel importante. El extracto del humo del cigarro (CSE) es dañino y da lugar a la síntesis de citocinas en el epitelio del tracto respiratorio. La quimiocina CCL2, se encuentra, por lo general, elevada en lavado broncoalveolar de pacientes con FPI. Sin embargo, se desconoce si el CSE induce su efecto profibrótico a través de CCL2. El objetivo de este estudio fue evaluar el rol de CCL2 en la migración y expresión de moléculas profibróticas al usar un modelo *in vitro* de células epiteliales pulmonares A549 transfectadas con el gen de *CCL2* y los fibroblastos CCD25 estimulados con el CSE. Los resultados indican que el CSE incrementa la expresión de *CCL2*, y modula la migración y proliferación de las células A549, así como la expresión y síntesis de TGF- β 1. El medio condicionado proveniente de las células A549 que sobreexpresan a *CCL2* induce a la migración y a la sobreexpresión del gen *IL6* en los fibroblastos. Así mismo, la sobreexpresión de *CCL2* en las células A549 estimuladas con el CSE da lugar a un efecto profibrótico en células CCD25, fungiendo como un orquestador en el desarrollo de la FPI.

Palabras clave: fibrosis pulmonar idiopática, células alveolares epiteliales, células A549, fibroblastos pulmonares, *CCL2*, extracto de humo de cigarro.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a form of idiopathic interstitial pneumonia (IIP) that is progressive and fatal. IPF occurs in adults over 50 years of age, and its etiology is unknown (Raghu *et al.*, 2011). Although the pathogenetic mechanism is not fully known, the evidence suggests that the fibrotic response is the result of abnormal function of alveolar epithelial cells (AECs) (King, Pardo & Selman, 2011). AECs produce mediators that induce the proliferation, migration, and activation of resident mesenchymal cells, the recruitment of fibroblasts, and induction of the epithelial-mesenchymal transition (Kim *et al.*, 2006). AECs are susceptible to stimuli such as cytokines released into the local airspace environment. Smoking is strongly associated with IPF disease, and it has been shown that cigarette smoke extract (CSE) increases the expression of profibrotic genes, mainly *CCL2*, in AECs (Checa *et al.*, 2016). It has been described the expression of *CCL2* in lung tissues of patients with IPF (Agostini & Gurrieri, 2006; Antoniadis *et al.*, 1992; Iyonaga *et al.*, 1994; Rose, Sung & Fu, 2010), which is expressed in epithelial tissue and vascular endothelium. Other studies in experimental models have associated the expression of *CCL2* with the development of fibrosis (Inoshima *et al.*, 2004; Mercer *et al.*, 2009; Yang *et al.*, 2020). Liu suggests that *CCL2* mediates fibroblast survival by inhibiting apoptosis through modulation of interleukin 6 (*IL6*) expression (Chen, Gao, Han, Pan & Fan, 2015; Liu *et al.*, 2007). Other evidence shows that when using the murine model of pulmonary fibrosis, a profibrotic pathway is induced that involves *IL-13*, which can modulate *CCL2*; *CCL2*, in turn, can modulate transforming growth factor β 1 (TGF- β 1) in pulmonary fibroblasts (Murray *et al.*, 2008). *CCL2* has been shown to induce collagen synthesis and *TGF β 1* expression in pulmonary fibroblasts (Gharaee-Kermani, Denholm & Phan, 1996). A study of potential clinical biomarkers in idiopathic interstitial pneumonia found that higher levels of *CCL2* circulating in their plasma combined with KL-6 and CXCL13 can improve the diagnostic efficacy of IIPs (Xue, Guo, Cai, Sun & Wang, 2019). Additionally, in a study carried out by Yang *et al.*, 2020 a *CCL12* knockout mouse (*CCL12* is the homologous cytokine of *CCL2* in humans) had no protection against fibrosis, while a mouse with specific deletion of *CCL12* in AECs exhibited a protective effect against fibrosis (Yang *et al.*, 2020).

Currently, the role of smoking in modifying the micro-environment to contribute to the development of a profibrotic phenotype in alveolar epithelial cells and fibroblasts is not clearly understood.

In this study, we hypothesize that *CCL2* induced by CSE in alveolar epithelial type II cells (A549 cells) may be an important mediator in the proliferation, migration, and expression of profibrotic factors such as *TGF β 1*, as well as an inducer in the expression of *IL6* in pulmonary fibroblasts.

Here we found that CSE increases *CCL2* expression and the proliferation of A549 cells. The expression of *CCL2* can modulate the migration and proliferation of A549 cells and the expression and synthesis of TGF- β 1. The conditioned medium of *CCL2*-overexpressing A549 cells can induce the overexpression of *IL6* in fibroblasts and the induction of migration. Our findings suggest that *CCL2* overexpression in CSE-stimulated epithelial alveolar cells has a profibrotic effect on fibroblasts, altering pulmonary homeostasis and serving as an essential orchestrator in the development of IPF when stimulated by cigarette smoke.

MATERIALS AND METHODS

Cell culture

Human A549 pulmonary epithelial cells (ATCC, Manassas, VA, USA) (the cell line was characterized as being representative of the alveolar type II pneumocytes of the human lung) and CCD25 human pulmonary fibroblasts (ATCC, Manassas, VA, USA) were cultivated in DMEM (Gibco Life Technologies, Grand Island, NY, USA) and EMEM (Lonza, Alpharetta, GA, USA), respectively; both media were supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Grand Island, NY, USA) and antibiotic-antimycotic (100 \times) (Gibco Life Technologies).

Stimulation of A549 cells with CSE

CSE was prepared using a modification of the method described by Liu (Liu, Gao & Zhang, 2010). Briefly, one filterless commercial cigarette (Oldham, DeSoi, Rimmer, Wagner & Morton, 2014) (Marlboro, Philip Morris, Richmond, VA, USA) was burned using a modified syringe-driven apparatus. Mainstream smoke was generated from one cigarette by drawing consecutive puffs into a 20 mL plastic syringe, with a stopcock connected through one port to a glass containing 25 mL of serum-free DMEM-F12 (Gibco Life Technologies).

A 20-mL puff drawn in 1 second was obtained in 10-second intervals; each puff was held for 3 seconds and then bubbled through DMEM for 5 seconds. One cigarette yielded an average of 45 puffs by this procedure. The resulting suspension was adjusted to pH 7.4 with concentrated NaOH and filtered through a 0.2- μ m-pore filter to remove bacteria and large particles (Barnant, Barrington, IL, USA). The resulting solution was designated a 100% CSE solution. The CSE solutions were prepared by the same person using the same method and were used within 30 minutes of preparation. This stock was diluted to 5% in DMEM, supplemented with 1% FBS and penicillin-streptomycin (Gibco Life Technologies). Exposure of A549 epithelial cells to CSE was standardized to 6.72×10^6 cells in T75 culture flasks (Merck KGaA, Darmstadt, Germany). The medium was changed every 24 h until one week was completed. A549 cells were stimulated with 5% CSE for 6 days.

CCL2 transfection in A549 cells

The overexpression of *CCL2* was achieved through stable transfection with the entry vector pCMV6 (Origene, Rockville, MD, USA), which has a TrueORF with a Myc label and a DDK sequence in the carboxyl-terminal region. According to the company's instructions, the plasmid was amplified in BL21 (DE3) chemically competent *Escherichia coli* cells (Merck KGaA) and then inserted using heat shock. The transfected bacteria were kanamycin resistant (Merck KGaA). The plasmid was isolated with a purification kit (QIAGEN, Germantown, MD, USA) according to the manufacturer's instructions. The *CCL2* plasmid was inserted into A549 cells with turbofectin (Origene; 90 μ L) in a 3:1 ratio with DNA. Cells were incubated with antibiotic G418 (Merck KGaA; 800 μ g/mL).

Knockdown of CCL2 in A549 cells

A549 cells were seeded in 12-well culture plates (Merck KGaA) with 7.5×10^5 cells in F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS. The infection was done according to the instructions of the company, with a mixture of polybrene (Santa Cruz Biotechnology, Dallas, TX, USA; 5 μ g/mL) and *MCP-1* shRNA-(h) lentiviral particles (Santa Cruz Biotechnology; 15 μ L), and this mixture was applied to A549 cells. The infection efficiency was evaluated using a positive control of lentiviral particles with a green fluorescent protein (GFP; Santa Cruz Biotechnology; 0.1 μ g/ μ L). Serial dilutions were made to obtain stable clones that were selected with puromycin (Santa Cruz Biotechnology; 7.5 μ g/mL). Plasmid-A (Santa Cruz Biotechnology; 0.1 μ g/ μ L) was used as a negative control, and we denoted it as the scramble control. The knockdown of *CCL2* in transfected cells was evaluated by real-time PCR and Western blotting using *CCL2* antibody.

CCL2 recombinant Protein Stimulation to CCL2 Knockdown A549 Cells

A549 cells were seeded in 6-well culture plates (Merck KGaA) with 2×10^5 cells in F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS and 3 mL of human *CCL2* (100 ng/ml, R&D Systems) was added to the culture of *CCL2* knockdown cells, for 12 hours.

Fibroblast stimulation

CCD25 fibroblasts were cultured (1×10^5) in a 12-well dish and stimulated with a conditioned medium from CSE-stimulated A549, CSE-stimulated and *CCL2*-inhibited A549, *CCL2*-transfected A549 cells, and empty vector, stimulated for 48 hours.

RNA extraction and real-time PCR

The mRNA of A549 cells and CCD25 fibroblasts was extracted with Trizol (Merck KGaA), and 1 μ g of RNA was reverse transcribed according to the instructions of the company, yielding a cDNA chain (Advantage RT-for PCR kit; Clontech, Palo Alto, CA, USA). For real-time PCR, the Step-One system

thermocycler (Applied Biosystems, Foster City, CA, USA) was used, with a Taqman probe labeled with fluorescein amidites (FAM), the expression of *CCL2* (Thermo Fisher Scientific, Waltham, MA, USA), and *TGFBI* (Thermo Fisher Scientific) in A549 cells was evaluated. In fibroblasts stimulated, we evaluated the expression of *IL6*, *ACTA2*, and *POLR2A* (all from Thermo Fisher Scientific). All PCRs were done in a 15- μ L volume containing 2 μ L of cDNA and 13 μ L of 2 \times Mastermix (Thermo Fisher Scientific). The PCR conditions were 2 minutes at 94 $^{\circ}$ C and 60 $^{\circ}$ C for 1 minute. The results are expressed as means \pm SD of the $2^{-\Delta\text{CT}}$ (delta of cycle threshold of the gene of interest, normalized with a housekeeping gene, *POLR2A*).

Proliferation analysis with WST-1

The proliferation rate was evaluated in the WST-1 assay (Merck KGaA) during 1 hour of incubation. According to the methods of the manufacturers and Van der Poel (Van Der Poel, 2004) A549 cells at a concentration of 1×10^3 cells/well were incubated in 96-well microplates, in a final culture medium volume of 100 μ L/well. Cells were incubated for 6 days with CSE, at 37 $^{\circ}$ C and 5% CO_2 . An amount of 10 μ L/well of WST-1 was added and incubated for 1 hour. The absorbance was analyzed in an ELISA reader (Biotek, Winooski, VT, USA) with Gen 53.0 software at 450 nm with a reference of 620 nm. The same method was applied for transfected and inhibited A549 cells.

To verify the same number of live cells, the method with trypan blue was used, for this, A549 cells were trypsinized and mixed with trypan blue solution according to Louis and Siegel (Louis & Siegel, 2011). Briefly, 0.4% trypan solution was prepared in 0.81% sodium chloride and 0.06% dibasic potassium phosphate (Merck KGaA). A 1:1 mixture was made of the cell suspension (1×10^6 cells/mL) and the 0.4% trypan blue solution. Finally, the mixture was transferred to an automated cell counter (Invitrogen, Carlsbad, CA, USA).

Wound healing assay

A549 cells at 9×10^5 cell/well density were seeded into six-well plates and incubated in DMEM with 1% FBS. After overnight culture, the A549 cells were scratched with 200 μ L micropipette tips to form straight lines. The floating cells were removed using $1 \times$ PBS three times. To further reduce the risk of cell proliferation confounding the study of migration, mitomycin C (Merck KGaA; 3 μ g/mL) was added every 24 h. The wound closure was monitored at 0, 24, 48, and 72 h, and it was photographed under an inverted microscope (Olympus, Richmond Hill, Ontario, Canada). The recolonized area was calculated by manually tracing the cell-free area in captured images using the ImageJ public domain software (NIH, Bethesda, MD, USA). The migration rate was expressed as the percentage of wound closure (% Wound closure = $[(A_{t=0h} - A_{t=\Delta h}) / A_{t=0h}] \times 100\%$) according to Ayman et al (Grada, Otero-Vinas, Prieto-Castrillo, Obagi & Falanga, 2017).

ELISA

Protein levels of CCL2 and TGF- β 1 were evaluated in the conditioned medium of CCL2-overexpressing A549 cells and CCL2 knockdown A549 cells with an ELISA kit (R&D Systems, Minneapolis, MN, USA). Per the manufacturer's directions, 200 μ L of the medium was used, the ELISA plate was incubated for 2 hours at room temperature, the plate was washed four times, a conjugated antibody was added, and the mixture was incubated for 1 hour at room temperature (the antibody is polyclonal, binds specifically to CCL2, and is coupled to horseradish peroxidase [HRP]). A 200- μ L volume of substrate solution was used, which contained hydrogen peroxide and a stabilizing chromogen (tetramethylbenzidine); this solution was incubated for 30 minutes, and the reaction was stopped with sulfuric acid, 2 N. The samples were read in a spectrophotometer at 450 nm (Biotek plate reader), using the Gen 5 software (Biotek). We added 300 ng of CCL2 recombinant protein (R&D Systems) to knockdown CCL2 in A549 cells, and then TGF- β 1 protein levels were evaluated.

Western blot

Conditioned medium from A549 epithelial cells not expressing CCL2 was concentrated with filtration units (Merck KGaA). The proteins obtained were quantified using the Bradford method (Bradford assay reagent; Bio-Rad Laboratories, Hercules, CA, USA), adjusting to 30 μ g. Equal amounts of protein from each sample and a molecular weight marker (Precision Plus Protein Dual Xtra Standards, Bio-Rad) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was exposed to a blocking solution overnight at 8 °C. An anti-CCL2 monoclonal antibody (R&D Systems) was used at a 1:1000 dilution. The production of TGF- β 1 (Cell Signaling Danvers, MA, USA) was detected at a 1:1000 dilution after incubation for 1 h 30 minutes. This primary antibody incubation was followed by incubation with HRP-conjugated anti-rabbit or anti-mouse antibody (both from Invitrogen, Carlsbad, CA, USA; used at 1:20,000 dilution) as the secondary antibody for 1 hour at room temperature. The chemiluminescence was detected using the Super Signal West Femto maximum sensitivity substrate developer solution (Thermo Fisher Scientific) and read on a transilluminator ChemiDoc XRS+ (Bio-Rad). To process the images, we used Image Lab software (Bio-Rad). Ponceau S solution was used as a loading control (Merck KGaA).

Cell migration and invasion assay

The fibroblast transmigration assay was performed in QCM Haptotaxis cell migration assay-collagen I, 24-well, colorimetric plates (Merck KGaA). A density of 2×10^5 cells/well was treated with an anti-CCL2 antibody (R&D Systems; 5 μ g/mL) or without any treatment and suspended in 200 μ L of serum-free DMEM containing 5% bovine serum albumin (BSA) and placed onto the top of chambers (Merck KGaA), and the lower chambers were filled with 600 μ L of medium from

A549 cells that overexpress CCL2 in DMEM with 5% BSA, or medium from A549 cells that had empty vector or platelet-derived growth factor (PDGF; PeproTech, Cranbury, NJ, USA; 50 ng/mL) as a positive control (is a migration inducer). After 12 h, non migratory CCD25 cells were removed with a cotton swab from the top surface of the chamber, and the lower filter of the chamber was stained with crystal violet solution for 20 min at 37 °C. The upper insert was dipped into a beaker of water several times to rinse. The dry stained inserts were transferred in a clean well containing 200 μ L of extraction buffer (acetic acid, 1 N) for 15 min at room temperature. Finally, 100 μ L of the dye mixture was transferred to a 96-well microtiter plate suitable for colorimetric measurement. The optical density was read in a BioTek plate reader at 560 nm.

Statistical analysis

Three tests were performed independently, each in triplicate. Data from all experiments were analyzed by using Prism 8.3.0 software (GraphPad, San Diego, CA, USA) and are shown as the arithmetic means \pm SD. The multiple two-tailed Student's t-test was performed to assess the differences between experimental groups and controls over time. One-way analysis of variance (ANOVA) followed by a Tukey post hoc test was used to analyze differences between groups for multiple comparisons. The level of significance was set at $p < 0.05$, and a high level of significance was set at $p < 0.01$.

RESULTS

CSE increases CCL2 expression and proliferation in A549 pulmonary AECs

It has been described that A549 alveolar epithelial cells are metabolically very active, which is reflected in a high production of cytokines and growth factors. For this reason, we decided to evaluate if the expression of CCL2 is increased when stimulated with CSE.

An increase in the expression and production of CCL2 was confirmed under CSE exposure from the second day of treatment (Figure 1A-B). CCL2 levels were up-regulated on day 4. On day 6, there was a high expression of CCL2. Simultaneously, we evaluated the cytotoxic effect of CSE on the proliferation of A549 cells in a WST-1 assay (Figure 1C), we observe that on day 6 under the stimulus of CSE there was an increase in proliferation.

CCL2 modulates the migration of A549 cells

We examined if the expression of CCL2 has any role in the migration of AECs. Real-time PCR and ELISA (Figure 2A-B) revealed that CCL2 was effectively overexpressed in A549 cells and was increased compared with empty vector and control A549 cells. Then, the efficiency of the inhibition of CCL2 was confirmed; notable decreases in the expression and synthesis of CCL2 were observed compared to the control cells and the scramble (negative) control (Figure 2C-D). The scramble control

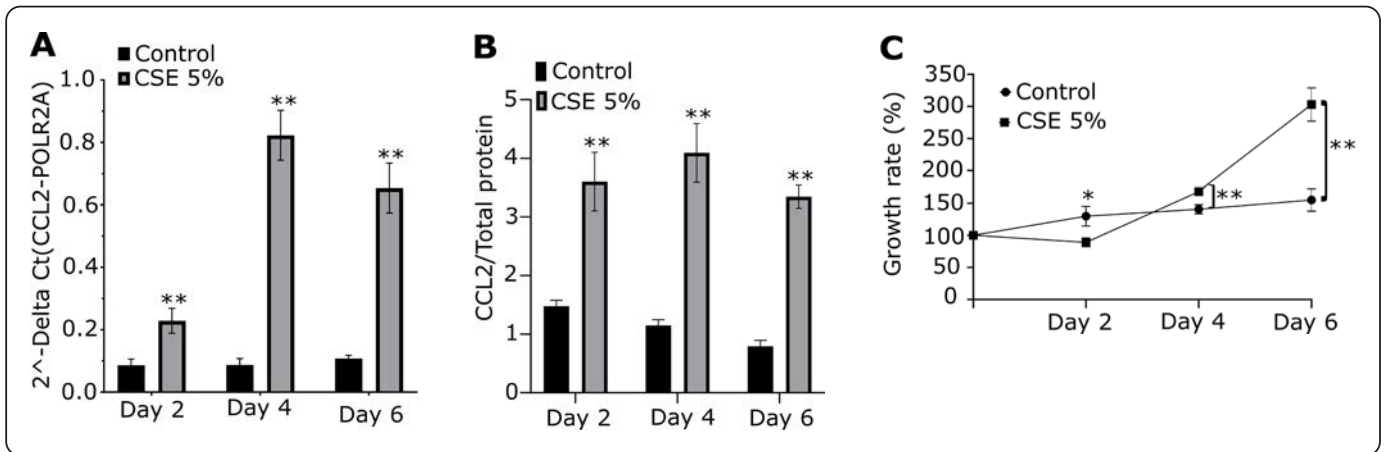


Figure 1. Effects of CSE on CCL2 expression and synthesis in A549 cells. A) Real-time PCR showing CCL2 gene expression in A549 lung epithelial cells stimulated with 5% CSE for 2, 4, and 6 days. B) CCL2 levels in the supernatant were quantified by ELISA. C) The WST-1 assay, in which we evaluated the proliferation of control A549 cells and cells stimulated with 5% CSE (** p<0.01 against the control without treatment).

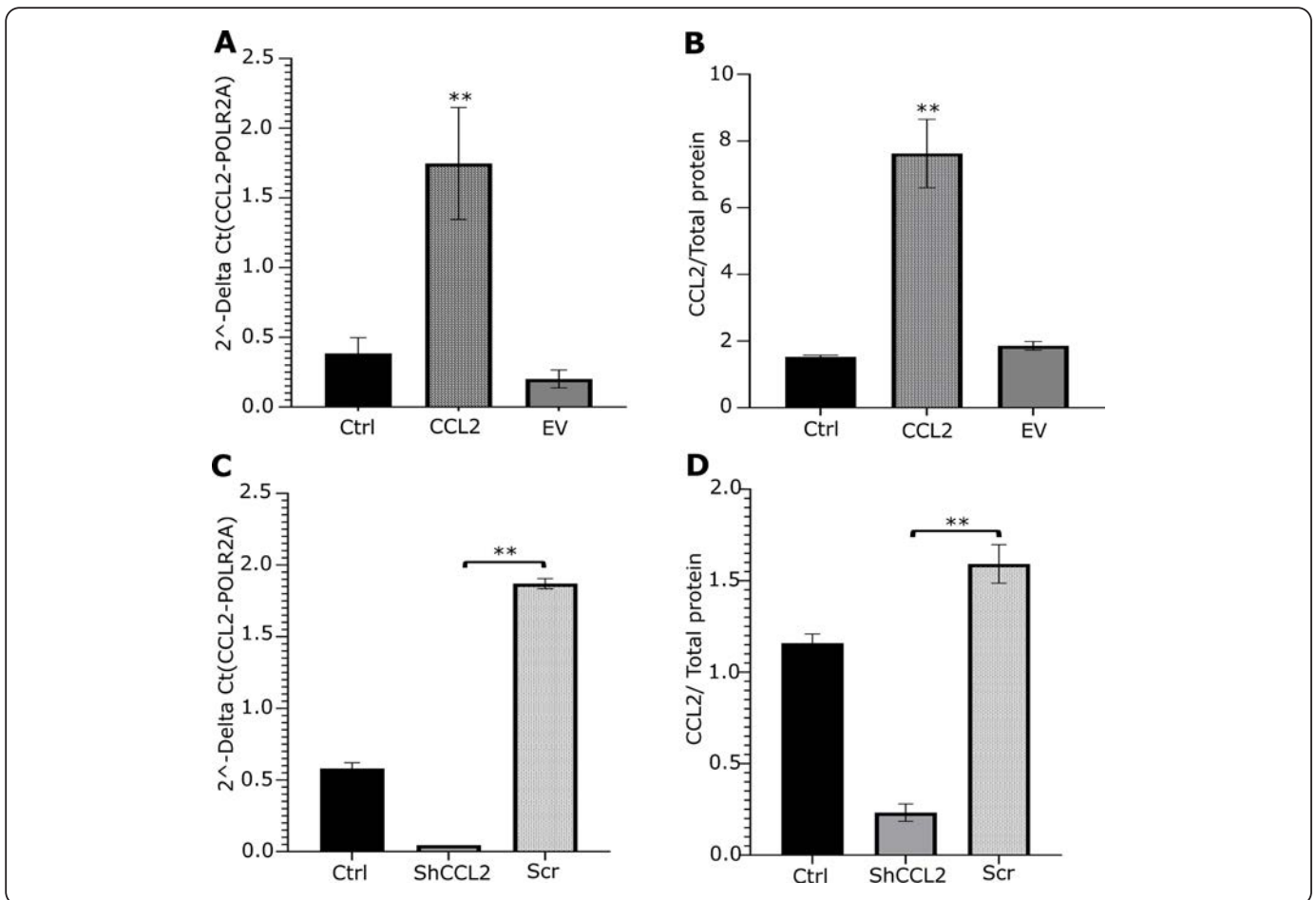


Figure 2. Modulation of CCL2 expression. A) Real-time PCR in cells that overexpress CCL2. B) The content in the supernatant shows the transfection efficiency quantified by ELISA. C) Real-time PCR of expression by CCL2 knockdown A549 cells. D) CCL2 content in CCL2 knockdown A549 cell supernatant. Ctrl: Control; CCL2: Cells that overexpress CCL2; EV: Empty vector; shCCL2: CCL2 knockdown A549 cells; Scr: scramble control (** p<0.01 against no-treatment control).

allows the comparison of knockdown with no transduced cells with a huge variable (virus infection), and this scramble will inevitably induce transcriptional programs that differ from no transduced cells. This explains why scramble cells overexpress the *CCL2* gene and protein synthesis.

Subsequently, the wound assay showed that control cells and cells with an empty vector moved slightly, while overexpression of *CCL2* induced a significant increase in the migration rate: cells reached near the middle of the scratch, indicating that *CCL2*

caused more mesenchymal-like characteristics and markedly promoted A549 cell mobility (Figure 3A-B). Additionally, the wound assay showed that *CCL2* inhibition is associated with the rate of tissue repair being slower.

The expression of *CCL2* affects the proliferation of human AECs in A549 cells

To determine the role of *CCL2* in cell proliferation, an assay with WST-1 was performed where cells that overexpress *CCL2* (or not) were evaluated. These experiments revealed that

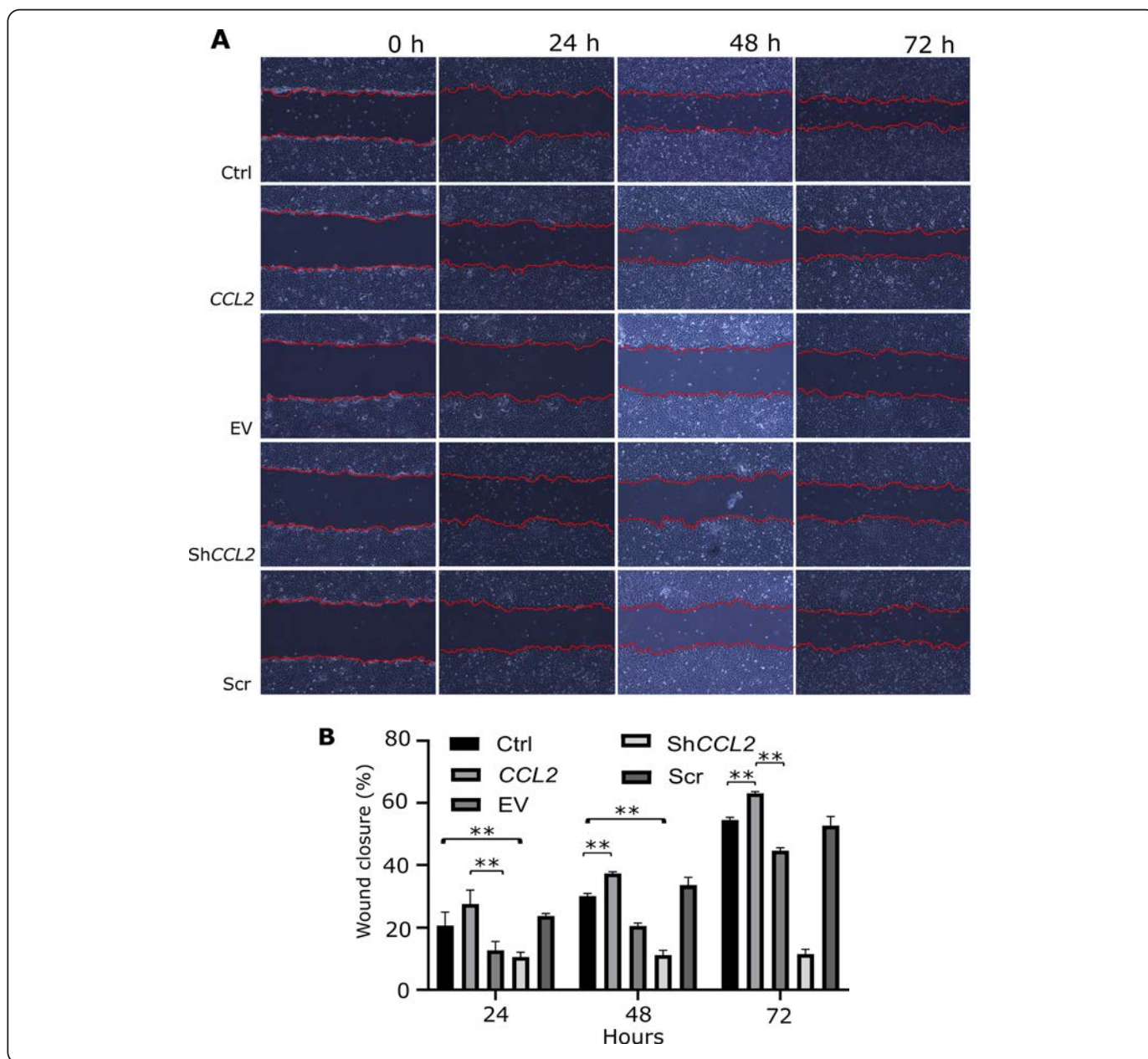


Figure 3. Evaluation of the rate of wound closure in A549 cells. A) Wound-healing assay in A549 cells that overexpress *CCL2* and in *CCL2* knockdown A549 cells. B) Graphical representation of the area covered by cells in the wound assay (** p<0.01).

proliferation is partially mediated by *CCL2*. Figure 4A details the 50% increase in the proliferation of *CCL2*-overexpressing A549 cells, and this effect remained until day 6 (compared to all conditions). For A549 *CCL2* knockdown cells, on day 4,

an increase in cell proliferation was observed compared to control cells and scramble cells, and there was a significant decrease compared to cells that overexpress *CCL2*, which was maintained until day 6.

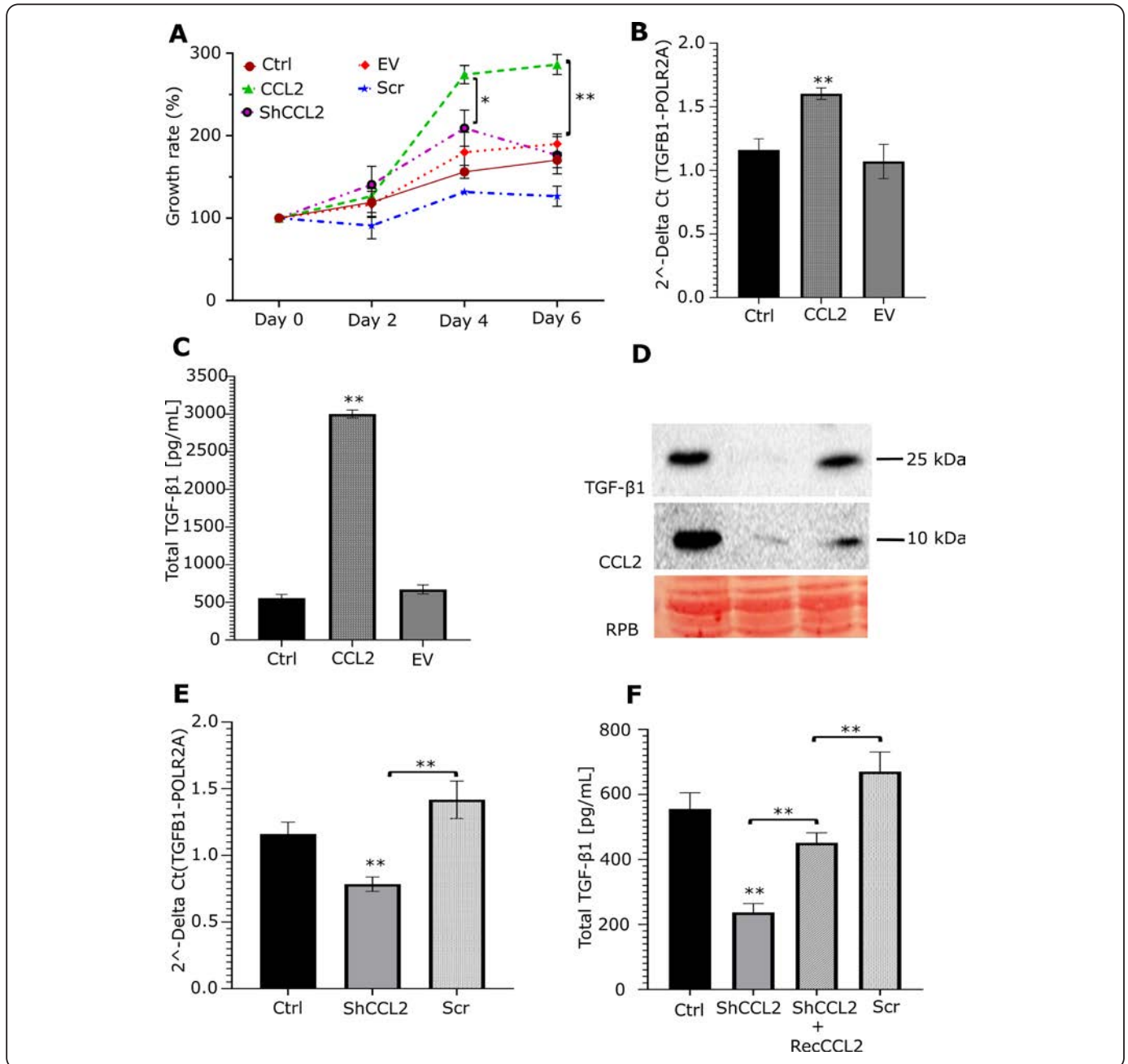


Figure 4. Assessment of proliferation and expression of TGFβ1 in CCL2-transfected A549 cells. **A)** WST-1 assay that shows epithelial cell proliferation. **B)** Real-time PCR showing TGFβ1 expression in A549 cells that overexpress CCL2. **C)** ELISA of total TGF-β1 in A549 cells that overexpress CCL2. **D)** Western blot showing the production of TGF-β1 and CCL2 in conditioned medium of CCL2 knockdown A549 cells; a red ponceau blot (RPB) was used as a loading control. **E)** Real-time PCR denoting *TGFβ1* expression in *CCL2* knockdown A549 cells. **F)** ELISA of TGF-β1 in A549 cells (** p<0.01). Ctrl: Control, *CCL2*: Cells that overexpress *CCL2*; EV: Empty vector; *shCCL2*: *CCL2* knockdown A549 cells; *ShCCL2* + RecCCL2: Addition of recombinant CCL2 protein to *CCL2* knockdown A549 cells; Scr: Scramble cells as a negative control.

CCL2 modulates TGF-β1 production in human AECs

Several studies have identified *CCL2* as a potent profibrotic mediator (Yang *et al.*, 2020); however, it is unknown how it induces these effects. To evaluate this, the expression of TGF-β1 mRNA and protein were measured in *CCL2*-overexpressing A549 cells (Figure 4B-C), and significant increases in its expression and synthesis were registered. A decrease in TGF-β1 protein and mRNA levels in A549 *CCL2* knockdown cell supernatants compared to control cells and scramble was detected (Fig. 4D-E). The ELISA showed that when recombinant *CCL2* protein was added to the culture of *CCL2* knockdown cells, there was a significant induction of TGF-β1 synthesis compared to cells that do not express *CCL2* (Figure 4F).

Conditioned medium from *CCL2*-overexpressing A549 cells modulates the expression of profibrotic genes in pulmonary fibroblasts

Epithelial and interstitial cells are important in the lung microenvironment, particularly fibroblasts, which are involved in the production of extracellular matrix components (Kuhn & McDonald, 1991). Pulmonary fibroblasts stimulated with supernatants of A549 cells stimulated with CSE, or *CCL2*-overexpressing A549 cells showed a significant increase in the expression of *TGFBI* compared with the control and the empty vector (Figure 5A). The *ACTA2* gene, a myofibroblast marker, also indicated marked overexpression under these conditions compared to the control and the empty vector (Figure 5B).

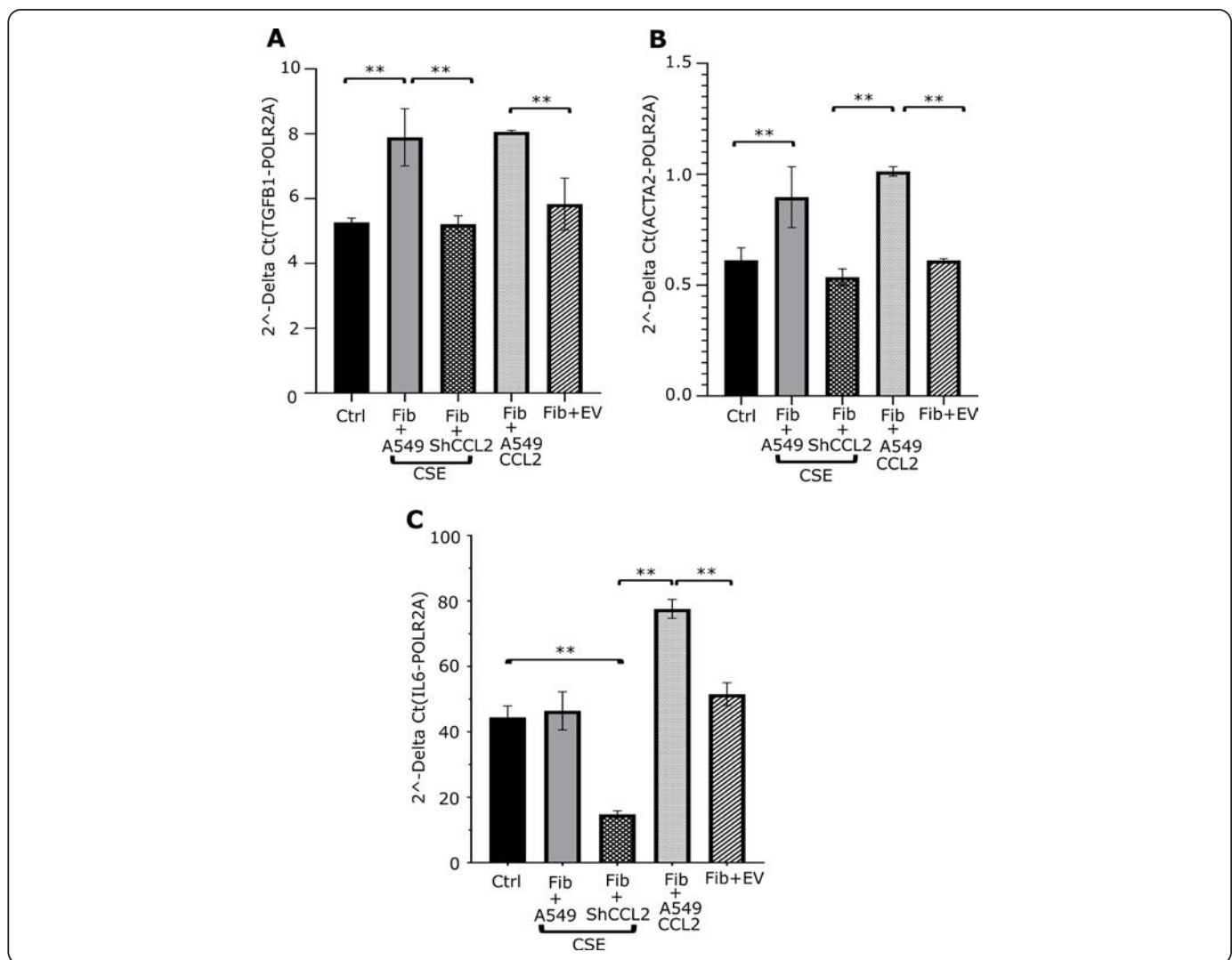


Figure 5. The response of pulmonary fibroblasts to the conditioned medium of A549 epithelial cells exposed to CSE or transfected with *CCL2*. A) Real-time PCR of *TGFBI* in fibroblasts. B) Real-time PCR of *ACTA2* in fibroblasts. C) Real-time PCR of *IL6* in fibroblasts (** p < 0.01). Ctrl: Control cells; Fib + A549: Fibroblasts stimulated with conditioned medium from A549 cells exposed to CSE; Fib + *shCCL2*: Fibroblasts stimulated with conditioned medium from A549 cells that do not express *CCL2* and that were exposed to CSE; Fib + A549 *CCL2*: Fibroblasts stimulated with conditioned medium from A549 cells that overexpress *CCL2*; Fib + EV: Fibroblasts exposed to conditioned medium of cells A549 transfected with the empty vector.

In contrast, on stimulation with the supernatants of A549 *CCL2* knockdown and CSE cells, there was no increase in the expression of *TGFBI* and *ACTA2*. Regarding the expression of *IL6* (Figure 5C), an increase in its expression was observed in fibroblasts stimulated with A549 medium, which overexpress *CCL2*. We also observed a decrease in fibroblasts stimulated with medium from A549 cells that do not express *CCL2*, even when stimulated with CSE.

Pulmonary epithelial cells modulate the migratory fibroblast phenotype through CCL2

To examine the migratory capacity of pulmonary fibroblasts in response to *CCL2* from lung epithelial cells, fibroblasts were treated with a medium from A549 cells that overexpress *CCL2*. Compared with the control and the empty vector, a significant increase in migration was detected (Figure 6). In contrast, anti-*CCL2* antibody treatment caused decreased fibroblast migration, compared to cells stimulated with empty vector medium and control fibroblasts.

DISCUSSION

As mentioned in the introduction, smoking has been strongly associated with IPF pathology. Various studies in human and animal models have shown that *CCL2* plays an important role in the development of fibrosis (Agostini & Gurrieri, 2006; Gharaee-Kermani *et al.*, 1996; Inoshima *et al.*, 2004; Liu *et al.*, 2007; Murray *et al.*, 2008; Stainer *et al.*, 2021; Yang *et al.*, 2020).

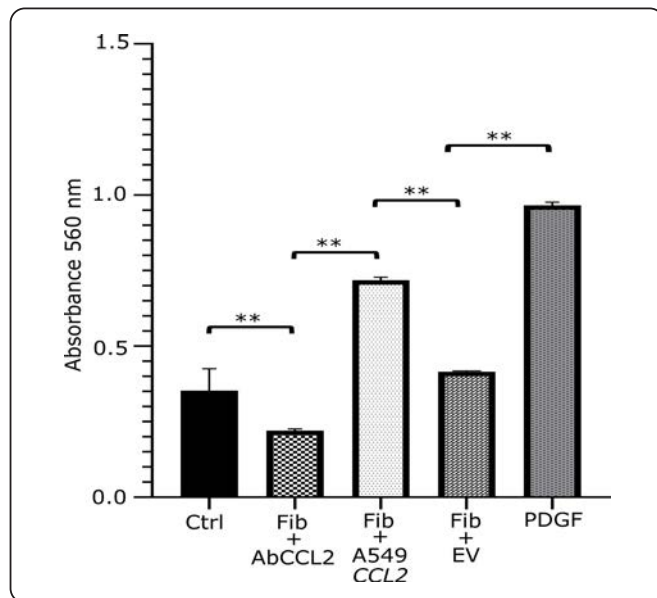


Figure 6. Assay in Boyden chambers in pulmonary fibroblasts (** $p<0.01$). Ctrl: Control cells; Fib + AbCCL2: Fibroblasts stimulated with the antibody against *CCL2*; Fib + A549 *CCL2*: Fibroblasts stimulated with conditioned medium from A549 cells that overexpress *CCL2*; Fib + EV: Fibroblasts exposed to conditioned medium of cells A549 transfected with the empty vector; PDGF: Fibroblasts stimulated with PDGF as positive control.

This is the first study that describes the role of *CCL2* in the proliferation, migration, and expression of *TGFBI* in lung epithelial cells stimulated with CSE, as well as its participation as a modulator in the production of profibrotic mediators and migration in fibroblasts. In our experimental model with CSE-stimulated A549 cells, increased proliferation and migration are associated with the expression of *CCL2*.

The response of type II AECs to injury is a key determinant of the initiation and progression of lung fibrosis in IPF (Zoz, Lawson & Blackwell, 2012). However, the pathways involved in *CCL2* signaling and its profibrotic function are not clear.

In previous studies, it has been reported that *CCL2* increases the proliferation of microglial cells (Hinojosa, Garcia-Bueno, Leza & Madrigal, 2011), and in prostate cancer, it can be done through the PI3K kinase/Akt pathway (Loberg *et al.*, 2006). We showed that cells that overexpress *CCL2* can increase the rate of wound closure, according to Tang and Tsai (Tang & Tsai, 2012); it is known that *CCL2* can induce chondrosarcoma cell migration via Ras/Raf/MEK/ERK/NF- κ B. Additionally, it was determined that *CCL2* induces its effects through TGF- β 1; it is known that regulation between *CCL2* and TGF- β 1 is carried out indirectly, and it can be through the transcription factor NF- κ B (Chompre, Martinez-Orengo, Cruz, Porter & Noel, 2019; Ekert *et al.*, 2011; Gharaee-Kermani *et al.*, 1996; Hinojosa *et al.*, 2011; King *et al.*, 2001; Paccosi, Giachi, Di, Guglielmotti & Parenti, 2016), which induces the expression of *CCL2* (Binder *et al.*, 2009; Deng *et al.*, 2013; Viedt *et al.*, 2002). *CCL2* deficiency in A549 cells was shown to decrease TGF- β 1 synthesis and expression. After stimulating the A549 *CCL2* knockdown cells with recombinant *CCL2* protein, there was a partial recovery in TGF- β 1 production, indicating the existence of an indirect regulation between *CCL2* and TGF- β 1 (Ruigrok, Frijlink, Melgert, Olinga & Hinrichs, 2021).

Epithelial cells and fibroblasts maintain their communication through various growth factors and pro-inflammatory cytokines, such as TGF- β 1, PDGF, *CCL2*/monocyte chemoattractant protein-1, and CXCL12 (Selman & Pardo, 2020).

Furthermore, there is a great diversity of monoclonal antibodies that specifically inhibit some of these cytokines (Sgalla *et al.*, 2020; Van Geffen *et al.*, 2021). In a clinical trial, it was observed that when blocking *CCL2* with a monoclonal antibody (Carlumab) it was observed that serum *CCL2* levels increased in patients from 24 to 52 weeks under treatment with the antibody, which established the hypothesis that there is a compensatory phase of *CCL2* synthesis when it is blocked (Raghu *et al.*, 2015).

In the alveoli, fibroblasts are in close contact with the AECs and are believed to support the maintenance of AEC2 (Shiraishi, *et al.*, 2019). In our experimental model with fibroblasts, we found a significant induction of the *TGFBI* and *ACTA2* genes

(myofibroblast marker) (Nielsen *et al.*, 2019) when stimulated with the medium of A549 cells exposed to CSE, and a similar behavior was observed when fibroblasts were exposed to the conditioned medium of A549 cells that overexpress *CCL2*. This finding is associated with the evidence that fibroblasts derived from IPF patients have a more profibrotic phenotype that includes faster growth and increased expression of *ACTA2*, type I collagen (*COL1A1*), and *TGFBI*, compared to normal fibroblasts (Deng *et al.*, 2013).

In contrast, we observed that stimulating fibroblasts with CSE and conditioned medium from A549 cells that do not express *CCL2* did not induce an increase in the expression of any of these genes. This tells us about a protective effect on fibroblasts, by specifically inhibiting *CCL2* in epithelial cells, which, upon exposure to CSE, do not show induced expression of these genes; this finding also reinforces the idea of Yang *et al.* (Yang *et al.*, 2013) that the protective effect induced by the inhibition of *CCL2* against any inducer of profibrotic damage is through its specific cellular inhibition.

Regarding the expression of *IL6*, an increase in its expression was seen, and it is known that there is a positive feedback loop between *IL6* and *CCL2* (Heinrich *et al.*, 2003; Moodley *et al.*, 2003; Roca, Varcos, Sud, Craig & Pienta, 2009); however, stimulation of fibroblasts with the medium of cells that do not express *CCL2* did not increase the expression of *IL6*, even when stimulated with CSE. These results confirm that there is a regulation between *CCL2*, *TGFBI*, *IL6*, and *ACTA2* that is responsible for inducing a profibrotic environment within the lung.

Finally, we observed a significant increase in the migration rate when we stimulated lung cells with medium from A549 cells that overexpress *CCL2*. This reinforces the idea that *CCL2* is an important recruiter of various cell types, such as fibrocytes (Ekert *et al.*, 2011), fibrotic and normal macrophages (Nakatsumi, Matsumoto & Nakayama, 2017), fibroblasts, and epithelial cells, since adding the recombinant antibody against *CCL2* significantly reduced the migration rate.

CONCLUSIONS

According to the findings and evidence obtained in the present work, we can establish that *CCL2* is an important inducer of proliferation once pulmonary epithelial cells have been stimulated with CSE, and it is an inducer of migration which can recruit more epithelial cells to the site of damage; cells in close contact with fibroblasts can induce them to acquire a profibrotic and invasive phenotype. *CCL2* can be an important orchestrator in the turnover of the extracellular matrix, due to all the intermediate molecules that it induces in pulmonary fibroblasts, and that participate in such a process, such as TGF- β 1, α -SMA, and *IL6*. For this reason, it is relevant to consider *CCL2* as a potential therapeutic target for the treatment of IPF.

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