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-β1. 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.
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 inuction 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; Antoniades 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 by 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 β1 (TGF-β1) in pulmonary fibroblasts (Murray et al., 2008). CCL2 has been shown to induce collagen synthesis and TGFβ1 expression in pulmonary fibroblasts (Gharaei-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 microenvironment 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 TGFβ1, 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-β1. 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×) (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-μm-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 × 10⁷ cells in T75 culture flasks (Merc 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 µL) in a 3:1 ratio with DNA. Cells were incubated with antibiotic G418 (Merck KGaA; 800 µg/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 µg/mL) and MCP-1 shRNA-(h) lentiviral particles (Santa Cruz Biotechnology; 15 µL), 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 µg/µl). Serial dilutions were made to obtain stable clones that were selected with puromycin (Santa Cruz Biotechnology; 7.5 µg/mL). Plasmid-A (Santa Cruz Biotechnology; 0.1 µg/µl) 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 (1x10^5) 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 µg 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 TGFB1 (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-µL volume containing 2 µl of cDNA and 13 µl of 2× Mastermix (Thermo Fisher Scientific). The PCR conditions were 2 minutes at 94 °C and 60 °C for 1 minute. The results are expressed as means ± SD of the 2^−ΔΔT (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 1×10^5 cells/well were incubated in 96-well microplates in a final culture medium volume of 100 µL/well. Cells were incubated for 6 days with CSE, at 37 °C and 5% CO2. An amount of 10 µL/well of WST-1 was added and incubated for 1 hour. The absorbance was analyzed in en 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 (1×10^5 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 µL micropipette tips to form straight lines. The floating cells were removed using 1× PBS three times. To further reduce the risk of cell proliferation confounding the study of migration, mitomycin C (Merck KGaA; 3 µg/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=Δh)/A_t=0h] × 100%) according to Ayman et al (Grada, Otero-Vinas, Prieto-Castrillo, Obagi & Falanga, 2017).
ELISA
Protein levels of CCL2 and TGF-β1 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 µL 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-µL 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-β1 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 µg. 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 increase in the expression and production of cytokines and growth factors. For this reason, we decided to evaluate if the expression of CCL2 is increased when stimulated with CSE.

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 ± 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
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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 TGFB1 in CCL2-transfected A549 cells. A) WST-1 assay that shows epithelial cell proliferation. B) Real-time PCR showing TGFB1 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 TGFB1 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](image-url)

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.
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; Gharaei-Kermani et al., 1996; Inoshima et al., 2004; Liu et al., 2007; Murray et al., 2008; Stainer et al., 2021; Yang et al., 2020).

In contrast, on stimulation with the supernatants of A549 CCL2 knockdown and CSE cells, there was no increase in the expression of TGFB1 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.

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 & Madrignal, 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-κB. Additionally, it was determined that CCL2 induces its effects through TGFB-β1; it is known that regulation between CCL2 and TGFB-β1 is carried out indirectly, and it can be through the transcription factor NF-κB (Chompre, Martinez-Orrego, Cruz, Porter & Noel, 2019; Ektet al., 2011; Gharaei-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 TGFB-β1 synthesis and expression. After stimulating the A549 CCL2 knockdown cells with recombinant CCL2 protein, there was a partial recovery in TGFB-β1 production, indicating the existence of an indirect regulation between CCL2 and TGFB-β1 (Ruigrok, Frijlink, Melger, Olinga & Hinrichs, 2021).

Epithelial cells and fibroblasts maintain their communication through various growth factors and pro-inflammatory cytokines, such as TGFB-β1, PDGF, CCL2/monocyte chemotactic 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 TGFB1 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 TGFβ1, 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, TGFβ1, 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-β1, 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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