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# Homeostatic chemokines in Idiopathic Pulmonary Fibrosis: Novel therapeutic candidates?

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## Abstract:

Idiopathic Pulmonary Fibrosis (IPF) is a fatal chronic Interstitial Lung Disease (ILD) affecting mechanical functions and gas exchange, caused by an increased fibroblast activity and collagen deposition that compromise the alveolar-capillary barrier. Identifying an effective therapy for IPF remains a clinical challenge. Chemokines are key proteins for cell communication with biological functions in immunity, tissue homeostasis, or damage, and repair. Chemokine receptor signaling induces lung resident cell activation and proliferation, including alveolar macrophages (AMs) and fibroblasts. AMs are an important chemokine and cytokine source during IPF. Here, we highlight the complexity of this system, and based on insights from genetic and transcriptomic studies, we propose a new role for homeostatic chemokines imbalance in IPF, with implications for putative therapeutic targets.

**Keywords:** Chemokines, Mucosal Immunology, Interstitial Lung Disease, Idiopathic Pulmonary Fibrosis, Therapeutic Targets.

## Glossary:

- **Adenylate Cyclase:** is a regulatory cytosolic enzyme present in almost every cell that generates the second messenger cyclic AMP.
- **Alarmins:** Alarmins are multifunctional endogenous molecules that are sensed by the host immune system and contribute to host defense.
- **Chemokines:** Chemokines are signaling proteins secreted by cells that induce directional movement of leukocytes during homeostasis and immunity.
- **Cyclic AMP:** Cyclic adenosine monophosphate is a second messenger or cellular signal important in many cell biological processes.
- **Cytokines:** Cytokines are a group of signaling molecules produced by cells that control the immune system and diseases.
- **ECM:** Extracellular Matrix is a network consisting of extracellular macromolecules and minerals, such as collagen, enzymes, and glycoproteins, that provide structural and biochemical support to surrounding cells and tissue organization.
- **FGF:** Fibroblast Growth Factor is a family of cell signaling proteins produced by macrophages.
- **Fibrocytes:** are hematopoietic-derived cells that directly contribute to tissue fibroblast pool and ECM producer following injury, and during disease, produce connective tissue proteins such as collagens.
- **GPCRs:** G protein-coupled receptors are heptahelical seven transmembrane domain receptors that form a large group of evolutionarily related proteins that detect molecules outside the cell.
- **iBALT:** Induced Bronchus-Associated Lymphoid Tissue is a mucosal lymphoid organ in the lungs.
- **Inositol triphosphate (IP3):** IP3 is a second messenger molecule used in biological cell signal transduction.
- **Interstitial Lung Disease (ILD):** Interstitial lung disease is the name for a lung disorder that affects a part of the lungs called the interstitium, where the gas exchange occurs.
- **Intracellular Calcium:** Calcium signaling is the use of calcium ions ( $\text{Ca}^{2+}$ ) to communicate and drive intracellular processes.
- **MS4A6A:** A class of four transmembrane-spanning proteins, a member of the CD20-like family.
- **NF- $\kappa$ B:** Nuclear factor kappa-light-chain-enhancer of activated B cells is a protein complex that controls the transcription of DNA, cytokine production, and cell survival.
- **NOTCH3:** Neurogenic locus notch homolog protein 3 plays a critical role in maintaining the phenotypic stability of vascular smooth muscle cells.
- **NLRP6:** NLR family pyrin domain containing 6 is an intracellular pattern recognition receptor (PRR) that plays a role in the immune system.
- **Phospholipase-C $\beta$ :** is a class of membrane-associated enzymes that cleave phospholipids just before the phosphate group, which play an essential role in eukaryotic cell signal transduction pathways.
- **PI3K:** Phosphoinositide 3-kinases are a family of enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking.

- **RANKL:** Receptor Activator for Nuclear Factor  $\kappa$  B Ligand is a member of the tumor necrosis factor (TNF) cytokine family that regulates osteoclast differentiation and activation.
- **TLR9:** This is an endosomal receptor that detects viral and bacterial CpG DNA and self-DNA.
- **VEGF:** Vascular endothelial growth factor is a potent angiogenic factor and is described as an essential growth factor for vascular endothelium.

## **Chemokines in the context of Idiopathic Pulmonary Fibrosis (IPF).**

Tissue repair is a protective response after injury, replacing inflammation by tissue healing with regenerative potential that restores homeostasis[1, 2]. While fibrosis repairs the chronic injury, it may also lead to tissue degeneration and dysfunction. Functional repair occurs when connective tissue replaces damaged tissues, leading to excessive extracellular matrix (ECM) deposition and abnormal tissue architecture[3]. Fibrosis is an orchestrated process determined by sequences of molecular and cellular mechanisms. The persistent tissue injury and parenchymal cell death provoke soluble mediators release (alarmins, cytokines, chemokines), activation of resident macrophages and chronic inflammation[4-6]. This complex molecular and cellular signaling activates collagen-producing mesenchymal cells (pericytes, myofibroblasts, fibrocytes)[4-6] contributing to excessive scarring. Pulmonary fibrosis belongs to Interstitial Lung Disease (ILD), lung disorder with similar clinical manifestation, classified by pathological and radiographic changes with varying degrees of morbidity[7, 8]. Among them, Idiopathic Pulmonary Fibrosis (IPF), a chronic lethal ILD of unknown origin and ineffective therapy, is characterized by a fibroproliferative nature and excessive extracellular matrix deposition in the pulmonary parenchyma[8-12]. The clinical signs of IPF include worsening dyspnea, progressive loss of lung volumes, and abnormal gas exchange[13-15], leading to respiratory collapse[9-12]. IPF has an average prevalence of 30 per 100,000 persons, with an average incidence of 34,000 new cases annually in the United States[8, 16]. Generally, the death associated with IPF is due to respiratory collapse by progressive loss of lung function, with an average survival of 3 to 5 years post- diagnosis[8, 12, 13].

The chemokine system comprises a complex network that orchestrates various functions in diverse cell populations, maintaining physiological homeostasis and pathology[17, 18], regulating pulmonary pathologies, such as acute lung injury and infection[19-23], asthma[24], and pulmonary fibrosis[25, 26]. Chemokines control fibrogenesis, leukocyte and fibrocyte influx, angiogenesis, alternative macrophage, and fibroblast collagen production, that sustain IPF development[3, 27]. The elucidation of chemokine-related mechanisms that orchestrate the aberrant tissue repair allows novel pharmacological strategies to treat IPF. Insights derived from recent genetic and transcriptomic studies have given a better interpretation of the molecular complexity of IPF. Here, we provide an update on the emerging cellular and molecular mechanisms involving the AMs and chemokine system in the onset of IPF, and propose that homeostatic chemokines sustain IPF disease, opening new putative therapeutic opportunities for IPF.

## Physiological importance of the chemokine system in health and disease.

The chemokines are “chemoattractant cytokines”[28] governing inflammation, primarily by recruitment and activation of leukocytes into inflammatory sites[29-33], but also angiogenesis[34], resolution of inflammation, and tissue repair[3, 31, 35]. Chemokines are small molecules (8-12 kDa) essential in leukocyte biology, controlling cell communication and activation during basal or inflammatory states orchestrating the immune response[28, 32, 36]. Epithelial, endothelial, fibroblasts, and smooth muscle cells can produce and respond to chemokines[36, 37]. Physiological properties are attributed to chemokine activity on their respective receptors in cell subsets (leukocyte recirculation, lymph node organization, and embryogenesis). Chemokines and receptors contribute to antigen presentation, chronic inflammation, vascular remodeling, fibrogenesis, and tumorigenesis[28, 32, 33, 36-39]. Thus, chemokines emerge as pleiotropic factors involved with homeostasis and disease[31, 32, 34, 37, 39].

The chemokine system is complex in vertebrates and mammals. In humans it comprises about 50 ligands (homeostatic, constitutively expressed or inflammatory, induced by stimulus) acting potentially on 20 receptors (**Table-1**) belonging to class A of Rhodopsin-like G-Protein-Coupled Receptors (GPCRs) with seven transmembrane domains[29, 33, 38]. Chemokines are structurally related and classified according to the position of conserved cysteine residues in the protein amino-acid sequence. CC-chemokines have the first two cysteines adjacent[33, 38]. CXC-chemokines have the first two cysteines separated by a single amino acid. Some CXC-chemokines contain a motif immediately before the first amino-terminal cysteine, called ELR motif (Glutamate-Leucine-Arginine), divided in chemokines CXC-ELR<sup>-</sup> and CXC-ELR<sup>+</sup>[33, 38]. The CX3C and C chemokines are characterized by having the first two cysteines separated by three amino acids and the absence of the first and third cysteine, respectively[30-33]. The chemokine receptors are broadly distributed on various cell subtypes, leukocytes, and non-leukocytes. The specific combination of cellular chemokine receptor repertoire confers migration properties on these cells by chemokine gradient, contributing to an alteration of receptor structure and signaling that direct cells during the diverse stages of leukocyte maturation and activation[29, 33, 37, 38].

Chemokine receptors are distributed in lipid rafts on the membrane, acting as extracellular sensors that dynamically transmit intracellular signals[33, 38]. Chemokines are promiscuous in binding to receptors but with non-redundant signaling pathways and activities[33]. One receptor interacts with multiple chemokines, and one chemokine interacts with more than one receptor[33, 36, 38]. Currently, 28 CC-chemokines (CCL1-28), 17 CXC-chemokines (CXCL1-17), 2 C-chemokines (XCL1-2) and 1 CX3C-chemokine (CX3CL1) are known, which interact with 10 CCR (CCR1-10), 6 CXCR (CXCR1-6), 1 XCR (XCR1), and 1 CX3CR (CX3CR1) chemokine receptors (**Table-1**)[33, 37, 38]. The signaling mediated by the chemokine-receptor complex causes receptor conformational

change and endocytosis, triggering intracellular signaling[33, 38]. The dissociation of the subunits  $G\alpha$  and  $G\beta\gamma$  activates adenylate cyclase, phospholipase- $C\beta$ , PI3K, and small GTPases, increasing AMP cyclic (adenosine monophosphate), inositol triphosphate (IP3), and intracellular calcium, causing cell activation[32, 33, 38, 40]. Chemokine receptor activation promotes actin filament polymerization and cell polarization, enhancing phagocytic capacity and cell survival by translocating NF- $\kappa$ B and *de novo* transcription of chemokines and receptors[29-31]. Chemokine-receptor interactions alter cytoplasmic organelles and vacuoles, needed for (i) recycling of chemokine receptors and (ii) degradation of chemokines and their receptors, important in homeostasis and pathology[31, 33, 38, 41, 42].

The chemokine system also features atypical chemokine receptors (ACKRs), functionally classified as scavengers, presenting promiscuous binding[31, 33, 38, 41-45]. ACKRs are structurally identical to other GPCRs, however, while using an alternative activation pathway as a consequence of changes in the sequence motif DRYLAIV[31, 38, 41-44]. ACKRs act as decoy receptors, bind to chemokines, sequestering and degrading, and decreasing the local or systemic levels. ACKRs compete with conventional chemokine receptors as a negative regulator of inflammatory chemokines[38, 46-48]. Six ACKRs have been described (**Table-1**)[33, 38, 43, 44]. ACKR1 binds to CC and CXC inflammatory chemokines and is found in erythrocytes. ACKR1 involves chemokine transport and movement in vascular endothelium, mediating leukocyte trans-endothelial migration[38, 43, 44, 46, 49]. ACKR2 recognizes inflammatory CC and CXC chemokines. ACKR2 is distributed on the lymphatic endothelial cells, controlling leukocyte influx into the lymph node. ACKR2 is expressed in mucosal sites (skin, lungs, intestine) and leukocytes[31, 38, 44, 46, 47, 50-54]. Unlike ACKR1 and ACKR2, the ACKR3 and ACKR4 are scavenger receptors of homeostatic CXC and CC chemokines[33, 38, 43, 44, 55]. ACKR5 and ACKR6 were recently added to the ACKR group, but their roles are still unclear[38, 43, 44]. Chemokine receptors and ACKRs might have roles in a specific disease[38, 43, 44, 46, 49, 56] tuning the tissue homeostasis and immune response, however only ACKR2 was investigated in context of pulmonary fibrosis[57].

### **Homeostatic chemokines in IPF: New insights from transcriptional data.**

Insights derived from genetics and transcriptomics have given a better appreciation of the molecular complexity involved in IPF. Microarray studies revealed several chemokines and receptors up-regulated in IPF samples: 21 genes in GSE32537, 14 genes in GSE47460, and 24 genes in GSE53845 (**Table-2**), as illustrated in **Figure-1A-B**. These datasets converge in showing the up-regulation of homeostatic and inflammatory chemokines such as CCL11, CCL13, CCL19, CCL21, CXCL6, CXCL12, CXCL13, CXCL17, or CCR7 (**Figure-1C**). On the other hand, RNA sequencing (RNAseq) analysis from IPF lung tissue samples revealed 33 up-regulated transcripts in GSE99621

and 19 in GSE92592 related to the chemokine system (**Table-2**), as illustrated in **Figure-1A-B**. Both RNAseq studies indicate up-regulated homeostatic and inflammatory chemokines such as CCL7, CCL13, CCL18, CCL19, CCL21, CCL22, CXCL12, CXCL13, CXCL14, CCR7, CCR8, and XCR1 in IPF lung tissue (illustrated in **Figure-1D**). The 3 microarrays and 2 RNAseqs from IPF lung tissue samples (GSE32537, GSE47460, GSE53845, GSE99621 and GSE92592) pointed to 5 shared, up-regulated transcripts, namely for CCL13, CCL19, CXCL12 and CXCL13, and CCR7 (summarized in **Figure-1E**). From these, 4 chemokines are classically classified as homeostatic chemokines[17, 38] and CCL13 as inflammatory[38], thus, suggesting that IPF could be caused by abnormal homeostatic chemokine expression in the lungs.

### **Chemokine system and IPF: AMs as a central player in fibrosis.**

Lung macrophages are the most abundant innate immune cells in the lungs, with essential roles in tissue homeostasis, host defense, and immune regulation[58]. Alveolar macrophages (AMs), including tissue-resident AMs (TR-AMs), monocyte-derived AMs (Mo-AMs), and interstitial macrophages (IMs), are the major macrophage populations in the lungs[59] in steady-state and pathology[58]. They are defined by their origins and immunological milieu, which endow these cells with distinct features and plasticity[58-60]. It has been suggested that various cells contribute to fibrotic processes[6], but in particular, macrophages may be a pivotal regulator of fibrogenesis during IPF[61-63]. RNAseq of BAL-derived AMs from IPF patients (GSE79544) revealed a distinct chemokine signature, with 8 up-regulated transcripts for CCL8, CCL13, CCL24, CXCL10, CXCL14, XCL2, CCR8 and ACKR1 (as illustrated in **Figure-1A-B/Figure-2/Table-2**). Therefore, AMs could be an essential chemokine source contributing to pulmonary fibrogenesis. RNAseq from lung tissue and BAL-derived AMs from IPF (GSE99621, GSE92592, and GSE79544) commonly expressed 3 up-regulated transcripts, the homeostatic chemokines CCL13 and CXCL14[17, 38], and CCR8 receptor (**Figure-1F**), expressed mainly by monocytes and macrophages[38]. Only CCL13 was up-regulated and conserved in all datasets evaluated (**Figure-1/Table-2**), pointing to CCL13 as a shared IPF chemokine signature in the samples analyzed.

AMs from IPF showed a distinct chemokine signature and may sustain a "hot fibrosis" state in a fibrogenic environment[2], supporting macrophage-myofibroblast molecular interaction. In particular, transcripts of BAL-derived AMs from patients with IPF (GSE79544) revealed the specific contribution of AMs pathways for interpreting the microenvironment and actively sourcing chemokines and cytokines (**Figure-2**), thus as key actors for sensing tissue stimulus (chemokines and cytokines) and stress that trigger inflammatory and fibrogenic processes (**Figure-3, Key Figure**). AMs can potentially control lung chemokine levels by producing (CCL8, CCL13, CCL24, CXCL10, CXCL14, XCL2) and scavenging (ACKR1), while CCR8 may contribute to AMs residency (**Figure-**



2). Cytokine receptors found in BAL-derived AMs (IL7R, IL-12RB1, IL18RAP, and FGFR1) suggest that AMs from IPF can potentially respond to an inflammatory stimulus (IL-7, IL-12, IL-18, FGF) derived from tissue milieu (**Figure-3**). Moreover, BAL-derived AMs from IPF could sense the tissue stress by receptors (NOTCH3, MS4A6A, CD24, CD36, CD69, TLR9, NLRP6) (**Figure-3/Box-1**). Despite the contribution of AMs triggering the inflammatory and fibrogenic process, cytokines (IL-9, IL-34, IL-38, RANKL, VEGF) are related to several phenomena, such as lung inflammation, angiogenesis, and fibrogenesis in IPF (**Figure-3/Box-1**). Therefore, AMs could sense the tissue microenvironment but exert regulatory functions by cytokine production, chemokine source and scavenging, tuning fibrogenic pathways during IPF.

### **Chemokine system and IPF: Targeting therapeutic potential of chemokine-receptor axis.**

Chemokine organizes lung immune response and plays a critical role in activating fibroblasts, governing IPF manifestation. Recent findings described that therapy directed to GPCRs[64] and chemokines may be a novel approach for IPF treatment. Based on what has been shown here, we highlighted a repertoire of chemokines involved in IPF with **therapeutic potential**:

- **CCL1/CCL18-CCR8 axis:** The inflammatory chemokine CCL1 binds to CCR8, related to the Th2 immune response[33, 38]. CCL18 binds to ACKR6 and CCR8, related to DC chemoattraction of T and B cells[33, 38]. CCL18 has a predictive value in IPF and is helpful for the clinical management of IPF patients[65, 66]. CCL18 genetic variation influences the survival of IPF patients[66, 67]. AMs and myofibroblasts derived from fibrocytes are sources of CCL18 in fibrotic lungs[68, 69], mediating lung fibroblast collagen production[70-73]. Activation of CCR8<sup>+</sup> AMs may increase the ability to perpetuate the fibrogenesis (**Figure-3**). Thus, CCR8 becomes therapeutic and attractive for IPF. Therefore, future studies may clarify the role of the CCL1/CCL18-CCR8 axis in IPF. While some CCR8 antagonists are available, no clinical trials in IPF have been reported yet[38].
- **CCL8-CCR2 axis:** The inflammatory chemokine CCL8 binds to CCR2, which is related to Th2 immune response[33, 38]. CCL8 is expressed by fibroblasts derived from IPF, and elevated BAL CCL8 is correlated to shorter survival[74]. CCL8 activates CCR2<sup>+</sup> macrophages and fibrocytes/fibroblasts, contributing to collagen deposition[75]. CCL8 was found in AMs from IPF (**Figure-2**). However, the precise mechanism of IPF is still unknown. Therefore, CCL8 may be an alternative putative target related to CCR2 activation since administration of the humanized anti-CCL2 antibody Carlumab worsened the progression of IPF[76].
- **CCL13-CCR2:** The inflammatory chemokine CCL13 binds to CCR2, which is related to Th2 immune response[33, 38]. CCL13 was increased in all datasets (**Figure-2**), however, it is

poorly studied in IPF. Blood circulating CCL13 levels is a prognostic biomarker for progression outcomes in IPF patients treated with pirfenidone[77].

- **CCL19-CCR7 axis:** The homeostatic chemokines CCL19 and CCL21 bind to CCR7, related to T cells and DC homing to lymph nodes[33, 38]. Alveolar epithelial, endothelial cells and fibroblasts express CCL19 in IPF lung samples[78]. The lymphatic endothelium is the source of CCL19 and CCR7, and CCR7 is also expressed by CD68<sup>+</sup> macrophages, suggesting that the CCL19-CCR7 axis is involved with lymphangiogenesis in IPF[79]. The CCR7 receptor is expressed by fibroblasts, showing activation and chemotactic activities induced by CCL21[80]. Lymphatic endothelial cells and lymphocytes in lymphoid follicles also express CCL21 in IPF[78]. CCL19 and CCR7 are homeostatic chemokines, however, they are up-regulated in IPF lung samples (**Figure-1**). Therefore, CCL19 and CCR7 may represent critical putative pharmacological targets for their activities in IPF, while sparing their homeostatic functions. Future studies will determine the potential of therapy for IPF based on the delicate balance of the CCL19-CCR7 axis. However, antagonists have not yet been identified for CCR7[38].
- **CCL24-CCR3 axis:** The inflammatory chemokines CCL11, CCL24, and CCL26 bind to CCR3, related to eosinophil trafficking and Th2 immune response[33, 38]. CCL11 has a selective profibrogenic effect on CCR3<sup>+</sup> lung fibroblasts, enhancing migration[81]. The role of CCL24 and CCL26 in IPF is unclear. CCL24 stimulates human lung fibroblasts to proliferate and synthesize collagen[82], however CCL26 promotes chemotaxis[82]. CCL24 was increased in IPF AMs (**Figure-2**). CCL24 inhibition can be useful for IPF. The CCR3 available inhibitors are antagonists, monoclonal antibodies, and antisense oligonucleotides. While some already entered clinical trials, such as the neutralizing monoclonal anti-CCR3 (ASM8/Pharmaxis), CCR3 antagonist (GSK766994/GlaxoSmithKline), CCR3 antagonist (DPC168/Dupont), or CCR3 antagonist (BMS-639623/BMS)[38], none of them has been tested for IPF as yet.
- **CXCL10-CXCR3 axis:** The inflammatory chemokine CXCL10 binds to CXCR3, related to the Th1 immune response and negative regulator of angiogenesis[33, 38]. CXCL10-CXCR3 axis promotes the sIL-13R $\alpha$ 2 decoy receptor expression by pulmonary fibroblasts[83] tuning ECM production. CXCL10 was found in AMs from IPF (**Figure-2**). Lymphocytes express CXCR3, and CXCL10 contributes to leukocyte influx, leading to tissue damage and scarring. CXCR3 antagonist (AMG487) inhibited leukocyte infiltration into the lungs in bleomycin-induced inflammation in mice[84]. Several synthetic CXCR3-specific small-molecule antagonists that show efficacy in humans have been developed, and future studies are needed to evaluate the CXCL10-CXCR3 axis in IPF.

- **CXCL12-CXCR4 axis:** The homeostatic chemokine CXCL12 binds to CXCR4, related to lymphopoiesis and hematopoietic progenitor cell circulation/homing[33, 38]. CXCR4 drives tissue fibrosis through CXCL12 in IPF. CXCL12 is higher in IPF patients, and increased CXCR4 was detected in lung epithelial or myeloid cells from IPF and other fibrotic ILDs[85]. CXCR4 is found in vessels close to fibrotic areas in IPF lungs[78, 86]. CXCR4 is expressed by epithelial cells and macrophages in areas with intense fibrotic remodeling [87]. In IPF lungs, the CXCL12 is produced by alveolar epithelial[88], hyperplastic epithelial cells, and fibroblasts in fibroblastic foci[78]. A negative correlation of plasma CXCL12 and lung diffusing capacity for carbon monoxide (DLCO) in IPF[88]. CXCL12 is up-regulated in IPF lung samples (**Figure-1**). Circulating fibrocytes increase the fibroblast pool and tissue fibrosis in patients with IPF. A positive correlation between the number of fibroblastic foci and the amount of CXCR4<sup>+</sup> lung fibrocytes is also related to increased blood CXCL12[88]. Fibrocytes are increased in the circulation of patients with IPF and express CXCR4, indicating that fibroblast and myofibroblast precursors can be recruited from the circulation to the focus of fibrosis[88], contributing to IPF progression. The treatment with AMD3100, a CXCR4 antagonist, reduced lung collagen, leukocyte, and fibrocyte infiltration[89-91]. However, CXCR4 antagonists have not yet been approved for IPF.
- **CXCL13-CXCR5 axis:** The homeostatic chemokine CXCL13 binds to CXCR5, which is involved in B and T-cell trafficking in lymphoid tissues[33, 38]. CXCL13 is produced by CD68<sup>+</sup> and CD206<sup>+</sup> AMs from patients with IPF[92]. In IPF, moderate or high levels of CXCL13 blood correlated with annual changes of forced vital capacity (FVC), the diffusion lung capacity for carbon monoxide (DLCO), the total lung capacity (TLC), suggesting that CXCL13 may be a predictive biomarker for the outcomes of IPF patients[89]. CXCL13 mediates the CXCR5<sup>+</sup> B-cell[93] and the CXCR5<sup>+</sup> T Follicular Helper (Tfh) cell[94] influx implicated in IPF pathogenesis. As highlighted here, CXCL13 is a homeostatic chemokine and its transcription is induced in pulmonary samples from IPF patients (**Figure-1**). CXCL13 is involved in CXCR5<sup>+</sup> expressing lymphocyte recirculation, maintaining chronic inflammation implicated with the IPF pathophysiology. Therefore, the CXCL13-CXCR5 axis becomes an essential and attractive putative pharmacological target for IPF, but no CXCR5 antagonists have been developed yet[38].
- **CXCL14:** CXCL14 is related to macrophage migration, but its receptor is still unknown[33, 38]. The role of CXCL14 in the context of IPF is unclear. CXCL14 was observed in Type II alveolar epithelial cells, fibroblast foci, and macrophages in IPF lungs. CXCL14 BAL levels correlated with increased macrophages in IPF patients, and serum levels correlated with spirometry parameters, suggesting that CXCL14 may participate in the progression of

IPF[95]. CXCL14 is found in IPF lungs and expressed in regions on the periphery of fibrotic foci and dense fibrosis, and CXCL14 may recruit and activate  $\alpha$ -SMA<sup>+</sup> cells [96]. Transcriptomic microarray and RNAseq analysis revealed that CXCL14 had a substantial differential gene expression in IPF lung[27, 96, 97]. CXCL14 was found in AMs from IPF (**Figure-2**). Circulating CXCL14 protein levels are higher in plasma from IPF patients than controls[98], and CXCL14 may represent a systemic biomarker to identify IPF patients.

- **XCL2-XCR1:** The homeostatic chemokines XCL1 and XCL2 bind to XCR1, related to antigen cross-presentation by CD8<sup>+</sup> DCs[33, 38]. XCL1 is lowly expressed in the lungs of humans[99], but their role in IPF remains unexplored. XCL2 was found in AMs from IPF (**Figure-2**). No significant functional difference has been shown between XCL1 and XCL2[38], and the molecular properties of XCL2 need to be explored in the context of IPF.

### **The pharmacological challenge of targeting the chemokine system in IPF.**

Chemokines are pleiotropic proteins involved in various biological functions, which regulate tissue homeostasis and response to damage or stress and repair[33, 38]. Targeting a single chemokine or chemokine receptor in IPF will benefit from a specific interaction, with decreased potential to modify other systems. The drug development for lung diseases, including IPF, has failed in many instances. Therefore, intervention on the chemokine system in lung diseases has been considered for phase I and II clinical trials such as COPD (CCR1 antagonist AZD4818/AstraZeneca, CXCR1/2 antagonist SCH527123/Schering-Plough, CXCR2 antagonist SB656933/GlaxoSmithKline, CXCR2 antagonist GSK1325756B/GlaxoSmithKline), asthma (neutralizing monoclonal anti-CCR3 ASM8/Pharmaxis, CCR3 antagonist GSK766994/GlaxoSmithKline, CCR3 antagonist DPC168/Dupont, CCR3 antagonist BMS-639623/BMS, CCR4 antagonist GSK2239633/GSK), pulmonary fibrosis (anti-CCL2 Carlumab/Centocor), cystic fibrosis (SB656933/GlaxoSmithKline) and bronchiectasis (CXCR2 antagonist AZD-5069/AstraZeneca)[38, 76]. However, some trials have shown no efficacy, such as CCR1/AZD4818 for COPD, CCR3/GSK766994 for asthma, and CCL2/Carlumab for IPF. Therefore, broad targeting of chemokine receptors may enhance anti-inflammatory coverage in lung diseases, but with the potential to modify other systems and lead to enhanced risk of side effects, such as immunosuppression or secondary infection[33]. Future therapies targeting chemokines requires understanding the diversity and complexity of the chemokine system in IPF.

## **Concluding remarks and Future Perspectives**

Chemokines are produced by a wide range of leukocyte and non-leukocyte (inducers) cells in lungs. The expression of chemokine receptors can trigger diverse cellular phenomena in various cell populations, thus having multiple targets (responders). Chemokine pathways seem to be shared in IPF samples, indicating emerging putative pharmacological targets. However, due to the intrinsic properties (complexity of the relationships between the families of chemokines and receptors) and extrinsic properties (complexity based on the production of chemokines and expression of chemokine receptors by several cells) during pulmonary fibrogenesis, it is difficult to propose a single chemokine target for IPF control. Finally, AMs may contribute to the chronicity and progression of IPF[2] as a source of chemokines and cytokines and through secondary pathways triggered by chemokine-receptor interaction, with complementary functions induced by specific stimulus and cytokines from tissue milieu. Thus, chemokines may enhance IPF by abnormal homeostatic chemokine production, which could activate multiple targets, suggesting that chemokine signaling can be central for perpetuating fibrogenesis. The chemokines highlighted here open new directions for future research on pharmacological targets involving the homeostatic chemokine fine-tuning as therapeutic opportunities for IPF disease management.

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**Author contribution:**

RCR designed the hypothesis, discussion, and conclusions, and wrote the manuscript. VFJQ and BR wrote the manuscript and conclusions.

**Declaration of interest:**

The authors declare no competing interests.

**Ethics approval:**

Ethical review and approval were waived for this study due to the use of public-deposited data, found below: <https://www.ncbi.nlm.nih.gov/geo/>, GSE32537, GSE47460, GSE53845, GSE99621, GSE92592, and GSE79544.

**Consent to participate:**

Patient consent was waived due to the use of public-deposited data, found below: <https://www.ncbi.nlm.nih.gov/geo/>, GSE32537, GSE47460, GSE53845, GSE99621, GSE92592, and GSE79544.

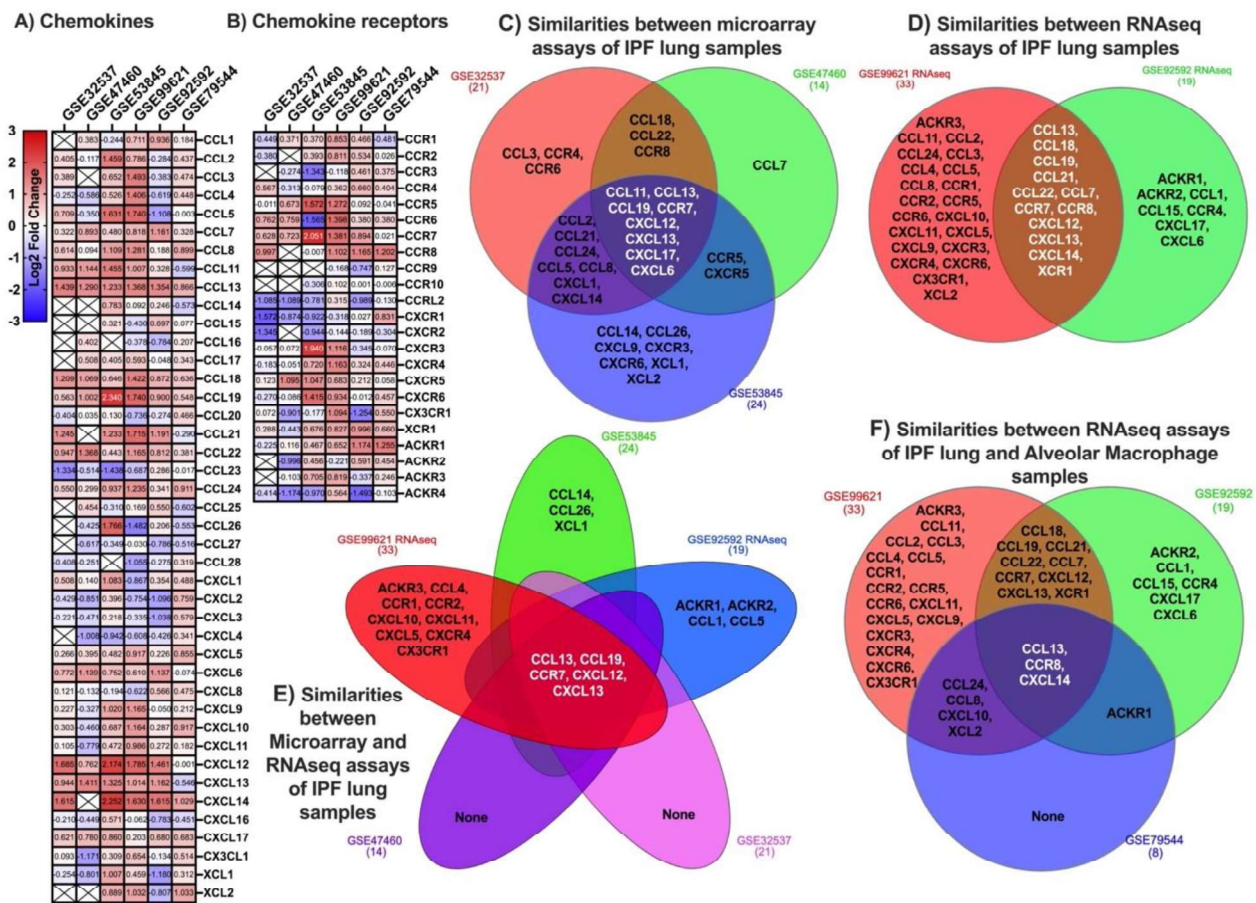
**Availability of data and material:**

The datasets presented in this study are public-deposited data and can be found in online repositories. The name of the repository and accession numbers can be found below: <https://www.ncbi.nlm.nih.gov/geo/>, GSE32537, GSE47460, GSE53845, GSE99621, GSE92592, and GSE79544.

**Box-1: The AMs tissue stimulus sensing regulates inflammation and fibrogenesis in IPF.** NOTCH signaling triggers myofibroblast differentiation and pulmonary fibrosis [100]. Recently, results highlighted the relevance of NOTCH3 in macrophage activation by TLR signaling [101], suggesting that NOTCH3 controls inflammation. Lung macrophages of Fibrotic Hypersensitivity Pneumonitis (FHP) express MS4A6A within the predominant M2 phenotype, exhibiting higher inflammatory activity [102] and MS4A6A<sup>+</sup> macrophages may be relevant in IPF. Migration and accumulation of CD24<sup>+</sup>CD64<sup>+</sup> interstitial macrophages occur in the chronic phase of pulmonary fibrosis [103], suggesting an involvement of CD24 with fibrogenesis. The apoptotic cells sensing by macrophage enhances M2 phenotype and fibrosis [104]. Interstitial macrophage expresses high CD36 in IPF [105], and CD36 has been implicated in efferocytosis of apoptotic cells during resolution triggering fibrosis [106]. CD69 interacts with carbohydrate moieties of Gal-1 expressed in dendritic cells and macrophages [107]. CD69 regulates macrophage activation, and CD69-deficient mice displayed attenuated lung fibrosis [108], supporting CD69-mediated macrophage development of IPF. TLR9 is widely expressed in immune, epithelial, and mesenchymal cells and recognizes unmethylated CpG DNA motifs present in bacterial and viral DNA and self-DNA [109]. In myofibroblasts, TLR9 drives the progressive IPF [110], and activates AMs converging TLR9 and PI3K $\gamma$  signaling inducing silicosis [111]. Nucleotide-binding oligomerization and Leucine-rich repeat Receptor (NLR) protein family consists of immune sensors that form inflammasomes and caspase-1 activation [112, 113]. NLRP6 receptor signaling form inflammasome [113], in neutrophils, macrophages, and epithelial cells from lungs during pneumonia [112], and may NLRP6 is involved in IPF progression. BAL-AMs-derived IL-9 may sustain the pulmonary Th9 activation that promotes fibroblast differentiation, activation, and collagen secretion in IPF [114, 115]. IL-34 has a prominent role in intestinal fibrogenesis. The fibroblast stimulation with IL-34 enhanced COL1A1 and COL3A1 expression through p38 MAP kinase-dependent mechanism [116], and can be involved in IPF. IL-38, an IL-1 family member, is overexpressed in patient lungs with IPF acute exacerbation and diffuse alveolar damage [117], suggesting that IL-38 may control chronic lung inflammation. Silica-induced osteoclast-like differentiation of AMs and recruited monocytes, driven by RANKL in pulmonary cells, and antibody anti-RANKL suppressed silica-induced pulmonary fibrosis [118], supporting that RANKL constitutes a promising target in IPF. VEGF has been implicated in vascular remodeling and fibrosis. VEGF sourced from ATII cells is critical in the development of IPF [119], indicating that VEGF/Src signaling mediated subpleural pulmonary fibrosis [120] and VEGF serum levels may reflect the severity of the disease's progression [121].

## Figures and legends:

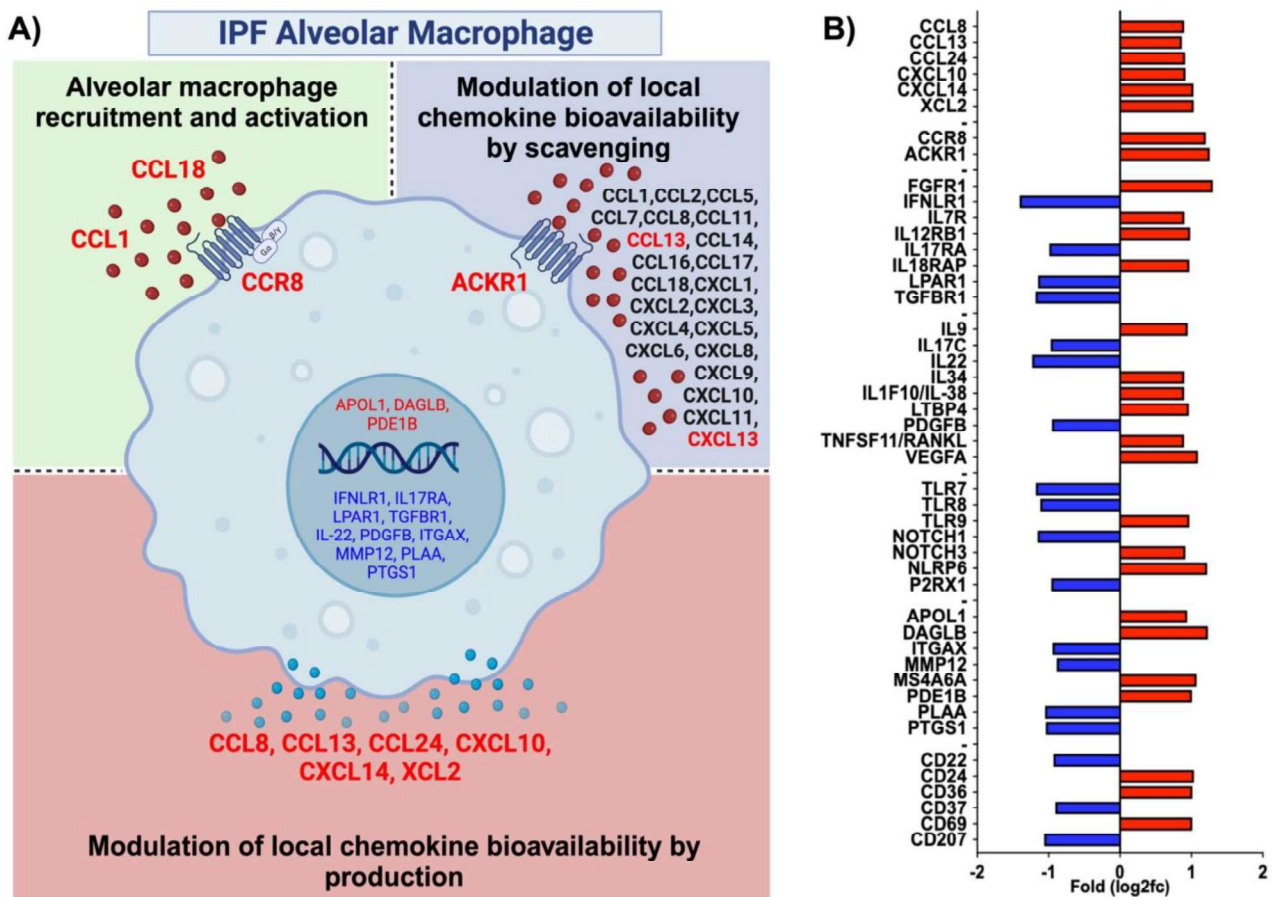
**Figure-1:**



**Figure-1: Chemokine signatures in IPF: The chemokine pathways in IPF samples.** Illustrative microarray analysis of lung tissue from IPF patients under GEO databases GSE32537[122] (IPF n=119 and healthy n=50), GSE47460[123] (IPF n=254 and healthy n=108) and GSE53845[124] (IPF n=40 and healthy n=8), and from RNAseq analysis of lung tissue from IPF patients under GEO databases GSE99621[125] (IPF n=18 and healthy n=8), GSE92592[126] (IPF n=20 and healthy n=19), and RNAseq of BAL-derived AMs from IPF patients under GEO database GSE79544[127] (IPF n=16 and healthy n=7) using *Phantasus*[128] (<https://genome.ifmo.ru/phantasus>), as previously described[129]. Multiple chemokines and receptors are up-regulated as depicted in Heatmaps of chemokine (A) and receptors (B) expression in IPF samples (up-regulated – red and down-regulated – blue). Schematic representation of expression patterns (C) from microarray GSE32537, GSE47460, and GSE53845 of lung tissue from IPF patients, indicating that CCL11, CCL13, CCL19, CCR7, CXCL6, CXCL12, CXCL13 and CXCL17 are jointly expressed. Schematic representation of expression patterns (D) of RNAseq GSE99621 and GSE92592 of lung tissue from IPF patients, indicating that CCL7, CCL13, CCL18, CCL19, CCL21, CCL22, CCR7, CCR8, CXCL12, CXCL13, CXCL14 and XCR1 are jointly expressed. Schematic representation of similarities (E) between microarray GSE32537, GSE47460, GSE53845 and RNAseq GSE99621 and GSE92592 of lung tissue from IPF patients, indicating that CCL13, CCL19, CCR7, CXCL12, and CXCL13 are commonly expressed. Similarities (F) between RNAseq GSE99621 and GSE92592 of lung tissue and GSE79544 of BAL-derived AMs from IPF patients, indicating that CCL13, CCR8, and CXCL14 are commonly expressed. Differences were considered significant at P value <0.05.

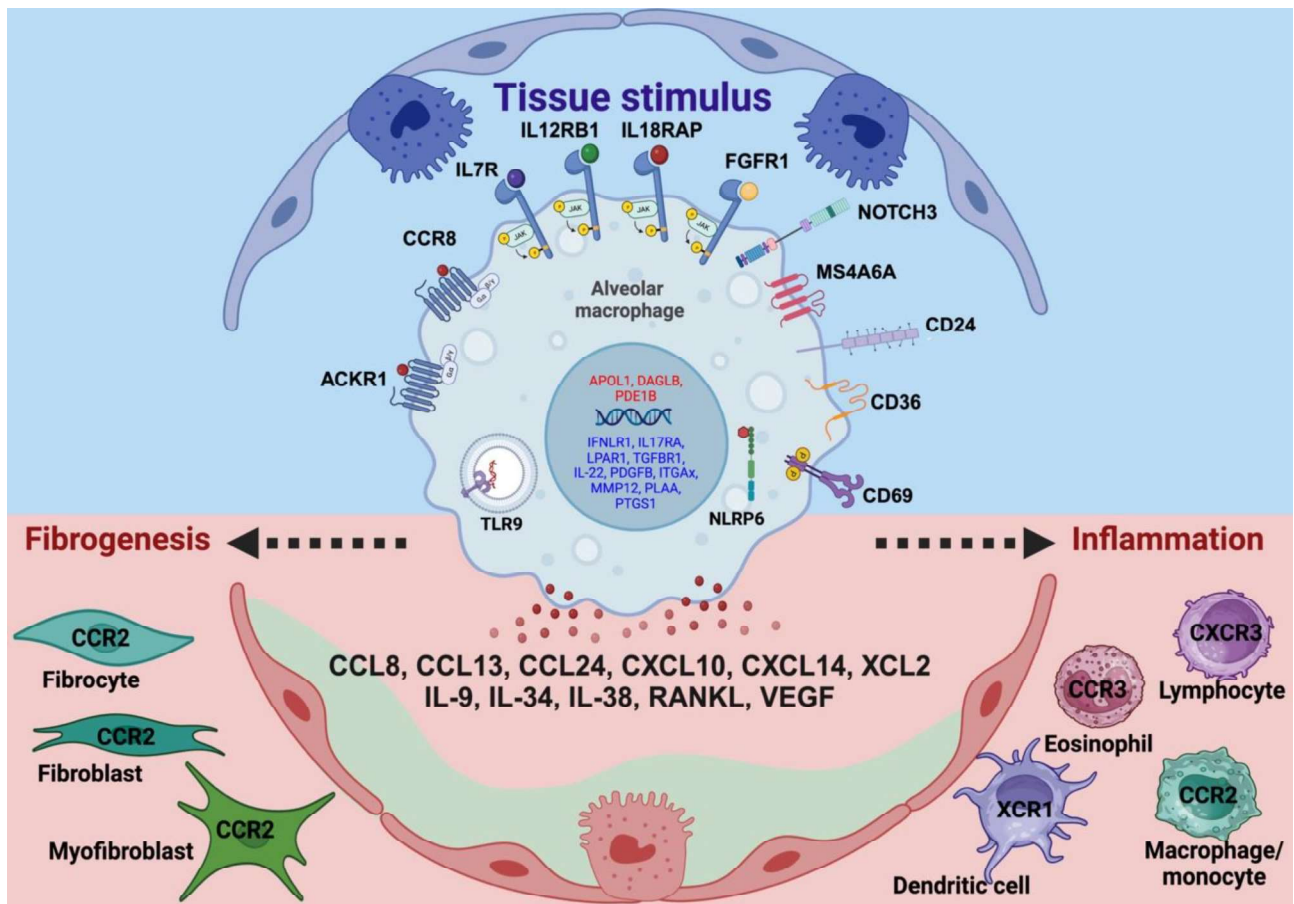


Figure-2:



**Figure-2: Deciphering alveolar macrophage (AMs) chemokine pathways in IPF: AMs modulates the lung tissue, tuning chemokine-dependent inflammation and fibrosis by chemokine production and scavenging.** Based on cytokine, chemokine, and receptors expressed by AMs from IPF (GSE79544[127]), AMs may be critical players sustaining a fibrogenic microenvironment during pulmonary fibrosis. Mechanistically, AMs expressing the CCR8 receptor (A) could contribute to AMs migration, cell activation, and lung residency maintained by local CCL1 and CCL18 response in lung tissue from IPF. Since AMs are resident lung cells, they potentially (A) produce inflammatory and fibrogenic chemokines (CCL8, CCL13, CCL24, CXCL10, CXCL14, and XCL2) in the pulmonary microenvironment. These chemokines have multiple targets, negatively regulating angiogenesis (CXCL10), acting on endothelial cells, inducing chronic inflammation (XCL2, CCL13, CCL24, and CXCL10), contributing to leukocyte influx and activation, and fibrogenesis by fibrocyte recruitment (CCL8) and fibroblast activation (CXCL14). Furthermore, the expression of ACKR1 (A) may have a modulating function by scavenging and chemokine clearance, post-transcriptionally controlling the CCL1, CCL2, CCL5, CCL7, CCL8, CCL11, CCL14, CCL16, CCL17, CCL18, CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL8, CXCL9, CXCL10, and CXCL11 chemokines, but specifically CCL13 and CXCL12 (red) commonly expressed by IPF lung tissues (Figure-1E). Illustrative example of the expression pattern of AMs cell markers (B) highlighting the up-regulated (red) and down-regulated (blue) transcripts, from RNAseq of BAL-derived AMs from IPF patients under GEO database GSE79544 (IPF n=16 and healthy individuals n=7) analyzed by *Phantasus*[128] (<https://genome.ifmo.ru/phantasus>), as previously described[129]. Differences were considered significant at P value <0.05.

Figure-3:



**Figure-3: Alveolar macrophages (AMs) in IPF: From tissue stimulus sensing to chronic inflammation and fibrogenesis orchestration.** In particular, some up-regulated transcripts in BAL-derived AMs from patients with IPF revealed specific contributions of AMs pathways (GSE79544[127]) related to (i) tissue stimulus and stress sensing that may trigger the (ii) inflammatory and/or (iii) fibrogenic processes, thus interpreting the microenvironment and actively expressing chemokines and cytokines. The GSE79544 data showed that cytokine receptors are up-regulated (IL7R, IL-12RB1, IL18RAP, and FGFR1), suggesting that BAL-derived AMs, hypothetically, can respond to inflammatory tissue stimulus (i) such as cytokines IL-7, IL-12, IL-18 and FGF derived from tissue milieu. The BAL-derived AMs from IPF could sense the tissue stress (ii) by expression of NOTCH3, MS4A6A, CD24, CD36, CD69, TLR9, and NLRP6 receptors (**Box-1**), up-regulated in this data set. Despite the contribution of AMs triggering the (ii) inflammatory and (iii) fibrogenic processes, the AMs produce chemokines (CCL8, CCL13, CCL24, CXCL10, CXCL14, and XCL2) in the pulmonary microenvironment, orchestrating chronic lung inflammation by leukocyte recruitment and activation (CXCR3<sup>+</sup> Lymphocytes, CCR3<sup>+</sup> Eosinophils, XCR1<sup>+</sup> Dendritic cells and CCR2<sup>+</sup> myeloid cells) and fibrosis (CCR2<sup>+</sup> fibrocytes, fibroblasts and myofibroblasts). AMs are essential sources of cytokines (IL-9, IL-34, IL-38, RANKL, and VEGF) related to several phenomena involved in fibrosis, such as immune functions, chronic lung inflammation, angiogenesis, and fibrogenesis (**Box-1**).

**Tables:**

**Table -1. Chemokine System: The complexity of chemokines and their receptors**

<b>Chemokines</b>	<b>Other name (s)</b>	<b>Chemokine Receptors</b>
CCL1	I-309, TCA-3	CCR8
CCL2	MCP-1, JE	CCR2, CCR2
CCL3	MIP-1 $\alpha$	CCR1
CCL4	MIP-1 $\beta$	CCR1, CCR5
CCL5	RANTES	CCR5
CCL6	C10, MRP-2	CCR1
CCL7	MARC, MCP-3	CCR2
CCL8	MCP-2	CCR1, CCR2B, CCR5
CCL9/CCL10	MRP-2, CCF18, MIP-1 $\gamma$	CCR1
CCL11	Eotaxin	CCR2, CCR3, CCR5
CCL12	MCP-5	CCR2
CCL13	MCP-4, NCC-1	CCR2, CCR3, CCR5
CCL14	HCC-1, MCIF, NCC-2	CCR1
CCL15	Leukotactin-1, MIP-5, HCC-2, NCC-3	CCR1, CCR3
CCL16	LEC, NCC-4, LMC,	CCR1, CCR2, CCR5, CCR8
CCL17	TARC, ABCD-2	CCR4
CCL18	PARC, DC-CK1, AMAC-1, MIP-4	Unknown
CCL19	ELC, Exodus-3	CCR7
CCL20	LARC, Exodus-1	CCR6
CCL21	SLC, 6Ckine, Exodus-2, TCA-4	CCR7
CCL22	MDC	CCR4
CCL23	MPIF-1, MIP-3, MPIF-1	CCR1
CCL24	Eotaxin-2, MPIF-2,	CCR3
CCL25	TECK	CCR9
CCL26	Eotaxin-3, MIP-4 $\alpha$ , IMAC, TSC-1	CCR3
CCL27	CTACK, ILC	CCR10
CCL28	MEC	CCR3, CCR10
CXCL1	Gro- $\alpha$ , GRO1, NAP-3, KC	CXCR2
CXCL2	Gro- $\beta$ , GRO2, MIP-2 $\alpha$	CXCR2
CXCL3	Gro- $\gamma$ , GRO3, MIP-2 $\beta$	CXCR2
CXCL4	PF-4	CXCR3B
CXCL5	ENA-78	CXCR2
CXCL6	GCP-2	CXCR1, CXCR2
CXCL7	NAP-2, CTAPIII, PEP	Unknown
CXCL8	IL-8, NAP-1, MDNCF, GCP-1	CXCR1, CXCR2
CXCL9	MIG, CRG-10	CXCR3
CXCL10	IP-10, CRG-2	CXCR3
CXCL11	I-TAC, IP-9	CXCR3
CXCL12	SDF-1, PBSF	CXCR4
CXCL13	BCA-1, BLC	CXCR5
CXCL14	BRAK	Unknown
CXCL15	Lungkine, WECH	Unknown
CXCL16	SRPSOX	CXCR6
CXCL17	DMC, VCC-1	Unknown
XCL1	Lymphotoctin $\alpha$	XCR1
XCL2	Lymphotoctin $\beta$	XCR1

CX3CL1	Fractalkine, Neurotactin	CX3CR1
<b>Atypical Chemokine Receptors</b>	<b>Other name (s)</b>	<b>Chemokines</b>
ACKR1	DARC, CD234, Duffy antigen	CCL1, CCL2, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL16, CCL17, CCL18, CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL8, CXCL9, CXCL10, CXCL11, CXCL13
ACKR2	D6, CCBP2, CMKBR9	CCL2, CCL3, CCL3L1, CCL4, CCL5, CCL6, CCL7, CCL8, CCL11, CCL12, CCL13, CCL14, CCL17, CCL22, CCL23, CCL24, CCL26, CXCL2, CXCL10
ACKR3	CXCR7, RDC1, CMKOR1	CXCL11, CXCL12, vCCL2
ACKR4	CCX-CKR, CCRL1, CCR11	CCL19, CCL20, CCL21, CCL22, CCL25, CXCL13
ACKR5	CCRL2, CRAM, GPR182	CCL2, CCL16, CCL17, CCL19, CCL20, CCL21, CCL22, CXCL4, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14
ACKR6	PITPNM3	CCL18
<b>References: [33, 38, 45]</b>		

**Table -2. The chemokines and receptors induced in IPF samples.**

Microarray			RNAseq		
GSE32537	GSE47460	GSE53845	GSE99621	GSE92592	GSE79544
CCL2	CCL7	CCL2	CCL2	CCL1	CCL8
CCL3	CCL11	CCL5	CCL3	CCL7	CCL13
CCL5	CCL13	CCL8	CCL4	CCL13	CCL24
CCL8	CCL18	CCL11	CCL5	CCL15	CCR8
CCL11	CCL19	CCL13	CCL7	CCL18	CXCL10
CCL13	CCL22	CCL14	CCL8	CCL19	CXCL14
CCL18	CCR5	CCL19	CCL11	CCL21	XCL2
CCL19	CCR7	CCL21	CCL13	CCL22	ACKR1
CCL21	CCR8	CCL24	CCL18	CCR4	
CCL22	CXCL6	CCL26	CCL19	CCR7	
CCL24	CXCL12	CCR5	CCL21	CCR8	
CCR4	CXCL13	CCR7	CCL22	CXCL6	
CCR6	CXCL17	CXCL1	CCL24	CXCL12	
CCR7	CXCR5	CXCL6	CCR1	CXCL13	
CCR8		CXCL9	CCR2	CXCL14	
CXCL1		CXCL12	CCR5	CXCL17	
CXCL6		CXCL13	CCR6	XCR1	
CXCL12		CXCL14	CCR7	ACKR1	
CXCL13		CXCL17	CCR8	ACKR2	
CXCL14		CXCR3	CXCL5		
CXCL17		CXCR5	CXCL9		
		CXCR6	CXCL10		
		XCL1	CXCL11		
		XCL2	CXCL12		
			CXCL13		
			CXCL14		
			CXCR3		
			CXCR4		
			CXCR6		
			CX3CR1		
			XCL2		
			XCR1		
			ACKR3		

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Figure 1

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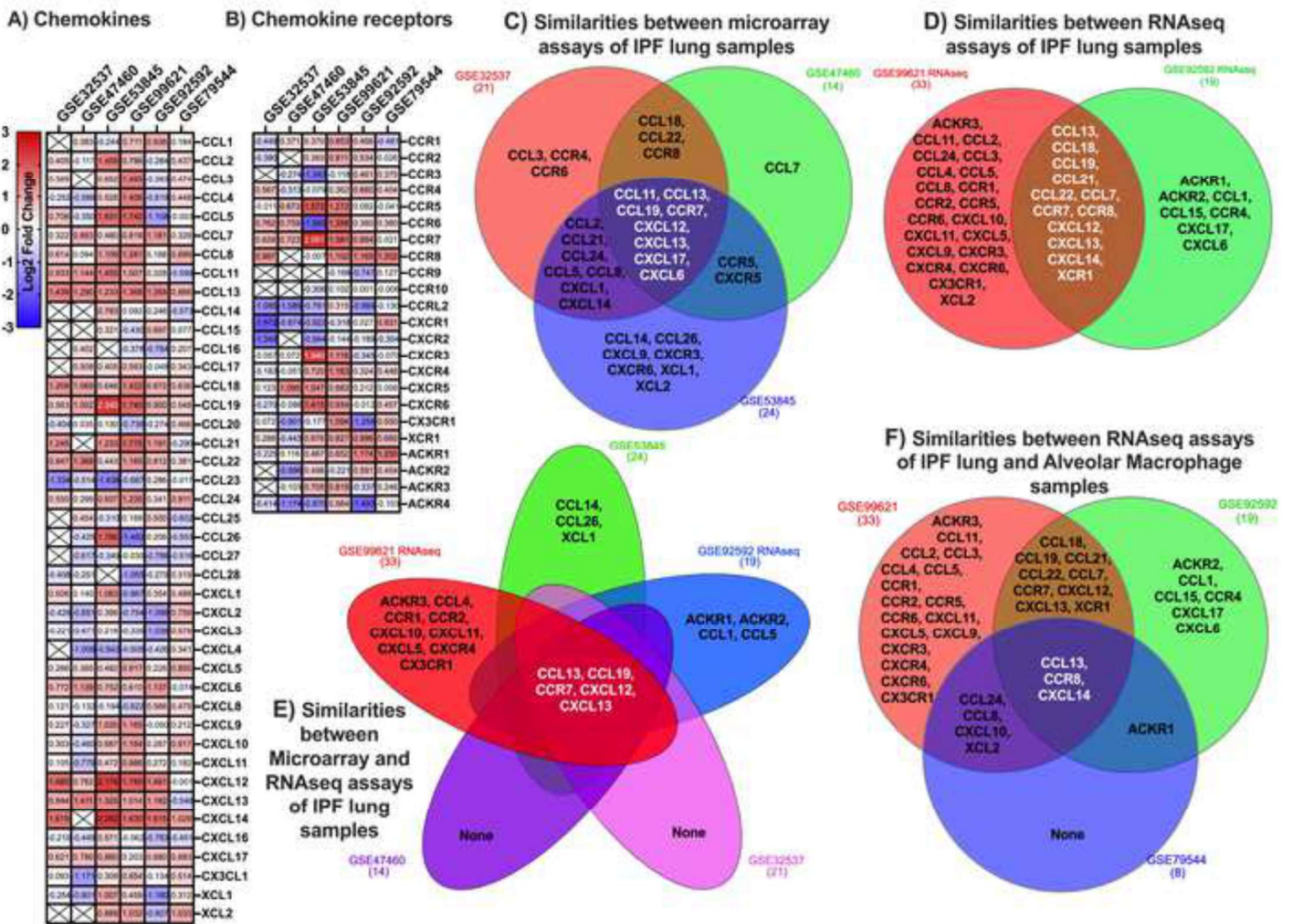
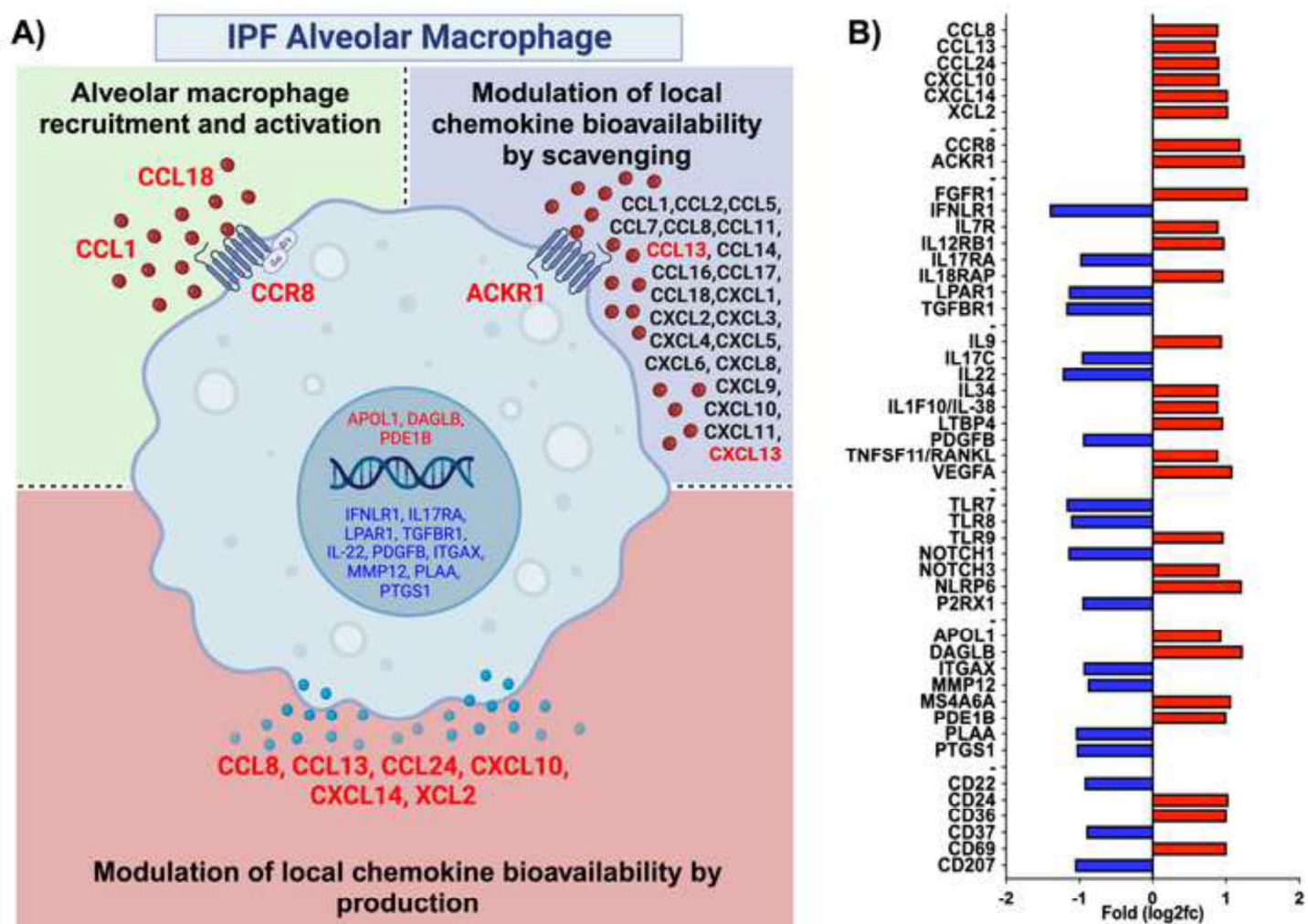
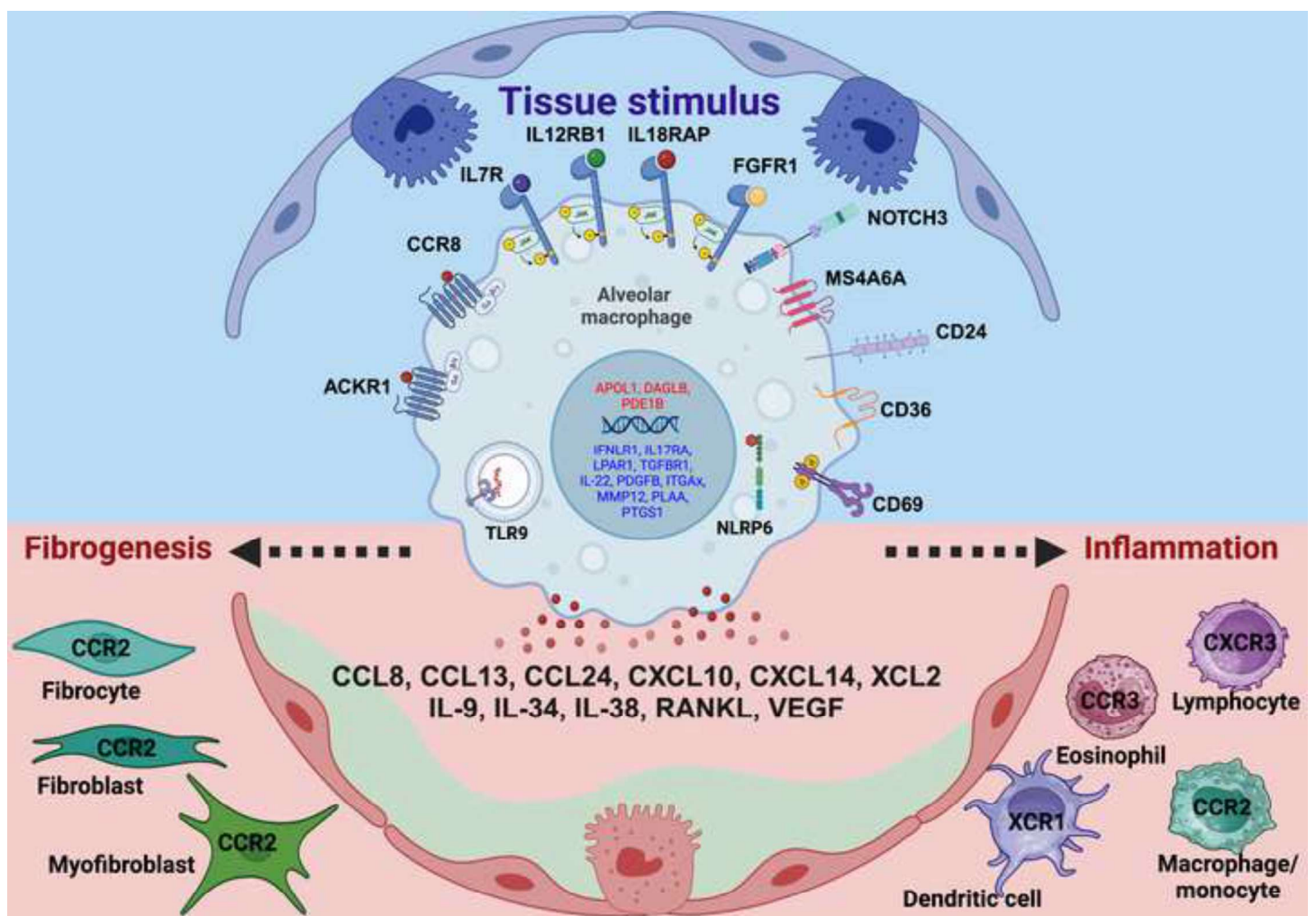


Figure 2

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**Outstanding Questions Box:**

- Elevated levels of homeostatic chemokines have been found in patients with IPF. Could IPF be a lung disease caused by abnormal levels of homeostatic chemokines in the lungs? Narrowing the implication of chemokines to certain sub-classes should help better defining putative therapeutic targets.
- Some therapies aimed at inhibiting a single target in IPF have failed from a clinical point of view. Could broad-spectrum chemokine receptor inhibitors be more effective for treating IPF? In the vast interplay of chemokines and their receptors, targeting more than one receptor may be beneficial.
- Homeostatic chemokines coordinate essential tissue and immune functions. Would it be possible to pharmacologically interfere with homeostatic chemokines without causing side effects on the basic functions of the immune system, such as immunodeficiency-like or susceptibility to viral and bacterial infections? This will directly impact the risk/benefit balance of putative therapeutic interventions.
- How could we interfere with homeostatic chemokine levels locally in the lungs of patients with IPF without causing systemic alterations and/or side effects? The role of homeostatic chemokines beyond IPF will determine the feasibility and safety of putative therapeutic interventions.
- Alveolar macrophages are important myeloid-resident lung cells that sense tissue signals and coordinate immune and fibrotic functions: could they regulate local chemokine levels in the lungs of patients with IPF? Targeting chemokine expression in the alveolar macrophages may provide another, transcriptional level for putative therapeutic interventions.

Idiopathic Pulmonary Fibrosis (IPF) is a fatal condition of unbalanced immune responses associated with physiological manifestations such as lung mechanical dysfunction and dyspnea. Recent studies show that chemokines are important regulators of immune response and fibrogenesis in the context of pulmonary fibrosis, with both elevation of chemokine levels and defective clearance. Thus, understanding mechanistically the contribution of the complex chemokines system to pathology progression is needed to appreciate their potential as pharmacological targets and therapeutic opportunities in IPF disease management.