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Review

Role of Chemokines in Non-Small Cell Lung Cancer: Angiogenesis and Inflammation

Selma Rivas-Fuentes[1]✉, Alfonso Salgado-Aguayo[2], Silvana Pertuz Belloso[3], Patricia Gorocica Rosete[1], Noé Alvarado-Vásquez[1], Guillermo Aquino-Jarquin[4]

1. Department of Biochemistry Research, National Institute of Respiratory Diseases "Ismael Cosío Villegas", Mexico City, Mexico.
2. Laboratory of Research on Rheumatic Diseases, National Institute of Respiratory Diseases "Ismael Cosío Villegas", Mexico City, Mexico.
3. Department of Comparative Biology, Faculty of Sciences, National Autonomous University of Mexico, Mexico City, Mexico.
4. Laboratory of Research on Genomics, Genetics and Bioinformatics. Tower of Haemato-oncology, Children´s Hospital of Mexico "Federico Gomez", Mexico City, Mexico.

✉ Corresponding author: Department of Biochemistry Research, National Institute of Respiratory Diseases "Ismael Cosío Villegas", Mexico City, Mexico. Calzada de Tlalpan 4502, Tlalpan, Ciudad de México, D.F. 14080, México. Phone +52 (55) 5487 1705; E-mail: selma.rivas@iner.gob.mx.

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Abstract

Non-small cell lung cancer (NSCLC) is one of the most common types of aggressive cancer. The tumor tissue, which shows an active angiogenesis, is composed of neoplastic and stromal cells, and an abundant inflammatory infiltrate. Angiogenesis is important to support tumor growth, while infiltrating cells contribute to the tumor microenvironment through the secretion of growth factors, cytokines and chemokines, important molecules in the progression of the disease. Chemokines are important in development, activation of the immune response, and physiological angiogenesis. Chemokines have emerged as important regulators in the pathophysiology of cancer. These molecules are involved in the angiogenesis/angiostasis balance and in the recruitment of tumor infiltrating hematopoietic cells. In addition, chemokines promote tumor cell survival, as well as the directing and establishment of tumor cells to metastasis sites. The findings summarized here emphasize the central role of chemokines as modulators of tumor angiogenesis and their potential role as therapeutic targets in the inflammatory process of NSCLC angiogenesis.

Key words: Chemokines, cytokines, angiogenesis, inflammation, non-small cell lung cancer.

1. Introduction

Lung cancer is the main cancer-related cause of death worldwide in both men and women [1]. It is classified into two types according to the size of the transformed cells: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The latter is the most common type, accounting for 85 to 90% of diagnosed cases, and although NSCLC spreads more slowly than SCLC, both have a poor prognosis [2].

NSCLC is a cancer of epithelial origin that groups together various histological subtypes that differ in their cytology, embryonic origin, anatomical location, and associated oncogenes [3]. The most common subtypes of NSCLC are adenocarcinoma (40% of all forms of lung cancer), squamous-cell carcinoma (25-to 30%) and large-cell carcinoma (10 to 15%) [2].

In NSCLC, the tumor stroma is characterized by active angiogenesis and abundant inflammatory infiltrate, which is mainly composed of tumor-associated macrophages (TAM). It is also characterized by the presence of tumor-infiltrating lymphocytes (TIL), including T, B and NK cells, and tumor-associated neutrophils (TAN) [4, 5]. The differences in the inflammatory infiltrate are attributable to the local production of chemokines, which are also important regulators of the angiogenesis that accompanies tumor growth [6].

2. Chemokines

Chemokines are a family of soluble proteins that

direct the migration of leukocytes under physiological conditions and during inflammation [7]. They are important in embryonic development, activation of the immune response, and in driving both physiological and pathological angiogenesis. Chemokines exert their biological effects on leukocytes through the activation of seven transmembrane domain receptors coupled to heterotrimeric G proteins [8]. The activation of the classical chemokine receptors initiates protein phosphorylation events, including PI3K, and the generation of second messengers such as IP3 and DAG, along with increased intracellular calcium concentration ($[Ca^{2+}]_i$) and activation of transcription factors such as NF-κB, which regulates the expression of various inflammatory response-related genes [8-10]. About fifty chemokines and twenty chemokine receptors are currently known [11]. Some chemokines can bind with high affinity to more than one receptor, and some receptors can be activated by more than one chemokine [12] (Table 1). The biological significance of this promiscuity of ligands and receptors may be related to the diversity of functions of these proteins [13]. Furthermore, some chemokine receptors have been described that, after binding to ligands, do not contribute to cell migration or to cell functions related to cell activation; these receptors are called atypical receptors, and are negative regulators of the activity of chemokines [14, 15]. Atypical chemoattractant receptors (Table 1), such as ACKR1 (DARC), ACKR2 (D6), ACKR3 (CXCR7) and ACKR4 (CCRL1), limit the amount of available ligands in the microenvironment due to their ability to internalize and degrade them; for this reason they are also known as "decoy receptors" [14, 15]. Chemokines are currently classified according to a systematic nomenclature based on the position of the cysteines located nearest to the N-terminal end of the protein. These are usually two cysteines that are together, or separated by 1 or 3 amino acid residues (X). Four types of arrangements of cysteines have been described so far, according to which chemokines are divided into four sub-families: CC, CXC, CX_3C and XC [16]. The CXC subfamily is divided into two groups, CXC (ELR^+) and CXC (ELR^-), according to the presence or absence of the amino acid motif consisting of glutamic acid, leucine and arginine (ELR motif)[7, 16].

Chemokines and their receptors are expressed in immune system cells, endothelial and epithelial cells, fibroblasts and keratinocytes, among others [17]. Some chemokines and their receptors have a constitutive expression, while others are affected by changes in the cellular microenvironment and therefore are categorized as inducible [18]. For example, stimulation with tumor necrosis factor (TNF-α) increases the expression of the chemokine CCL5 and its receptor CCR5 in the central nervous system [15], whilst interferon gamma (INF-γ) is a potent inducer of the expression of CXC-ELR^- chemokines [19-21].

3. Chemokines and their receptors in the cancer process.

For the past 20 years, there have been studies aimed at understanding the role of chemokines in the pathophysiology of cancer. It is currently accepted that the system of chemokines and their receptors has direct and indirect effects on the pathophysiology of cancer and that these molecules are important in the development and progression of the disease. Chemokines and their receptors are regulators of angiogenesis, which allows tumor growth and metastasis [22]. Furthermore, chemokines and their receptors mediate the recruitment of cells of the immune system to the tumor microenvironment. These cells actively modify the microenvironment; for example, macrophages are recruited by a pro-inflammatory environment and contribute to perpetuate inflammation through the production of angiogenic factors such as VEGF-A [23]. Finally, it has been shown that chemokines induce the proliferation of cancer cells and promote the metastasis of tumor cells by inducing a more motile phenotype [24, 25].

Table 1. Complexity of the ligand-receptor system in the chemokine family. There are 4 subfamilies of chemokines, classified according to the position of the N-terminal cystein residues: CC, CXC, CX3C and XC. The chemokine-chemokine receptor system is quite complex, since some chemokines can activate several receptors, and a single receptor can have several ligands (bold text). To date, 5 atypical receptors have been characterized, which do not induce activation after ligand binding (last column).

CC	CXC	CX3C	XC	ATYPICAL CHEMOKINE RECEPTOR
CCR1 (CCL**3**,**5**,**7**,**8**,14,**15**,16, 23)	CXCR1 (CXCL**1**,**6**,**7**,**8**)	CX3CR1 (CX3CL1)	XCR1(XCL1,2)	ACKR1
CCR2 (CCL2,7,**8**,12,**13**)	CXCR2(CXCL**1**,2,3,5,**6**,**7**,**8**)			ACKR2
CCR3 (CCL**5**,**7**,**8**,11,**13**,**15**,24,26)	CXCR3 (CXCL4, CXCL4L1, CXCL9,10,11)			ACKR3
CCR4 (CCL**17**,22)	CXCR4 (CXCL12)			ACKR4
CCR5 (CCL**3**,**4**,**5**)	CXCR5 (CXCL13)			ACKR5
CCR6 (CCL20)	CXCR6 (CXCL16)			
CCR7 (CCL19,21)	CXCR7 (CXCL12)			
CCR8 (CCL1,**4**,**17**)				
CCR9 (CCL25)				
CCR10 (CCL27, 28)				
CCR11 (CCL**8**,**13**)				

4. Angiogenesis, chemokines and cancer

Angiogenesis is a key process in cancer development. It is a multi-step process coordinated by several types of molecules (including the chemokines), most of them soluble and secreted by immune, stromal and neoplastic cells, as well as by activated endothelium [26]. In the adult, physiological angiogenesis is involved in wound repair and formation of the endometrium [26, 27]. Aberrant angiogenesis occurs in cancer as a result of alterations in the expression of molecules controlling the process, such as the chemokines [28-30]. At the cellular level, angiogenesis is a complex process involving several stages. It begins with the activation of endothelial cells and the destabilization of capillary structures. Activated endothelial cells produce matrix metalloproteinases (MMPs); initiate cell proliferation programs and acquire migratory properties [28, 30]. Angiogenesis is coordinated by many types of molecules, which together are called angiogenic and angiostatic factors, according to whether they promote or inhibit angiogenesis [31-33].

CXC chemokines have a dual role in angiogenesis, since some members of this subfamily are angiogenic and others are angiostatic [34, 35] (Table 2). In 1995, Strieter *et al* showed that CXC-ELR$^+$ chemokines have angiogenic properties, can induce *in vitro* the chemotaxis of endothelial cells, and neovascularization in the rat cornea model; while CXC-ELR$^-$ chemokines have angiostatic properties, and inhibit neovascularization even in the presence of angiogenic chemokines and FGF-2[34, 36]. Furthermore, the use of chemokines with mutations on the ELR motif, or the addition of an ELR sequence to CXC-ELR$^-$ chemokines, showed that the ELR motif is central to the angiogenic/angiostatic activity of CXC chemokines [36]. The only known exception is CXCL12, which lacks the ELR motif but has angiogenic activity mediated through its receptors CXCR4 and CXCR7 [37, 38] (Table 3). The expression of CXC-ELR$^-$ chemokines is finely regulated by INF-γ, produced mainly by lymphoid cells during both innate and adaptive immune responses [26].

Table 2. Major angiogenic and angiostatic molecules. The table shows the major angiogenic and angiostatic molecules and their receptors, and their participation in the angiogenic process. One of the most important angiogenic factors are VEGF-A, FGF-2, Ang-2, MMP2 & MMP9, TNF-α, TGF-β, and the CXC-ELR$^+$ chemokines. On the other hand, the major angiostatic factors are IFN-γ, Angiostatin, Thrombospondin-2, TIMP, (mainly TIMP-2 and TIMP-3), and the CXC-ELR$^-$ chemokines.

TYPE OF MOLECULES	MOLECULES	RECEPTOR	FUNCTION
ANGIOGENIC MOLECULES			
VEGF Family	VEGF-A	VEGFR-1 VEGFR-2	↑ Proliferation (EC, P) ↑ Migration (EC) ↑ Formation of tubular structures
FGF Family	FGF-2	FGFR-1 FGFR-2 FGFR-3 FGFR-4	↑ Proliferación (EC, F) ↑ Migration (EC) ↑ VEGF Expression ↑ Formation of capillary structure ↑ Inflammation- related genes
Angiopoietin (Ang)	Ang-1* Ang-2	Tie-1 Tie-2	↑ Sprouting*, proliferation, migration, vessel stabilization* (EC) ↑ Recruitment and activation of mural cells
Metalloproteinases (MMP)	MMP-2 MMP-9	None Substrate: Basement Membrane (BM) Extracellular Matrix (ECM)	↑ Degradation of BM and ECM ↑ Migration (EC)
Cytokines	TNF-α TGF-β	TNFR TβRI TβRII	↑ Migration (EC) ↑ Tube formation ↑ Vessel stabilization ↑ Inflammation ↑ Tube formation
CXC Chemokines	CXC-ELR $^+$	CXCR2 CXCR4 CXCR7	↑ Proliferation, migration, inflammation (EC) ↑ Secretion and activation of MMPs
Transcription factors	HIF-1α* NF-κB** AP-1***		↑ *Expression of VEGF, VEGFR, CXCL8 ↑ bFGF ↑ **Expression of CXCR2, CCL5, CXCL8 ↑ ***CXCL8
ANGIOSTATIC MOLECULES			
Angiostatin	---	None.	↓ Proliferación (EC)

		Angiosotatin binds several proteins: Angiomotin ATP synthase Integrins Annexin II NG2 proteoglycan	↓ Migration (EC) ↓ Formation of capillary structure ↓ VEGF Expression
Endostatin	---	None Endostatin binds several proteins	↓ Proliferación (EC) ↑ Apoptosis ↓ Migration (EC) Inhibition MMPs Acivation ↓ VEGF Expression
CXC Chemokines	CXC-ELR-	CXCR3B	↓ Migratory form ↓ Proliferation ↓ Formation of capillary structures
Cytokines	INF-γ	INFgammaRI INFgammaRII	↑ *Expression of angiostatic chemokines ↓ Angiogenesis
MMPs Inhibitors: TIMP	TIMP-2 TIMP-3	Inhibition of MMPs	↓ Migratory form ↓ Proliferation ↓ Formation of capillary structures

Abbreviations: AP-1, Activator Protein 1;BM, Basement Membrane; EC, Endothelial Cells; EpC Ephitelial Cells, ECM, Extracellular Matrix; FGF, Fibroblast Growth Factor; F, Fibroblasts; HIF-1, Hypoxia-Inducible Factor 1;MMP, Matrix Metalloproteinase; NF-κB, Nuclear Factor κB; TIMP, Tissue Inhibitors of Metalloproteinases; VEGF, Vascular Endothelial Growth Factor.

Table 3. Chemokines and chemokine receptors involved in angiogenesis and associated inflammation. The main chemokines and chemokine receptors that play a dual role in angiogenesis and the recruitment of immune cells into tissues were classified according to the major receptors, cellular source and target cells.

Sub- Family	Systematic Name	Classic Name	Major Receptors	Cell Source	Target Cell	Effect
CXC ELR+	CXCL1	Gro-α	CXCR1, CXCR2	Ne, Ma, EpC	Ne, Mo, EC	Angiogenic
	CXCL2	Gro-β	CXCR2	Ne, Ma	NK, Mo, DC, Ba, T	
	CXCL3	Gro-γ	CXCR2	Ne, EpC, Ma	EpC	
	CXCL5	ENA-78	CXCR2	Ne		
	CXCL6	GCP-2	CXCR1, CXCR2	Ne, Ma		
	CXCL7	NAP-2	CXCR1, CXCR2	Ne, Ma		
	CXCL8	IL-8	CXCR1, CXCR2	Ne, Ma, T, EC, EpC, T, F		
CXC ELR-	CXCL4	PF4	CXCR3A, CXCR3B	P	F, Ne, Mo	Angiostatic
	CXCL4L1	PF4alt	CXCR3A, CXCR3B	EC	F, Ne, Mo	
	CXCL9	MIG	CXCR3A, CXCR3B	EC, Th1, NK	Th1, NK	
	CXCL10	IP-10	CXCR3A, CXCR3B	EC, Th1, NK, Mo, F	Th1, NK	
	CXCL11	I-TAC	CXCR3A, CXCR3B	Th1, NK	Th1, NK	
	CXCL14	BRAK	Unknown		Mo, Ma, iDC, NK	
	CXCL12	SDF-1	CXCR4, CXCR7		Leukocytes	Angiogenic
CC	CCL2	MCP-1	CCR2,4	Mo, Ma, T, NK, iDC, B, Ba, EpC	Mo, Ma, T, NK, iDC, B, Ba, Ne	Angiogenic
	CCL5	RANTES	CCR5,1,3, 4	EC, Mo, Ma, T, NK, iDC, Ba, Eo	EC, Mo, Ma, T, NK, iDC, Ba, Eo	

Abbreviations: B, B cells; Ba, basophils; ENA-78, Epithelial cell-derived Neutrophil-Activating peptide; EC, endothelial cells; Eo, eosinophils; EpC, epithelial cells; F, fibroblasts; Granulocyte Chemotactic Protein 2,GCP; Gro-α, Growth-Regulated Oncogene; Interferon-inducible T-cell Alpha-Chemoattractant, iDC, immature dendritic cells; I-TAC;IL-8, Interleukin 8; Interferon-gamma-inducible Protein 10, IP-10; Ma, Macrophages;MCP-1, Monocyte Chemoattractant Protein; MIG, Monokine Induced by Gamma interferon; NAP-2, Neutrophil Activating Protein; Ne, neutrophils; NK, Natural killer cells; P, platelets; PF-4, Platelet Factor 4; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; T, T cells; Th1, T helper 1.

In some diseases such as cancer, hypoxic conditions can alter angiogenesis, since the expression of several angiogenic molecules, including VEGF, VEGFR and the chemokine CXCL8, are under transcriptional regulation of Hypoxia Inducible Factor (HIF-1) [30, 39], which, as its name suggests, activates the transcription of various genes in response to low oxygen levels. In addition, there are reports that HIF-1 can be activated independently of hypoxia; this activation is related to the expression of oncogenes, growth factors and chemokines [40, 41].

In the neoplastic process, alterations in angiogenesis have important implications. It has been shown that the growth of tumors larger than 2-3 mm^3 is dependent on angiogenesis [42]. In addition, angiogenesis facilitates the invasion of malignant cells into the circulation and is also important in the establishment of these cells at the site of metastasis [22, 29, 43].

In turn, chemokines regulate and are also regulated by other angiogenic factors. For example, the metastatic potential of some tumors correlates with the expression levels of some MMPs [44], and CXCL8 induces the secretion and activation of MMP-2 in endothelial cells [45, 46]. Furthermore, CCL7 is cleaved by MMP-2, and thus loses the capacity to induce chemotaxis and calcium fluxes, but retains its ability

to bind to CCR1, CCR2 and CCR4 receptors. Thus, CCL7, digested by MMP-2, acts as a chemokine antagonist [47, 48]. In short, the relationship between chemokines and MMPs may have important implications in the development of angiogenesis and inflammation, and have an indirect impact on the evolution of the neoplastic process [45].

5. Inflammation, chemokines and cancer

The composition of cell subpopulations in the tumor microenvironment is important for the evolution of the neoplastic process. The tumor tissue is composed of tumor cells, stromal cells and infiltrating leukocytes [49]. These cells secrete chemokines that orchestrate the recruitment of cells of the immune system to the tumor microenvironment. In prostate cancer, CCL2 is important for infiltration of TAM into the neoplastic tissue [50]. These cells have high plasticity and are often polarized in the tumor towards a phenotype known as M2, which favors angiogenesis, because it is associated with the secretion of TGF-β, FGF, VEGF and CXC-ELR$^+$ chemokines [51]. In addition, TAM M2 produces IL-10, a cytokine that represses the cytotoxic immune response and can contribute to tumor escape mechanisms [51, 52]. TAM produce the CXCL1, CXCL3, CXCL5, and CXCL8 chemokines, which are chemoattractant for neutrophils and could be responsible for the infiltration of TAN; meanwhile CXCL17 is a chemoattractant for immature myeloid cells and macrophages [53, 54].

Although it is not clear yet how important is the infiltration of TAN for the prognosis of the neoplastic process [5, 55], it is known that some of the compounds released by neutrophils, such as hypochlorous acid (HOCl), are genotoxic[56]. *In vitro* tests have shown that HOCl is mutagenic in lung adenocarcinoma cells A549 [56]. In addition, the local production of HOCl actives MMP- 2, 7, 8 and 9, and inactivates the metalloprotease inhibitor TIMP-1, which may favor the invasion process [57, 58].

Another cell type that is frequently found in the transformed tissue is composed by regulatory T cells, which are increased in several types of cancer, including esophageal squamous cell carcinoma and gastric cancer [59-61]. In the murine model of Lewis lung carcinoma, CCL22 was identified as a chemokine involved in the recruitment of regulatory T cells; this chemokine is produced by NK cells and TAM [62].

6. Chemokines in non-small cell lung cancer

In the last decade, several clinicopathological studies have focused on establishing whether there are associations between the expression level of chemokines and/or their receptors in tumor tissue, patient survival or development of NSCLC. In this regard, it has been reported that the increased expression of the chemokine CCL5 in grade I lung adenocarcinoma correlates with an increase in the survival rate [63], but, as will be discussed later, these results are controversial. On the other hand, the high expression of CXCL8 is related to a worse outcome of the disease [64]. In the studies mentioned above, the pro-angiogenic effect of CXCL8 could stimulate the neoplastic process through increased tumor cell survival and tumor growth, while the higher expression of CCL5 could be related to a more efficient anti-tumor response through an augmented recruitment of T lymphocytes [65]. There seems to be significant differences in the expression of chemokine receptors in the stromal region and in the tumor foci of neoplastic tissue. Ohri and colleagues analyzed the expression of CXCR2-5 and CCR1 receptors, and their correlation with the survival, in a cohort of 20 patients with NSCLC (mainly squamous cell carcinoma). The increased expression of CXCR2, CXCR3 and CCR1 in the foci of tumor cells was associated with greater survival, while the increased expression in stromal cells of CXCR2, CXCR3 and CXCR4 was associated with a lower survival [66]. Further studies are needed to understand the significance of this phenomenon.

7. Relevant CC chemokines in NSCLC

CCL2

The CCL2/CCR2 axis is important in several aspects of tumorigenesis and one of the most relevant is the generation of new vascular structures that allow tumor growth [67]. Treatment with CCL2-neutralizing antibodies showed that this chemokine is important in tumor vascularization and growth [68]. At least two mechanisms are involved in angiogenesis mediated by CCL2. First, CCL2 directly activates endothelial cells and induces their migration and the formation of capillary structures [68, 69]. Second, CCL2 indirectly promotes angiogenesis by recruiting TAM precursor cells (which are a major source of angiogenic molecules) and/or influencing their polarization [50, 70]. In NSCLC, the chemokine CCL2 is expressed by tumor and stromal cells [71, 72]. It has been demonstrated that tumor tissue homogenates are monocyte chemoattractant, and that the use of neutralizing antibodies against CCL2 significantly reduces this effect [71]. These results strongly suggest that CCL2 is crucial in the infiltration of monocytes, which are TAM precursor cells. However, *in vivo* murine models of tumorigenesis showed that the neutralization of CCL2 did not alter the number of TAM, although it promoted the polarization of TAM towards the M1 phenotype (associated with an an-

ti-tumor response mediated by CD8+T cells)[73], while the presence of CCL2 favored the polarization towards the M2 phenotype, which produces angiogenic molecules. In addition, CCL2 induces the recruitment of myeloid suppressor cells (MDSC) [74], which are associated with tumor progression and promotion due to their immunosuppressive activities and are also a source of angiogenic factors. Furthermore, it has been reported that the recruitment of these cells through CCL2/CCR2, is important in the metastasis of colorectal cancer [75]. It is also known that in breast and prostate cancer, the CCL2/CCR2 axis mediates metastasis to bone and lung tissue [76]. In addition, the use of CCL2$^{-/-}$ mice in a model of breast cancer (4T1 cell line) showed that stromal CCL2 favors metastasis of transformed cells to the lung [72]. Although *in vitro* studies and humanized animal models indicate that the presence of CCL2 favors the progression of the neoplastic process, a recent clinicopathological study of 65 patients with advanced NSCLC concluded that the expression of CCL2 in tumor tissue is related to greater survival [77].

CCL5

This chemokine is an important biomarker due to its ability to distinguish patients with NSCLC from healthy controls when measured in serum [78]. The role of this chemokine in tumor progression is unclear, as there is contradictory evidence as to whether or not it favors the progression of cancer [63, 79-82]. The overexpression of chemokine CCL5 in breast and cervical-uterine cancer correlates with a poor prognosis, while low plasma levels of CCL5 in patients with late stages of NSCLC correlated with long-term survival [80]. Some evidence suggests that CCL5 might contribute to the progression of NSCLC. For example, it was observed that in a TIMP2 $^{-/-}$ murine model there is more metastasis of lung carcinoma cell lines. TIMP2 is a metalloprotease inhibitor that also inhibits the transcription of cytokines and chemokines (including CCL5) necessary for the growth of myeloid-derived suppressor cells (MDSCs) [83]. Since there is more expression of CCL5 in TIMP 2$^{-/-}$ mice, there is more recruitment of MDSCs [83]. Furthermore, it is known that the expression level of TbRII, a TGF-β receptor, is decreased in different NSCLC cell lines [84], and this reduction is reflected in an increase in the invasive capacity of these cells. There is greater expression of CCL5 in invasive tumors and in cells with decreased expression of TbRII and inhibiting CCL5-mediated signaling abrogates the invasion of these cells [81].

Contradictorily, it seems that the expression of CCL5 also has a protective effect due to its ability to chemoattract immune effector cells to the tumor [63]. In fact, it has been reported that in response to the CCL5 secreted by tumor cells, CD8+ T cells migrate to the tumor, where they can perform their effector functions. Patients with an active lymphocytic response (ALR) have better prognosis, and, among patients with ALR, CCL5 is a good predictor of survival [63]. This chemokine is released in the lung in response to many noxious stimuli and it has been reported that it might have antitumor activity [63]. Recently, Skachkova *et al.* found that patients with NSCLC who had no relapse after surgical resection, had a significant increase in the CCL5 mRNA compared to patients with relapse [79]. Finally, it is worth mentioning that 4T1 cells constitutively produces CCL5 and spontaneously metastasize to the lungs [85]. The expression of CCL5 could favor the formation of premetastatic niches since CCL5 induces the release of members of the family of chemoattractant molecules S100 [82]. In particular, tumor cells expressing CCL5 had a significant decrease in lung metastasis in S100A4$^{-/-}$ mice, indicating that metastasis to this organ is strongly dependent on the interaction between CCL5 and S100A4 [86].

CCL19 and CCL21

CCL19 and CCL21 are homeostatic chemokines that regulate lymphocyte migration and bind to the receptor CCR7, which is expressed by naïve T cells and dendritic cells [87]. These chemokines are important in the activation of the local immune response, which comprises the activation of dendritic cells, the recruitment and activation of naïve T cells, and the formation of lymphoid structures [87]. Similarly to other neoplasms, in NSCLC the expression of chemokines CCL19 and CCL21 is important for the formation of lymph node-like structures associated with tumor tissue [88-90]. In these structures T and B lymphocytes are segregated into two adjacent regions, T zone and follicles, respectively, that are surrounded by specialized blood vessels called high endothelial venules [90]. CCL19 is located in the extra-follicular area, which is the concentration site of mature dendritic cells, while CCL21 is restricted to the lymphatic vessels [88]. Lymphoid structures associated to the tumor have been related to an increase in the antitumor response and with improved survival of patients [88, 89]. In murine models of lung cancer, the presence of lymphatic structures causes a reduction of the tumor [91]. *Ex vivo* studies with cells from cancer patients showed that the antitumor response associated with overexpression of CCL21, depends partly on the activation and recruitment of dendritic cells and the release of chemokines CXCL9 and CXCL10[91, 92], which are induced by IFN-γ and inhibit angiogenesis[93]. In addition, these chemokines bind to CXCR3 on T cells, increasing the secretion of

IFN-γ (establishing a positive feedback loop in angiostasis) and decreasing TGF-β; the latter is related to invasion processes [94]. Clinicopathological studies in patients with pulmonary adenocarcinoma have reported that the increased expression of CCR7 or CCL19 is associated with a higher life expectancy after surgical resection [95]. Immunotherapy strategies with CCL21 have been tested in NSCLC. These strategies involve the transfer of dendritic cells that overexpress this chemokine, obtaining a promising antitumor response through the activation of local dendritic cells [96-98]. In addition, nanocapsules carrying CCL21 have been injected intra-tumorally, inhibiting the growth of lung cancer [99]. However, it is worth noting that CCL21 has also been implicated in the metastasis and inhibition of apoptosis of tumor cells [100]. In this regard, microarray approaches in NSCLC showed that CCL19 could be a prognostic marker of the course of the disease associated with better survival [101].

CCL25

This chemokine specifically binds the CCR9 receptor, forming a non-promiscuous chemokine/chemokine receptor axis [11]. Recent studies show that the CCL25/CCR9 axis plays an important role on the pathophysiology of lung cancer [102, 103]. On different types of cancer (colorectal, prostatic, ovarian and breast) [104-106], the CCL25/CCR9 axis is related to the severity of the disease. In NSCLC, human neoplastic adenocarcinoma cancer and squamous cancer cells have a robust and equivalent CCR9 expression [102]. However, these two cancer subsets show differential plasma levels of CCL25, with squamous cell carcinoma having higher levels of the chemokine. *In vitro* studies with NSCLC cell lines show that these cells increase their migration and invasive capacity when stimulated by CCL25, with the invasion process mediated by MMPs. In squamous cell carcinoma, MMP mediates invasion, while in adenocarcinoma, both MMP2 and MMP9 play a role in this process [102]. On the other side, it has been reported that the activation of the CCL25/CCR9 axis decreases apoptosis through the positive regulation of antiapoptotic signals, and the negative regulation of proapoptotic molecules [107]. In an *in vivo* model, silencing of this axis with small interfering RNAs diminished the size of the tumor. This data shows that in NSCLC, like in other types of cancer, the activation of the CCL25/CCR9 axis increases the neoplastic process [107].

8. Relevant CXC chemokines in NSCLC

Strieter *et al.* showed that the angiogenic/angiostatic activity of CXC chemokines is determined in most cases by the presence of the ELR amino acid motif. Thus, CXC-ELR$^+$ chemokines are angiogenic, while CXC-ELR$^-$ chemokines are angiostatic [36]. It is worth noting, however, that the chemokine CXCL12, which is an ELR$^-$ chemokine, has angiogenic activity. The CXC-ELR$^+$ group includes the chemokines CXCL1-3 and CXCL5-8 while the CXC-ELR$^-$ group includes CXCL4, CXCL9-11 and CXCL14 [29, 108] (table 3).

8.1 ELR$^+$ Chemokines

CXCL8

This chemokine has angiogenic and pro-inflammatory activity; it induces the proliferation, survival and migration of endothelial cells through its binding to CXCR1 and CXCR2 receptors, and the recruitment of neutrophils during inflammatory processes [46, 109, 110]. In contrast to SCLC cells, NSCLC cells produce substantial amounts of CXCL8 [111]. In human lung tumor tissue, the increased expression of CXCL8 is accompanied by increased vascularization and tumor growth, as well as metastasis to lymph nodes [112]. Furthermore, it has been reported that CXCL8 also has an effect on tumor cells, inducing the proliferation of lung cancer cells through CXCR1 [111] in human cells and through CXCR2 in animal models of tumor cell transfer [113]. It was recently reported that cell proliferation induced by CXCL8 involves the transactivation of the Epidermal Growth Factor Receptor (EGFR)[24], a protein overexpressed in 40-80% of NSCLC and associated with poor prognosis [2], as is also the increased expression of CXCL8 [114]. In addition, a recent report focused on grade IV lung adenocarcinoma, found that the expression of CXCL8 was associated with nutritional deterioration in patients [115]. The expression of CXCL8 is regulated by inflammatory cytokines such as TNF-α and IL-1 [116, 117], angiogenic molecules such as EGF [118], hypoxia [119] and the KRAS oncogene [120]. Cell lines with mutations in KRAS and EGFR have an increased expression of CXCL8, while the silencing of these molecules and treatment with tyrosine kinase inhibitors, decreases its expression [120]. Furthermore, studies on a model of human NSCLC carcinoma (H460) in immunodeficient rats suggested that the increase in serum levels of CXCL8 was associated with a decrease in the survival of animals [121].

CXCL5

This pro-inflammatory chemokine with angiogenic properties, induces neutrophil chemotaxis and is produced by epithelial cells [122]. It has been shown that density of blood vessels is greater in tumors expressing CXCL5 [123]. Arenberg *et al* found that

CXCL5 is an important angiogenic factor for NSCLC and that its expression is correlated with an increase in tumor mass [124]. By immunohistochemical analysis of fresh human biopsy samples of NSCLC it was found that CXCL5 was increased. In other cancers, such as in gastric hepatocellular carcinoma cell lines, an upregulated expression of CXCL5 is associated with a high metastatic potential [125].

It is important to mention that CXCL5 has a central role in the recruitment of leukocytes in lung inflammation induced by tobacco smoke, which is the most important risk factor for developing lung cancer [126]. In this regard, it has been described that CXCL5 (along with other ligands of CXCR2) promotes the migration of pro-tumor neutrophils and induces angiogenesis [127]. In this sense, the infiltration of neutrophils promoted by CXCL5 could have some cytotoxic activity. Unfortunately, this activity is especially effective in cells with low metastatic activity [128]. It has been recently shown that HB-EGF (heparin-binding EGF-like growth factor), in combination with CXCL5, has a synergistic effect on the proliferation, migration and invasion of lung cancer [129] and in some cases, this effect is dependent on the PI3K-Akt and ERK1/2 pathway [127].

Interestingly, a study on tumor tissue obtained from patients with stage I and II NSCLC found that out of 23 genes assessed by real time PCR, only CXCL5 showed a statistically significant difference, which led to propose it as a prognostic element in these patients [130].

Other CXCR2 ligands

It is important to note that CXCL8 and CXCL5 share the receptor CXCR2 with the chemokines CXCL1-3, 6-7[11]. Little is known about the implication of these chemokines in the pathophysiology of NSCLC, even though CXCR2 is a very important receptor in NSCLC [131]. Studies using tumorigenesis models in $CXCR2^{-/-}$ mice showed that this receptor has a central role in tumor growth, since CXCR2-deficient mice showed a significant decrease in tumor mass (associated with an increase in necrotic tissue), compared to wild type mice, while levels of the chemokines CXCL1-3 were increased [131].

8.2 ELR- Chemokines

CXCL9, CXCL10 and CXCL11, and their receptor CXCR3, are negative regulators of angiogenesis and are also involved in recruiting activated T cells and NK cells [132, 133]. There are reports that show differences in the expression of CXCL10 in different types of NSCLC. For example, the expression of this chemokine in adenocarcinoma is equivalent to normal tissue, while in lung squamous cell carcinoma the expression of CXCL10 is increased compared to normal tissue [113], and this chemokine is expressed mainly by tumor cells. It has been reported that the neutralization of CXCL10 augments vascularization in lung squamous cell carcinoma [134]. In addition, it was shown that plasma levels of CXCL10 are inversely proportional to the size of the tumor in patients and in tumorigenesis models of NSCLC in SCID mice [134]. In animal models, the intra-tumoral administration of CXCL10 for 8 weeks has an antineoplastic effect in which the size of the tumor decreases through a reduction in vascularization [113]. The metastatic activity is also partially abated, while there is an increase in apoptosis in the primary tumor. This effect appears to be dependent on the stage of the disease; if the treatment is prolonged for 10 weeks, the size of the tumor increases [134]. Interestingly, the antineoplastic effect of CXCL10 results in an increase in the survival of animals [135].

Recent studies in Lewis lung carcinoma, colon carcinoma (CT26) and breast carcinoma (4T1) murine models, tested the antitumor effect of a chimeric protein with CXCL10 and CXCL11 domains [136]. The CXCL10-CXCL11 chimeric protein was more effective than CXCL10 or CXCL11 separately in reducing the size of the tumor and inhibiting the recruitment of immune cells to the tumor infiltrate [136]. An important aspect is that in some cancers, such as colorectal carcinoma, CXCL10 has been reported to promote the invasion process through an increase in cell motility, although this does not seem to occur in primary cultures [137]. Furthermore, the chemokine CXCL9 is another ligand for the receptor CXCR3 with angiostatic function, and responsible for the recruitment of CD4+ and CD8+ T lymphocytes [138]. Apparently, there are no differences in the expression of CXCL9 in NSCLC compared with healthy lung tissue. However, in experimental models the expression of CXCL9 is associated with a decrease in vascularization and tumor size [138, 139].

CXCL14

This chemokine has angiostatic properties and a strong chemotactic activity for monocytes, macrophages and immature DCs [140]. CXCL14 differs from other chemokines in that it is ubiquitously distributed on normal tissue, and rarely expressed by cell lines or primary carcinomas [141, 142]. To date no receptor for this chemokine has been found [11], however recent results suggest that CXCL14 can bind glycoproteins with heparan sulfate or sialic acid, and induce proliferation and migration of NCI-H460 human lung cancer cells [143] Recently CXCL14 has appeared as an important tumor suppressing gene. The gene that codes for CXCL14 is silenced (by DNA hypermetila-

tion) in up to 80% of colorectal carcinomas in human, and its silencing seems to contribute towards an aggressive phenotype of neoplastic cells (i.e. increased motility and invasiveness) [144]. Furthermore, a recent study showed that transgenic mice overexpressing CXCL14 had a diminished increase in the size of transplanted tumor and number of metastases, an effect probably due to NK cells since the depletion of these cells with GM1 antibodies attenuates these effects and partially restores the phenotype of wild type mice [145]. In lung cancer, the forced expression of CXCL14 in the H23 lung adenocarcinoma cell line through Decitabine (an inhibitor of DNA methylation) treatment, stimulates necrosis and tumor size, and alters the expression pattern of pro-apoptotic genes and genes related to inhibition of cell cycle and, such as caspase 4 and RBP7, respectively [108]

CXCL12

This is one of the most studied chemokines in cancer and has been shown to be important in the angiogenesis, survival and metastasis of the tumor [25, 146, 147]. It is strongly expressed in several types of tumor, including breast, pancreas and lung cancer [148]. CXCL12 binds mainly to the CXCR4 receptor, which is up-regulated by hypoxia in various cell types, such as tumor-associated macrophages [149], endothelial cells and cancer cells [149-151]. It is also regulated by inflammatory stimuli which converge into the activation of NF-κB [152]. Recent studies have shown that CXCL12 also binds with high affinity to CXCR7 [153]. In breast cancer, both receptors are overexpressed in the primary tumor and the metastases [154]. In vitro studies of breast cancer showed that the activation of the CXCL12/CXCR7 axis mainly induces angiogenesis and a moderate chemotactic and invasive response, suggesting an important role of these molecules in metastasis [38]. However, in studies on murine models, only the pharmacological inhibition of the CXCL12/CXCR4 axis was effective in reducing metastasis to lymph nodes and lung, indicating that the metastasis is mainly mediated by the CXCL12/CXCR4 axis [25].

A high expression of CXCR4 in cancer cells was reported in NSCLC, while the chemokine CXCL12 was strongly expressed in the organs affected by metastasis such as bone marrow, adrenal glands, and liver [155]. Thus, it would be possible to form chemotactic gradients of CXCL12 that direct the migration of tumor cells to metastatic sites [25]. It has been shown *in vitro* that CXCL12 induces chemotaxis in lung cancer cell lines, while the neutralization of CXCL12 with antibodies in animal models reduces primary tumor metastasis [155]. Furthermore, human lung adenocarcinoma A549 cells transfected with CXCL12 have greater motility and increased expression of MMP-2 and MMP-9, which are associated with the invasion process [156]. In addition, it has been reported that the activation of CXCR4 by CXCL12 induces cancer cell survival [147].

Clinical studies in patients with grade IV pulmonary adenocarcinoma indicate that the increased expression of CXCR4 correlates with a decrease in survival of approximately 50% [157]. Furthermore, in patients with NSCLC who underwent surgical resection, increased levels of CXCR4 were associated with brain metastasis [158], denoting the metastatic role of this chemokine.

9. Relevant CX_3C chemokines in NSCLC

CX_3CL1 is the only known member of the CX_3C subfamily. In contrast to other chemokines, CX_3CL1 can be found in two forms: tethered to the cell membrane by a mucin-like stalk, or soluble [159] after digestion of the complete protein by metalloproteases such as ADAM7 and ADAM10 [160, 161]. Each form exhibits different properties: while soluble CX_3CL1 is mainly a chemotactic molecule (attracting predominantly NK cells, monocytes and CD8+ T cells), membrane bound CX_3CL1 is able to mediate the binding of monocytes and NK cells to the endothelium [162-164], since CX_3CL1 is expressed on the surface of activated endothelial cells [165]. CX_3CR1 is the only known receptor for CX_3CL1 [11]. The CX_3CL1/CX_3CR1 axis plays a role in the recruitment of immune cells in inflammation, angiogenesis, proliferation and survival of endothelial and smooth muscle cells [166-168]. The role of the CX_3CL1/CX_3CR1 axis in the neoplastic process is controversial. On the one hand, its activation has been related to pro-tumoral processes such as an increase of migration of clear cell renal cell carcinoma and proliferation in breast cancer [169, 170], while high levels of CX_3CR1 or CX_3CL1 expression has been associated with the metastatic status and a reduced patient survival [169, 171]; on the other hand it has also been reported that a high expression of CX_3CL1 in human breast cancer cells correlates with the infiltration of cytotoxic cells ($CD8^+$ T cells and NK cells) and DCs, and with higher overall patient survival [172]. The antitumor effect of the CX_3CL1/CX_3CR1 axis is supported by studies in $CX_3CR1^{-/-}$ mice inoculated with B16 metastatic melanoma cells [173]. In this model, the lack of CX_3CR1 was associated with larger tumors, neoplastic process-associated cachexia, and a significant reduction of recruitment of NK cells to the lungs of these mice [173]. Several studies show that, in NSCLC, the activation of the CX_3CL1/CX_3CR1 axis has an important antitumor effect. Using the Lewis Lung Carcinoma (LCC) transfer model with cells expressing CX_3CL1

(3LL-FK) or mock transfected cells (3LL-mock) injected into the lung of C57BL/6, it was found that mice that received 3LL-FK cells had smaller tumors, less metastasis (up to 10 times less), and prolonged survival compared with mice inoculated with 3LL-mock cells [174, 175]. *In vivo* depletion of certain cell subtypes indicate that $CD8^+$ T cells and NK cells have a role in the inhibition of the growth of the tumor in mice that received 3LL-FK cells [176]. Regarding the antitumoral mechanisms induced by the transfer of 3LL-FK cells, it was found that the cytotoxic activity of CTL was increased against LCC, due to DCs and NK cells. Mice that received 3LL-FK cells had an augmented number of infiltrating DCs and NK cells in the tumor. These cells were potentially recruited through the CX_3CL1/CX_3CR1 axis, since conditioned media from 3LL-FK cells induced an *in vitro* migration of these cells that could be blocked with a neutralizing antibody against CX_3CL1, and it has been found that membrane bound CX_3CL1 mediates the binding of NK cells [176]. Coculture of DCs with 3LL-FK induces the maturation of DCs [174], while coculture of NK cells with 3LL-FK increases cytotoxic activity against 3LL and the production of IL-12 [176].

According to a recent publication by Savai et al., coexpression of CX_3CR1 and CCR2 by tumor associated macrophages could have important therapeutic roles in NSCLC [177]. The authors explore *in vitro, in vivo* and *ex vivo* the role of TAM in the growth and metastasis of NSCLC mediated by these receptors. Macrophages from Lewis lung carcinoma infected mice were cocultured with several human NSCLC lines, resulting in an upregulation of the CX_3CL1/CX_3CR1 and CCL2/CCR2 axis both in neoplastic cell lines and in macrophages. This upregulation correlated with an increase in carcinogenesis, particularly related to the proliferation and migration of the neoplastic cell lines. *In vivo* assays showed that higher expression of the CX_3CL1/CX_3CR1 and CCL2/CCR2 axis increases the polarization of TAMs toward an M2 phenotype. When macrophages were depleted using clodronate liposomes or FAS-induced apoptosis, or when concurrently blocking the expression of CCR2 and CX_3CR1, tumor growth and metastasis were inhibited; this effect was accompanied by a polarization toward the M1 phenotype, and an increase in survival of the mice [177], and the switch toward an M1 phenotype inhibited tumor growth, an effect probably related to the profile of cytokines, chemokines and growth factors secreted by macrophages.

10. Relevant XC chemokines in NSCLC

Chemokines belonging to this family have only two of the four conserved cysteine residues present in other chemokines. In humans there are only two members in this subfamily: XCL1 (Lymphotactin) and XCL2 (SCM1-β); both proteins have a very similar structure, differing in only two residues, and they have a slightly different affinity for heparin [178]. XCL1 is expressed by different lymphoid cells, including activated CD8+ T cells, CD4+ T cells and NK cells [179, 180]; while XCL2 is expressed by macrophages, NK cells and CD8+ T Cells. Both chemokines activate the same receptor, XCR1, with virtually identical functional profiles *in vitro*, leading to calcium mobilization and chemotaxis [178]. In humans, the mRNA for this receptor has been detected in placenta, spleen, thymus and neutrophils [181, 182]. It is known that the activation of XCR1 by XCL1/2 induces the migration and proliferation of cells from human epithelial ovarian carcinoma [183]. However, the evidence about the role of XCL1/2 or XCR1 on NSCLC is scarce or non-existent. XCL1 seems to have natural adjuvant properties that might help antitumor responses by both adaptive and innate immune response, and therefore could be important in immunotherapies directed against cancer [184, 185]. For example, Cairns et al. transfected the SP2/0 myeloma cell line with the XCL1 gen before testing their ability to form tumors in mice. In this model, XCL1 expressing SP2/0 tumors regressed and became infiltrated with lymphocytes and neutrophils [185]. On the other hand Zhang et al. showed that DCs transduced with the XCL1 gene are better than WT DCs at inducing protective and therapeutic antitumor immunity through improved chemotaxis of T cells towards DCs in the tumor model of 3LL lung carcinoma [184]. These findings mean that XCL1 could play a role in the gene therapy of NSCLC.

11. Regulation of the chemokine system through the expression of atypical receptors in NSCLC

Accumulating evidence indicates that the atypical chemokine receptors may be relevant in cancer. For example, the chemokine receptor ACKR2 (also known as the Duffy antigen) inhibits growth and metastatic potential in breast cancer [186]. *In vitro,* the overexpression of ACKR2 in breast cancer cells (MDA-MB-231 and MDA-MB-435) inhibits proliferation and invasion; while the chemokine ligands for ACKR2 (CCL2, CCL4, CCL13 and CCL22) decreased in conditioned culture media. *In vivo*, D6 overexpression inhibits vessel density, tumor growth, metastasis, angiogenesis and TAM infiltration [186]. Moreover, recent studies showed that the expression of CCX-CK is involved in the metastasis of mammary carcinoma, promoting a more aggressive and motile phenotype through an increase in the expression of TGF-β in the

transformed cells [187].

In NSCLC studies on the A549 lung cancer line, it was shown that the overexpression of ACKR2 leads to a moderate but significant decrease in cellular proliferation [188]. In addition, the authors found a decrease in the concentration of CCL2, CCL4 and CCL5 in the conditioned culture medium, while no changes were found in the messenger RNA levels of these chemokines. Importantly, although the chemokines CCL3 and CXCL12 were tested in this study, no changes were observed in the level of these proteins as a result of the overexpression of ACKR2 [188]. Additionally, studies on a tumor transfer model using BALB/c mice showed that the overexpression of ACKR2 is accompanied by a decrease in tumor size [188]. Together, these studies indicate that the ACKR2 receptor controls the local availability of chemokines through the specific sequestration of CCL2, CCL4 and CCL5, suggesting that deregulation of the expression of this receptor could lead to changes in the tumor microenvironment, with important consequences.

12. Concluding remarks

In cancer, the involvement of chemokines and their receptors comprises several aspects. First, chemokines regulate, through the activation of endothelial cells, the angiogenesis that supports tumor growth. Furthermore, chemokines and their receptors contribute actively to the formation of the tumor microenvironment through the recruitment of infiltrating tumor cells such as tumor-associated macrophages. Infiltrating tumor cells change the tumor microenvironment by secreting cytokines, chemokines, growth factors, and other effector proteins (Figure 1). These molecules also contribute to the recruitment of other cell types, such as regulatory T cells, and are also important in the recruitment of TAN, which can contribute to genomic instability, thereby promoting the process of carcinogenesis. Moreover, it has been shown that many chemokines have direct effects on tumor cells and are able to regulate their proliferation, survival and migration. The role of angiogenesis in solid tumor growth has attracted a great deal of attention as a potential therapeutic target. Lung cancer is the main cancer-related cause of death worldwide in both men and women. Although much is still unknown about the role of chemokines in NSCLC, the evidence shown here indicates that these molecules and their receptors play a major role in the pathophysiology of this disease. Additional studies that examine the role of specific chemokine/receptor axes at different stages of lung cancer would be of great importance to understand the role of these molecules in the course of the disease and to establish the differences in the activation of chemokine receptors through different ligands.

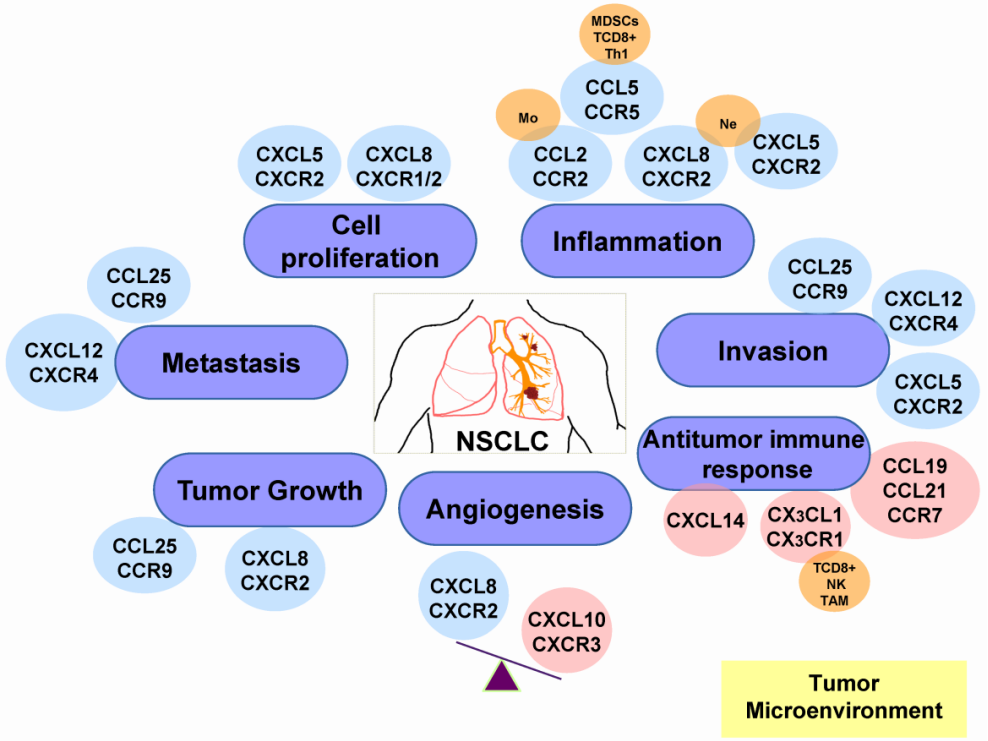

Figure 1: Chemokine ligand/receptor axis involved in the pathophysiology of NSCLC. The pathophysiology of NSCLC involves several processes, including tumor growth, angiogenesis, cell proliferation, recruitment of immune cells, invasion, metastasis, and occasionally antitumoral immune response. The figure shows the main Chemokine ligand/receptor axes involved in these processes.

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Competing Interests

No competing interests were disclosed.

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