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Elucidating the impact of IL-17A/F in lung cancer

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“In the middle of difficulty lies opportunity.”

Albert Einstein

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ABSTRACT

Lung cancer is the leading cause of cancer mortality with an incidence of approximately 1.6 million new cases per year. More recently, several reports have been associating inflammation with development of lung carcinogenesis. The inflammation arises from the interplay between host cells and signaling molecules, such as cytokines.

Despite their well-characterized proinflammatory functions, the role of interleukin-17 (IL-17) cytokine family members in tumor pathogenesis remains controversial. While interleukin-17 A (IL-17A) exhibited both pro and anti-tumor roles, interleukin-17 F (IL-17F), which shares the strongest sequence homology to IL-17A, has been suggested as a candidate for cancer therapy. As for lung cancer, while IL-17A has been found to augment tumor growth, the relevance of IL-17F has not been addressed. The re-analysis of lung cancer ribonucleic acid (RNA) expression of public data sets showed a positive correlation between IL-17F expression and patient survival. Thus, the aim of this project was to analyze in depth the effects of IL-17A and IL-17F in lung cancer. Specifically, we addressed the pro or anti-tumoral function of both cytokines in several cell features, such as migration, cell viability and metabolism.

To address whether IL-17A/F effect is specific to tumoral cell, we stimulated human and mice lung cancer cell lines *in vitro*. No alteration in viability, migration and metabolism was observed upon stimulation with IL-17A/F. Macrophages are one of the most abundant immune cells in tumor microenvironment (TME) with a significant role in tumor progression in several cancers. We have previously observed that IL-17F induce a quiescent state on murine bone marrow macrophages. Therefore, we hypothesized that the effect of IL-17F on lung cancer might be indirectly via immune cells, specifically macrophages. To evaluate the effect of IL-17A/F on the crosstalk between the lung cancer cells and macrophages, we submit Lewis Lung Carcinoma 1 (LLC1) cell line to conditioned media (CM) of IL-17A or F stimulated macrophages. Interestingly, CM from IL-17F stimulated macrophages, promoted murine lung cancer cells progression through a enhance migration capacity and both CM enhanced *in vivo* tumor growth. Contrarily of The Cancer Genome Atlas (TCGA) analysis, our results indicate a pro-tumoral role of both cytokines through macrophage manipulation. Future studies will evaluate the intratumoral overexpression of each cytokine in immunocompetent murine models. Alternatively, the effect of each cytokine on other immune cell types should be aimed to provide a global picture of potential pro- or anti-tumoral effect.

RESUMO

O cancro do pulmão é a principal causa de morte por cancro, com uma incidência de aproximadamente 1,6 milhões de novos casos por ano. Recentemente, vários estudos relacionaram o envolvimento da inflamação no desenvolvimento da neoplasia pulmonar. A inflamação é caracterizada pela interação entre as células do sistema imune e as moléculas de sinalização, como as interleucinas.

Apesar das suas funções pró-inflamatórias estarem bem caracterizadas, o papel da família da Interleucina-17 (IL-17) na progressão tumoral permanece controverso. Enquanto a Interleucina-17A (IL-17A) demonstrou funções pró- e anti- tumorais, a Interleucina-17F (IL-17F), que compartilha forte homologia com a IL-17A, tem sido sugerida como um candidato para a terapia do cancro. Relativamente ao cancro do pulmão, enquanto a IL-17A foi associada ao crescimento tumoral, a relevância da IL-17F não foi abordada. A reanálise da expressão de ácido ribonucleico (RNA) a partir de dados públicos, demostrou uma correlação positiva entre a expressão de IL-17F e uma melhor sobrevida nos pacientes. Assim, o objetivo deste projeto é analisar a função da IL-17A e F no cancro de pulmão. Especificamente, abordamos o efeito de ambas interleucinas em várias características celulares, tais como a viabilidade, migração e metabolismo.

Para abordar se o efeito da IL-17A/F é específico para células tumorais, estimulamos, *in vitro,* linhas humanas e de ratinho. Nenhuma alteração na viabilidade, migração e metabolismo foi observada após estimulação com IL-17A/F. Os macrófagos são uma das células imunes mais abundantes no microambiente tumoral, com um papel significativo na progressão tumoral em vários tipos de cancro. De facto, observamos que a IL-17F induz um estado quiescente em macrófagos da medula óssea de ratinho. Portanto, sugerimos que o efeito da IL-17F pode ser indiretamente via macrófagos. Para tal, submetemos a linhagem Lewis Lung Carcinoma 1 (LLC1) ao meio condicionado (CM) de macrófagos estimulados com IL-17A ou F. Curiosamente, o CM proveniente da estimulação com IL-17F, promoveu a capacidade migratória das células tumorais, e ambos os CM aumentaram o crescimento tumoral *in vivo*. Ao contrário da análise do Atlas do Genoma do Cancro (TCGA), nossos resultados indicam um papel pró-tumoral de ambas as interleucinas através da manipulação de macrófagos. Estudos futuros avaliarão a sobreexpressão intratumoral de cada interleucina, em modelos murinos imunocompetentes. Alternativamente, o efeito de cada interleucina em outros tipos de células imunológicas deve ser estudado, de modo a fornecer uma visão global do potencial efeito pró- ou anti- tumoral.

CONTENTS

List of Abbreviations

Acetyl-CoA	acetyl coenzyme A
ACT	Adoptive cell transfer
AdenoCA	Adenocarcinoma
AIS	Adenocarcinoma *in situ*
AKT/PKB	Protein kinase B
ALK	Anaplastic lymphoma kinase
AMP	Antimicrobial Peptides
ANOVA	Analysis Of Variance
AP-1	Activator Protein-1
APC	Antigen-presenting cell
Arg-1	Arginase-1
BAFF	B-cell Activating-Factor
BCL-2	B-cell lymphoma 2
BCL-xL	B-cell lymphoma-extra large
BMDCs	Bone marrow-derived cells
BMDM	Bone Marrow Derived Macrophages
C/EBP	CCAAT-Enhancer Binding Protein
CAFs	Cancer Associated Fibroblasts
CAIX/CA9	Carbonic Anhydrase IX
CAM	Chorioallantoic Membrane
CBAD	C/EBP Beta Activation Domain
CCL20	Chemokine (C-C motif) ligand 20
CCL7	Chemokine (C-C motif) Ligand 7
cDNA	complementary DNA
CM	Conditioned Media
CO_2	Carbon dioxide
COPD	Chronic Obstructive Pulmonary Disease
CSF-1	Colony Stimulating Factor-1
CTL	Cytotoxic T Lymphocytes
CTLA-4	Cytotoxic T Lymphocyte Associated- antigen 4
CTLA-8	Cytotoxic T Lymphocyte Associated- antigen 8
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL10	Chemokine (C-X-C motif) ligand 10
CXCL2	Chemokine (C-X-C motif) ligand 2
CXCL5	Chemokine (C-X-C motif) ligand 5

CXCL7	Chemokine (C-X-C motif) ligand 7
CXCL9	Chemokine (C-X-C motif) ligand 9
DAMP	Damage-Associated Patterns
DAPI	4′,6-Diamidino-2-Phenylindole
DC	Dentritic cells
DMEM	Dulbecco's Modified Eagle Medium
EAE	Experimental Autoimmune Encephalomyelitis
EBT	External Beam Therapy
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-Mesenchymal Transition
ERK-1	Extracellular signal–regulated kinase- 1
ERK-2	Extracellular signal–regulated kinase- 2
ES-SCLC	Extensive-stage small cell lung cancer
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FN	Fibronectin
GC	Germinal Centers
G-CSF	Granulocyte-Colony Stimulating Factor
Glut1	Glucose Transporter 1
Glut2	Glucose Transporter 2
GM-CSF	Granulocyte Macrophage- Colony Stimulating Factor
HK2	Hexokinase 2
HPLC	High Perfomance Liquid Chromatography
Hsp90	Heat shock protein 90
HSV13	Herpes Simplex Virus 13
HuR	Human antigen R
ICAM-1	Intercellular Adhesion Molecule-1
IECs	Intestinal epithelial cells
IFN	Interferon
IFN-α	Interferon- alpha
IFN-β	Interferon- beta
IFN-γ	Interferon- gamma
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-15	Interleukin-15
IL-17	Interleukin-17
IL-17B	Interleukin-17B
IL-17C	Interleukin-17C

IL-17D	Interleukin-17D
IL-17E	Interleukin-17E
IL-17R	Interleukin-17 Receptor
IL-17RB	Interleukin-17 Receptor B
IL-17RC	Interleukin-17 Receptor C
IL-17RD	Interleukin-17 Receptor D
IL-17RE	Interleukin-17 Receptor E
IL-1β	Interleukin-1 β
IL-2	Interleukin-2
IL-21	Interleukin-21
IL-22	Interleukin-22
IL-23	Interleukin-23
IL-6	Interleukin- 6
IL-8	Interleukin- 8
ILC3	Type 3 Innate Lymphoid Cells
KO	Knockout
LC	Lung Cancer
LCAC	Large Cell Anaplastic Carcinoma
LCCM	L929- Cell Conditioned Medium
LDHA	Lactate Dehydrogenase A
LLC1	Lewis Lung Carcinoma 1
LS-SCLC	Limited-stage small-cell lung cancer
LTi	Lymphoid Tissue inducer cells
MAPK	Mitogen-Activated Protein Kinase
MCA	3 Methylcholanthrene
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage- Colony Stimulating Factor
MCT1	Monocarboxylate Transporter 1
MCT4	Monocarboxylate Transporter 4
MDSC	Myeloid- Derived Supressor Cells
MEF	Mouse embryonic fibroblasts
MIA	Minimally Invasive Adenocarcinoma
MICA/B	Major histocompatibility complex class I-related chain A/B
miR-192	microRNA-192
MMP	Matrix metalloproteinase
MMP1	Matrix metalloproteinase 1
MMP13	Matrix metalloproteinase 13
MMP2	Matrix metalloproteinase 2
MMP3	Matrix metalloproteinase 3

MMP8	Matrix metalloproteinase 8
MMP9	Matrix metalloproteinase 9
mRNA	messenger RNA
NF-kB	Nuclear Factor Kappa B
NK	Natural killers
NO	Nitric Oxide
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NSCLC	Non-Small Cell Lung Cancer
OS	Overall Survival
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate Buffered Saline
PD-1	Programmed cell Death-1
PDH	Pyruvate dehydrogenase
PDK	Pyruvate Dehydrogenase Kinase
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PFS	Progression-free survival
PGE2	Prostaglandin E2
RA-FLS	Rheumatoid arthritis- Fibroblast-like synoviocytes
RFA	Radiofrequency ablation
ROS	Reactive Oxygen Species
SBRT	Stereotactic body radiotherapy
SCC	Small Cell Carcinoma
SCLC	Small-cell lung cancer
SCO2	Cytochrome c oxidase (COX) assembly
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
SQCLC	Squamous Cell Lung Cancer
SRB	Sulforhodamine B
STAT	Signal Transducer and Activator of Transcription
STAT3	Signal transducer and Activator of Ttranscription 3
TAM	Tumor-Associated Macrophages
TBS-T	TBS-Tween
TCA	Trichloroacetic Acid
TCGA	The Cancer Genome Atlas
TCR	T-cell Receptor
TFF1	Trefoil factor 1
TGF-β	Transforming Growth Factor- beta
Th1	Helper T cells 1
Th17	Helper T cells 17

Th2	Helper T cells 2
TKI	Tyrosine kinase inhibitor
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
TNF-α	Tumor Necrosis Factor- alpha
TNM	Tumor Node Metastasis
TRAF2	TNF Receptor Associated Factor 2
TRAF4	TNF Receptor Associated Factor 4
TRAF5	TNF Receptor Associated Factor 5
TRAF6	TNF Receptor Associated Factor 6
Treg	regulatory T cells
VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular Endothelial Grow Factor
VEGF-C	Vascular Endothelial Growth Factor C
WHO	World Health Organization
WT	Wild Type
ZEB1	Zinc Finger E-Box Binding Homeobox 1
γδT	Gamma delta T cells

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CHAPTER 1: INTRODUCTION

1. General Introduction

Accordingly to the World Health Organization (WHO), cancer was accountable for 8.2 million deaths in 2012, being classified as the leading cause of death globally[1]. This class of diseases, responsible for nearly 1 in 6 deaths worldwide, is characterized by uncontrolled growth and spread of abnormal cells. Through a series of genetic and epigenetic alterations, these modified cells start to grow uncontrollably developing tumors that can become malignant if it spreads to others parts of the body, this latter process is the major cause of death from cancer[2] .

Underlying these alterations, tumor cells acquires the capacity to sustain proliferative signaling, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis and activate invasion and metastasis. These six hallmarks of cancer, complementary to each other, enables tumor cells growth and metastatic propagation[3]. The progress made during the last decade in cancer biology allowed the addition of two new emerging hallmarks: the reprogramming of energy metabolism and evading immune destruction[4].

In opposition to normal cells that prefers to oxidize glucose when oxygen is present, cancer cells prefers the conversion of glucose to lactic acid even in the presence of oxygen. This phenomenon is known as "aerobic glycolysis" or Warburg effect[5], but in the opposition to Otto Warburg hypothesis, tumor cells can access a hybrid state with coexistence of both metabolic modes, oxidative phosphorylation (OXPHOS) and glycolysis. This hybrid phenotype contributes to their metabolic plasticity, allowing cancer cells to adapt to microenvironment area, making it difficult to identify effective metabolic targets[6,7].

One of the mechanisms of the aerobic glycolysis phenotype is through the activation of a transcription factor, termed as hypoxia-inducible factor-1 alpha (HIF-1α), which is upregulated by several stressors factors, such as hypoxic, oncogenic, inflammatory, metabolic and oxidative stress. In turn, HIF-1α increases the expression of several key intermediators in conversion of glucose to lactate, such as glucose transporter isoform 1 and 3 (GLUT1 and GLUT3, respectively), hexokinase 1 and 2 (HK1 and HK2, respectively) that are responsible for the initial steps of glycolysis, and lactate dehydrogenase A (LDHA) that transforms pyruvate into lactate. Also, this transcription factor enhances the extrusion of this metabolite by enhancing the lactate-extruding enzyme monocarboxylate transporter 4 (MCT4)[8,9]. In addition another monocarboxylate transporter regulated by c-myc expression, the monocarboxylate transporter 1 (MCT1), is important regulator of intracellular pH homeostasis as it regulates both influx

and efflux of lactate[10]. Furthermore, HIF-1α inhibits the OXPHOS pathway by increasing the expression of pyruvate dehydrogenase kinase 1 (PDK1) that targets pyruvate dehydrogenase (PDH), and consequently decreases the conversion of pyruvate to acetyl coenzyme A (acetyl-CoA)[11]. This metabolic phenotype confers an adaptation to intermittent hypoxia in pre-malignant lesions, as they grow progressively apart from the blood supply. Moreover, the constant release of lactate that contributes to microenvironmental acidosis, requires a phenotype capable of dealing with acid-induced cell toxicity. In fact in response to hypoxia, tumor cells upregulates the expression of a transmembrane enzyme, the carbonic anhydrase isoform 9 (CAIX/CA9), that maintains a normal intracellular pH by the hydration of extracellular carbon dioxide (CO_2)[12].Therefore, tumor cells that present this particular phenotype have a powerful growth advantage compared to normal cells, exhibiting unconstrained proliferation and invasion capacity, by inducing damage to adjacent normal cells[13].

The importance of the immune system in the control of tumor growth and in the modulation of their variants, has been demonstrated[14,15]. Has Burnet and Lewis proposed[16,17], immune system can play a vital role in the eradication of tumor before it clinical appearance . This hypothesis, termed as "cancer immunosurveillance" was supported by studies demonstrating the importance of interferon- gamma (IFN-γ) through increased incidence of lymphomas in mice presenting a knockout (KO) for this cytokine[18] or by lymphocytes action through a KO for perforin, an important glycoprotein that mediates lymphocyte-dependent killing[19], with a more incidence of tumors as compared with perforin-sufficient mice treated in the same conditions[20–22]. Several more evidences (reviewed in [15]) supports this hypothesis, however it does not explain the appearance of tumors in immunocompetent hosts. With the idea that cancer cells can be modulated by the immune system in a way to eliminate highly immunoreactive cells clones and to maintain the cells clones presenting less immunoreactive phenotype, it can explain the tumor development in individuals with functional immune system. In fact, this theory was demonstrated though transplantation of tumors developed in immunodeficient mice that grew more slowly than tumors developed in wild-type mice (reviewed in [15]).

The concept of immunoprotection against tumor cells and the shape of tumor immunogenicity set the basis to understand the cancer immunoediting hypothesis that postulates the dual role of immune system in tumor development but also in host-protection[23]. In this hypothesis a sequential process proceeds from "elimination", "equilibrium" to "escape" (Figure 1). Although, in some cases there is only a phase of escape or equilibrium without passing through an earlier phase or changes in the direction of the flow by external factors. These alterations might be due to influences of environmental stress, immune

system deterioration with aging, and also by alteration through immunotherapeutic intervention in human patients[23].

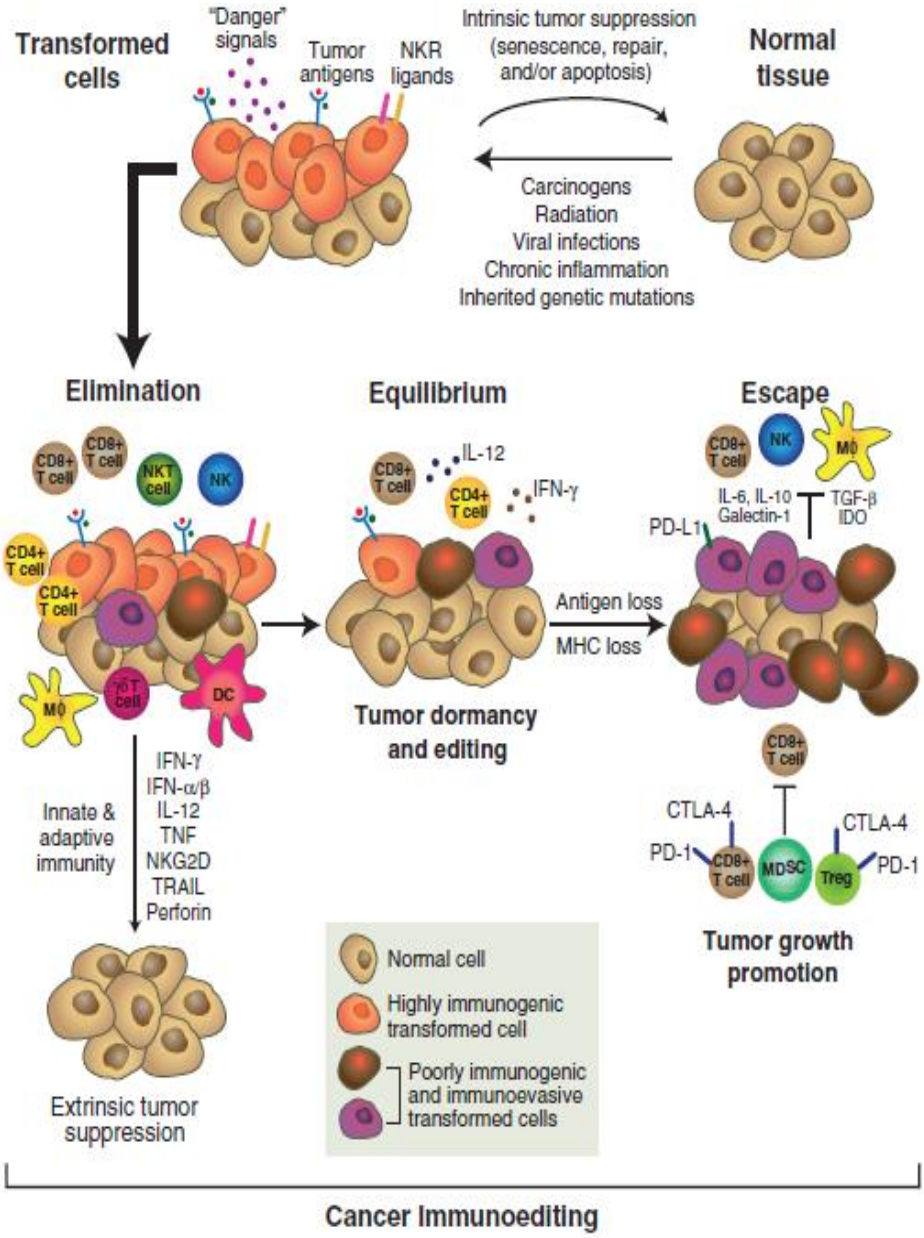


Figure 1. **The overview of cancer immunoediting hypothesis.** After transformation of normal cells to cancer cells and without intrinsic tumor suppressors, cancer cells undergo through immunoediting process, which consists three sequential phases. In the first phase, the elimination phase, both innate and adaptive cells works together in order to eradicate the tumor growth, if the tumor is eliminated then the process is finished. However, sometimes some cancer cells survive under a strict control by the adaptive immune cells. This happens at equilibrium phase where tumor cells are in state of dormancy and can last for the lifetime of the host. Also, in this phase is where the cells are modulated by the immune system, and if the tumor cells gain characteristics that are selective benefits, these cells will expand and enter in the last phase, the escape phase, in which the immune system cannot control and can promote the tumor growth, as they become clinically apparent disease [23].

During the early development of cancer, the induction of danger "signals" such as releasement of molecules by dying tumor cells or from damaged tissues knowing as damage-associated patterns molecules (DAMPs), induces the activation of receptors of both innate and adaptive system. This leads to a secretion of cytokines and chemokines, which in turn, recruits the immune cells at tumor site in an attempt of an anti-tumoral response towards a suppression or elimination of tumor before become clinically visible [24]. If the tumor is eradicated, then the process of immunoediting is consisted only by the elimination phase. However, variants of tumor cells can survive through a state of dormancy (equilibrium phase), in which immune cells controls the tumor outgrowth but also shapes their immunogenicity[23]. The tumor latency in the equilibrium phase was demonstrated in mice rechallenged with same tumor or in immunocompetent mice receiving low doses of 3´-methylcholantrene (MCA), a carcinogen. After depletion of T cells and IFN-γby injection of monoclonal antibodies in these mice, the tumor emerged in half of the mice, presenting a highly immunogenic phenotype, which highlights the importance of the immune system as a master modulator of the tumor. Moreover, there was a high presence of adaptive immunity, especially interleukin-12 (IL-12), IFN- γ, $CD4^+$ and $CD8^+$ T cells in this phase, in contrast to innate immunity showing a more relevant function of the adaptive immune response in this stage [25] .

This constant pressure of immune system in tumor cells can lead to a selection of immunoresistant clones responsible for the tumor outgrowth. Loss of surface antigens, resistance to immune cell cytotoxic effects or a persistent activation of proto-oncogenic transcription factors as signal transducer and activator of transcription 3 (STAT3) to reduce the apoptotic effects, are some examples of mechanisms of tumor evasion from the immune system[23]. Moreover, tumor cells can also induce an immunosuppressive state within tumor microenvironment[26], through a release of immunosuppressive cytokines as vascular endothelial grow factor (VEGF)[27] and/or by recruitment of immunosuppressive leukocytes comprising regulatory T (Treg) cells and myeloids-derived suppressor cells (MDSCs).

Inflammation is an immune system response to reestablish tissue homeostasis from stressors such as infection or tissue damage[28]. This physiological process has been associated with development of cancer. In fact, epidemiological studies showed a long-term inflammation with development of dysplasia, being accountable for 15 % of cancer incidence and mortality worldwide[29,30].In a particular case of the lung cancer (LC), there was an independent connection between inflammatory molecules and its effectors, with tumor progression and survival in LC patients with advanced stage[31,32].

Despite the improvements in diagnosis and therapy, the prognosis of LC is still poor. Understanding how molecules and effectors are evolved in the relation between the immune system and cancer progression, can open new avenues of immunotherapeutic approaches in lung cancer.

2. LUNG CANCER

2.1 Epidemiology and Etiology

Lung cancer is the most prominent cause of cancer mortality in the world, presenting 1.8 million new cases and 1.59 million deaths in 2012 [33,34]. Among men, this cancer type is the most common and the primary cause of cancer death, while in women is presented as the third most common and the second leading cause of cancer death [33].

Regarding global geographic distribution, a higher incidence rates were observed in Central and Eastern Europe (53.5 per 100,000) and Eastern Asia (50.4 per 100,000) for men. Contrarily, low incidence rates were noticed in Middle and Western Africa (2.0 and 1.7 per 100,000, respectively). In the case of women, the incidence rates are lower although presenting a difference in geographic distribution according with different historical exposure to tobacco. Therefore, higher estimated rates were in Northern America (33.8) and Northern Europe (23.7) and in Eastern Asia (19.2), while the lowest rates were found in Western and Middle Africa (1.1 and 0.8, respectively). In the case of lung cancer mortality, the overall ratio of mortality to incidence is 0.87, presenting similar geographic patterns with those in incidence[35].

Regardless of new diagnostic and genetic technologies, progressions in surgical techniques and new treatments, such as targeted and immunotherapeutic treatments, the overall 5-year survival rate for lung cancer is still very reduced, being 15.6% in United states (2005-2011), and even worse in Europe, China and developing countries with only 8.9% [36]. Risk factors for acquiring lung cancer includes radon exposure, air pollution, harmful occupational exposure, hereditary susceptibility, radiation exposure and unbalanced diet [35]. Most importantly, the incidence of lung cancer is highly connected with addiction to cigarettes worldwide[37], being 80% of cases in men and 50% in women associated to smoking[38]. Numerous

researches have suggested more risk factors, such as immunodeficiency, inflammation and virus infection, but this remains debatable[35,36].

2.2 Diagnosis and classification

Described as highly heterogeneous tumors, LC can arise in different locations in the bronchial tree, thus presenting variable symptoms according with their anatomic location. Furthermore, LC are characterized by its histological features, dividing mainly into non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). NSCLC is further subdivided in adenocarcinoma (AdenoCA), squamous cell lung cancers (SQCLC) and large cell anaplastic carcinoma (LCAC) (table1)[39].

Table 1. **Types of lung cancer.** Adapted from[39].

Lung Cancer Type	Anatomic Location	% of All Lung Cancer
Adenocarcinomas (AdenoCA)	Arise in peripheral bronchi	40 %
Squamous cell lung cancers (SQCLC)	Arise in main bronchi and advance to the carina	25-30%
Large cell anaplastic carcinomas (LCAC)	Tumors lack the classic glandular or squamous morphology	10 %
Small cell lung cancers (SCLC)	Derive from the hormonal cells.Disseminate into submucosal lymphatic vessels and regional lymph nodes almost without a bronchial invasion	10-15 %

Inside NSCLC, the most predominant form is AdenoCA accounting for approximately 40% of all LC that is characterized by the appearance in peripheral bronchi (Table 1). Particularly, it is further subdivided according with invasiveness into adenocarcinoma *in situ* (AIS) and minimally invasive adenocarcinoma (MIA). The second most abundant form of NSCLC is SQCLC, primarily caused by smoking that represents about 25%-30% of all lung cancers. SQCLC arise initially in the main bronchi and advance to the carina. LCAC that represents 10 % of all NSCLC can grow in any part of the lung and grows and spreads quickly. However, the most aggressive histological type are SCLCs, comprising about 10%-15% of all lung cancers. It derives from hormonal cells of the lung, being the most dedifferentiated cancers and have a tendency to be mediastinal tumors [39].

Given the importance of cancer staging in diagnosis process, LC is categorized into stages according with international TNM (Tumor Node Metastasis)-based staging system (Figure 2), which describes the anatomical extent of the disease by the size and range of the primary tumor, the presence or absence of distant metastatic extent and the involvement of regional lymph nodes. NSCLC are staged from one to four, being the one with lower cancer spread and four with higher spread. Regarding SCLC, it is defined by two stages: Limited-stage disease (LS-SCLC), restricted to the hemithorax of origin, the mediastinum, or the supraclavicular lymph nodes and extensive-stage disease (ES-SCLC) which is spread outside of supraclavicular areas[40].

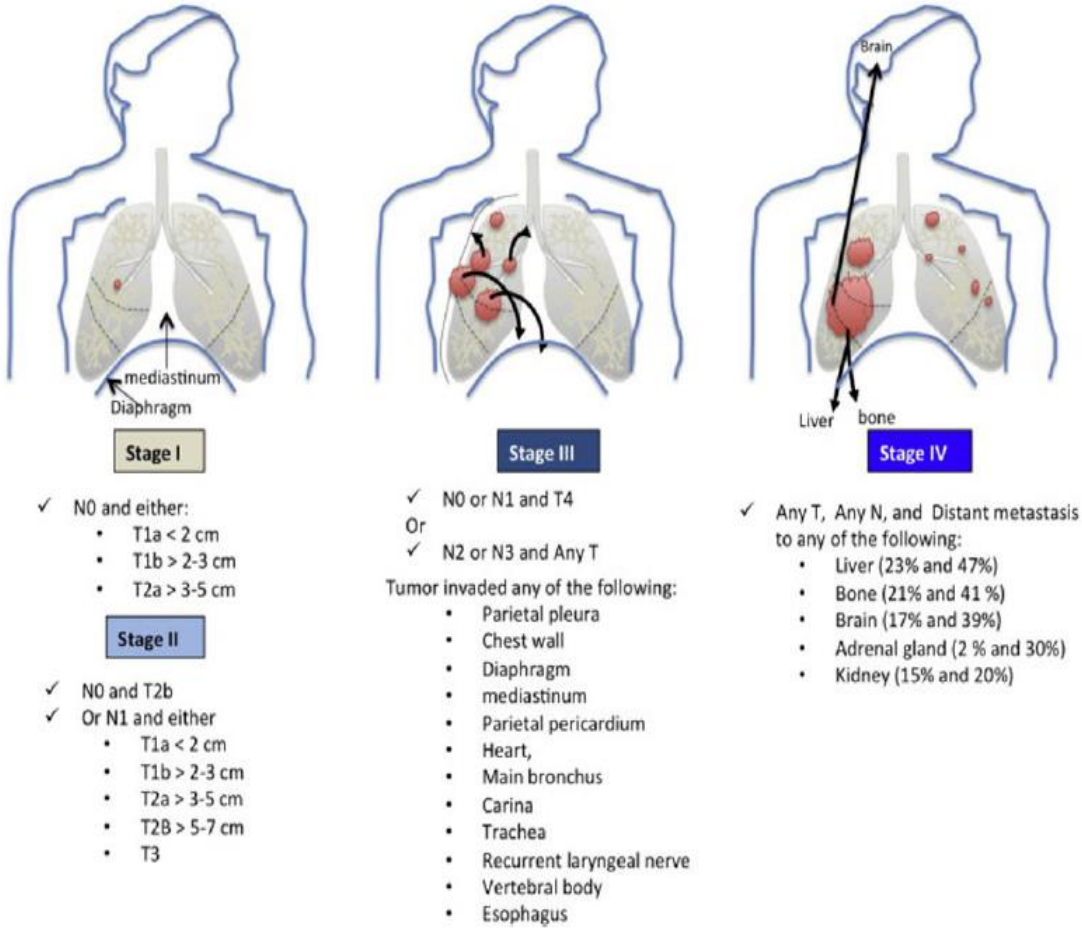


Figure 2. **Schematic representation of NSCLC staging**. Of note, in stage IV is represented the incidence of distant metastases to extrathoracic organs. Moreover, for each organ, is depicted the percentage of distant metastasis for SQCLC and AdenoCA, respectively [39].

2.3 Therapy and outcomes

The biggest challenge in lung cancer is the fact that about 70% of patients are diagnosed with advanced stage disease (stage III or IV)[41]. For about 25 % of patients that represents the stage I and II of NSCLC, the first line of therapy approach is surgery which is associated with five-year survival rates of 60%-80% in stage I and 30%-50% for stage II, respectively[42]. Despite the efficiency of this treatment, the outcome is dependent on tumor location and whether is resectable. Patients that cannot undergo to surgical resection, mainly because of compromised lung function, relies on nonsurgical treatment options. These treatments includes conventional therapy, stereotactic body radiotherapy (SBRT) and radiofrequency ablation (RFA). Regarding conventional therapy, the 5-year survival rates ranges from 15% to 48% with a local failure rate of about 50%[43], mainly because of lung injury with higher radiation doses. In the case of SBRT, the approach is to deliver a high dose of radiation directly into defined volume of tumor, improving the tumor eradication by the higher dose of therapy and avoiding damage of surrounding normal tissue. In fact, this method achieved a local control of 85% to 96% and 5-year survival rates of more than 50% in patients with stage I NSCLC[44]. Another option is through RFA treatment, which consists in thermal damage of tumor mass through an electromagnetic energy. A study in patients with NSCLC showed a complete excision rate of 80% for tumors less than or equal to 3.5 cm, presenting 1-year and 2-year survival rates of 70% and 48%, respectively[45]. Despite these treatment options a distant relapse may occur, being the major cause of death in patients who dies within 5 years of complete surgical resection of tumor. Considering this fact, adjuvant platinum-based chemotherapy is a recommended treatment for completely resected patients, demonstrating an enhancement of 5% to 15% in the 5-year survival rate for patients with stage II and III NSCLC[46].

Representing up to 35 % of NSCLC patients, the stage III is characterized as a heterogeneous disease varying from resectable tumors with microscopic metastases to unresectable with multiple nodal locations[39,47]. For resectable the treatment pass through chemotherapy after surgery resection and for unresectable tumors standard treatment include either a sequential or concurrent combination of chemotherapy and radiation therapy. The concurrent method was shown to be more efficacious than a sequential administration, however it is accompanied with increased toxicity, primarily esophagitis and pneumonitis[47]. For patients that cannot tolerate these combined therapies, external beam therapy (EBT) represents an alternative approach[39].

In stage IV that accounts for 40 % of NSCLC patients, the disease is characterized by metastaic dissemination to several sites, including contralateral lung, brain, bone, liver, and andrenal glands. For the patients presenting this advanced stage, the treatment option take in consideration many factors, such as, comorbidity, histology, and molecular genetic features of the cancer[39,47]. As the first line of treatment the standard option is combined chemotherapy, as it demonstrated higher response rates and better overall survival than treatment with a single-agent[48]. Moreover, for patients that harbors specific genetic mutations, the targeted therapy has been shown to be more effective than chemotherapy[47]. Examples of targeted therapy comprises Epidermal Growth Factor Receptor (EGFR) tyrosine kinase inhibitors (TKI), such as erlotinib and gefitinib; and crizotinib, an inhibitor of the anaplastic lymphoma kinase (ALK). In patients with EGFR-activating mutations, that can trigger the abnormal activation of this receptor causing uncontrolled cell growth, the treatment with EGFR TKIs showed a response rates of 60% to 80% and median progression-free survivals of 9 to 11 months. In the case of patients harboring rearrangement of the ALK gene, crizotinib showed an objective response rate of nearly 60% and a median progression-free survival of 10 months (reviewed in [47]) . Another first-line option have focus on the VEGF, as an important regulator of angiogenesis. The use of bevacizumab, an monoclonal antibody that inhibits new vessels formation by targeting VEGF, together with chemotherapy showed a higher response rate and improved overall survival when in comparison with chemotherapy alone[49]. However it is not recommended for patients that presents a SQCLC histology, due to a high probability for pulmonary hemorrhage[50]. For patients that does not respond to the first line of treatment, a single-agent therapy are recommended as a second line of treatment [47]. In fact several chemotherapeutic and targeted therapy drugs, such as docetaxel, pemetrexed, erlotinib, and gefitinib are approved by the Food and Drug Administration (FDA), as the second- line of treatment in advanced stage NSCLC patients[39,47].

Among SCLC patients, for 30 % that are diagnosed with LS-SCLC, the treatments options includes platinum-based chemotherapy and radiation therapy, combination chemotherapy alone, surgery followed by chemotherapy or chemoradiation therapy, and prophylactic cranial irradiation. In the case of 70 % of patients with ES-SCLC patients, treatment references include combination chemotherapy, radiation therapy and prophylactic cranial irradiation[51]. Despite of the initial response to chemotherapy and other treatments, this LC type is more challenging because of its wide dissemination and aggressive growth[39].

Regardless of improvements made in chemotherapeutic field, there are still limited benefits with low response rates and presenting a median survival of about 10 months for metastatic NSCLC[52]. Along with appearance of molecular-targeted therapies, a progress in the outcomes of patients harboring

mutations in EGFR (15-18% of unselected NSCLC patients) and ALK translocation (2-8% of unselected NSCLC patients) was seen[53,54]. However, a large group of NSCLC and SCLC patients does not have a benefit from this treatment as it does not present these genomic alteration.

Advancements in immunotherapy approach has been made, especially in the treatment of NSCLC patients. Focusing on immune checkpoints that regulates the immune system in an immunosuppressive mechanism, the most studies are about the inhibitory receptors cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and PD-1 (programmed cell death-1) (Figure 3). In fact, several trials are testing inhibitors of CTLA-4 (ipilimumab) and PD-1 (nivolumab) alone or in combination with chemotherapy in NSCLC patients[55]. Data from phase III CheckMate-017 trial, showed an improvement of 41 % in overall survival (OS) with nivolumab when in comparison with docetaxel treatment in patients presenting SQCLC histology[56]. Moreover, in the phase III CheckMate-057 trial, the OS for patients without receiving nivolumab treatment was 27%[57]. Taking into consideration these trials, nivolumab (Opdivo) was accepted by FDA for treatment of patients with SQCLC histology[58]. Regarding ipilimumab, an phase II trial showed that the concurrent combination of carboplatin or paclitaxel with this CTLA-4 inhibitor enhanced the progression-free survival (PFS) of SQCLC patients with advanced stage, when in comparison with chemotherapy treatment alone[59].

Others immune-oncology approaches in NSCLC includes (Figure 3): therapeutic vaccines in order to stimulate the immune system in the recognition of tumor antigens, enhancing the antitumor activity; the use monoclonal antibodies that binds to specific tumor-associated antigens, thus blocking the oncogenic signaling pathways; the immunotoxin therapy that promotes cell death by the use of antibodies to deliver effective toxins inside the cancer cells; and the adoptive cell therapy (ACT), in which immune cells from the patients are educated to target the tumor cells and then are infused again into the patients, allowing a better recognition of the tumor cells (reviewed in[60]).

Despite the improvement of immunotherapy in lung cancer treatment, there are still some limitations as demonstrated in NSCLC Phase III clinical trials[60] , and study of this approach in SCLC patients is scarce[61] . The challenge pass through the complexity of TME and the not fully elucidated LC biology, thus more studies are needed to understand not only lung cancer biology but the surrounding environment. This will potentiate the efficacy of current available therapy or create alternative therapeutic strategies.

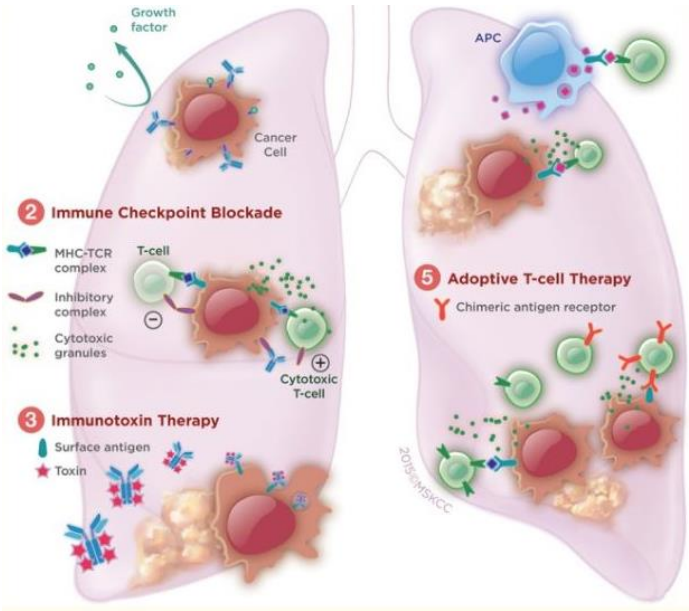


Figure 3. **Immunotherapeutic approach for NSCLC.** The current immunotherapy strategies for NSCLC includes monoclonal antibody therapy (1), immune checkpoint blockade (2), immunotoxins (3), anticancer vaccines (4), and ACT (5). MHC: major histocompatibility complex; TCR: T-cell receptor; APC: antigen presenting cell[60].

3. LUNG CANCER AND INFLAMMATION

3.1 Inflammation in lung cancer

The relation between inflammation and cancer was first described in 1863 by Rudolf Virchow. The pathologist observed that some chemical irritants that causes tissue injury and therefore inflammation were associated with an increase in cellular proliferation and neoplastic development[62].

As mentioned previously, inflammation is natural process that acts in response of tissue injury or affronts as microbial infections, chemicals or particulate exposure. It begins with leukocytes (mainly macrophages and neutrophils) recognition of signs of tissue damage or infecting microorganisms, through a family of specific receptor molecules (Pattern recognition receptors), leading to an increase of signaling molecules, namely cytokines and chemokines. These molecules in turn amplify the inflammatory response by recruiting more leukocytes. These early events are denominated as innate immune response, but a more antigen-specific response will follow, through a stimulation of B cells and T cells, in a process that called as adaptive immune response. If this acute inflammatory response eliminates the causative agent, the homeostasis is restored, on the contrary, if the immune system fails to respond, it can lead to a chronic inflammation and prolonged tissue damage[63,64].

Lungs are one of the organs presenting more vulnerability towards environmental factors, such as tobacco smoke, which provokes lung inflammation[65]. As such, its anatomic localization promotes a constant and sometimes chronic inflammation affronts damage. Epidemiological studies evidenced a positive correlation between an increased risk for lung cancer and several measurements of inflammation, including polymorphisms that induces or modulate inflammation, biomarkers of inflammation, and diseases associated with inflammation, such as tuberculosis[66]. As an example, infection caused by *Chlamydia pneumoniae* which is associated with asthma, was demonstrated as a possible risk factor[67]. Another disease known as chronic obstructive pulmonary disease (COPD), has been implicated to increased incidence and a poor prognosis in LC[35,36]. Additionally, COPD has been long established as risk for lung cancer showing two- to fivefold greater risk for LC when compared with smokers without COPD[68]. Despite these evidences pointing a connection between inflammation and lung cancer, further studies are needed to clarify this relation.

3.2 Tumor microenvironment

Anticancer therapies strategies were initially focused in targeting malignant cells, ignoring the surrounding non-neoplastic components of the tumor, or TME, which has been demonstrated to promote all the features of tumor aggressiveness. TME comprises a complex cross-talk between tumor cells and cancer associated fibroblasts (CAFs), tumor vasculature composed by endothelial cells and pericytes, immune and inflammatory cells, bone marrow-derived cells (BMDCs) and extracellular matrix (ECM) (Figure 4)[69].

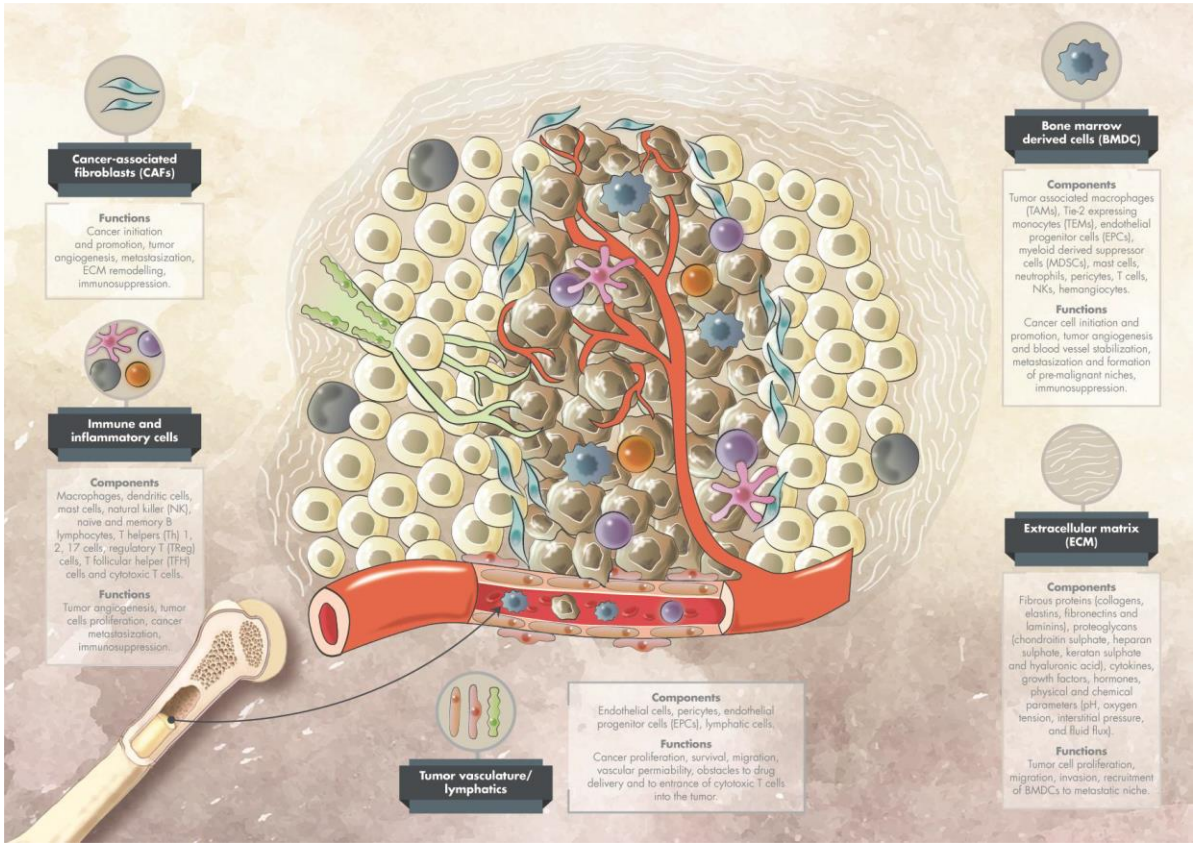


Figure 4. **Components of TME and their function.** Through a convoluted cross-talk with neighboring non-malignant components, tumor cells modifies them in a way to support their capacity to proliferate, migrate, to resist therapy and other features, allowing tumor progression[69].

Nowadays, TME has been gained attention due to the important role of their component with tumor progression and treatment outcomes. One of the mechanisms of TME to promote tumor growth is by supporting tumor escape from immunosurvellaince. This is achieved by an increase of tumor-associated immune cells compressed by tumor-associated macrophages (TAMs), dendritic cells (DC) subsets, cytotoxic T lymphocytes (CTLs) and Treg cells, natural killers (NK) cells and MDSCs[70]. MDSCs were found increased in the peripheral blood of advanced stage NSCLC patients when compared to healthy controls[71]. MSDCs exerts their pro-tumoral action through the inhibition of T-cell proliferation and stimulation by enhancing nitric oxide (NO) synthase and arginase-1 (Arg-1) levels[72,73], interleukin-10 (IL-10) release[74] and reactive oxygen species (ROS)[75]. TAM is another type of myeloid cells, which play an important role in tumor cell survival and metastatic phenotype through the release of growth factors as VEGF. Similarly to MDSCs, TAMs levels was associated with poor patient outcome and decreased overall survival in NSCLC patients (reviewed in[76]).

Besides immune cell content, cytokines and chemokines also play important roles in the tumor process. Cytokines can play either pro-tumoral or anti-tumor roles, unrelatedly with their source, by activation of key transcriptional factors such as nuclear factor kappa B (NF-kB), activator protein 1 (AP-1), signal transducer and activator of transcription (STAT) and SMAD, or directly can have an effect on cancer cell growth and survival[77]. The importance of these molecules has been validated by the approval of interferon-alpha (IFN-α), interferon- beta (IFN-β), interleukin-2 (IL-2) and granulocyte macrophage-colony stimulating factor (GM-GSF) in anticancer treatment, while others, such as IL-12, interleukin-15 (IL-15) and interleukin-21 (IL-21), are under clinical evaluation[78]. In recent times, IL-17 has been widely explored, denoting a debatable role of this molecule in tumor immunity[79,80].

4. INTERLEUKIN-17 FAMILY

4.1 Overview of Interleukin-17 function

Using a murine hybridoma, Rouvier and colleagues described and cloned a new gene named as cytotoxic T-lymphocyte-associated antigen 8 (CTLA-8), which showed homology with Herpes Simplex Virus 13 (HSV13) gene[81]. The transcription product of these genes presented cytokine like activity but with no similarity to other already described cytokines families. Moreover, cloning of CTLA-8 binding receptor suggested that this receptor was structurally different from the others known cytokines receptors. From these findings, a new pro-inflammatory cytokine emerged and was named as IL-17A[82].

IL-17A is a homodimeric glycoprotein constituted by 155 amino acids with a molecular weight of 35 KDa[82]. Based on genomic sequencing, other cytokines were added and created the IL-17 family of cytokines: Interleukin-17B (IL-17B), Interleukin-17C (IL-17C), Interleukin-17D (IL-17D) , Interleukin-17E (IL-17E) and IL-17F[83]. IL-17F presents the highest homology with IL-17A (around 60%)[84], as both are clustered on chromosome 1A4 in murine and 6p12 in human[85].

Several immune cells are responsible for the production of IL-17, including gamma delta T cells ($\gamma\delta$ T)cells, NK cells, group 3 innate lymphoid cells (ILC3s) and lymphoid tissue inducer (LTi) cells[86,87].However, particular diseases such as fungal infections and breast cancer provides conditions for IL-17 releasement from neutrophils[88,89], or from alveolar macrophages during allergic lung inflammation related to asthma[90] (Figure 5). Moreover, the characterization of IL-17 family of cytokines came alongside with the discovery of a new T helper subset, the main producer of IL-17 cytokines: type 17 helper T cells

(Th17) cells[91–93]. The factors known to induce the differentiation of naïve T cells into Th17 cells include: combination of interleukin-6 (IL-6) and transforming grow factor- beta (TGF- β), IL-21 and TGF- β and interleukin-1beta (IL-1β), IL-6 and interleukin-23(IL-23)[85,94,95].

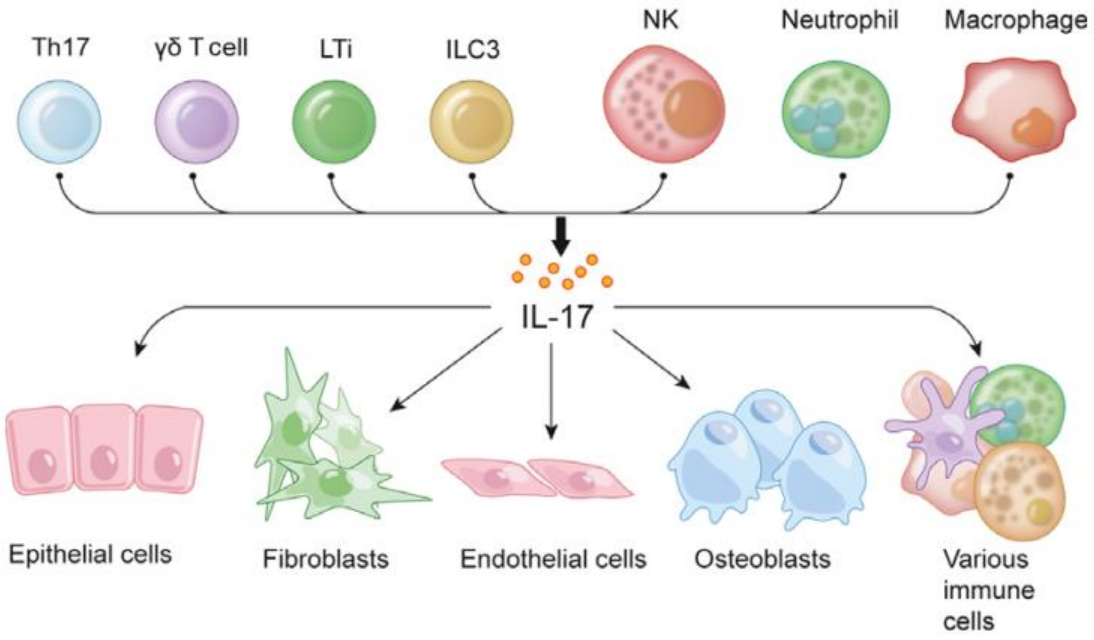


Figure 5. **Major sources of cells responsible for IL-17 secretion and main targets**. Cellular sources of IL-17 family comprises Th17 cells and others immune cells such as γδT, LTi, ILC3 and NK cells. When inflammation occurs, macrophages and neutrophils can also produce IL-17. The main targets of IL-17 family are composed by nonhematopoietic cells, such as endothelial cells, fibroblasts, epithelial cells and osteoblasts. Others cells from immune system can also be a target from IL-17[96].

Inside the IL-17 family, IL-17 F presents the highest homology with IL-17A. In fact, both signal via the same interleukin-17 receptor A (IL-17RA)- interleukin-17 receptor C (IL-17RC) combination, having the capacity to connect and form heterodimers[97]. Others members of IL-17 family signals through an obligate IL-17 RA receptor subunit connected with others members of interleukin-17 receptor (IL-17R) family: interleukin-17 receptor B (IL-17RB), IL-17RC, interleukin-17 receptor D (IL-17RD) and interleukin-17 receptor E (IL-17RE)[98]. In the cytoplasmic tail, these receptors contain a conservative region denominated as SEFIR domain[99] , and a distal domain named as C/EBP-βactivation domain (CBAD), whose purpose is to downregulate IL-17 signaling[100,101]. Additionally, IL-17 RA and IL-17 RC present a non-conserved region, SEFEX domain, which is also required for IL-17 signaling[100,102]. In the extracellular compartment, it is composed by two fibronectin (FN) III- like domains, in which IL-17 signaling is initiated[99,103,104]. Although both IL-17 A and IL-17F signals through the same receptor complex, IL-17F shows about 100-1000 times lower affinity to IL-17RA subunit than IL-17A, which could explain their distinct function[97,105].

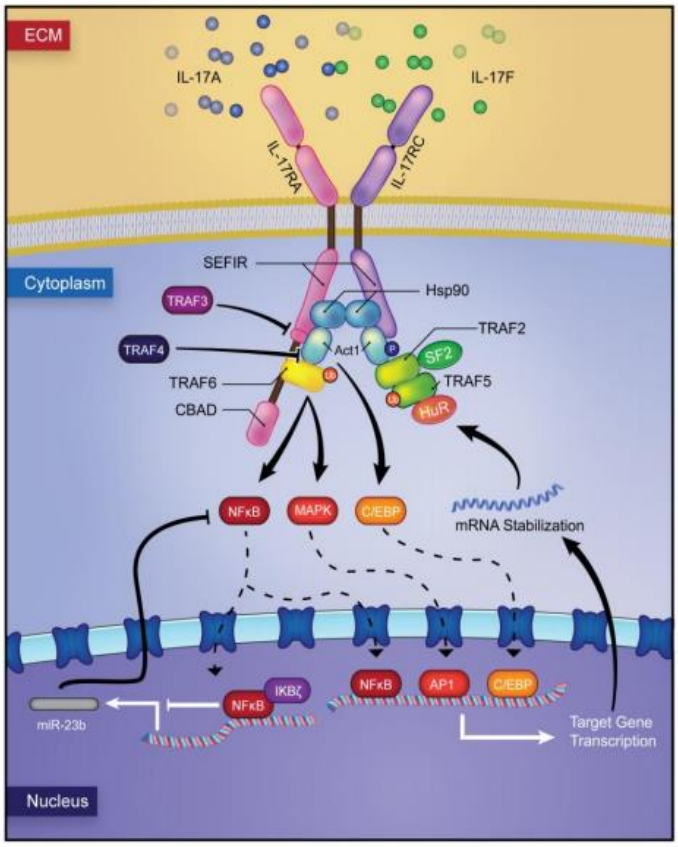


Figure 6.**Signalling of IL-17A, IL-17F and IL-17A/F.** After biding to IL-17RA-RC complex, Act1 is recruited and this protein acts trough ubiquitination of Tumor Necrosis Factor (TNF) receptor associated factor 6 (TRAF6).This leads to activation of pathways namely, NF-kB, mitogen-activated protein kinase (MAPK) and CCAAT-enhancer binding proteins (C/EBP), with transcription of IL-17 target genes. Positive regulators of this cascade includes: heat shock protein 90 (Hsp90), that stabilizes actin1; TNF receptor associated factor 2 (TRAF2) and TNF receptor associated factor 5 (TRAF5) that combines with SF2 to recruit human antigen R (HuR), resulting in the messenger RNA (mRNA) stabilization .On the other hand, the negative regulators comprises: TNF receptor associated factor 3 (TRAF3) that inhibits the connection of IL-17R and ACT1; TNF receptor associated factor 4 (TRAF4), responsible for inhibition of act1 recruitment by TRAF6; miR-23b inhibits NF-kB activation[96].

IL-17 A, IL-17F and IL-17A/F acts through upregulation of inflammatory genes via MAPK and NFkB activation, or by stabilizing target mRNA transcripts[98] (Figure 6). Although, IL-17A stimulation is considered to be a weak inducer of inflammation[106], a synergistic enhancement of the pro-inflammatory response and to recruit trough chemokines the immune cells occurs when signals with other pro-inflammatory cytokines[107]. For example, tumor necrosis factor alpha (TNF-α) produces highly unstable pro-inflammatory mRNAs and IL-17 enhances TNF-α effect by stabilizing these mRNAs[108].

As abovementioned, IL-17 has a vital role in promoting the recruitment of immune cells by inducing the expression of chemokines, such as chemokine (C-X-C motif) ligand 1,2,5,7(CXCL1, CXCL2, CXCL5 and CXCL7, respectively) and C-C motif chemokine ligand 7 (CCL7), that acts as chemoattractants

for neutrophils and monocytes[109]. Moreover, access to site of inflammation is facilitated trough induction of matrix-metalloproteinases (MMPs), including MMP-1,-2,-3,-8,-9 and -13[110].In addition, the number of polymorphonuclear cells may be enhanced by the presence of IL-17A in the site of inflammation, through induction of vasodilatation via prostaglandin E2 (PGE2) production and maintenance of cell functions through induction of granulocyte-colony stimulating factor (G-CSF) and GM-CSF[111,112] (Figure 7).

Additionally, inflammation process orchestrated by IL-17 can be sustained through a positive feedback loop by promoting IL-6,IL-1β, and TNF that are responsible for the induction of Th17 cells[113].

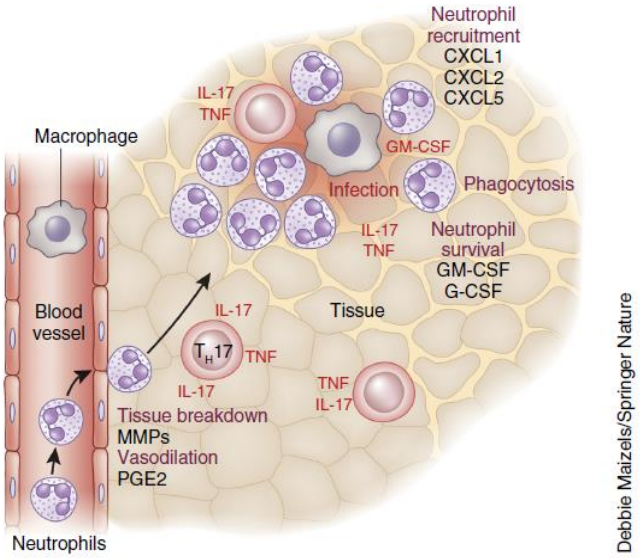


Figure 7. **IL-17A mechanism during inflammation.** One of main function of IL-17A is the recruitment of immune cells. This occurs through PGE2-mediated vasodilation, MMP expression, the promotion of chemoattractants, and the sustainment of phagocytic cells through G-CSF and GM-CSF induction[107].

IL-17 receptors are ubiquitously expressed in non-hematopoietic cells, namely endothelial cells, fibroblasts, epithelial cells and osteoblasts[96] (Figure 5), which are the first responders to this cytokine[114]. In fact, IL-17 plays an important role at epithelial barrier sites, such as lung and skin[115] . One of the mechanisms demonstrating this importance, is through the synergistic activity with interleukin-22 (IL-22) that leads to production of antimicrobial peptides (AMPs), such as β-defensins and lipocalin-2, which are responsible for eliminating foreigner microorganisms[116].Another example, is through sequestration of Act1 from B-cell activating factor (BAFF) receptor, leading to a retention of B cells in germinal centers (GCs), which in turns increases the high-affinity antibody releasement, offering a lifelong protection response[117].

Altogether, showing the importance of IL-17 A and F in cooperating with other cytokines to promote inflammation and the capacity to recruit and maintain immune cells function, these cytokine plays both beneficial and potential dangerous role.

4.2 Interleukin-17 family in cancer

Although the extensive study in determining the function of IL-17A in tumor biology, this subject remains debatable. Several studies appoints to an oncogenic role of this cytokine, due to the high levels of IL-17A in tumor tissues from gastric carcinoma to breast cancer, which positively correlates with tumor aggressiveness[118,119]. Adding to this, Th17 cells are also found gathered in tumor infiltrates when in comparison with health tissues[120]. Moreover, this cytokine has been associated with a higher capacity to inhibit apoptosis and promote malignant cell proliferation, as studies show a higher apoptotic rate and diminished tumor growth and proliferation in tumor cells with KO for IL-17 RA[121,122].

Other authors demonstrated that the pro-tumoral effect is correlated with the capacity to inhibit antitumor response, reviewed in[80] . By KO of IL-17A in tumor-bearing mice, the tumor growth was reduced along with increase of intratumoral expression of $CD8^+$ and $CD4^+$ T cells, and interestingly these cells from IL-17 KO mice produced more IFN-γ than wild type (WT) mice[121,123,124]. Furthermore, immunosuppressive cells, as Tregs, MSDCs and macrophages were accumulated at tumor sites with IL-17 stimulation[121,125-127] .

The capacity to promote angiogenesis, which consists the formation of new blood vessels necessary for the growth and tumor dissemination throughout the body was enhanced with an endogenous IL-17A stimulation in several reports[128,129], whereas IL-17A KO mice presented a less vascular density in tumor area when in comparison with WT mice[130]. Moreover, a correlation between VEGF and IL-17 expression was found in colorectal cancer tissues and in osteosarcoma cells[131,132], with the suppression of IL-17A associated with a decrease of this factor[133].

An aggressive malignancy phenotype, characterized by tumor metastization and invasion was also shown to be associated with IL-17A expression and increased levels of MMP-2 and MMP-9 in hepatocellular carcinoma[134] and expression of intercellular adhesion molecule 1 (ICAM-1) in several tissue cells[135]. The biological process that enables a cell to acquire migratory and invasive capacity requires the loss of cell polarity and cell-cell adhesion, termed as epithelial-mesenchymal transition (EMT), can be upregulated by inhibition of microRNA-192 (miR-192) trough p65 pathway, or by increasing Zinc Finger E-Box Binding Homeobox 1 (ZEB1) expression via NFkB activation. IL-17 exposure to myeloma cells was

shown to enhance EMT by downregulating miR-192[136], and through upregulation of ZEB1 in A549 human NSCLC cells[137].

This cluster of data suggest the role of IL-17 as a pro-tumoral cytokine. However, some articles show contradictory results, as IL-17A overexpression do not alter the number of $CD4^+$ and $CD8^+$ T cells[130] in tumor tissue or that KO of IL-17 RA does not modifies blood vessel density[122]. Additionally, other reports suggest IL-17 A as anti-tumoral. Data from gastric adenocarcinoma[138] and several others carcinomas[139-142], correlates high IL-17 expression with higher five-year survival rates, when compared to patients with low IL-17 expression. Several factors may explain this, as the promotion of $CD8^+$ T, $CD4^+$ T cells and DCs at tumor site among others. A study showed a decrease of $IFN\gamma^+$ $CD4^+$ T cells, $IFN\gamma^+$ $CD8^+$ T cells and $IFN\gamma^+$ NK cells in IL-17 KO mice, and depletion of $CD4^+$ T cells and $CD8^+$ T cells diminished the effect of IL-17 in inhibition of tumor growth[143]. Additionally, IL-17 can enhance antitumor response by chemoattracting NK and $CD8^+$ T cells via induction of C-X-C motif ligand 9,10 (CXCL9 and CXCL10, respectively) in tumor cells[140,144]. Another type of cell associated with anti-tumor functions, CD8 T cells, has been found to be upregulated upon IL-17 stimulation, leading to a suppression of hematopoietic tumors, as mastocytoma and plasmoma. This IL-17-driven effect is thought to be indirect and associated with a higher production of IL-6 or IL-12 from macrophages, which enhances CTLs killing activity[145].

Others authors have suggested that anti-tumoral effect of IL-17 is mediated by neutrophil recruitment, via the release of pro-inflammatory intermediaries by stromal cells[146]. IL-17 may also act as an anti-tumor cytokine through inhibition of tumor invasion. In fact, some studies reports that high levels of IL-17 originate lower tumor invasion in cancers, such as cervical adenocarcinoma and esophageal cancers[142,147].

Contradictory results have suggested a pro-tumoral role whereas others suggests IL-17 an anti-tumor role of IL-17A (Figure 8). Thus, further studies are needed to clarify the involvement of this cytokine in tumor progression.

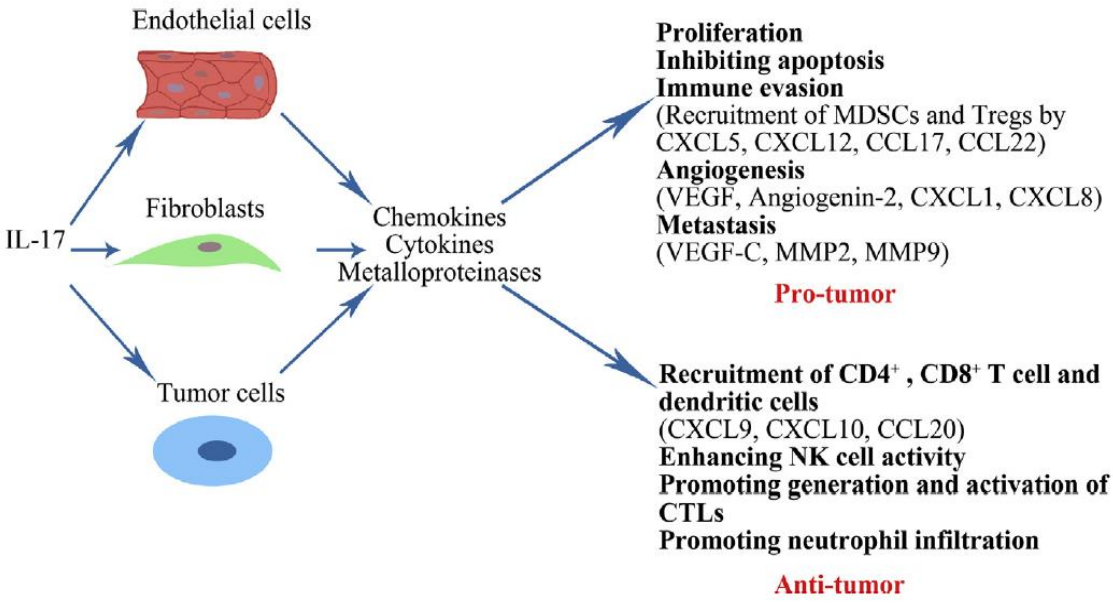


Figure 8. **Function of IL-17A in tumor microenvironment**. IL-17A stimulates both stromal and tumor cells underlying the tumor microenvironment in order to promote tumor development or inhibit its progression[80].

Regarding the others members of IL-17 family, these cytokines were also been implicated with tumor progression. In the case of IL-17B, the enhanced IL-17RB/IL-17B signaling was associated with poor prognosis of breast cancer patients. Furthermore, though the induction of extracellular-signal-regulated kinase 1 (ERK1)/ extracellular-signal-regulated kinase 1 (ERK2) pathway, the upregulation of anti-apoptotic proteins of the B-cell lymphoma 2 (BCL-2) family led to resistance of breast cancer cell lines to paclitaxel treatment. Moreover, in pancreatic cancer, IL-17RB expression augment postoperative metastasis and negatively correlated with PFS in pancreatic cancer patients. Additionally, through ERK1/2 activation, the C-C motif ligand 20 (CCL20)/CXCL1/Interlekin-8 (IL-8)/Trefoil factor-1 (TFF1) chemokine enhancement led to cancer cell invasion, macrophage and endothelial cell recruitment at primary sites. In the gastric cancer, IL-17RB was significant more expressed in the cancer tissues than in noncancerous tissues, being associated with poor prognosis in gastric cancer patients. Also, IL-17B/IL-17RB signal was found to enhance cell grow, migration and stemness through the stimulation of Protein Kinase B (AKT)/ß-catenin pathway. Altogether these studies indicates a pro-tumor role of IL-17B (reviewed in[148]).

About the IL-17C and its relation with tumorigenesis, few studies were made. Concerning colorectal cancer, Song et al. suggests a pro-tumor role through microbiota alteration. They saw that change in microbiota led to IL-17C enhancement, promoting cell survival and tumorigenesis in both chemically induced and spontaneous intestinal tumor models through BCL-2 and B-cell lymphoma-extra large (BCL-xL) activation in intestinal epithelial cells (IECs)[149]. In the case of NSCLC, IL-17C was identified

as a negative prognostic factor in patients with lymph node metastasis. Furthermore, Jungnickel et al. demonstrated that IL-17C induces lung tumors growth through neutrophils recruitment. Interestingly, these researches argues that IL-17C might connect a pathologic microbiota present in COPD patients with enhanced tumor growth[150].

Associated with anti-tumor role, IL-17D functions through stimulation of chemoattractant protein-1 (MCP-1) from tumor endothelial cells, leading NK cells activation[151]. Moreover, it was found that transcription factor nuclear factor erythroid-derived 2-like 2 (Nrf2) activation, by stress surveillance pathway, induced IL-17D in tumor cells causing tumor regression[152].

Another cytokine from IL-17 family was associated with anti-tumor function. Through investigation of several tumor xenograft models, namely melanoma, breast, lung, colon, and pancreatic cancers, IL-17E function in tumorigenesis was evaluated. Significant antitumor activity was detected through injection of recombinant IL-17E in these tumors. Moreover, combination of chemotherapy or immunotherapy treatment with IL-17E in human tumor xenograft models, enhanced the antitumor efficacy as compared to either approach alone (reviewed in [153]).

Regarding IL-17F, its function in tumorigenesis has not yet been clarified. Tong et al. saw a decreased expression of this cytokine in colon cancer tissues. Moreover, they saw a protective role in this type of cancer, through an inhibition of tumor angiogenesis as demonstrated by increased levels of VEGF in Il-17F KO mice[154]. In addition, a multivariate analysis of oral tongue squamous cell carcinoma (SCC) specimens showed a positive correlation between extracellular mast cell-derived IL-17F and better disease-specific survival in patients of oral tongue SCC[155].

4.3 Interleukin-17 A/F and its role in lung cancer

Despite the similarity between IL-17A and F, as mentioned previously, IL-17F has been shown to have a weaker effects than IL-17A during infections[156-158], being IL-17A alone sufficient to reduce pathology in autoimmune models[159,160]. Moreover, IL-17A is more effective in inducing cytokine production in macrophages than IL-17F[107].With similar gene expression profile, both cytokines can act in synergy with TNF-αand both are able to activate epithelial innate immune response[87,161]. Furthermore, only in the absence of both cytokines in mice models with extracellular bacteria *Staphylococcus aureus* and *Citrobacter rodentium* stimulus, the mice were more susceptible to infection[156]. Despite these similar actions of IL-17A/F in infection, some studies identified divergent roles for IL-17A and IL-17F (reviewed

in[162]). Although IL-17A and F being both required at earlier stage of *C.rodentium* infection (day 7), at later stages (days 14 and 21), IL-17F has showed a more important role than IL-17A in the control of bacterial burden[156]. Another study, using experimental autoimmune encephalomyelitis (EAE) model, demonstrated only reduced infection in IL-17A KO mice whereas IL-17F KO mice did not impact the initiation of EAE. Regarding asthma model, a reduction of eosinophil infiltration was observed in IL-17A KO mice whereas IL-17F knockout mice presented a higher Th2 cytokine and eosinophil infiltration, demonstrating the importance of IL-17F but not IL-17A in asthma suppression. Contrarily, IL-17A was described to have a protective role in dextran sulfate sodium model of colitis, whereas IL-17F presented a pathogenesis role in this infection[163].

The role of IL-17A and IL-17F cytokines in tumor progression are still not clear, including in LC. Regarding IL-17A, several reports determine it as a pro-tumor cytokine. Human studies demonstrate high levels of IL-17A in serum of NSCLC patients[164], being studied as a possible diagnostic marker[165].

In murine studies, IL-17A deficient mice presented a reduced numbers of lung tumors, proliferation, angiogenesis, recruitment of myeloid cells and pro-inflammatory mediators[166,167]. Moreover, IL-17A promoted lymphangiogenesis via upregulation of lymphangiogenic factor vascular endothelial growth factor-C (VEGF-C), in murine cancer cells[168]. Using an *in vitro* approach, it was observed that A549 and H520 NSCLC cell lines stimulated with IL-17A had a upregulation of VEGF through phosphorylation of STAT3[169]. However, it was suggested that IL-17A may have an anti-tumor role, as a reduction of lung metastasis trough promotion of NK cell activity and enhanced tumor growth and lung tumor metastasis in IL-17A KO mice was observed[143].

Regarding the function of IL-17F in lung cancer, there is only a study focusing on genetic variation of IL-17F given its relevance in COPD[170], which is highly connected with lung cancer. The authors showed a positive relation between a single nucleotide polymorphism (SNP), IL-17F 7488G allele, with a higher risk for lung cancer in Tunisian population[171].A possible explanation to this association, is through a reduction of immune responses or stimulation of cytokines and chemokines production in comparison with wild-type IL-17F.In addition, using Kras-induced lung cancer mouse model (CC-LR) crossed with IL-17A or IL-17 deficient mice, the researchers observed a significant reduction in lung tumors numbers in IL-17A deficient mice but not in IL-17F mice[167].

Overall, although the increase amount of evidence indicating IL-17A as an anti-tumor role, there are still some evidences suggesting otherwise. In opposition, knowledge about the function of others

members of IL-17 family are still shallow, thus further exploration of IL-17 family and its role on tumor development are needed.

CHAPTER 2: OBJECTIVES

OBJECTIVES

Based on previous data obtained from the analysis of lung cancer RNA expression of public datasets, intratumoral IL-17F expression was correlated with a better patient overall survival. Taking this in consideration and given the exploration of IL-17 as a therapeutic target in cancer, the aim of this thesis is to elucidate the impact of IL-17A and F in lung cancer. To address this, we evaluated the direct effect of each cytokine on human and mice lung cancer cells, or the potential indirect effect through manipulation of immune cells. With our experimental approaches we expect to shed light in the relevance of IL-17 A and F in lung cancer.

CHAPTER 3: MATERIALS AND METHODS

Cell lines

Two distinct cell lines, A549 and LLC1, were used during this work. LLC1 is a cloned line of cells isolated from the Lewis Lung Carcinoma, established from a C57BL mice in 1951, by Dr.M.R.Lewis[172]. This cell line present both adherent and suspension populations, display a doubling time of 21 hours and exhibit rounded loosely attached or floating morphology. A549 is a human pulmonary adenocarcinoma cell line isolated in 1973[173]. This cell line is comprise only of an adherent population, present a doubling time of about 22 hours and display epithelial like morphology. A549 and LLC1 cell lines were obtained from American Type Culture Collection (ATCC®, CCL-185™ and ATCC® CRL1642™, respectively). These lung cancer cell lines were cultured in Dulbecco´s Modified Eagle Medium (DMEM, Biochrom®) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biochrom®) and 1% antibiotic Penicillin-Streptomycin (Gibco®) in a humidified incubator at 21% O2, 5% CO2 and 74% N2 at 37°C. For hypoxic conditions, cells were placed inside hypoxic chambers (Modular Incubator Chamber (MIC-101), billups-rothenberg.inc) with gas mixture 0% O_2, 5% CO_2 and 95% N_2, and placed in an incubator at 37°C for a specific period of time accordingly to the assay.

Bone marrow derived macrophages (BMDM) Conditioned Media

To obtain conditioned media (CM) from BMDM (Figure 9), bone marrow cells were collected from C56BL/6 mice between 6 and 8 weeks of old. Briefly, after mice being euthanized by cervical dislocation, femur and tibia bones were dissected, by removing the skin around the paw and muscles around the bones, and placed on cold phosphate buffered saline (PBS) on ice. After this, bones were flushed with cold DMEM until whitening in a petri dish, and the suspension were homogenized by the use of 21g needle (Terumo®) and a 40 µm filter (Falcon®). Following centrifugation at 1200 rpm for 5 minutes, cells were counted using Trypan Blue (Gibco®), in a Neubauer chamber. Bone marrow cells ($8x10^6$ cells) were seeded in a 100 mm petri dish (Sterilin, ThermoScientific®) in complete DMEM containing 10% FBS, 1% Glutamine (Gibco®), 1% HEPES 1M (Gibco®), 1% Sodium Pyruvate (Gibco®) and 1 % Penicillin-Streptomycin supplemented with 30% of L929-cell conditioned medium (LCCM), as a source of

macrophage colony-stimulating factor (M-CSF). Cells were maintained in a humidified incubator at 21% O2, 5% CO2 and 74% N2 at 37°C. At day 3 of culture additional 3ml of differentiated medium was added and allowed to differentiate until day 6.

At day 6 macrophages were harvested with 4 ml of ice cold PBS. The dish was scrapped using the plunger of a syringe and cells were placed into a falcon tube. The suspension was centrifuged at 1200 rpm for 5 minutes and the number of cells was determined using Trypan Blue in a Neubauer chamber. Then, macrophages were seeded at density of $1x10^6$ cell/well in a 6 well plate, with complete DMEM medium. To stimulate the cells, after letting the cells settled down at 37°C for 1 hour, media with 50 ng/ml of IL-17A (210-17, Peprotech®) or IL-17F (210-17F, Peprotech®) diluted in complete DMEM were added into the cells. Media with only complete DMEM media was used as control conditioned media (CM (MO)). After 24 hours of stimulation, the medium was discharged and cells were incubated with DMEM containing 1 % of FBS for 24 hours. Supernatant was collected and cell-free CM was obtained by centrifugation of 1200 rpm for 5 minutes and stored at -20°C until use. Thus, from this experiment we obtained the following CM:

1. CM from macrophages stimulated with complete DMEM - **CM (MO);**
2. CM from macrophages stimulated with 50 ng/ml of IL-17A - **CM (MO+IL-17A);**
3. CM from macrophages stimulated with 50 ng/ml of IL-17F - **CM (MO+IL-17F)**.

This assay was performed according to the European Union Directive 2010/63/EU, and approved to the local ethics committee (074/2016).

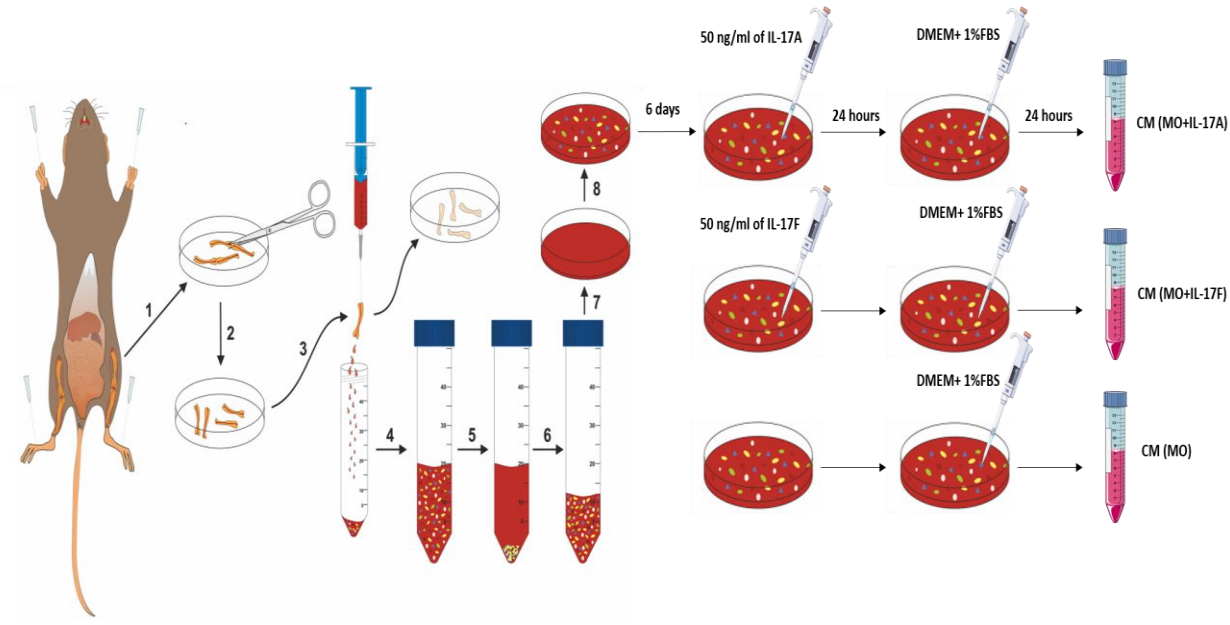


Figure 9. **Overview of the protocol for isolation of bone marrow derived macrophages and conditioned medium preparation**. The hind legs are exposed with helps of needles, and the femur and tibia bones are dissected out (1) and separated (2). Then

bone ends are chopped off, and the bones flushed with cold DMEM (3) and collected in a falcon tube (4).The cells are enriched followed by centrifugation (5) and the pellet is dissolved in complete DMEM medium (6) and added into Sterilin petri dishes filled with complete DMEM medium (7-8). After 6 days, differentiated macrophages are stimulated with 50 ng/ml of IL-17A or IL-17F or without stimulation for 24 hours. Then the media is removed to addition of DMEM with 1% of FBS to production of different conditioned medium. Adapted from[174].

Preparation of LCCM

For differentiation of macrophages, LCCM was used as a source of M-CSF. For that, $9{,}4x10^5$ L929 cells were added in a 175 cm^2 flask containing 110 ml of medium (DMEM, 10% FBS, 1% HEPES, 1% P/S), and allowed the cells to grow for 7 days. Then, medium was collected, centrifuged to remove the cells and stored at -20ºC.

Immunofluorescence staining- IL-17R

A549 and LLC1 cells were seeded on cover slips at a density of $8x10^4$ and $4x10^4$ cells/well respectively and allowed to adhere overnight. Cells were fixed and permeabilized with cold methanol and incubation primary antibody-mouse monoclonal antibody IL-17R (1:100 dilution, sc-376374, Santa Cruz Biotecnology,Inc.) was executed in a humified chamber, overnight at room temperature. Polyclonal goat anti-mouse IgG antibody (1:500 dilution, 406618, Biolegend,Inc.) was used as secondary biotinylated antibody, which was incubated for 1 hour at room temperature. To enhance the signal, Streptavidin-AF647 (1:500 dilution, 405237, Biolegend,Inc.) was added and incubated for 1 hour, at room temperature. Cells were also stained with mounting media (F6057, Sigma-Aldrich) containing DAPI (4′,6-diamidino-2-phenylindole) that bind regions in DNA, staining the nuclei. The immunofluorescence photos were taken with an Olympus BX61 microscope using CellSens Dimension software at 200x magnification.

Viability assay

Cell viability was evaluated in A549 and LLC1 cells stimulated with 10 or 50 ng/ml of IL-17A or IL-17F diluted in DMEM 1%FBS , and in LLC1 stimulated with the different CM for 24 and 48 hours. This assay was performed by sulforhodamine B (SRB) protocol (Figure 10), which consists on the binding of the dye to amino acids of cellular proteins given a colorimetric evaluation of total protein mass, which corresponds to an estimation of viable cells. A549 and LLC1 cells were plated in 48-well plate, at a density of $1.3x10^4$ and $8x10^3$ cells/well, respectively. After treatment with different conditions, the protocol of SRB

was performed as described in. After treatment, cell viability was determined by the SRB assay, as described previously[175].

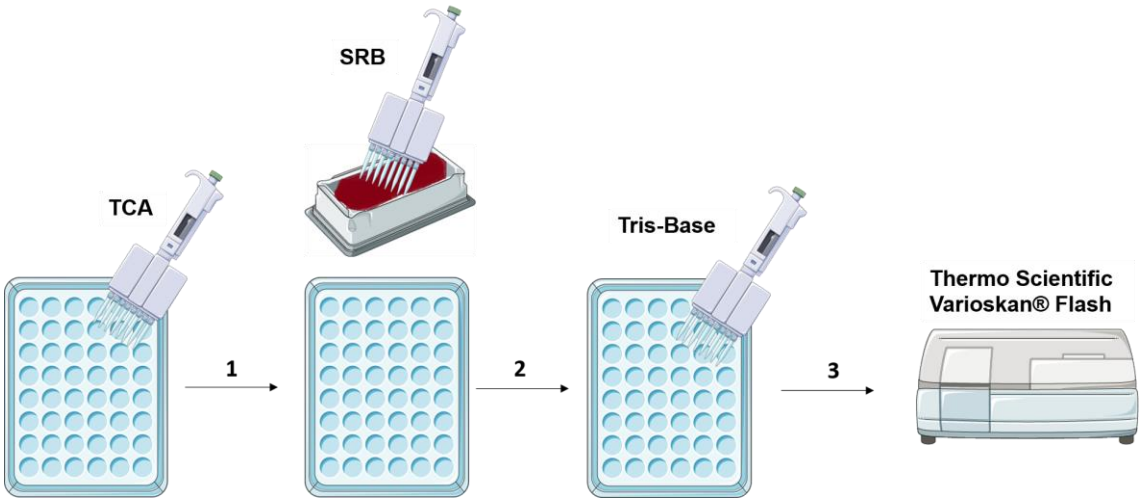


Figure 10.**SRB assay protocol.** After treatment with different conditions, the media is removed and cells are fixed with Trichloroacetic acid (TCA) overnight or 1 hour at 4°C. After fixation, cells are washed with distillated water and SRB is added (1). Thirty minutes later of incubation with SRB, the excessive bound is washed out with 1% acetic acid and 10 mM of Tris-Base (pH 10.5) is added to dilute the bound between the dye and cellular proteins (2). Lastly, the plate is read at 530 nm in Thermo Scientific Varioskan® Flash, using the SkanIt™ software (3).

Migration assay

To evaluate migration capacity in A549 cells wound healing protocol was performed, whereas Boyden chamber assay was used in LLC1 cells. For wound healing assay, cells were plated at a density of $8x10^5$ cells/well and after a monolayer structure was formed, the wound was made. Then, after removal of suspended cells with sterile PBS, the media containing 10 or 50 ng/ml of IL-17A (200-17, Peprotech®) or IL-17F (200-25, Peprotech®) diluted in DMEM 1%FBS was given to the cells. The wound areas were photographed at 0, 24, 48 and 72 hours in Olympus IX51 microscope. The relative migration distances were analyzed using beWound™ Software[176]. The relative migration for A549 was expressed as percentage relative to the respective control conditions (cells without stimulation).

Following boyden chamber protocol, LLC1 cells ($2.5x10^4$) diluted with DMEM 1%FBS were placed in the upper chamber of transwell culture inserts with 8 μM pore size (Corning® Costar® Transwell® cell culture inserts, 3464). Inserts were placed in well containing CM from stimulated macrophages described above and the plates were incubated at 37°C, until 48 hours. Viable migrated cells were identified using concentrated hematoxylin and images were taken in Olympus BX61 microscope using CellSens Dimension software at 100x magnification. The number of migrating cells were counted based on three different images from the same condition.

Metabolism assay

A549 and LLC1 cells were plated in 48-well plate, at a density of $1.3x10^4$ and $8x10^3$ cells/well, respectively. Following overnight incubation, either cell lines were exposed to 10 or 50 ng/ml of IL-17A or IL-17F diluted in DMEM 1%FBS, and with different CM for LLC1 cells. After 24 and 48 hours, glucose and lactate quantification were measured by High Perfomance Liquid Chromatography (HPLC) or with enzymatic kits from SpinReact®, for each metabolite.

For determination of extracellular glucose and lactate production in A549 stimulated with 10 or 50 ng/ml of IL-17A or IL-17F, cultured supernatants were collected and kept at -20°C, until be run in the HPLC apparatus. For that, Sulfuric Acid (H_2SO_4) 0.0025 M was used as mobile phase and the metabolites were detected by Gilson Bomb with HypeREX XP Carbohydrate H^+ 8 µL column and refractive index detector (IOTA2, Reagents). Each sample was analyzed for 15 minutes, at a temperature of 54°C.

In relation to LLC1 under stimulation with IL-17 A/F or with different CM and A549 under hypoxic conditions, the consumed glucose and extracellular lactate were quantified with enzymatic kits from SpinReact®, accordingly with manufacturer´s protocol. Briefly, the metabolic intermediates were quantified by the Thermo Scientific Varioskan® Flash readout at 490 nm of absorbance. The results were normalized against total biomass (µg of metabolite/total biomass).

Western Blot Analysis

For detection of glycolytic protein levels, total cellular extracts were collected from A549 stimulated with 10 ng/ml of IL-17A or IL-17F diluted in 10% DMEM and LLC1 stimulated with 10 ng/ml of IL-17A or IL-17F diluted in 10% DMEM, or with different CM. After 24 hours of stimulus, cells were lysed and scraped with proteases inhibitors (Roche®) diluted in lysis buffer (50mM Tris pH 7.6-8.0; 150mM NaCl; 5mM EDTA; 1mM Na3VO4; 10mM NaF; 10mM Na4P2O7; 1% NP-40) in a proportion of 1:7, for protein extraction. Proteins were quantified using Bio-Rad DC Protein Assay (BioRad®), which consists in a reaction of protein with an alkaline copper tartrate solution and Folin reagent.30 µg of total protein were ran in a 10% acrylamide SDS gel and transferred for 30 min in Trans-Blot Turbo® transfer system (BioRad®) to a nitrocellulose membrane (Amersham Biosciences®). Membranes were blocked in TBS-Tween (TBS-T) containing 5% skim milk (Molico ®, Nestlé) and incubated overnight at 4°C with

primary antibodies (Table 2). Then, the membranes were washed with TBS-T and incubated with the corresponding secondary antibodies (Table 3) during 1 hour. Proteins levels were detected after incubation with Supersignal™ West Fento Maximum Sensitivity Substrate (Thermo Scientific™) using a Molecular Imager®Chemidoc™ XRS System from BioRad®.

Table 2. **Information about primary antibodies used for Western-Blot.**

Antibody	Reference	Target	Size	Clone	Dilution
PDK	SC-28783	Rabbit	49/46/47 KDa	G1411	1/200
CAIX	ab15086	Rabbit	49.7/55 kDa	GR273986-4	1/2000
HK2	ab104836	Mouse	102 kDa	GR164816-6	1/2000
Glut1	ab15309	Rabbit	55 KDa	GR207686-2	1/500
MCT4	SC-50329	Rabbit	43 kDa	C1915	1/500
MCT1	SC-365501	Mouse	40-48 KDa	E0317	1/500
Actin	SC-8432	Mouse	43 KDa	E1314	1/500
Glut3	ab41525	Rabbit	45-47 KDa	GR41399-18	1/500
LDHA	SC-137243	Mouse	35 KDa	H1314	1/1000

Table 3. **Information about secondary antibodies used for Western-Blot.**

Antibody	Reference	Clone	Dilution
Anti-mouse	SC-516102	I2017	1/2500
Anti-rabbit	SC-2357	K2017	1/2500

Spheroids Growth

Since 3D- model mimics *in vivo* cyto-architectural conditions, presenting a more physiological relevance than 2D dimensional culture, LLC1 cells were used for spheroids formation. Concisely, 3.000 cells were plated in 48 well plate containing 1% of agarose at the bottom of the well. After 3 days of incubation, the spheroids were treated with different CM from macrophages, and images were taken in Olympus IX51 microscope until 6 days after treatment. The area was assessed using Image J software.

Chicken chorioallantoic membrane (CAM) assay

To assess *in vivo* LLC1 tumor growth upon treatment with CM from macrophages with different IL-17 stimulations, CAM assay which consists in tumor implantation in a highly vascularized extraembryonic membrane and immunodeficient environment, was performed.

Fertilized eggs were incubated at 37°C and 70% of humidity for 3 days. Afterwards, a window was made into the eggshell and air chamber was punctured, and then the window was closed with a tape and eggs were incubated. On 9th day of development, 1x10^{6} cells in 10 µL of CM and 8 µL of matrigel were injected into CAM. At day 11 and 13, the medium was refreshed by adding 10 µL of CM. Finally, on the 16th day of development, the formed tumors were photographed *ex ovo* and *in ovo*, in a stereo-microscope (Olympus S2916) using a digital camera (OlympusDP71). Tumor perimeter and area were evaluated with Image J software. The embryos were sacrificed at -80°C for 20 min with formaldehyde.

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism version 6. Metabolism data, namely metabolites quantification and glycolytic proteins levels, were analyzed with Student´s t test. Also, statistical analysis tumor area and perimeter in CAM assay, were analyzed with the same test. Regarding spheroids growth and cell viability, two-way analysis of variance (ANOVA) and multiple comparisons between the control and the groups, was performed. All the data are reported as the mean ± standard deviation (SD), and p-value lower than 0.05 was assumed as statistically significant difference.

CHAPTER 4: RESULTS

4.1. Metabolic and functional effects of IL-17A/F on lung cancer cell lines.

Accumulating evidence indicates that a dynamic crosstalk between tumor cells and the immune system cells can regulate tumor growth and metastasis. Based on the previous data from TCGA analysis, we have strong evidence showing intratumoral expression of IL-17F, but not IL-17A, positively associated with patient survival. A controversial role of the IL-17 family of cytokines in several cancers has been observed with reports describing both pro- and anti-tumoral functions. This seems to be the case for IL-17A in lung cancer, where both functions have already been described (reviewed in[177]) suggesting that the functional effects may depend on other contributing factors. Yet, the role of IL-17F, the closest member of the IL-17 family of cytokines, is yet to be addressed.

Tumor environment is characterized by different oxygen levels, dependent on the proximity to blood vessels. The "oxygenated" cells are localized at proximity of oxygen supplies, in normoxic regions. As the tumor cells starts to proliferate and move away from the capillaries, the oxygen levels starts to be scarce in the hypoxic regions[178]. This last region has been characterized in most tumors, being associated with tumor progression through tumor stroma remodulation and favoring immune suppression role [179].

Given the ubiquitously expression of IL-17 RA and IL-17RC[96] and the ability of IL-17 to induce key molecules, such as pro-inflammatory cytokines and metalloproteinases, our first aim was to evaluate the direct effects of these cytokines on tumor cells in both normoxic and hypoxic conditions, which composes the tumor microenvironment.

4.1.1. IL-17 R is expressed in A549 and LLC1 cell lines.

Although the expression of the receptor in A549 was already described in the literature, nothing was described regarding LLC1 cells. Thus, we confirm the expression levels of the IL-17 receptors on human (A549) and murine (LLC1) cell lines. The protein expression (Figure 11) was detected in both cell lines making these cells prone to receive IL-17 A/F stimulus.

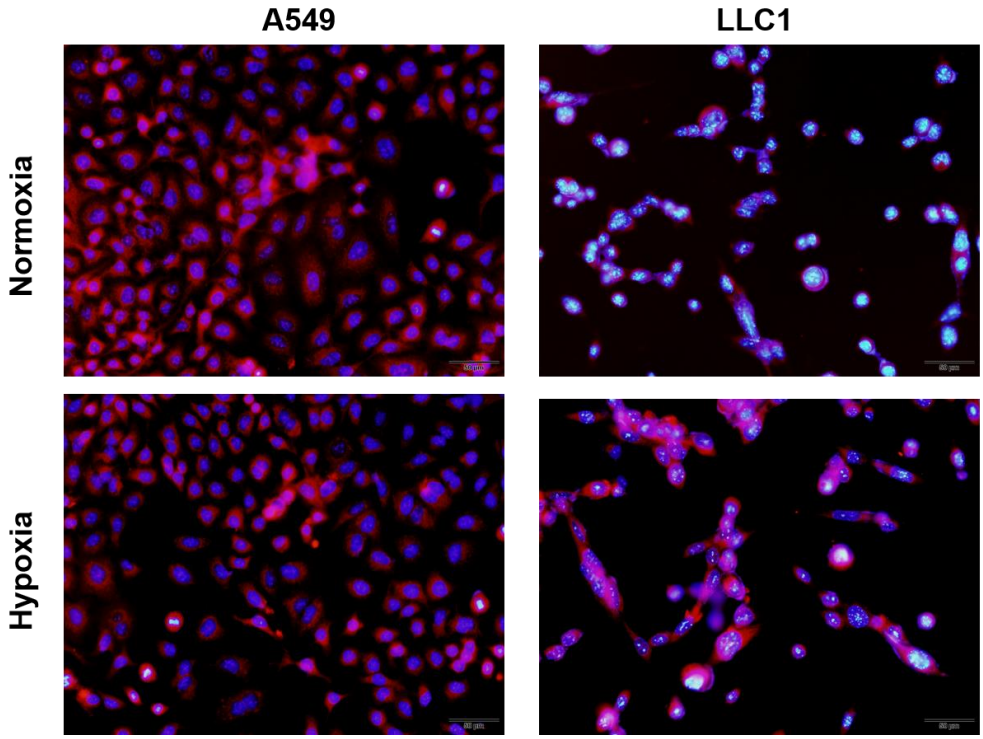


Figure 11. **Immunofluorescence for IL-17R using a specific anti-IL-17R antibody.** In left side is the expression of IL-17R in A549, and on the right side for LLC1. The expression was analyzed in normoxic (top) and hypoxic (bottom) conditions. The cell nucleus were counterstained with DAPI and the pictures were taken at 200x in an Olympus fluorescence microscope.

4.1.2. IL-17A/F do not interfere with lung cancer cell viability.

To evaluate the effects of IL-17 A/F in cell viability, A549 and LLC1 cells were stimulated with 10 or 50 ng/ml of IL-17 A or IL-17F, under normoxic and hypoxic conditions and cell viability was assessed using SRB protocol.

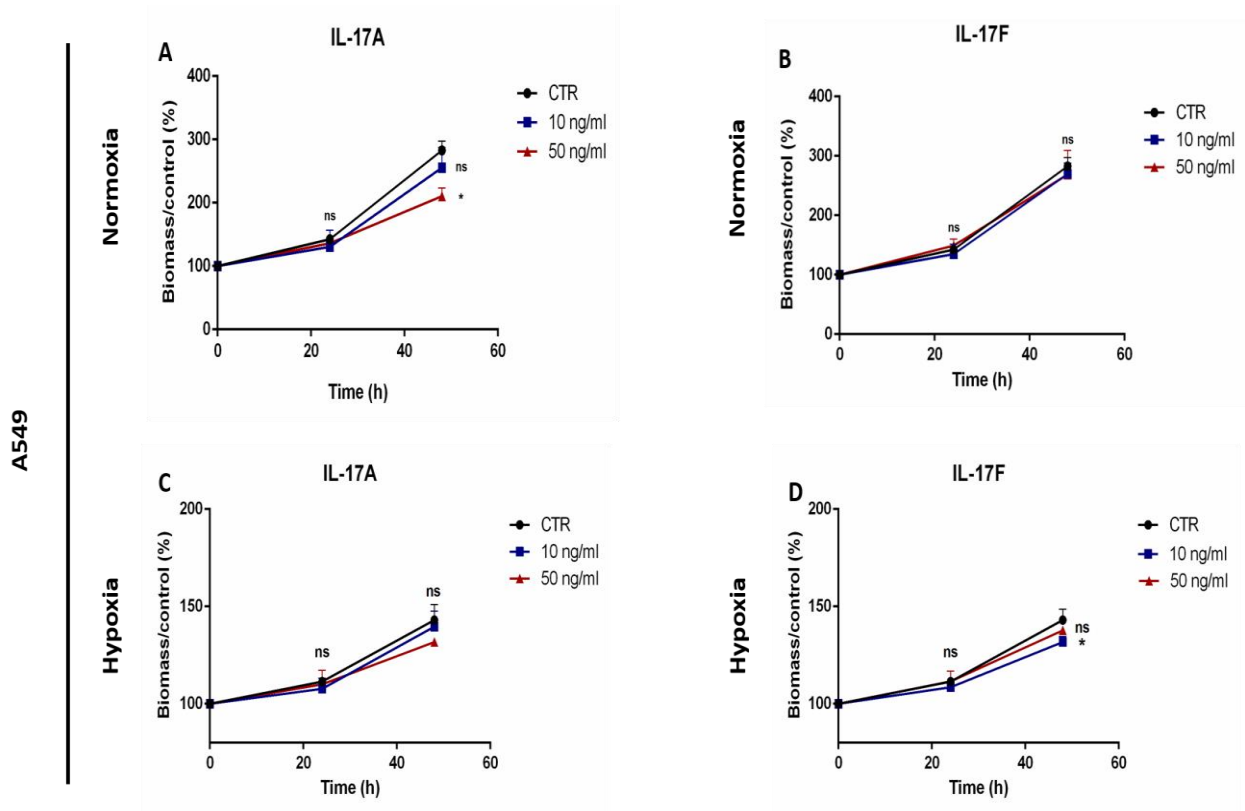


Figure 12. **Cell viability measurement of A549 cell under 10 (blue line) or 50 (red line) ng/ml of IL-17A (A, C) or IL-17F (B, D) stimulus.** Under normoxic (top) and hypoxic (bottom) conditions, cells were incubated with IL-17 A or IL-17F and cell viability was assessed until 48 hours, by SRB protocol. Results represent the mean ± SD of triplicates from two independent experiments. * $p < 0.05$; ns- non significant; compared to untreated cells (CTR).

Concerning A549 cells (Figure 12), it was observed a decrease in cell viability in a dose-dependent manner with IL-17A in normoxia conditions (Figure 12A) but not in hypoxia (Figure 12C), whereas regarding IL-17F only the concentration of 10 ng/ml was able to reduce cell viability in hypoxic conditions (Figure 12D).

In the case of LLC1 cells (Figure 13), neither IL-17A nor IL-17F affected cell viability in both normoxic (Figure 13 A, B) and hypoxic (Figure 13 C, D) conditions.

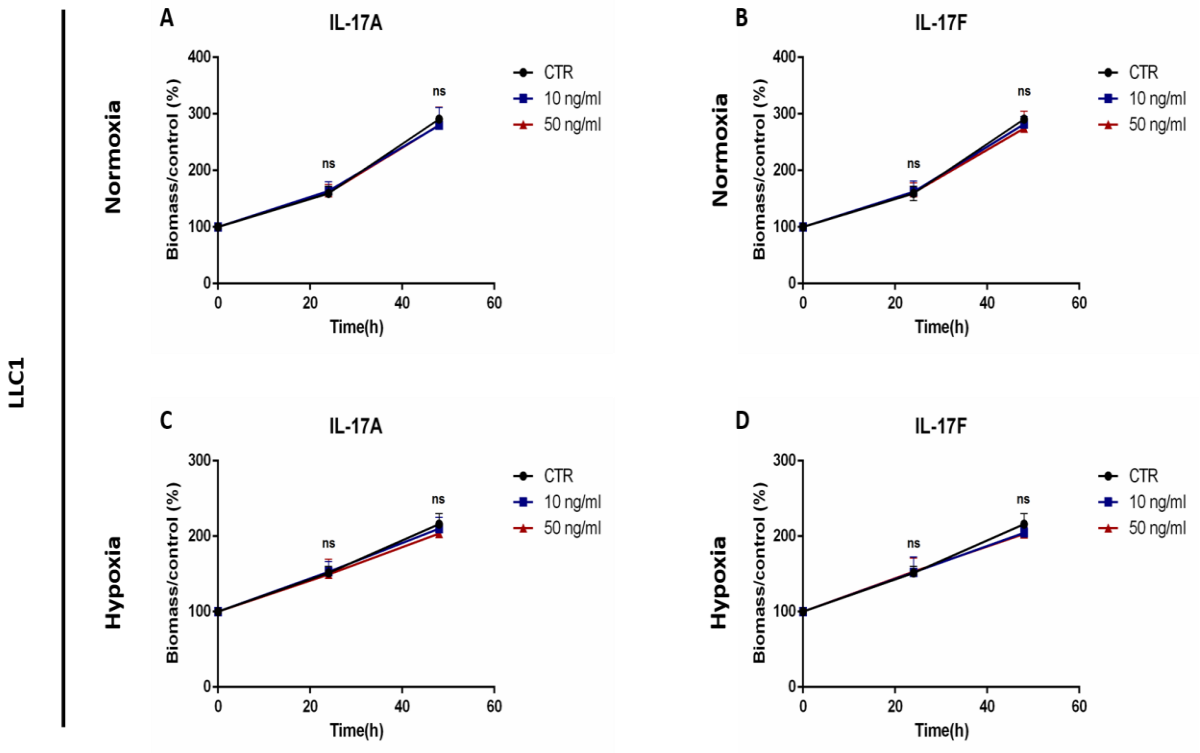


Figure 13. **Cell viability measurement of LLC1 cell under 10 (blue line) or 50 (red line) ng/ml of IL-17A (A, C) or IL-17F (B, D) stimulus**. Under normoxic (top) and hypoxic (bottom) conditions, cells were incubated with IL-17 A or IL-17F and cell viability was assessed until 48 hours, by SRB protocol. Results represent the mean ± SD of triplicates from three independent experiments. $p < 0.05$; ns- non significant; compared to untreated cells (CTR).

Altogether, despite of a slight decrease observation in A549 viability under IL-17A stimulation in normoxic conditions and with IL-17F in hypoxic conditions, our data showed not a significant impact of IL-17 A and F in the growth of lung cancer cells directly. This goes in accordance with previous study using SQCLC and AdenoCA cells (Sq-19 and A549 cells, respectively) under IL-17A stimulation[129], in which they saw no direct effect on the *in vitro* growth. However, they observed an enhanced *in vivo* growth in NSCLC, through CXCR-2 dependent angiogenesis.

4.1.3. IL-17A/F does not alter lung cancer cell metabolism.

Given the importance of altered metabolism in tumor cells, conferring them the capacity to adapt to various microenvironments, we went to analyze the effect of IL-17 A/F in this emerging hallmark.

Differently from normal cells that prefers OXPHOS pathway to generate the energy for cellular functions, most tumors have a preference on fermentation process even in the presence of oxygen[7]. This pathway that enables the rapid growth of tumor cells, is characterized by high rates of glucose

consumption and lactic acid production. To approach this question, we analyzed the presence of glucose and lactate metabolites in supernatant of IL-17A/F stimulated cell lines.

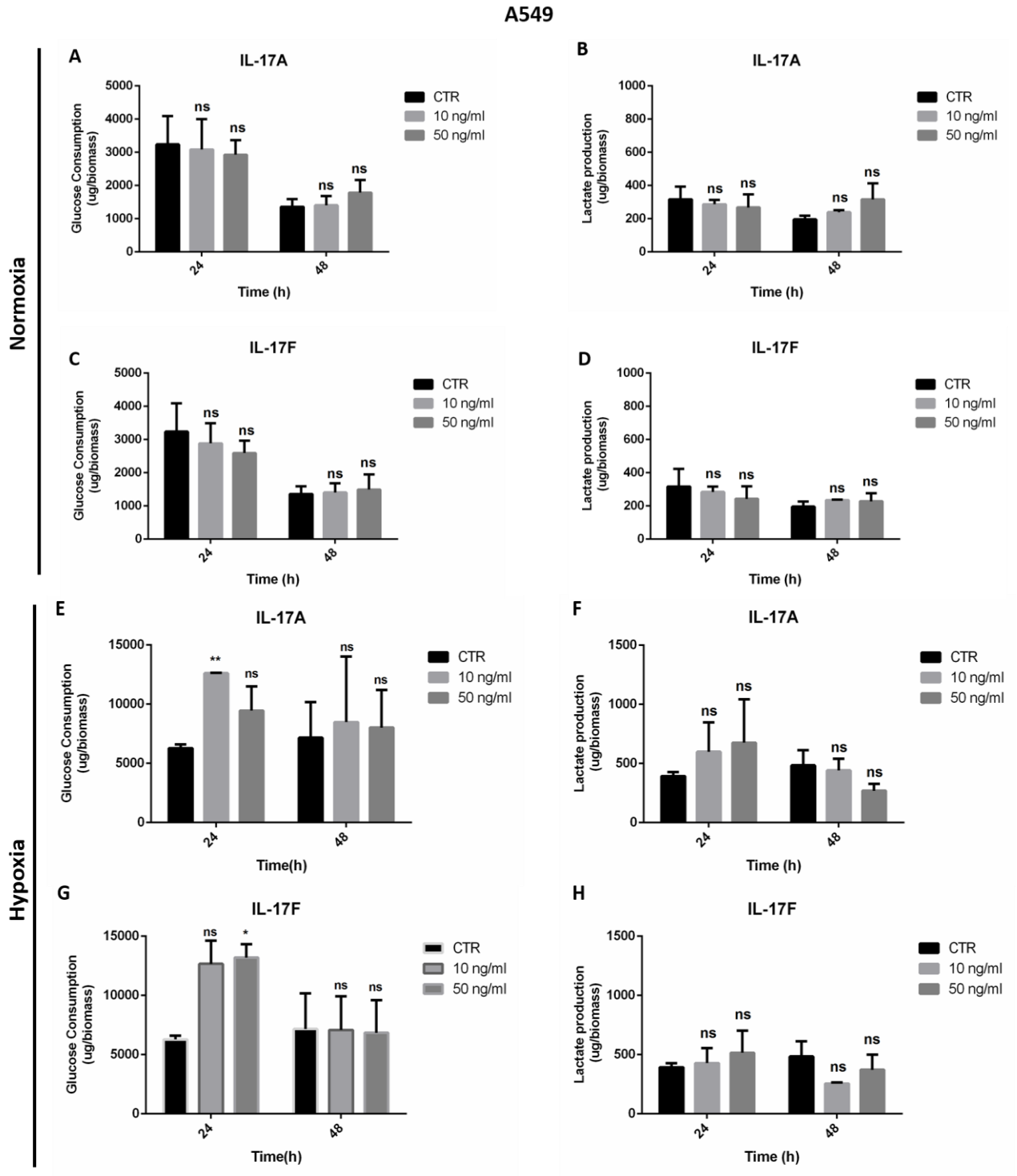
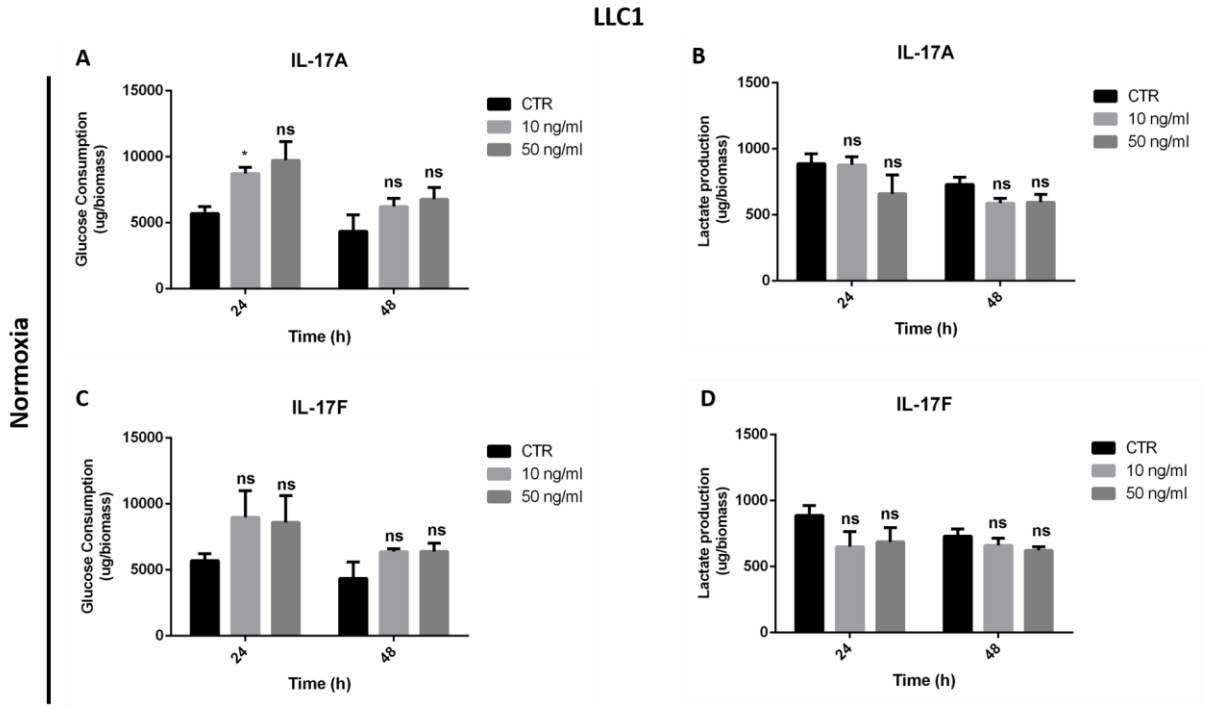


Figure 14. **Assessment of A549 metabolism stimulated with IL-17A/F.** A-D: Glucose (A, C) and lactate (B, D) quantification, by HPLC, of cells stimulated with 10 and 50 ng/ml of IL-17 A/F or without stimulation (CTR) under normoxic conditions. (E-H) Evaluation of glucose consumption (E, G) and extracellular lactate (F, H) of cells stimulated with 10 and 50 ng/ml of IL-17 A/F or without stimulation, by enzymatic kits under hypoxic conditions. Results represent the mean ± SD of triplicates from two independent experiments.* $p < 0.05$, **$p < 0.01$; ns- non significant; compared to untreated cells (CTR).

IL-17A/F stimulation of A549 cells cultivated in normoxic conditions did not modify the rate of glucose consumption or lactate secretion when compared to unstimulated cells (Figure 14 A-D). However, under hypoxic conditions, both IL-17A/F stimulated cells presented a significant increase in glucose consumption (Figure 14 E, G) with no impact on lactate production (Figure 14 F, H). This enhanced glucose consumption, can be explained by the activation of downstream pathways of IL-17 signaling. For instance, NF-kB activation led to enhanced glucose consumption through an upregulation of GLUT3 expression in Mouse Embryonic Fibroblasts (MEF) model[189]. Moreover, a study showed a synergistic combination of hypoxia and IL-17A stimulation with the enhanced expression of HIF-α, a transcriptional factor responsible of enzymes involved in the glycolysis pathway [184].



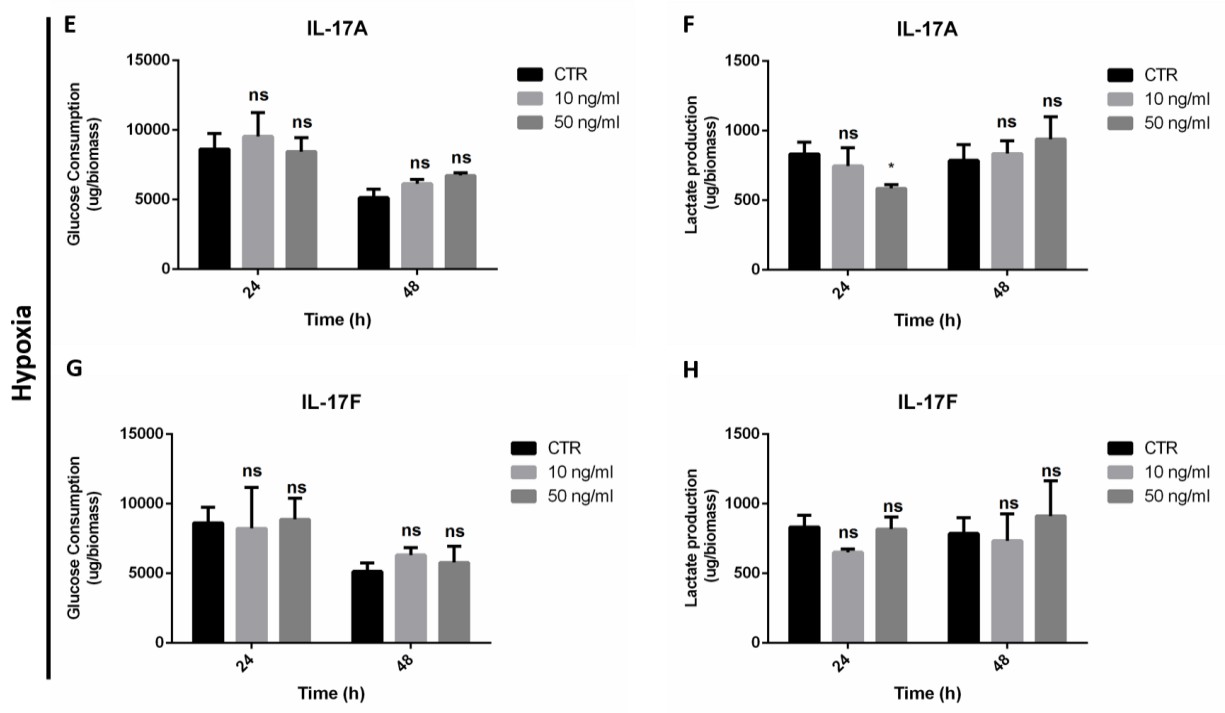


Figure 15. **Assessment of LLC1 metabolism stimulated with IL-17A/F**. A-D: Glucose consumption (A, C) and lactate (B, D) production of cells stimulated with 10 and 50 ng/ml of IL-17 A/F or without stimulation (CTR) under normoxic conditions. (E-H) Evaluation of glucose consumption (E, G) and extracellular lactate (F, H) of cells stimulated with 10 and 50 ng/ml of IL-17 A/F or without stimulation, by enzymatic kits under hypoxic conditions. Results represent the mean ± SD of triplicates from three independent experiments.* $p < 0.05$; ns- non significant; compared to untreated cells (CTR).

Nonetheless, the observed phenotypes were not maintained in LLC1 cell line. An increase in glucose consumption was indeed observed in LLC1 stimulated with IL-17A and IL-17F but in normoxic conditions (Figure 15 A, C). However, in this case the increase of glucose consumption was not reflected on lactate production (Figure 15 B, D). Moreover, in hypoxic conditions, a significant decrease in extracellular lactate was seen in cells stimulated 50 ng/ml of IL-17A (Figure 15F). Overall, both cell lines behave metabolically different upon IL-17A or F stimulation.

The decrease in lactate production may be explained by the fact NFkB can also activate the OXPHOS pathway, via p53-dependent upregulation of synthesis of cytochrome c oxidase 2 (SCO2)[180].

To gain insight on the proteins involved in the glycolytic pathway (Figure 16), western blot analysis was performed using a set of proteins: Glut1 and Glut3, responsible for glucose uptake across the plasma membrane; HK2 that catalyzes the phosphorylation of glucose; MCT1 and MCT4, respectively, acting as carries of monocarboxylates such as lactate; LDHA, that converts pyruvate into lactate; and PDK that acts as regulator of OXPHOS, through decrease of pyruvate oxidation in mitochondria.

Moreover CAIX, an important enzyme responsible for intracellular pH maintenance and a marker of hypoxia, was also evaluated (Figure 16).

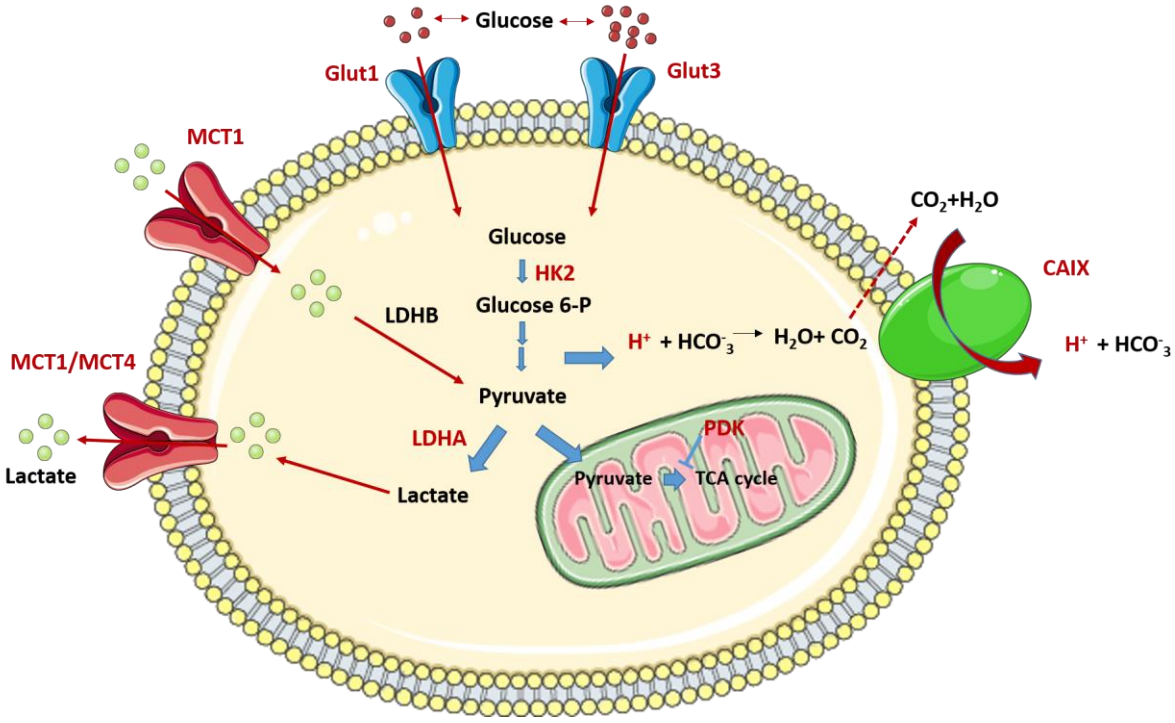


Figure 16.**Squematic representation of the proteins (marked in red) analyzed by western-blot.**

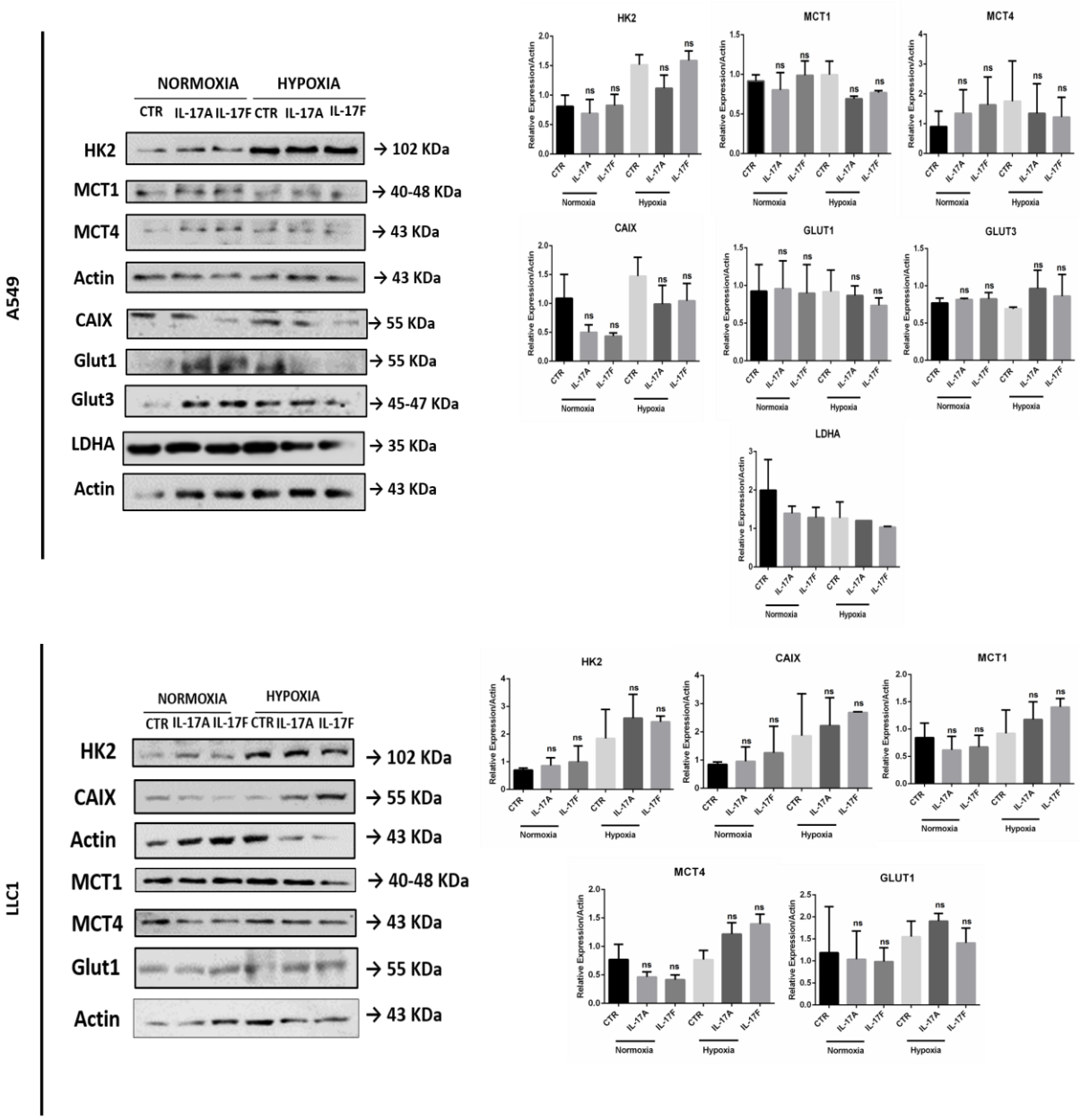


Figure 17. **Analysis of key glycolytic proteins in lung cancer cell lines with and without IL-17 A/F stimulation by Western Blot.** After 24 hours of stimulation with 10 ng/ml of IL-17 A/F in A549 (top) and LLC1 (bottom) cells, proteins involved in glycolytic pathway were analyzed. At left are representative images of two independent assays, and at right the respective relative quantification, normalized against actin expression. ns- non significant.

Interestingly, although some differences were found in glucose consumption and lactate secretion in A549 and LLC1 stimulated cells, we did not observed any relevant and significant modification of several enzymes involved in glucose metabolism (Figure 17).

In the case of LLC1 cells, despite the tendency of increase in glycolytic proteins, especially in hypoxic conditions, the results were not significant different between cells without stimulation and the cells with IL-17A /F treatment. Altogether, these data showed no significant impact of IL-17A/F directly in NSCL metabolism.

4.1.4. Migration of A549 cells were not affected directly with IL-17A/F stimulus.

Previous studies have demonstrated a connection between IL-17A stimulation and increase in cell migration capacity of NSCLC cells[137,181]. Thus, we further investigated this relationship by performing wound healing assay in A549 cells under stimulation with IL-17 A/F in a dose-dependent manner (Figure 18). However, no significant differences were found between cells stimulated with IL-17 A/F stimulation and the control groups.

Overall, our data demonstrate that direct IL-17A/F stimulation in both A549 and LLC1 cell lines did not modify tumor growth, cell viability or cell migration capacity, despite the presence of IL-17 receptors.

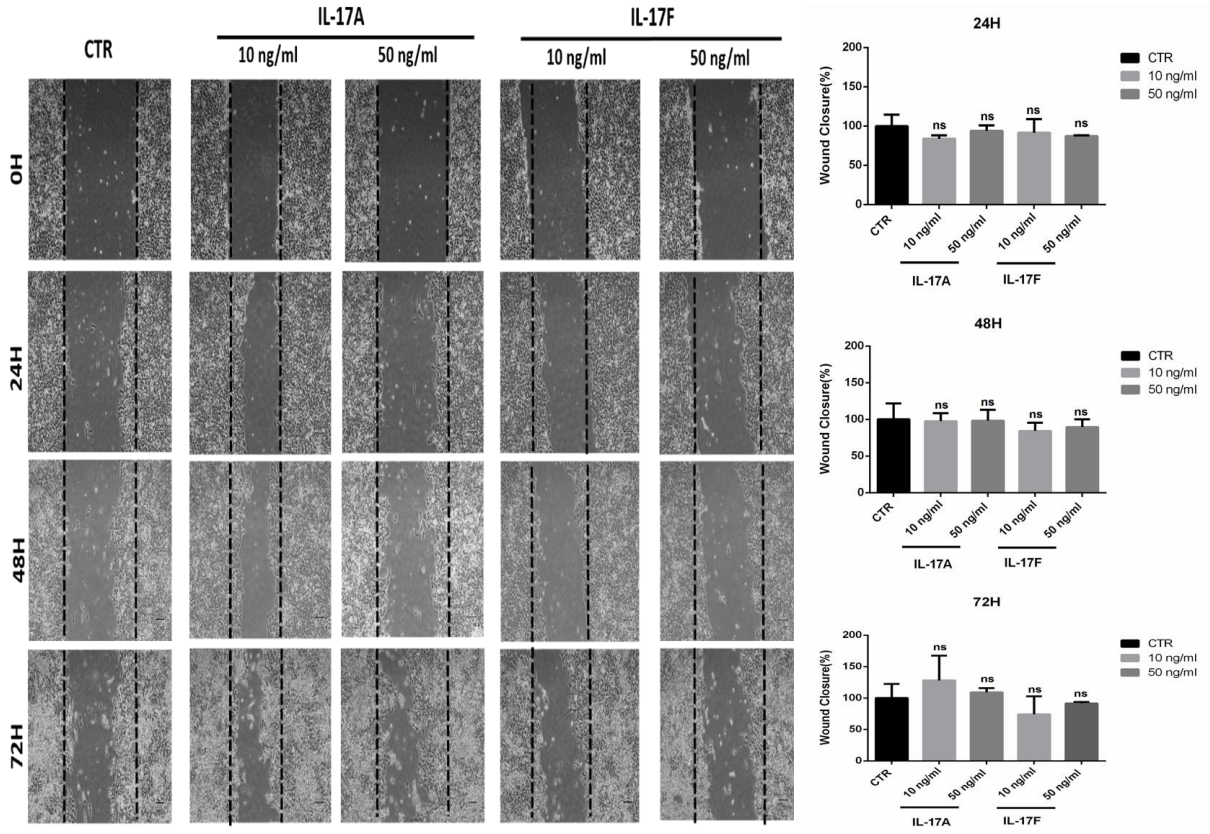


Figure 18. **Migration of A549 cells under IL-17 A/F stimulation.** Assessment of migration capacity by wound healing assay after 24, 48 and 72 hours of stimulation with 10 or 50 ng/ml of IL-17 A/F, or without stimulation (CTR). At the left are representative pictures of wounds (40x magnification). The results were analyzed from two independent assays. ns- non significant.

4.2. Evaluation of an indirect impact of IL-17A/F on tumor cells via macrophage stimulation.

Macrophages represent the dominant leukocyte population in the tumor microenvironment. Accumulating evidences suggest that TAMs actively participate in all aspects of tumor initiation, growth and progression (reviewed in[182]). Yet, TAMs phenotype/polarization and function vary depending on the local inflammatory state and/or tumor microenvironment mediators. Based on preliminary unpublished data that shows modulation of macrophages metabolism and function upon IL-17A and IL-17F stimulation, we hypothesized that the mechanism of action of these cytokines in lung cancer may pass through macrophages manipulation.

To address this question, we culture murine lung cancer cell line with a CM composed by the supernatant of cultured bone marrow macrophages stimulated with 50 ng/ml of IL-17A or IL-17F. Several tumorigenicity characteristics were further evaluated.

4.2.1. Media from IL-17 A/F stimulated macrophages did not impact LLC1 cells viability.

We started to evaluate the effect of IL-17 A (CM (MO+IL-17A)), IL-17 F (CM (MO+IL-17F)) stimulated macrophages- conditioned media in cell viability (Figure 19). No effect was observed in cell mass in any of the tested conditions.

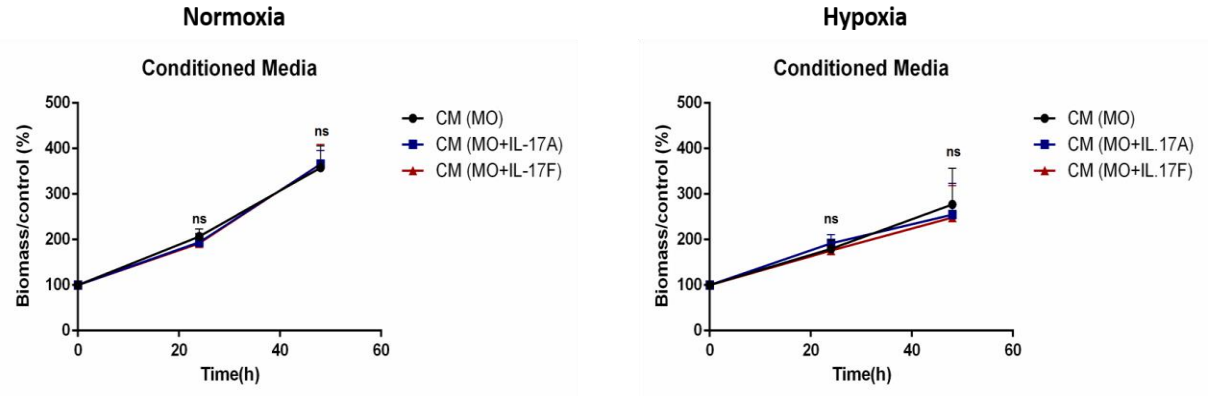


Figure 19.**Evaluation of LLC1 viability until 48 hours with CM (MO+IL17A), CM (MO+IL-17F) and CM (MO) stimulation, by SRB assay.** The results were analyzed from two independent assays in hypoxic conditions (right) and three independent assays in normoxic (left) conditions, done in triplicated per condition. ns- non significant.

4.2.2. Conditioned media from macrophages under IL-17 A/F does not change tumor spheroids area.

We further addressed the influence of IL-17A/F in LLC1 growth capacity, using a different *in vitro* model. Tumor spheroids has the advantage of mimic avascular *in vivo* tumors presenting both hypoxic region at the center and normoxic regions at surface region, making this 3D-culture more physiologically relevant than 2D-monocultures. No significant differences were observed in the area of tumor spheroids between the groups with IL-17A/F and the CM (MO) group (Figure 20).

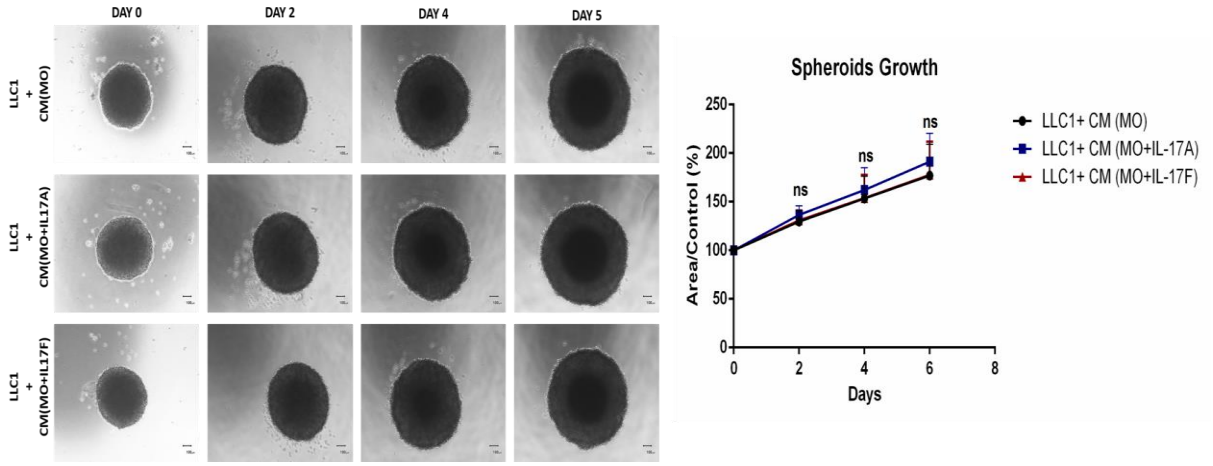


Figure 20. **Tumor spheroids area across time, under stimulation with CM (MO+IL-17A), CM (MO+IL-17F) and CM (MO) evaluation**. At left are representative pictures of spheroids (40x magnification) and at right are the respective spheroid area normalized with the area of spheroids before CM stimulation. The results are from three independent assays. ns-non significant.

4.2.3. Media from IL-17 A/F stimulated macrophage did not significant impact LLC1 metabolism.

Given that we previously observed some tendencies in glucose consumption in lung cancer cells stimulated with IL-17 A/F, we evaluated if this response is maintained or enhanced by the IL-17 A/F macrophage manipulation. No significant differences were found in the majority of the conditions (Figure 21). Yet, contrary to what was observed in LLC1 stimulated directly with IL-17A/F, extracellular lactate was significant increased with CM (MO+IL-17A) (Figure 21B) under normoxic conditions. Interestingly, a tendency for a decrease in glucose consumption was observed in all conditions, although a significant decrease was only observed in cells with 48 hours of stimulation with CM (MO+IL-17A) and CM (MO+IL-17F).

Despite the alteration in glucose and lactate metabolites quantification, no alteration in glycolytic proteins was observed (Figure 22). Overall, these results suggest that another carbon source is produced by stimulated macrophages.

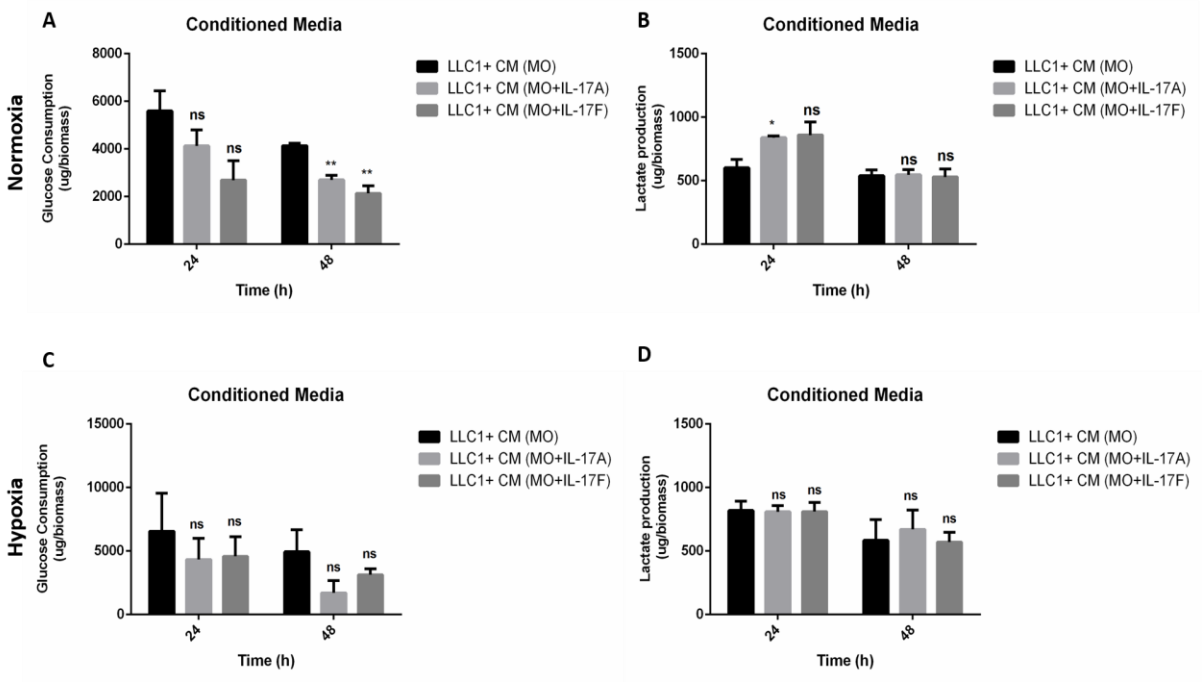


Figure 21 **Assessment of LLC1 metabolism stimulated with CM from IL-17A/F stimulated macrophages**. Glucose consumption (A, C) and lactate (B, D) production of cells stimulated with CM(MO), CM(MO+IL-17A) and CM(MO+IL-17F) stimulation (CTR), under normoxic conditions (top) and hypoxic conditions (bottom). Results represent the mean ± SD of triplicates from three independent experiments in normoxic conditions and two in hypoxic conditions. * $p < 0.05$; $^{**}p < 0.01$; ns- non significant; compared to cells treated with medium from macrophages without stimulation (LLC1+CM (MO)).

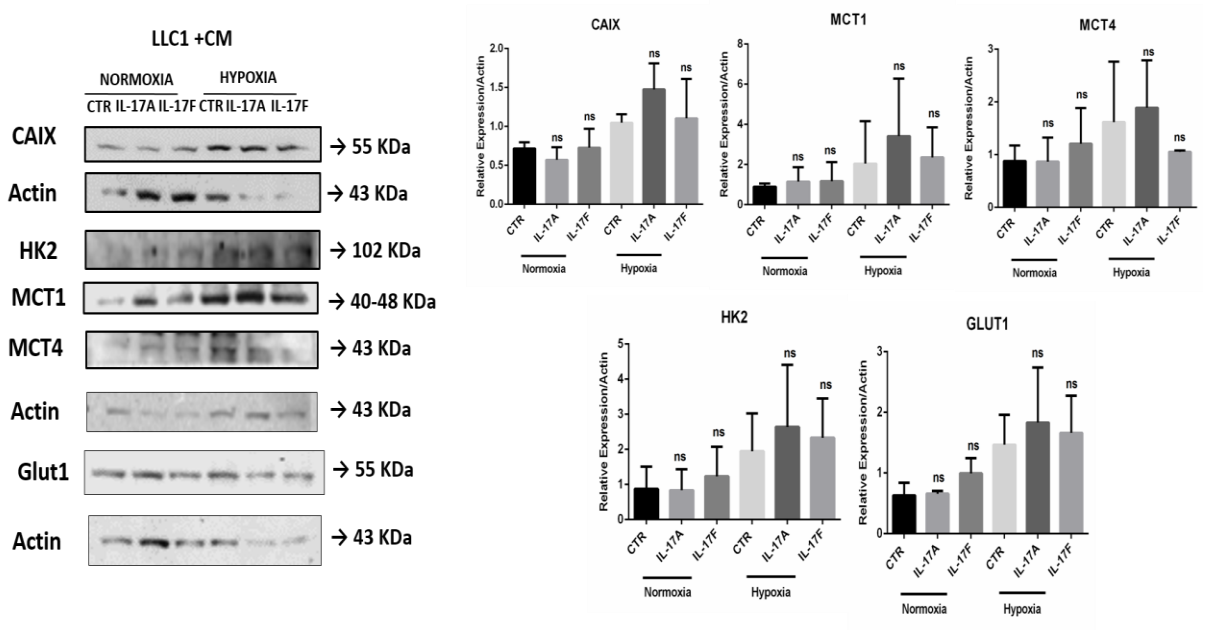


Figure 22. **Evaluation of key glycolytic proteins in LLC1 cells with and without IL-17 A/F stimulated with different conditioned media, by Western Blot.** After 24 hours of stimulation with different CM from IL-17A/F stimulated macrophages, proteins involved in glycolytic pathway were analyzed. At left are representative images of two independent assays, and at right the respective relative quantification, normalized against actin expression.ns-non significant.

4.2.4. LLC1 stimulated with media from IL-17 F stimulated macrophage presented an enhanced migration capacity.

Since IL-17A/F did not impact cell migration of A549 cells directly, we further explore if this characteristic is altered via macrophage stimulation. LLC1 cells under stimulation with CM (MO+IL-17F) presented an increased migratory capacity when in comparison with CM (MO) stimulated LLC1 cells (Figure 23). This data suggests a pro-tumoral characteristic of IL-17F through immune system alteration.

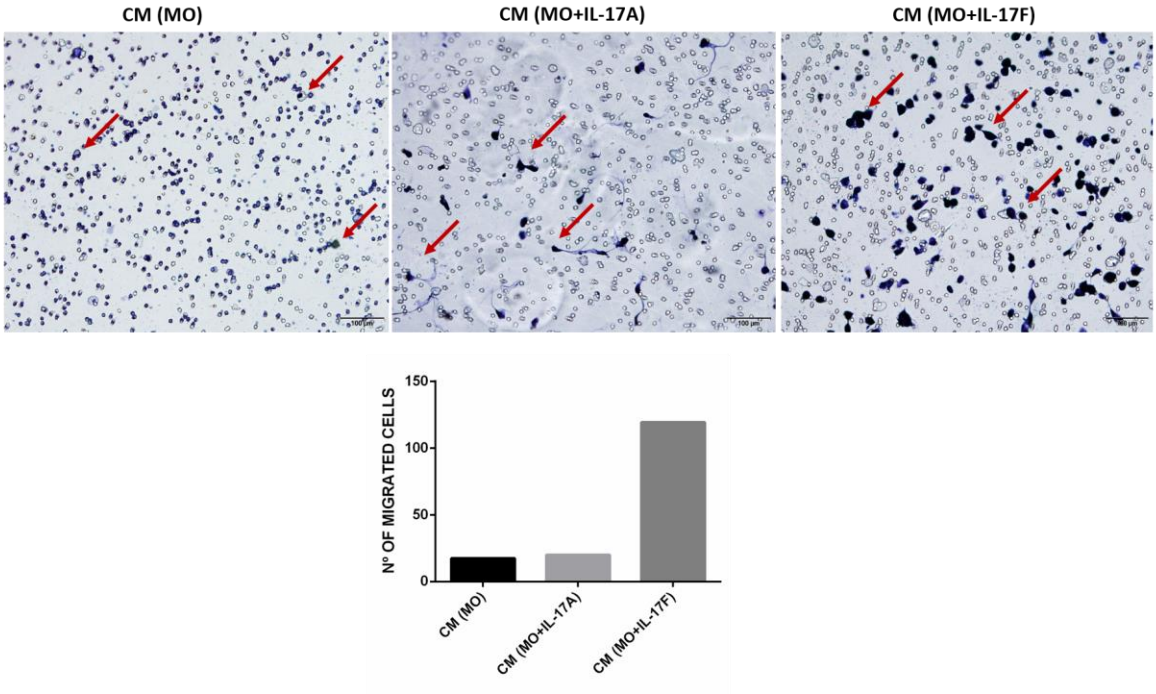


Figure 23. **Migration of LLC1 cells under different CM stimulation.** Assessment of migration capacity by boyden chamber after 48 hours of stimulation with CM(MO),CM(MO+IL-17A) and CM(MO+IL17F).At the top are representative pictures of migrated cells area (100x magnification). Migrated cells (indicated with red arrow) were quantified by analyze of three different areas of the membrane. Results represent the mean of one independent experiment

4.2.3. LLC1 with conditioned media from macrophages with IL-17A or IL-17F displayed a higher tumor growth, *in vivo*.

To gain further insight on the potential pro-tumoral effect of IL-17F, LLC1 cells were injected in combination with different CM into the CAM of chick embryos. After 7 days of treatment, embryos were sacrificed to analyze tumor growth.

As depicted in figure 24, *ex vivo* images were taken after excision of tumors from the CAM, showing a significant increase of tumor growth in tumors treated with CM(MO+IL-17A) or CM (MO+IL-17F) when in comparison with tumors with media from unstimulated macrophages CM (MO). Altogether, our data suggests an indirect pro-tumoral function of IL-17 A and IL-17F in tumor progression of LLC1 cells *in vivo*.

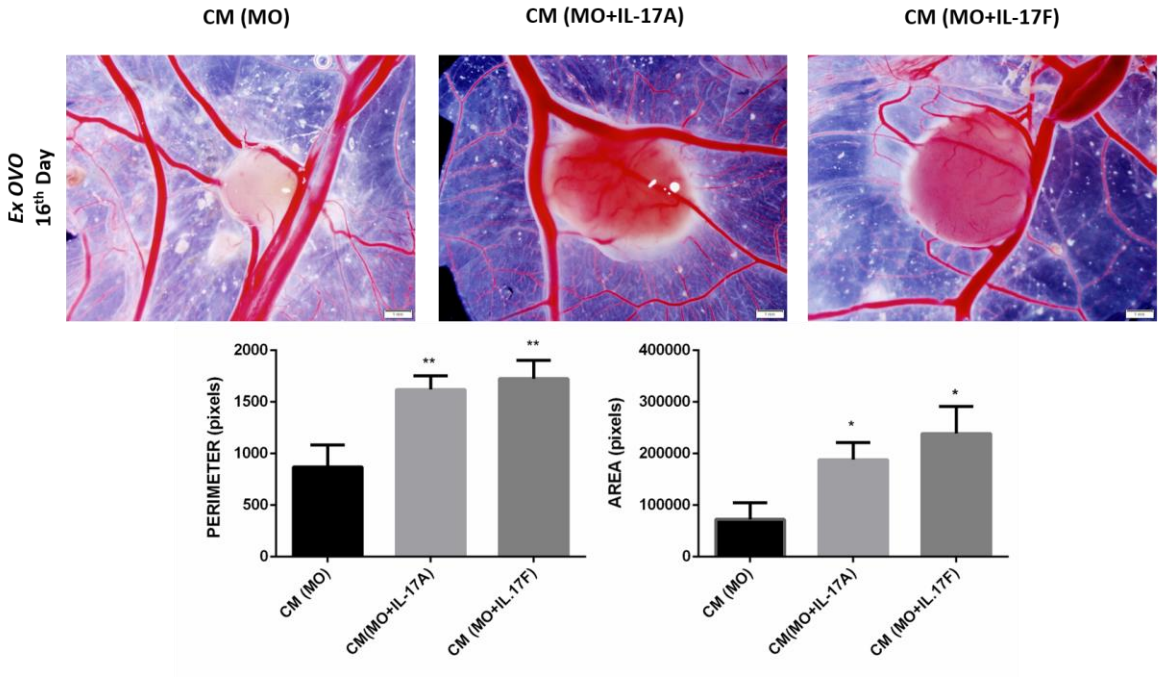


Figure 24. ***In vivo* role of different conditioned media from IL-17A/F stimulated macrophages.** Representative pictures (top) were taken *ex ovo*, after 16 days of development, using a stereomicroscope. Tumor growth (bottom) is represented as the mean of tumors areas and perimeter (pixels). The results are representative of n=6 for CM (MO) and n=8 for CM (MO+IL-17A) and CM(MO+IL-17F).

CHAPTER 5: DISCUSSION

DISCUSSION

Lung cancer is the principal cause of cancer-related deaths worldwide, presenting one of the lowest 5-year survival rates of all malignancies[28,29]. One of the reasons that explain this high mortality rate, is the fact that the majority of patients are diagnosed at an advance stage of disease progression that do not allow surgery resection of tumor, which is the best curative treatment for this type of cancer. The current treatment for these patients is based on platinum-based doublet chemotherapy, however it has been proven to have a modest contribution on survival rate. Given the lack of effective treatment for advanced-stage lung cancer patients, advances in understanding the lung cancer biology are imperative towards the improvement of patient survival.

The last years have been portraying the connection between immune system and tumorigenesis, which is now considered one of hallmarks of cancer[4]. Moreover, lung cancer is a highly immunogenic tumor, and as such a number of immunotherapeutic strategies are in clinical trials as adjuvants or first-line therapy in treatment of lung cancer[183]. However, tumors can become resistant to these agents over time, being important to understand the TME to overcome tumor resistance and enhance the capacity of the current treatments.

The IL-17 family of proinflammatory cytokines have been acting as a double-agent in tumor progression in a widely type of cancers [79]. Nevertheless, the crosstalk between lung cancer and these group of cytokines has been discussed, being IL-17A pointed as a pro-tumor factor[136-142] despite some studies suggesting otherwise[123]. Regarding IL-17F, only one study addressed a genetic polymorphism as a risk factor for lung cancer, in Tunisian population[144].

Preliminary data of TCGA analysis demonstrated a positive correlation between the intratumoral expression of IL-17F, but not IL-17A, and patient survival. Therefore, we firstly aimed to understand if IL-17 A/F affects directly lung cancer cells using *in vitro* approaches.

After confirming the expression of IL-17 R in the human and murine cell lines by protein levels, we stimulated in a dose-dependent manner these cells with 10 or 50 ng/ml of IL-17 A/F and evaluated several tumorigenic characteristics. Although a small decrease on cell viability in A549 cells stimulated with IL-17A in a dose dependent manner in normoxic conditions and with 10ng/ml of IL-17 F in hypoxic condition, in overall there was not a significant impact of IL-17A/F in A549 cell viability. Along with the

absence of significant impact on cellular viability in LLC1 cell line, our data shows that IL-17A/F stimulation does not affect *in vitro* growth rate, in accordance with study from Numasaki M et al. In their study, using the MTT assay approach that determines cell viability by metabolic activity, no significant impact on NSCLC cells SQCLC and AdenoCA cell (Sq-19 and A549 cells, respectively) growth was observed upon culture with and without human IL-17 ranging from 0.1 to 1000 ng/ml for 5 or 7 days. Furthermore, the researchers explored the relation between IL-17 and growth of NSCLC, by transfecting A549 and Sq-19 cells with human IL-17 gene expression plasmid. Again, until 6 days of culture, IL-17 did not affect the grow rate of NSCLC [129]. The fact that the authors tested a wide range of IL-17A concentration, it seems for certain that IL-17 does not have a direct effect on NSCLC growth.

Regarding cell migration, in contrast with a study presented by Ye et al. and Kuo Gu et al., IL-17 A/F exogenous stimulus did not alter migratory capacity of A549 cells. In the research presented by Kuo Guo et al[137], the capacity of migration under IL-17 stimulation was evaluated in A549 cells by two different approaches. Firstly, they performed a wound healing assay in which they cultured the cells at density of $1x10^5$ cells per well, in order to analyze the stimulation with and without IL-17A; with IL-17A plus an inhibitor of NF-kB activity; and IL-17A stimulus in A549 cells silenced for ZEB-1. After 24 hours the wound healing capacity was higher in cells treated with IL-17A in comparison to the others groups. Furthermore, by using Transwell chambers, after 24 hours with treatment they saw the same response with IL-17A stimulation. Therefore, the researchers concluded that the enhanced migratory favored by IL-17A stimulation via the NF-κB/ZEB1 signaling pathway. Many variables can explain the difference between this response and our results. Firstly, it might be explained by the different density of A549 cells used to perform the wound healing assay, which is important to this assay as cells need to form a monolayer structure. If the cell density is low, the wound closure might be due to the growth of cells as it has space and not enhanced wound healing capacity. On the contrary, if the density is too high the inhibition of migration ability might occur, and the stress caused can even result in cell detached from the well, impeding wound healing measure. Another variable is that the authors could have used another concentration, however, there was no mention of the concentration of IL-17 that was used during A549 stimulation. Furthermore, the percentage of FBS that given to the cells was not mentioned. This factor is important since higher concentrations of FBS can enhance the cell migration capacity and thus, it can have an influence in wound healing results. In relation to Ye et al. [137,181] study, they assessed the migration using a different approach. Through 8-μm pore polycarbonate filters in 24-well Transwell chambers, A549 cell migration was evaluated using 50 ng/ml of IL-17A. An increased in migratory capacity was observed upon IL-17 stimulation. However, a potential bias comes from the coating the membranes with

fibronectin, which has been described as chemoattractant including in tumor cells [184]. Therefore, the increase of A549 migration capacity with IL-17 compared with control might be due to the combination of IL-17 and fibronectin, and not IL-17 itself.

As a recent added hallmark, we evaluated the capacity of cancer cells to reprogramme their metabolic activity to sustain their proliferative and aggressiveness ability [4]. Given the several advantages that a metabolic reprogramming towards a highly fermentative pathway conferred to malignant cells, we investigate whether IL-17A/F could impact lung cancer metabolism. We observed a higher consumption of glucose in A549 with IL-17A or IL-17F stimulus in hypoxic conditions, and in LLC1 cells with 10 ng/ml of IL-17A under normoxic condition. Despite this increase, glucose consumption was not translated in lactate production, showing even a decrease in some groups. Moreover, despite a slight increase on GLUT3 in A549 and GLUT1 in LLC1 in hypoxic conditions, this increase was not statistically significant similarly to the rest of analyzed glycolytic proteins. Interestingly, combination of IL-17 and TNF-α promoted glucose metabolism in human colorectal cancer cells[185].Thus, we suggest that IL-17 needs to synergize with another cytokine to potentiate the glycolytic phenotype of lung cancer cells.

Although no significant impact of lactate production under hypoxic conditions was observed, some studies connected the relationship between IL-17 and indirectly with glucose metabolism, through HIF- α expression. Study from Li et al.[186] demonstrated an enhanced invasion and migration capacity of fibroblast-like synoviocytes-rheumatoid arthritis (RA-FLSs) cells through upregulation of MMP2 and MMP9, via NF-κB/HIF-1α pathway. Moreover, a synergy of cobalt chloride (CoCl2) induced hypoxia with IL-17 resulted in protein and mRNA expression of HIF-1α and MMP-9 in rat synovial macrophages[187]. Additionally, through a decrease of miR-497 expression in experimental mouse autoimmune encephalomyelitis (EAE) model, high HIF-1α expression was observed by IL-17 simulation[188]. Altogether, HIF-1α, a known transcription factor responsible for upregulation of several glycolytic key enzymes[189], is upregulated by IL-17 alone or in combination with hypoxia environment. This connection might lead to a enhance glucose metabolism. Thus, it would be important to analyze if the HIF- α expression is altered in NSCLC cells stimulated with IL-17A or IL-17F.

Another impact of IL-17 in metabolism may pass through NF-kB or MAPK signaling. Novellasdemunt et al.[190] found a relation between glycolysis and MAPK pathway. Through MAPK- activated protein kinase 2 (MK2) phosphorylation action, MK2 activated 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 3 (PFKFB3), a key glycolysis-promoting enzyme.

In the case of NF-kB, their effect on cell metabolism might be dependent on p53 status[191]. Tanaka et al, showed an increase of glucose uptake though GLUT3 in $p53^{-/-}$ MEF, and even was responsible for

high glycolytic flux maintenance [192]. Also, NF-κB has a connection with mitochondrial respiration, through stimulation of OXPHOS through SCO2 upregulation[180]. Therefore, it would be interesting to perform a genome sequencing of LLC1 cells to analyze the p53 expression to understand if our cell line is mutated for this gene. Regarding A549 cells, these cells presents a WT p53 which can explain the decrease of lactate production by the enhance oxidative phosphorylation via NF-kB pathway.

Altogether, our data support that neither IL-17A nor IL-17F can influence directly lung cancer cells. Given the knowledge that IL-17 A is not a powerful pro-inflammatory cytokine by itself and normally acts in synergy with another cytokines in inflammation, the tendency of glucose metabolism in NSLC cell lines could be enhanced by the combination with another pro-inflammatory cytokines. In fact, many studies have been combined with TNF-α to evaluate IL-17 in tumor progression. In breast cancer, the synergistic combination these cytokines, led to an enhancement of HIF-1α gene. Moreover, Vasodilator-stimulated phosphoprotein (VASP) was also upregulated. This protein acts through promotion of actin polymerization[193,194] and in the regulation of adherence junctions in epithelial cells[195]. Thus, VASP upregulation reduce the adhesion of breast cancer MDA-MB-231 cells. Curiously, this protein has been connected with invasive biological behavior of AdenoCAs, possibly due to focal adhesion, intracellular actin filament formation and cell migration regulation[196].

In the absence of direct influence of IL-17 on tumor cells, we next explored if the effect of IL-17A/F pass through manipulation of the dominant leukocyte population in the lung, the macrophages[197]. These immune cells were also chosen due to the unpublished preliminary data from our group, which demonstrate a quiescent state on macrophages upon IL-17F stimulation. To address this question, media from macrophages stimulated with IL-17A or IL-17F and media from unstimulated macrophages were given to LLC1 cells. Analysis of LLC1 growth with different CM was assessed in monolayer and a 3D structure. No difference in cell viability and tumor spheroids growth was observed regardless of the CM from IL-17A/F stimulated macrophages given to the cells. Unexpectedly, when tumor cell metabolism was evaluated upon CM treatment, a decrease in glucose consumption was observed whereas a significant increase in lactate production in LLC1 stimulated with CM (MO+IL-17A) was detected. This suggests that another energy source than glucose present in the CM may promote lactate production in LLC1 cells. A previous study identified IL-6, PGE2 and stromelysin among the most present products in the culture supernatant of human macrophages stimulated with IL-17A[198]. Stromelysins, also known as MMP-3, functions through degradation of ECM proteins. Therefore, further analysis of CM is necessary to understand the potential carbon source for the observed enhanced lactate production. As before, no significant alteration of glycolytic proteins was found, suggesting that IL-17A/F do not alter the glycolytic

pathway at least, though macrophage manipulation. If the products described in human IL-17 stimulated macrophages translate to murine macrophages, it could partially explain the enhanced number of migrated cells under CM from IL-17F stimulated macrophages via stromelysin expression.

Finally, we addressed the *in vivo* tumor growth role of the different CM after 16 days in chick embryos. Both CM from IL-17A or Il-17F stimulated macrophages, showed an increase in tumor area and perimeter when in comparison with tumor with stimulus from CM (MO).

The migration and CAM assay suggests that both IL-17A and IL-17F acts as pro-tumoral cytokines through macrophage stimulation. In fact, the combination of PGE2 produced by epithelial cells under IL-17 stimulation can induce a M2-macrophage differentiation[199], which are associated with pro-tumor functions.

CHAPTER 6: CONCLUSIONS AND FUTURE PERSPECTIVES

CONCLUSIONS AND FUTURE PERSPECTIVES

The connection between IL-17 family of cytokines and cancer is still a debatable question. Some authors suggest a pro-tumoral role whereas other demonstrates an anti-tumoral function, depending on cancer type.

Although our study still did not clarify the putative differential role for both cytokines in lung cancer, the analysis of the function of these cytokines should be further evaluated in murine model. Since the TCGA analysis assessed the intratumoral expression of IL-17F in patients with better prognostic, it would be interesting to analyse the effect of both IL-17A/F intratumorally. To do that, genetic manipulation of LLC1 cell line to overexpress IL-17A or IL-17F under a stringent control of an antibiotic, would be an approach since it allow the increase the levels of each cytokine inside the tumor avoiding any relevant systemic effects and the possibility to control temporally their induction. Following injection of these genetically engineered LLC1 to secrete IL-17A or IL-17F in WT mice, several readouts can be made. Firstly in a subcutaneous model, it would be important to measure tumor growth and analyze the temporal effect of these cytokines, inducing cytokine overexpression during the first days or during last days upon tumor injection. Following, tumor metastasis should be evaluated by dissecting different organs (lung, liver, spleen) for histology analysis.

In orthotopic model, upon the injection in the left lung of the mice, tumor progression could be assessed by the life span of the mice and survival curves should be designed. These *in vivo* approaches would be beneficial to evaluate the impact of IL-17A/F on tumor growth in different stages of tumor evolution, and also the effect in metastization and overall survival impact.

The demonstration that IL-17 A and F stimulation alone does not impact lung cancer cells biology, the action mechanism may be via immune system orchestration as demonstrated by the effect of macrophage manipulation in promoting, *in vivo* LLC1 tumor growth. It would be interesting to investigate which immune cell population is recruited upon IL-17A/F stimulation in lung cancer tumor bearing mice, by flow cytometry analysis. Moreover, analysis of culture supernatant should be performed, as for example ELISA quantification, to identify potential cytokines combination that could increase their effect.

Since cancer cells can use other pathways than glycolysis such as glutaminolysis, which supports lipid production, amino acids synthesis and pentose phosphate pathways, it would be important to address the function of IL-17 A/F in lung cancer metabolism. To approach this, metabolomics experiments should be performed to characterize and quantify the metabolites in supernatant of NSLC cells stimulated with IL-17A/F. Also, to address the altered metabolic fluxes, measurements through Seahorse analysis would be important since it allows a real time analysis of the metabolic rate upon stimulation.

Finally, to understand the biological effect of IL-17 inhibition, a study using an established mice model presenting an endogenous KO for IL-17, or the administration of FDA approved IL-17 antibodies, such as Secukimumab and Ixekizumab, would be an interesting approach.

This master thesis provided new insights of how IL-17F acts in lung cancer progression. Future studies are in order with a murine *in vivo* approach to further evaluate the function of this cytokine within immunocompetent system.

CHAPTER 7: BIBLIOGRAPHY

BIBLIOGRAPHY

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