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# Impact of respiratory viral infections on tumor progression in Non-Small-Cell Lung Carcinoma (NSCLC)

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# **Impact of respiratory viral infections on tumor progression in Non-Small-Cell Lung Carcinoma (NSCLC)**

**PhD Thesis**

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## **Abbreviations**

### **A**

ADC: lung adenocarcinomas

AMs: alveolar macrophages

APCs: antigen-presenting cells

ARG1: Arginase 1

### **B**

BAL : bronchoalveolar lavage

B cells: lymphocytes B cells

Bcl-2: B-cell lymphoma 2

### **C**

CAFs: cancer- associated fibroblasts

CCL: CC chemokine ligand, CXCL: CXC chemokine ligand

CDS: cytosolic DNA sensors

COPD: chronic obstructive pulmonary disease

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

### **D**

DAMPs: Damage-associated molecular patterns

DCs: dendritic cells, pDCs: Plasmacytoid dendritic cells, mDC: Myeloid dendritic cells

### **E**

ECM: extracellular matrix

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

EMT: epithelial to mesenchymal transition

### **F**

FGF: fibroblast growth factor

### **G**

$\gamma\delta$  T cells: Gamma delta ( $\gamma\delta$ ) T cells

GM-CSF: granulocyte–macrophage colony-stimulating factor

### **H**

HA: influenza hemagglutinin glycoprotein

HIF-1: hypoxia-inducible factor 1

## **I**

ICIs: immune checkpoint inhibitors

IFN: Interferon

IFNAR: interferon- $\alpha/\beta$  receptor

IFNLR: IFN  $\lambda$  receptor

IDO: indoleamine 2,3-dioxygenase

IL : Interleukin

iNOS2: inducible Nitric oxide synthase 2

irAEs: immune-related adverse events

IRF: IFN-regulatory factor

ISGs: interferon-stimulated genes

ISREs: IFN-stimulated response elements

IV: influenza virus, IAV: influenza A virus, IBV : influenza B virus, ICV : : influenza C virus

## **J**

JAK1/2: Janus Kinase 1/2

## **L**

LAG3: lymphocyte activation gene 3

LGP2: laboratory of genetics and physiology 2

## **M**

MAPK: mitogen-activated protein kinase

MDA5: melanoma differentiation-associated protein 5

MAVS: Mitochondrial antiviral-signaling protein

MDSCs: Myeloid-derived suppressor cells, PMN-MDSCs: polymorphonuclear MDSCs, M-MDSCs: monocytic MDSCs

MHC-I/II: major histocompatibility complex type I/II

MyD88: myeloid differentiation primary response gene 88

## **N**

NA: Influenza neuraminidase glycoprotein

NADPH: Nicotinamide adenine dinucleotide phosphate

NETs: neutrophil extracellular traps

NF- $\kappa$ B: nuclear factor kappa-light-chain-enhancer of activated B cells

NK cells: natural killer cells

NLRC5: NOD-like receptor family CARD domain containing 5

NLRP3: NOD-, LRR- and pyrin domain-containing protein 3

NLRs: NOD-like receptors

NSCLC: non–small-cell lung cancer

## **P**

PAMPs: Pathogen-associated molecular pattern molecules

PD-1: programmed death 1

PD-L1/L2: programmed death ligand 1/ ligand 2

PGE2: prostaglandin E2

PRRs: pattern recognition receptors

## **R**

Raf Kinase: Rapidly Accelerated Fibrosarcoma Kinase

RANTES: Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted

RIG-I: retinoic acid-inducible gene-1

RLRs: RIG-I like receptors

ROS: Reactive oxygen species

RSV: respiratory syncytial virus

RSV-F: RSV- fusion protein

## **S**

SCC: squamous cell carcinoma

SCLC: small-cell lung cancer

STAT1/2/3: Signal transducer and activator of transcription 1/2/3, pSTAT1: phosphorylated STAT1

## **T**

TAMs: tumor-associated macrophages

T cells: lymphocytes T cells

TECs: tumor endothelial cells

TEVs: Tumor cell-derived extracellular Vesicles

TGF- $\beta$ : transforming growth factor- $\beta$

TH1/2: T helper 1/2

TIGIT: T cell immunoreceptor with Ig and ITIM domains

TIL: tumor-infiltrating lymphocyte

TIRAP: TIR domain-containing adapter protein

TLRs: Toll-like receptors

TLS: tertiary lymphoid structures

TME: tumor microenvironment

TRAIL: TNF-related apoptosis-inducing ligand

T-Reg: regulatory T cells

TRIF: TIR domain-containing adaptor inducing interferon IFN- $\beta$ .

TYK2: Tyrosine Kinase 2

## **U**

UPR: unfolded protein response

## **V**

VEGF: Vascular endothelial growth factor

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## Summary

NSCLC is the most common malignancy and the most common cause of cancer deaths. Lung TME is a dynamic, highly complex and heterogeneous network structure that can be modulated by external stimuli such as microbial infections. Influenza virus (IV) and respiratory syncytial virus (RSV) infections in lung cancer patients are among the most common respiratory viral infections. While IV infection in NSCLC patients is associated with severe disease, more complications, prolonged viral shedding, and higher mortality, little is known about the epidemiology of RSV infection in these patients.

Despite their frequency, the impact of acute IAV and RSV on NSCLC tumor progression and anti-tumor immune response is largely unexplored.

Herein we provide evidence that acute RSV infection delays tumor progression in a murine model of orthotopic NSCLC. This impact can be mediated by modifying tumor cell hallmarks and tumor immune infiltrate. RSV infection of murine NSCLC cells induces late immunogenic apoptotic cell death that may induce anticancer immune responses, and it also inhibits cell proliferation. It results in an upregulation of RNA viral sensors (NLRP3, TLR7, TLR3, MDA5 and RIG-I), production of several proinflammatory cytokines, chemokines, and type I IFNs, which are involved in inflammation and consequently may modify the TME. Infection of NSCLC cells leads to the upregulation of MHC-I and Fas molecules at the cell surface of infected cells, as well as non-infected cells, mediated by type-I IFN production. In addition, RSV infection of murine NSCLC tumors causes a selective and reproducible increase in the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells expressing the T cell activation/memory/resident memory/less exhausted phenotypes, as compared to non-infected NSCLC tumors.

Despite many similarities between the two viruses, we found that IV induces viral sensors upregulation, inflammatory environment and some immune escape mechanisms, in addition to EMT and angiogenesis markers without inhibiting cell proliferation or lysing tumor cells, which may suggest a protumoral effect.

These results highlight the importance of developing new therapeutical approaches to treat NSCLC patients, which involve less pathologic engineered RSV, and they strongly encourage personalized lung cancer therapeutical protocol, in the case of viral respiratory infection, and emphasize the importance of IV vaccine in cancer patients.

**Keywords:** RSV, IV, NSCLC, TME, Oncolytic virotherapy, IV vaccine

## Chapter 1

### Lung cancer

Worldwide, lung cancer is the most common malignancy and the most common cause of cancer deaths in the past few decades. In 2012, a total of 1.8 million new cases were estimated, accounting for 12.9% of all new cancer diagnoses (Wong et al., 2017).

It represents about 20% of all cancer deaths (Carioli et al., 2020). Its five-year survival rate (17.8%) was much lower than that of other leading cancers (Wong et al., 2017). In the EU, while lung cancer mortality rates decline by 9.2% between 2015 and 2020 in men, they increase by 6.0% in women (due to changes in the rate of tobacco consumption) (Carioli et al., 2020).

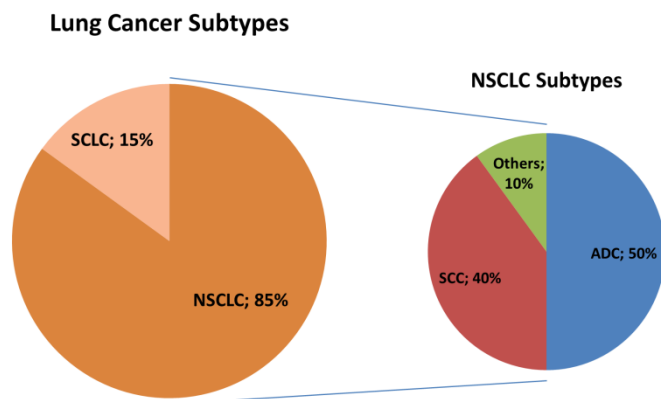
#### 1. Lung Cancer Subtypes:

Lung cancer is made up of distinct subtypes, including non–small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). Based on cell origin, about 80–85% of lung cancers are of non-small-cell lung cancer (NSCLC).

NSCLCs are a group of distinct diseases with genetic and cellular heterogeneity. They are further divided into lung adenocarcinomas (ADC; ~50%), squamous cell carcinoma (SCC; ~40%) and other cancers, including large cell carcinoma (~10%)

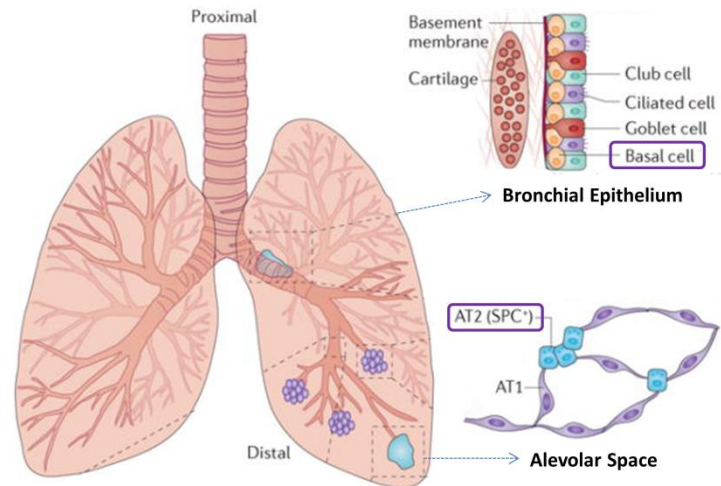
based on their histological features (Chen et al., 2014; Yuan et al., 2019) (Fig. 1).

Despite sharing many biological features, NSCLC subtypes differ in their cell of origin, location within the lung, and growth pattern. In the alveolar space (Fig. 2), it was suggested that type II pneumocytes (also called alveolar epithelial type 2 - AT2) cells can function as facultative stem cells and it has been proposed to be the cell(s) of origin for ADC. On the other hand, SCC is



**Figure 1 | Lung cancer subtypes.** Lung cancers are divided in small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLCs are subdivided into adenocarcinoma (ADC), squamous cell carcinoma (SCC) and other cancers.

thought to arise from basal cells which function as adult stem cells for the epithelium of trachea, main bronchi and upper airways (Chen et al., 2014) (Fig. 2).



**Figure 2 | Different respiratory epithelial cells in the bronchi and alveolar spaces.** Basal cells and alveolar type 2 (AT2) cells, the cells origin of SCCs and ADCs respectively, are marked (adapted from Chen *et al.*, 2014).

## 2. Classification, prognosis, clinical presentation and diagnosis of NSCLC:

NSCLCs are classified in stages (I–IV) by using the TNM staging system, which is based on the characteristics of the primary tumor (T), the degree of lymph node involvement (N) and the presence or absence of metastasis (Lim et al., 2018). The TNM staging system guides patient management, provides information regarding prognosis and eligibility for clinical trials, and allows international comparisons.

NSCLCs have bad prognosis in general, with a five-year overall survival of 15%, all stages combined, despite treatments, due to late diagnosis, frequent metastasis occurrence, and relapse. However, all stages lung cancer–specific survival improved from 26% among men with NSCLC that was diagnosed in 2001 to 35% among those in whom it was diagnosed in 2014 in the USA (Howlader et al., 2020). The most frequent stage at diagnosis is stage IV (46.6%), followed by stage III (24.2%) (Ruano-Raviña et al., 2020). The high percentage of late diagnoses is largely due to the non-specificity of most of the symptoms, and to the fact that they are not as frequent as would be expected.

### 3. Etiology and risk factors of lung cancer:

**Genetic risk factors:** A positive family history of lung cancer has been found to be a risk factor for early-onset lung cancer (Malhotra et al., 2016). Several studies have shown an association between genetic variants and lung cancer. The three main susceptibility loci identified are in the 15q25, 5p15 and 6p21 regions (Amos et al., 2008).

**Driver mutations:** NSCLC is a heterogeneous disease marked with a high rate of somatic mutations. Several genes altered (by mutations, amplification, or rearrangements) have been identified in ADCs and SCCs. EGFR (Epidermal Growth Factor Receptor), KRAS (Kirsten ras oncogene), ALK (Anaplastic lymphoma kinase), BRAF, ERBB2 (Erb-B2 Receptor Tyrosine Kinase 2) and Ros1 alterations were detected in about 22%, 25%, 9%, 7%, 2% and 2% of adenocarcinoma patients respectively (Carper and Claudio, 2015; Chu, 2020; Sholl et al., 2015). FGFR (Fibroblast Growth Factor Receptor), SOX2 (SRY related HMG box gene 2), PTEN (phosphatase and tensin homolog), PIK3CA (phosphoinositide-3-kinase catalytic subunit alpha isoform), DDR2 (discoidin domain receptor 2) and MET alterations were detected in 25%, 21%, 20%, 15%, 4% 3% respectively (Carper and Claudio, 2015). Thus, identifying the mutations that constitute good targets for therapeutic intervention in NSCLC is highly recommended.

**Tobacco Smoking:** Tobacco smoking is the major cause of all major histological types of lung cancer. Continuous smokers have 20- to 50-fold increase in lung cancer risk, relative to that among never-smokers. Duration of smoking is the strongest determinant of lung cancer risk in smokers (Malhotra et al., 2016). Even second-hand exposure to cigarette smoke increases lung cancer risk in nonsmokers, as nonsmoker married to a smoker has 20–30% more risk (Hackshaw et al., 1997).

**Occupational exposures:** The proportion of lung cancer cases attributable to occupational agents is 12.5% in men in France. Asbestos, polycyclic aromatic hydrocarbons, and chromium VI are the main occupational carcinogens (Boffetta et al., 2010).

**Chronic inflammation concomitant to other lung pathologies:** chronic obstructive pulmonary disease (COPD) affects an estimated 40% to 70% of patients with lung cancer. Patients with COPD, especially men, are at increased risk for lung cancer. The prevalence of COPD in newly

diagnosed lung cancer was 6-fold greater than matched smokers, suggesting that COPD itself is an important independent risk factor with potential relationship to the pathogenesis of lung cancer (Dela Cruz et al., 2011). In addition, Interstitial fibrosis has also been associated with an increase in lung cancer risk, even after adjustment for smoking (Hubbard et al., 2000).

**Infections:** Pulmonary infections commonly present in lung cancer patients are linked to clinical outcomes. Patients with pulmonary tuberculosis have been found to be at increased risk of lung cancer. The relative risk of lung cancer in the subjects with a history of tuberculosis is 2.0 after 20 years from the diagnosis of tuberculosis. Whether the excess risk is caused by the chronic inflammatory status of the lung parenchyma or by the specific action of the Mycobacterium is not clear (Malhotra et al., 2016).

Chlamydia pneumonia, a common cause of acute respiratory infection, might be involved in lung cancer carcinogenesis (Littman et al., 2005). As Chlamydia is not a known oncogenic pathogen, the inflammation resulting from the infection may lead to reactive oxygen species formation that can cause DNA damage, cell injury, and repair, increasing the risk of mutations, which can confer selective advantages that lead to cancer. Such infection can also act synergistically with cigarette smoking to increase the risk of lung cancer (Littman et al., 2005).

HIV-infected patients are associated with a hazard ratio of 3.6 for lung cancer after controlling for smoking status. The potential direct effects of the HIV virus and the consequences of long-term immunosuppression are the different hypothesized mechanisms for this association (Stebbing et al., 2009).

The lung is a mucosal tissue colonized by a diverse bacterial community. Recently, it was shown that lung microbiota may contribute to lung adenocarcinoma development by provoking inflammation in a mechanism that activates lung-resident  $\gamma\delta$  T cells (Jin et al., 2019a). In addition, lung microbiome has been linked to the establishment of lung metastasis from other primary cancers (Ramírez-Labrada et al., 2020).

On note, very few studies investigated the role of respiratory viral infections, which are very frequent, in the occurrence and progression of lung cancer. Several evidences suggest that infection may play a role in lung cancer; however, definite proof of a causal relationship is currently lacking. This will be further developed in the chapter 3.



#### 4. Management of NSCLC :

NSCLC is currently treated with therapeutic approaches based on classical surgery, chemotherapy and radiotherapy, and completed since several years by targeted therapies (that target driver mutations and genetic alterations) and immunotherapy for advanced stages. Patients are evaluated in a multidisciplinary tumor board. Therapeutic decisions are guided by an understanding of the molecular features of patient’s tumor tissues. All patients with metastatic NSCLC should undergo molecular testing for relevant mutations, and expression of the protein PD-L1 (programmed death ligand 1) (Majem et al., 2019).

Lung cancer cells have multiple immunosuppressive mechanisms, including PD-1 / PD-L1 pathway, which allow them to escape of the immune system and survive. Anti PD-1 and anti PD-L1 antibodies have showed activity in NSCLC patients with significant benefit in overall survival, long lasting responses and good safety profile, including naïve and pretreated patients regardless of the histological subtype (Rolfo et al., 2017). Immune therapy can increase 5 year survival rate of NSCLC up to 40% in PD-L1 high expressing patients (Horvath et al., 2020).

The clinical guidelines for the treatment of NSCLCs depend on the classification of cancer, and it’s summarized in table 1

**Table 1 | Summary of the clinical guidelines for the treatment of NSCLCs** (Chu, 2020; Majem et al., 2019; Yuan et al., 2019)

<b>Stage I–II</b>	
<b>Stages I–II medically fit for surgery</b>	Surgery
<b>Stages I–II medically inoperable and NSCLC tumors ≤ 4 cm</b>	Stereotactic ablative radiotherapy (SART)
<b>Stages I –II NSCLC patients with tumors &gt; 4 cm</b>	Adjuvant cisplatin-based chemotherapy
<b>Stage III</b>	
<b>Stage III and completely resected</b>	Surgery and adjuvant cisplatin-based chemotherapy ± Postoperative radiotherapy (PORT)
<b>Unresectable stage III</b>	Medically fit: cisplatin-based chemotherapy.

	PD-L1 inhibitor Durvalumab if no progressive disease after chemoradiotherapy
<b>Stage IV</b>	
<b>1. Stage IV without driver mutations</b>	
<b>PD-L1 ≥ 50%</b>	anti-PD1 antibodies (Pembrolizumab) ± chemotherapy
<b>PD-L1 &lt; 50% or unknown</b>	based on tumor histology:
<b>Squamous cell carcinoma (SCC)</b>	Immunotherapy (anti-PDL1 antibodies (Atezolizumab) or Pembrolizumab) and carboplatin plus paclitaxel or nab-paclitaxel)
<b>Non-squamous-cell carcinoma (non-SCC)</b>	Cisplatin/pemetrexed + Bevacizumab Pemetrexed maintenance Immunotherapy (atezolizumab <sup>(#)</sup> or pembrolizumab) plus standard chemotherapy
<b>2. Stage IV with driver mutations</b>	
<b>EGFR positive</b>	TKIs (tyrosine kinase inhibitors) such as Erlotinib, gefitinib, afatinib, dacomitinib, osimertinib
<b>EGFR T790M mutation positive</b>	Osimertinib (if not previously given)
<b>EGFR T790M mutation negative</b>	Platinum-based chemotherapy
<b>Anaplastic lymphoma kinase (ALK) rearrangements positive</b>	Alectinib, brigatinib, crizotinib or ceritinib
<b>ROS-1 rearrangements Positive</b>	Crizotinib
<b>BRAF alterations Positive</b>	Dabrafenib plus trametinib
<b>3. Oligometastatic disease</b>	Systemic therapy and local ablative strategies Local ablative strategies and TKI-continuation if clinical benefit is still retained (if actionable mutation)

Take Home Messages



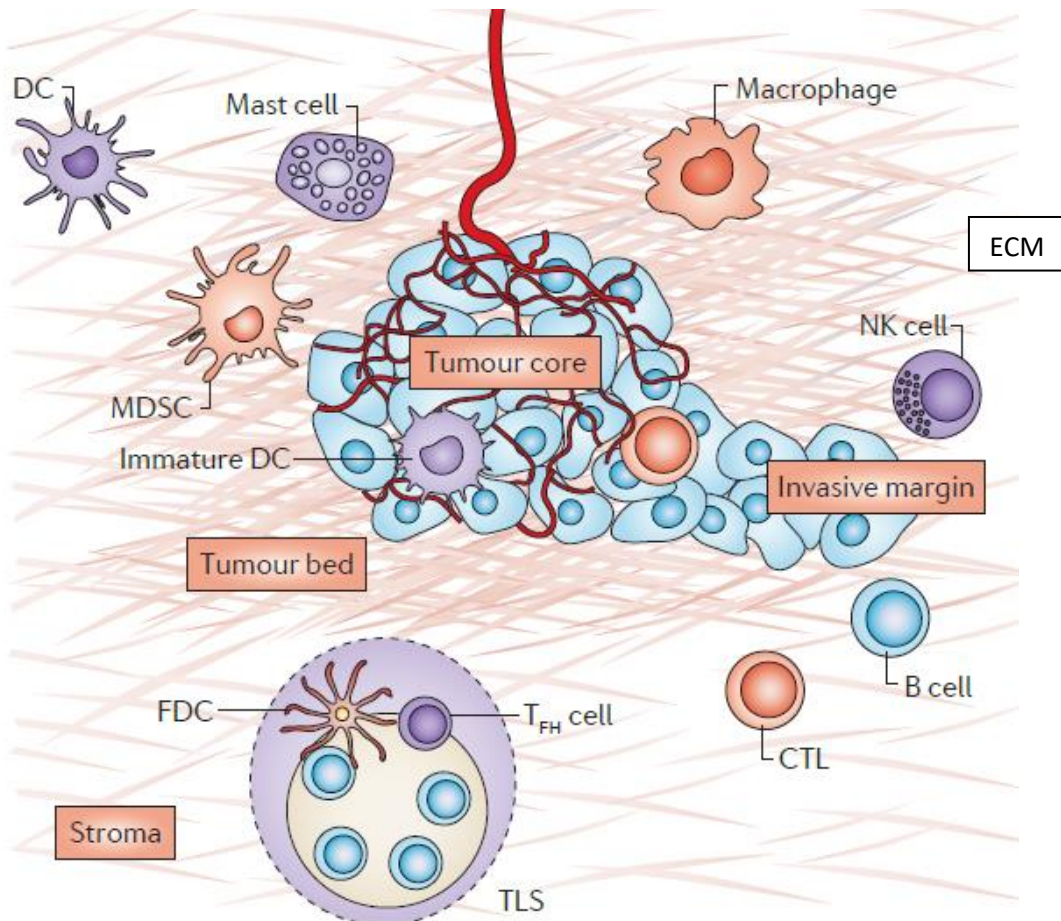
1. NSCLC is the most common malignancy and the most common cause of cancer deaths.
2. Infections are among the factors implicated in the etiology and development of NSCLC.
3. NSCLC is currently treated with therapeutic approaches, according to their stage, including immunotherapies that improve the survival rate.

## Chapter 2

### The tumor microenvironment (TME) in NSCLC

The lung tumor microenvironment (TME) plays an important role in the initiation and progression of primary de novo lung carcinoma and metastasis by contributing to inflammation, angiogenesis, immune modulation and response to therapies (Chen et al., 2014).

The TME is a complex environment composed of tumor cells, stressed normal epithelial cells, fibroblasts, endothelial cells and extracellular matrix (ECM) in addition to immune cells. All immune cell types may be found in a tumor, including macrophages, dendritic cells, mast cells, natural killer (NK) cells, naive and memory lymphocytes, B cells and effector T cells (including



**Figure 3 | The components of the lung tumor microenvironment (TME).** Tumour anatomy showing the features of the immune contexture, including the tumour core, the invasive margin, tertiary lymphoid structures (TLS) and the tumour microenvironment. The distribution of different immune cells is also shown. Adapted from (Fridman et al., 2012)

various subsets of T cell: T helper cells, T helper 1 (TH1) cells, TH2 cells, TH17 cells, regulatory T (T-Reg) cells, T follicular helper (TFH) cells and cytotoxic T cells. These immune cells can be located in the core (the centre) of the tumor, in the invasive margin or in the adjacent tertiary lymphoid structures (TLS) (Fridman et al., 2012) (**Fig. 3**). The fact that functional populations of immune cells are located in different areas of a tumor suggests that different immune cell populations may have different roles (discussed later).

These components play multifaceted roles during cancer progression which leads to rapid proliferation, metastasis, and drug resistance, or serves as a tumors-suppressor, e.g., through tumor-immune cell interaction (Fridman et al., 2012; Werb and Lu, 2015) (**Fig. 3**).

For instance, cancer-associated fibroblasts (CAFs) can mediate cancer cell proliferation, angiogenesis, invasion, metastasis, modulation of immune responses and cisplatin chemoresistance (Altorki et al., 2019). The ECM, composed of collagens, proteoglycans and glycosaminoglycans, mediates interactions between cancer cells and stromal cells to promote carcinogenesis. ECM density has also been shown to control the positioning and migration of T cells into tumor beds in human NSCLC (Salmon et al., 2012). Tumor-derived angiogenic factors contribute to the formation of new blood capillaries by tumor endothelial cells (TECs) that support tumor progression, invasion and metastasis. However, a subset of NSCLCs exploit pre-existing blood vessels, without inducing new vessels (Pezzella et al., 1997).

Thus, while lung cancer cells can reprogram the tumor-infiltrating stromal cells, which in turn contribute to carcinogenesis, immune cells try to eliminate cancer cells (Fridman et al., 2012; Werb and Lu, 2015). However, The lung TME is a dynamic, highly complex and heterogeneous network structure that can be modulated by external stimuli such as microbial infections (Jin et al., 2019b; Ramírez-Labrada et al., 2020).

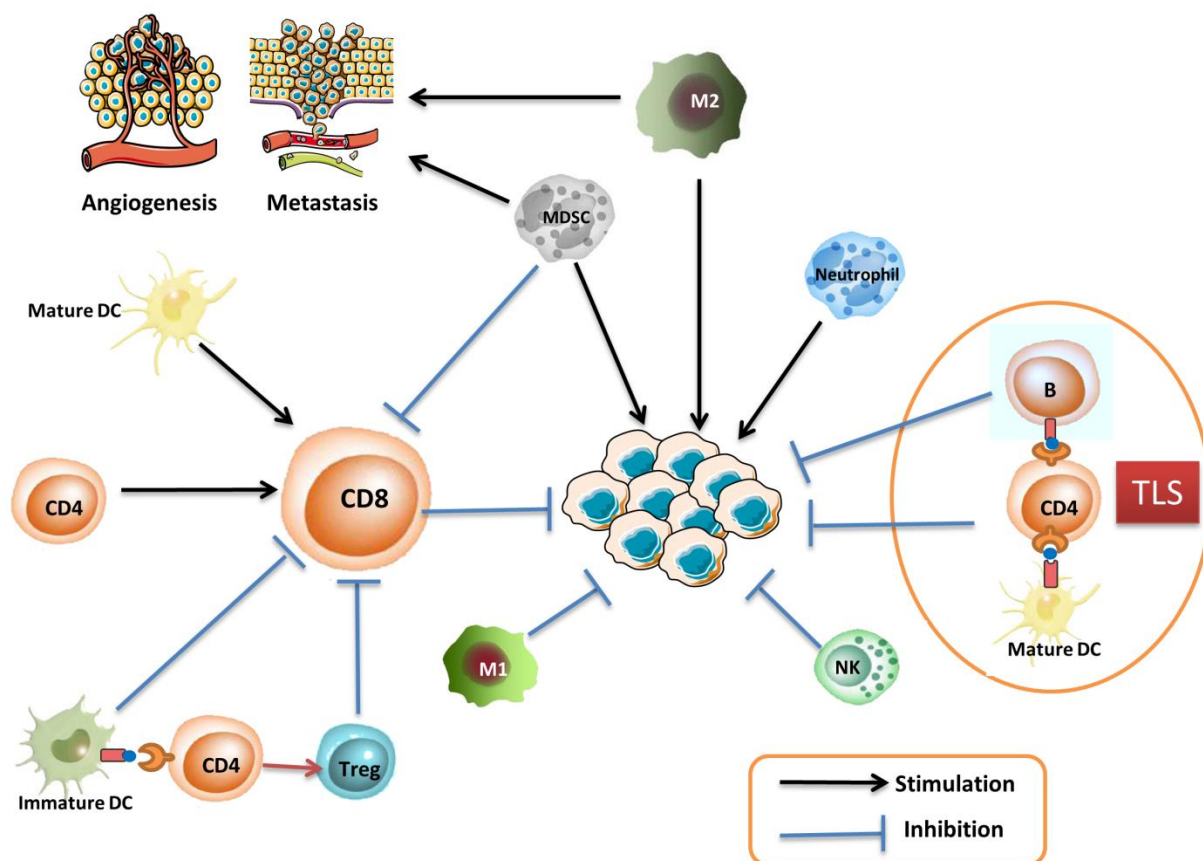
### **1. The immune components of the lung TME**

The precise characterization of the immune cells in the TME – composition, proportion, activation or functional states and spatial distribution - is therefore critical for diagnosis and therapeutic intervention (Anichini et al., 2018; Corredor et al., 2019). We, and others have largely contributed to the characterization of the TME of non-small cell lung cancer (NSCLC) and the impact of tumor-infiltrating

cells on clinical outcomes (Biton et al., 2018b, 2018a; De Chaisemartin et al., 2011; Djenidi et al., 2015; Fucikova et al., 2016; Ganesan et al., 2017; Germain et al., 2014; Gillard-Bocquet et al., 2013; Goc et al., 2014; Guo et al., 2018; Kargl et al., 2017; Kim et al., 2019b; Lambrechts et al., 2018; Lavin et al., 2017a; Maier et al., 2020; Mei et al., 2016; Platonova et al., 2011; Shang et al., 2015).

Lymphoid and myeloid cells harbor both tumor-promoting and tumor-suppressing activities in the TME of both mouse and human NSCLC.

While tumor and stromal mature dendritic cells (DCs), natural killer (NK) cells, CD8<sup>+</sup> cytotoxic T cells and CD20<sup>+</sup> B cells are generally associated with improved prognosis, inactive pDCs, tumor PD-L1 and T-Reg cells are associated with poorer prognosis in NSCLC (Soo et al., 2018; Tuminello et al., 2019).



**Figure 4 | Role of different immune populations in the lung TME.** Neutrophils, MDSCs and M2 macrophages support tumor growth, angiogenesis and metastasis. CD8<sup>+</sup> T-cells, M1 macrophages and NK cells suppress tumor growth. In the lung tertiary lymphoid structures (TLS), mature DCs and activated B cells present endogenous tumor antigens to tumor-infiltrating CD4<sup>+</sup> T-cells to induce anti-tumor response. B cells secrete tumor-specific antibodies that target tumor cells. NK cells and CD8<sup>+</sup> T-cells secrete granzyme A, granzyme B, perforin, and IFN-γ to lyse tumor cells. CD4<sup>+</sup> T-cells and mature DCs activate CD8<sup>+</sup> T-cells. By contrary, MDSCs, immature DCs and T-reg suppress CD8<sup>+</sup> T-cells function.

## 1.1. Myeloid cell populations

While myeloid cells regulate adaptive immunity by controlling tumor-infiltrating lymphocyte (TIL) composition, activation and antitumor function, they contribute to carcinogenesis in various ways.

### 1.1.1. Dendritic cells (DCs)

DCs are central orchestrators of antitumoral immunity by virtue of their ability to cross-present tumor antigens to prime T lymphocytes in the draining lymph nodes. Mature DCs express a higher level of co-stimulatory molecules (CD40/80/86), and an elevated cytokine levels indicate a potent T cell activating ability (Wang et al., 2019a).

However, NSCLC-induced DC suppression, through different mechanisms, can impede immune clearance:

1. Lung cancers dynamically exclude functional DCs from the tumor region to support malignant progression. Indeed, human CD141<sup>+</sup> DCs, which mainly interact with CD8<sup>+</sup> T-cells, is significantly lower in tumorous tissues than on-tumorous lung tissues (Lavin et al., 2017b).
2. The recruiting of immunosuppressive pDCs into the surrounding tissues of lung tumors, which express CD33 and PD-L1, and secret produce high levels of IL-1 $\alpha$ . They did not exert cytotoxic activity against tumor cells but instead promoted their proliferation (Sorrentino et al., 2015).
3. DCs cocultured with NSCLC cells secret Immunosuppressive molecules, such as IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ), which induces the differentiation of CD4<sup>+</sup> T cells into regulatory T (T-Reg) cells that suppress T cell proliferation (Dumitriu et al., 2009).
4. DCs, cocultured with NSCLC cells or serum from lung tumor patients, upregulate the expression of co-inhibitory molecule (such as B7-H3) and downregulate the expression of effector molecules such as CD40, CD80, CD86, MHC type II and IL-12 (Lu et al., 2019b; Zong et al., 2016).

### **1.1.2. Neutrophils**

In human NSCLC, neutrophils dominate the immune landscape, accounting for nearly 20% of all CD45+ cells (Kargl et al., 2017). Depleting neutrophils is associated with a pronounced reduction in murine NSCLC models, suggesting a protumoral role for neutrophils (Altorki et al., 2019) (**Fig. 4**). However, a unique subset of tumor-infiltrating neutrophils that expressed markers of both neutrophils and antigen-presenting cells (APCs), are able to cross-present antigens, as well as trigger and augment anti-tumor T cell responses in human NSCLCs (Singhal et al., 2016).

### **1.1.3. Myeloid-derived suppressor cells (MDSCs)**

MDSCs are a heterogeneous cell population that is usually divided into two subpopulations: polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs). They are recruited to the tumour site by the CC chemokine ligand CCL2, CXC chemokine ligand CXCL 12, and CXCL5, in a mechanism that involves stimulation of PRRs, such as TLR7 (Dajon et al., 2019).

MDSCs are involved in promoting tumor progression through different mechanisms:

1. Activated MDSCs produce IL-10 and TGF- $\beta$ , Adenosine, ARG1, iNOS2, IDO, NADPH oxidase and immunosuppressive cytokines that have the potential to inhibit cytotoxic T lymphocytes, B cells, DC, and NK cells as well as expand T-regs (Yang et al., 2020).
2. The hypoxia in the TME induces the differentiation of M-MDSCs to tumor-associated macrophages (TAMs), while some PMN-MDSCs die due to ER stress, releasing factors that promote immunosuppressive mechanisms (Yang et al., 2020).
3. MDSCs can promote tumor growth, angiogenesis and metastasis by producing VEGF, MMPs, and exosomes (Dajon et al., 2019; Deng et al., 2017).
4. MDSCs are associated with chemotherapy resistance, and as they express PD-L1, they predict the efficacy of anti-PD-1 therapy (Kim et al., 2019a; Li et al., 2017).

### **1.1.4. Tumour-associated macrophages (TAMs)**

TAMs are abundant components of the NSCLC immune infiltrate, which exhibit a continuum of phenotypes, with M1 and M2 phenotypic distinctions representing the extreme ends of this



spectrum. Given this heterogeneity of phenotypes, the prognostic relevance of TAMs in NSCLC remains unresolved (Altorki et al., 2019).

The phenotype of TAMs depends on the stage of tumor development, as M1-like macrophages are believed to predominate at tumor initiation, M2-like macrophages dominate the immune infiltrate of established tumors (Conway et al., 2016) (**Fig. 4**).

Human NSCLC macrophages express a unique phenotype compared to macrophages in normal lung. In particular, tumor macrophages expressed higher levels of PPAR $\gamma$ , a transcription factor known to drive an immunosuppressive program, lower levels of the co-stimulatory molecule CD86 and higher levels of IL-6, indicating that these tumor macrophages are pro-tumorigenic (Lavin et al., 2017b). In addition, PD-L1<sup>+</sup> macrophages reside at the tumor-invasive margin, suggesting their potential contribution to modulating T cell entry in the tumor lesion (Lavin et al., 2017b).

M1 macrophages support initiation of tumorigenesis through the generation of reactive oxygen and nitrogen intermediates, which induce DNA damage in proliferating cells, predisposing them to neoplastic transformation (Conway et al., 2016).

M2 macrophages promote tumor progression, angiogenesis, epithelial to mesenchymal transition, and immune suppression, through their secreted factors, including growth factors and cytokines, such as the epidermal growth factor (EGF), and TGF- $\beta$  (Messex et al., 2020).

## **1.2. Lymphoid cell populations:**

### **1.2.1. Natural killer (NK) cells**

NK cells are innate lymphoid cells that play a key role in antitumor immunity. Cytotoxic NK cells act early in the antitumor immune response by controlling tumor burden and stimulating adaptive T cell immune responses, through secretion of various cytokines and chemokines including TNF- $\alpha$ , IFN- $\gamma$ , CCL3 and granulocyte–macrophage colony-stimulating factor (GM-CSF), allowing them to attract and interact with other immune cells (Russick et al., 2020).

However, CD16<sup>+</sup> NK cells are reduced at the tumor site in human NSCLCs, due to the reduction of CD16<sup>+</sup> monocytes, which promote NK cell recruitment to tumors (Lavin et al., 2017b).

In addition, tumor NK cells in human NSCLCs display altered phenotype and functional impairment, relative to NK cells in non-tumorous lung tissues (Platonova et al., 2011). They have a distinct phenotype and cytokine secretion profile, altered expression of migratory receptors, as well as a higher cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Russick et al., 2020). In addition, NK cells are poorly cytolytic, with low expression of granzyme B and less IFN- $\gamma$ , and negatively regulate DC maturation (Lavin et al., 2017a; Russick et al., 2020).

### 1.2.2. T-Cells

The efficient antitumor immune response requires the cooperation of both CD8<sup>+</sup> and CD4<sup>+</sup> T-cells (**Fig. 4**). CD8<sup>+</sup> cytotoxic T cells target tumor cells for destruction through production of IFN $\gamma$ , tumor necrosis factor (TNF) and granzyme B after ligation of their T cell receptors (TCRs) (Biton et al., 2018a; Djenidi et al., 2015; Fucikova et al., 2016; Ganesan et al., 2017; Germain et al., 2014; Gillard-Bocquet et al., 2013; Guo et al., 2018; Kargl et al., 2017; Kim et al., 2019b; Lambrechts et al., 2018; Lavin et al., 2017a; Maier et al., 2020; Mei et al., 2016; Shang et al., 2015).

However, T-cells in progressing NSCLCs display altered number, phenotype and functional impairment:

1. NSCLC lesions have a strongly reduced CD8<sup>+</sup> T effector/Treg ratio compared to normal Lung (Ganesan et al., 2013).
2. T-Reg cells can directly suppress the antitumour function of CD8<sup>+</sup> T cells, and inhibit their expression of granzyme A, granzyme B, perforin, and IFN- $\gamma$  (Ganesan et al., 2013).
3. Persistent antigen exposure can lead to T cell dysfunction or exhaustion characterized by loss of effector and memory functions. In patients with NSCLC, T cell coexpress multiple inhibitory receptors, including PD1, T cell membrane protein 3 (TIM3; also known as HAVCR2), cytotoxic T lymphocyte-associated antigen 4 (CTLA4), lymphocyte activation gene 3 (LAG3) and B and T lymphocyte attenuator (BTLA) (Thommen et al., 2015).

### 1.2.3. B-Cells

In patients with NSCLC, tumour-infiltrating B cells and CD4<sup>+</sup> T cells reside within the tertiary lymphoid structures (TLS), and these structures correlate with better prognosis (Germain et al., 2014).

Tumor-infiltrating B cells present endogenous tumor antigens to tumor-infiltrating CD4<sup>+</sup> T-cells. Activated tumor-infiltrating B cells are associated with activated IFN $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cells, and exhausted tumor-infiltrating B cells are associated with immunosuppressive T-Reg cells (Bruno et al., 2017).

## **2. Inflammation in the TME:**

Chronic inflammation is considered an important hallmark of cancer; it promotes tumor development and progression, and has been associated with chemoresistance, metastasis, higher tumor grades and a poor prognosis (Hanahan and Weinberg, 2011; Sato et al., 2009). Different mechanisms and signaling pathways are implicated in the protumoral effects of chronic inflammation in the lung TME:

### **2.1. Role of NF- $\kappa$ B**

NF- $\kappa$ B is considered as the master regulator of inflammation. Its activation in the TME is induced by the interaction of PRRs with DAMPS or PAMPs, issues from lung commensal microbiota or concurrent bacterial and viral lung infection. For example, it was shown recently that TLR ligands derived from the lung commensal microbiota promote murine lung cancer development via  $\gamma\delta$  T cells. TLR signaling leads to activation of NF- $\kappa$ B and the production of IL-1 $\beta$  and IL-23 from alveolar macrophages and neutrophils, thereby inducing the proliferation and activation of protumoral and proinflammatory lung-resident  $\gamma\delta$  T cells (Jin et al., 2019a).

NF- $\kappa$ B activation regulates the production of a large variety of proinflammatory cytokines, chemokines, growth factors, collagenases and antiapoptotic proteins that impact tumor, stromal and immune cells (Basith et al., 2012). Indeed, NSCLC has shown increased levels of IL-1 $\beta$ , IL-6, IL-8, IL-17, IL-22 in tumor specimens (Deng et al., 2020a), associated with low disease-free survival and overall survival (Deng et al., 2020a).

## **2.2. Role of Signal transducer and activator of transcription 3 (STAT3)**

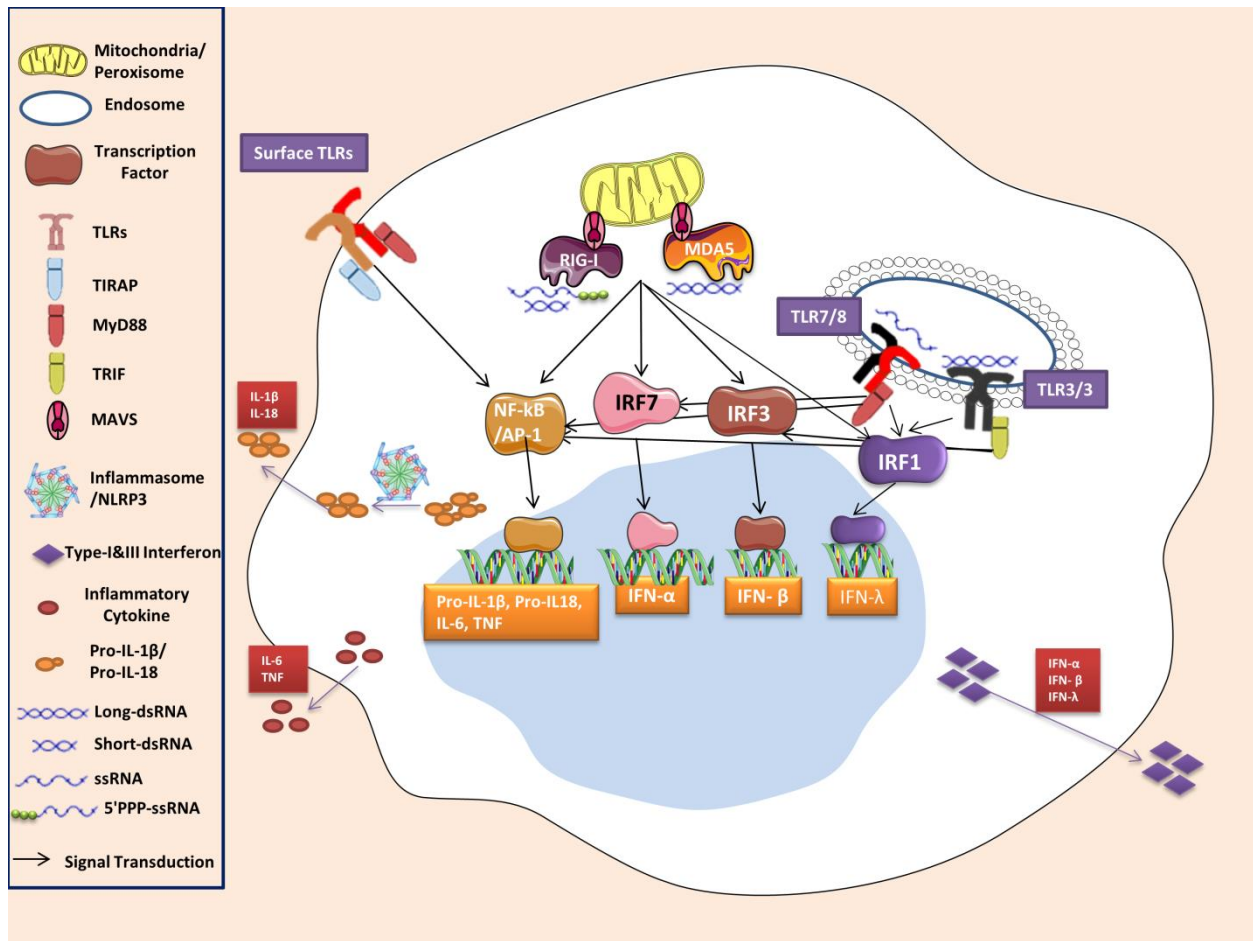
STAT3 is a transcription factor involved in cellular processes like apoptosis, angiogenesis, and metastasis (Deng et al., 2020a). The majority of NSCLC patient samples and cell lines harbor persistent STAT3 activation and high intra-tumoral pSTAT3 levels correlate with advanced disease stage, smoking and EGFR-mutation status (Mohrherr et al., 2020). STAT3 also plays a pivotal role in promoting cancer hallmarks, such as tumor cell proliferation, evasion of apoptosis and angiogenesis by controlling the expression of pro-tumorigenic genes like Cyclin D1/2, c-MYC, MCL1, Survivin, BCL-XL, HGF, HIF-1 $\alpha$  and VEGF, in addition to escape from anti-tumor immunity (Mohrherr et al., 2020).

The chronic inflammation, as well as the communication between cancerous and surrounding stromal cells is a two-way process and can be partially mediated by receptors, called pattern recognition receptors (PRRs), whose function may impact the complex TME.

### **3. Pattern recognition receptors (PRRs):**

PRRs are members of innate immunity that act as a bridge between innate and adaptive immunity. PRRs are highly expressed in the TME, not only in innate and adaptive immune cells, but also in stromal cells such as fibroblasts, endothelial cells and most remarkably in tumor cells, where they can have anti- or pro-tumoral effects, depending the context (Angrini et al., 2020).

PRRs includes several families: Toll-like receptors (TLRs), RIG-I like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin and cytosolic DNA sensors (CDS) (Bai et al., 2020) (Fig. 5). The first three receptors are highly implicated in the carcinogenesis of NSCLCs and are responsible for the recognition of RNA respiratory viruses such as influenza and RSV, as will be discussed later.



**Figure 5 | Pattern recognition receptors, and their signaling pathways.** Following binding of a specific ligand, PRRs recruit adaptor protein (MAVS in the case of RLRs, and MyD88, TIRAP and TRIF in the case of TLRs). This starts the signal transduction cascade (details of the cascade are not shown here, because it is out of the scope of my thesis). The cascade leads to the production and secretion of inflammatory cytokines in the case of membranous TLRs and NLRP3 inflammasome, while it leads to the production of type-I & III IFNs as well as inflammatory cytokines in the case of RLRs and endosomal TLRs. TLRs: toll-like receptors, RIG-I: retinoic acid-inducible gene-1, MDA5: melanoma differentiation-associated protein 5, MAVS: Mitochondrial antiviral-signaling protein. MyD88: myeloid differentiation primary response gene 88, TIRAP: TIR domain-containing adaptor protein, TRIF: TIR domain-containing adaptor inducing interferon IFN- $\beta$ .

PRRs, localized on cell surface or in intracellular space, recognize pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs). PAMPs include various viral and bacterial compounds such as lipopolysaccharides, complex lipids, various carbohydrates, nucleic acids etc. DAMPs include products of host's own metabolism including uric acid, bile acid salts, remnants of cell death, some minerals and others (Żeromski et al., 2019) (Table 2).

**Table 2 | List of Natural TLR Ligands (DAMPs and PAMPs) for endosomal TLR and RLRs, which are the most important PRRs in the context of NSCLC and viral infections** (Angrini et al., 2020; Iurescia et al., 2018)

PRRs	DAMPs	PAMPs
TLR3	<ul style="list-style-type: none"> <li>• Self double-stranded RNA (dsRNA), mRNA</li> <li>• Heat shock proteins (HSP27)</li> </ul>	Viral dsRNA
TLR4	Self cellular glycoproteins such as: <ul style="list-style-type: none"> <li>• Heat shock proteins (HSP22, HSP60, HSP70, HSP96)</li> <li>• Fibrinogen &amp; Fibronectin</li> <li>• Heparan sulfate</li> <li>• Hyaluronic acid (Hyaluronan)</li> <li>• HMGB1, <math>\beta</math>-defensin 2</li> <li>• Surfactant-protein A</li> <li>• Glycoprotein 96 (gp96)</li> <li>• Peroxiredoxin</li> </ul>	<ul style="list-style-type: none"> <li>• Heat shock proteins (HSP60 of Chlamydia)</li> <li>• Lipopolysaccharides (LPS)</li> <li>• RSV fusion protein</li> <li>• MMTV envelope proteins</li> <li>• Paclitaxel</li> <li>• Glycoinositolphospholipids</li> <li>• Taxol: Plant product.</li> </ul>
TLR7/8	Self ssRNA	Viral ssRNA
TLR9	<ul style="list-style-type: none"> <li>• Self DNA</li> <li>• mitochondrial DNA (mtDNA),</li> <li>• IgG-chromatin complexes</li> <li>• HMGB1</li> </ul>	<ul style="list-style-type: none"> <li>• Bacterial and viral Unmethylated CpG DNA motifs</li> <li>• Hemozoin</li> </ul>
RIG-I	Self 5'-triphosphate RNA and short dsRNA released by dying, damaged, cancer cells	Viral 5'-triphosphate RNA and short dsRNA
MDA5	Self long dsRNA	Viral long dsRNA

### 3.1. Toll-like receptors (TLRs) shape the TME:

As the largest family of pattern recognition receptors, Toll-like receptors (TLRs) are among the major activators of NF- $\kappa$ B and are involved in inflammation in the TME. TLR1, 2, 5, 6 and 10 (extracellular TLRs) are largely localized on the cell surface, while TLR3, 7, 8 and 9 (endosomal

TLRs) are localized in intracellular organelles, such as endosomal/lysosomal compartments and the endoplasmic reticulum (ER). TLR4 is unique as it is localized to both the plasma membrane and endosomal vesicles (Angrini et al., 2020). After binding their specific legends, TLRs activate signaling pathways that lead to the production of proinflammatory cytokine genes, and to the production of type I & III IFNs (Angrini et al., 2020) (**Fig. 5**).

TLRs are widely expressed on tumor cells in NSCLC. The expression of TLR4, 5, 7, 8 and 9 in tumor cells are markedly higher than that in normal lung tissue (Gu et al., 2018). In a cohort of NSCLC patients, high expression of TLR7 on tumor cells is associated with poor clinical outcome in patients treated or not with neoadjuvant chemotherapy (Cherfils-Vicini et al. 2010; Chatterjee et al., 2014). While TLR3 expression on tumor cells predicted a favorable outcome of early stage NSCLC, patients with TLR3-positive immune infiltrating cells, notably macrophages, show a worse prognosis compared with all other patients (Bianchi et al., 2019).

Recognition of natural TLR ligands (DAMPs or PAMPs) shapes the TME by mediating both pro- and anti-tumorigenic pathways (**Table 2**). TLR signaling may culminate in different outcomes depending on the type of tumor, the implicated TLR, type of TLR ligands, and most importantly, the main type of cell(s) that are targeted by TLR ligands (Angrini et al., 2020). On the one hand, in preclinical studies, TLR agonists incite anti-tumor responses. Nonetheless, most TLR agonists used as stand-alone antitumor drugs have yielded limited success in clinical trials. On the other hand, activation of TLRs in tumor cells generally serve as positive regulators of cancer (Angrini et al., 2020), with different mechanisms suggested to explain these pro-tumoral effects in tumor cells.

### **3.1.1. Impact of TLR signaling on tumor cells**

- Inflammation: TLR signaling upregulates the NF- $\kappa$ B cascade and thus mediates the release of cytokines and chemokines by cancer cells that enhance inflammation in the TME and contribute to carcinogenesis and cancer cell proliferation (Sato et al., 2009) (**Fig. 5**).
- Cell survival: TLR activation enhances tumor cells survival. Our team previously showed that stimulation of human NSCLC cell lines with TLR7 or TLR8 agonists leads

to NF- $\kappa$ B activation, upregulation of the antiapoptotic protein Bcl-2, and increase of tumor cell survival (Cherfils-Vicini et al., 2010). TLR4 ligation induces resistance of human lung cancer cells to TNF- $\alpha$  or TRAIL-induced apoptosis (He et al., 2007). Silencing TLR4 in NSCLC cells with siRNA inhibits proliferation and survival of tumor cells and drastically decreases inflammatory cytokine secretion, such as IL-6, TNF- $\alpha$  and IL-8 (Li et al., 2014).

- Cancer stem cell stimulation: NF- $\kappa$ B activation by TLR signaling is associated with the expansion, invasion, and tumorigenesis of cancer stem cells (CSCs). CSCs possess self-renewal and differentiation abilities, which promote tumor progression and metastasis and are responsible for treatment resistance and cancer relapse (Yeh et al., 2016).
- Metastasis: Following TLR activation, tumor cells can undergo epithelial-to-mesenchymal transition (EMT) and epithelial-to-leucocytic transition (ELT) - by which tumors develop the ability to activate leucocytic traits otherwise inaccessible to epithelial cells - and gain the ability to; (1) evade the immune system at the primary tumor site, (2) access the lymphatic system, (3) metastasize through the vasculature and (4) avoid destruction by the immune system at the site of metastasis (Luddy et al., 2014). Our team showed that TLR7 stimulation results in accelerated NSCLC growth and metastasis, accompanied by a gene expression signature linked to aggressiveness and metastatic dissemination; tumors expressing high levels of TLR7 display an EMT phenotype with high expression of vimentin and low abundance of E-cadherin (Dajon et al., 2019).
- Immunosuppression: TLR ligands may induce the production of immunosuppressive cytokines and molecules, including IL-10, IL-8, transforming growth factor- $\beta$ , IDO, proangiogenic VEGF and iNOS by lung tumor cells, which can counteract the activation of innate immunity (Ito et al., 2015; Ninomiya et al., 2015).
- Metabolism: TLRs can directly regulate cell metabolism affecting tumor and immune cell behavior and function in several cancers. For example, TLR3 ligands increase



glycolysis and lactate production, decrease the level of tricarboxylic acid cycle (TCA) (Warburg effect), promote amino-acid synthesis and HIF-1 $\alpha$  activity (Huang et al., 2018).

- Resistance to therapy: TLR ligands can induce resistance to cancer therapy, either by acting directly on tumor cells, or indirectly by inhibiting the immune system. TLR7 high expression on tumor cells is a marker of chemoresistance to cisplatin combined with gemcitabine or vironelbine (Chatterjee et al., 2014). In NSCLC cells lines, A549 and H1299, Pellino-1, a critical mediator for NF- $\kappa$ B activation in TLR3 and TLR4 signaling, is up-regulated following treatment with TLR agonists conferring chemoresistance to cisplatin or paclitaxel and increased cell viability. The upregulation of cIAP1 and cIAP2 by Pellino-1, reduces cisplatin-induced cleavage of caspase-3, caspase-7, and PARP accounting for the chemoresistance (Jeon et al., 2016).

### **3.1.2. Impact of TLR signaling on immune cells**

TLR stimulation and signaling may also result in modulation of intratumoral immune cell recruitment or differentiation, leading to anti- or pro-tumoral effects:

- Impact on myeloid-derived suppressor cells (MDSCs): Our team demonstrated that TLR7 stimulation of tumor cells results in the recruitment of MDSCs to the TME, leading to accelerated tumor growth and metastasis (Dajon et al., 2019). Versican, an ECM-derived peptide and a DAMP, upregulated in many human tumors including lung cancer, activates TLR2/6 on MDSCs to enhance TNF- $\alpha$  production leading to tumor cell proliferation and metastasis (Kim et al., 2009).
- Impact on macrophages: Chronic TLR signaling plays a role in the conversion of macrophages from the M1 to the M2 phenotype in the TME. The DAMP HSP27 released from lung cancer cells is recognized by TLR3 in monocytes and inhibits their differentiation into DCs while skewing them towards M2 macrophages (Thuringer et al., 2013). Tumor cell-derived extracellular Vesicles (TEVs) can bind to TLRs in immune cells and activate them in a paracrine loop leading to the production of cytokines that increase cell

proliferation and metastatic potential. TEVs carry cargo containing NSCLC tumor-associated miR-21 and miR-29a that bind to TLR7 and TLR8 in immune cells including macrophages, activating NF- $\kappa$ B and triggering a pro-metastatic inflammatory response (Fabbri et al., 2012).

- Impact on T cells: SMP-105, a cell wall skeleton preparation of *M. bovis* BCG, has anti-tumoral effects in a model of subcutaneous murine NSCLC. By activating the NF- $\kappa$ B promoter in a TLR2-dependent manner, SMP-105 leads to a T-helper (Th) 1 type immune response and the proliferation of cytotoxic T lymphocytes (CTL) (Murata, 2008).

### 3.2. RIG-I like receptors (RLRs):

RLRs include RIG-I, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RIG-I binds preferentially to short 5'PPP-double-strand RNAs (dsRNAs), while MDA5 senses preferentially long dsRNA (Žeromski et al., 2019) (**Table 2**). Both cytoplasmic receptors interact with mitochondrial adaptor molecule (MAVS). This interaction results in activation of transcription factors IRF3, IRF7, IRF1 (IFN-regulatory factors) and NF- $\kappa$ B that enter cell nucleus and promote activation of IFN $\alpha$ , IFN $\beta$ , IFN- $\lambda$ s and several pro-inflammatory cytokine genes respectively (Brisse and Ly, 2019a; Žeromski et al., 2019) (**Fig. 5**). While type I IFNs are induced by mitochondrion-associated MAVS, activation of peroxisome-associated MAVS triggers the production of IFN- $\lambda$ s (Kotenko and Durbin, 2017).

RIG-I and MDA5 are expressed in all cell types, especially innate immune cells, such as macrophages, neutrophils, and dendritic cells, as well as in other cells like mucosal epithelial cells, including NSCLC tumor cells (Brisse and Ly, 2019b). It was shown that the expression of MDA5, RIG-1 and IRF3 in human NSCLC tumors is higher in tumor tissues compared with the adjacent normal tissues (Yi et al., 2018). The prognostic value of RIG-I expression in tumors varies according to tumor type, as high expression of RIG-I is associated with better and worse prognosis in hepatic and ovarian cancers respectively (Hou et al., 2014; Wolf et al., 2020). There is a need to further investigations in the context of NSCLC.

Poly(I:C) transfection in A549 cells, NSCLC cell line, triggers apoptosis via the extrinsic apoptotic pathway, and activates the innate immune response by promoting IFN- $\beta$  and C-X-C

motif chemokine ligand 10 (CXCL-10) expression. Following treatment, cleaved caspase-3, caspase-8 and TRAIL are significantly increased (Sato et al., 2018; Yi et al., 2018). Of note, Poly(I:C) is a well-known activator of MDA5/RIG-I/IRF3 axis that can activate TLR3 as well.

### 3.3. NOD-like receptors (NLRs):

Among all 22 known NLRs, NOD1, NOD2 and inflammasomes are the most studied members of the NLR family. NOD1 and NLRP3 are widely expressed by a variety of cell types such as epithelial cells, immune cells, stromal cells, and endothelial cells. The expression of NOD2 is limited to certain cell types such as hematopoietic cells and lung epithelial cells (Caruso et al., 2014).

The intracellular receptors NOD1 and NOD2 sense fragments of peptidoglycan (PGN) that contain diaminopimelic acid (DAP) and muramyl dipeptide (MDP) respectively, which are found in various Gram-negative bacteria and Gram-positive bacteria. However, recent studies showed that RNA and DNA viruses, such as RSV and IAV can activate NOD2 also, which results in the activation of MAVS, IFN regulatory factor 3 (IRF3) and NF- $\kappa$ B pathway (Liu et al., 2019a).

Inflammasomes are cytoplasmic multimeric protein complexes. Their formation is triggered by PAMPs or DAMPs that results in the activation of caspase-1, which leads to either the processing and maturation of proinflammatory cytokine interleukin (IL)-1 $\beta$  and IL-18 or an inflammatory cell death termed pyroptosis (Kong et al., 2015) (**Fig. 5**).

Different studies have shown that NLRs are implicated in the carcinogenesis of NSCLC:

- NOD1 expression predicts poor prognosis and is associated with worse overall survival in lung squamous cell carcinoma (SCC) patients (Liu et al., 2020).
- An antagonist of NOD2 signaling, MDP Derivative, enhances the antitumor and antimetastatic efficacy of Paclitaxel, via inhibiting both NF- $\kappa$ B and MAPK inflammatory signaling in a murine model of NSCLCs (Dong et al., 2017).
- NLRP3 inflammasome is upregulated in lung adenocarcinoma (ADC) tissues and cell lines, and is associated with high-grade ADCs in resected lung cancer tissues (Kong et al., 2015). In addition, activation of NLRP3 inflammasome enhances the proliferation and migration

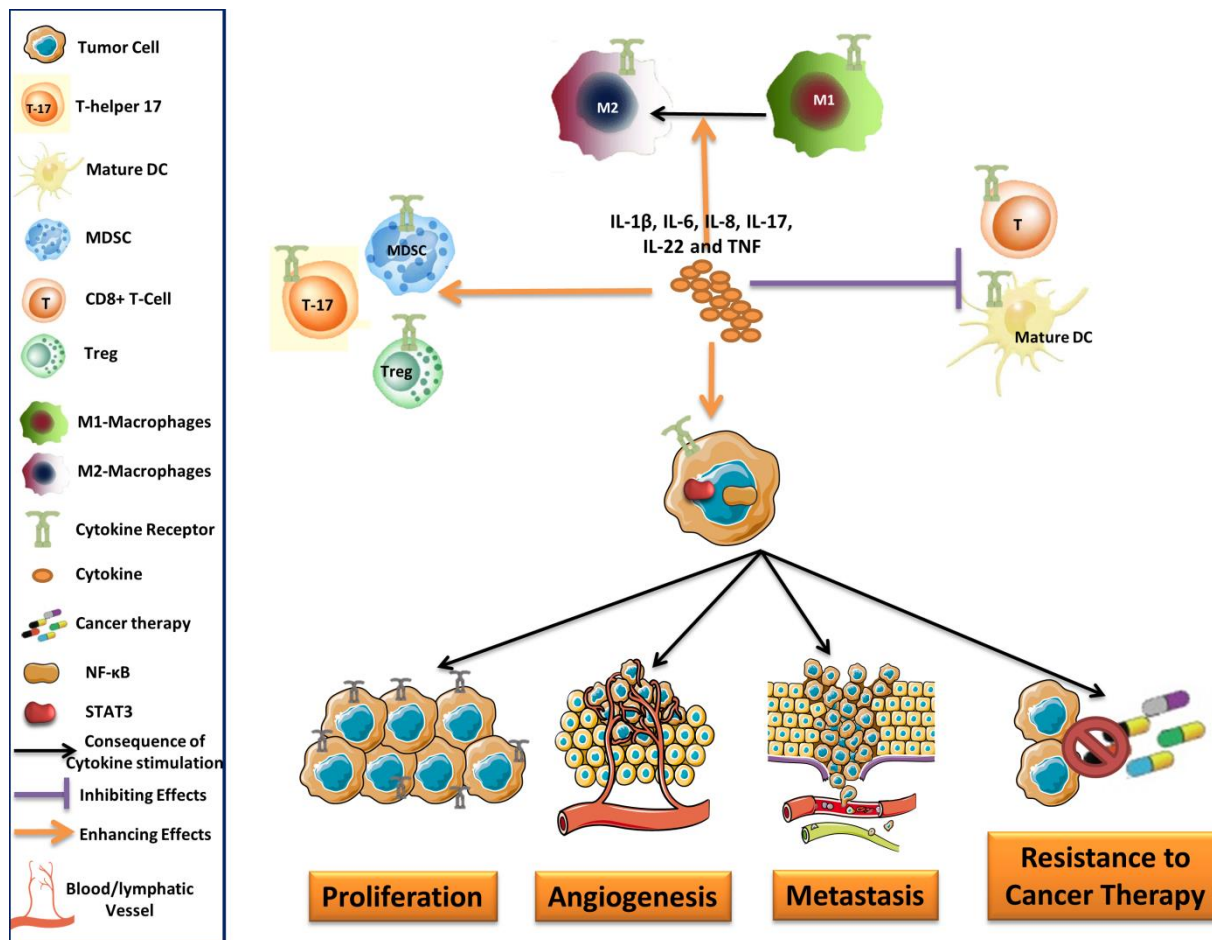
of A549 lung cancer cells. It increases also the expression of Snail, and decreases the expression of E-cadherin, which suggests an EMT (Wang et al., 2016). In addition, Polydatin inhibits the proliferation and migration of NSCLC cells (A549 and H1299 cells) by inhibiting NLRP3 inflammasome activation via NF- $\kappa$ B pathway (Zou et al., 2018).

#### 4. Cytokines and their receptors

High levels of inflammatory cytokines may impact both tumor cells and immune cells in the lung TME:

##### 4.1. The impact of inflammatory cytokines on tumor cells

It was shown that IL-6 supports the proliferation, invasiveness, angiogenesis, EMT, and



**Figure 6 | Effects of inflammatory cytokines in lung TME.** IL-1 $\beta$ , IL-6, IL-8, IL-17, IL-22 and TNF, secreted from various cells in the lung TME are regulated by NF- $\kappa$ B and STAT3 pathways. These cytokines exert protumoral effects on tumor and immune cells.

metabolism of NSCLC cells (Caetano et al., 2016; Lee et al., 2016). IL-1 $\beta$  and IL-8 augments levels of VEGF, TGF- $\beta$  and fibroblast growth factor (FGF), supporting angiogenesis and metastasis (Deng et al., 2020a) (**Fig. 6**).

Additionally, a subset of NSCLC cells undergoes a gradually progressing EMT phenotype, following a long exposure to IL-1 $\beta$ , an abundant proinflammatory cytokine in NSCLC (Li et al., 2020). Indeed, chronic IL-1 $\beta$  exposure leads to epigenetic modifications, including accumulation of H3K27, H3K9, and DNA methylation, in the CDH1 (E-cadherin) promoter (Li et al., 2020). Finally, IL-22 mediates the activation of different cytokines including IL-6 and IL-17; and it triggers upregulation of stemness markers (Khosravi et al., 2018).

#### **4.2. The impact of inflammatory cytokines on immune cells**

IL-1 $\beta$ -deficient mice show increased DC infiltration, immunosuppressive macrophages and increased CD8<sup>+</sup> T lymphocytes supporting antitumor cytotoxic responses (Kaplanov et al., 2019). IL-6 educates the lung TME toward a protumoral phenotype by inducing a decrease in CD8<sup>+</sup> T cell responses and cytotoxicity, a polarization from M1 to M2 macrophage, a triggering of T helper 17 (Th17) and Treg cell responses, as well as increased migration of MDSCs to tumor (Caetano et al., 2016; Lee et al., 2016). IL-8, IL-17 and TNF- $\alpha$  increase recruitment of MDSCs to lung TME (Deng et al., 2020a) (**Fig. 6**).

#### **4.3. The impact on NSCLC therapy**

High levels of IL-8 have been associated with resistance to lung cancer therapies such as EGFR inhibitors, conventional chemotherapy and anti-PD-1 immunotherapy (Deng et al., 2020a). IL-17 has been found to promote resistance to immune checkpoint blockade in lung cancer through a neutrophil-dependent modification of the lung TME (Akabay et al., 2017) (**Fig. 6**).

### **5. Interferons (IFNs) and IFN-stimulated genes in the lung TME:**

Interferons (IFNs) are important pleiotropic cytokines recognized due to their roles in viral infections, cancer and inflammatory and autoimmune diseases. IFN signaling through IFN

receptors ultimately results in transcriptional activation of genes called interferon-stimulated genes (ISGs). Remarkably, approximately 10% of the genes in the human genome have the potential to be regulated by IFNs (Schoggins, 2019). IFNs are classified into three major classes (Type I, II, III IFNs), according to their primary structures and their influence on different dimeric target receptors (**Table 3**).

### **5.1. Type I and III IFNs:**

Respiratory tract epithelial cells respond to both type I and type III IFNs, as they express IFN receptors, such as IFNAR (IFN  $\alpha$  &  $\beta$  receptor) and IFNLR (IFN  $\lambda$  receptor) (Kotenko and Durbin, 2017). Type I and III IFNs share similar downstream signaling pathways, as both IFNs signal through the JAK-STAT pathway to activate the heterotrimeric transcription factor complex ISGF3, comprised of phosphorylated STAT1 and STAT2, and interferon regulatory factor 9 (IRF9) (**Table 3**). Activated ISGF3 translocates to the nucleus and binds to IFN-stimulated response elements (ISREs) in the upstream promoter regions of IFN-stimulated genes (ISGs), which encode proteins that act via a variety of mechanisms (Lazear et al., 2019). ISGs encode many proteins, such as these implicated in the proliferation, apoptosis, antiviral response (such as viperin, interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), Mx1, (protein kinase R (PKR), oligoadenylate synthetase, (OAS), RNaseL, Cholesterol-25-hydroxylase (CH25H), ArfGAP Domain-Containing Protein 2 (ADAP2), DEXD/H box helicase 60 (DDX60), SERPINE1 and Interferon Alpha Inducible Protein 6 (IFI6), cytokines, chemokines, and their receptors, TLRs, RIG-I-like receptors, in addition to IFN signaling modulators. While IRF7, STAT1 and STAT2 positively modulate IFN production, ISG15 (G1P2), SOCS, and TAM receptors (Tyro3, Axl, and Mer) can negatively modulate it (Lazear et al., 2019; Schoggins, 2019).

Despite the similarity in the signaling pathways and the overlapped activated genes between type I & III IFNs, differences in cell type specificity and signaling kinetics result in distinct responses. Type I IFN response through induction of interferon stimulated genes (ISGs) is more systemic, inflammatory, potent, and transient, whereas the type III IFN response is not systemic (more strained to barrier surfaces), less inflammatory, less potent, and sustained (Lazear et al., 2019; Pervolaraki et al., 2018).

## 5.2. Type II IFNs

Likewise, IFN- $\gamma$  could induce inflammatory responses, cellular growth and proliferation in airway epithelial cells (Zhu et al., 2019). IFN- $\gamma$  binds to the IFN- $\gamma$  receptor, leading to phosphorylation of JAK1 and JAK2, followed by phosphorylation of STAT1 in most cells and STAT3 in some cells. The activated STAT dimers then accumulate in the nucleus to act as transcription factors, where they bind to the Gamma interferon activation site (GAS) elements present in most ISGs (Alspach et al., 2019a) (**Table 3**).

Although the Induction of type II IFN expression persists longer than type I IFN, strong convergence and synergy exist between type I and type II IFNs, as most differentially expressed ISGs are actually inducible by both of them. However, temporal changes in IFN expression patterns identifying phase-specific ISGs, as ISGs induced during the acute phase are either expressed at lower levels during the chronic phase, or not at all (Echebli et al., 2018).

**Table 3 | Summary of different IFNs families, including members, secreting and targeted cells, receptors and the transcription factors implicated in the signaling pathways** (Alspach et al., 2019a; Lazear et al., 2019).

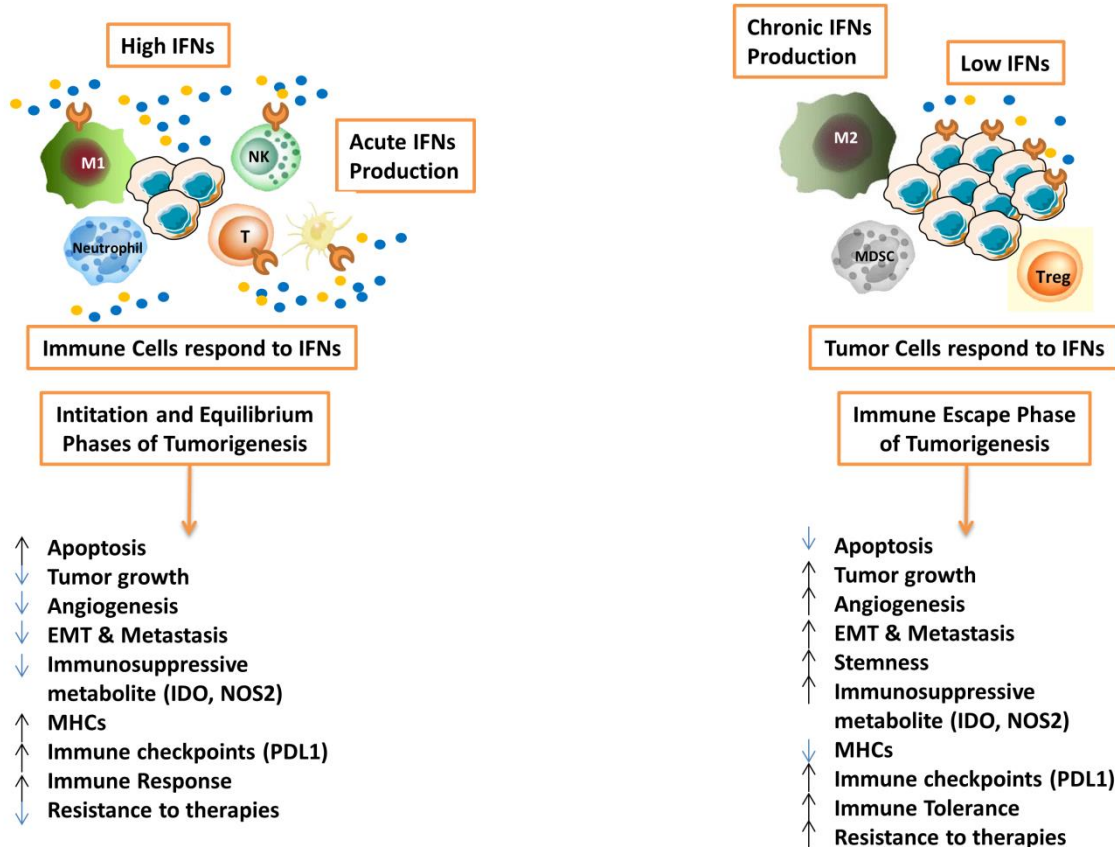
	Type I IFNs	Type II IFN	Type III IFNs
Family members	IFN- $\alpha$ family, IFN- $\beta$ , IFN- $\epsilon$ , IFN- $\kappa$ , IFN- $\omega$	IFN- $\gamma$	IFN- $\lambda$ 1 (IL-29), IFN- $\lambda$ 2 (IL-28A), IFN- $\lambda$ 3 (IL-28B), and IFN- $\lambda$ 4
Secreting cells	All cell types, including epithelial, tumor and immune cells, especially DCs	NK, natural killer T (NKT) cells and T cells	Epithelial & tumor cells are the main source of type III IFNs. Macrophage, monocytes & DC can also produce type III IFNs
Receptors	IFNAR-1 and IFNAR-2 heterodimer	IFNGR-1 and IFNGR-2 heterodimer	IFNLR1 (also termed IL28R $\alpha$ & IFN- $\lambda$ R1) and IL-

			10R $\beta$ (IL-10R2) heterodimer
<b>Implicated kinases</b>	JAK1 and TYK2	JAK1 and JAK2	JAK1 and TYK2
<b>Transcription factor</b>	ISGF3 (composed of pSTAT1, pSTAT2 and IRF9)	GAF (homodimer of pSTAT1)	ISGF3 (composed of pSTAT1, pSTAT2 and IRF9)
<b>Target cells</b>	Receptors are ubiquitously expressed	Receptors are ubiquitously expressed	Receptors are preferentially expressed on epithelial, tumor and some immune type (neutrophils & pDCs)



### 5.3. Impact of IFNs in the lung TME

Increasing data show a complex role of IFNs in cancer behaving as a double-edged sword (Snell et al., 2017).



**Figure 7 | Interferons impact on the TME.** High quantities of acute IFNs production activate ISGs in immune cells, leading to immune surveillance during the initiation and equilibrium phases of tumorigenesis. Low quantities of chronic IFNs production activate ISGs in tumor cells, leading to immune tolerance during the escape phase of tumorigenesis

#### 5.3.1. IFN impact on tumorigenesis

Physiological low levels of IFNs can be constitutively produced locally without systemic effects (Cheon et al., 2014). Endogenous type I IFNs and IFN- $\gamma$  are critical in preventing the initiation of tumorigenesis through tumor surveillance; as loss of sensitivity to endogenous IFNs can contribute to escape of tumor cells from host immune surveillance (Cheon et al., 2014). During the initiation and the equilibrium phases of tumorigenesis, IFN- $\gamma$  inhibits the

proliferation of tumor cells, and induces their expression of MHC-I and antigen presentation. Furthermore, it promotes myeloid cell activation, costimulatory molecules expression on APCs, induces directed cellular migration through production of a variety of chemokines, and facilitate CD4<sup>+</sup> T-cell polarization to Th1 and maturation of CD8<sup>+</sup> T cells into cytolytic T cells (Alspach et al., 2019). However, in the escape phase of tumorigenesis, IFN- $\gamma$  drives the up-regulation of PD-L1 on tumor cells and host myeloid cells, promotes the expression of the immunosuppressive metabolite IDO in these cells, thereby helping to establish a state of resistance that leads to suppression of tumor-specific T cells and to the formation of an immunosuppressive TME (Alspach et al., 2019). Cancer cells develop strategies to escape from IFN- $\gamma$ -dependent cancer immunosurveillance. This includes the recruitment of immunosuppressive cells, secretion of immunosuppressive factors, and suppression of cytotoxic T lymphocyte responses (Lin et al., 2017) (**Fig. 7**).

### **5.3.2. The duration of IFNs secretion**

IFNs activates antitumor immune responses, but when chronically secreted, it leads to deleterious effects and increases PD-L1 expression by tumor cells (Morimoto et al., 2018) (**Fig. 7**). Persistent type I and II interferon signaling allows tumors to acquire STAT1-related epigenomic changes and increases intratumoral accumulation of regulatory T cells (Treg) and myeloid cells, and the expression of iNos2 and ligands for multiple T cell inhibitory receptors (such as PDL1) in both tumor and dendritic cells (DC), and thus leading to increased resistance to immune checkpoint blockade (Benci et al., 2016; Jacquelot et al., 2019). In addition, the strength and duration of IFN signaling can differentially impact other cancer therapies. An interferon-related gene signature (IRDS), composed of few ISGs, is linked to resistance to adjuvant chemotherapy and radiotherapy (Weichselbaum et al., 2008).

### **5.3.3. The quantity of IFNs and their receptors**

The pattern of ISG expression is correlated to the concentration of IFNs present in the TME. Constitutive low levels of IFNs are able to induce steady-state upregulation of ISGs in cancer cells, including STAT1, STAT2, and IRF9, IFI27, ISG15, and BST2, with no increase of proapoptotic and anti-proliferative ISGs, which are induced by the higher doses of IFNs used

therapeutically (Cheon et al., 2014). Low levels of IFN- $\gamma$  endowed cancer stem-like properties via the intercellular adhesion molecule-1 (ICAM1)–PI3K–Akt–Notch1 axis in NSCLC cells, and is closely associated with poor prognosis, increased expression of cancer stem cells (CSC) markers and increased risk of tumor metastasis during immunotherapy in patients with NSCLC (Song et al., 2019). Low levels of IFN- $\gamma$  is associated with downregulation of major histocompatibility complexes, upregulation of IDO, and checkpoint inhibitors such as PDL1 (Jorgovanovic et al., 2020) (**Fig. 7**).

By contrary, administration of therapeutic quantities of type I and III IFNs induce apoptosis and inhibit tumor growth of some, but not all, human NSCLC cells engrafted in severe combined immunodeficiency mice (Fujie et al., 2011). Likewise, therapeutic quantities of IFN-  $\gamma$  inhibit tumor angiogenesis and potentiates Fas- and TRAIL-mediated apoptosis by increasing caspase-8 expression in NSCLC cells (Deng et al., 2018; Kim et al., 2002).

Similarly, while a low number of type I and III IFN receptors on an individual cancer cell suffices for antiviral response, a high number of receptors is required for antiproliferative and apoptotic response (Levin et al., 2011; Li et al., 2012b).

#### **5.3.4. The impacted cell type**

Recently, Benci and colleagues also showed that a high ratio of ISGs in tumor cells and low ratio of ISGs in immune cells (T cells, NK cells, and macrophages) correlates with resistance to radiation, chemotherapy and immune checkpoint blockade. This ratio is independent of tumor mutational burden (TMB)(Benci et al., 2019) and is concurrent with T cell exhaustion via upregulation of PD-L1 by tumor cells. In addition, inhibiting tumor IFN- $\gamma$  signaling decreases ISGs in cancer cells, allowing their killing by T cells after checkpoint therapy (Benci et al., 2019). Tumor cells that constitutively overexpress the IFN/STAT1 pathway are selected by the lung TME. STAT1-overexpressing tumor cells demonstrate metastatic potential and resistance to IFN- $\gamma$ , ionizing radiation (IR), and doxorubicin. A subset of ISGs (about 30 ISGs) is consistently overexpressed in therapy-resistant cancer cells. This subgroup of ISGs includes IFI27 (ISG12), IFIT1, Mx1, ISG15, BST2, OAS1, OAS3, OASL, and others (Khodarev et al., 2009). A high ISGs score is found in a group of The Cancer Genome Atlas Program (TCGA) primary lung tumors that

correlates with upregulation of multiple immunosuppressive factors thereby evading immune control (Liu et al., 2019b) (**Fig. 7**).

#### Take Home Messages



1. The different components of the lung TME either promote cancer proliferation, metastasis, and drug resistance, or serve as a tumors-suppressor.
2. Lymphoid and myeloid cells harbor both tumor-promoting and tumor-suppressing activities in the lung TME.
3. Chronic inflammation promotes tumor development and progression, and has been associated with chemoresistance, metastasis occurrence.
4. PRRs are highly implicated in the carcinogenesis of NSCLCs and are responsible of the activation of NF- $\kappa$ B, the master regulator of inflammation.
5. NF- $\kappa$ B activation regulates the production of a large variety of proinflammatory cytokines that impact both tumor cells and immune cells to support tumor progression
6. IFNs in NSCLC behave as a double-edged sword, as low quantities of chronic IFNs production activate ISGs in tumor cells, leading to immune tolerance

## Chapter 3

### Respiratory viral infections

Respiratory viral infections are an important cause of morbidity and mortality in elderly persons, and in patients suffering from chronic diseases. They can be divided into upper respiratory tract infection (URTI), influenza-like illness (ILI) and lower respiratory tract infection/pneumonia (LRTI). URTI can be assumed if a patient has a new onset of symptoms including at least one of cough, coryza, sore throat or shortness of breath. LRTI, which includes acute bronchitis, pneumonia and acute exacerbations of chronic pulmonary disease, requires clinical and radiological evidence. ILI is diagnosed in patients with a sudden onset of new symptoms including at least one of fever, malaise, headache or myalgia and at least one of the respiratory symptoms cough, sore throat or shortness of breath (Mahashur, 2018).

Accumulating evidence shows that a heterogeneous group of viruses such as respiratory syncytial virus (RSV), influenza virus (IV), parainfluenza virus (PIV), metapneumovirus (HMPV), rhinovirus (RhV), and coronavirus (CoV), including the recently emerged SARS-CoV-2 are associated with a more complicated and severe disease in cancer patients (Chemaly et al., 2012; Hijano et al., 2018; Liang et al., 2020; Volling et al., 2014). Respiratory viral infections in cancer patients are characterized by a high rate of progression from upper to lower respiratory tract infection (30 to 50%) and prolonged viral shedding, and for those who develop pneumonia, mortality can be as high as 75% (Hijano et al., 2018).

Among all of them, IAV and RSV infections in cancer patients are among the most common and the best studied viruses, and thus, serves as a model to study the impact of respiratory viral infections in this disease, notably in lung cancer.

#### **1. Influenza virus (IV):**

Influenza is a self-limiting acute respiratory illness that represents a significant burden on society in terms of morbidity, mortality, hospitalizations and lost working time. Influenza virus

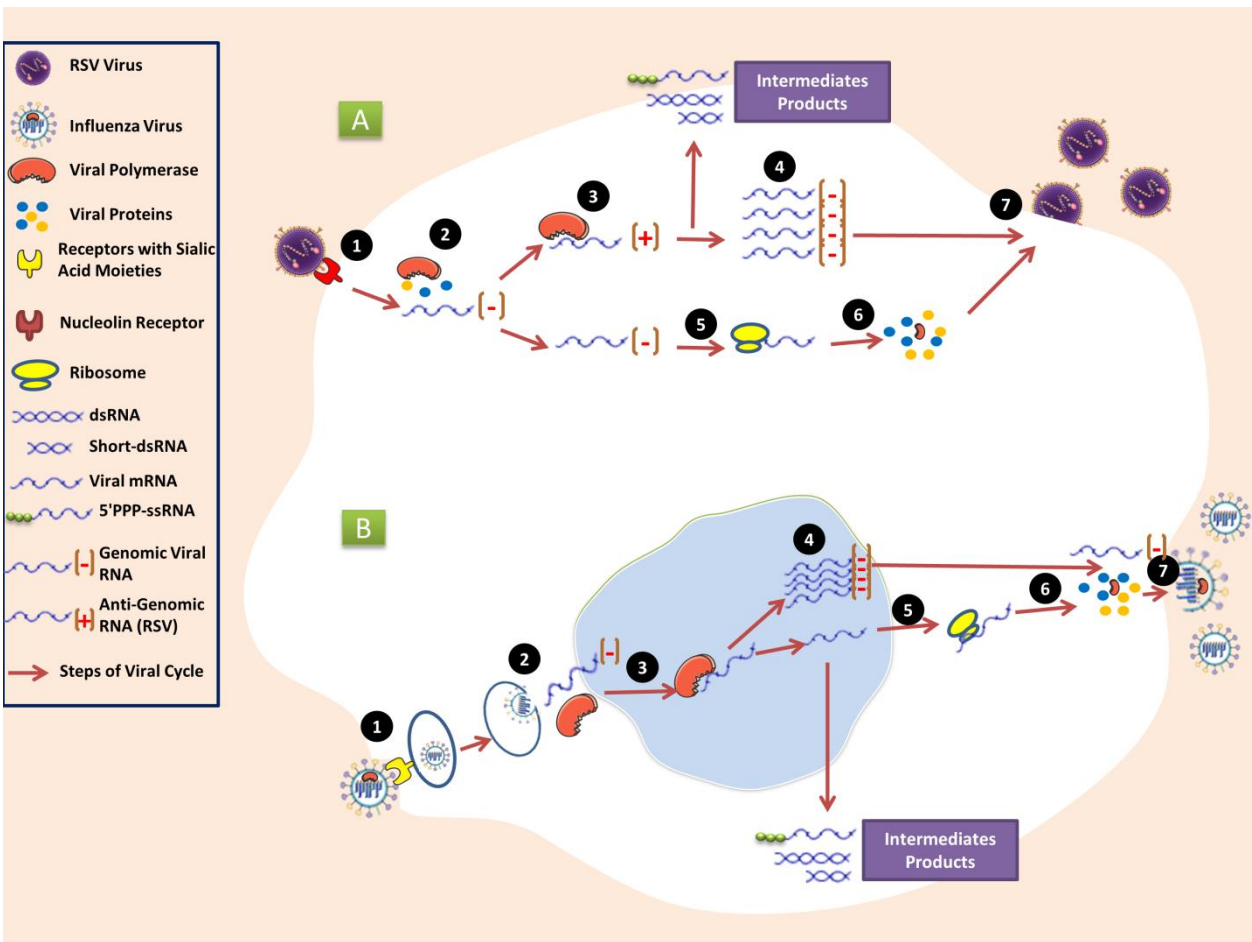
affects 2% to 10% of the global population, resulting in 250,000 to 500,000 deaths annually. However, the mortality may rise greatly in pandemic periods (Gasparini et al., 2012).

**1.1. IV subtypes:** There are four known subtypes of influenza virus (IV): groups A, B, C and D. While IBV and ICV are classified only by strains, IAVs can be subtyped according to the antigenic properties of their hemagglutinin (HA) and neuraminidase (NA) glycoproteins (Shao et al., 2017). There are 18 known subtypes of HA (H1–18) and 11 of NA (N1–11) (Allen and Ross, 2018). Despite the numerous possible subtype combinations, currently, only the pandemic IAV 2009 H1N1 (H1N1pdm09) is co-circulating with IAV (H3N2) and IBVs (Victoria and Yamagata lineages), to form the endemic strains in the human population (Adam et al., 2019).

**1.2. Structure of IV virion:** Influenza viruses are enveloped particles that contain a single-stranded, segmented RNA genome. IAV and IBV contain eight, negative-sense, single-stranded viral RNA (vRNA) gene segments, that encode at least 17 proteins, including HA, NA, matrix 1&2 (M1, M2), nucleoprotein (NP), viral polymerase subunits PB1, PB2, and PA, non-structural protein 1&2 (NS1, NS2) and nuclear export protein (NEP) (Dou et al., 2018).

**1.3. Host cells of IV:** Generally, IAV viruses replicate productively in differentiated human alveolar type II cells, alveolar type I cells, and alveolar macrophages (Yu et al., 2011). Also, IAV infection of human blood monocytes, macrophages, dendritic cells (DCs), lymphocytes, natural killer (NK) cells, fibroblasts, endothelial cells has been shown to result in productive infection with release of infectious particles (Armstrong et al., 2012; Guo et al., 2009; Hao et al., 2008; Hoeve et al., 2012; Li and Karlsson, 2017; Mock et al., 2018; Reading et al., 2000). Tumor cells of most cancer types could also be infected by influenza, as will be shown in details later. Importantly, A549 cells, as a well-known model of NSCLC (lung adenocarcinoma), are susceptible to the infection with influenza viruses and permissive for viral replication and they are widely used as a model for influenza infection too (Laske et al., 2019).

**1.4. Viral cycle:** HA has an important role in determining host tropism, as it binds to host cell receptors that contain terminal  $\alpha$ -2,6-linked or  $\alpha$ -2,3-linked sialic acid ( $\alpha$ -2,6-SA or  $\alpha$ -2,3-SA) moieties to mediate cell entry (Medina and García-Sastre, 2011). The IV viral cycle is summarized in **(Fig. 8)**.



**Figure 8 | Comparison between RSV and IV viral cycles, and the formation of viral intermediates. (A) RSV viral cycle. (B) IV viral cycle.** The cycle begins with the attachment of viral glycoprotein to the receptors on cell membrane (step 1) to induce fusion of viral envelope and host cell membrane and release of the viral genome into the cytoplasm (step 2). While IV RNA genome and viral RNA polymerase trafficked to the cell nucleus, RSV genome produces positive sense anti-genome RNA by viral RNA polymerase in the cytoplasm (Step 3). RSV and IV RNA genomes are replicated in the cytoplasm and nucleus consequently, that includes formation of dsRNA Intermediate (Step 4). The negative-sense RNA genome undergoes primary transcription to produce mRNAs that are translated in the cytoplasm by the cellular ribosomes to viral structural and non-structural proteins (step 5-6). Through a series of interactions, the M protein co-ordinates the assembly of the envelope proteins with the nucleocapsid proteins (NP) prior to budding of the mature virion (step 7).

### **1.5. Epidemiology of IV infection in cancer patients:**

Cancer patients (mean age occurrence of 70 years) may be infected by seasonal IVs in addition to the rare, but more severe pandemic influenza strains. Patients with cancer are more prone to longer and more complicated disease, severe morbidity and higher mortality in all age groups, following influenza infection (Memoli et al., 2014; El Ramahi and Freifeld, 2019). The rate of seasonal influenza-associated hospitalizations is approximately 12-fold higher in cancer patients compared to the general population (440.8 and 36.8 per 100,000 patients respectively) (Cooksley et al., 2005; Thompson et al., 2004). In addition, the annual estimates of confirmed influenza with pneumonia-related patient deaths are 7.6% and 4.0% in cancer patients and in the general population, respectively (Cooksley et al., 2005; Thompson et al., 2004). Cancer patients display prolonged viral shedding that increases the risk of transmission and which most importantly reflects higher persistent viral replication (El Ramahi and Freifeld, 2019).

Of note, the impact of IV infection in cancer patients is related to the age of the patient and the type of cancer: the outcome of infection in cancer is about 3-fold worse in patients aged  $\geq 65$  years, compared to those aged  $< 65$  years in terms of hospitalization and mortality rates (Cooksley et al., 2005). Increased influenza severity is associated with a higher rate of hospitalizations, length of stay, higher mechanical ventilation requirements and increased case-fatality rates for non-small-cell lung carcinoma (NSCLC) patients, the more common type of lung cancer (Cooksley et al., 2005; Kinslow et al., 2019). Interestingly, recent reports point at a higher risk of developing lung cancer with cumulative exposure to the virus (Weng et al., 2019). A study on a cohort of Japanese patients found a link between increase in prevalence of influenza infection and the formation of malignant cancer, notably one year before the detection of cancer (Inaida and Matsuno, 2020).

Altogether these epidemiologic data reveal that influenza viral infection is more severe in lung cancer patients. The reciprocal impact of viral infection on tumor progression and on the anti-tumor immune response will be discussed later.



## **2. Respiratory syncytial virus (RSV):**

Human RSV is a major cause of acute lower respiratory tract infection in infants, children and elderly. RSV infections in adults are usually mild to moderate in severity unless patients have an underlying risk factor such as being immunocompromised or having an underlying chronic pulmonary or circulatory disease (Griffiths et al., 2017). In the USA, 2–10% of older adults are infected and approximately 11,000 persons die due to the illness each year, with approximately 10 times that number of patients hospitalized in each year (Griffiths et al., 2017; Yoon et al., 2020).

**2.1. RSV subtypes:** Two major viral groups, designated A and B, each with numerous subgroups, co-circulate in the community, with group A viruses tending to be responsible for the majority of cases (Walsh, 2017).

**2.2. Structure of RSV virion:** RSV is an enveloped virus with non-segmented negative-sense RNA genome that encodes for 11 proteins. The viral lipid bilayer envelope contains the viral attachment glycoprotein (G) and fusion glycoprotein (F) that mediates entry of the viral genome into cells (Walsh, 2017). The nucleocapsid proteins, which include the viral polymerase and the nucleoprotein (N), surround the viral ssRNA genome (Cervantes-Ortiz et al., 2016a). The matrix (M) protein acts as a bridge between the nucleocapsid and the lipid bilayer envelope, and plays a central role in RSV assembly (Shahriari et al., 2016). Two nonstructural RSV proteins (NS1 and NS2) inhibit cellular antiviral innate type I interferons providing defense against the early host immune response (Walsh, 2017).

**2.3. Host cells of RSV:** RSV primarily targets ciliated and basal airway epithelial cells (AECs), but immune cells such as dendritic cells (DCs), monocytes, macrophages, lymphocytes B and even CD4<sup>+</sup> and CD8<sup>+</sup> T cells can also be infected with RSV (Bohmwald et al., 2017a; Cervantes-Ortiz et al., 2016a; Raiden et al., 2017a; Zhivaki et al., 2017). Depending on the strain of virus, different cellular receptors have been proposed to facilitate RSV entry (Griffiths et al., 2017). It has been demonstrated that binding of the RSV G and F glycoproteins with the cellular receptors, CX3C chemokine receptor 1 (CX3CR1) and insulin-like growth factor-1 receptor (IGF1R) consequently, can lead to the induction of nucleolin gene and the recruiting of nucleolin protein from the

nuclei of cells to the plasma membrane, where it also binds to RSV-F on virions to enable viral entry (Anderson et al., 2020; Griffiths et al., 2020) (**Fig. 8**).

**2.4. Viral cycle:** Unlike IAV, RSV replicates within the host cytoplasm and doesn't enter host nucleus (**Fig 8**). The attachment of RSV to the host cell and fusion of the viral membrane to the host cell membrane are facilitated by the viral glycoprotein (G), and most importantly glycoprotein (F) (Shahriari et al., 2016). Unlike the case of IV, the RSV viral cycle is entirely cytoplasmic, and is summarized in (**Fig. 8**).

Transcription and replication of RSV and IAV genomes generate specific RNA intermediates, such as short and long dsRNA and 5'PPP-ssRNA, that constitute pathogen-associated molecular patterns (PAMPs), which are sensed by pattern recognition receptors (PRRs) to trigger the interferon (IFN)-mediated antiviral response and the expression of proinflammatory cytokines (Cervantes-Ortiz et al., 2016a).

## **2.5. Epidemiology of RSV infection in cancer patients**

Both childhood and adult cancer patients remain particularly susceptible to clinically severe RSV infections, owing to their compromised immune system. However, it has to be kept in mind, that most data regarding RSV in cancer patients originate from hematological malignancies (HM) and very little is known regarding patients with other forms of malignancy. RSV infection is associated with the need for extended hospital stay, ICU care, lower respiratory tract complications and mortality in adults of all ages with chronic underlying conditions, including cancer patients (Volling et al., 2014). Solid cancer and hematologic malignancy are shown to be risk factors for ICU admission and RSV pneumonia need for invasive ventilation and mortality (Chatzis et al., 2018; Yoon et al., 2020). Of note, the impact of RSV infection on cancer progression has never been studied.

### Take Home Messages



1. Respiratory viral infections in cancer patients are associated with severe disease, more complications, prolonged viral shedding, and higher mortality.
2. IAV and RSV infections in cancer patients are among the most common respiratory viral infections.
3. Despite being both single-stranded RNA viruses, IV and RSV have different primary target epithelial cells and distinct viral cycles and they use different receptors for cell entry. While IV replicates in the host nucleus, RSV replicates within the host cytoplasm and doesn't enter host nucleus.
4. Unlike IV, very little is known about the epidemiology of RSV infection in NSCLC patients.

## Chapter 4

### Impact of IV and RSV infections on NSCLC

#### 1. Persistence of RSV and IV in the lungs

In cancer patients, persistence of influenza virus was observed as patients were unable to clear viral infection in the usual delay observed for the global population (McMinn et al., 1999; Souza et al., 2010). Interestingly, a recent study also showed that IAV RNA can be present in the lungs of infected mice for at least 105 days post infection and demonstrated the capacity of IAV to persist as active viral RNA remnants causing a switch to chronic lung disease (Keeler et al., 2018).

Similar observations also indicate RSV persistence in humans and mice, as RSV genomic RNA and mRNA can still be detected after 100 days in the murine lungs (Rivera-Toledo and Gómez, 2012). RSV-infected mice may exhibit abnormal chronic inflammatory mononuclear infiltrate, 13 altered genes expression correlated with airway hyperreactivity, and persistence of RSV RNA, up to 42 days post-infection in the lungs, which may contribute to long-term airway disease induced by RSV (Mejías et al., 2008). RSV can establish persistent infection in macrophages, which leads to the production of excessive level of cytokines and chemokines that are associated with chronic inflammation, enhanced phagocytosis, preserved ability to process and present other viral antigens, as well as upregulated expression of proteins related to cell survival anti-apoptotic (Bcl-2 family) (Rivera-Toledo and Gómez, 2012).

This supports the possibility that persistent viral RNA may maintain local inflammation in the lung TME, and modulate this complex ecosystem, with subsequent impact on tumor progression, clinical outcome and response to treatments - chemotherapy, radiotherapy and immunotherapy.

Considering that IV and RSV are primarily respiratory pathogens, I will highlight the specific effects of IAV and RSV infections of different cells that constitute the lung tumor to understand the global impact of infection on the TME. However, it is important to note that severe seasonal and pandemic influenza and RSV infections can cause viremia (Rohwedder et al., 1998; Tse et

al., 2011), and thus cancer cells of different origins are susceptible to IAV and RSV infections. In addition, these infections can alter anti-cancer responses, even in distant tumors as discussed later.

## **2. IV and RSV sensing in the TME**

Following virus entry into host tumor, stromal and immune cells of the TME, viral RNA is recognized by membrane or cytosol pattern-recognition receptors (PRRs), which cooperate to induce an innate immune response that aims to control the replication of IV and RSV. Both viruses can be sensed by TLRs, RLRs and NLRs, that are expressed by both immune and tumor cells (**Table 4**). Following viral replication and sensing, signaling pathways are triggered to elicit the interferon (IFN)-mediated antiviral response and the expression of proinflammatory cytokines and chemokines. The differential expression of IFNs, cytokines and chemokines following infection with different IV/RSV viruses could be a key determinant, depending the tumor context, for clinical outcome.

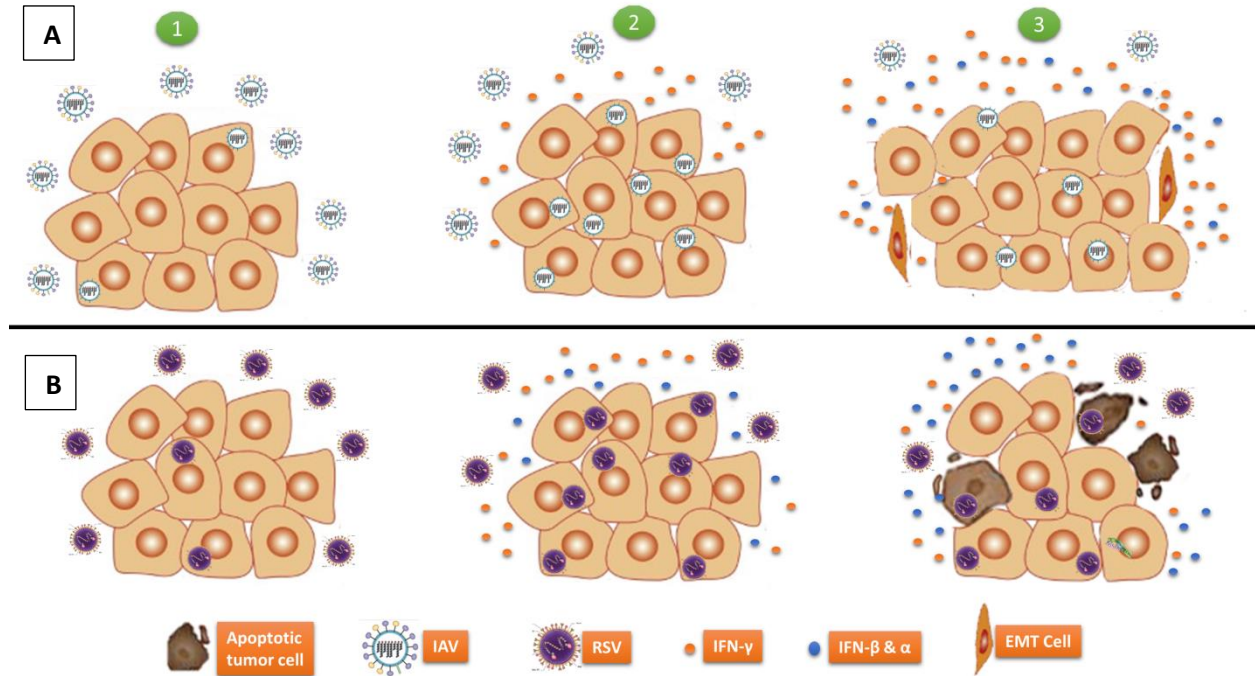
### **2.1. IFNs production:**

While type-I IFN is produced mainly by tumor, normal respiratory epithelial cells and pDCs, type-III IFN is restricted to tumor and epithelial cells (Denney and Ho, 2018). Both RIG-I and TLR3, but not TLR7 or NOD2 are required for type III IFNs induction (Wu et al., 2015), whereas all of them are required for type I IFNs production. Type I IFNs have different kinetics, as they are produced later and/or to a lesser extent, most importantly in the case of high viral burden. Another difference between the two types is that Inflammatory cytokines (e.g., TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and CCL-2) are induced predominantly by IFN- $\alpha$  and not IFN- $\lambda$  (**Fig. 9**). Accordingly, mice deficient for type III IFNs exhibited greater inflammatory burdens in the lungs than did wild-type (WT) or mice deficient for IFNAR1, when infected with IAV (Galani et al., 2017).

A549 cells are more permissive to RSV infection, compared to non-tumorous bronchial epithelial BEAS-2B cells. In addition, RSV infection of A549 cells leads to the production of both some type I & III IFNs (IFN- $\beta$ 1, IFN-  $\lambda$ 1, IFN-  $\lambda$ 2 and IFN-  $\lambda$ 3), comparing with induction of IFN- $\lambda$ ,

without IFN- $\beta/\alpha$ , by RSV infection in primary nasal epithelial cells (NECs) (Hillyer et al., 2018; Okabayashi et al., 2011) (**Fig. 9**) (**Table 4**).

Activation of both type I and III IFN induce hundreds of ISGs. ISGs inhibit viral replication, trigger apoptosis, shut down protein synthesis and activate key components of the innate and adaptive immune systems.



**Figure 9 | Impact of viral infections on NSCLC cells.** Schema represents Influenza (A) and RSV (B) infections in the beginning of the infection (1), early stages of infection (2) and late stage of infection (3). While IAV infection results in the production of type III IFNs in early stages, RSV infection leads to type I & III IFNs. RSV infection induces apoptosis in late stage of infection. IAV promotes EMT markers.

## 2.2. Cytokines and chemokines production:

IAV infection leads to rapid expression of IL-1 $\beta$ , IL-6, CXCL10, TGF- $\beta$ , TNF- $\alpha$ , IL-8 and CCL2 in tumor and respiratory epithelial cells, as well as resident and recruited immune cells (Lam et al., 2010).

RSV leads to an increase in the BALFs of RSV infected individuals are mainly IL-2, IL-12, IFN- $\gamma$ , IL-8, IL-6, and TNF- $\alpha$ . RSV infection of A549 cells induces production of IL-1 $\alpha$ , IL-6, CCL2, CCL5, CXCL5 and CXCL8 (Hillyer et al., 2018). Interestingly, exosomes isolated from RSV-infected A549 cells induce significantly higher secretion of Monocyte chemoattractant protein-1 (MCP-1/CCL2), IP-10, and RANTES in non-infected monocytes and A549 cells, compared to mock exosomes, which suggests the ability of these exosomes to induce proinflammatory mediator secretion in by-stander cells (Chahar et al., 2018).

**Table 4 | PRRs implicated in IAV and RSV sensing.** RIG-I: Retinoic acid-inducible gene I; MDA5: NOD2: Nucleotide-binding oligomerization domain-containing protein 2; NLRP3: NLR family pyrin domain containing 3; MDA5: Melanoma Differentiation-Associated protein 5; TRIF: TIR-domain-containing adapter-inducing interferon- $\beta$ ; MyD88: Myeloid differentiation primary response protein 88; MAVS: mitochondrial antiviral signaling protein; ASC: apoptosis-associated speck-like protein containing a CARD; IRF3/7: interferon regulatory factor 3/7 (Iwasaki and Pillai, 2014; Marr et al., 2013; Sabbah et al., 2009; Shirey et al., 2016; Tate et al., 2016)

Family	Pattern-recognition receptors (PRRs)	Putative IAV/RSV Ligand	Adapter Proteins	Consequences
Toll-like receptors (TLRs)	TLR3	Unidentified dsRNA structures (Viral replication intermediates)	TRIF $\rightarrow$ NF- $\kappa$ B/IRF3/IRF7/IRF1	<ol style="list-style-type: none"> <li>1. Pro-inflammatory cytokines and chemokines (such as pro-IL-1<math>\beta</math>, IL-2, IL-6, IL-15, and pro-IL-18, TNF-<math>\alpha</math>, IP-10 (CXCL10) and CCL-5 (RANTES).</li> <li>2. Type-I IFNs</li> <li>3. Type-III IFNs</li> <li>4. MCP1/IL-8 and MIP: recruit monocytes and</li> </ol>
	TLR4	RSV F protein. IAV-Induced HMGB1 (DAMP)	MyD88 $\rightarrow$ NF- $\kappa$ B/IRF3/IRF7	
	TLR7/8	Viral ssRNA genome	MyD88 $\rightarrow$ NF- $\kappa$ B/IRF3/IRF7	
RIG-I-like	RIG-I	5'-triphosphate viral	MAVS $\rightarrow$ NF-	

receptors (RLRs)		ssRNA	κB/IRF3/IRF7/IRF1	neutrophils
	MDA5	Unidentified Long ds-RNA (Viral replication intermediates)	MAVS → NF-κB/IRF3/IRF7	
Nod-like receptors (NLRs)	NOD2	ssRNA genome/ cellular damage (DAMPS)	MAVS → IRF3	IFNβ
	NLRP3 (Inflammasome)	ssRNA /proton flux	ASC/Caspase-1	production of pyrogenic IL-1β and IL-18

### 3. Direct effect of IV/RSV infection on the hallmarks of tumor cells

#### 3.1. Effects on cellular stress responses and cell death

##### 3.1.1. Influenza infection

Influenza infection incites several cellular stress responses, which can be protective in nature (such as unfolded protein response (UPR)), pro-cell death, (such as apoptosis, necroptosis, pyroptosis or necrosis), or both such as autophagy which helps cells adapt to stress by either preventing cell death or promoting it (autophagic cell death) (Fulda et al., 2010).

IAV infection of A549 cells induces ER stress and ROS generation, leading to oxidative stress and an inflammatory response (Jung et al., 2019). UPR program is activated early under stress conditions in cancer cells, enabling cancer cells to survive and adapt to adverse environmental conditions, enhances angiogenesis, and promotes resistance to conventional chemotherapy (Corazzari et al., 2017).

There are three main programmed cell death pathways: apoptosis (non-inflammatory cell death), necroptosis (DAMPs-releasing necrotic cell death), and pyroptosis (inflammatory cell death, mediated by inflammasome, which induce immune response) (Wang et al., 2019b). The impact of influenza infection on apoptosis and pyroptosis is cell type-, viral strain-, dose- and



time-dependent, via the induction of extrinsic and intrinsic apoptosis pathways (El-Sayed et al., 2016; Shahsavandi et al., 2013). IAV induces little apoptosis without pyroptosis in lung tumor cells by contrary to non-tumoral cells (Daidoji et al., 2008; Lee et al., 2018). This suggests that signaling pathways to trigger immunogenic pyroptosis may decline along with the tumor development, thereby allowing tumor cells to escape from cancer immunosurveillance. Influenza virus, through NS1, initiates anti-apoptotic PI3k-Akt signaling in A549 cells at early and middle phases of infection to protect cells from fast apoptotic death, through the inhibition of proapoptotic Bcl-2 family proteins (Lee et al., 2018; Zhirnov and Klenk, 2007) and Scribble (Liu et al., 2010). The interaction of viral NA protein with carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) protein enhances cell survival in A549 cells infected with IAV, leading to minimal apoptosis, following IAV infection (Gaur et al., 2012).

Necroptosis is only mildly induced in cancerous cell lines (Lee et al., 2018). However, IAV (H1N1) infection induces strong necroptosis in infiltrated immune cells and, to a lesser degree, normal lung alveolar epithelial cells in murine lungs on day 7 post-infection (Wang et al., 2019). Thus, it is possible that necroptosis is stimulated by IAV infection in a species- and/or cell type-dependent manner.

In addition, IAV, possibly through HA and NS1, induces cellular autophagy that may lead to induce autophagic cell death in non-tumorous alveolar epithelial cells (Sun et al., 2012; Zhu et al., 2015). However, IAV, through Matrix protein 2 (M2), keeps autophagy at moderate levels, by blocking autophagosome-lysosome fusion in A549 cells, resulting in limited cell death. In addition, IAV-induced inhibition of macroautophagy induces A549 cell death with low immunogenicity (apoptosis) (Gannagé et al., 2009), and helps the evasion of viral antigen presentation (Mehrbood et al., 2019). However, induced autophagy in NSCLC is associated with the tumor proliferation, poor survival (Nakashima et al., 2018) and resistance to paclitaxel (Chen and Shi, 2016).

Taken together, these data suggests that IV infection of NSCLC cells leads to minor cell death, yet mostly non immunogenic which is not expected to induce immune response.

### 3.1.2 RSV infection

RSV activates ER stress in the A549 cells, which antagonizes RSV replication through mechanisms that need further investigation (Cervantes-Ortiz et al., 2016b). Similar to IV, RSV infection in A549 cells leads to a production of ROS, likely by the NADPH oxidase 2 (NOX2), which positively regulates the signaling pathways leading to expression of antiviral IFNs and proinflammatory cytokines. However, RSV induces late apoptosis after 48h pi in A549 cells. RSV F protein mediates the phosphorylation of tumor suppressor p53, activation of proapoptotic Bax and the induction of both extrinsic and intrinsic apoptosis pathways (Eckardt-Michel et al., 2008). Indeed, RSV infection of A549 cells also induces several proapoptotic factors of the Bcl-2 family and caspases 3, 6, 7, 8, 9, and 10, representing both the death receptor- and mitochondrion-dependent apoptotic pathways, in later stages of infection, 48h pi and later) (Kotelkin et al., 2003a) (**Fig. 9**).

The delay in RSV-induced apoptosis is mediated by viral NS proteins through NF- $\kappa$ B-dependent mechanism (Bitko et al., 2007), which includes early upregulation of antiapoptotic members of the Bcl-2 family, such as Mcl-1, which maximizes at 24h pi (and diminishes later) (Kotelkin et al., 2003b) and induction of autophagy and mitophagy, in order to promote viral replication (Li et al., 2018).

RSV infection of A549 cells strongly up-regulates the expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its functional receptors death receptor 4 (DR4) and DR5 (Kotelkin et al., 2003a). These findings suggest that RSV sensitized infected tumor cells in vivo to killing through the TRAIL pathway by immune cells such as natural killer, CD4<sup>+</sup> T lymphocytes, macrophages, and dendritic cells that bear membrane-bound TRAIL. Moreover, RSV infection of A549 cells upregulates Fas expression, which suggests potential interaction with CD8<sup>+</sup>T cells which express FasL (O'Donnell et al., 1999). TRAIL and FasL are inducers of the death receptor (external) pathway of apoptosis.

Taken together, RSV infection of NSCLC cells leads to delayed cell death, accompanied by TRAIL and Fas upregulation, suggests the induction of immune response.

### **3.2. Effects on tumor cell proliferation**

IAV inhibits the proliferation of tumor cells and results in G0/G1-phase accumulation of infected cells caused by the prevention of cell cycle entry from G0/G1 phase into S phase, at 24h pi. Indeed, G0/G1-phase cell cycle arrest in infected cells provides favorable conditions for viral protein accumulation and virus production (He et al., 2010). In addition, this arrest is caused by inhibition of the activity of the Ras homolog gene family member A (RhoA) in a NS1-dependent manner (Jiang et al., 2013), and it is accompanied by cyclin D3 relocalization and degradation, which, in turn, enhances the assembly of progeny virions (Fan et al., 2017). How much time will last this cell cycle arrest, is a question yet to answer.

RSV infection of tumor cells induces cell cycle arrest (in G0/G1 phase), mediated by TGF- $\beta$ , in order to enhance its replication (Gibbs et al., 2009). In fact, Cdc2 (also known as cyclin-dependent kinase 1 (cdk1)) cyclins D2, A, E, and B1 are less abundant, following RSV infection (Munday et al., 2015).

### **3.3. Effects on Epithelial to Mesenchymal Transition (EMT)**

During cancer progression, the EMT process is necessary to the conversion of benign tumor to aggressive and highly invasive cancer, and thus induces metastasis and even chemoresistance (Iwatsuki et al., 2010).

IAV infection of NSCLC cells downregulates epithelial cell junctions markers such as the E-cadherin, Occludin, and Claudin, through upregulating the transcription factors, Snail and Slug. This is associated by upregulation the expression of mesenchymal markers, such as N-cadherin, and Vimentin, in a time and dose-dependent manner (Ruan et al., 2020) (**Fig. 9**).

The EMT is driven by growth factors, including TGF- $\beta$  (Gonzalez and Medici, 2014). Most IAV subtypes activate latent TGF- $\beta$  (LTGF- $\beta$ ) in vitro and in vivo, which can converted to active TGF- $\beta$ , by a mechanism mediated by viral NA protein (Carlson et al., 2010). Meshcheryakova and her colleagues identified a sphingolipid/EMT-associated gene signature in lung adenocarcinoma tissues, which is also induced by IAV infection of A549 cells. This suggests that influenza infection of A549 may induce EMT signature (Meshcheryakova et al., 2016).

RSV doesn't induce EMT markers in infected NSCLC cells. RSV infection upregulates E-cadherin expression in infected A549 cells, mainly on the RSV-induced syncytial giant cells (Wang et al., 2000). In addition, it doesn't change the expression of mesenchymal markers, such as  $\alpha$ -SMA and Vimentin (Kaltenborn et al., 2012).

### **3.4. Effects on angiogenesis**

IAV (H1N1) infection increases the concentrations of HIF-1 $\alpha$  protein, in the serum of infected patients, and 6 days pi in the lungs of infected mice, comparing with healthy persons and mock infected mice consequently. Additionally, it was shown that IAV (H1N1) infection of A549 results in HIF-1 $\alpha$  accumulation, through proteasome inhibition and decreasing the expression of factor inhibiting HIF-1 (FIH-1), which mimics a hypoxic response in normoxia condition, during the first 24h pi. Correspondingly, this leads to increase the expression of HIF-1-responsive genes VEGF and GLUT-1, that are involved in angiogenesis (Ren et al., 2019).

It's well known that FGF2 promotes the tumor angiogenesis, epithelial–mesenchymal transition (EMT) and invasiveness. Indeed, FGFR overactivations are detected in a variety of human cancers, including lung cancers (Wesche et al., 2011). Normal and tumoral epithelial cells, but not innate immune cells, secrete basic fibroblast growth factor 2 (FGF2) following IAV infection. Administration of the recombinant FGF2 protein significantly alleviates the severity of IAV-induced lung injury, reduces viral replication and promoted the survival of IAV-infected mice. This suggests that FGF2 secretion from tumor cells, following IAV infection, may lead to promote the tumor angiogenesis, EMT, invasiveness and protect tumor cells (Wang et al., 2018).

RSV infection of patients and A549 cells results in elevated levels of pro-angiogenic VEGF-A protein isoforms in the nasal washings from patients and cellular supernatant respectively (Lee et al., 2000). In addition, RSV infection of A549 leads to increase the secretion of fibroblast growth factor basic (FGFb), 24h post-infection (Dosanjh et al., 2003).

### **3.5. Effects on metabolism**

IV infection can stimulate a metabolism akin to that seen in tumor cells in their host cells and even in neighboring cells, via transfer of virally modified extracellular vesicles. Cells show reduced oxidative phosphorylation rate, higher extracellular acidification and, thus, increased aerobic glycolysis in the absence of hypoxia (Magalhaes et al., 2019). These observations are consistent with the induction of Warburg Effect, which in tumor cells enhances cells survival.

In addition, IAV infection of A549 cells induces alteration in the expression of genes implicated in cellular function and metabolic processes, such as purine metabolization, lipid metabolization, and glutathione metabolization, which serves in oxidation-reduction process (Cao et al., 2019; Tian et al., 2019).

RSV stimulates both glycolysis and oxidative phosphorylation by the TCA cycle, leading to the accumulation of ATP, nucleotides, amino acids, and fatty acids that are necessary for the generation of the viral progeny, while a general downregulation of lipids is observed in RSV-infected A549 cells and nasal pharyngeal aspirates from infected patients (Martín-Vicente et al., 2020; Rezinciuc et al., 2020). RSV may promote lipid degradation to obtain additional energy for virus production and also to control excessive oxidative stress (Martín-Vicente et al., 2020).

## **4. Effects of viral infection on immune cells**

### **4.1. Influenza**

IV infection impacts the immune cells in the TME by several mechanisms, mediated either by direct infection of different immune cell types or by indirect effects mediated by infection of tumor and other stromal cells. For instance, the immune protection mediated by monocytes, dendritic cells, and macrophages can be compromised during IV infection when the cells are also targeted by the virus, leading to impaired cytokine responses and altered interactions with other immune cells (Vangeti et al., 2018).

#### **4.1.1. Efficiency of infection**

Most immune cells are susceptible to IV infection, but the efficiency of infection differs according to immune cell type. IAV replicates productively mainly in monocyte-derived

macrophages, monocytes, alveolar macrophages and NK cells (Mao et al., 2009, 2017; Yu et al., 2011). IAV also infects lymphocytes, yet less effectively compared with macrophages. Moreover, the presence of monocytes-macrophages is required to attain infection of both resting and proliferating CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes, as the formation of immune cell clusters is necessary for lymphocytes infection (Mock et al., 2018).

Neutrophils, mDCs and pDCs are susceptible to IAV infection. However, whether it is productive or abortive is a matter of debate, as different studies reported contrasting results (Malachowa et al., 2018; Thomas et al., 2014a) (**Table 5**).

**Table 5 | Summary of the infectability of immune cells by IV and RSV**

Immune Cell	Influenza	RSV
Monocytes & Macrophages	Reproductive infection	Abortive infection
Neutrophils	Debatable (Reproductive/Abortive)	Abortive infection
mDCs & pDCs	Debatable (Reproductive/Abortive)	Abortive infection
T-Cells	Low efficient & requires immune clusters	Low efficient infection
NK Cells	Reproductive infection	Low efficient infection

**4.1.2. Immune cell death**

In addition to apoptotic human alveolar epithelial cells, numerous apoptotic leukocytes are observed in the lungs of a patient who died on day 6 of a fatal IAV infection, accompanied by severe leucopenia (Uiprasertkul et al., 2007). As immune cells play key roles in viral clearance and tumor surveillance, it's expected that depletion of immune cells during IAV infection

significantly compromises the host response and increases infection lethality and tumor progression.

IAV infection induces caspase-dependent apoptosis of monocytes and alveolar macrophages in a dose and time dependent manner, with increased activation of caspase 9, 3/7 and decreased anti-apoptotic proteins Mcl-1 and Bcl-xL (Hoeve et al., 2012; Marriott et al., 2009).

IAV infection of pDCs results in limited apoptosis (Thomas et al., 2014). The impact of flu infection on mDCs depends on the viral strain, as two different mechanisms are suggested:

Seasonal IAVs induce profound immunogenic RIPK3-mediated mDC death (necroptosis), that leads to the enhancement of the maturation of uninfected DC and T cell proliferation (Hartmann et al., 2017). Pandemic and severe strains suppress immunogenic mDC death and disseminate via DC (Hartmann et al., 2017; Thitithanyanont et al., 2007).

During IAV infection, infected and non-infected neutrophils are prone to early apoptosis (Malachowa et al., 2018). Likewise, IAV infection of lymphocytes is accompanied by apoptosis in Fas-FasL dependent mechanism, even for non-infected lymphocytes (Nichols et al., 2001).

IAV infection induces marked NK cell apoptosis, mediated by viral replication, as intact inactivated IAV virion and viral HA protein don't induce NK apoptosis (Mao et al., 2009, 2017).

To conclude, IV infection induces the death of different immune cell types (**Table 6**), which may alter the TME and modifies the immune response towards NSCLC tumor cells, and may limit the anti-tumor response.

**Table 6 | Summary of impact of IV and RSV infections on immune cell death**

Immune Cell	Influenza	RSV
Monocytes & Macrophages	Caspase-dependent apoptosis	Suppression of early apoptosis, late necroptosis
Neutrophils	Early apoptosis	Delayed apoptosis

mDCs/ pDCs	Necroptosis/ limited apoptosis	No apoptosis
T-Cells	Apoptosis in Fas-FasL mechanism	No apoptosis
NK Cells	Marked apoptosis	No Data

#### 4.1.3. Production of cytokines and IFNs

Macrophage flu infection is accompanied by a production of inflammatory cytokines and chemokines such as IL-1, IL-6, IL-12, TNF- $\alpha$  and CXCL10 (Hoeve et al., 2012; Yu et al., 2011). Likewise, infection of monocytes produces large amounts of IFNs, in addition to an array of cytokines and chemokines; (such as IL-1 $\beta$ , MIP-1 $\alpha$ , IL-8, IL-18, and CCL3) (Lamichhane and Samarasinghe, 2019).

Also, during IAV infection, infected and non-infected neutrophils upregulate expression of type-I IFNs and ISGs, in addition to proinflammatory cytokines and chemokines (such as, IL-8, IL-6, MIP-1 $\beta$ , and MCP-1, CCL2, CCL4, CCL4L2, and CXCL10) (Malachowa et al., 2018). Furthermore, IAV infection of pDCs results in producing important quantities of type I IFN (Thomas et al., 2014).

Type I interferons (IFNs) curtail the spread of IAV and promote the elimination of infected cells. However, primary peripheral blood monocytes from elderly healthy human donors show a marked defect in the IAV-induced secretion of type I IFNs, but not proinflammatory cytokines, due to an impaired primary RIG-I signaling pathway, thereby reducing antiviral resistance to IAV while sustaining inflammation in older patients, who are more prone to cancers (Molony et al., 2017).

Altogether, this suggests that IV infection induces the production of proinflammatory cytokines from different immune cells, which may modify the TME and contribute to the chronic inflammation observed in NSCLC.

#### 4.1.4. Impacting the function of immune cells



IV infection impacts multiple immune cell functions that are indispensable for anti-tumor response, such as Ag presentation, immune cell infiltration and immune cytotoxicity, as well as inducing some immunosuppression mechanisms.

In order to evade immune recognition, different strains of IAV and IBV downregulate MHC-I expression. Interestingly, the two influenza types employ different mechanisms toward MHC-I downregulation. Following IAV infection, infected (NP<sup>+</sup>) cells have significantly reduced levels of both surface and intracellular MHC-I, in a mechanism that is independent of endocytosis but dependent on proteasomal degradation, while IBV causes a loss of surface MHC-I, but not intracellular MHC-I, due to delayed MHC-I trafficking to the cell surface. Importantly, downregulation of MHC-I molecules at the cell surface can help infected cells to evade immune recognition, thereby delay viral clearance by CD8<sup>+</sup> T cells (Koutsakos et al., 2019). It is well established that tumor immune escape, especially from T-cell mediated immunosurveillance, is associated with MHC-I downregulation, as seen in different human and experimental tumors (Garrido et al., 2016). In addition, Infected mDCs efficiently present endogenous IAV antigens on MHC class II, but not on MHC class I, which impairs their ability to cross-present antigen to CD8<sup>+</sup> T-cells (Smed-Sørensen et al., 2012; Thomas et al., 2014a).

IV infection induces neutrophils migration to the site of infection, where they release neutrophil extracellular traps (NETs) and other damaging inflammatory signals, in order to inactivate viral particles and prevent viral dissemination (Hemmers et al., 2011). Importantly, NETs have tumor-promoting roles and promote metastatic progression (Rayes et al., 2019). Moreover, excessive trafficking of neutrophils to the lungs is associated with poor outcomes, NSCLC cancer progression and poorer prognosis (Aggarwal et al., 2019; Moroy et al., 2012; Tang et al., 2019).

IAV infection of primary airway epithelial cells and tumor cells enhances PDL-1 expression, in an IFNAR signaling-dependent manner (McNally et al., 2013). Also, IAV infection results in high expression of PD-1 on effector CD8<sup>+</sup> T cells, which interact with PD-L1, expressed in the TME of some NSCLC patients, leading to T cell exhaustion (McNally et al., 2013; Nikolaev et al., 2019). In addition, In a model of melanoma, IAV infection of the lung promotes tumor growth and

accelerated tumor-related death due to induction of T cell exhaustion and a disruption in the trafficking of anti-tumor CD8<sup>+</sup> T cells to the site of infection (Kohlhapp et al., 2016).

Following IAV infection, NK cells show impairments in cytotoxicity and IFN- $\gamma$  production, as well as, decreased virus-specific killing mediated by cytotoxic T lymphocytes (CTLs), which weaken both innate and adaptive immune responses (Liu et al., 2018). Moreover, the viral NA protein in IAV prevents HA recognition by activating NKp44 and NKp46 receptors resulting in reduced killing of infected cells by NK cells. Since lung tumor cells can be infected by IAV, a similar mechanism of escape from NK cell cytotoxicity may occur in the TME (Bar-On et al., 2014).

In addition to direct effects on immune cells, IAV induces an immunosuppressive environment since it results in the accumulation of MDSCs and memory regulatory T cells (mTregs) in the lungs of mice and human infected with IAV, which can be capable of suppressing T cell proliferation and cytokine production and promoting tumor growth (Betts et al., 2012; Lu et al., 2019a; O'Connor et al., 2017). IAV infection also increases indoleamine 2,3-dioxygenase (IDO) activity in the murine lung parenchyma, as well as in infected A549 cells. In cancer, high IDO1 expression dampens the immune response via inhibition of T-cell responses (Van Der Sluijs et al., 2006) - it reduces the numbers of Th1-CD4<sup>+</sup> T cells and effector memory CD8<sup>+</sup> T cells in infected lungs (Fox et al., 2013), increases IL-10 production (Van Der Sluijs et al., 2006) and suppresses NK cell function by inhibiting NKG2D and NKp46 expression (Park et al., 2019). Increased IDO activity is associated with poor clinical outcome in adults hospitalized with IAV infection (Pett et al., 2018). Drugs targeting IDO are being tested in clinical trials with the aim of reverting cancer-induced immunosuppression (Platten et al., 2015). In addition, IAV infection of A549 cells results in a time-dependent accumulation of prostaglandin E2 (PGE2), following NS1-induced activation of the COX-2 enzyme (Li et al., 2008). IAV infection markedly increases PGE2 in plasma and bronchoalveolar lavage (BAL) fluid of IAV-infected mice. PGE2 was showed to inhibit blood monocyte recruitment to lungs and their differentiation into DCs. In addition, PGE2 inhibits T cell recruitment and delays the onset of IAV-specific T cell responses in the lungs, impairs the control of viral replication by macrophages and suppresses MHC class II expression reducing their capacity to present antigens to T cells (Coulombe et al., 2014).

Altogether, these data support a role of influenza infection in worsening lung tumor disease, by modulating the TME, subsequently leading to pro-tumoral effects. This highlights a crucial role of prevention to protect patients from influenza infection.

**Table 7 | Comparison of the impact of IV and RSV infections on immune cells function**

	Influenza	RSV
<b>Cytokines Secretion</b>	Produce inflammatory cytokines and chemokines from macrophages and neutrophils, type I IFN by pDCs and type II IFN by T-cells and NK cells	
<b>Antigen presentation</b>	Downregulate MHC-I expression, impairs mDCs ability to cross-present antigen on MHC class I to CD8+ T-cells	Upregulation of costimulatory molecules in mDCs and pDCs (CD80, CD83, CD86) molecules, upregulation of NLRC5, class I MHC transactivator and class II MHC proteins
<b>Neutrophils</b>	Neutrophils are <b>not</b> necessary to limit virus replication, and are <b>not</b> responsible of severe infections	Neutrophils are necessary to limit virus replication, and are implicated in severe infections
	Neutrophils secrete proteolytic enzymes, neutrophil elastase, myeloperoxidase and ROS-dependent NETosis	
<b>T-cells</b>	Upregulates PD-1 on effector CD8 <sup>+</sup> T cells, PDL-1 expression on epithelial cells, disruption in the trafficking of anti-tumor CD8 <sup>+</sup> T cells, decreased T-cell cytotoxicity, T cell exhaustion	Upregulates PD-1 on effector CD8 <sup>+</sup> T cells, PDL-1 expression on epithelial cells, increase CD8 <sup>+</sup> T cell activation, proliferation, and antiviral function, expansion of tissue-resident memory CD4 <sup>+</sup> and CD8 <sup>+</sup> T Cells
<b>T-regs and MDSCs</b>	Expansion of MDSCs and memory	No impact

	regulatory T cells	
<b>NK Cells</b>	Reduced cytotoxicity and production of IFN- $\gamma$ and NKp44 and NKp46 receptors	Increase activation of lung NK cells, NK cells express activating receptors NKG2D and CD27 and produce a large amount of gamma interferon (IFN- $\gamma$ )
<b>Immunosuppressive mediators</b>	Increases IDO production, prostaglandin E2 and TGF- $\beta$	

## **4.2. RSV**

### **4.2.1. Efficiency of infection**

RSV results in an abortive and non-replicative infection of murine and human alveolar macrophages (AMs), mDCs in addition to blood and lung (Bohmwald et al., 2017b; de Graaff et al., 2005; Halfhide et al., 2011; Le Nouën et al., 2009). However, RSV infects and replicates in a small percentage (up to 20%) of NK cells, CD4<sup>+</sup> and CD8<sup>+</sup> T-cells (van Erp et al., 2019; Raiden et al., 2017b).

### **4.2.2. Immune cell death**

The impact of RSV on immune cell apoptosis depends on immune cell type. RSV infection leads to a delayed apoptosis of neutrophils in the course of the infection (Deng et al., 2020b; Lindemans et al., 2006). The delay is mediated by PI3K and NF- $\kappa$ B pathways (Lindemans et al., 2006). RSV abortive infection in mDCs results in maturation, without inducing mDC apoptosis (Le Nouën et al., 2009).

RSV suppresses intrinsic and external apoptotic pathway in macrophages during the first 24h pi, through reducing the activities of caspases 3, 8 and 9 and increasing the expression of anti-apoptotic proteins, such as Bcl-2, Bcl-X, and XIAP (Nakamura-Lopez et al., 2015; Nakamura-López et al., 2011). However, a recent study shows that RSV infection triggers necroptosis in primary mouse alveolar macrophages and human monocytes (Santos et al., 2020).

Likewise, RSV infection of T-cells doesn't lead to more apoptosis or cell death, compared with uninfected cells (Raiden et al., 2017b).

#### **4.2.3. Production of cytokines and IFNs**

RSV infection of different immune cells results in the production of different proinflammatory cytokines and IFNs that could impact the anti-tumor response.

RSV abortive infection of macrophages and neutrophils leads to the secretion of several proinflammatory cytokines, such as IL-1 $\beta$ , IL-1 $\alpha$ , IL-6, IL-8, TNF $\alpha$ , IL-17, as well as chemokines such as IL-8, CCL2, CCL3, CXCL8 and CXCL12 that are essential to recruit and activates inflammatory monocytes, CD8<sup>+</sup> T-cells, and NK cells that contribute to viral clearance (Bohmwald et al., 2017b; Sebina and Phipps, 2020).

During RSV infection, pDCs generate high levels of type I-IFNs and play immune regulatory role in reducing pulmonary inflammation and viral replication (Wang et al., 2006). Likewise, RSV Infection of NK Cells Induces IFN- $\gamma$  Production (van Erp et al., 2019).

Although RSV infection impairs the production of IL-2 by CD4<sup>+</sup> T cells, it doesn't change IFN- $\gamma$ , IL-5 and IL-17 by CD4<sup>+</sup> T and CD8<sup>+</sup> T-cells (Raiden et al., 2017a).

#### **4.2.4. Impacting the function of immune cells**

RSV infection leads to up-regulation of costimulatory molecules in mDCs and pDCs (CD80, CD83, CD86) and MHC class II molecules, and increase activation of naive T cells (Le Nouën et al., 2009; Schwarze, 2008; Wang et al., 2006).

Although RSV infection doesn't impact non-stimulated memory CD8<sup>+</sup> T-cells (Antunes et al., 2019), it leads to a peak in the expansion of tissue-resident memory CD4<sup>+</sup> and CD8<sup>+</sup> T Cells at day 8 pi in mice (Luangrath et al., 2021). This suggests that RSV may enhance the proliferation of T-cells in the TME.

RSV infection of primary human bronchial epithelial cell results in strong expression of PD-L1 expression and increase of CD8<sup>+</sup> T cell activation, proliferation, and antiviral function, which is even more increased by blocking PD-L1 (Telcian et al., 2011). An increased of PD-1 expression

on lung CD4<sup>+</sup> and CD8<sup>+</sup> T cells following RSV infection in mice after RSV infection of mice (Yao et al., 2015). This suggests a potentially critical role of PD-L1 and PD-1 interactions in the lung for controlling host inflammatory responses and disease progression in clinical RSV infection (Yao et al., 2015), and this may impact the anti-tumor response.

RSV infection also promotes the accumulation and activation of functional lung NK cells at the early stage of infection in mice (Li et al., 2012a).

RSV infection of A549 cells promotes upregulation of NLRC5, class I MHC transactivator and class II MHC proteins, mediated by IFN- $\beta$  and RIG-I signaling. Elevated expression of MHC-I molecules can sensitize tumor cells to T lymphocyte-mediated cytotoxicity or immunopathologic damage (Guo et al., 2015).

However, Similar to IV, RSV infection of A549 cells and mDC upregulates IDO transcripts and PGE2 secretion, (Ajamian et al., 2015; Rajan et al., 2017), which supports viral replication and the production of proinflammatory mediators (RANTES, MCP-1, and IL-8) (Liu et al., 2005). In addition, RSV infection of A549 cells induces TGF- $\beta$  mRNA and protein expression (Gibbs et al., 2009).

To conclude, despite some similarities in the cytokines secretion and immunosuppressive mediators, IV and RSV infections impact differently the immune cells in terms of cell death, antigen presentation, immune cells activation proliferation and cytotoxicity. This may suggest different outcomes on the anti-tumor response and tumor progression.

## Take Home Messages



1. IV and RSV leads to persistent infection in human and murine lungs, which suggests possible subsequent impact on the TME and tumor progression.
2. IV and RSV can be sensed by different PRRs, which leads to the production of relatively similar profile of proinflammatory cytokines and IFNs
3. While IV infection of NSCLC cells leads to minor cell death, yet mostly non immunogenic which is not expected to induce immune response, RSV infection of NSCLC cells leads to delayed cell death, accompanied by TRAIL and Fas upregulation, suggests the induction of immune response.
4. While IAV infection of NSCLC cells upregulates EMT markers, RSV infections doesn't upregulate them.
5. IV infection induces the death of different immune cell types which may alter the TME and may limit the anti-tumor response. By contrary, RSV infection doesn't lead to cell death of immune cell types, except neutrophils and macrophages (delayed apoptosis).
6. IV infection impacts multiple immune cell functions that are indispensable for anti-tumor response, such as Ag presentation, immune cell infiltration and immune cytotoxicity, as well as inducing some immunosuppression mechanisms, which may results in protumoral effects. RSV infection enhances DC maturation, proliferation and activation of NK cells and T-cells.

## Chapter 5

### Viral vaccine and oncotherapy in NSCLC patients

#### 1. Influenza Vaccine

Considering the multiple, deleterious impacts of influenza infection on cancer progression, strategies to optimize influenza prevention among patients with cancer are highly warranted. Influenza vaccines and specific anti-flu medications are two suggested preventive therapeutics to be considered (Angrini et al., 2021).

The optimal strategy to prevent influenza infection in cancer patients is vaccination. Vaccine-induced HA-specific antibodies can neutralize antigenically matched IAV by preventing attachment of the virus to its receptor on airway epithelial cells (Vollaard et al., 2017).

As the use of the live attenuated vaccine is contra-indicated in cancer patients, inactivated Influenza vaccines are the only available vaccine formula.

Two influenza inactivated vaccine formulas are available:

**A.** Trivalent influenza vaccine (TIV) which is formulated annually and it consists of hemagglutinin (HA) antigens derived from three inactivated influenza viruses, two different influenza type A strains (H1N1 and H3N2) and one influenza type B strain, either B/Victoria or B/Yamagata (Khurana et al., 2019).

**B.** Quadrivalent influenza vaccines (QIVs) which includes strains from both B lineages (Khurana et al., 2019), thus may provide broader protection, but it has not yet been incorporated in most national seasonal influenza vaccination campaigns.

It's still unclear which inactivated vaccine formula is the optimal for NSCLC patients. Further studies are needed to confirm.

Influenza vaccination might be associated with lower mortality and reduced infection-related outcomes (Bersanelli et al., 2018; Bitterman et al., 2018; Bosaeed and Kumar, 2018; Vollaard et



al., 2017). Influenza vaccination correlates with reduced incidence of lung cancer as was shown in patients with chronic obstructive pulmonary disease (COPD), which is a well-known risk factor for lung cancer (Chen et al., 2019). In patients undergoing curative surgery for solid cancers, giving a flu vaccine in the postoperative period results in less overall mortality and cancer-related mortality, compared with patients never receiving a vaccine (Gögenur et al., 2020). Vaccination not only prevents influenza from modulating the TME directly, but it could also promote anti-tumor immunity in cancer patients. This has been demonstrated in murine studies where intra-tumoral flu vaccination reactivates CD8<sup>+</sup> T-cell-mediated antitumor immunity and sensitizes resistant tumors to immune checkpoint blockade (anti-PD-L1) (Newman et al., 2020).

However, few studies have shown that the immunogenicity of available inactivated Influenza vaccines in cancer patients varies between 10 and 60%, whereas vaccination prevents clinical infection in 70–90% of healthy individuals and lowers the rate of influenza-related hospitalization, and mortality (Volllaard et al., 2017). This suggests that healthy individuals, especially patient relatives and healthcare providers should also be encouraged to get the flu vaccine to prevent those with cancer from catching the virus. In order to improve influenza vaccine immunogenicity in lung cancer patients, different vaccination strategies should be investigated, such as using adjuvanted vaccine, two vaccine doses in the same influenza season, intranasal vaccine injections, different timing of vaccination, and high-dose vaccines.

Of note, receiving active chemotherapy doesn't change significantly the vaccine effectiveness among patients with solid tumor cancer (Blanchette et al., 2019; Volllaard et al., 2017). In addition, flu vaccination reduces the number of oncologic treatment interruptions compared to non-vaccinated cancer patients. Of note, receiving active chemotherapy doesn't change significantly the vaccine effectiveness among patients with solid tumor cancer (Volllaard et al., 2017). In addition, in a multi-center study with a broad range of cancers, influenza vaccine administration was associated with a non-significant increase in overall survival among patients on immune checkpoints inhibitors (Bersanelli et al., 2018). However, further studies that investigate the impact of Flu vaccination on targeted or immunotherapy are needed to bring a strong conclusion.

The association of influenza vaccine with an increase in immune-related adverse events (irAEs) among cancer patients treated by immune checkpoint inhibitors (ICIs) is still debatable. One recent study suggested that about 50% of Lung cancer patients, treated by PD-1 blockade, show irAEs at 3.2 months after influenza vaccination, with 26% of patients show severe grade 3/4 irAEs (Läubli et al., 2018). In contrast, another study found that Influenza vaccination in patients with lung cancer receiving anti-PD-1 immunotherapy does not induce significantly higher irAEs (Wijn et al., 2018).

For cancer patients who are not vaccinated, antiviral treatment such as neuraminidase inhibitor (oseltamivir: Tamiflu), is recommended as early as possible for cancer patients with confirmed or suspected influenza, to reduce viral load and consequently limit the pro-tumoral effect of virus, with a probable impact on morbidity and mortality. However, because of viral persistence in cancer patients, the virus accumulate mutations during the course of infection, which leads to acquire resistance against antiviral treatments (Mitha et al., 2019). This emphasizes the recommendation of timely identifying resistant mutations that arise in the neuraminidase in infected cancer patients, and the importance to prevent the transmission of resistant influenza strains among cancer patients.

Despite efforts to understand the impact of influenza infection in lung cancer patients, there are still many unanswered questions:

- What are the short and long-term outcomes of influenza infection in lung cancer patients?
- How should physicians personalize lung cancer therapeutic protocols, in the case of influenza infection?
- What is the best anti-influenza medication to be added to the therapy strategy of lung cancer patients? How can we avoid the development of drug resistance?
- Considering that influenza can cause viremia, does influenza infections influence the progression of other cancers?

## 2. RSV Vaccine:

Despite the urgency of reducing this RSV global health burden and the developing understanding of the molecular mechanism of RSV infection, there is no safe or effective vaccine against RSV. However, the RSV vaccine landscape has rapidly expanded to include 19 vaccine candidates and monoclonal antibodies in clinical trials, reflecting the prioritization of RSV vaccine development (Noor and Krilov, 2018).

## 3. Influenza virus in oncotherapy

Given the stability of influenza and its tropism to epithelial tissues, the exploitation of the favorable replication and lytic effects of unmodified or recombinant influenza strains in particular types of tumors (pancreatic, lung, and melanoma) has attracted many teams in the field of oncolytic virus therapy since the beginning of the 21st<sup>th</sup> century. The different strategies and their outcomes are summarized in table 2.

**Table 8: Summary of the different strategies to use IAV in oncotherapy**

Strategy	Tumor model	Virus	Outcome	References
Unmodified IAV	Pancreatic ductal adenocarcinoma (PDA) (compromised in type-I IFN signaling)	IAV (H1N1) and Low pathologic avian IAV (H7N3), HP IAV (H5N1)	Apoptosis of tumor cells/ reduced tumor growth	(Huo et al., 2018; Kasloff et al., 2014)
	Spontaneous murine NSCLC tumors (express Raf kinase)	IAV (H1N1)	Efficient oncolysis, due to reversion of M2 into M1 macrophages	(Masemann et al., 2018)

	Subcutaneously-administrated murine LLC cells (NSCLC) in C57BL/6 mice	Mice experienced IAV infection, prior to tumor inoculation	Flu infection leads to the release of TAA that elicit specific and lasting immune memory	(Iheagwara et al., 2014)
Recombinant influenza that target tumor cells	PDA cells (BxPC-3)/ palpable BxPC-3 tumors in a SCID mouse model	Deleted NS1-IAV virus	Reduction in tumor growth	(Pizzuto et al., 2016)
	C57BL/6 mice and athymic nude mice inoculated subcutaneously with mouse melanoma B16-F1 cells and human PDA consequently	Partially deleted NS1 fused to GFP IAV (H1N1) and an elastase-sensitive HA cleavage site	Inhibition of tumor outgrowth, and improve mice survival	(Kuznetsova et al., 2017)
Recombinant influenza that stimulates the antitumor immune response	Subcutaneously-administrated murine melanoma B16-F1 in C57BL/6 mice	IL-15-expressing oncolytic IAV (H1N1), NS1-truncated virus	Increases mice survival, decreases tumor growth, and increases intratumoral T- and NK cells	(Hock et al., 2017; Kuznetsova et al., 2017)
Using flu in Combinational Therapy	Spontaneous murine NSCLC tumors (express Raf kinase)	low-pathogenic IAV (H1N1) + B7-H3 immune-checkpoint	Efficient oncolysis, due to reversion of M2 into M1 macrophages,	(Masemann et al., 2021)

		inhibitors	increased lung infiltration by cytotoxic T-cells	
Metastasis of murine B16-F10 melanoma cells to mice lungs	Recombinant IAV+ PD-1 checkpoint blockade		Sustained reduction of 50% of metastatic tumor size. IAV alone leads to unsustained anti-tumor efficacy	(Sitnik et al., 2020)
Intradermal murine melanoma (Colo-679 cells)	cisplatin and deleted-NS1 IAV		increased oncolytic effects in comparison to either single treatment alone	(van Rikxoort et al., 2012)
Intradermal murine B16-F10-derived melanoma in C57BL/6 mice	Transgenic IAV (H1N1) expresses CTLA4 antibody		Increased overall survival and delayed tumor growth (enhance systemic anti-tumor immune response)	(Hamilton et al., 2018)
Murine model of B16-F10 melanoma tumors in lungs	IAV (H1N1 that expresses OVA peptide) + PD-1 checkpoint blockade	FLU-OVA	Reduced melanoma foci in the lung	(Newman et al., 2020)

#### **4. RSV in oncotherapy**

Some studies have introduced additional evidence that RSV imposes a potent oncolytic effect on different malignancies.

RSV infection of A431 cells, a human cell line of skin cancer, resulted in a considerable cell death, early and late apoptotic cells and inhibition of cell growth, in a dose- and time-dependent manner. This decrease in viability results from a productive replication of RSV (Salimi et al., 2013).

Intratumoral and intraperitoneal injections of RSV lead to a significant regression of human prostate tumor xenografts in nude mice, caused by growth inhibition and activation of intrinsic and external apoptosis pathways in cancer cells (Echchgadda et al., 2009, 2011).

In addition, RSV infection of hepatocellular carcinoma cell-lines result in decreased cell growth, cell migration and apoptosis (Choi et al., 2015).

Finally, intratumoral expression of RSV- fusion (RSV-F) protein, encoded by a replication-defective adenovirus vector, in combination with local coexpression of cytokines can induce tumor-specific immune responses in a syngeneic murine colorectal cancer model (Hoffmann et al., 2007).

Taken together, using oncolytic IV and RSV might be an innovative future approach to target NSCLCs. However, only specific strains of IV are shown to be oncolytic in certain models (ex. cells express Raf kinase), and thus it could be an interesting tool in individualized cancer therapies, but not in all NSCLC cases.

### Take Home Messages



1. The optimal strategy to prevent influenza infection in cancer patients is vaccination, as it's associated with lower mortality and reduced infection-related outcomes.
2. Further studies are needed to determine the optimal vaccine formula and vaccination protocol to enhance the immunogenicity of IV vaccine in cancer patients.
3. There is a need to personalize lung cancer therapeutic protocols, in the case of influenza infection.
4. For cancer patients who are not vaccinated, antiviral treatment is recommended as early as possible, accompanied by timely identifying resistant mutations.
5. So far, there is no safe or effective vaccine against RSV.
6. RSV and certain strains of IV have shown to be effective in oncolytic therapy in certain types of cancers.

## **Hypothesis and Objectives of the Study**

Based on published literature and previous discussion, we hypothesize that acute respiratory viral infections, exemplified by IV and RSV, can cause persistent infections in NSCLC tumors, which suggests possible subsequent impact on the TME and tumor progression. This impact (in case of its existence) could be mediated by impacting the hallmarks of cancer cells, anti-tumor immune response, or both. In addition, despite some similarities, previous studies have suggested that IV and RSV could impact NSCLC tumors differently.

We hypothesize that sensing of IV/RSV infections by different RNA sensors in tumor and immune cells leads to the production of inflammatory cytokines and type-I IFNs, which leads to the induction of different subsets of ISGs in tumor and immune cells. Consequently, this may impact tumor cell death, proliferation, EMT and angiogenesis markers, and may modify the TME and the anti-tumor immune response in murine NSCLC models.

Therefore, we investigated the effect of RSV and IV infections on murine in vivo and in vitro models of NSCLC, and explored the infectivity of resected human tumors by these viruses. Considering the burden of the work and the limit of the time, I was mainly responsible for investigating the impact of RSV on murine in vivo and in vitro models, as well as the impact of IV infection on in-vitro model of NSCLC tumors.

### **Objectives of the Study**

1. To investigate the impact of RSV infection on tumor progression in NSCLC orthotopic mouse model.
2. To search for potential mechanisms that can explain the in vivo results on tumor cell level, through investigating the impact of RSV infection on the hallmark of NSCLC cell lines in-vitro, including tumor cells proliferation, viability/apoptosis, markers of angiogenesis and Epithelial–mesenchymal transition (EMT) and cytokines profile production upon viral infection.
3. To investigate the impact of RSV infection on immune cell infiltration and signaling pathways in murine tumors.



4. To investigate whether other viruses (IAV) can induce similar changes in the hallmark of NSCLC cell lines.
5. To investigate whether human NSCLC can also be infected by RSV.

## Materials and Methods

### 1. Cell lines

LLC-Luc cells (gift from Vincenzo di Bartolo, Institut Pasteur, Paris) were obtained from LLC cells (Lewis Lung Carcinoma, ATCC CRL-1642) that were stably transfected with firefly luciferase. LLC-Luc cells were cultured in DMEM+ GlutaMax (Gibco) with 10% heat-inactivated FCS, 1% penicilin/streptomycin mixture, and 0.5mg/ml hygromycin B.

LKR cells, obtained from Marie Wislez (Wislez M et al. Cancer Research 2005) and TC1 cells were cultured in DMEM+GlutaMax (Gibco) with 10% heat-inactivated FCS and 1% penicilin/streptomycin mixture.

KP cells, derived from tumor-bearing lungs of  $Kras^{LSL-G12D/+}Trp53^{flox/flox}$  mice, were cultured in RPMI-1640 Medium with 10% heat-inactivated FCS, 1% penicilin/streptomycin mixture.

A549 cells were cultured in DMEM/F12K with 10% heat-inactivated FCS, 1% penicilin/streptomycin mixture, 1% L-Glutamine and 1% HEPES.

The cumulative culture length of the cells was fewer than 6 months after resuscitation. Early passage cells were used for all experiments and they were not reauthenticated.

### 2. Virus

Influenza A/Scotland/20/74 (H3N2 strain) was provided by Jean-Michel Sallenave's Lab (INSERM UMR 1152, Bichat Université Paris Diderot, Paris, France). From the supernatants of infected MDCK cells, influenza virus was purified by centrifugation in sucrose gradient, aliquoted and kept at  $-80^{\circ}\text{C}$  until use (Sanchez-Guzman et al., 2019). The virus was previously adapted to C57Bl/6 mice through serial passage (Sanchez-Guzman et al., 2019). Purified virus was used for infection at different MOIs in appropriate complete culture medium.

Recombinant human RSV, subgroup A, strain Long (GenBank accession: AY911262), in which the red fluorescent protein (mCherry) or the firefly luciferase (Luc) genes are inserted into the RSV

genome, were provided by Jean-François Eléouët's lab (Unité de Virologie et Immunologie Moléculaires (UR892), INRA, Jouy-en-Josas, F78352, France). Viruses were amplified and titrated on HEp-2 cells at 37 °C using a plaque assay and virus stocks were frozen at passages 3 and 4 (Rameix-Welti et al., 2014). RSV was used for infection at different MOIs in appropriate complete culture medium.

### **3. Mice**

Wild-type C57Bl/6J mice were purchased from Janvier Laboratories. All the animals were housed at CEF (Centre d'Explorations Fonctionnelles of Centre de Recherche des Cordeliers, Agreement no. A75-06-12). All mice were housed in specific pathogen free (SPF) conditions in full accordance with FELASA recommendations. All procedures received approval from Institutional and Regional Ethical Review Boards.

All experiments were conducted in 8-12 week-old female mice in accordance with the institutional guidelines and the recommendations for the care and use of laboratory animals put forward by the Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes (project has been approved by a user establishment's ethics committee and the Project Authorization).

### **4. In vitro viral infection**

50,000 LLC-Luc cells or 75,000 KP-DBY cells were cultured in 1 ml of complete growth medium in 24-well plate for 24 hours. Cells were then counted and infected with optimal MOI for different time points in a volume of 1.5 ml of complete growth medium. Non-infected cells were used as control. At different time points, cells were collected for flow cytometry and RT-qPCR analysis, and the supernatants were collected for type-I IFNs titration. For IFNAR1 blocking experiment, mouse IFNAR1 monoclonal antibody, functional Grade (eBioscience, n. 16-5945-85) was used to block IFNAR receptors.

#### **4.1. Flow cytometry:**

Apoptosis was investigated by using Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Thermofisher). Click-it Plus EdU Pacific Blue Flow Cytometry Assay Kit (Invitrogen) was used in cell proliferation assay.

For the other analyses, cells were incubated for 20 minutes with blocking antibody (24G2, 1mg/ml, 1/100). Cells were then stained for 30 minutes with LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (Life Technologies) MHC Class-I, PDL1, and Calreticulin antibodies, detailed in **(Table 9)**.

Later, cells were fixed by fixation/permabilisation solution (eBiosciences), as recommended by the manufacturer, and then they were stained for 30 minutes with Influenza A Virus Nucleoprotein, MDA5, TLR3 and pSTAT1 antibodies. The staining was assessed with a FACS Fortessa cytometer, and flow cytometry data were analyzed using Kaluza software.

**Table 9: List of antibodies used for flow cytometry in in-vitro experiments**

Target protein	Fluorochrome	Clone	Source (company)	Reference
<b>Influenza A Virus Nucleoprotein Antibody</b>	FITC	D67J	Abcam	ab210526
<b>Respiratory Syncytial Virus Glycoprotein F Antibody</b>	Alexa Fluor 647	5A6	Novus Biological	NBP2-50396AF647
<b>Mouse MHC Class-I (H-2Kb) Antibody</b>	PerCP-eFluor 710	AF6-88.5.5.3	eBioscience	46-5958-82
<b>Mouse Calreticulin antibody</b>	PE	FMC 75	Abcam	ab83220
<b>Mouse IgG1 Isotype</b>	PE	B11/6	Abcam	Ab91357

<b>Control</b>				
<b>Mouse CD274 (PD-L1) Antibody</b>	PE	MIH5	eBioscience	12-5982-82
<b>IgG2a kappa Isotype Control</b>	PE	eBR2a	eBioscience	12-4321-81
<b>Rabbit MDA5 antibody</b>			Abcam	ab79055
<b>Rabbit IgG Isotype Control</b>			Abcam	ab37415
<b>Donkey Anti-Rabbit IgG H&amp;L</b>	Alexa Fluor 647		Abcam	ab150067
<b>Mouse CD283 (TLR3) Antibody</b>	PE	11F8	Biolegend	141904
<b>Mouse IgG2a, κ Isotype Ctrl Antibody</b>	PE	RTK2758	Biolegend	400508
<b>Mouse IFNAR1 Antibody, Functional Grade</b>		MAR1-5A3	eBioscience	16-5945-85
<b>Mouse IgG1 kappa Isotype Control</b>		P3.6.2.8.1	eBioscience	14-4714-82
<b>Mouse IFNAR1 Antibody</b>	APC	MAR1-5A3	Biolegend	127314
<b>Mouse Phospho-Stat1 (Ser727) Antibody</b>	APC	Stat1S727-C6	Invitrogen	MA5-28056

#### 4.2. RNA expression analysis

For individual gene expression analysis, RNA extraction from LLC-Luc or KP-Dpy cells, infected or not by IAV or RSV, was performed by using the Qiagen RNeasy mini kit. The quality and quantity of extracted RNA were tested by using Agilent RNA 6000 Nano Kit and Agilent Bioanalyzer

(Agilent Technologies) according to the manufacturers' guidelines. Reverse transcription of RNA to cDNA was performed using 1 µg of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and Thermocycler Eppendorf nexus Mastercycler. All qPCRs were done by using FastStart universal Probe master mix 2X (Roche) using inventoried TaqMan gene expression assays probes specific for murine studies. All used probes are listed in (Table 11). IAV and RSV infections were assayed by using designed primers and probes to amplify M2 gene of influenza A or Nucleocapsid Gene of RSV-A (Eurofins Genomics) (Table 10).

**Table 10: List of probes and primers used to detect viral infections of NSCLC cell lines**

Virus	Studied Gene	Gene Code	Probe	Forward Primer	Reverse Primer
IAV	Matrix Protein	M2-IAV	5'- FAM-TTT GTG TTC ACG CTC ACC GT-TAMRA	5'- AAGACCAATCCTGTACCTCTGA	5'-CAAAGCGTCTACGCTGCAGTCC
RSV	Nucleocapsid Gene	N-RSV-A	5'- FAM-CAC CAT CCA ACG GAG CAC AGG AGA T-TAMRA	5'-AGA TCA ACT TCT GTC ATC CAG CAA	5'-TTC TGC ACA TCA TAA TTA GGA GTA TCA AT

In addition, quantitative gene expression analysis of cytokines expression following RSV and IAV infection of LLC-Luc cells was done by using TaqMan™ Array Mouse Immune Panel (Applied Biosystems, Assay ID 4367786) and Applied Biosystems 7900HT Fast Real-Time PCR System.

**Table 11: List of PCR probes used in the in-vitro experiments**

Protein Name	Gene Name	TaqMan Assay ID	Dye	Source (company)
GAPDH	Gapdh	Mm99999915_g1	FAM-MGB	Thermofisher
HPRT	Hprt	Mm03024075_m1	FAM-MGB	Thermofisher

<b>TLR7</b>	Tlr7	Mm00446590_m1	FAM-MGB	Thermofisher
<b>TLR3</b>	Tlr3	Mm01207404_m1	FAM-MGB	Thermofisher
<b>MDA5</b>	Ifih1	Mm00459183_m1	FAM-MGB	Thermofisher
<b>RIG-I</b>	Ddx58	Mm01216853_m1	FAM-MGB	Thermofisher
<b>NLRP3</b>	Nlrp3	Mm00840904_m1	FAM-MGB	Thermofisher
<b>PDL1</b>	Cd274	Mm03048248_m1	FAM-MGB	Thermofisher
<b>Cdh1</b>	Cadherin 1 (E-cadherin)	Mm01247357_m1	FAM-MGB	Thermofisher
<b>Cdh2</b>	Cadherin 2 (N-cadherin)	Mm01162497_m1	FAM-MGB	Thermofisher
<b>Snai1</b>	Snai1	Mm00441533_g1	FAM-MGB	Thermofisher
<b>Snai2</b>	Snai2	Mm00441531_m1	FAM-MGB	Thermofisher
<b>Vim</b>	Vimentin	Mm01333430_m1	FAM-MGB	Thermofisher
<b>Krt7</b>	Keratin 7	Mm00466676_m1	FAM-MGB	Thermofisher
<b>Twist1</b>	M-Twist	Mm00442036_m1	FAM-MGB	Thermofisher
<b>Vegf</b>	VEGF-A	Mm00437306_m1	FAM-MGB	Thermofisher
<b>Pdgf b</b>	PDGF B	Mm00440677_m1	FAM-MGB	Thermofisher
<b>Fgf2</b>	Basic FGF	Mm01285715_m1	FAM-MGB	Thermofisher

#### 4.3. Type-I IFNs detection by ELISA

Following RSV infection of LLC-Luc and KP-Dpy cells, the supernatants were collected at different time points for type-I IFNs titration. The supernatants were stored at  $-80^{\circ}\text{C}$  until the day of performing the Elisa test.

IFN- $\alpha$  and IFN- $\beta$  production was quantified by using mouse IFN beta ELISA Kit (Abcam, ab252363) and mouse IFN alpha 1 ELISA Kit (Abcam, ab252352), as recommended by the manufacturer guidelines.

## **5. In vivo study**

LLC-Luc cancer cells ( $3 \times 10^5$  in 30 $\mu$ l PBS/injection), infected by RSV or not, were injected intratracheally in the recipient mice. Mice were anesthetized by Ketamin+Xylysine before the injection.

### **5.1. Tumor growth and metastasis**

Tumor growth in the lungs of mice was followed by an IVIS imaging system (Lumina II). Mice were injected (i.p.) by 225mg/kg body weight D-luciférine K+ (Interchim), then were anesthetized by isofluran, 10 minutes before imaging. Mice were euthanized between days 15 and 20. The lungs were harvested and washed with 1X PBS.

### **5.2. Preparation of single cell suspensions and flow cytometry analyzes**

Mouse lungs were harvested in RPMI and digested with the Miltenyi tumor dissociation kit (ref. 130-096-730) according to manufacturer's instructions using the gentleMACS™ Octo Dissociator with heaters following the 37C\_m\_TDK\_2 program. After digestion, pieces were manually filtered through a 100  $\mu$ m filter with the help of a syringe head. Enzymatic digestion was halted with 10% fetal calf serum followed by 2 washes with PBS. Approximately 2 million cells were transferred into conical wells in a 96 well plate for flow cytometry staining. Fc receptors were blocked with anti-CD16/CD32 antibody for 30 mins at 4°C. Cells were washed with FACs buffer (PBS containing 2 mM EDTA) followed with a staining of cell surface proteins by LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (Life Technologies) and specific antibody staining in 20  $\mu$ l of FACs buffer for 30 minutes at 4°C. All antibodies used are detailed in (**Table 12**). After staining, cells were washed 3 times with PBS, fixed by fixation/permabilisation solution (eBiosciences), as recommended by the manufacturer, and staining was analysed by using a BD Fortessa X20 flow cytometer. Results were plotted and analysed using the FlowJo 10 software.



**Table 12:** List of antibodies used for flow cytometry in in-vivo experiments

Target protein	Fluorochrome	Clone	Source (company)	Reference
Influenza A Virus Nucleoprotein	FITC	D67J	Abcam	ab210526
Mouse MHC Class-I (H-2Kb)	PerCP-eFluor 710	AF6-88.5.5.3	eBioscience	46-5958-82
Mouse CD45	PE	30F11	BD Pharmingen	553081
Mouse CD45	PE-Cy7	30F11	BD Pharmingen	552848
Mouse CD3	Alexa Fluor 700	500A2	BD Pharmingen	557984
Mouse CD3	APC-eflour780	500A2	eBioscience	47-0033-82
Mouse CD3	FITC	145-2C11	BD Pharmingen	553062
Mouse CD4	PE-TexasRed	RM4-5	Invitrogen	MCD0417
Mouse CD8a	PE	53-6.7	BD Pharmingen	553032
Mouse CD8a	FITC	53-6.7	BD Pharmingen	553031
Mouse CD69	BV650	H1.2F3	Biolegend	104541
Mouse PD1	Percp-eflour710	J43	eBioscience	46-9985-82
Mouse Lag3 (CD223)	eF450	C9B7W	eBioscience	48-2231-82
Mouse TIGIT	BV650	GIGD7	eBioscience	12-9501-

				82
<b>Mouse CD39</b>	APC	Duha59	Biolegend	143810
<b>Mouse CD103</b>	Alexa Fluor	2E7	Biolegend	121442
<b>Mouse CD44</b>	APC-eflour780	IM7	eBioscience	47-0441-82
<b>Mouse CD62L</b>	APC	MEL-14	eBioscience	17-0621-82
<b>Mouse CD127</b>	Brilliant Violet 785	A7R34	Biolegend	135037
<b>Mouse CD25</b>	PE	3C7	BD Pharmingen	553075
<b>Mouse CD64</b>	Brilliant Violet 605	X54-5/7.1	Biolegend	139323
<b>Mouse IA/IE</b>	Brilliant Violet 650	M5/114.15.2	BD Horizon	563415
<b>Mouse CD11b</b>	Brilliant Violet 785	M1/70	Biolegend	101243
<b>Mouse CD11c</b>	APC	N418	eBioscience	17-0114-81
<b>Mouse Ly6G (Gr-1)</b>	APC-eFluor 780	RB6-8C5	eBioscience	47-5931-82
<b>Mouse SiglecF</b>	PE-eflour594	E50-2440	BD Horizon	562757
<b>Mouse CD24</b>	PE-Cy7	M1/69	Biolegend	101822
<b>Mouse CD274 (PD-L1)</b>	Brilliant Violet	MIH5	BD Horizon	564716

	421			
<b>Mouse NK1.1</b>	Brilliant Violet 605	PK136	BD Horizon	563220
<b>Mouse CD19</b>	APC-Cy7	1D3	BD Pharmingen	557655
<b>Mouse CD38</b>	APC	90	eBioscience	17-0381- 81
<b>Mouse IgM</b>	PE-CF594	R6-60.2	BD Horizon	56-25-65
<b>Mouse CD49b</b>	Percp- eflour710	DX5	eBioscience	46-5971- 82
<b>Mouse IgD</b>	PE/Dazzle 594	11-26c.2a	Biolegend	405742
<b>Mouse anti-CD16/CD32 (Fc Block)</b>	Purified	2.4G2	BD Pharmingen	553141
<b>Mouse MHC Class-I (H- 2Kb)</b>	PerCP-eFluor 710	AF6-88.5.5.3	eBioscience	46-5958- 82

## 6. Human tumors

A prospective cohort of 11 NSCLC tumors of stage I-III patients who underwent primary surgery and who were operated between 2018 and 2020 was obtained from Cochin hospital (Paris, France). Human tumors and adjacent tissues were cut into small pieces (~ 2 mm diameter), and distributed equally in 12-well plate in 1,5ml of RPMI-1640 Medium with 10% heat-inactivated FCS and 1% penicilin/streptomycin mixture. Human tumors and adjacent tissues were infected by  $10^6$ pfu of RSV virus or not for 48h.

At 48h pi, tumor and adjacent tissues were washed with PBS followed by overnight incubation with 4% paraformaldehyde at room temperature before transferring them into 70% ethanol for 24h. Then, tissue was embedded in paraffin using Leica ASP300/Histocore Arcadia H

instruments and 4  $\mu\text{m}$  sections were cut. Sections were warmed overnight at 56°C, deparaffinized, rehydrated. For hematoxylin and eosin staining, slides were sequentially stained with Dako hematoxylin solution (Agilent; CS70030-2) and an aqueous eosin Y solution 0,5% containing glacial acetic acid (Millipore; 1.09844.1000). After clarification with xylene, slides were mounted using the Dako Toluene-Free Mounting Medium (Dako CS705). Stained sections were scanned using a nanozoomer (Hamamastu manufacturer) and analyzed with NDP.view2 viewing software.

For immunofluorescence staining, slides were warmed overnight at 56°C and then at 97°C for 30 min at pH9 using LT-Link instrument. After washing, slides were incubated in 3% hydrogen peroxide for 15 min and then blocked in 5%BSA, Triton 100X at 0,1% and protein block (Dako, X0909). Primary antibody staining was performed using a primary anti-CD45 antibody (clone EP322Y, Abcam ab40763 at 1/100), a anti-panCK antibody (clone AE1/AE3 + 5D3 at 1/50) or an Anti-Respiratory Syncytial Virus antibody (Clone 2F7, Abcam ab43812, at 1/200) for 15 min at room temperature followed by Opal HRP polymer Ms+Rb for 10 min at room temperature for the RSV and panCK primary antibody. The anti-CD45 antibody was revealed with ImmPRESS® HRP Goat Anti-Rat IgG Polymer Detection Kit (MP-7404) used at room temperature for 10 min and Opal 520. The anti-RSV antibody was detected with Opal 690 and panCK with Opal 780 (Akoya, PerkinElmer). All sections were stained with DAPI (1 $\mu\text{g}/\text{mL}$ ) and mounted in Prolong Diamond anti-fade mounting (Life Invitrogen). Slides were scanned using Axioscan (Zeiss) and the analysis was done using Halo software.

### **Statistical analysis**

Statistical analyses for in-vitro and in-vivo experiments were performed by GraphPad Prism 5 (GraphPad Software). Significance threshold for p values was set to 0.05. One-way ANOVA method, with Post hoc analyses by Bonferroni corrections were applied.

# Results

## Part I: Impact of RSV infection on NSCLC progression and tumor cell hallmarks

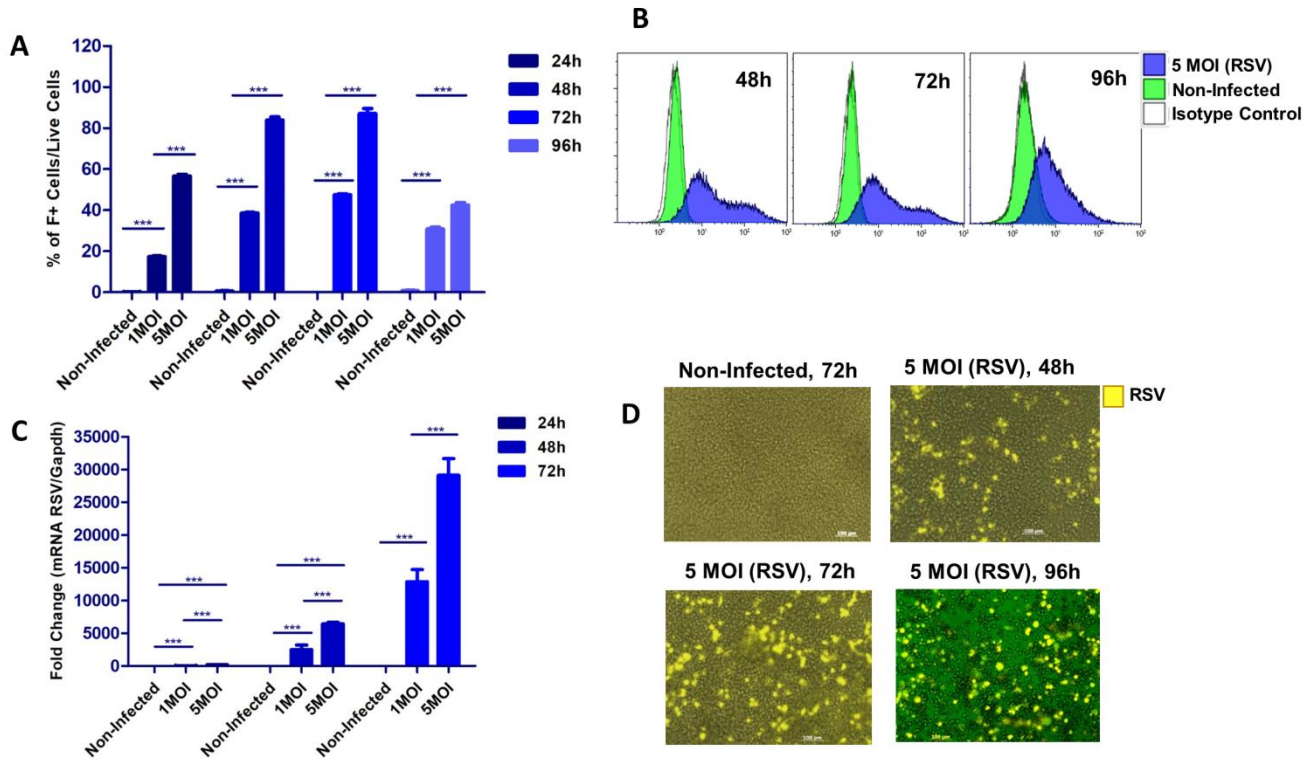
The impact of RSV infection on NSCLC progression can be mediated by directly affecting the tumor cells or by modifying the TME or both. The direct impact of RSV infection on NSCLC tumor cells can be envisaged if they could be infected by RSV. So, we first investigated whether NSCLC cell lines and resected human tumors can be infected by RSV. Later, we studied the impact of RSV infection of NSCLC cell lines on cancer hallmarks, such as inducing inflammation, cell proliferation, cell death, angiogenesis, and EMT.

As it's not possible to investigate the impact of RSV on the progression of human tumors, we investigated this impact on the progression of a model of orthotopic murine NSCLC tumors and the tumor immune infiltrate.

### 1. RSV infects tumor cells

In order to study the direct impact of RSV infection on tumor cells, we first need to investigate whether NSCLC cells are permissive to RSV. We infected human and murine lung tumor cells lines with increasing multiplicity of infection (MOI) of mCherry-RSV and quantified the level of infection by different assays. We found that RSV can productively infect multiple murine NSCLC cell lines. Indeed, RSV can infect murine NSCLC cell lines: LLC (**Fig. 10**), KP (**Fig. 11A, B, D**) and LKR-Luc (**Fig. 11C, D**), TC1 cells (**Fig.11 D**) as well as human A549 cells (**Fig. 11D**). RSV infects LLC in a time and dose-dependent manner; as RSV fusion protein was detected by flow cytometry in about 20% and 40% of live LLC cells, in the 1 MOI RSV condition, compared with about 60% and 90% of live cells in the 5 MOI RSV condition, at 24h and 48h pi respectively (**Fig.10A**). Similarly, the expression of RSV nucleocapsid mRNA in 1 and 5 MOI samples increases by time and dose during the first 72h pi (**Fig.10C**). Of note, the level of expression of F (RSV) protein by infected LLC cells is heterogenous, as infected cells express different amounts of F protein at 48 and 72h pi (**Fig. 10B**).

At 72h pi, we detected the maximum of infection on protein and mRNA levels (**Fig.10A, C, D**), as about 50% and 95% of LLC cells express RSV-F protein in 1 and 5 MOI RSV (**Fig.10A**). At 96h, the infection rate reduces, reflecting the role of anti-viral mechanisms in controlling the infection (**Fig.10A, D**), as only about 40% of cells express F (RSV) protein in the 5MOI sample (**Fig.10A**). At 48, 72 pi, LLC cells don't show important cellular cytopathic changes, except of the formation of some syncytia that increases at 72h, compared with 48h pi (**Fig. 10D**). However, the presence of detached cells (dead cells), notably among mCherry expressing cells, was observed at 96h pi, in the 5MOI RSV sample (**Fig. 10D**).

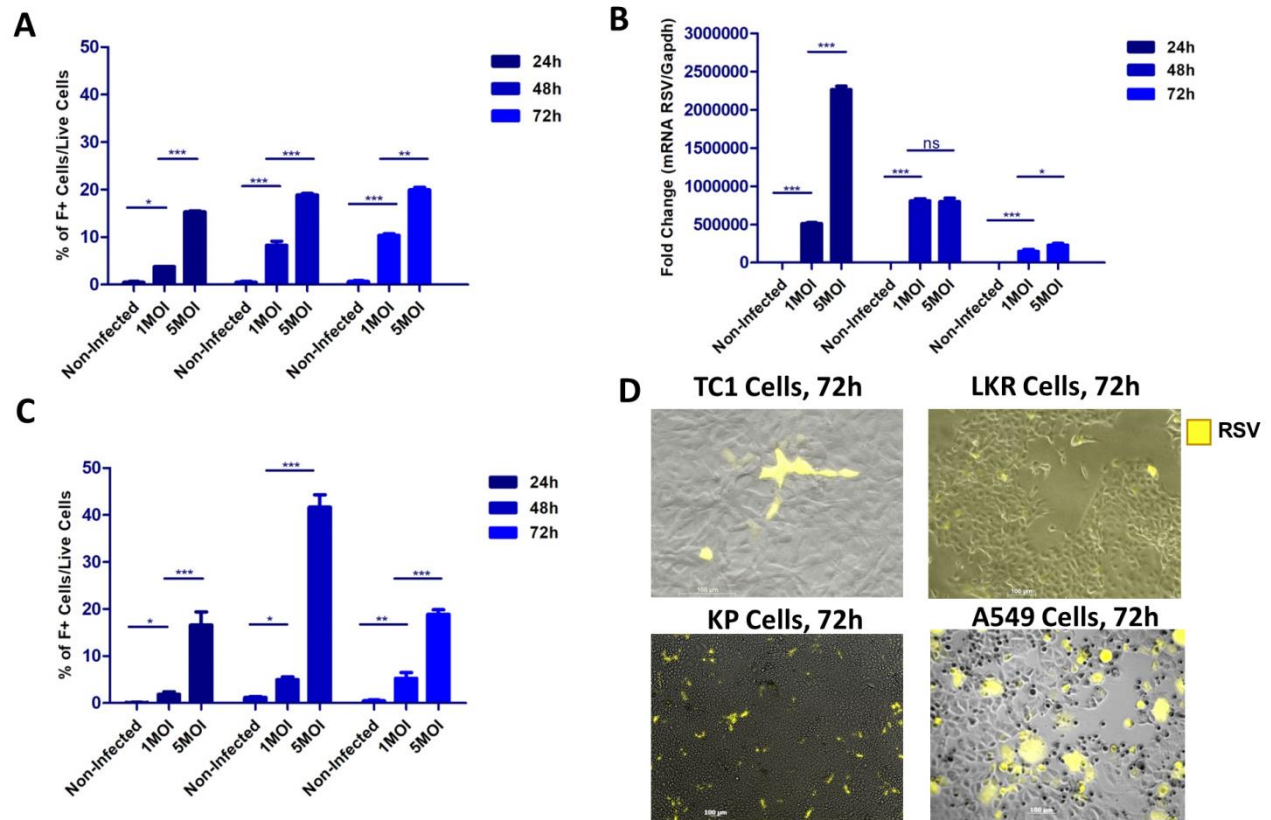


**Figure 10 | RSV infects LLC-Luc cells in a time and dose dependent way by different assays. LLC-Luc cells were infected by 1 and 5MOI mCherry-RSV and the infection was assayed by immunofluorescence microscopy, flow cytometry and RT-qPCR analysis at different time points 24, 48, 72h and 96h pi A):** Percentage of infected LLC cells (F+) by 1 and 5 MOI, measured by flow cytometry. **B):** Histograms show the infection of LLC cells, infected by 5MOI RSV, at 48, 72 and 96h pi, compared with non-infected sample and isotype control. **C):** Expression of RSV nucleocapsid gene on mRNA level by RT-qPCR. Results are shown as fold change compared with non-infected sample. GAPDH is used as housekeeping gene. **D)** Infection of LLC cells by 5 MOI mCherry-RSV is confirmed through the detection of mCherry by immunofluorescence microscope at 48, 72 and 96h pi., compared with non-infected sample. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.0001$ . Each experiment was repeated three times at least.

In addition, mCherry-RSV infects KP-Luc cells (**Fig. 11A, 2B, 2D**); as 5% and 15% of KP cells were infected by 1 and 5 MOI at 24h pi, compared with about 10% and 20% at 48h pi, which show dose-dependent effect. However, the rate of infection is lower than that observed with LLC cells, which reflects a better and earlier anti-viral response in KP cells. This observation was confirmed on viral mRNA level, as mRNA expression of RSV nucleocapsid gene is reduced over time after 24h pi (**Fig. 11B**), contrary to what we observed with LLC cells (**Fig.10 C**). In addition, mCherry-RSV infection of LKR-Luc cells follows similar pattern (**Fig. 11 C, D**); as about 2% and 18% of LKR cells were infected by 1 and 5 MOI at 24h pi, compared with about 5% and 40% at

48h pi. At 72h pi, the rate of infection by 5MOI RSV is reduced to about 20% of LKR cells, which show earlier anti-viral response in LKR cells, as compared with LLC cells.

Moreover, we also found that murine TC1 cells and human A549 cells can be infected (**Fig. 11D**). Infection of A549 cells results in cytopathic changes, including remarkable multiple syncytia formation and cell death (**Fig. 11D**).



**Figure 11 | RSV infects other NSCLC cells in a time and dose dependent way by different assays. KP-Luc and LKR-Luc cells were infected by 1 and 5MOI mCherry-RSV and the infection was assayed by immunofluorescence microscopy, flow cytometry and RT-qPCR analysis at different time points 24, 48, 72h pi** **A)** Percentage of infected KP cells (F+) by 1 and 5 MOI, measured by flow cytometry. **B)** Expression of RSV nucleocapsid gene on mRNA level by RT-qPCR in KP cells, infected or not. Results are shown as fold change compared with non-infected sample. GAPDH is used as housekeeping gene. **D)** Infection of TC1, LKR, KP and A549 cells by 1 MOI mCherry-RSV is confirmed through the detection of mCherry by immunofluorescence microscope at 72 pi, compared with non-infected sample. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.0001$ . Each experiment was repeated three times at least.

**These results confirm that murine and human NSCLC cells can be infected by RSV in a dose and time dependent manner; however the rate of infection and the velocity to control viral**

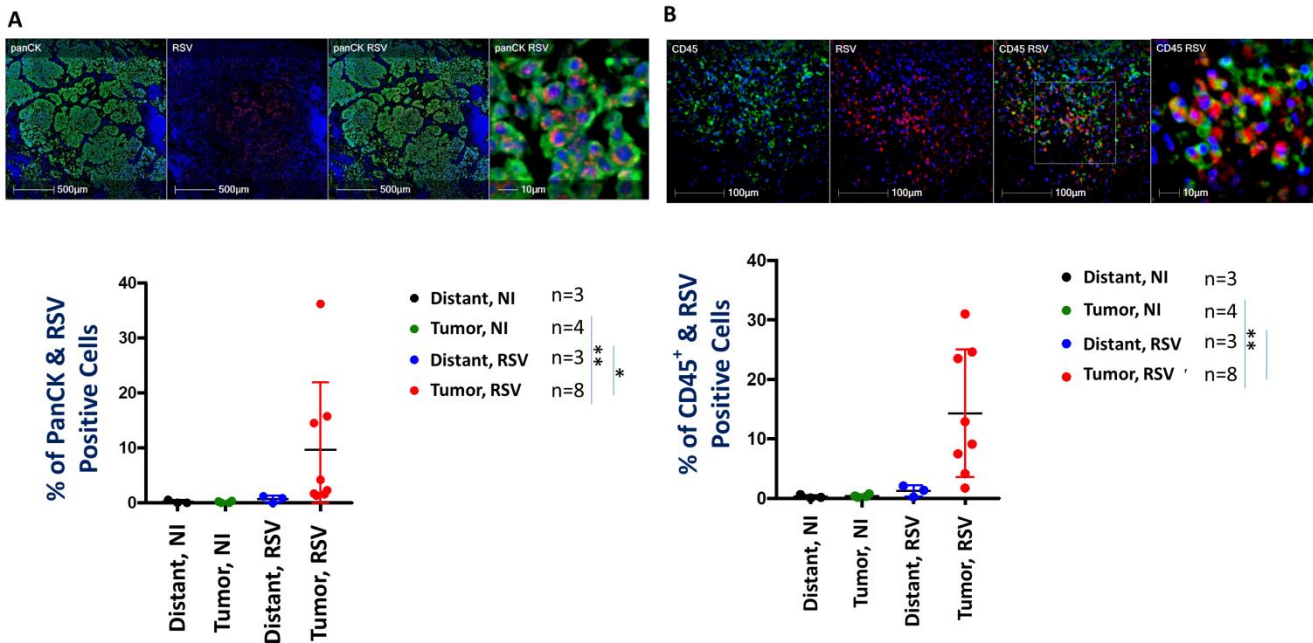


**replication depends on the cell type.** Despite being a virus that targets human, our results show that murine NSCLC cells are susceptible to RSV infection, and thus can be used as a model to study the impact of RSV infection on NSCLC.

Finally, we incubated pieces of resected human NSCLC and distant tissues ex-vivo with  $10^6$  pfu mCherry-RSV for 48h (**Fig 12**). We found that about 10% of the cells of epithelial origin (Pancytokeratin positive) (supposedly mostly tumor cells) in the resected tumor tissues are infected by RSV, whereas very low percentage (1-2%) of epithelial cells in the distant tissues (stressed normal cells) is infected (**Fig 12A**). This may suggest that either human tumor cells are more susceptible to RSV infection than normal respiratory epithelial cells, or that the high density of cells in the tumor tissue facilitates RSV dissemination, compared with low cellular density in the distant tissues. Of note, in this model, we hypothesize that the cells that are located in the core of each piece of tumor and distant tissue are less accessible to RSV infection, as compared to the cells located in the periphery. Also, as it's not possible to count the cells in each tumor and distant tissues before infecting them with the virus, it's impossible to determine the specific MOI used in the infection, however, we hypothesize that  $10^6$  pfu of RSV leads to a low MOI infection.

In addition, in the tumor tissues, about 15% of immune cells were infected by RSV, while about only 2% of immune cells in the distant tissues were infected (**Fig 12B**). This discrepancy may be due to the presence of denser immune infiltrate in the tumor tissues, in addition to the dissemination of different phenotypic immune cell subtypes that could be more susceptible to RSV infection than that in the distant tissues.

Altogether, we conclude that RSV productively infects murine and human NSCLCs cells, in addition to human NSCLC tumor tissues and consequently may directly impact cancer hallmarks, such as tumor cell proliferation, evasion of apoptosis, induction of inflammation, Immune escape mechanisms, EMT and angiogenesis, as well as the tumor infiltrate and the TME.



**Figure 12 | RSV infects human NSCLC tumors.** Resected human NSCLC tumors were cut into small pieces and incubated by  $10^6$  pfu RSV for 48h. Then, tumors were fixed, put in paraffin and stained with anti-PanCytokeratin, anti-CD45 and anti-RSV antibodies, and assayed by immunofluorescence microscopy and quantified by Halo **A**): Representative photos of resected human NSCLC tumors, stained by anti-PanCytokeratin and anti-RSV antibodies (Up). Percentage of RSV-infected cytokeratin double positive cells in the distant tissues (Distant RSV) and tumor tissues (Tumor RSV), as compared with non-infected distant tissues (Distant NI) and non-infected tumor tissues (Tumor NI) as controls (Down). **B**): Representative photos of resected human NSCLC tumors, stained by anti-CD45 and anti-RSV antibodies (Up). Percentage of RSV-infected CD45 double positive cells in the distant tissues (Distant RSV) and tumor tissues (Tumor RSV), compared with non-infected distant tissues (Distant NI) and non-infected tumor tissues (Tumor NI) as controls. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.0001$ . The number of patient samples (n) for each condition is indicated to the right of each figure.

## 2. RSV infection induces late cell death of and inhibits proliferation of LLC cells

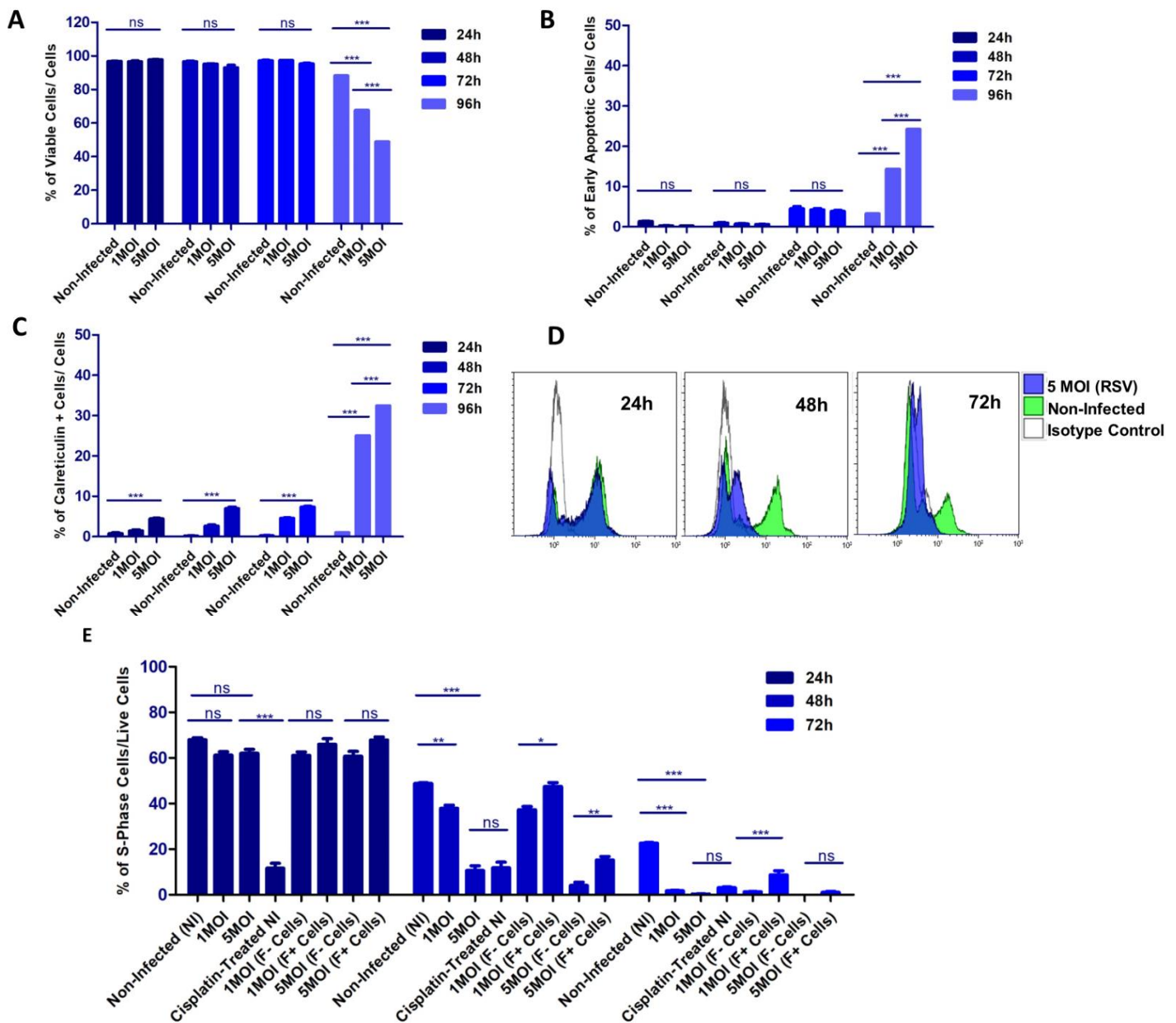
After confirming the productive infectivity of NSCLC cells and human tumors by RSV, we wanted to investigate whether RSV infection lyses NSCLC cells. We found that RSV infection of LLC cells

didn't impact the cell viability during the first 72h pi. However, it remarkably reduces cell viability to about 65% and 50% in the 1 and 5 MOI samples at 96h pi (**Fig. 13 A**). The reduction in cell viability was largely due to apoptosis induction; as 15% and 25% of LLC cells were in early apoptosis state (Annexin-V+, Dapi-) in the 1 and 5 MOI samples at 96h pi (**Fig. 13 B**). The rest of dead cells were in late apoptosis state (Annexin-V+, Dapi+) (results not shown).

As the preapoptotic translocation of calreticulin (CRT) to the cell surface is an indicator of immunogenic cell death and anticancer immune responses (Obeid et al., 2007), we were interested in investigating whether apoptosis is accompanied by an increase in membranous expression of calreticulin. Of note, RSV infection of LLC cells leads to a mild induction of calreticulin surface expression in the first 72h pi (a bit less than 10% in the 5MOI sample), followed by remarkable increase to more than 30% of cells at 96h pi (**Fig. 13 C**). **This indicates that RSV infection of LLC cells induces late immunogenic apoptotic cell death that may induce anticancer immune responses.**

In addition, RSV infection induces remarkable inhibition of LLC cells proliferation at 48h and 72h pi, as compared with non-infected LLC cells. The percentage of LLC cells in the S-phase in the control non-infected cells, which reflects cellular proliferation, decreases over time, from about 60%, to 50% to 25% at 24, 48 and 72h pi (**Fig. 13 D and E**). This inhibition is probably caused by the contact inhibition effect.

The inhibition correlates with RSV dose, as the percentage of LLC cells in the S-phase is about 40% and 10% in the 1 and 5 MOI RSV at 48h pi, as compared with 50% of cells in the non-infected sample, and decreases further to less than 5% at 72h pi (**Fig. 13 D and E**). The inhibition in the cell proliferation in the 5MOI RSV sample is similar to the positive control, the cisplatin-treated non-infected sample at 48 and 72h pi. F- LLC cells (non-infected cells in the infected sample) proliferate a little bit slower than F+ cells (infected cells in the infected sample) at 48h and 72h pi, yet much less than non-infected control. This suggests that the inhibition in cell proliferation is not limited to infected LLC cells, where RSV replicates, and could be mediated by secreted metabolites in the supernatant, as indirect effect of viral replication.



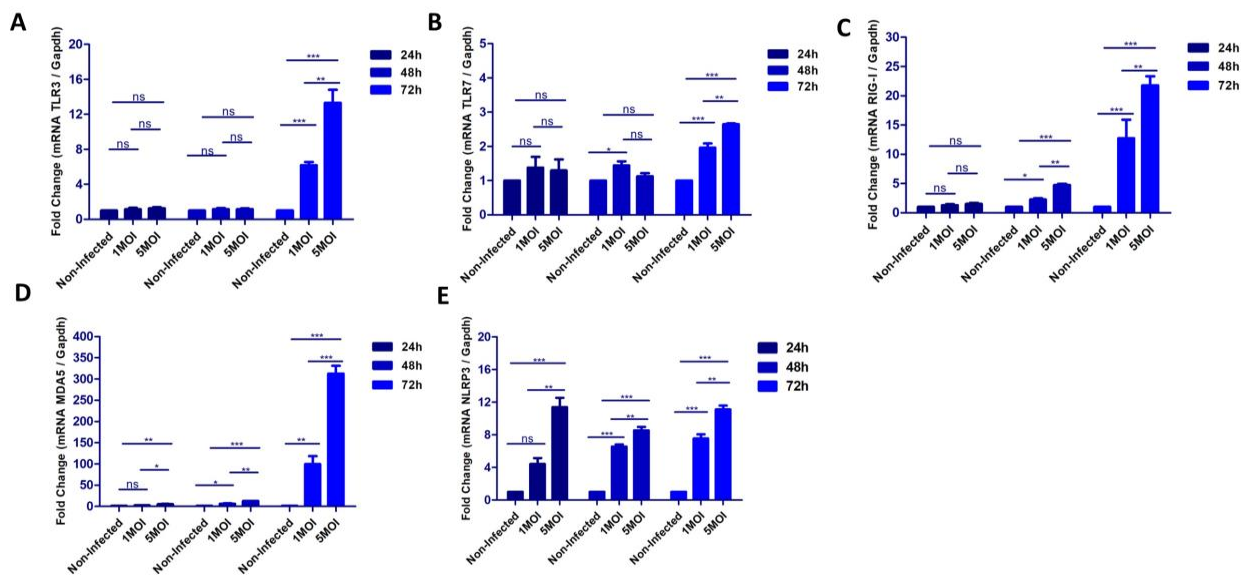
**Figure 13 | Impact of RSV infection on cell viability, apoptosis, calreticulin expression and proliferation of LLC cells.** LLC cells were infected by 1 and 5 MOI RSV, and collected at 24, 48, 72 and 96h pi, for flow cytometry analysis. **A)** Percentage of viable LLC cells, stained by Live/Dead stain **B)** Percentage of early apoptotic LLC cells (Annexin V+ cells). **C)** Percentage of calreticulin expression on cell membranes **D)** Histogram shows LLC cells, infected by 5MOI RSV, vs non-infected and isotype control **E)** LLC cells in the S-phase of cell cycle, which reflects cell proliferation. Cisplatin-treated non-infected cells serve as control positive of cell proliferation inhibition. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.0001$ . Each experiment was repeated three times at least.

### 3. RSV infection induces the upregulation of viral RNA sensors expression

The role of the innate immune receptors in detecting RNA viruses is crucial for the establishment of proper inflammatory and antiviral responses, which can impact cancer hallmarks. These receptors, known also as viral RNA sensors, have the capacity to sense the presence of RNA as PAMPs, which leads to the induction of type I IFNs as well as inflammatory cytokines and chemokines, which also results in the upregulation of the expression of these sensors. Thus, investigating the expression of these sensors serves as an indicator of the activation of these signaling pathways and reflects the importance of individual sensors in viral sensing.

RSV infection of different NSCLC cell lines results in the upregulation of all RNA viral sensors, including TLR3, TLR7, RIG-I, MDA5 and NLRP3, in a time and dose dependent manner 5 (**Fig. 14**).

Of note, RSV infection of LLC cells leads to a rapid upregulation of NLRP3 on mRNA level at 24h pi, while upregulation of all other sensors starts at 48h pi and maximizes at 72h pi. As TLR3 and RIG-I have the highest basal expression among all sensors in LLC cells (data not shown), we

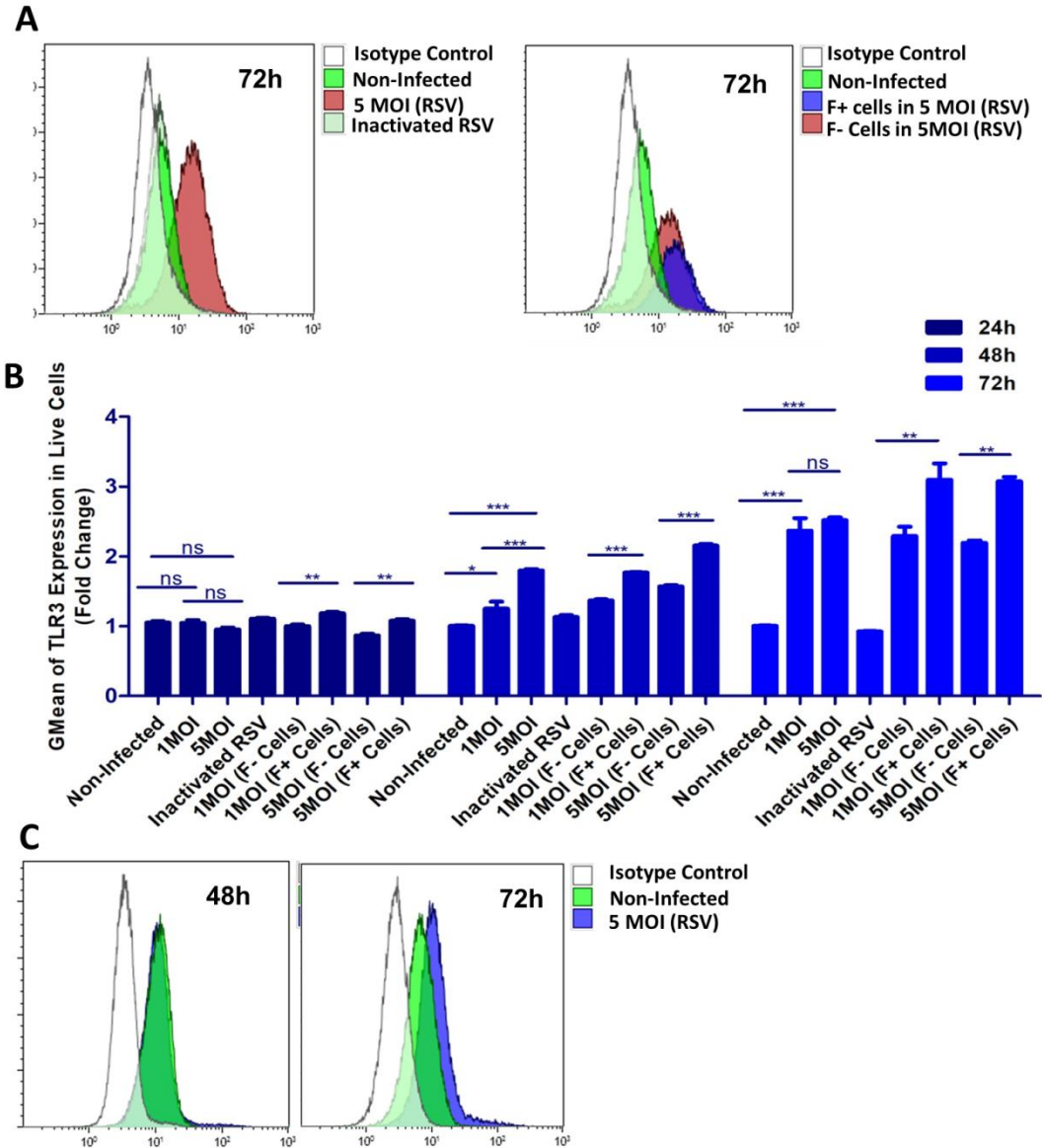


**Figure 14 | Impact of RSV infection of LLC cells on the expression of viral RNA sensors.** LLC cells were infected by 1 and 5 MOI and collected at 24, 48 and 72h pi for RT-qPCR analysis. Results are shown as fold change that relates mRNA sensors to mRNA of housekeeping gene for **A):** TLR3 **,B):** TLR7, **C):** RIG-I, **D):** MDA5, **E):** NLRP3. Gapdh is used as housekeeping gene. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.0001$ . Each experiment was repeated three times at least.

suggest that the upregulation of TLR3 and RIG-I (to about 14 and 22 -fold change) at 72h is the most impacted by RSV infection.

As it was shown that RIG-I, TLR3 and TLR7 expression is mainly mediated by the induction of type I IFN (Hayakari et al., 2013; Miettinen et al., 2001), we hypothesize that the late upregulation of viral sensors is due to the time needed for the production and the accumulation of type I IFNs by tumor cells in the supernatant. Of note, NLRP3 expression is mainly mediated by the activation of NF- $\kappa$ B (He et al., 2016), which explains why NLRP3 is unregulated at early stages of infection (at 24h pi).

We validated the upregulation of TLR3 and MDA5 on protein level, as the intracellular expression of TLR3 receptors is upregulated, as compared to non-infected cells, at 48h pi, and maximizes at 72h pi (about 2.5 fold increase) in a dose and time dependent manner (**Fig 15 A and B**). Inactivated RSV didn't lead to any upregulation in TLR3 expression, which suggests that the active viral replication is indispensable to any upregulation in TLR3 expression. Of note,

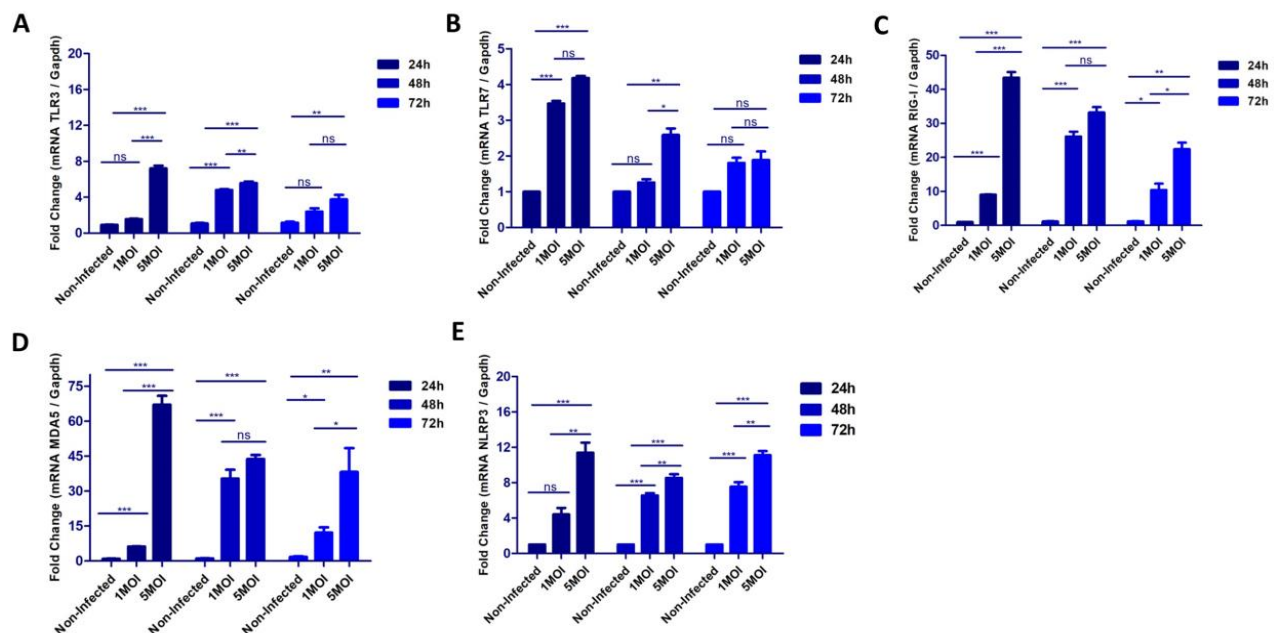


**Figure 15 | Impact of RSV infection of LLC cells on the expression of TLR3 and MDA5 proteins.** LLC cells were infected by 1 and 5 MOIs mCherryRSV and collected at 24, 48 and 72h pi for flow cytometry **A)**: TLR3, **B)**: TLR7, **C)**: RIG-I, **D)**: MDA5, **E)**: NLRP3. Gapdh is used as housekeeping gene. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean ± SEM. ns = not significant, \*: p < 0.05; \*\*: p < 0.01; \*\*\*: p < 0.0001. Each experiment was repeated three times at least.

infected (F+) cells in the 5MOI sample express higher TLR3 than non-infected (F-) cells in the 5MOI RSV sample. In a similar way, yet less evident, infected LLC cells express higher intracellular MDA5, as compared to non-infected sample, at 72h pi (**Fig 15 C**).

Similarly, RSV infection of KP cells enhances the mRNA expression of NLRP3 as soon as 24h pi (**Fig 15 E**). Surprisingly, the mRNA expression of other viral sensors (TLR3, TLR7, RIG-I and MDA5) (**Fig. 16 A, B, C, D**) maximizes at 24h pi, and diminishes at 48 and 72h pi. This different kinetics of viral sensors upregulation, along with lower infection rate in KP cells, as compared to LLC cells (**Fig 11A**) reflects early activation of anti-viral control mechanisms, and possibly early production of type I & III IFNs. As TLR3, RIG-I and MDA5 have the highest basal expression among all sensors in KP cells (data not shown), we suggest that the upregulation of TLR3, RIG-I and MDA5 (to about 8-, 45- and 70-fold change respectively) at 24h is the most impacted by RSV infection in KP cells.

**Altogether, this suggests that RSV infection of LLC cells results in an early upregulation of NLRP3 (probably due to NF- $\kappa$ B activation), and late upregulation of other RNA sensors, TLR3 and RIG-I, probably due to the accumulation of type I and III IFNs secreted by tumor cells in the culture medium.**



**Figure 7 | Impact of RSV infection of KP cells on the expression of viral RNA sensors.** KP cells were infected by 1 and 5 MOIs mCherryRSV and collected at 24, 48 and 72h pi for RT-qPCR analysis. Results are shown as fold change that relates mRNA sensors to mRNA of housekeeping gene for **A):** TLR3, **B):** TLR7, **C):** RIG-I, **D):** MDA5, **E):** NLRP3. Gapdh is used as housekeeping gene. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.0001$ . Each experiment was repeated three times at least.

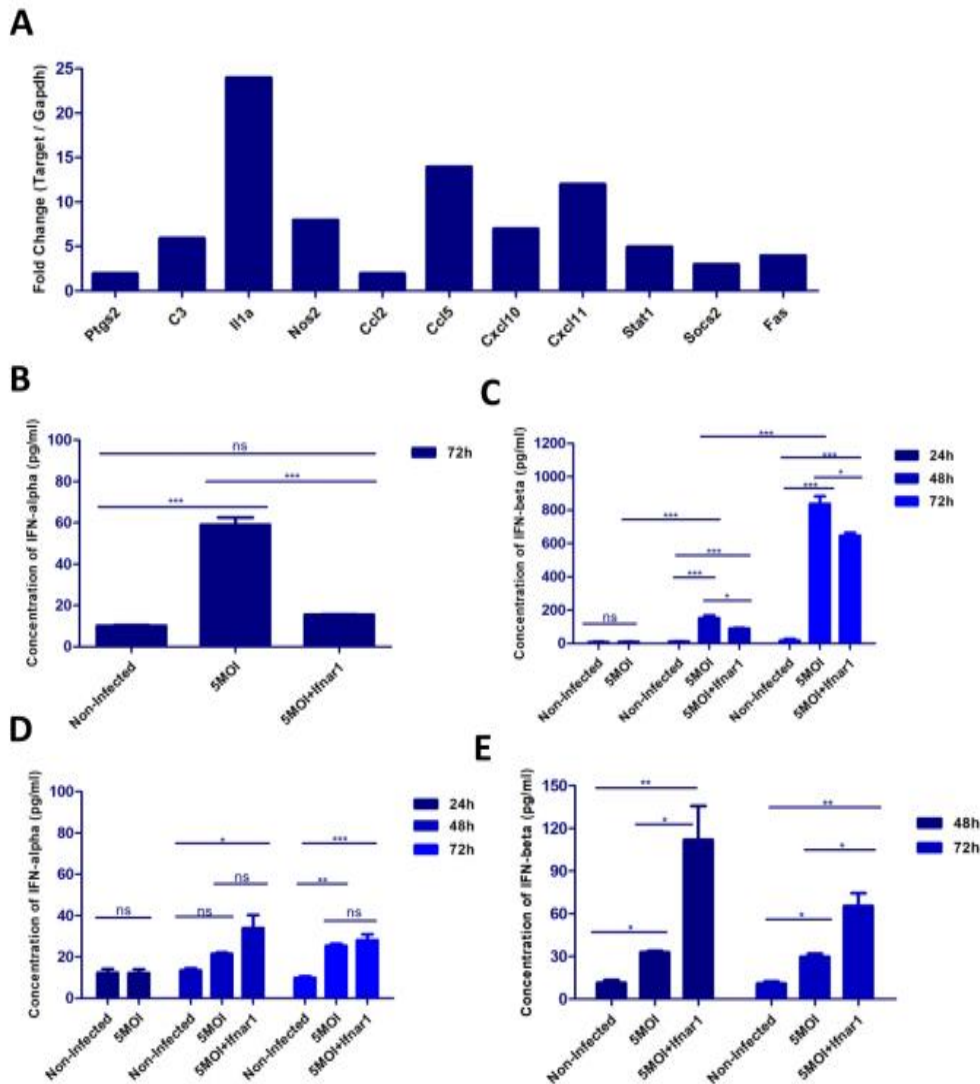


#### **4. RSV infection induces the upregulation of proinflammatory cytokines and chemokines**

As previously discussed in the introduction, NF- $\kappa$ B activation regulates the production of a large variety of proinflammatory cytokines and chemokines.

RSV infection of LLC cells enhances the mRNA expression of several proinflammatory cytokines and chemokines (Fig. 8), mediated by the activation of NF- $\kappa$ B and type I IFNs. Among the most upregulated cytokines are IL-1 $\alpha$ , CXCL11 and CCL5 (more than 10 fold change), in addition CXCL10, STAT1, C3, NOS2, and Fas (more than 5 fold change) (Fig. 8A). CXCL10, CXCL11, CCL5 and CCL2 are implicated in the migration of various leucocytes and the modulation of their phenotype, including T-cells, granulocytes and monocytes.

RSV infection of LLC cells results in the production of type-I IFNs in the culture medium, notably IFN- $\beta$ . The accumulation of type-I IFNs in the supernatant maximizes at 72h pi, as the concentration of IFN- $\alpha$  and IFN- $\beta$  reaches about 60 and 900 pg/ml in the 5MOI RSV sample (Fig. 17B and C). On the other hand, RSV infection of KP cells results in the accumulation of lower quantities of type-I IFNs in the culture medium, as the concentration of IFN- $\alpha$  and IFN- $\beta$  reaches



**Figure 17 | Impact of RSV infection of LLC and KP cells on the expression of several proinflammatory cytokines and chemokines, as well as type-I IFNs.** LLC or KP cells were infected by 5 MOI RSV and collected at 48h pi for RT-qPCR analysis. The supernatant was collected for type-I IFNs titration. RT-qPCR results are shown as fold change that relates mRNA target genes to mRNA of housekeeping gene Gapdh. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.0001$ . Each IFN experiment was repeated three times at least.

about 25 and 30 pg/ml in the 5MOI RSV sample at 72h pi (**Fig. 17D and E**), probably as a consequence of lower infection rate in these cells, as compared with LLC cells (**Fig. 11A**). Blocking IFNAR1 receptors in KP cells results in the accumulation of more quantities of type-I IFNs at 48 and 72h pi, as compared to 5MOI RSV without blocking IFNAR1 receptors. This suggests that type-I IFNs are important to limit viral replication, as infection rate were higher following blocking IFNAR1 receptors (data not shown). In the case of LLC cells, blocking IFNAR1 receptors doesn't lead to an increase in the type-I IFNs, nor lead to an increase in the infection rate (results not shown). This is possibly due to the fact that the majority of LLC cells are infected at 72h, and that type-I IFNs may play an important role in LLC cells in the positive feedback to produce more IFNs.

**To conclude, the sensing of RSV, by several viral RNA sensors in NSCLC cells, enhances the expression and production of several proinflammatory cytokines, chemokines, and type I IFNs, which are involved in inflammation and consequently may modifying the TME.**

#### **5. RSV infection upregulates the expression of MHC-I and Fas in NSCLC cells**

To assess whether RSV infection affects MHC-I surface expression, we infected NSCLC cells with 1 and 5 MOI RSV. Without RSV infection, LLC cells downregulate surface MHC-I during the first 72h of culture, possibly as a mechanism to evade immune recognition and immune destruction by CD8<sup>+</sup> T-cells (Koutsakos et al., 2019). However, at 96h pi, non-infected LLC cells (control) start to express surface MHC-I (**Fig 18 A**), possibly due to the accumulation of basic type-I IFNs. This argument will be enforced later.

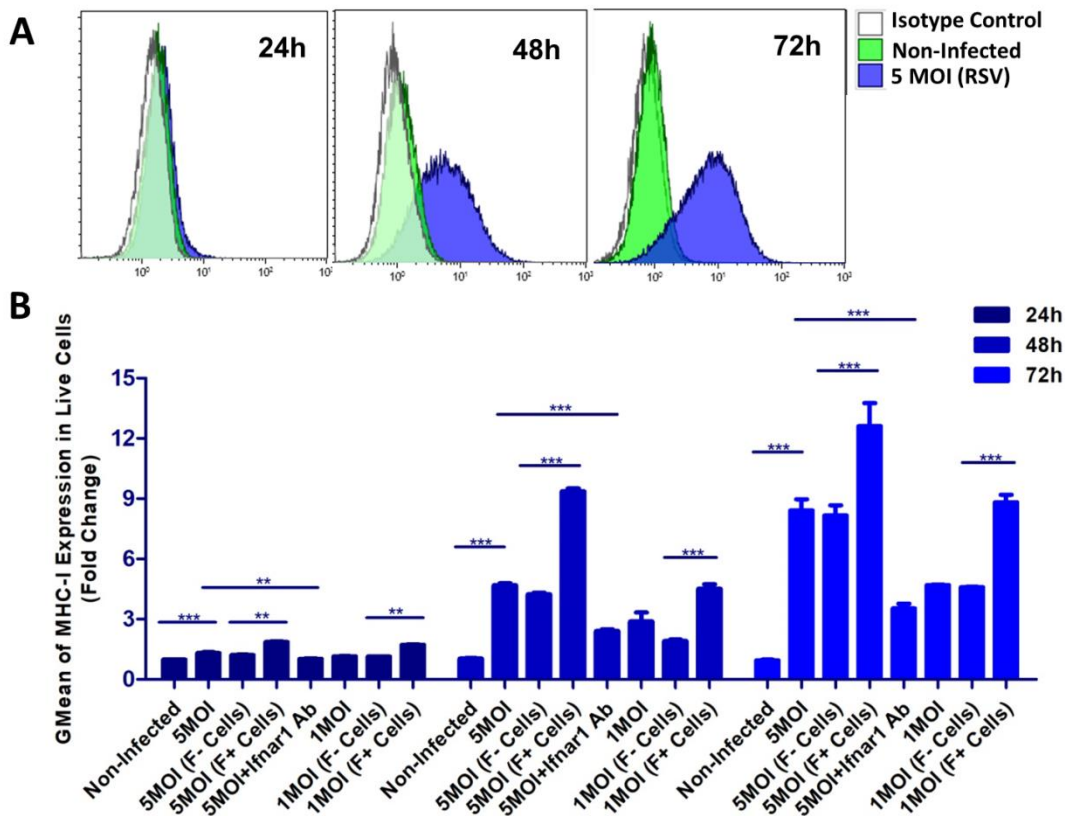
We found that RSV infection upregulates surface MHC-I expression in a time and dose dependent manner. The increase in MHC-I geometric mean fluorescence intensity (MFI) expression starts at 48h pi, and maximizes to 8 and 20 fold increase at 72h and 96h pi, as compared to non-infected condition. Both non-infected (F-) and infected (F+) cells in the 1 and 5MOI samples similarly express high surface MHC-I expression, as compared to non-infected sample (**Fig 18 A and B**). This suggests that viral replication doesn't directly induce MHC-I surface expression, but probably through secreting metabolites. Blocking IFNAR1 receptors by

blocking antibodies largely reverses MHC-I expression (**Fig 18 B**). This proves that MHC-I expression is mediated by type-I IFNs, secreted in the supernatant.

In addition, RSV infection of LLC cells upregulates surface Fas expression at 96h pi; as the geometric mean fluorescence intensity (MFI) of Fas expression is more than 5 fold change, as compared to non-infected cells (**Fig 18 C**). Infected LLC cells (F+) show greater surface Fas expression (more than 15 fold change) than non-infected LLC cells (F-) (about 5 fold change) in the 5MOI RSV, as compared to non-infected sample. Of note, the histogram shows that LLC cells express basal Fas at 96h pi, that is largely augmented by RSV infection (**Fig 18 C**).



compared to non-infected condition. Non-infected (F-) cells in the 1 and 5MOI samples express lower surface MHC-I expression than infected (F+) KP cells, but higher than non-infected sample (Fig 19 A and B). This suggests that viral replication doesn't directly induce MHC-I surface expression, but probably through secreting metabolites. Blocking IFNAR1 receptors by blocking antibodies largely abolished the upregulation of MHC-I expression (Fig 19 B). This proves that MHC-I expression is mediated by type-I IFNs, secreted in the supernatant.



**Figure 19 | Impact of RSV infection of KP cells on the surface expression of MHC-I.** KP cells were infected by 1 and 5 MOI, and collected at 24, 48 and 72 pi for flow cytometry analysis. Results are shown as fold change that relates GMean of membranous MHC-I to the GMean of MHC-I in the non-infected control. **A):** Histogram shows the surface expression of MHC-I in KP cells, infected or not, at different time points, **B):** GMean of MHC-I in KP cells, represented as fold change, as compared to non-infected LLC cells. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0,05$ , \*\*:  $p < 0,01$ , \*\*\*:  $p < 0,0001$ . Each experiment was repeated three times at least.

**To conclude, infection of NSCLC cells leads to the upregulation of MHC-I and Fas molecules at the cell surface of infected cells, as well as non-infected cells, mediated by type-I IFN production.** The upregulation continues until 96h pi, and this may possibly induce a CD8<sup>+</sup> T-cells immune response, that can lyse tumor cells.

#### **6. RSV infection upregulates the expression of PDL1 in infected NSCLC cell lines**

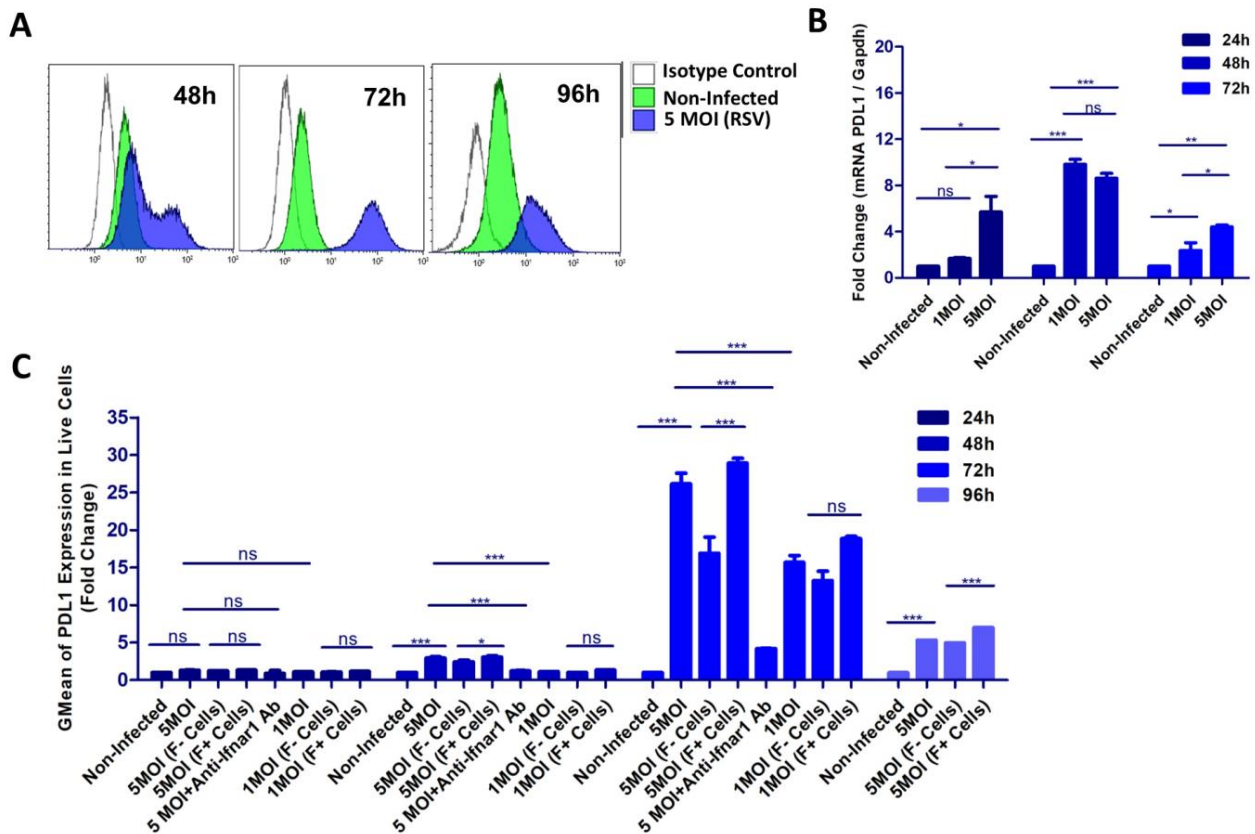
To assess whether RSV infection affects PDL1 cell surface expression, as a mechanism of immune escape, we infected NSCLC cells with mCherry-RSV. LLC and KP cells express basic PDL1 (**Fig 20A, Fig 21A**), as a mechanism to avoid immune destruction by CD8<sup>+</sup> T-cells.

We found that RSV infection of LLC upregulates mRNA levels of PDL1 at 24h and 48h pi (to about 6 and 9 fold change) in the 5MOI RSV, and then reduces to 4 fold change at 72h pi (**Fig 20 B**). The overall surface expression of PDL1 protein increases (to about 2 and 25 fold change) at 48h and 72h pi in the 5MOI RSV (**Fig. 20C**). In addition, the surface expression of PDL1 was lower in the non-infected (F-) cells, as compared to infected cells (F+ cells) in the 1 and 5 MOI RSV, but yet higher than non-infected control, at all time-points. For example, it was about 15 fold change more than PDL1 expression in the non-infected control at 72h pi (**Fig. 20C**). This suggests that viral replication doesn't directly induce PDL1 surface expression, but probably through secreting metabolites. Blocking IFNAR1 receptors by blocking antibodies abolishes PDL1 expression (**Fig 20 C**). This proves that PDL1 expression is mediated by type-I IFNs, secreted in the supernatant.

RSV infection of KP cells upregulates mRNA levels of PDL1 at all time-points and maximizes at 24h pi (to about 30 fold change in the high dose of RSV), followed by a reduction to about 12 and 5 fold change at 48 and 72h pi, and it upregulates the overall surface expression of PDL1 (to about 3 fold change in the high dose of RSV) at 48 and 72h pi (**Fig. 21B and C**). Of note, KP cells don't basally express PDL1 (**Fig 21A**), yet they express PDL1 following 72h of culture, possibly caused by the effect of basal IFN secretion.

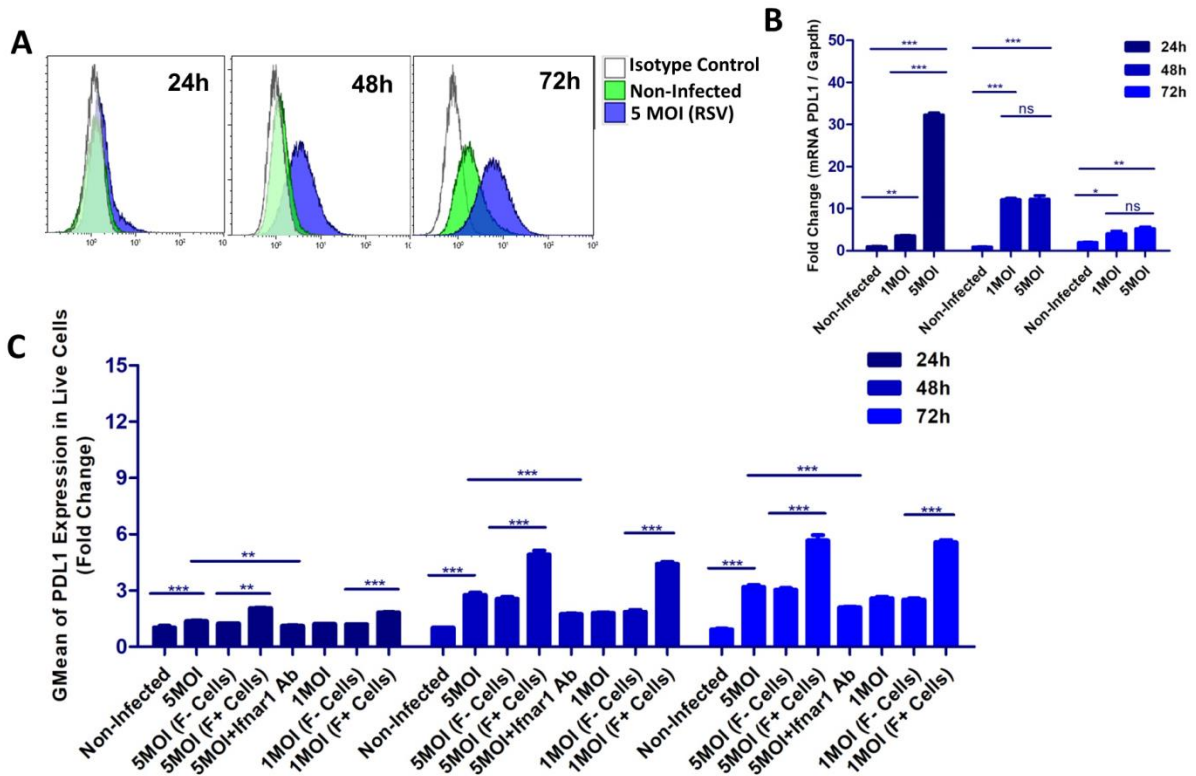
Similarly to LLC, the surface expression of PDL1 was higher in the infected (F+) cells, as compared to non-infected cells (F- cells) or non-infected control, at all time-points, and maximizes at 72h pi (to about 6 fold change in 1 and 5 MOI) (Fig. 21C).

Thus, RSV infection of NSCLC cells leads to a transient upregulation of PDL1, caused by the secretion of type-I IFNs, more remarkable in LLC than KP cells. Unlike the case with MHC-I, PDL1 expression diminishes at 96h pi.



**Figure 11 | Impact of RSV infection of NSCLC cells on the expression of PDL1.** LLC cells were infected 1 and 5 MOI, and collected at 24, 48, 72h and 96h pi for RT-qPCR and flow cytometry analysis. RT-qPCR Results are shown as fold change that relates mRNA PDL1 to mRNA of housekeeping gene Gapdh. Flow cytometry data are shown as fold change that relates the relative geometric mean fluorescence intensity (MFI) levels of surface PDL1 as compared to non-infected control. **A)** Histogram shows the surface expression of PDL1 in LLC cells, infected or not, at different time points **B)** mRNA levels of PDL1 in LLC cells **C)** relative geometric mean fluorescence intensity (MFI) of PDL1 in LLC cells. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0,05$ , \*\*:  $p < 0,01$ , \*\*\*:  $p < 0,0001$ . Each experiment was repeated three times at least.



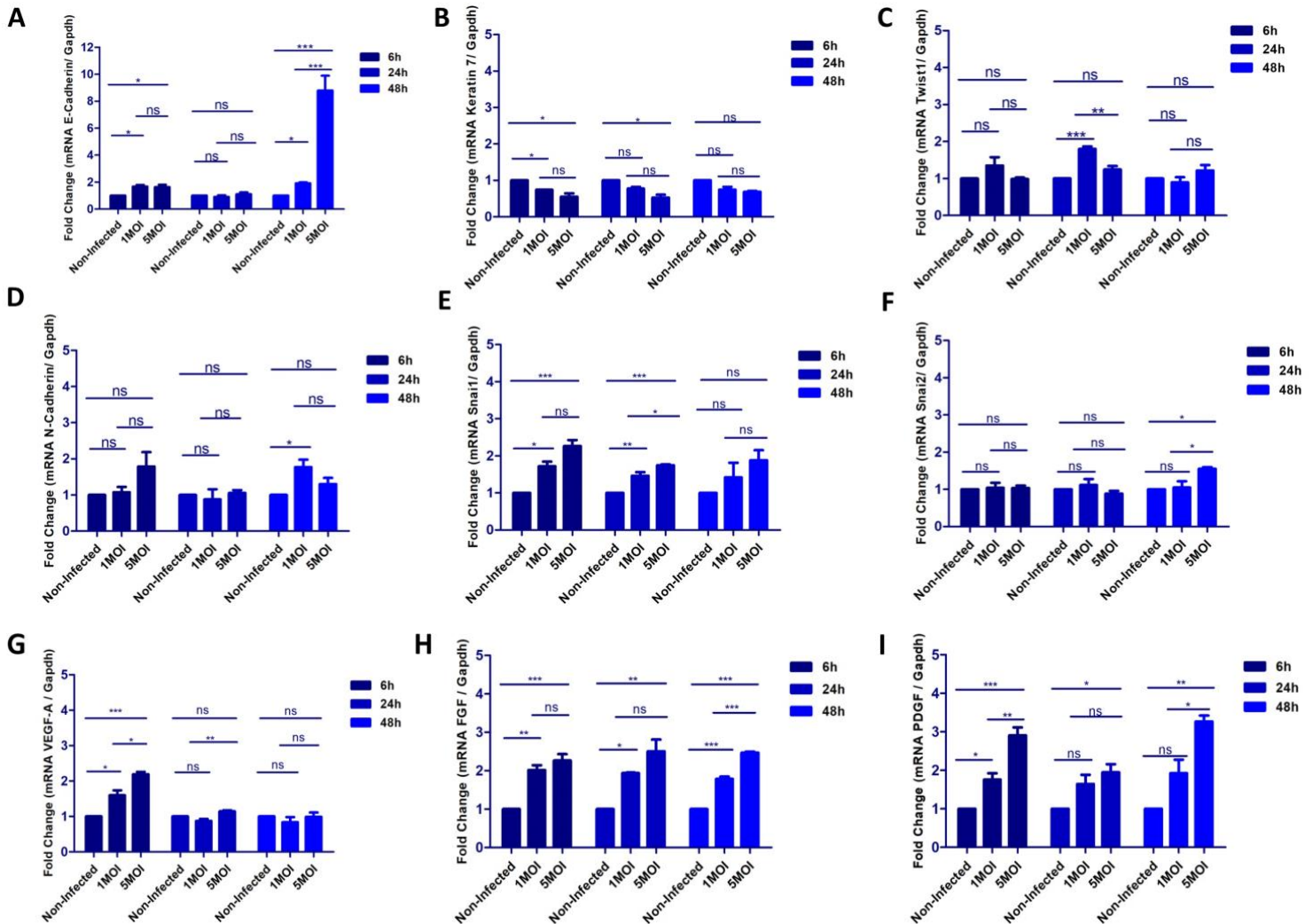


**Figure 12 | Impact of RSV infection of KP cells on the expression of PDL1.** KP cells were infected by 1 and 5 MOI, and collected at 24, 48 and 72h pi for RT-qPCR and flow cytometry analysis. RT-qPCR Results are shown as fold change that relates mRNA PDL1 to mRNA of housekeeping gene Gapdh. Flow cytometry data are shown as fold change that relates the relative geometric mean fluorescence intensity (MFI) levels of surface PDL1 as compared to non-infected control. **A)**: Histogram shows the surface expression of PDL1 in KP cells, infected or not, at different time points **B)**: mRNA levels of PDL1 in KP cells, infected or not **C)**: relative geometric mean fluorescence intensity (MFI) of PDL1 in KP cells. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0,05$ , \*\*:  $p < 0,01$ , \*\*\*:  $p < 0,0001$ . Each experiment was repeated three times at least.

## 7. RSV infection of LLC cells doesn't upregulate EMT markers, but induces some angiogenesis markers

Angiogenesis and EMT are important hallmarks of tumor cells and markers of disease severity and linked to poor outcome in human NSCLC (Iwatsuki et al., 2010). To therefore assessed whether RSV infection affects the expression of EMT and angiogenesis markers. We found that RSV infection of LLC upregulates mRNA levels of epithelial marker, E-Cadherin, at 48h pi (to about 9 fold change). With the exception of Snail1, It doesn't lead to stable upregulation of

mRNA levels of mesenchymal makers (N-Cadherin, twist1, snail2) at 6, 24 and 48h pi (Fig 22 A-F). Altogether, these changes suggest that RSV infection doesn't support the possibility of the occurrence of EMT in LLC cells.



**Figure 22 | Impact of RSV infection of LLC cells on EMT and angiogenesis markers.** LLC cells were infected by 1 and 5 MOI for and collected at 6, 24 and 48h pi for RT-qPCR. RT-qPCR Results are shown as fold change that relates mRNA target to mRNA of housekeeping gene Gapdh. **A):** E-cadherin **B):** Keratin 7 **C):** Twist1, **D):** N-cadherin **E):** Snai 1, **F):** Snai 2, **G):** VEGF-A, **H):** FGF2 (FGF- $\beta$ ), **I):** PDGF-B. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*: p < 0,05, \*\*: p < 0,01, \*\*\*: p < 0,0001. Each experiment was repeated three times at least.

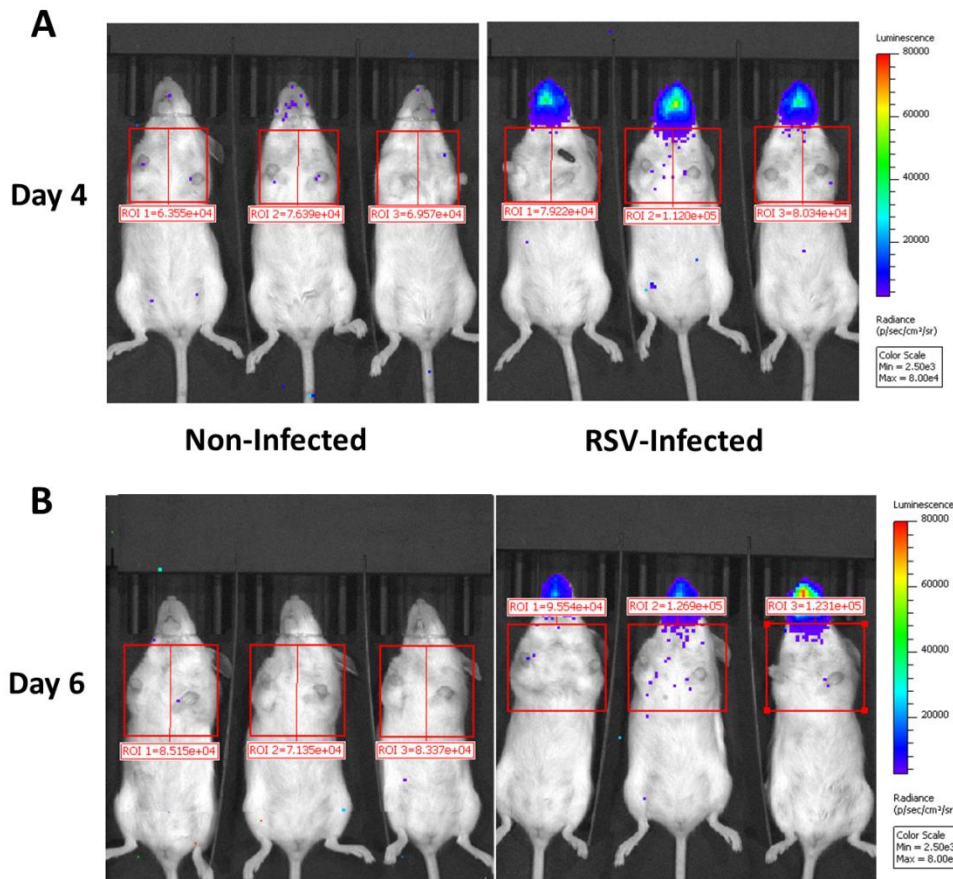
In addition, we found that RSV infection of LLC upregulates mRNA levels of VEGF-A at 6h pi, PDGF-B and FGF2 (FGF- $\beta$ ) at 24 and 48h pi (up to 3 fold change increase) (Fig 22 G-I). This

suggests that RSV infection of LLC cells may enhance the possibility of tumor cells to mediate the formation of new vessels in the TME.

**Altogether, we showed that RSV can infect NSCLC cells and tumors, inhibits cell proliferation and induces late immunogenic cell death, some cytokines, chemokines and type-I interferon production, some angiogenesis markers, in addition to MHC-I and Fas expression, without inducing EMT markers.**

### 8. Optimizing a model of RSV infection and tumor progression in in C57BL/6J mice

Despite their limitations, mice are the most commonly used animal species for in vivo modeling of RSV disease. Most inbred mouse strains are semi-permissive for RSV infection, which explains



**Figure 23 | Optimizing a model of RSV infection and tumor progression in C57BL/6J mice.** 8-12 week-old female C57BL/6J mice were intranasally inoculated by 80,000 pfu of Luc-RSV in 40 $\mu$ l. At day 4 and 6, mice were Intraperitoneally injected by 225mg/kg body weight of D-luciferin, then were anesthetized by isofluran, 10 minutes before imaging by IVIS imaging system. As a control, non-infected mice were inoculated by 40 $\mu$ l of PBS. **A)** representative images of non-infected vs RSV-infected mice at day 4 **B)** representative images of non-infected vs RSV-infected mice at day 6. Each experiment was repeated three times.

why infection should be done by inoculum with a high viral titer (van Erp et al., 2019). At present, BALB/c mice are widely used as animal model for RSV infection, however all commercially available NSCLC cell lines are compatible with C57BL/6J mice, not with BALB/C mice, so we tried to optimize the use of C57BL/6J mice in our model to study the impact of RSV infection on tumor progression.

As intranasal inoculation methods are most common within the RSV field, we inoculated C57BL/6J mice with 80,000pfu of Luc-RSV, recombinant RSV virus that express luciferase, to follow RSV infection. We followed the infection by IVIS system, which detects the bioluminescence produced by Luc-RSV, following luciferin injection. Surprisingly, we found that intranasal RSV inoculation in C57BL/6J mice leads to upper respiratory tract infections only, and spares the lungs (**Fig. 23**). Infection increases until day 6 pi, then starts to decrease and becomes undetectable by IVIS by day 11.

However, to study the impact of RSV infection on NSCLC progression in the lungs, we need RSV virus to replicate in the lungs, not in the upper respiratory tracts, so we decided to change the model, and opted to intratracheally inject LLC-Luc cells, already infected or not, to make sure that RSV replicates in the Tumor and stromal cells that constitute the lung TME.

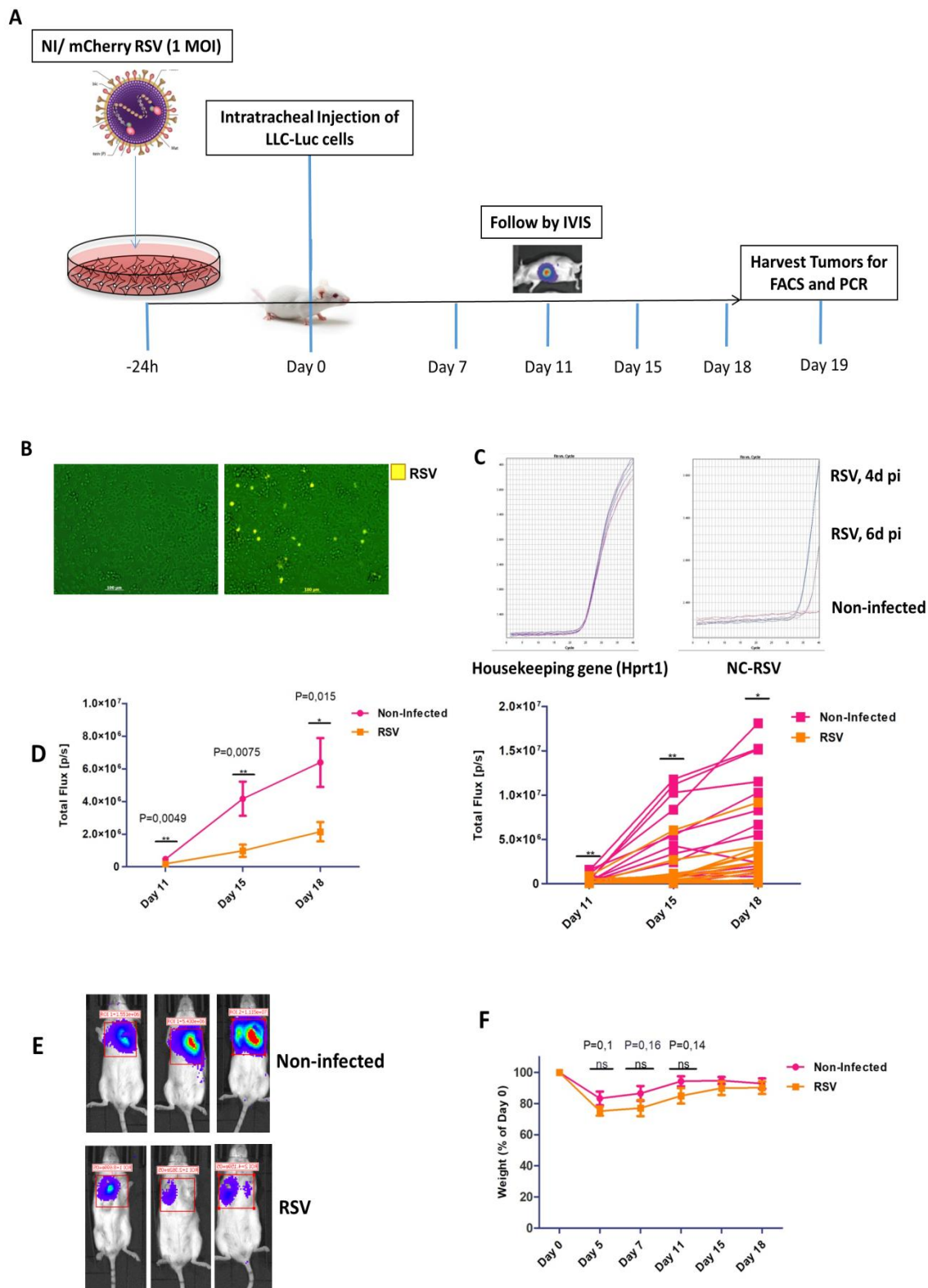
### **9. RSV infection delays tumor progression in in C57BL/6J mice**

To make sure that RSV replicates in the murine lungs, we infected LLC-Luc cells by 1 MOI mCherryRSV for 24h. Non-infected LLC-Luc cells were used as a control (**Fig 24A**). Then, we confirmed RSV infection of LLC-Luc cells by immunofluorescence microscopy, as RSV expresses mCherry (**Fig 24B**). After confirming RSV infection, we intratracheally injected 8-12 week-old female C57BL/6J mice by 300,000 LLC-Luc cells infected by 1MOI of mCherryRSV or non-infected. Tumor progression was followed for 18 days by IVIS, as LLC-Luc cells express luciferase. At day 4 and 6, mice from each group were euthanized, lungs were extracted and digested to get single cell suspension, and we confirmed the replication of RSV in the murine lungs, by RT-qPCR. Hprt1 was used as housekeeping gene (**Fig 24C**).

At day 19, mice were euthanized, lung tumors were extracted and digested to get single cell suspension, in order to characterize the immune infiltrate in infected group vs non-infected group. The protocol of the experiment is detailed in (**Fig 24A**).

We found that RSV infection delays tumor progression; as tumor growth, represented as total flux, was significantly lower in the RSV-infected mice vs non-infected mice (**Fig 24 D and E**). Indeed, the average of the total flux in the RSV-infected group was about  $1 \times 10^6$  and  $2 \times 10^6$  at day 15 and 18, vs  $4 \times 10^6$  and  $6 \times 10^6$  p/s in the non-infected group (**Fig 24 D left**). On individual level, tumor growth in both groups show heterogeneity, probably related to the model used in the experiment (**Fig 24D right**). Both groups of mice lose weight to about 85% after 7 days of IT inoculation, due to the procedure. However, mice recover their weight at day 11 (**Fig 24F**). RSV infection leads to non-significant small loss of weight, as compared to non-infected group, possibly due to the infection (**Fig 24F**).

**To conclude, we found that 1 MOI-RSV infection results in anti-tumoral effect, which could be due to RSV effects on tumor cells and immune cells. We previously showed that RSV inhibits LLC cell proliferation and induces late immunogenic cell death, type-I interferon production, in addition to MHC-I and Fas expression, without inducing EMT markers.** Thus, we need to study the impact of RSV infection on immune cell infiltrate, and to do that, we euthanized mice at day 19 and extracted lung tumor to characterize it.



**Figure 24 | Impact of RSV infection on tumor progression in C57BL/6J mice.** 8-12 week-old female C57BL/6J mice were intratracheally inoculated by 300,000 LLC-Luc cells infected by 1MOI of mCherryRSV, or not, in 40 $\mu$ l of full culture medium. **A)** Schema represents the protocol used in-vivo studies. **B)** At day 0, just before the inoculation, 1MOI mCherryRSV infection of LLC-Luc was confirmed by immunofluorescence microscopy **C)** At day 4 and 6, mice were euthanized, lungs were extracted and digested to get single cell suspension. mRNA was extracted and RT-qPCR was done to confirm RSV infection by amplifying the nucleocapsid gene, as compared to non-infected control **D)** Graph shows tumor progression, detected by IVIS at days 11, 15 and 18 days, presented as total flux (p/s). Left: average of total flux in the non-infected<sup>6</sup> and RSV infected groups. Right: Total flux of all mice in the non-infected and RSV infected groups. **E)** Representative images of mice IVIS at day 15. **F)** Figure shows changes in mice body weight during the experiment, represented as % of mice weight at day 0. T-Test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0,05$ , \*\*:  $p < 0,01$ , \*\*\*:  $p < 0,0001$ . Each experiment was repeated three times.

## 10. RSV infection alters the intratumoral lymphoid immune infiltrates

To study the impact of RSV infection on intratumoral immune cell infiltrate, we euthanized mice at day 19 and extracted lung tumor to determine the immune phenotyping by flow cytometry. We found that RSV infection of LLC tumors doesn't impact the percentages of myeloid compartment cells (**Fig 25 A**), including macrophages, monocytes, DC cells and granulocytes (Data not shown), at this time point (19 days post injection). However, it alters the lymphoid compartment, as it slightly increases the percentage of NK and B cells in RSV-infected LLC tumors (11% and 3%), as compared to non-infected LLC tumors (8% and 2%) (**Fig 25 A**). Although the percentage of T-cells (CD3<sup>+</sup>) didn't change, the percentage of CD8<sup>+</sup> T-cells among all T-cells was higher in RSV-infected tumors (about 50% of all T-cells), as compared to non-infected LLC tumors (40%)(**Fig 25 B**).

It has been demonstrated previously that naïve T cells express a CD62L<sup>hi</sup>CD44<sup>lo</sup> phenotype, whereas memory T cells exhibit a CD62L<sup>lo</sup>CD44<sup>hi</sup> phenotype (Gerberick et al., 1997). So we wanted to determine whether RSV infection of LLC tumors would induce a change in the phenotype of CD4<sup>+</sup> and/or CD8<sup>+</sup> T-cells. We found that RSV-infected LLC tumors have less naïve cells (CD62L<sup>hi</sup> CD44<sup>lo</sup>) (30% vs 35%), higher memory (CD62L<sup>lo</sup> CD44<sup>hi</sup>) (11% vs 8%) CD8<sup>+</sup> T-cells, as compared to non-infected LLC tumors (**Fig 25 C**). Similarly, we found less naïve cells (23% vs 31%), higher memory (CD62L<sup>lo</sup> CD44<sup>hi</sup>) (11% vs 8%) CD4<sup>+</sup> T-cells in RSV-infected tumors, as compared to non-infected LLC tumors (**Fig 25 E**).

Moreover, it was shown that CD8<sup>+</sup> T-cells expressing both CD69 and CD103, within the lung tissue, represent the canonical markers of resident memory CD8<sup>+</sup> T-cells (Schmidt et al., 2018). Resident memory T (TRM) cells are a unique subset of CD8<sup>+</sup> T-cells that are present within lung tissues and do not recirculate through the blood. Long term memory establishment and maintenance are dependent on tissue population of resident memory T cells. They are characterized by dual CD69/CD103 positivity, and play a role in both response to viral infection and local cancer immunosurveillance (Craig et al., 2020).

As a result, we analyzed the expression of CD69 and CD103 on CD8<sup>+</sup> T-cells, and we found that CD8<sup>+</sup> T-cells express higher CD69 and CD103 markers in RSV-infected LLC tumors (6.8%), as compared to non-infected tumors (4.6%) (**Fig 25 C**).

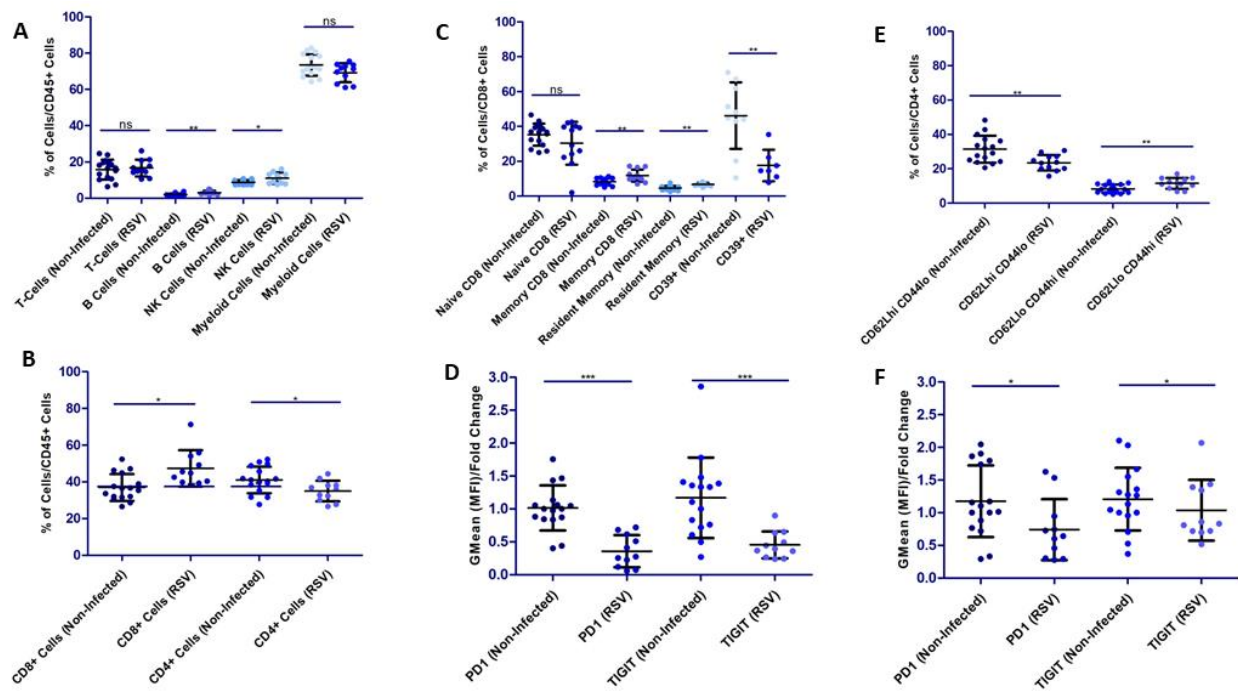
Simoni and his colleagues showed that CD8<sup>+</sup> tumor-infiltrating lymphocytes (TILs) can not only be specific for tumour antigens (for example, neoantigens), but also recognize a wide range of epitopes unrelated to cancer (such as viruses). These bystander CD8<sup>+</sup> TILs have diverse phenotypes that overlap with tumour-specific cells, but lack CD39 expression (Simoni et al., 2018). In addition, CD39+CD8<sup>+</sup> T-cells exhibit distinct phenotypes that represent severe exhaustion and terminal differentiation among tumor-antigen-specific CD8<sup>+</sup> T TILs (Lee et al., 2019).

Thus, we analyzed the expression of CD39 on CD8<sup>+</sup> T-cells, and we found that the percentage of CD39+ CD8<sup>+</sup> cells in RSV-infected tumors express is lower (17%), as compared to non-infected tumors (46%) (**Fig 25 C**). This indicates the possible presence of anti-RSV bystander CD8<sup>+</sup> T-cells in RSV-infected LLC tumors and that CD8<sup>+</sup> T-cells are less exhausted, as compared with non-infected tumors.

In addition, we found that CD8<sup>+</sup> T-cells in RSV-infected tumors express less immune checkpoint inhibitors (PD1 and TIGIT) (0.35 and 0.45 MFI fold change), as compared with non-infected tumors (1.01 and 1.17), which accounts for less than half the expression of these molecules in non-infected tumors (**Fig 25 D**). Similarly, we found that CD4+ cells express less PD1 and TIGIT molecules (0.74 and 1.03 MFI fold change), as compared to non-infected LLC tumors (1.2 and 1.2 MFI fold change) (**Fig 25 F**).

**Altogether, these data demonstrate that RSV infection of LLC tumors, causes a selective and reproducible increase in the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells expressing the T cell activation/memory/resident memory/less exhausted phenotypes, as compared to non-infected LLC tumors. To conclude, RSV infection of LLC tumors impact tumor and immune cells, leading to a delay in tumor growth that was observed in our murine NSCLC tumor model.**





**Figure 25 | Impact of RSV infection of LLC tumors on intratumoral immune cell infiltrate.** Tumor-bearing mice were euthanized at day 19 post-injection, and lung tumor were extracted to characterize the immune infiltrate. **A)**: Percentage of T, B, NK and myeloid cells in non-infected vs RSV-infected LLC tumors **B)**: Percentage of CD4<sup>+</sup> and CD8<sup>+</sup> cells among T-cells (CD3<sup>+</sup>) **C)**: Percentage of naïve (CD62L<sup>hi</sup> CD44<sup>lo</sup>), memory (CD62L<sup>lo</sup> CD44<sup>hi</sup>), resident memory (CD69+CD103+) and CD39+ CD8<sup>+</sup> cells among all CD8<sup>+</sup> cells in non-infected vs RSV infected LLC tumors **D)**: MFI expression of PD1 and TIGIT in CD8+ cells, shown by fold change in GMean values, as compared to non-infected sample **E)**: Percentage of naïve (CD62L<sup>hi</sup> CD44<sup>lo</sup>) and memory (CD62L<sup>lo</sup> CD44<sup>hi</sup>) CD4<sup>+</sup> cells in non-infected vs RSV infected LLC tumors, **F)**: MFI expression of PD1 and TIGIT in CD4+ cells, shown by fold change in GMean values, as compared to non-infected sample. T-Test "Mann Whitney test" was used. Data are mean ± SD. ns = not significant, \*: p < 0,05, \*\*: p < 0,01, \*\*\*: p < 0,0001. Each experiment was repeated twice.

## Part II: Impact of Influenza virus infection on murine lung tumor cell hallmarks

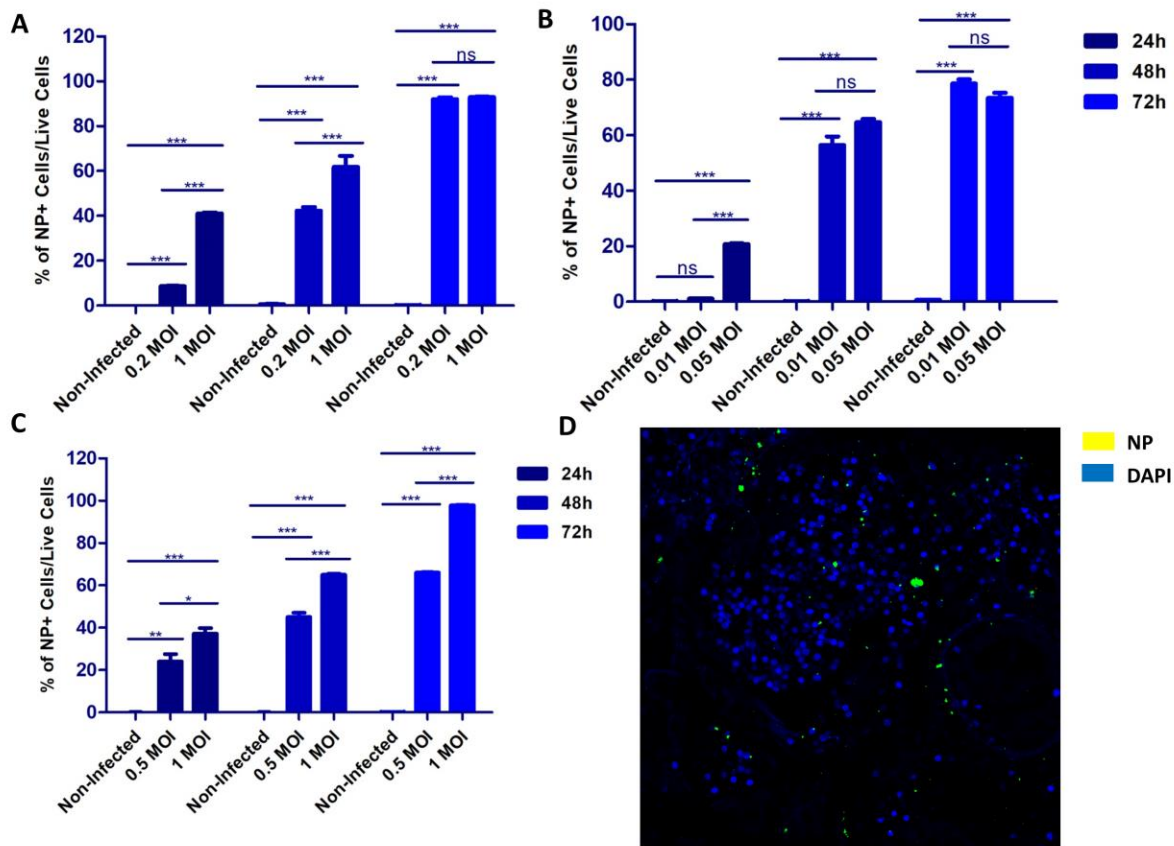
After studying the impact of RSV on NSCLC tumor progression, we wanted to investigate whether other respiratory viruses (such as IAV) can induce similar changes in the hallmark of NSCLC cell lines.

The direct impact of IAV infection on NSCLC tumor cells can be envisaged if they could be infected by IAV. So, we first investigated whether NSCLC cell lines and resected human tumors can be infected by IAV. Later, we studied the impact of IAV infection of NSCLC cell lines on cancer hallmarks, such as inducing inflammation, cell proliferation, cell death, angiogenesis, and EMT. We used the same murine cellular model (LLC-Luc and KP-Luc) that were used in RSV studies.

### 1. IAV infects tumor cells

In order to study the direct impact of IAV infection on tumor cells, we first need to investigate whether NSCLC cells are permissive to IAV. We infected murine lung tumor cells lines with increasing IAV multiplicity of infection (MOI) and quantified the rate of infected cells. We found that IAV can productively infect multiple murine NSCLC cell lines, by following the production of viral protein (NP) during the first 72h post-infection (pi). Indeed, IAV can infect LLC (**Fig. 26A**), KP (**Fig. 26B**) and LKR-Luc (**Fig. 26C**) cells in a dose and time dependent manner; as 0.2 MOI IAV can infect about 10% and 40% of LLC cells, 24h and 48h pi comparing with about 40% and 60% of infected cells in 1 MOI IAV condition, during the same time points (**Fig.26A**). At 72h pi, the majority of cells in both doses are infected (**Fig.26A**).

IAV infection of KP and LKR-Luc cells follows similar pattern (**Fig. 26B and 26C**). In addition, we also found that A549 human NSCLC cells are infected at similar levels (results not shown) and that human NSCLC pieces of tumors (obtained from surgery just after resection) can be infected ex-vivo by IAV (**Fig 26D**).



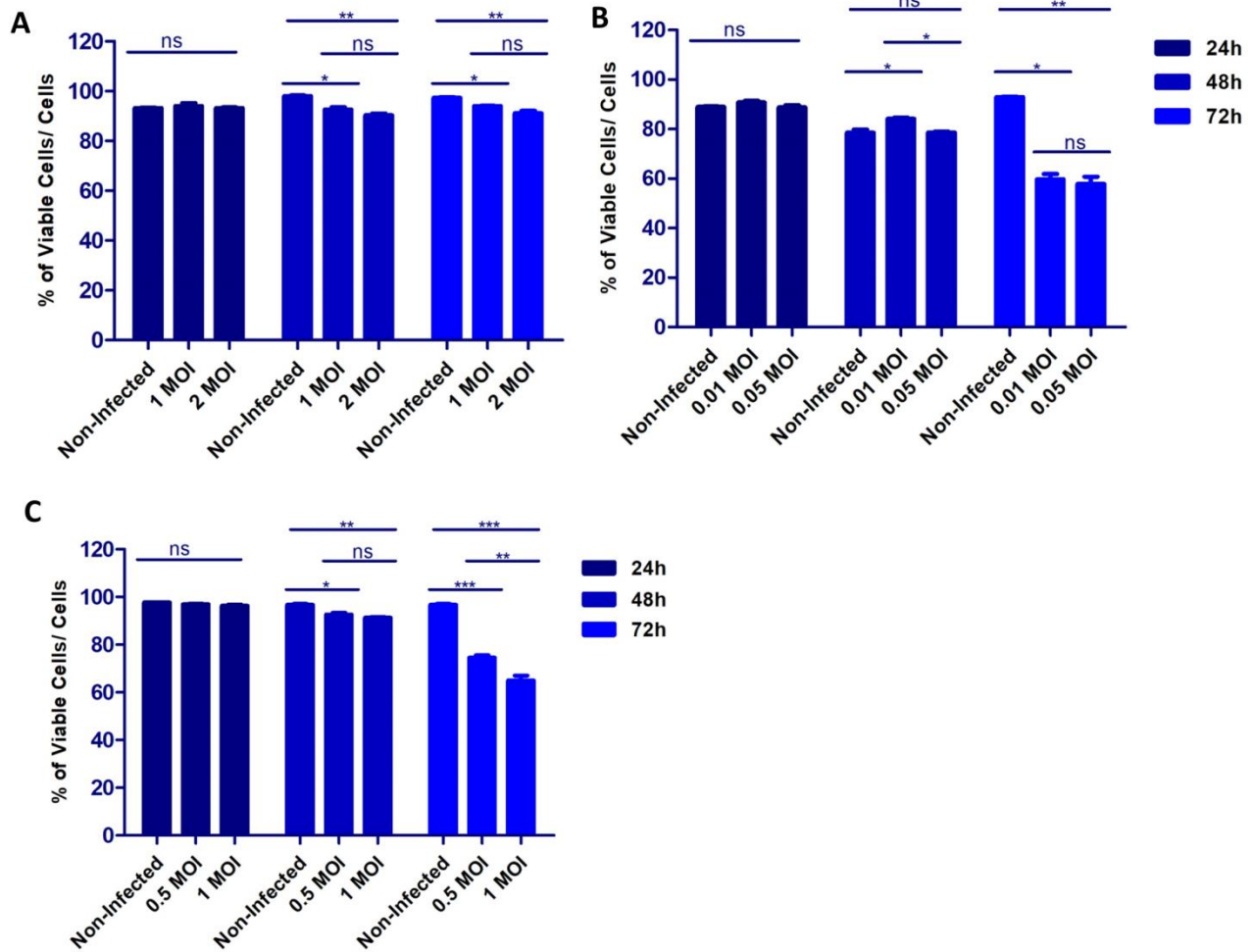
**Figure 26 | IAV infects NSCLC cell lines in a time and dose dependent way.** Percentage of infected cells (NP+ cells) at 24, 48 and 72h pi was measured by flow cytometry **A)**: Percentage of infected LLC cells (NP+) by two different IAV doses (0.2 and 1 MOI). **B)**: Percentage of infected KP cells (NP+) by two different IAV doses (0.01 and 0.05 MOI). **C)**: Percentage of infected LKR-Luc cells (NP+) by two different IAV doses (0.5 and 1 MOI). One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.0001$ . Each experiment was repeated three times at least. **D)**: Representative figure show IAV infection in human tumors. Resected human NSCLC cells were cut into small pieces and incubated by  $10^5$  pfu for 48h. Then, tumors were fixed, put in paraffin and stained with anti-NP (IAV) antibodies and DAPI, and assayed by immunofluorescence microscopy.

**Thus, IAV productively infects NSCLCs cells and resected human tumors and consequently may directly impact cancer hallmarks, such as tumor cell proliferation, evasion of apoptosis inflammation, Immune escape, EMT and angiogenesis.**

## 2. IAV infection reduces cell viability of some NSCLC cell lines at late stage of infection

After confirming the productive infectivity of NSCLCs by IAV, we wanted to know whether IAV behaves as a lytic virus to NSCLC cells. We investigated whether IAV infection lyses NSCLC cells

and found that IAV infection modestly reduces cell viability at 72h pi. This effect is dependent on cell line, as LLC-Luc cells (**Fig. 27 A**) were more resistant than KP-Luc (**Fig. 27 B**) and LKR-Luc cells (**Fig. 27 C**). At 72h pi, more than 90% of LLC cells were viable, comparing with about 60% of KP and LKR-Luc cells.

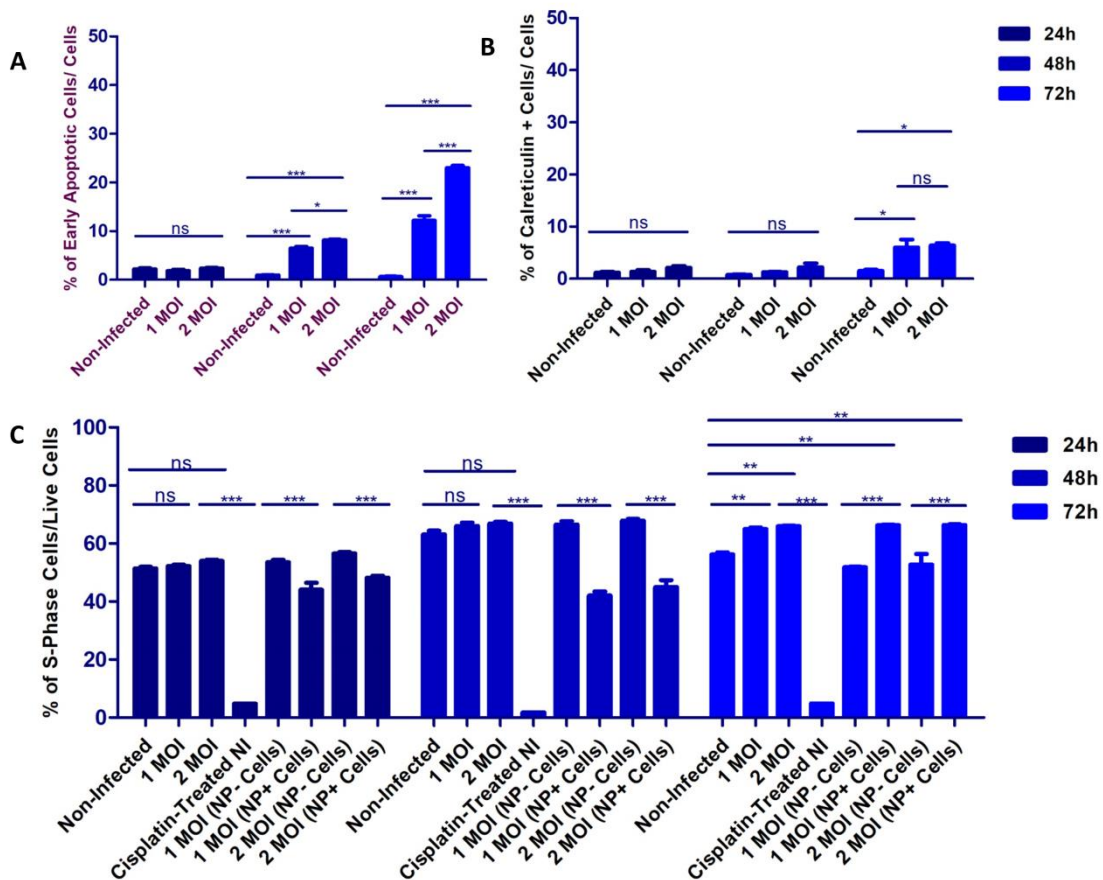


**Figure 27 | IAV infection reduces cell viability of some NSCLC cell lines.** At 24, 48 and 72h pi, NSCLC cells infected or not are stained by LIVE/DEAD™ Fixable Yellow Dead Cell Stain to assess viability by flow cytometry. **A):** -Percentage of viable LLC cells, infected by 0.2 and 1 MOI IAV. **B):** Percentage of viable KP cells, infected by 0.01 and 0.05 MOI of IAV. **C):** Percentage of viable LKR-Luc, infected by 0.5 and 1 MOI of IAV. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean ± SEM. ns = not significant, \*: p < 0.05; \*\*: p < 0.01; \*\*\*: p < 0.0001. Each experiment was repeated three times at least.

### 3. IAV infection induces modest apoptosis of some NSCLC cell lines at late stage of infection

We then investigated whether apoptosis and cell proliferation were affected by IAV infection and we found that IAV infection modestly induces cell apoptosis in NSCLC cells at 72h pi. However, this effect is dependent on cell line, as IAV induces about 10% of apoptosis in LLC cells, infected with 1 MOI (Fig. 28 A) vs about 25% in LKR-Luc cells, at 72h pi (Fig. 29 A). Even increasing the viral dose of IAV to 2 MOI increased the apoptosis to less than 25% of LLC cells (Fig.28 A).

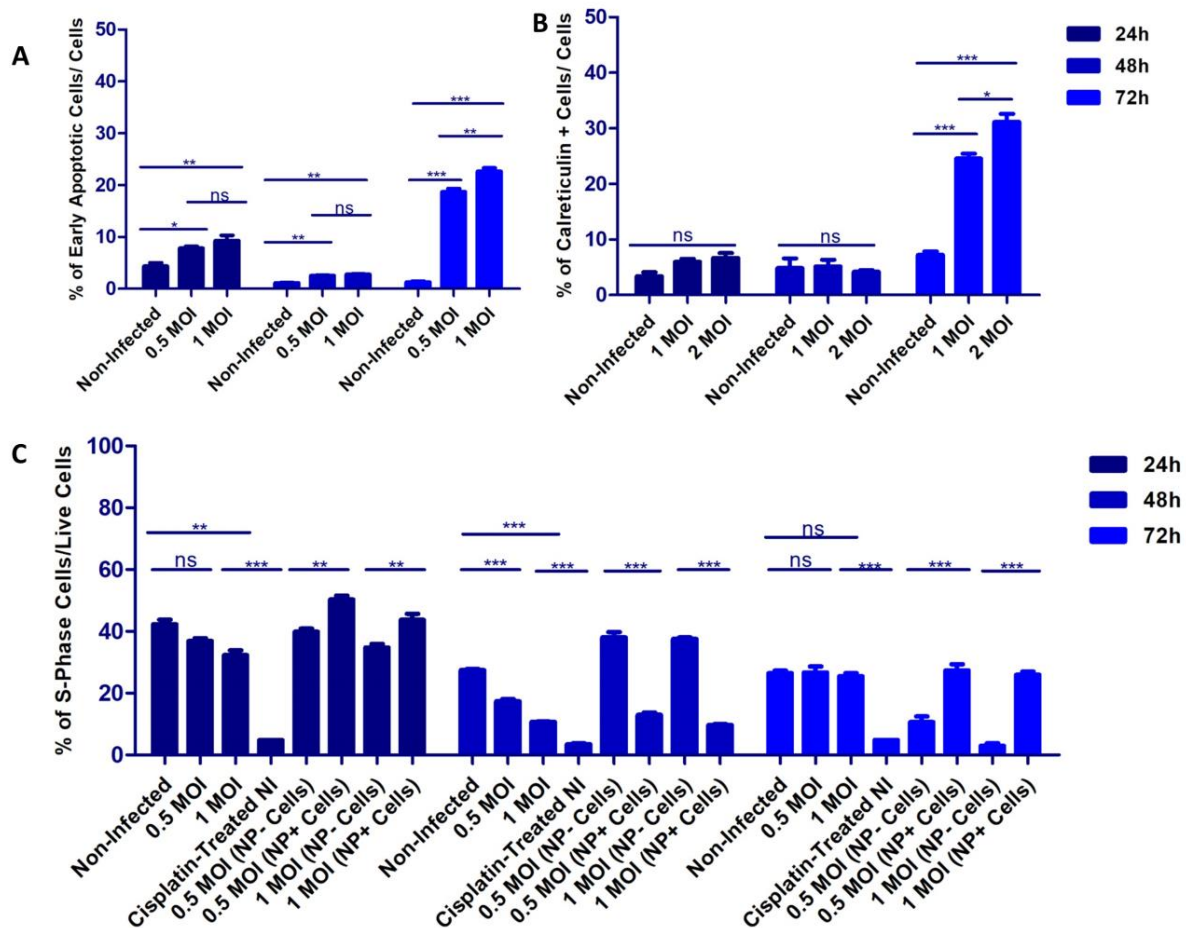
As the preapoptotic translocation of calreticulin (CRT) to the cell surface is an indicator of immunogenic cell death and anticancer immune responses (Obeid et al., 2007), we were interested in investigating whether apoptosis is accompanied by an increase in membranous expression of calreticulin. A modest increase in calreticulin expression was observed (to about



**Figure 28 | Impact of IAV infection on apoptosis, calreticulin expression and proliferation of LLC cells.** LLC cells were infected by 1 and 2 MOI IAV and collected at 24, 48 and 72h pi for flow cytometry analysis **A)**: Percentage of early apoptotic LLC cells (Annexin V+ cells). **B)**: Percentage of calreticulin expression on cell membranes **C)**: LLC cells in the S-phase of cell cycle, which reflects cell proliferation. Cisplatin-treated non-infected cells serve as control positive of cell proliferation inhibition. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.0001$ . Each experiment was repeated three times at least.

30% of LKR-Luc cells, vs less than 10% in LLC cells infected with the higher doses of IAV) (Fig.28 B, Fig.29 B).

In addition, IAV infection doesn't induce any inhibition in LLC cells proliferation, comparing with non-infected LLC cells. NP+ cells (infected cells) proliferate at slower rate than NP- cells (Non-infected cells in the infected sample) during the first 48h pi, whereas at higher rate at 72h pi (Fig. 28 C).



**Figure 29 | Impact of IAV infection on apoptosis, calreticulin expression and proliferation of LKR cells.** LKR-Luc cells were infected by 0.5 and 1 MOI and collected at 24, 48 and 72h pi for flow cytometry analysis **A)**: Percentage of early apoptotic LKR-Luc cells (Annexin V+ cells). **B)**: Percentage of calreticulin expression on cell membranes **C)**: LKR-Luc cells in the S-phase of cell cycle, which reflects cell proliferation. Cisplatin-treated non-infected cells serve as control positive of cell proliferation inhibition. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.0001$ . Each experiment was repeated three times at least.

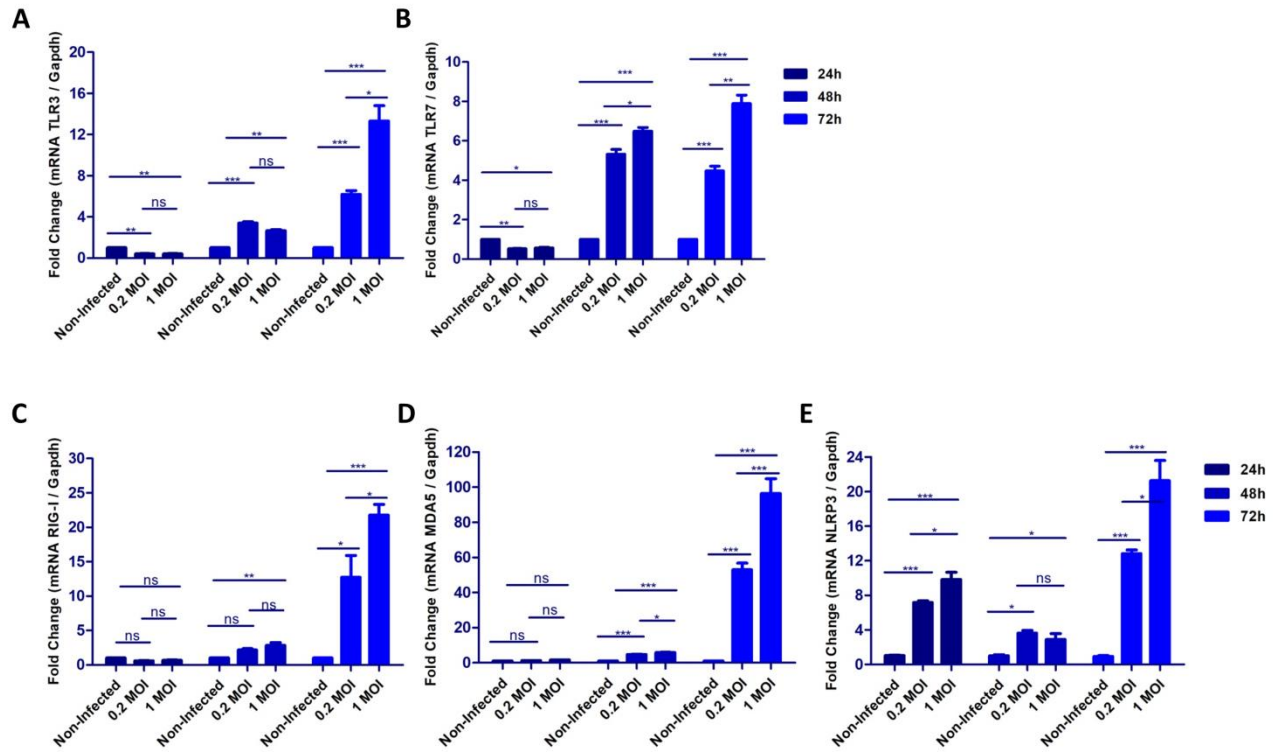
By contrary, IAV infection induces transient inhibition in LKR-Luc cells proliferation at 48h pi, comparing with non-infected LKR-Luc cells. However, infected cells proliferate at a similar rate, comparing with non-infected cells, at 72h pi. NP+ cells (infected cells) proliferate at slower rate than NP- cells (Non-infected cells in the infected sample) at 48h pi, and at quicker rate at 72h pi (**Fig 29 C**). This indicates that IAV doesn't induce steady inhibition of NSCLC cells proliferation.

**Thus, we conclude that IAV doesn't behave as a lytic virus and doesn't inhibit NSCLC cells proliferation, despite inducing mild apoptosis in addition to a modest increase in calreticulin expression at late stage of infection in some NSCLC cell lines.**

#### **4. IAV infection induces the upregulation of viral RNA sensors expression**

The role of the innate immune receptors in detecting RNA viruses is crucial for the establishment of proper inflammatory and antiviral responses, which can impact cancer hallmarks. These receptors, known also as viral RNA sensors, have the capacity to sense the presence of RNA as PAMPs, which leads to the induction of type I IFNs as well as inflammatory cytokines and chemokines, which also results in the upregulation of the expression of these sensors. Thus, investigating the expression of these sensors serves as an indicator of the activation of these signaling pathways and reflects the importance of individual sensors in viral sensing.

IAV infection of different NSCLC cell lines results in the upregulation of all RNA viral sensors, including TLR3, TLR7, RIG-I, MDA5 and NLRP3, in a time and dose dependent manner.



**Figure 30 | IAV infection results in the upregulation of viral RNA sensors.** LLC cells were infected by two different IAV doses (0.2 and 1 MOIs) and collected at 24, 48 and 72h pi for RT-qPCR analysis. Results are shown as fold change that relates mRNA sensors to mRNA of housekeeping gene for **A):** TLR3, **B):** TLR7, **C):** RIG-I, **D):** MDA5, **E):** NLRP3. Gapdh is used as housekeeping gene. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.0001$ . Each experiment was repeated three times at least.

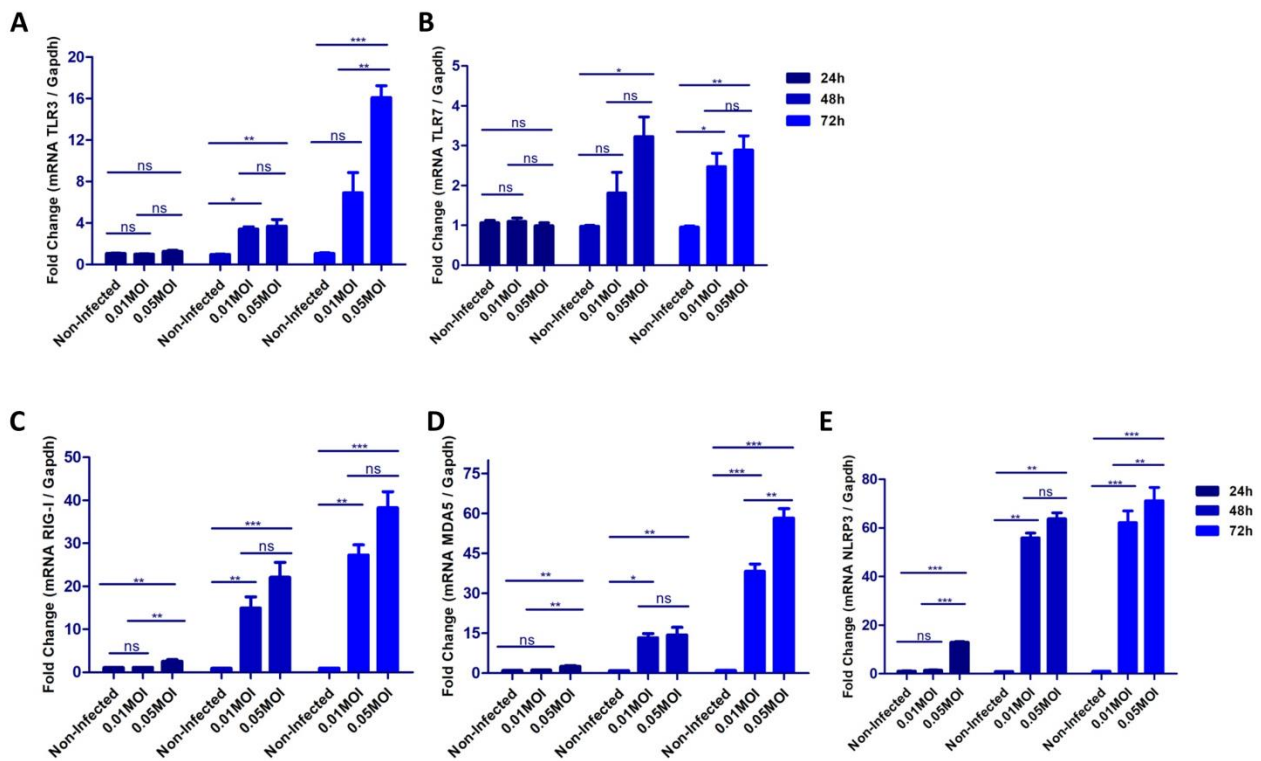
Of note, IAV infection of LLC cells leads to a rapid upregulation of NLRP3 on mRNA level at 24h pi (**Fig 30E**), while upregulation of all other sensors starts at 48h pi and maximizes at 72h pi (**Fig 30 A, B, C, D**). As TLR3 and RIG-I have the highest basal expression among all sensors in LLC cells (data not shown), we suggest that the upregulation of TLR3 and RIG-I (to about 16 and 20 -fold change) at 72h is the most impacted by IAV infection.

As it was shown that RIG-I, TLR3 and TLR7 expression is mainly mediated by the induction of type I IFN (Hayakari et al., 2013; Miettinen et al., 2001), we hypothesize that the late upregulation of viral sensors is due to the time needed for the production and the accumulation



of type I IFNs by tumor cells in the supernatant. Of note, NLRP3 expression is mainly mediated by the activation of NF- $\kappa$ B (He et al., 2016), which explains why NLRP3 is unregulated at early stages of infection (at 24h pi).

Similarly, IAV infection of KP cells enhances the mRNA expression of NLRP3 as soon as 24h pi (**Fig 31 A**), while the mRNA expression of other sensors (TLR3, TLR7, RIG-I and MDA5) (**Fig. 31 B, C, D, E**) starts at 48h and maximizes at 72h pi. As TLR3, RIG-I and MDA5 have the highest basal expression among all sensors in KP cells (data not shown), we suggest that the upregulation of TLR3, RIG-I and MDA5 (to about 16-, 40- and 60-fold change respectively) at 72h is the most impacted by IAV infection.



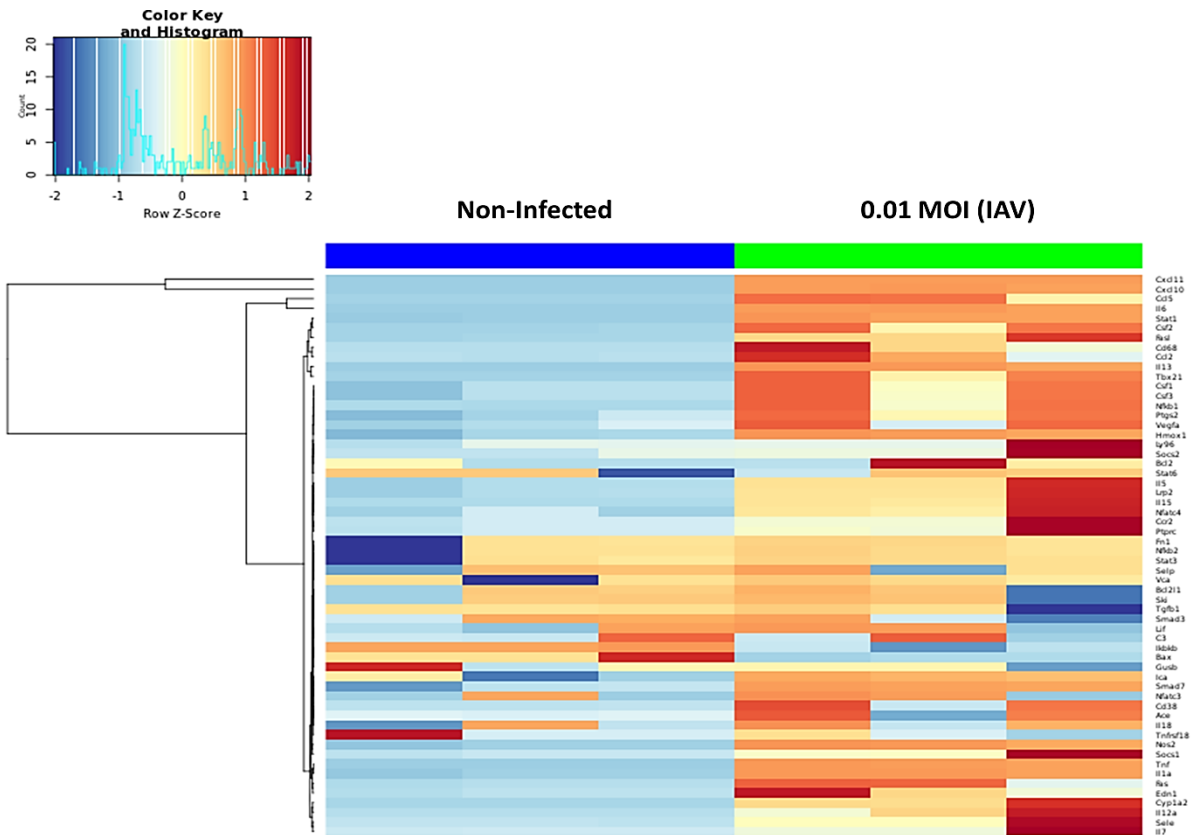
**Figure 31 | IAV infection of KP cells results in the upregulation of viral RNA sensors.** KP cells were infected by two different IAV doses (0.01 and 0.05 MOIs) and collected at 24, 48 and 72h pi for RT-qPCR analysis. Results are shown as fold change that relates mRNA sensors to mRNA of housekeeping gene for **A):** TLR3, **B):** TLR7, **C):** RIG-I, **D):** MDA5, **E):** NLRP3. Gapdh is used as housekeeping gene. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.0001$ . Each experiment was repeated three times at least.

IAV infection of LKR cells, results in similar profile and kinetics, regarding mRNA expression of TLR3, TLR7, RIG-I and MDA5 (data not shown).

Altogether, this suggests that IAV infection of NSCLC cells results in an early upregulation of NLRP3 (probably due to NF- $\kappa$ B activation), and late upregulation of other RNA sensors, notably TLR3 and RIG-I, probably due to the accumulation of type I and III IFNs secreted by tumor cells in the culture medium.

### 5. IAV infection induces the upregulation of proinflammatory cytokines and chemokines

As previously discussed in the introduction, NF- $\kappa$ B activation regulates the production of a large variety of proinflammatory cytokines and chemokines.



**Figure 32 | IAV infection of KP cells results in the upregulation of several proinflammatory cytokines and chemokines.** KP cells were infected by 0.01 MOI IAV and collected at 48h pi for RT-qPCR analysis. Results are shown as fold change that relates mRNA target genes to mRNA of housekeeping gene Gapdh. Each experiment was repeated three times at least.

IAV infection of KP cells enhances the mRNA expression of several proinflammatory cytokines and chemokines (**Fig. 32**), mediated by the activation of NF- $\kappa$ B and type I IFNs. Among the most upregulated cytokines are IL-6, CXCL10, CXCL11 and CCL5 (more than 200 fold change), in addition to IL-1 $\beta$ , TNF- $\alpha$ , IL-12a, IL-13, STAT1, CSF-2 (GM-CSF), and CCL2 (more than 10 fold change) (**Fig. 32**).

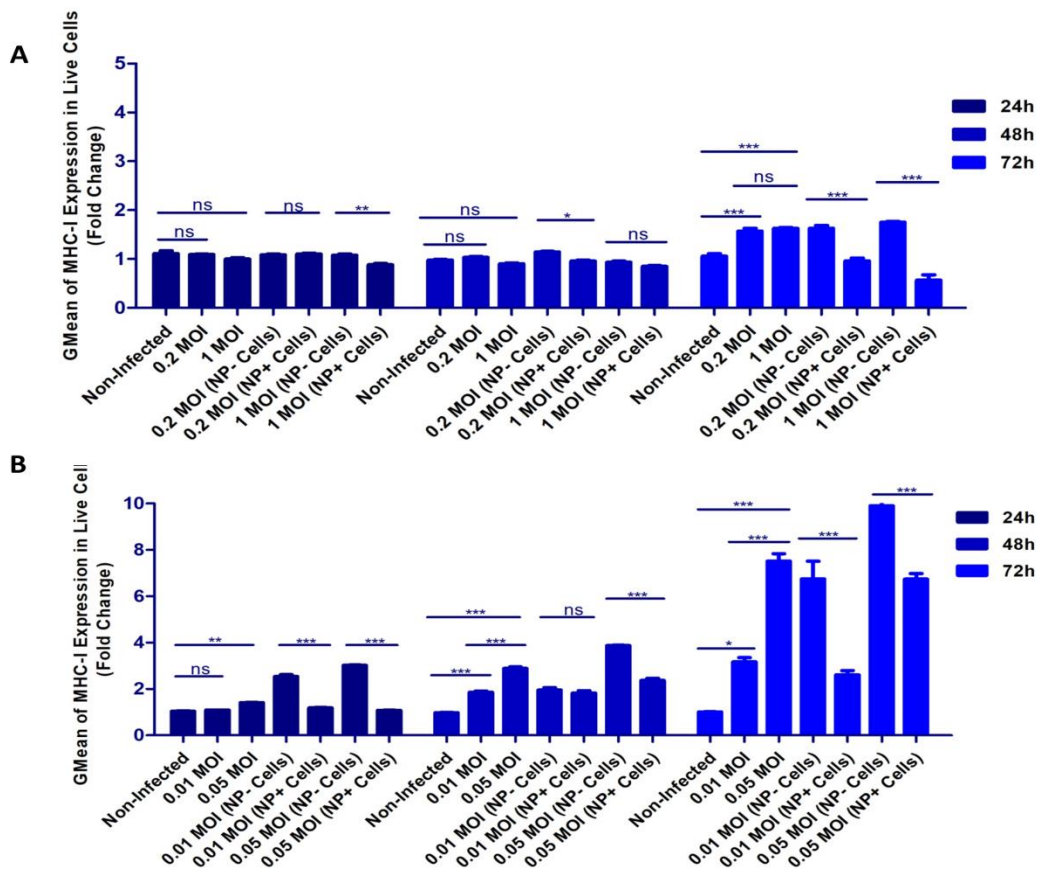
CXCL10, CXCL11, CCL5 and CCL2 are implicated in the migration of various leucocytes and the modulation of their phenotype, including T-cells, granulocytes and monocytes.

In addition, other cytokines and molecules implicated in the NF- $\kappa$ B and type I IFN signaling pathways are upregulated, yet to a lesser degree, such as IL-5, IL-7, IL-15, Nos2, NF- $\kappa$ B1 and Socs1. Finally, Bcl2, an antiapoptotic molecule, is also upregulated (**Fig. 32**).

**To conclude, the sensing of IAV, by several viral RNA sensors in NSCLC cells, enhances the expression and production of several proinflammatory cytokines, chemokines, and type I IFNs, which are involved in inflammation and consequently may modifying the TME.**

## 6. IAV infection downregulates the expression of MHC-I in infected LLC cell line

To assess whether influenza virus infection affects MHC-I cell surface expression, we infected NSCLC cells with IAV. Infected (NP+) LLC cells expressed significantly lower geometric mean fluorescence intensity (MFI) levels of surface MHC-I as compared to non-infected (NP-) cells



**Figure 33 | IAV infection of NSCLC cells results in the downregulation of MHC-I in infected cells.** LLC and KP cells were infected by two different IAV doses (0.2 and 1 MOIs for LLC and 0.01 and 0.05 MOIs for KP cells) and collected at 24, 48 and 72h pi for flow cytometry analysis. Results are shown as fold change that relates GMean of membranous MHC-I to the GMean of MHC-I in the non-infected control. **A):** GMean of MHC-I in LLC cells, **B):** GMean of MHC-I in KP cells. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0,05$ , \*\*:  $p < 0,01$ , \*\*\*:  $p < 0,0001$ . Each experiment was repeated three times at least.

during the first 72h pi. At 72h pi, NP+ cells in the higher dose of infection (1 MOI) expressed significantly lower GMean than non-infected control (**Fig 33 A**).

In contrast, NP- cells expressed variably higher MFI levels of MHC-I compared to the non-infected control (**Fig. 33 A**), likely due to the effects of accumulated type I IFN secreted by influenza-infected cells during 72h pi.

Infected (NP+) KP cells expressed significantly lower geometric mean fluorescence intensity (MFI) levels of surface MHC-I as compared to non-infected (NP-) cells at 48h and 72h pi (**Fig. 33 B**). However, IAV infection resulted in overall upregulation of surface MHC-I in the infected sample, as compared with non-infected control, in a time and dose-dependent manner, possibly due to the increasing accumulation of IFNs in the medium, similar to what we found with RSV. The overall upregulation of surface MHC-I (about 3 and 7 fold increase in the lower and higher IAV dose) is higher than that observed with LLC (less than 2 fold change), and this could be due to more activation of type-I IFN pathway, resulted from the production of bigger quantities of IFN or more expression of IFNAR receptors on the surface of KP cells. Koutsakos and his team showed that IAV infection of A549 cells results in a downregulation of surface MHC-I, compared with non-infected cells, during the first 16h pi. However, they didn't follow MHC-I expression, up to 72h pi (Koutsakos et al., 2019).

To conclude, infection of NSCLC cells leads to the downregulation of MHC-I molecules at the cell surface of infected cells, as compared to non-infected cells. This possibly helps infected tumor cells to evade immune recognition and immune destruction by CD8<sup>+</sup> T-cells (Koutsakos et al., 2019).

#### **7. IAV infection mildly upregulates the expression of PDL1 in infected NSCLC cell lines**

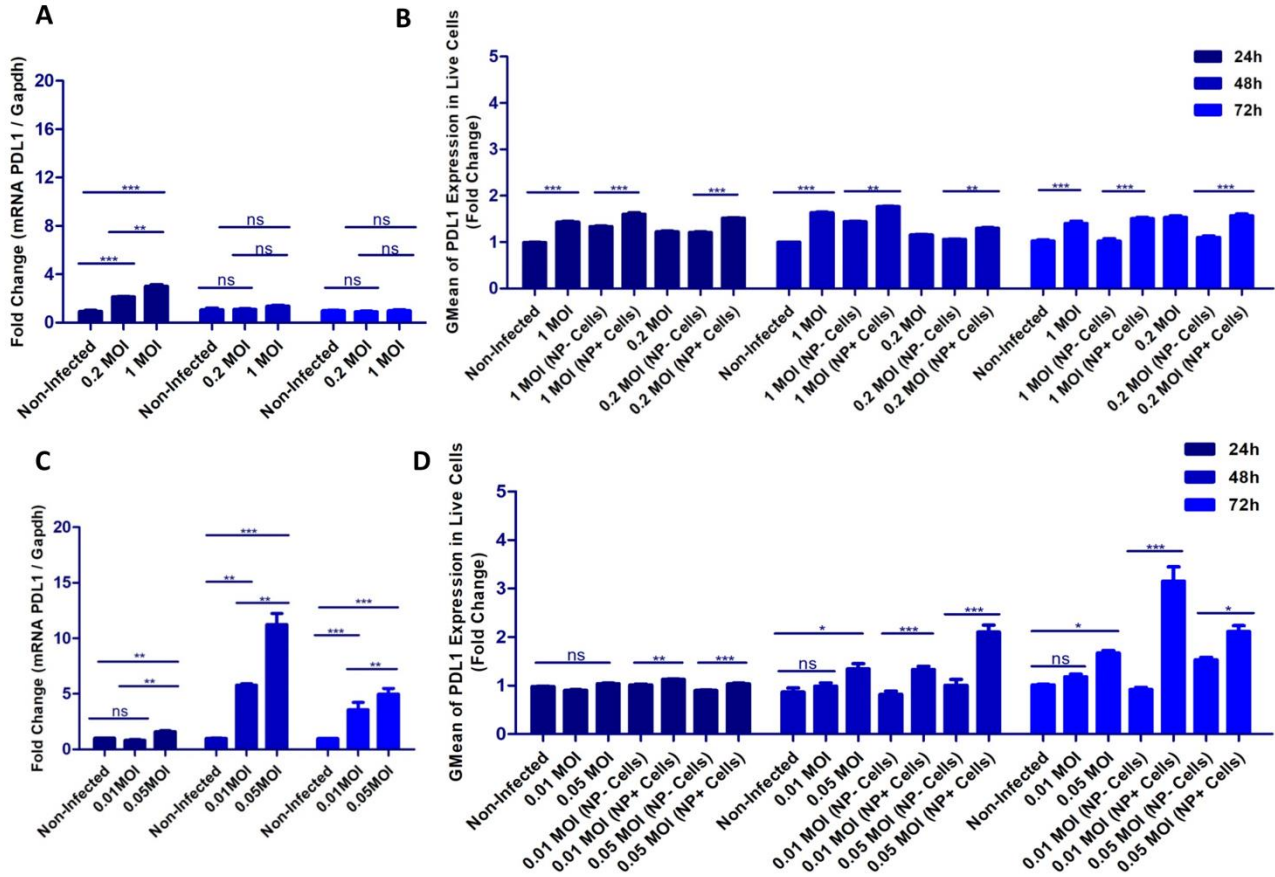
To assess whether influenza virus infection affects PDL1 cell surface expression, as a mechanism of immune escape, we infected NSCLC cells with IAV.

IAV infection of LLC mildly upregulates mRNA levels of PDL1 at 24h pi (to about 4 fold change in the higher dose of IAV) and the overall surface expression of PDL1 (to about 2 fold change) at 72h pi (**Fig. 34A and B**). In addition, the surface expression of PDL1 was higher in the infected

(NP+) cells, as compared to non-infected cells (NP- cells) or non-infected control, at all time-points.

IAV infection of KP upregulates mRNA levels of PDL1 at all time-points and maximizes at 48h pi (to about 12 fold change in the high dose of IAV) and it upregulates the overall surface expression of PDL1 (to about 2 fold change in the high dose of IAV) at 48 and 72h pi (**Fig. 34C and D**). Similarly to LLC, the surface expression of PDL1 was higher in the infected (NP+) cells, as compared to non-infected cells (NP- cells) or non-infected control, at all time-points, and maximizes at 72h pi (to about 4 fold change in the low dose of IAV) (**Fig. 34D**).

Thus, IAV infection of NSCLC cells enhances the possibility of infected tumor cells to escape CD8<sup>+</sup> T-lymphocytes, through downregulating surface MHC-I and upregulating PDL1.



**Figure 34 | IAV infection of NSCLC cells results in the upregulation of PDL1 in infected cells.** LLC and KP cells were infected by two different IAV doses (0.2 and 1 MOIs for LLC and 0.01 and 0.05 MOIs for KP cells) and collected at 24, 48 and 72h pi for RT-qPCR and flow cytometry analysis. RT-qPCR Results are shown as fold change that relates mRNA PDL1 to mRNA of housekeeping gene Gapdh. Flow cytometry data are shown as fold change that relates the relative geometric mean fluorescence intensity (gMFI) levels of surface PDL1 as compared to non-infected control. **A):** mRNA levels of PDL1 in LLC cells **B):** relative geometric mean fluorescence intensity (gMFI) of PDL1 in LLC cells. **C):** mRNA levels of PDL1 in KP cells **D):** relative geometric mean fluorescence intensity (gMFI) of PDL1 in KP cells. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0,05$ , \*\*:  $p < 0,01$ , \*\*\*:  $p < 0,0001$ . Each experiment was repeated three times at least.

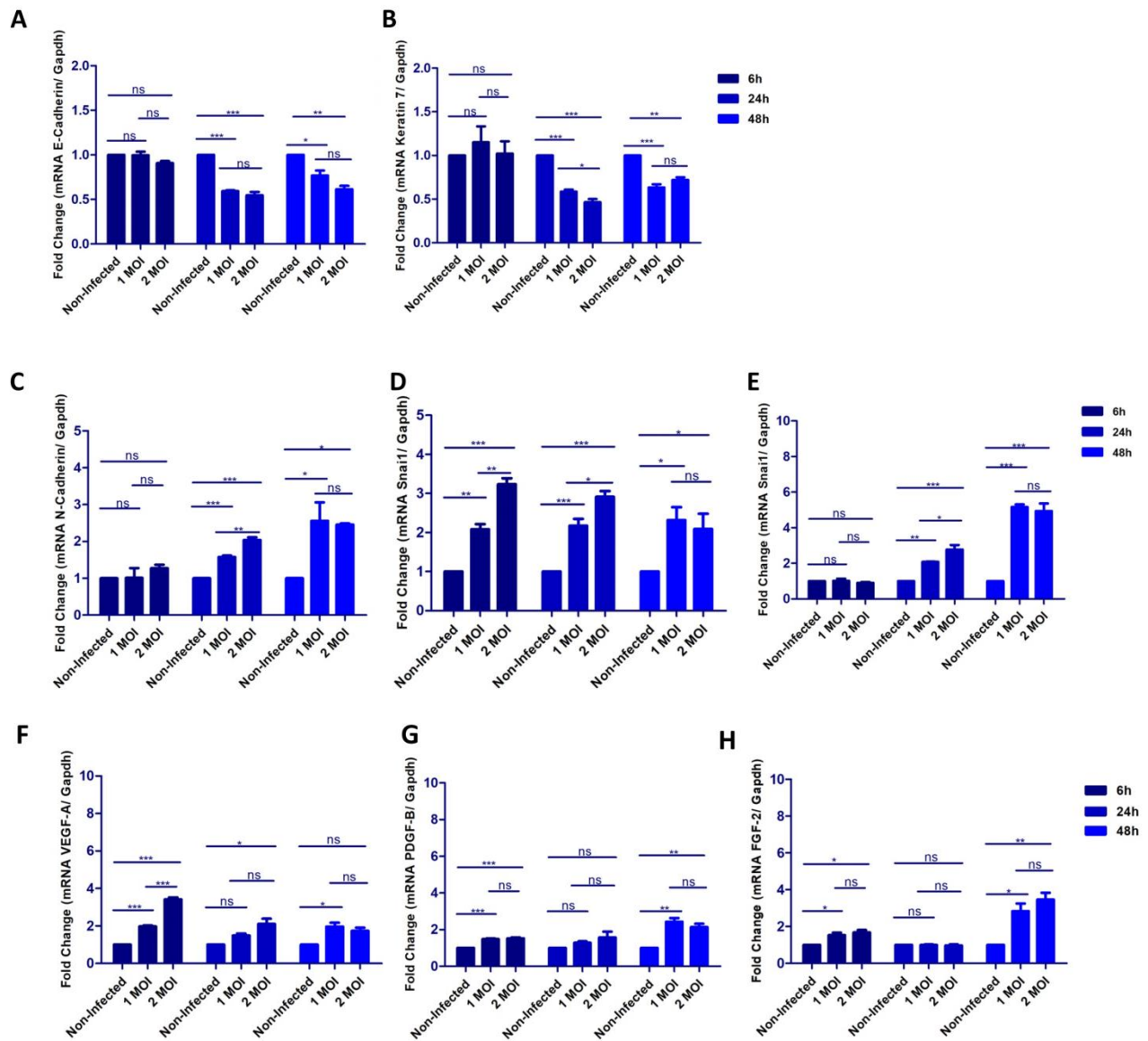
## **8. IAV infection upregulates EMT and angiogenesis markers in infected LLC cells**

Epithelial–mesenchymal transition (EMT) and angiogenesis are 2 critical factors influencing tumor growth and metastasis, and linked to poor outcome in human NSCLC (Jiang et al., 2018). To therefore assessed whether IAV infection affects the expression of EMT and angiogenesis markers, we found that while IAV infection of LLC represses mRNA levels of epithelial makers (E-Cadherin and Keratin 7) at 24 and 48h pi, it upregulates mRNA levels of mesenchymal makers (N-Cadherin, snail2) at 24 and 48h pi, in addition to snail1 as soon as 6h pi and at 24 and 48h pi (up to 6 fold change increase) (**Fig 35 A-E**). Altogether, these changes suggest that IAV infection enhances the possibility of the occurrence of EMT in LLC cells.

In addition, we found that IAV infection of LLC upregulates mRNA levels of VEGF-A, PDGF-B and FGF2 (up to 4 fold change increase at 48h pi) (**Fig 35 F-H**). This suggests that IAV infection of LLC cells enhances the possibility of tumor cells to mediate the formation of new vessels in the TME.

**Altogether, we showed that IAV can infect NSCLC tumors, and induces inflammatory environment and some immune escape mechanisms, in addition to EMT and angiogenesis markers without inhibiting cell proliferation or lyse cells.**





**Figure 35 | IAV infection of LLC cells upregulates EMT and angiogenesis.** LLC cells were infected by two different IAV doses (0.2 and 1 MOIs for LLC) and collected at 24, 48 and 72h pi for RT-qPCR. RT-qPCR Results are shown as fold change that relates mRNA PDL1 to mRNA of housekeeping gene Gapdh. **A):** E-cadherin **B):** Keratin 7 **C):** N-cadherin **D):** Snai 1, **E):** Snai 2, **F):** VEGF-A, **G):** PDGF-B, **H):** FGF2. One way Anova and Bonferroni's multiple comparison post-test was used. Data are mean  $\pm$  SEM. ns = not significant, \*:  $p < 0,05$ , \*\*:  $p < 0,01$ , \*\*\*:  $p < 0,0001$ . Each experiment was repeated three times at least.

## Conclusion, Discussion and Future Directions

Our current understanding of cancer progression and immune response relies principally on studies in which cancer is the only challenge to the immune system. However, in reality, the immune system is often tasked with responding to multiple concomitant challenges, including cancers and infections, and how one type of challenge impacts the other type and the immune response is not well understood.

Further, although acute non-oncogenic, non-lytic, non-immune-destructive respiratory viral infections and NSCLCs are two of the most common human maladies, and that NSCLC patients are at increased risk of short-term complications and mortality following viral respiratory infections, as we already discussed, very little information is available regarding the consequences of concomitant infection and NSCLC, and thus this subject is a matter of ongoing debate.

Accumulating evidence shows that a heterogeneous group of viruses such as respiratory syncytial virus (RSV), influenza virus (IV), parainfluenza virus (PIV), metapneumovirus (HMPV), rhinovirus (RhV), and coronavirus (CoV), including the recently emerged SARS-CoV-2 are associated with a more complicated and severe disease in cancer patients (Chemaly et al., 2012; Hijano et al., 2018; Liang et al., 2020; Volling et al., 2014).

Among all of them, IAV and RSV infections in NSCLC patients are among the most common and the best studied viruses, in addition to COVID-19, and thus, serves as a model to study the impact of respiratory viral infections in lung cancer.

### 1. Conclusion

The impact of these viruses on cancer progression could be mediated by impacting the hallmarks of cancer cells, anti-tumor immune response, or both. Thus we tried to investigate both mechanisms. Considering the burden of the work and the limit of the time, I focused on studying the effect of RSV and IV infections on in vitro models of NSCLC cells, and I also

explored the infectivity of resected human tumors by these viruses. In addition, I investigated the impact of RSV infection on a murine orthotopic model of NSCLC. At the same time, my colleagues investigated the impact of IV infection on a spontaneous murine NSCLC model.

On the one hand, we found that RSV productively infects murine and human NSCLCs cells, in addition to human NSCLC tumor tissues. RSV infection of murine NSCLC cells induces late immunogenic apoptotic cell death that may induce anticancer immune responses, and it inhibits cell proliferation. It results in an upregulation of RNA viral sensors (NLRP3, TLR7, TLR3, MDA5 and RIG-I), probably due to the accumulation of type I and III IFNs secreted by tumor cells in the culture medium. As a result, the sensing of RSV, enhances the expression and production of several proinflammatory cytokines, chemokines, and type I IFNs, which are involved in inflammation and consequently may modifying the TME. In addition, infection of NSCLC cells leads to the upregulation of MHC-I and Fas molecules at the cell surface of infected cells, as well as non-infected cells, mediated by type-I IFN production. Furthermore, RSV infection doesn't support the possibility of the occurrence of EMT in LLC cells (**Table 13**).

In parallel, in a murine orthotopic model, RSV infection results in a delay in the tumor progression, which could be due to RSV effects on the hallmark of tumor cells or/and intratumoral immune cells infiltration. RSV infection of murine NSCLC tumors, causes a selective and reproducible increase in the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells expressing the T cell activation/memory/resident memory/less exhausted phenotypes, as compared to non-infected NSCLC tumors. Thus, RSV infection of LLC tumors impacts tumor and immune cells, leading to a delay in tumor growth that was observed in our murine NSCLC tumor model.

On the other hand, I found that IAV induces viral sensors upregulation, inflammatory environment and some immune escape mechanisms, in addition to EMT and angiogenesis

markers without inhibiting cell proliferation or lyse tumor cells (**Table 13**), which possibly play a role in the protumoral effects that were found by my colleagues in the lab.

**Table 13:** Comparison between the impact of IV and RSV on NSCLC cells hallmarks

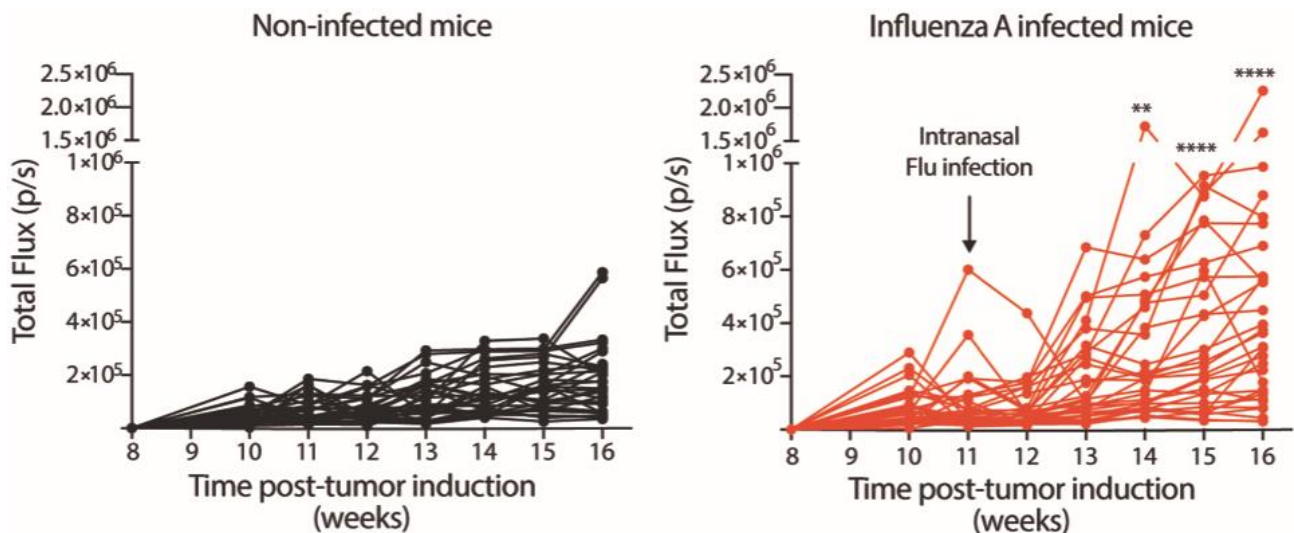
	IV	RSV
Cell Proliferation	No impact	Inhibits cell proliferation
Cell Death	Slightly impact cell death	Induces late cell death
EMT Markers	Induces EMT	Doesn't induce EMT
Angiogenesis Markers	Upregulates angiogenesis markers	Slightly upregulates angiogenesis markers
Promoting Inflammation	upregulates RNA sensors (RIG-I, MDA5, TLR3), Cytokines + type-I IFNs	
Avoiding Immune Destruction	downregulates MHC-I in infected cells	Upregulates MHC-I and Fas

## 2. Discussion

Lung cancer patients are more frequently prone to microbial infections, due to their compromised immune response and immunosuppression (DP et al., 2015). Among infections, IV and RSV infections in cancer patients are among the most common respiratory viral infections. However, the impact of these infections on NSCLC progression has received very little attention, especially in the case of RSV, and yet, it is still controversial, with substantial differences among different viruses.

Despite many similarities between the two viruses, we found that IAV and RSV impact differently NSCLC progression. As discussed before, I found that RSV and IAV infect NSCLC cells

and resected human tumors. However, IAV and RSV infections in murine NSCLC models lead to different outcomes, and this could be explained by their different impact on cancer cell hallmarks, as well as on TME of NSCLCs, including tumor immune infiltrate. Indeed, I found that RSV infection delays tumor progression in C57BL/6J mice. Interestingly, my colleagues in the lab, Aditi Varthaman, Irati Garmendia and Solenne Marmier demonstrated that IAV infection enhances tumor progression in a murine model of spontaneous NSCLC tumor (**Fig.36**) (submitted paper). In addition, my colleagues demonstrated that IV infection promotes a significant acceleration of tumor growth in the KP mouse model of spontaneous lung cancer, with an increased number of nodules and stage of tumors. In addition, IAV considerably alters the intratumoral T cell compartment, inducing the reduction of tumor-specific T cell responses and the expression of multiple negative immune checkpoints on tissue-resident memory CD8+ T cells as well as PD-L1 expression on tumor cells (submitted paper).



**Figure 36 | Impact of IAV infection on tumor progression in KP mice.** Lung adenocarcinoma was induced in 8 to 10 weeks old KP mice by intratracheal administration of  $2 \times 10^4$  lentiviral particles in a volume of  $60 \mu\text{L}$  and in vivo tumor progression was measured weekly by measuring bioluminescence. Twelve weeks post lentiviral inoculation, mice were infected with IAV by intranasal delivery of the H3N2 virus (150 pfu in  $40 \mu\text{L}$  PBS). Control mice received PBS alone. IAV infection was confirmed by measuring the body weight of the mice daily after viral infection. Mice were euthanized 16 weeks lentiviral delivery.

These protumoral effects of IV infection in a murine spontaneous model of NSCLC are in line with previous studies. Weng and his colleagues showed that there is an increased risk of developing lung cancer due to cumulative exposure to IAV (Weng et al., 2019). In addition,

Kohlhapp and colleagues demonstrated that IAV infection of the lung promotes tumor growth and accelerated tumor-related death due to induction of T cell exhaustion and a disruption in the trafficking of anti-tumor CD8<sup>+</sup> T cells to the site of infection (Kohlhapp et al., 2016). In addition, IAV infection of primary airway epithelial cells and tumor cells enhances PDL-1 expression, in an IFNAR signaling-dependent manner (McNally et al., 2013), and results in high expression of PD-1 on effector CD8<sup>+</sup> T cells, which interact with PD-L1, expressed in the TME of some NSCLC patients, leading to T cell exhaustion (McNally et al., 2013; Nikolaev et al., 2019).

Corresponding with these findings, our study provides evidence for the tumor-promoting impact of acute IAV infection in NSCLC. In addition, I found that IAV induces viral sensors upregulation, inflammatory environment and some immune escape mechanisms, in addition to EMT and angiogenesis markers without inhibiting cell proliferation or lyse tumor cells, which possibly play a role in the protumoral effects that were found by my colleagues in the lab. These results corresponds with previous studies which demonstrated that IV infection of NSCLC cells induces the production of TGF- $\beta$ , and a sphingolipid/EMT-associated gene signature, thus may enhances EMT (Carlson et al., 2010; Meshcheryakova et al., 2016), as well as enhances HIF-1 $\alpha$ , VEGF-A and FGF2 production leading to promoting tumor angiogenesis (Lee et al., 2000; Wang et al., 2018). However, it counteracts He et al who showed that IAV inhibits the proliferation of tumor cells and results in G0/G1-phase accumulation of infected cells caused by the prevention of cell cycle entry from G0/G1 phase into S phase, at 24h pi (He et al., 2010). These differences could be due to the use of different cell lines, and different time points, as they followed the proliferation of cells for 24h pi only.

On the other hand, Some studies have introduced additional evidence that RSV imposes a potent oncolytic effect on different malignancies, including colorectal, hepatocellular

carcinoma, prostate and skin cancers (Choi et al., 2015; Echchgadda et al., 2009, 2011; Hoffmann et al., 2007). However, this is the first time that the impact of RSV infection on tumor progression in murine *in vivo* model of NSCLC is investigated. Thus, this is the first time that such antitumoral effect of RSV infection in NSCLC tumors is shown.

In line with my results, it was previously shown that RSV infection of NSCLC cell lines result in delayed apoptosis accompanied by upregulation of proapoptotic proteins, Fas and TRAIL expression (Bitko et al., 2007; Kotelkin et al., 2003; O'Donnell et al., 1999). In addition, Gibbs et al showed that RSV infection of tumor cells induces cell cycle arrest (in G0/G1 phase) (Gibbs et al., 2009). In addition, few studies demonstrated that RSV doesn't induce EMT markers in infected NSCLC cells; as RSV infection upregulates E-cadherin expression in infected A549 cells (Wang et al., 2000), and doesn't change the expression of mesenchymal markers, such as  $\alpha$ -SMA and Vimentin (Kaltenborn et al., 2012).

It was shown that RSV infection of primary human bronchial epithelial cell results in strong expression of PD-L1 expression and increase of CD8<sup>+</sup> T cell activation, proliferation, and antiviral function, which is even more increased by blocking PD-L1 (Telcian et al., 2011). An increased of PD-1 expression on lung CD4<sup>+</sup> and CD8<sup>+</sup> T cells following RSV infection in mice after RSV infection of mice (Yao et al., 2015). In the context of murine NSCLC tumors, we found that RSV infection leads to a transient increase in PDL1 expression by tumor cells, which comes back to normal value at 96h pi, mediated by the production of type-I IFNs. In addition CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the lungs of infected mice express less PD1 at 19 days post-tumor inoculation, as compared with non-infected mice. This could be due to different timing of the experiment or the lysis of infected CD4<sup>+</sup> and CD8<sup>+</sup> T cells, who possibly express higher PD1.

However, further studies are needed to confirm the results of RSV infection in our murine *in vivo* model, as several **limitations** can be observed in the design of the model:

1. In our *in vivo* model, we injected LLC cells that were either infected or not (as a control) before the injection. In optimal design, we wanted to inject LLC cells first, wait for few

days, until the development of tumor and TME, and then infect the mice intranasally by RSV, which mimics the pathophysiology of RSV infection in human NSCLC patients. However, this was not possible, as RSV infection of C57BL/6J mice was restricted to upper respiratory tract and didn't reach the lungs to be able to infect tumor and immune cells in the TME. While it was shown in different studies that RSV infection of BALB/c mice leads to important infection in the lungs, there are no commercially available murine NSCLC cell lines, derived from BALB/c genetic background that can be used to induce tumor in BALB/c mice.

2. In our in-vivo model, we found some heterogeneity in the growth of non-infected LLC cells (control), which we overcame by increasing the number of mice and repeating the experiment. We tried another method to deliver LLC cells to lungs by directly injecting LLC cells in the intercostal space. Tumor growth in the control group was homogenous in this model, but the tumors developed in the plural cavity, not inside the lung parenchyma, so we preferred the intratracheal injections of LLC cells. In addition, the development of NSCLC tumors was fast (20 days until the need to euthanize mice, which doesn't reflect the evolutionary course of disease progression in human).
3. In optimal circumstances, to be able to compare the impact of IAV and RSV infections in murine NSCLC model, we should be able to use the same model. However, using the spontaneous KP NSCLC model for RSV infection was not possible, due to the fact that KP mice are developed from mice with C57BL/6J background.
4. Studying the impact of IAV and RSV infections in human NSCLC patients by infecting patients is apparently not possible due to ethical consideration. In addition, clinical data



of resected NSCLC patients doesn't include routine screening of RSV/IAV infections. However, we showed that resected tumors can be infected by RSV and IAV, which doesn't exclude our hypothesis of a possible impact of RSV and IAV on human NSCLC progression. My team showed influenza infection induces a distinct transcriptional profile in ex vivo cultures of primary human lung tumors, that is associated with immune antiviral response and immune response processes, including myeloid cell activation, granulocyte activation, T cell activation, cytokine production and antigen processing and presentation (Data under publication). Thus, IAV infection modulates the human lung TME, affecting both tumor-associated features (lipid metabolism, oncogenesis) and immune response, at early stages of the infection.

5. In addition, care must be taken when interpreting the results as cell lines do not always accurately replicate the primary cells. As serial passage of cell lines can further cause genotypic and phenotypic variation over an extended period of time and genetic drift can also cause heterogeneity in cultures at a single point in time. However, we tried to repeat the experiments on different cell lines, and we showed that the results are repetitive in majority of experiments.

To overcome these limitations, there is a need to find better in-vivo model. Two major categories exist; genetically engineered mouse models (GEMMs) and carcinogen-induced NSCLC. The optimal is to repeat the experiments that I did in a murine model of Balb/c background that has slow rate and homogenous NSCLC growth, with the ability to wait until the development of lung TME, and then followed by intranasal inoculation of RSV or IAV in the

same experiment for comparison purposes. Unfortunately I couldn't have access to such optimal model in my studies! One candidate is the Urethane induced lung adenocarcinoma in balb/c mice (Janker et al., 2018). However, it takes 4 months to develop tumors and it needs sophisticated techniques to follow tumor progression; as it's not possible to follow it by IVIS.

In addition, there is a need to repeat the in-vitro results on human and murine NSCLC organoids and spheroids that are cultured in 3 dimensions. Organoids are highly complex and are more in vivo-like when compared to spheroids, and they show to predict how well patients respond to cancer drugs to aid in personalized medicine (Gilazieva et al., 2020).

### **3. Future Directions**

Considering the high incidence and availability of quick diagnostic methods for RSV and IAV, regular testing of both viruses in NSCLC patients should be recommended in routine practice. This will be crucial to define the therapeutical strategy in these patients.

#### **RSV Infection**

In the case of RSV infections, most data regarding RSV in cancer patients originate from hematological malignancies (HM) and very little is known regarding patients with NSCLC. Thus, there is a need to investigate the impact of RSV infection in these patients in short-term, as well as long term.

#### **Oncolytic RSV virotherapy**

Although adenoviruses are the primary option of oncolytic virotherapy for NSCLS, other viruses, including herpes simplex virus, coxsackievirus, Newcastle disease virus, Seneca valley virus, and reovirus, have also been reported (Alvanegh et al., 2021).

As we and others have introduced additional evidence that RSV imposes a potent oncolytic effect on different malignancies, designing therapies that involve intratumoral delivery of non-pathogenic engineered strain of RSV or intratumoral expression of RSV- fusion (RSV-F) protein may lead to a significant regression of NSCLC tumors.

In fact, NSCLC cancer is an ideal therapeutic target for this approach owing to the possibility of delivering viral particles through the upper respiratory tract, the high infectivity of RSV in human patients, the induction of immune responses and the limited efficacy of current treatment protocols of complicated NSCLC tumors even after the introduction of immunotherapy.

Engineered RSV that can also deliver immunomodulatory genes, drug-metabolizing genes, pro-apoptotic genes, and even therapeutic miRNAs to tumor cells, can enhance the oncolytic abilities of RSV in NSCLC tumors. This promises to overcome many of the current shortages and difficulties of current NSCLC treatment methods, such as drug resistance.

Another benefit is that engineered RSV can act as a vaccine towards high pathogenic RSV strains which could cause a severe and complicated RSV disease on short term, in addition to treating NSCLC tumors. However, this immunogenicity can hinder the repetitive use of RSV in virotherapy.

Using RSV as an oncolytic virus could also be accompanied by other targeted and immune therapies to promote tumor elimination. However, further studies are needed to validate such utility in murine models, and later human patients.

### **Anti-RSV treatment**

If RSV infection is found to be associated with severe complications in NSCLC patients in the short-term, then there is a need to find the optimal anti-viral therapy in this group of patients. Inhaled ribavirin and palivizumab are currently the only registered treatment options for RSV in addition to supportive care (Zwart et al., 2020); however, inhaled ribavirin is rarely used in nonimmunocompromised adults because of the limited evidence for its efficacy, its price, and the occupational risk to healthcare workers exposed of ribavirin aerosols. Vaccines and new antivirals are being tested, but they are not yet available for daily practice (Zwart et al., 2020). However, oral ribavirin could be beneficial to prevent the short-term complications of RSV infection in NSCLC patients, if confirmed.

### **Influenza Infection**

As influenza infections have severe short-term complications and are associated with high mortality of in NSCLC patients, as well as their protumoral effects on tumor progression that we demonstrated in in-vitro and in-vivo murine models, it is indispensable to unravel the impact of these infections on cancer patients, to identify appropriate therapies to block tumor progression and metastasis. In addition, it is indispensable to define the optimal strategy to treat patients in such conditions. A better understanding of the mechanistic effects of influenza infection in different cells within the tumor-suppressive microenvironment may enable the development of novel therapeutic strategies to treat cancer patients. This would help adapt therapies in susceptible patients, for a better chance of success, and should be integrated into the arsenal of personalized therapies under development in lung cancer.

In fact, one of the mechanistic effects of influenza infection could be mediated by sensing of IV infection by tumor cells which may lead to protumoral effects, through the secretion of type-

I/III IFNs that leads to High ratio of ISGs in tumor cells. In support of this hypothesis, it was shown that high ratio of ISGs in tumor cells and low ratio of ISGs in immune cells (T cells, NK cells, and macrophages) correlates with resistance to radiation, chemotherapy and immune checkpoint blockade. This ratio is independent of tumor mutational burden (TMB)(Benci et al., 2019) and is concurrent with T cell exhaustion via upregulation of PD-L1 by tumor cells. STAT1-overexpressing tumor cells demonstrate metastatic potential and resistance to IFN- $\gamma$ , ionizing radiation (IR), and doxorubicin. A subset of ISGs (about 30 ISGs) is consistently overexpressed in therapy-resistant cancer cells. This subgroup of ISGs includes IFI27 (ISG12), IFIT1, Mx1, ISG15, BST2, OAS1, OAS3, OASL, and others (Khodarev et al., 2009). A high ISGs score is found in a group of The Cancer Genome Atlas Program (TCGA) primary lung tumors that correlates with upregulation of multiple immunosuppressive factors thereby evading immune control (Liu et al., 2019b).

In addition, finding strategies to prevent and treat these infections can improve infection-related mortalities, and reduce the health, social and economic burden that arise from them. Thus, it's highly recommended to vaccinate NSCLC patients, their families and health care staff, as well as administrating the anti-viral therapy in case of infection.

### **Anti-flu treatment and influenza vaccine**

Antiviral treatment is recommended as early as possible for any cancer patient with confirmed or suspected influenza, ideally within 48 hours of symptom onset. The neuraminidase inhibitor, oseltamivir, is the recommended antiviral for the treatment and prophylaxis of patients with severe, complicated, or progressive illness. Because of the prolonged influenza viral replication in immunocompromised patients, including cancer patients, the rate of developing a resistance

for antiviral treatments may be higher than otherwise healthy adult patients (Mitha et al., 2019). This emphasizes the importance of timely antiviral resistance testing in cancer patients, and the prevention of the transmission of resistant influenza strains among cancer patients.

A better strategy to prevent influenza infection in cancer patients is flu vaccine. As the use of the live attenuated vaccine is contra-indicated in cancer patients, inactivated Influenza vaccines are the only available vaccine platform. However, few scarce studies have shown that the immunogenicity of available inactivated Influenza vaccines in cancer patients is lower than for healthy controls, where vaccination results in the prevention of clinical infection in 70–90% of individuals and in lower rate of influenza-related hospitalization and mortality (Volllaard et al., 2017).

Importantly, a study that enrolled patients with chronic obstructive pulmonary disease (COPD) demonstrated that influenza vaccination reduced the incidence of lung cancer (Chen et al., 2019). Mechanistically, this has been confirmed in murine studies where intra-tumoral vaccination was shown to reactivate CD8<sup>+</sup> T cell-mediated antitumor immunity and to sensitize resistant tumors to checkpoint blockade (anti-PD-L1), and provides protection against subsequent active IAV lung infection (Newman et al., 2020) (Figure 1).

#### **Further Questions:**

Despite the remarkable efforts that we and others have tried to understand the impact of RSV and influenza infection in lung cancer, there are still many questions yet to be answered.

- What can be the short and long-term outcomes of influenza and RSV infections in lung cancer patients?

- How should physicians personalize lung cancer therapeutical protocol, in the case of viral respiratory infection? Will immunotherapy with anti-PD1/anti-PDL1 antibodies integrate in the therapeutical protocol of lung cancer patients, complicated by IV infection? At what bases, patients will be chosen for such therapy?
- Is it possible to improve influenza vaccine immunogenicity in lung cancer patients by trying different vaccination strategies, such as using adjuvanted vaccine, two vaccine doses in the same influenza season, intranasal vaccine injections, different timing of vaccination, and high-dose vaccines?
- What could be the best anti-influenza and anti-RSV medications to be added to the therapy strategy of lung cancer patients? How can we avoid the development of drug resistance?
- Considering that influenza and RSV can cause viremia, how may these infections impact the progression of other cancers?
- What could be the impact of other respiratory viral infections, such as COVID-19, on NSCLC patients, in terms of their complications and their impact on tumor progression? Lung cancer patients are identified as a particularly high-risk group for SARS-CoV-2 infection and its complications. Patients with cancer are more susceptible to infection than individuals without cancer because of their systemic immunosuppressive state caused by the malignancy and anticancer treatments, such as chemotherapy or surgery, and they have a poorer prognosis and more severe disease (Gupta et al., 2020; Liang et al., 2020). Inflammation, high neutrophil activation and a strongly impaired adaptive immune response are observed in patients who experience the more severe form of the disease (Russick et al.,

2021). However, the impact of SARS-CoV-2 infection on lung cancer progression is not studied yet, and it will be very essential to determine whether SARS-CoV-2 has protumoral or antitumoral effects. Another important question is: what will be the impact of different Covid-19 vaccines on the progression of NSCLC tumors? Apparently, further studies are needed to answer these questions.



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## Index 1 Review Article

Angrini, M., Varthaman, A., Garcia-Verdugo, I., Sallenave, J., Alifano, M., and I, C. (2021). To Vaccinate or not: Influenza Virus and Lung Cancer Progression. *Trends in Cancer* 7, 573–576.

### To vaccinate or not: Influenza virus and lung cancer progression

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#### Abstract

Influenza virus infection leads to severe and complicated disease particularly in lung cancer patients. It alters the tumor microenvironment (TME), which may potentiate lung cancer progression and disrupt responses to anti-tumoral treatments. Consequently, flu vaccination and antiviral treatments should be recommended to all lung cancer patients.

**Keywords:** Influenza virus, lung cancer, vaccine in cancer patients

## **Introduction**

Patients with cancer, and particularly those with lung cancer, are at a higher risk of morbidity and mortality from seasonal influenza. Monthly mortality rates for low and high flu months are 4.9 and 5.7% in lung cancer patients (Kinslow et al., 2019). Additionally, the appearance of malignant cancer is linked to the prevalence of influenza infection that occurred one year before the detection of cancer (Inaida and Matsuno, 2020) and a higher risk of developing lung cancer is related to cumulative exposure to the virus (Weng et al., 2019). Recent studies have suggested that treatment with antivirals or vaccination against influenza may provide clinical benefit, including reduced hospitalization and reduced mortality, to patients with cancer (Volvaard et al., 2017). Mechanistically, vaccination may limit tumor progression by modulating anti-tumor immune responses. Herein, we stress the importance of vaccination against influenza in reducing lung cancer progression and incidence and discuss the mechanisms that may contribute to this reduced mortality.

## **Influenza induced inflammation and associated tumor microenvironment changes**

Cancer patients display higher persistent viral replication that may result in chronic inflammation, which is considered a hallmark of cancer and is strongly associated with tumor induction and/or progression. In lung cancer, chronic inflammation induced by influenza is due to systemic and lung immunosuppression that results in lower viral clearance, and influenza specific RNA persistence in the lungs for more than one hundred days post-infection (Keeler et al., 2018). The prolonged infection and the resulting chronic inflammation lead to profound

alteration of the tumor microenvironment (TME), which worsens tumor progression and disrupts responses to treatments.

Toll-like receptor (TLR) signaling in immune cells plays an essential role in immune surveillance and activation through recognition of pathogen-associated molecular patterns and stimulation of downstream proinflammatory cytokines. However, when expressed by cancer cells similar stimulation can promote tumor progression and survival (Chatterjee et al., 2014). Viral RNA detected by TLR7, which is highly expressed by lung tumor cells in many patients (Chatterjee et al., 2014), activates interferon regulatory factor (IRF), nuclear factor-kappa B (NF- $\kappa$ B) and mitogen-activated protein kinase (MAPK) signaling pathways, followed by induction of inflammatory responses and the production of type I interferons (IFN-I), which promotes the expression of interferon-stimulated genes (ISGs). The persistence of viral RNA in the lungs following influenza infection may induce a prolonged stimulation of TLR7 in tumor cells – as we observed with repeated stimulation with synthetic TLR7 agonists- leading to overactivation of NF- $\kappa$ B and overexpression of Bcl2. This activation increases tumor cell survival and metastasis, and induces poor response to chemotherapy (Chatterjee et al., 2014).

Influenza may also alter the anti-tumor immune response through T cell exhaustion and immunosuppression. Influenza infection of tumor bearing mice shunted anti-tumor CD8<sup>+</sup> T cells to the infection site, where they express high levels of the inhibitory receptor programmed cell death 1 (PD-1) resulting in inhibition of T cell function (Kohlhapp et al., 2016). Influenza virus-infected cancer cells also downregulate class I major histocompatibility complex (MHC-I)

expression, which promotes both tumor escape from cytotoxic CD8<sup>+</sup> T cells leading to tumor progression and delayed viral clearance(Koutsakos et al., 2019).

Influenza virus favors the establishment of an immunosuppressed TME, which promotes tumor growth. In a cohort of lung cancer patients from the cancer genome atlas (TCGA), a high ISGs score correlates with upregulation of multiple immunosuppressive factors including PD-L1, PD-L2, IDO1 and LGALS9. This finding suggests that an additional mechanism by which infected lung cancer cells can evade immune control(Benci et al., 2016).

Viral persistence can also lead to development of resistance to anti-tumoral treatments via TME changes. Tumors resistant to immunotherapy exhibit sustained IFN-I signaling (Benci et al., 2016), similar to changes induced by viral persistence in the TME. Sustained IFN signaling in tumor cells induces STAT1-related epigenomic changes and increases the expression of PDL1 and NOS2 in antigen presenting cells, leading to increased resistance to immune checkpoint blockade of the PD1/PDL1 axis(Jacquelot et al., 2019). Moreover, a high ratio of ISGs in tumor cells versus ISGs in immune cells (T cells, NK cells, and macrophages) correlates with resistance to radiation, chemotherapy, and immune checkpoint blockade(Benci et al., 2019) (Figure 1).

### **Antiviral treatment and influenza vaccine**

Considering the multiple, deleterious impacts of influenza infection on cancer progression, strategies to optimize influenza prevention among patients with cancer are highly warranted. Influenza vaccines and specific antiviral, flu medications are two suggested preventive therapeutics to be considered.



The optimal strategy to prevent influenza infection in cancer patients is vaccination. As the use of the live attenuated vaccine is contra-indicated in cancer patients, inactivated Influenza vaccines are the only available vaccine platform. Influenza vaccination correlates with reduced incidence of lung cancer as exemplified in patients with chronic obstructive pulmonary disease (COPD), which is a well-known risk factor for lung cancer (Chen et al., 2019). In patients undergoing curative surgery for solid cancers, giving a flu vaccine in the postoperative period results in less overall mortality and cancer-related mortality, compared with patients never receiving a vaccine (Gögenur et al., 2020). Vaccination not only prevents influenza from modulating the TME directly, but it could also promote anti-tumor immunity in cancer patients. This has been demonstrated in murine studies where intra-tumoral flu vaccination reactivates CD8<sup>+</sup> T cell-mediated antitumor immunity and sensitizes resistant tumors to immune checkpoint blockade (anti-PD-L1) (Newman et al., 2020).

However, few studies have shown that the immunogenicity of available inactivated Influenza vaccines in cancer patients varies between 10 and 60%, whereas vaccination prevents clinical infection in 70–90% of healthy individuals and lowers the rate of influenza-related hospitalization, and mortality (Vollaard et al., 2017). This suggests that healthy individuals, especially patient relatives and healthcare providers should also be encouraged to get the flu vaccine to prevent those with cancer from catching the virus. In order to improve influenza vaccine immunogenicity in lung cancer patients, different vaccination strategies should be investigated, such as using adjuvanted vaccine, two vaccine doses in the same influenza season, intranasal vaccine injections, different timing of vaccination, and high-dose vaccines.

In addition, flu vaccination reduces the number of oncologic treatment interruptions compared to non-vaccinated cancer patients. Of note, receiving active chemotherapy doesn't change significantly the vaccine effectiveness among patients with solid tumor cancer (Vollaard et al., 2017). However, studies that investigate the impact of Flu vaccination on targeted or immunotherapy are lacking to bring a strong conclusion.

For cancer patients who are not vaccinated, antiviral treatment such as neuraminidase inhibitor, (oseltamivir: Tamiflu), is recommended as early as possible for cancer patients with confirmed or suspected influenza, to reduce viral load and consequently limit the pro-tumoral effect of virus, with a probable impact on morbidity and mortality. However, because of viral persistence in cancer patients, the virus accumulate mutations during the course of infection, which leads to acquire resistance against antiviral treatments (Mitha et al., 2019). This emphasizes the recommendation of timely identifying resistant mutations that arise in the neuraminidase in infected cancer patients, and the importance to prevent the transmission of resistant influenza strains among cancer patients.

### **Concluding remarks**

Influenza infection is more frequent, severe and complicated in cancer patients. Finding strategies to prevent and treat these infections can improve infection-related mortalities, and reduce the health, social and economic burden that arise from them. We must understand the impact of these infections on tumor progression, their short and long-term outcomes and define an optimal strategy to treat patients in such conditions. WE HAVE SAME ISSUES WITH COVID-19

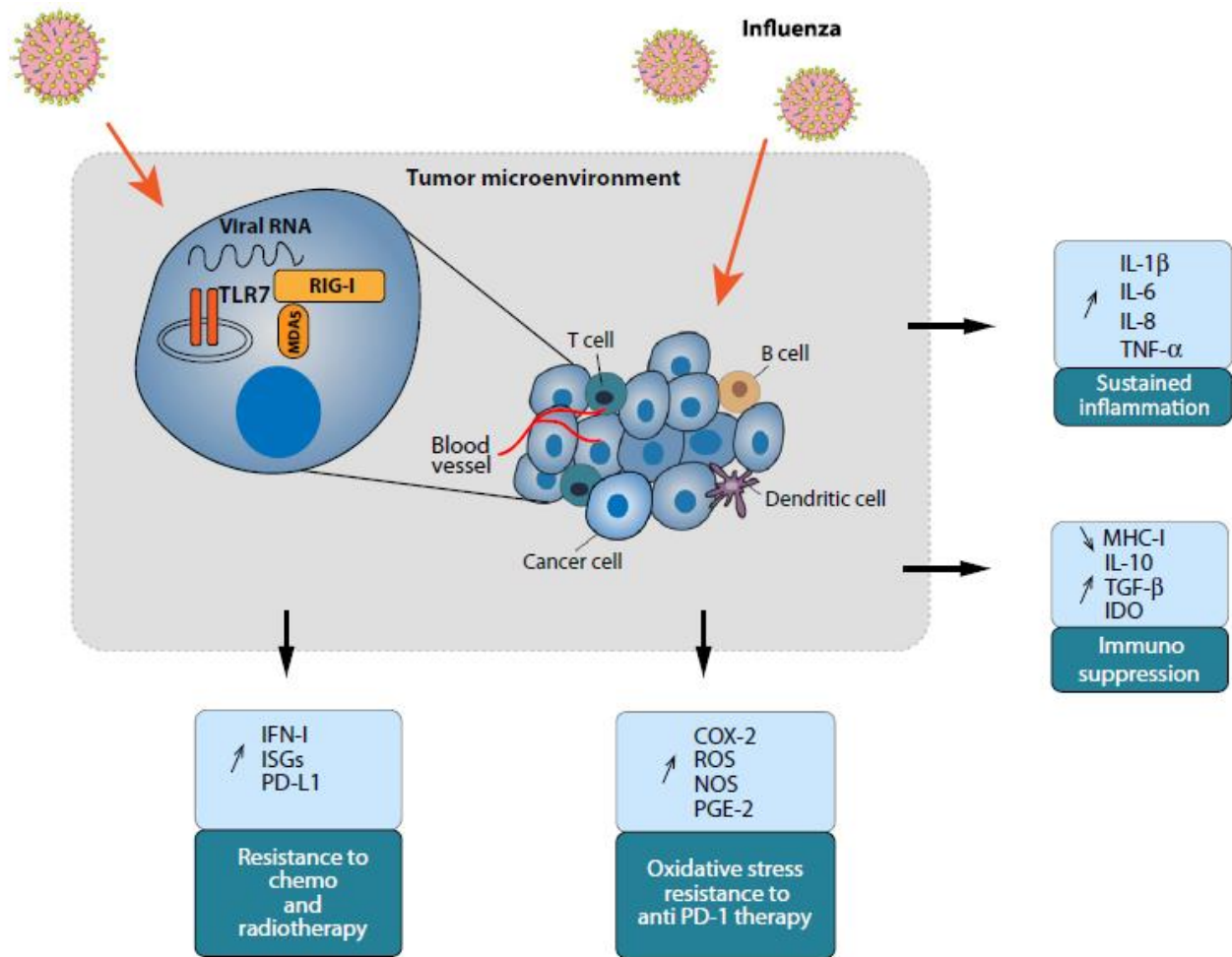
A better understanding of the mechanistic effects of influenza infection in different cells within the tumor-suppressive microenvironment may enable the development of novel therapeutic strategies to treat cancer patients. This knowledge would help adapt therapies in susceptible patients, for a better chance of success, and should be integrated into the arsenal of personalized therapies under development in lung cancer (Figure 2). Annual influenza vaccination, inexpensive and relatively safe preventive, in addition to early initiation of antiviral therapy should be highly recommended in this high-risk population, their relatives and healthcare providers. The capacity to improve the immunogenicity of vaccine is even expected to give a better protection in lung cancer patients.

Despite efforts to understand the impact of influenza infection in lung cancer patients, there are still many unanswered questions:

What are the short and long-term outcomes of influenza infection in lung cancer patients?

How should physicians personalize lung cancer therapeutic protocols, in the case of influenza infection?

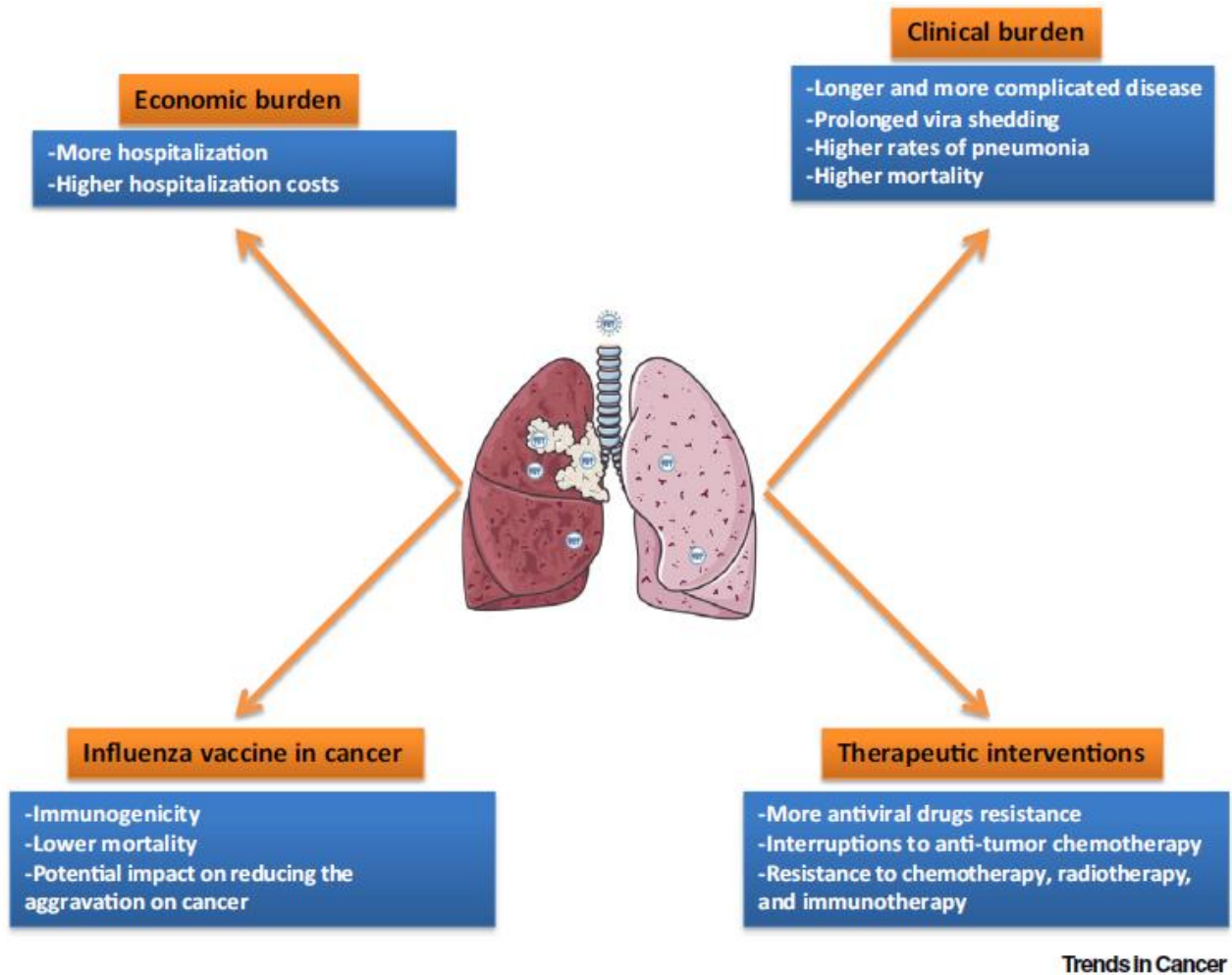
What is the best anti-influenza medication to be added to the therapy strategy of lung cancer patients? How can we avoid the development of drug resistance? Considering that influenza can cause viremia, does influenza infections influence the progression of other cancers?



Trends in Cancer

**Figure 1: Summary of the impact of influenza infection on the tumor immune microenvironment.**

Influenza virus may infect tumor and immune cells leading to modulation of numerous immune characteristics: viral infection increases chemokine secretion (that are involved in immune cell migration), increases sustained type I IFNs and ISGs production (leading to resistance to treatments), increases reactive oxygen species (ROS) (involved in oxidative stress), increases immunosuppressive molecules (leading to immunosuppression), and increases inflammatory cytokines.



**Figure 2: Consequences of influenza infection in patients with lung cancer.** Influenza infection of lung tumor patients has multifactorial impacts in economy, health, flu vaccine strategies and anti-viral and anti-tumoral treatments.

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## Index 2: Book Chapter

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# Toll-Like Receptors (TLRs) in the Tumor Microenvironment (TME): A dragon-Like Weapon in a Non-Fantasy Game of Thrones

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## Abstract

Toll-like receptors (TLRs) in the tumor microenvironment (TME) are expressed not only in innate and adaptive immune cells, but also in stromal cells such as fibroblasts, endothelial cells (EC) and tumor cells. The role of TLR signaling in the TME is complex and controversial due to their wide expression within the TME. Moreover, TLR signaling may culminate in different outcomes depending on the type of tumor, the implicated TLR, type of TLR ligands, and most importantly, the main type of cell(s) that are targeted by TLR ligands. Understanding to what extent these complex TLR signals impact on tumor progression merits further investigation, as it can help improve existing anti-cancer treatments or unravel new ones. In most cases, TLR signaling in tumor cells and in immune cells is associated with protumoral and antitumoral effects respectively. A better understanding of the relationship between TLRs and the TME,

especially in humans, is required to design better anti-cancer therapies, considering that most current TLR-involved treatments were disappointing in clinical trials.

In this chapter, we will discuss the impact of TLR signaling on the hallmarks of cancer, by highlighting their effects in tumor, immune and stromal cells within the TME. Furthermore, we will discuss how the understanding of the role of TLRs can pave the way to develop new anti-cancer treatments and even predict clinical outcome and chemotherapy efficacy.

**Keywords:** TLRs, Tumor microenvironment, DAMPs, PAMPs, CAFs, TAMs, MDSC, TECs, TLR agonist, Cancer, Anti-tumor immune response, chemoresistance, Angiogenesis, Metastasis, TLR polymorphisms, Immunotherapy, TLR-based treatment, microbiota, Cancer vaccine

## **Introduction**

In addition to cancer cells, the tumor microenvironment (TME) is composed of stromal cells, extracellular matrix and infiltrating immune cells. Stromal cells include fibroblasts, myofibroblasts, vascular and lymphovascular endothelial cells and stressed normal epithelial cells. These cells play multifaceted roles during cancer progression as, depending on local and systemic conditions, they can promote or inhibit tumor development (Werb et Lu., 2016)<sup>1</sup>.

Tumor antigens can activate the immune system eliciting an antitumor response which in some cases can result in tumor destruction. During early stages of tumor development, cytotoxic immune cells such as natural killer (NK) and CD8+ T cells recognize and eliminate the more immunogenic cancer cells. As the less immunogenic variants of cancer cells evolve to a clinically detectable tumor, different subsets of inflammatory cells impact on tumor fate (Gonzalez et al., 2018)<sup>2</sup>. Inflammation is considered an important hallmark of cancer; it promotes tumor development and progression, and has been associated with higher tumor grades and a poor prognosis (Hanahan et al., 2011<sup>3</sup>; Sato et al., 2009<sup>4</sup>). NF-



$\kappa$ B activation - which is seen in most tumor cells - plays a key role in tumor initiation, progression, metastasis and chemoresistance by mediating the production of a large variety of proinflammatory cytokines, chemokines, growth factors, collagenases and antiapoptotic proteins (Basith et al., 2012)<sup>5</sup>.

As the largest family of pattern recognition receptors, Toll-like receptors (TLRs) are among the major activators of NF- $\kappa$ B and are involved in inflammation in the TME. In addition to the well-described roles of TLRs in innate and acquired immunity against microbial infection, and their role in regulating tissue repair and regeneration, emerging studies now implicate TLRs in inflammation-associated cancers (Gribar et al., 2008)<sup>6</sup>.

TLRs are not only expressed in innate and adaptive immune cells, but also in stromal cells such as fibroblasts, endothelial cells and most remarkably in tumor cells. Recognition of natural TLR ligands shape the TME by mediating both pro- and anti-tumorigenic pathways. On the one hand, abnormal activation of TLRs in tumor cells can facilitate aberrant cytokine profiles associated with immune tolerance and tumor progression. On the other hand, in preclinical studies, TLR agonists incite anti-tumor responses. Nonetheless, most TLR agonists used as stand-alone antitumor drugs have yielded limited success in clinical trials (Huang et al., 2018)<sup>7</sup>.

In this chapter, we will describe the complex mechanisms underlying TLR responses in the TME, and how TLR signaling in different cells, including tumor cells, reshapes the TME. Understanding this will facilitate the development of more effective TLR-based therapeutic strategies in a wide variety of cancers.

## **1. What are Toll-like Receptors?**

TLRs are the largest and most well-studied family of pattern recognition receptors (PRRs). In 2011, Bruce Beutler and Jules Hoffmann were awarded the Nobel Prize in Physiology or Medicine for their

discoveries of the role of TLRs in innate immunity, highlighting the importance of TLRs in physiology as well as pathology.

TLRs are type I transmembrane glycoproteins characterized by the presence of an extracellular domain (ECD), a single transmembrane helix, and an intracellular Toll-like/interleukin-1 (IL-1) receptor (TIR) domain. The ECD domain recognizes TLR ligands while the TIR domain is responsible for downstream signaling (Manavalan et al., 2011)<sup>8</sup>.

To date, 10 and 12 functional TLRs have been identified in humans and mice, respectively. TLR1-9 are conserved in both species; however, mouse TLR10 is not functional, and TLR11-13 have been lost from the human genome (Kawai and Akira, 2010)<sup>9</sup>.

TLR1, 2, 5, 6 and 10 (extracellular TLRs) are largely localized on the cell surface while TLR3, 7, 8 and 9 (intracellular TLRs) are localized in intracellular organelles, such as endosomal/lysosomal compartments and the endoplasmic reticulum (ER). TLR4 is unique as it is localized to both the plasma membrane and endosomal vesicles (Kagan et al., 2008)<sup>10</sup> (Figure 1).

The adapter protein Unc-93 homolog B1 (UNC93B1) guides intracellular TLRs (Nucleic acid-sensing TLRs) from the ER to their respective endosomal signaling compartments, and the flagellin receptor TLR5 to the cell surface, whereas other cell-surface TLRs, such as TLRs 2 or 4, function independently of UNC93B1. Loss of UNC93B1 results in near-complete loss of TLR3 and TLR7 signaling in primary splenic mouse dendritic cells and macrophages (Pelka et al., 2018)<sup>11</sup>. Importantly, murine DCs with a non-functional mutation in Unc93b1 (3d mutation) exhibit compromised antigen cross-presentation and anti-tumor responses (Maschalidi et al., 2017). TLRs that reach endocytic compartments are cleaved by proteases in order to signal (Ewald, SE,2011<sup>12</sup>; Garcia-Cattaneo A, 2012<sup>13</sup>).

### **1.1. Exogenous and Endogenous TLR Ligands**

In addition to synthetic TLR agonists, two types of natural TLR ligands exist: exogenous pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) (**Table 1**).

PAMPs represent evolutionarily conserved microbiota/pathogen-specific sugars, lipoproteins or nucleic acids essential for the survival of entire classes of pathogens, and are distinguishable from “self” moieties (Mogensen, 2009<sup>14</sup>; Ellerman et al., 2007<sup>15</sup>). Below are a few examples of PAMP-associated effects in cancer:

- TLR signaling is implicated in an association between the microbiota and gastric, bladder, hepatobiliary, pancreatic, lung and colorectal cancer (Nagano et al., 2019). The microbiota and its metabolites activate TLRs and result in inflammation and critical signaling pathways, which promote the malignant behavior of host cells. The microbiota can also increase susceptibility to infections that upregulate the expression of certain TLRs promoting carcinogenesis (Mao et al., 2018)<sup>16</sup>.
- Gut microbiota can selectively activate mucosal endothelial cells in CRC to promote specific pro-angiogenic responses in a TLR-dependent fashion (Schirbel et al., 2013)<sup>17</sup>. Following activation by commensal microbiota-derived TLR ligands, epithelial calcineurin supports the survival and proliferation of tumor stem cells and promotes colorectal cancer (CRC) (Peuker et al., 2016)<sup>18</sup>.
- TLR4 and the intestinal microbiota promote Hepatocellular Carcinoma (HCC) by increasing proliferation, expression of the hepatomitogen epiregulin, and by preventing of apoptosis (Dapito et al., 2012)<sup>19</sup>. Lipoteichoic acid, a component of cell walls of gut microbiota and a ligand of TLR2, increases in liver cancer and upregulates cyclooxygenase-2 (Cox-2) expression, which in turn leads to the overproduction of prostaglandins (Loo et al., 2017)<sup>20</sup>.

- Lung commensal microbiota promotes murine lung cancer development via  $\gamma\delta$  T cells in a mechanism that could be mediated by TLR ligands derived from the lung microbiota. TLR signaling leads to the production of IL-1 $\beta$  and IL-23 from alveolar macrophages and neutrophils, thereby inducing the proliferation and activation of pro-inflammatory lung-resident  $\gamma\delta$  T cells (Jin et al, 2019)<sup>21</sup>.

DAMPs constitute structurally diverse and evolutionarily unrelated multifunctional endogenous danger signals, that communicate cell injury to the host and promote early innate and adaptive immune responses important for the mobilization of repair mechanisms. Interestingly, DAMPs are hidden from recognition by the immune system under normal physiological conditions. But, under conditions of cellular stress/tissue injury, such as cancer, these molecules can be actively produced by stressed immune cells, epithelial cells and cancer-associated fibroblasts (CAFs). In addition, neo-antigens and tumor-associated antigens can be passively released into the TME from necrotic and apoptotic cells or from the damaged extracellular matrix to act as DAMPs (Land, 2015<sup>22</sup>; Srikrishna et al., 2009<sup>23</sup>) that activate TLRs (Ouyang et al., 2012)<sup>24</sup>. These DAMPs interact with TLRs on infiltrating inflammatory cells, fibroblasts, tumor endothelial cells (TECs) and tumor cells, and play critical roles in both extrinsic and intrinsic pathways of cancer-related inflammation. Below are a few examples of DAMP-associated effects on cancer:

- HMGB1, the most abundant DAMP, is released by dying hypoxic tumor cells in the TME, binds to TLR2, TLR4 and TLR9 and contributes to tumor progression (He et al., 2017)<sup>25</sup>. During hypoxia, HMGB1 translocates from the nucleus to the cytosol, binds to mitochondrial DNA (mtDNA) released from damaged mitochondria and further activates TLR9 promoting the proliferation of hepatocellular carcinoma cells (Liu et al., 2015)<sup>26</sup>.

- The HMGB1-TLR4 interaction leads to the production of pro-inflammatory and proangiogenic cytokines and activation of ECs, macrophages, endothelial progenitor cells (EPC), and mesoangioblasts, all of which could contribute to vessel formation (Yang et al., 2014)<sup>27</sup>. Furthermore, by inducing autophagy, HMGB1 accumulation in the TME promotes the survival, differentiation and suppressive activity of myeloid-derived suppressor cells (MDSCs) (Parker et al., 2014)<sup>28</sup>, which are known to inhibit anti-tumor immunity and promote tumor progression (Parker et al., 2016)<sup>29</sup>.

**Table 1: A List of Natural TLR Ligands (DAMPs and PAMPs) for Each TLR ((Sato et al., 2009), (Das et al., 2016), (Basith et al., 2012), (Henrick et al., 2019)).**

TLR	DAMPs	PAMPs
TLR1		<ul style="list-style-type: none"> <li>• Triacyl lipoproteins</li> <li>• Soluble factors: Neisseria</li> </ul>
TLR2	<ul style="list-style-type: none"> <li>• Heat shock proteins (HSP60, HSP70, HSP96)</li> <li>• High-mobility group protein B1 (HMGB1)</li> <li>• Hyaluronic acid</li> <li>• pancreatic adenocarcinoma upregulated factor (PAUF)</li> <li>• monosodium urate</li> <li>• versican</li> <li>• matrix metalloproteinase-2 (MMP-2)</li> </ul>	<ul style="list-style-type: none"> <li>• Peptidoglycan</li> <li>• Lipoprotein/lipopeptides</li> <li>• Lipoteichoic acid</li> <li>• Phenol-soluble modulins</li> <li>• Heat-killed bacteria</li> <li>• Porins</li> <li>• Structural viral proteins</li> <li>• Zymosan</li> </ul>
TLR3	<ul style="list-style-type: none"> <li>• Self double-stranded RNA (dsRNA), mRNA</li> <li>• Heat shock proteins (HSP27)</li> </ul>	viral dsRNA
TLR4	<ul style="list-style-type: none"> <li>• Heat shock proteins (HSP22, HSP60, HSP70, HSP96)</li> <li>• Fibrinogen &amp; Fibronectin</li> <li>• Heparan sulfate</li> <li>• Hyaluronic acid (Hyaluronan)</li> <li>• HMGB1, <math>\beta</math>-defensin 2</li> <li>• Surfactant-protein A</li> <li>• Glycoprotein 96 (gp96)</li> <li>• Peroxiredoxin</li> </ul>	<ul style="list-style-type: none"> <li>• Heat shock proteins (HSP60 of Chlamydia)</li> <li>• Lipopolysaccharides (LPS)</li> <li>• RSV fusion protein</li> <li>• MMTV envelope proteins</li> <li>• Paclitaxel</li> <li>• Glycoinositolphospholipids</li> <li>• Taxol: Plant product.</li> </ul>
TLR5	HMGB1	Flagellin (Gram-positive or Gram-negative bacteria)
TLR6		<ul style="list-style-type: none"> <li>• Lipoteichoic acid</li> <li>• Diacyl &amp; Triacyl lipoproteins</li> <li>• Zymosan</li> </ul>

		<ul style="list-style-type: none"> <li>• Heat-labile soluble factor</li> <li>• Phenol-soluble modulin</li> </ul>
TLR7/8	Self ssRNA	Viral ssRNA
TLR9	<ul style="list-style-type: none"> <li>• Self DNA</li> <li>• mitochondrial DNA (mtDNA),</li> <li>• IgG-chromatin complexes</li> <li>• HMGB1</li> </ul>	<ul style="list-style-type: none"> <li>• Bacterial and viral Unmethylated CpG DNA motifs</li> <li>• Hemozoin</li> </ul>
TLR10	Unknown	HIV-1 gp41
TLR11 (not expressed in human)		Profilin-like molecule

## 1.2. TLR signaling

Signal transduction commences with the binding of a specific ligand that leads to: a. the formation of heterodimers of two receptor chains that are structurally similar (TLR2/1, TLR2/6, TLR2/10), b. the formation of homodimers (TLR4/4, TLR3/3, TLR5/5) and even, c. reorientation of the TIR domains in the case of TLR7, 8 and 9 (Liu et al., 2008<sup>30</sup>; Ridnour et al., 2012, Fan et al., 2010<sup>31</sup>). This interaction is followed by the recruitment of downstream signaling adapter proteins. There are five adaptor proteins containing TIR domains, which function in TLR signaling; myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adapter protein (TIRAP), TIR domain-containing adaptor inducing interferon IFN- $\beta$  (TRIF), TRIF-related adaptor molecule (TRAM) and sterile  $\alpha$ - and armadillo motif-containing protein (SARM) (Basith et al., 2011)<sup>32</sup> (Figure 1). All TLRs recruit the canonical MyD88-dependent signaling pathway - with the exception of TLR3 and endosomally-localised TLR4 which exclusively use the TRIF pathway - to induce the nuclear translocation of transcription factors, NF- $\kappa$ B, AP1 and CREB, leading to the expression of proinflammatory cytokine genes ((Medzhitov et al., 1998)<sup>33</sup>, (O'Neill et al., 2013)<sup>34</sup>). The noncanonical TRIF adaptor-dependent pathway leads to the nuclear translocation of transcription factors, IRF3 and IRF7, leading mainly to the secretion of type I IFNs

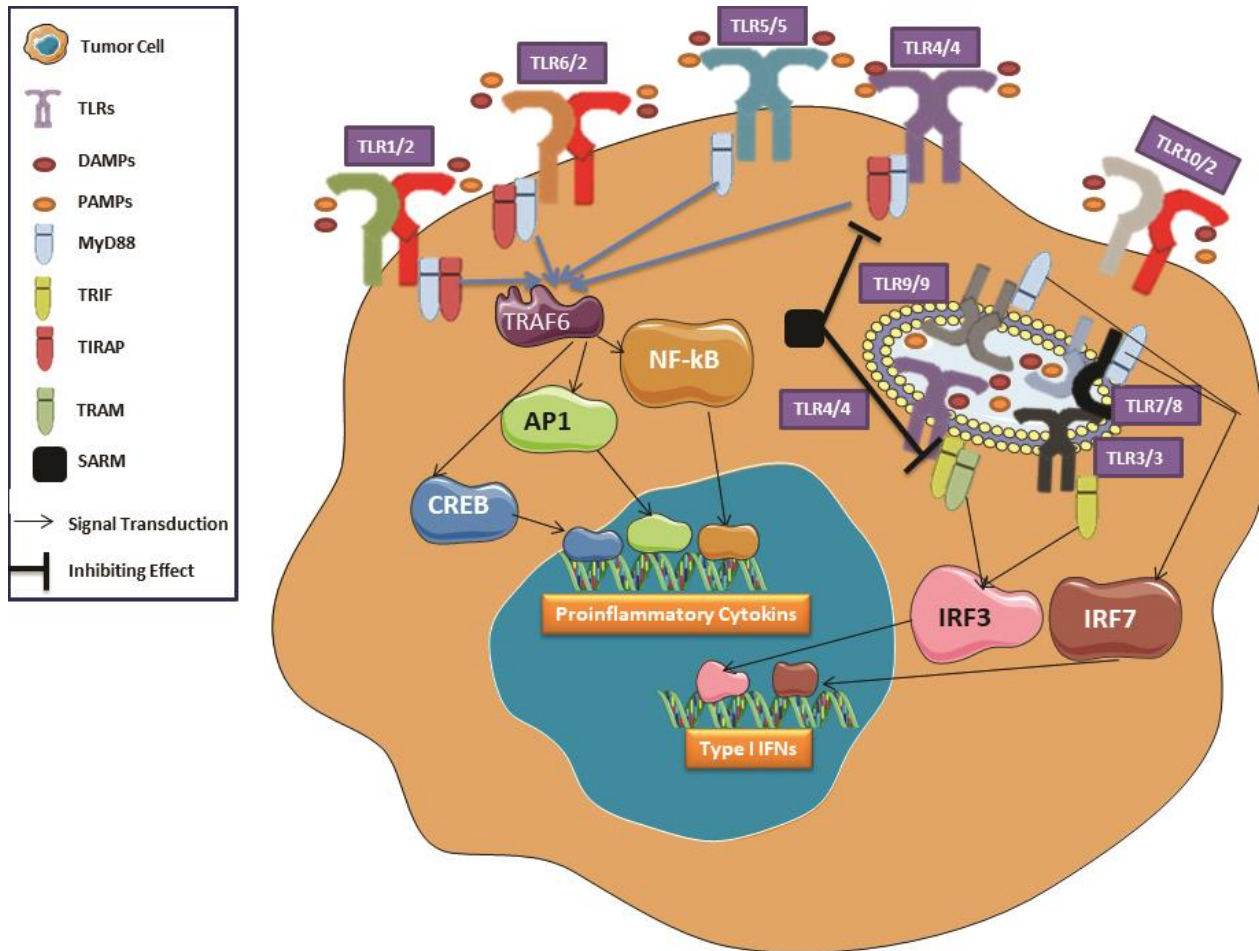
(O'Neill et al., 2013). In humans, SARM functions as a specific inhibitor of TRIF-dependent TLR signaling (Belinda et al., 2008)<sup>35</sup> (Figure 1).

TLR4 is unique among TLRs in its ability to activate two distinct signaling pathways; firstly, the canonical pathway, dependent on TIRAP and MyD88, and then, upon endocytosis of TLR4, the noncanonical pathway dependent on adaptors, TRIF and TRAM (Kagan et al., 2008)<sup>36</sup>.

Of note, while NF- $\kappa$ B activation in macrophages is a common theme to all TLR ligands, the MAPK signaling pathways are more significantly enriched in gene sets induced by TLR9 and TLR1/2 agonists (CpG and Pam3CSK4), and type I interferon pathways are enriched in TLR3 and TLR4 agonists (Poly(I:C) and LPS)-driven gene sets (Butcher et al., 2018)<sup>37</sup>.

## **2. Effects of TLR Signaling in Tumor Cells**

TLRs are widely expressed on tumor cells and normal epithelial cells. The expression of some TLRs in tumor cells is even higher than their normal tissue counterparts. For example, TLR4, 5, 7, 8 and 9 in Non-small cell lung carcinoma (NSCLC) was markedly higher than in normal lung tissue (Gu et al., 2018)<sup>38</sup>.



**Figure 1: TLR Signaling Pathways in Tumor and Immune cells.** Following binding of a specific ligand, TLRs form homo- or hetero-dimers and recruit adaptor protein(s). This starts the signal transduction cascade (details of the cascade are not shown here, because it is out of the scope of this review). TLRs: toll-like receptors, PAMPs: pathogen-associated molecular patterns, DAMPs: damage-associated molecular patterns, MyD88: myeloid differentiation primary response gene 88, TIRAP: TIR domain-containing adapter protein, TRIF: TIR domain-containing adaptor inducing interferon IFN- $\beta$ , TRAM: TRIF-related adaptor molecule, SARM: sterile  $\alpha$ - and armadillo motif-containing protein.

## 2.1. TLRs and the Stage of Cancer



Expression of TLRs in tumor cells depends on the type and stage of cancer, and varies from one patient to another. The expression of TLR4 is positively correlated with the degree of tumor differentiation, stage and metastasis in lung cancer (Li et al., 2014)<sup>39</sup>. A strong expression of TLR4 is characteristic of advanced-stage (TNM III or IV) gastric cancer (GC), whereas moderate or weak staining is characteristic of early-stage (TNM I or II) GC tumors (Yuan et al., 2013). The expression of TLR9 and TLR5 is absent or weak in normal cervical squamous epithelial cells but gradually increases with the histopathological grade with invasive cervical squamous cell carcinoma (ISCC) showing a moderate to strong expression of TLR9 in 70% of cases (Lee et al., 2007<sup>40</sup>; Kim et al., 2007<sup>41</sup>). Similarly, in esophageal carcinomas, TLR9 expression is positively associated with tumor size, location and TNM stage (Zhang et al., 2014). In oral tongue squamous cell carcinoma, cytoplasmic TLR2 and TLR4 correlate significantly with higher tumor grade, whereas high TLR5 expression associates with lower tumor grade (Mäkinen et al., 2015)<sup>42</sup>.

## **2.2. Correlation with Worse Prognosis**

Many studies find a correlation between TLR expression in tumors and a worse clinical outcome. TLR4 is highly expressed in gastric cancer cells and is associated with tumor aggressiveness (Yuan et al., 2013)<sup>43</sup>. TLR7 expression in NSCLC is markedly associated with poor clinical outcomes (Chatterjee et al., 2014)<sup>44</sup>. A meta-analysis including 2,812 patients with various cancers found that higher expression levels of TLR4 or TLR7, but not TLR9, in tumor tissues predicts poor survival and bad prognosis (Wang et al., 2018)<sup>45</sup>. In oral tongue squamous cell carcinoma, high TLR2, 4, and 9 expression correlates with deeper tumor invasion and high expression of TLR9 correlates with advanced tumor size (Mäkinen et al., 2015). Of note, breast carcinomas with high TLR3 expression in tumor cells are significantly associated with higher probability of metastasis and bad prognosis (González-Reyes et al., 2010)<sup>46</sup>.

## **2.3. TLR polymorphisms**

A new controversial field investigates TLR polymorphisms in cancer. Studies show that:

- Polymorphisms in TLRs are linked to susceptibility to cancer; higher frequencies of certain polymorphisms of TLR2, TLR4 and TLR9 increase susceptibility to development and progression of colorectal cancer (Messaritakis et al., 2018)<sup>47</sup>, melanoma (Gast et al., 2011)<sup>48</sup>, breast cancer (Theodoropoulos et al., 2012)<sup>49</sup>, nasopharyngeal carcinoma (Yang et al., 2012)<sup>50</sup> and cervical cancer (Tian et al., 2018)<sup>51</sup>. Certain TLR3 polymorphisms increase risk of cancer incidence, and are associated with larger tumor size in breast (Fan et al., 2019)<sup>52</sup> and rectal cancer (Slattery et al., 2012)<sup>53</sup>.
- TLR polymorphisms may influence outcomes of therapeutic interventions; some TLR4 polymorphisms indicate resistance to chemotherapy and serve as prognosis markers of head and neck squamous cell carcinomas (HNSCC) (Bergmann et al., 2011)<sup>54</sup>.
- Certain polymorphisms of TLR2, TLR5 and TLR9 show a significantly decreased risk of gastric cancer, and may play important roles in *H. pylori*-related gastric carcinogenesis (Zeng et al., 2011)<sup>55</sup>; De Re et al., 2019<sup>56</sup>). TLR4 single nucleotide polymorphisms may play an important protective role in the development of hepatocellular carcinoma (Minmin et al., 2011)<sup>57</sup>.

Of note, some TLR polymorphisms are not associated with increased risk of cancer; a TLR7 variant is not involved in the susceptibility to basal cell carcinoma (BCC) (Russo et al., 2016)<sup>58</sup>, and TLR4 and TLR1, 6 and 10 polymorphisms are not significantly associated with the risk of prostate cancer (Lindström et al., 2010<sup>59</sup>; Weng et al., 2014<sup>60</sup>).

#### **2.4. Mechanisms of protumoral effects of TLRs in tumor cells**

TLRs in tumor cells generally serve as positive regulators of cancer or, in other words, have pro-tumoral effects. Different mechanisms for these effects are suggested:

- TLR signaling upregulates the NF-κB cascade and induces anti-apoptotic proteins contributing to carcinogenesis and cancer cell proliferation. TLRs mediate the release of cytokines and chemokines by cancer cells that enhance inflammation in the TME - a hallmark of cancer (Sato et al., 2009). NF-κB

activation by TLR signaling is associated with the expansion, invasion, and tumorigenesis of cancer stem cells (CSCs). CSCs possess self-renewal and differentiation abilities, which promote tumor progression and metastasis and are responsible for treatment resistance and cancer relapse (Yeh et al., 2016)<sup>61</sup>.

- Following TLR activation, tumor cells can undergo epithelial-to-mesenchymal transition (EMT) and epithelial-to-leucocytic transition (ELT) - by which tumors develop the ability to activate leucocytic traits otherwise inaccessible to epithelial cells - and gain the ability to; (1) evade the immune system at the primary tumor site, (2) access the lymphatic system, (3) metastasize through the vasculature and (4) avoid destruction by the immune system at the site of metastasis (Luddy et al. 2014)<sup>62</sup>.
- TLR ligands may induce the production of immunosuppressive cytokines and molecules, including IL-10, transforming growth factor- $\beta$ , IDO and iNOS by tumor cells, which can counteract the activation of innate immunity (Ito et al., 2015)<sup>63</sup> (Ninomiya et al., 2015)<sup>64</sup>.
- TLRs can directly regulate cell metabolism affecting tumor and immune cell behavior and function in melanoma, prostate, head and neck carcinoma and breast cancer (Huang et al., 2018).

#### **2.4.1. Effects of TLRs on proliferation and apoptosis of tumor cells**

A primary way to enhance tumor progression is to induce cancer cell proliferation or inhibit apoptosis.

- In the majority of gastric cancer patients, elevated TLR2 expression is associated with a tumor-potentiating gene signature predicting poor patient outcome. High TLR2 expression in human and murine gastric cancers correlates with the up-regulation of six anti-apoptotic genes (BCL2A1, BCL2, BIRC3, CFLAR, IER3, TNFAIP3) and the down-regulation of two tumor suppressor (PDCD4, TP53INP1) genes (West et al., 2017)<sup>65</sup>. In highly invasive breast cancer cells (MDA-MB-231), a TLR2 agonist (Porphyromonas gingivalis lipopolysaccharides, pg-LPS) promoted cell invasion and increased NF- $\kappa$ B signaling, IL-6, TGF- $\beta$ , VEGF and MMP9 secretion, while TLR2 blockade diminished this outcome

(Xie et al., 2009)<sup>66</sup> and thus their invasion potential. Highly metastatic B16 melanoma release heat shock protein 60 (hsp60), that causes persistent activation of TLR2, increasing their invasion capacity, and the release of immunosuppressive cytokines and chemokines (Yang et al., 2009)<sup>67</sup>.

- Furthermore, binding of lipopolysaccharide (LPS) to TLR4 on gastric cancer cells stimulates the generation of mitochondrial ROS (mROS), the activation of Akt phosphorylation and NF- $\kappa$ B p65 nuclear translocation, which in turn enhances proliferation without affecting apoptosis (Yuan et al., 2013). Exposure of human melanoma cells to Hyaluronan fragments induces TLR4-mediated activation of NF- $\kappa$ B leading to the enhanced expression of matrix metalloproteinase 2 (MMP2) and interleukin (IL)-8 - factors that contribute to melanoma progression (Huang et al., 2005)<sup>68</sup>, (Voelcker et al., 2008)<sup>69</sup>. LPS activation of TLR4 promotes production of the immunosuppressive cytokine TGF- $\beta$ , proangiogenic VEGF and chemokine IL-8 by human lung cancer cells. TLR4 ligation also induces resistance of human lung cancer cells to TNF- $\alpha$  or TRAIL-induced apoptosis (He et al., 2007). Of note, LPS stimulation increases the proliferation of glioma CD133+ cancer stem cells (CSCs) isolated from human glioma samples in a time- and dose-dependent manner (Che et al., 2017)<sup>70</sup>.
- TLR3 and TLR9 are expressed in about 90% of hepatocellular carcinoma tissues. While stimulation of TLR3 with polyI:C activates the NF- $\kappa$ B pathway without affecting cell viability, stimulation of TLR9 with CpG-ODNs promotes cell proliferation, and reduces cytotoxicity of the anticancer drug Adriamycin (Nojiri et al., 2013)<sup>71</sup>, via up-regulation of apoptosis inhibitors such as survivin, Bcl-xL, XIAP and cFLIP (Tanaka et al., 2010)<sup>72</sup>. In esophageal carcinomas, high TLR9 expression is positively associated with tumor size, location and TNM stage, and the TLR9 ligand, CpG ODN, significantly enhances the invasion of esophageal cancer cell line, TE10 cells. TLR9 activation also leads to NF $\kappa$ B activation and enhances expression of pro-invasive matrix metalloproteinase (MMP)-2, MMP-7 and cyclooxygenase-2 (COX-2) (Zhang et al., 2014)<sup>73</sup>.

- TLR7 and TLR8 stimulation with the agonist R848 increases proliferation of a human pancreatic cancer cell line (PANC1) by inducing the NF- $\kappa$ B pathway and COX-2. Furthermore, high TLR7 and TLR8 expression leads to increase in PANC1 tumor growth in Balb/c nude mice (Grimmig et al., 2015)<sup>74</sup>. Our team previously showed that stimulation of human NSCLC cell lines with TLR7 or TLR8 agonists leads to NF- $\kappa$ B activation, upregulation of the antiapoptotic protein Bcl-2, and increase of tumor cell survival (Cherfils-Vicini et al., 2010)<sup>75</sup>.

TLRs in tumor cells can also directly or indirectly act as a 'double-edged sword'. (Figure 2) Several TLR ligands, such as the TLR7 agonist imiquimod, TLR3 ligand Poly (I:C) and TLR9 ligand CpG, can directly induce tumor cell apoptosis or enhance the function of tumor-infiltrating innate cells and tumor-specific T-cells. Treating a human breast cancer cell line (MCF-7) with synthetic commercial agonists, poly(I:C) and imiquimod, decreases growth rate and increases sensitivity to radiotherapy (Kang et al., 2010)<sup>76</sup>. However, polyI:C has many disadvantages in addition to the fact that, besides TLR3, it also triggers cytosolic sensors RIG-I and MDA. Its undefined chemical structure and very poor homogeneity leading to unpredictable pharmacokinetics and high toxicity in many clinical trials (Naumann et al., 2013)<sup>77</sup> necessitates careful interpretation of the results.

#### **2.4.2. Effects of TLRs on Metastasis**

Modulation of the TME and of the tumor cells themselves is required for tumor cells to metastasize to distant sites. Endogenous and exogenous ligands inducing TLR signaling are implicated during this process (Figure 2).

- Versican, an extracellular matrix (ECM)-derived peptide and a DAMP, upregulated in many human tumors including lung cancer, activates TLR2/6 on MDSCs to enhance TNF- $\alpha$  production leading to tumor cell proliferation and metastasis (Kim et al., 2010)<sup>78</sup>.

- Although the chemotherapeutic effect of paclitaxel treatment is largely efficacious in controlling TLR4-negative breast cancers, paclitaxel significantly increased the incidence and burden of pulmonary and lymphatic metastasis in TLR4-positive breast tumors. Indeed, TLR4 activation by paclitaxel induces de novo generation of deep intratumoral lymphatic vessels that are highly permissive to invasion by malignant cells (Volk-Draper et al., 2014)<sup>79</sup>.
- Specific stimulation of highly expressed TLR2, TLR3, and TLR4 on human melanoma cells induces cell migration (Goto et al., 2008)<sup>80</sup>. In HCC cells, LPS significantly enhances their invasive potential and induces EMT with the upregulation of Vimentin, N-cadherin, Snail and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and downregulation of epithelial markers (E-cadherin and  $\beta$ -catenin) (Jing et al., 2012)<sup>81</sup>.
- Stimulation with various TLR agonists (MALP-2 for TLR2/6, LPS for TLR4, and poly I:C for TLR3) induces mesenchymal characteristics and invasion potential in SK-OV-3 cells, a metastatic epithelial ovarian cancer cell line. TLR-mediated PI3K activation promotes the secretion of EMT-related cytokines in SK-OV-3 cells (TGF- $\beta$ 1, TNF- $\alpha$ , VEGF, IL-6, IL-8, and IL-10). Interestingly, TLR4-mediated galectin-1 production induces EMT in SK-OV-3 cells (Park et al., 2017)<sup>82</sup>.
- TLR7 stimulation results in accelerated NSCLC growth and metastasis, accompanied by a gene expression signature linked to aggressiveness and metastatic dissemination; tumors expressing high levels of TLR7 display an EMT phenotype with high expression of vimentin and low abundance of E-cadherin (Dajon et al., 2018)<sup>83</sup>.

#### **2.4.3. Effects of TLRs on Angiogenesis**

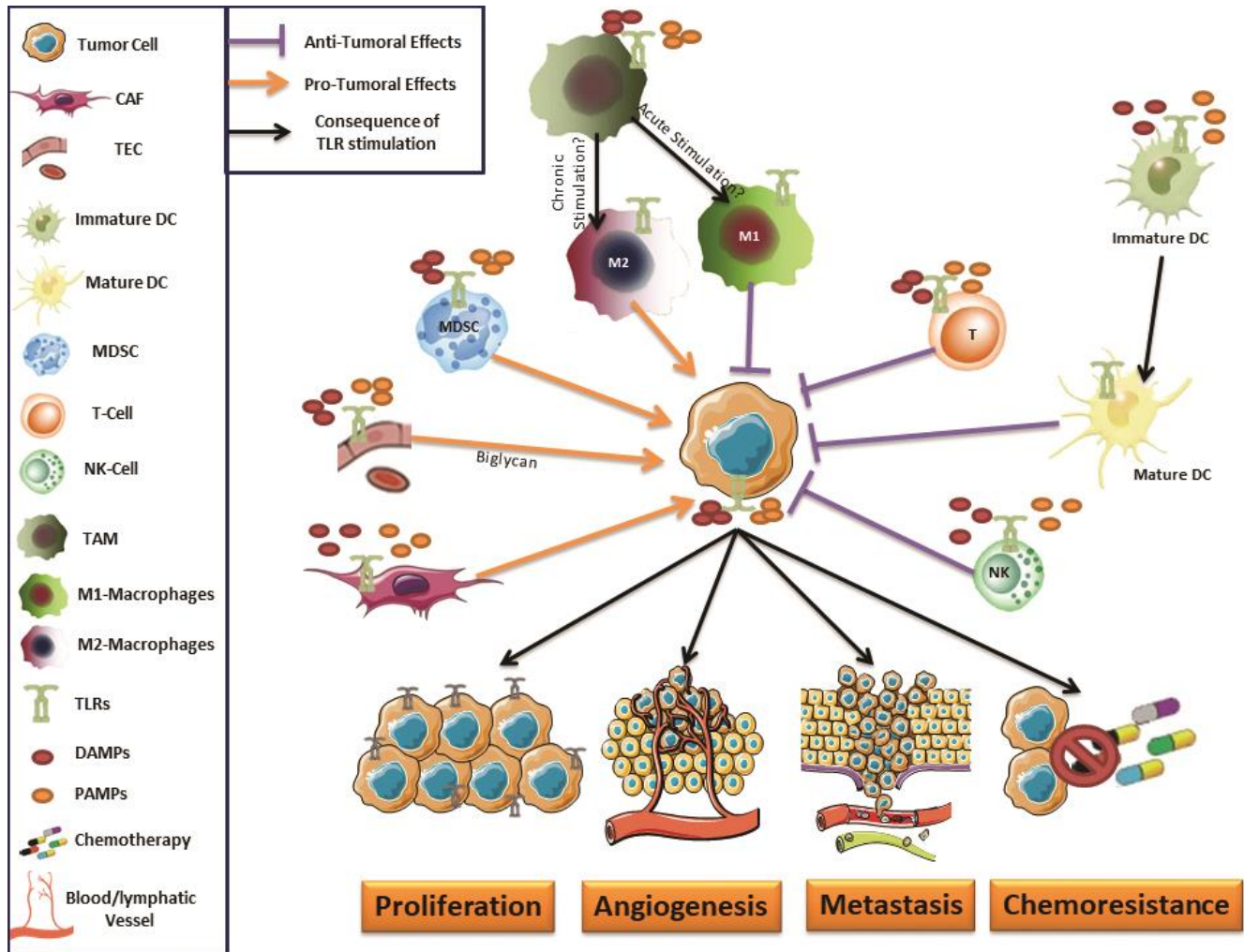
Tumor invasion and progression depends on angiogenesis. Subsets of endothelial cells with specialized functions exhibit different levels of TLR expression. TLR2 and TLR4 are ubiquitously present within the vasculature, whereas TLR7, TLR8 and TLR9 are sparse (Salvador et al., 2016)<sup>84</sup>. Human primary dermal

and lung lymphatic endothelial cells (LECs) express TLR1– 6 and TLR9, but not TLR7, TLR8, and TLR10 (Pegu et al., 2008)<sup>85</sup>. TLRs and their ligands contribute to the process of angiogenesis in different ways:

- Tumor endothelial cells (TECs) interact with tumor cells by secreting a small leucine-rich repeat proteoglycan known as biglycan, which acts as a DAMP binding TLR2 and TLR4 in neighboring tumor cells. Through the activation of NF- $\kappa$ B and ERK signaling (Maishi et Hida, 2017)<sup>86</sup>, the biglycan stimulates tumor cells to metastasize, promotes gastric cancer cell migration and induces upregulation of VEGF in neighboring TECs, resulting in enhanced capacity for tube-formation, migration, motility and proliferation in gastric and colon cancer (Hu et al., 2016<sup>87</sup>; Xing et al., 2015<sup>88</sup>; Hojo et al., 2017<sup>89</sup>) (Figure 2).
- Peroxiredoxin-1 (Prx1), a chaperone protein and a DAMP secreted by prostate cancer cells, activates TLR4 resulting in the secretion of VEGF from tumor cells, macrophages and ECs thus stimulating TECs to proliferate, migrate, and differentiate (Riddell et al., 2011)<sup>90</sup>. LPS induces growth and lung metastasis of breast cancer cells through increased angiogenesis, vascular permeability, and tumor cell migration (Ling et al., 2011)<sup>91</sup>.
- In highly vascularized tumors, in both murine and human melanoma, oxidative stress, exemplified by the accumulation of lipid oxidation,  $\omega$ -(2-carboxyethyl)pyrrole (CEP), is recognized by TLR2 on endothelial cells, leading to a pro-angiogenic response independent of VEGF (West et al., 2010)<sup>92</sup>.
- Activation of TLR3 by HSP27 released by tumor cells induces NF- $\kappa$ B activation leading to VEGF-mediated cell migration and angiogenesis (Thuringer et al., 2013)<sup>93</sup>. In prostate cancer cell lines (PC3 cells), TLR3 activation leads to the increase of the specific 1.3 isoform of HIF-1 $\alpha$  and nuclear accumulation of the HIF-1 complex, resulting in reduced apoptosis and secretion of functional VEGF (Paone et al., 2010)<sup>94</sup>.

- Some studies observe a pro-lymphangiogenic role of TLR4. TLR4 ligands LPS, HMGB1 and paclitaxel induce inflammatory lymphangiogenesis while TLR4-deficient mice form lesser lymphatic vessels as compared to wild-type mice. TLR4 ligands induce pro-lymphatic reprogramming of human and mouse myeloid cells into Myeloid/Monocyte-derived Lymphatic Endothelial Cell Progenitors (M-LECP) responsible for the formation of lymphatic vessels and increased lymphatic metastasis in a model of breast cancer (Volk-Draper et al., 2017)<sup>95</sup>. Indeed, LPS induces a lymphatic phenotype in endogenous macrophages in vivo, by increasing VEGFR-3 expression. These macrophage-derived lymphatic endothelial cell progenitors (M-LECPs) contribute to new lymphatic vessel formation (Hall et al., 2012)<sup>96</sup>.





**Figure 2: Effects of TLR Signaling on the Cellular Components of the TME.** In tumor cells, DAMPs and PAMPs induce TLR signaling leading to enhanced proliferation, angiogenesis, metastasis, and chemoresistance. In CAFs, TECs, and MDSCs, TLR signaling also leads to protumoral effects. Within immune cells, TLR signaling induces the maturation of DCs, and the activation of T and NK cells resulting in anti-tumoral responses. In macrophages, TLR signaling can result either in the shift of TAMs into the anti-tumoral M1 phenotype, or into the protumoral M2 phenotype (possibly through acute or chronic TLR signaling activation respectively). CAFs: cancer-associated fibroblasts, TECs: tumor endothelial cells, NK cells: natural killer cells, DCs: dendritic cells, MDSC: myeloid-derived suppressor cells, TAMs: tumor-associated macrophages, TLRs: toll-like receptors, PAMPs: pathogen-associated molecular patterns, DAMPs: damage-associated molecular patterns.

#### 2.4.4. TLRs and Resistance to therapy

TLR ligands can induce resistance to cancer therapy, either by acting directly on tumor cells, or indirectly by inhibiting the immune system (Figure 2).

1. Paclitaxel is a potent anti-cancer drug commonly used for the treatment of advanced breast cancer and melanoma. However, Toll-like receptor 4 (TLR4) expression in these tumors confers resistance to paclitaxel by promoting the production of pro-inflammatory cytokines (IL-6, IL-8) and the anti-apoptotic protein XIAP (Sootichote et al., 2018)<sup>97</sup>. LPS-induced TLR4 stimulation in ovarian cancer (OvCa) cells leads to activation of the NF-κB pathway, promotes IL-8, IL-6 and VEGF secretion, and confers resistance to paclitaxel-induced apoptosis (Szajnik et al., 2009)<sup>98</sup>.
2. In Gliomas, LPS-dependent TLR4 stimulation in CD133+ cancer stem cells (CSCs) induces resistance to chemotherapy and evasion from cytotoxic T lymphocyte-induced cytolysis (Che et al., 2017).
3. In NSCLC cells lines, A549 and H1299, Pellino-1, a critical mediator for NF-κB activation in TLR3 and TLR4 signaling, is up-regulated following treatment with TLR agonists conferring chemoresistance to cisplatin or paclitaxel and increased cell viability. The upregulation of cIAP1 and cIAP2 by Pellino-1, reduces cisplatin-induced cleavage of caspase-3, caspase-7, and PARP accounting for the chemoresistance (Jeon et al., 2016)<sup>99</sup>.
4. TLR7 or TLR8 stimulation induces chemoresistance to 5-fluorouracil in human pancreatic cancer cell line (PANC1) (Grimmig et al., 2015). In a cohort of NSCLC patients, high expression of TLR7 on tumor cells is associated with poor clinical outcome in patients treated or not with neoadjuvant chemotherapy. TLR7 expression on tumor cells is a marker of chemoresistance to cisplatin combined with gemcitabine or vironelbine (Chatterjee et al., 2014).
5. Co-treatment with LPS and cisplatin induces immune-suppressive tolerogenic DCs via abundant IL-10 production, thereby skewing T helper cell differentiation towards Th2 and Tr1 cells. Together, this may provide cancer cells with an opportunity to evade the immune system (Kim et al., 2016)<sup>100</sup>.

## **2.5. TLRs and Tumor Cell Metabolism**

In the TME, tumor cells preferably use aerobic glycolysis instead of OXPHOS (the Warburg effect) to meet their demands for growth and proliferation, especially for rapid ATP generation and biosynthesis (Huang et al., 2018). The excess nutrient consumption and waste excretion by tumor cells, in addition to insufficient oxygen delivery in TME, result in dysfunction and loss of potential antitumor activity of tumor infiltrating immune cells (Sugiura and Rathmell, 2018)<sup>101</sup>. TLRs can directly regulate cellular metabolism by affecting tumor cell function. TLRs are also redox sensitive receptor proteins and are implicated in the response to oxidative stress (Karki et al., 2015)<sup>102</sup>.

Stimulation with the TLR3 specific ligand Poly (A:U) dsRNA promotes metabolic reprogramming of head and neck carcinoma cells, resulting in increased tumor growth. TLR3 stimulation enhances the capacity of carcinoma cells to switch from OXPHOS to anabolic glycolysis, accompanied by the upregulation of the transcription factor HIF-1 $\alpha$  enabling better adaptation to hypoxia and oxidative stress in the TME (Veyrat et al., 2016)<sup>103</sup>.

In patients with breast carcinoma, TLR9 regulates lipid peroxidation by trace elements (selenium, copper, zinc, magnesium and iron) in response to oxidative stress (Karki et al., 2015).

TLR signaling not only impacts directly on tumor metabolic reprogramming and tumor growth, but also indirectly influences the antitumor immune responses in the TME by modulating cancer cell metabolites. TLR2, 3, 4, 5, 7 and 9 activation in tumor cells induces T-cell senescence in naïve/effector T cells by releasing cyclic adenosine monophosphate (cAMP) (Ye et al., 2014)<sup>104</sup>. However, activation of TLR8 signaling in tumor cells downregulates cAMP thereby reversing the immunosuppression of senescent naïve/effector T.

TLRs can modulate cancer cell metabolites by inducing indoleamine 2,3-dioxygenase (IDO) and induced nitric oxide synthase (iNOS). Intra-tumoral administration of the TLR7 agonist, imiquimod, significantly increases IDO and iNOS expression in tumor-draining lymph nodes, leading to the inhibition of the tumor

antigen-specific Th1 response (Ito et al., 2015). Inhibition of IDO or iNOS enhances the therapeutic efficacy of TLR agonists via the increase of the Th1 immune response (Ito et al., 2015).

### **3. Effects of TLR signaling in Immune Cells in the TME**

TLRs on immune cells play a major role as initiators of the innate immune response to defend against pathogens, and are also required for an effective secondary immune response. Following pathogen recognition by TLRs, antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs) are activated (Huang et al., 2008)<sup>105</sup> inducing a program of maturation that leads to the migration of APCs into lymph nodes, and the release of pro-inflammatory cytokines such as TNF $\alpha$ , IL6, IL12 (that directs the T cell response toward a Th1 phenotype), and IFNs, that are crucial for the activation of NK cells and cytotoxic T lymphocytes (CTLs) (Kopp and Medzhitov, 2003)<sup>106</sup>. The idea of inciting an immune response to fight cancer dates back to more than a 100 years, when Dr. William Coley in the late 19th century showed that crude microbial extracts (killed *Streptococcus pneumoniae* and *Serratia marcescens*, commonly known as Coley's toxin), can promote an antitumor response in different types of cancers (Basith et al., 2012). The same principle is still applied today in the form of *Bacillus Calmette-Guerin* (BCG), which is used for the treatment of bladder cancer (Bassi, 2002)<sup>107</sup>. It is likely that these agents trigger an immune response through multiple TLRs to promote antitumor immunity. However, the impact of TLR signaling in the TME is more complicated and illusive today than what Coley imagined.

#### **3.1. TLRs and Macrophages**

During tumor progression, the TME often induces the shift of tumor-associated macrophages (TAMs) from their protective classically activated M1-phenotype to pro-tumorigenic alternatively activated M2-subtype. Within the TME, M2-macrophages are associated with tumor growth and progression by impacting on inflammation, immune regulation, angiogenesis, invasion, and metastasis. The interaction between macrophages and tumor cells results in an autocrine/paracrine loop that enhances their pro-tumorigenic properties (Poh and Ernst, 2018)<sup>108</sup>. The impact of TLR stimulation in TAMs is controversial,

as different studies report different outcomes. However, it seems that the duration of TLR stimulation can explain the different outcomes (Figure 2).

- On the one hand, upon LPS treatment, TAMs from ascites of ovarian cancer patients polarize toward an M1-like phenotype as demonstrated by the disappearance of membrane-bound IL-18 and upregulation of CD80 and CCR7. The LPS-activated TAMs release immunostimulatory cytokines (IL-12, soluble IL-18) that efficiently trigger the cytolytic activity and IFN- $\gamma$  release of co-cultured NK cells (Bellora et al., 2014)<sup>109</sup>. Intratumoral administration of poly I:C in mice bearing colon adenocarcinoma tumors triggers an IFN- $\alpha\beta$ -dependent (MC38) reversion of M2-macrophages to the M1 phenotype and leads to tumor regression (Vidyarthi et al., 2018)<sup>110</sup>.
- On the other hand, chronic TLR signaling plays a role in the conversion of macrophages from the M1 to the M2 phenotype in the TME. The DAMP HSP27 released from lung cancer cells is recognized by TLR3 in monocytes and inhibits their differentiation into DCs while skewing them towards M2 macrophages (Thuringer et al., 2013)<sup>111</sup>. Tumor cell-derived extracellular Vesicles (TEVs) can bind to TLRs in immune cells and activate them in a paracrine loop leading to the production of cytokines that increase cell proliferation and metastatic potential. TEVs carry cargo containing NSCLC tumor-associated miR-21 and miR-29a that bind to TLR7 and TLR8 in immune cells including macrophages, activating NF- $\kappa$ B and triggering a pro-metastatic inflammatory response (Fabbri et al., 2012)<sup>112</sup>. Stimulation of murine macrophages (BMDM) with Pam3CSK4 (TLR1/2), Poly(I:C) (TLR3), LPS (TLR4), and CpG DNA (TLR9) induces a transcriptomic profile that shows a robust activation of known inflammatory genes, including IL6, TNF and chemokines (Butcher et al., 2018). However, re-stimulation 24 hours later of the macrophages with the same doses of previous TLR agonists, induces TLR tolerance shifting away from a pro-inflammatory response toward a pro-resolution and anti-inflammatory response (Butcher et al., 2018).

### **3.2. TLRs and Dendritic Cells (DCs)**

DCs are professional APCs that play a pivotal role in the link between innate and adaptive immunity. Two major subtypes of DCs are recognized in mouse and human tissues: Conventional/Myeloid DCs (cDCs/mDCs) and plasmacytoid DCs (pDCs). cDCs encompass several subsets: cDC1 (which express CD141) and cDC2 (which express CD1c), in addition to monocyte-derived DC (Mo-DCs) (Collin and Bigley, 2018)<sup>113</sup>. While cDC subtypes produce large amounts of proinflammatory cytokines, including IL-12 and induce strong Th1 and CTL responses, pDCs produce large amounts of type I IFN in response to viral and bacterial stimuli (Banchereau et al., 2000)<sup>114</sup>. Different DC subsets exhibit distinct TLR repertoires; pDCs selectively express endosomal TLR7 and TLR9 (Mathan et al., 2013)<sup>115</sup>, cDC2 express all TLRs except TLR9 and, cDC1 highly express TLR3, TLR9 and TLR10, in addition to low expression levels of TLR1-2, TLR6, and TLR8 (Hemont et al., 2013)<sup>116</sup>.

Within the TME, heterogenous DCs are frequently recruited by tumor-derived and stroma-derived factors. However, DCs are often show impaired maturation, differentiation, and function, resulting in DC-mediated tolerance, immune suppression and deficient anti-tumor immune responses (Zong et al., 2016)<sup>117</sup>. Following long exposure with TLR3 and TLR4 ligands, bone marrow DCs (BMDCs) downregulate MHCII and co-stimulatory molecules CD40 and CD86, as well as the cytokines IL-12, TNF-alpha and IL-6. Tumor cells induce inhibitory molecules (B7-DC, B7-H1 and CD80) on spleen DCs in vivo and on BMDCs, even in the presence of TLR ligands. This has implications for the development of DC-based cancer immune therapies using TLR ligands as adjuvants (Idoyaga et al., 2007)<sup>118</sup>, (Dong et al., 2014)<sup>119</sup>.

TLRs appear to play an important, yet controversial, role in the development of an anti-tumor immune response..

- On one hand, specific interaction between TLRs and their ligands (Table 1) results in DC maturation (Figure 2), which boosts MHC-I cross-presentation (Maschalidi et al., 2017)<sup>120</sup>. In Glioblastoma multiforme (GBM), intra-tumoral delivery of adenoviral vectors (Ad) expressing the DC-differentiation

cytokine Fms-like tyrosine kinase 3 ligand (Flt3L) and thymidine kinase (TK), results in infiltration of mDCs, a clonal expansion of antitumor T cells, and an induction of an effective anti-GBM immune response in a TLR2-dependent manner. In fact, HMGB1, an alarmin protein released from dying tumor cells, is recognized by Toll-like receptor 2 (TLR2) and is responsible of tumor regression observed in this murine model (Curtin et al., 2009)<sup>121</sup>. Recognition of HMGB1 by TLR4 in DCs increases their maturation, processing and cross-presentation of tumor antigen released from dying tumor cells during radio and chemotherapy (Apetoh et al., 2007)<sup>122</sup> enhancing the infiltration and clonal expansion of T cells and tumor killing. HSP70, released from tumor cells, activates tumor cells through TLR4 to produce chemokines that attract DCs and T cells (Chen et al., 2009)<sup>123</sup>. In addition, repeated intra-tumoral injections of Poly A:U, a TLR3 agonist, activates CD103+ cDC1 cells inside murine B16-OVA melanoma resulting in higher tumor-specific immunity, with an increase in infiltrating granzyme B+ CD8+ T cells and a decrease in IL-10-producing M2-like macrophages, impacting on the ability of the immune system to control tumor growth (Roselli et al., 2019)<sup>124</sup>.

- On the other hand, studies have found protumoral effects for TLR signaling in DCs in the TME. Murine and human tumors secrete TLR2 ligands, such as Versican, that promote differentiation of IL-10-producing cDCs. DCs become more responsive to IL-6 and IL-10 stimulation, following TLR2 ligation, by increasing the activation of STAT3. TLR2 blockade using anti-TLR2 antibodies *in vivo* improves intra-tumor DC function, enhances anti-tumor CTL and the efficacy of immunotherapy (Tang et al., 2015)<sup>125</sup>. Matrix metalloproteinase 2 (MMP-2), another DAMP, interacts with TLR2 resulting in an increase in tumor angiogenesis and dampening of the immune response. Activating TLR2 by MMP-2 upregulates an immunoregulatory receptor OX40L on DCs and leads to the secretion of inflammatory cytokines that polarizes T cells to an immunosuppressive Th2 phenotype (Godefroy et al., 2014)<sup>126</sup>. Finally, intra-tumoral Mo-DCs express high levels of TLRs (except TLR3) in metastatic breast cancer patients with circulating tumor cells (CTC), compared to CTC-negative patients (Green et al., 2014)<sup>127</sup>.

### 3.3. TLRs and myeloid-derived suppressor cells (MDSCs)

MDSCs are immature and pathologically-activated cells that have potent immune suppressive activity in tumors. MDSC can also support tumor progression by promoting tumor cell survival, angiogenesis, invasion and metastasis. MDSC are classified into two different types, as identified in mice and humans: polymorphonuclear MDSC (PMN-MDSC) are morphologically and phenotypically similar to neutrophils, whereas monocytic MDSC (M-MDSC) are similar to monocytes (Kumar et al., 2016)<sup>128</sup>. The effect of TLR stimulation in MDSCs is controversial, and requires further investigation.

- TLR 7/8 agonists reverse the suppressive activity of human blood MDSC from cancer patients inducing their differentiation into tumoricidal M1-like macrophages. In contrast, agonists targeting TLR 1/2 cause M-MDSC to mature into immunosuppressive M2-like macrophages (Wang et al., 2015).
- On the other hand, our team demonstrated that TLR7 stimulation of tumor cells results in the recruitment of MDSCs to the TME, leading to accelerated tumor growth and metastasis. Depleting MDSC indicated that these cells are involved in the pro-tumoral effect of TLR7 stimulation (Dajon et al., 2018) (Figure 2). In addition, tumor-derived exosomes (TDEs) activate human MDSCs and trigger their suppressive function in an Hsp72/TLR2-dependent manner (Chalmin et al., 2010)<sup>129</sup>.

### 3.4. TLRs and NK cells

In solid malignancies, tumor-associated NK cells (TA-NK cells) in peripheral blood and tumor-infiltrating NK (TI-NK) cells display phenotypes of anergy or reduced cytotoxicity (Platonova., 2011)<sup>130</sup>. Besides their well-established anti-tumor effect, NK cells may support cancer both by immunosuppression and by supporting tumor angiogenesis (Bassani et al., 2019)<sup>131</sup>. Considering that almost all TLRs can be expressed on human NK cells (Guo and Zhang, 2012)<sup>132</sup>, many TLR agonists stimulate NK cell function directly or through accessory cells in a cytokine or contact dependent manner (Figure 2).

Poly I:C through TLR3 can directly activate human NK cell lines, NK92, YTCl2 and YTS (Pisegna et al., 2004)<sup>133</sup> promoting the secretion of IFN- $\gamma$  and CXCL10 and up-regulating the cytotoxic activity of NK cells



against human erythromyeloblastoid leukemia and B-lymphoblastoid cell lines, which do not express the major histocompatibility complex class I (MHC-I) (Pisegna et al., 2004). Poly I:C impairs the internalization of TLR3 and leads to the activation of NK cells within the Head and neck squamous cell carcinoma (HNSCC) microenvironment (Xie et al., 2007)<sup>134</sup>. In addition, TLR7/8 stimulation indirectly promotes NK cell activation and production of IFN-  $\gamma$ , granzyme B and perforin exerting powerful anti-tumor immunity in melanoma and HNSCC tumors (Ma et al., 2010<sup>135</sup>; Pries et al., 2008<sup>136</sup>).

### **3.5. TLR and T-Cells**

Certain TLRs are expressed in T lymphocytes, and their ligands can directly modulate T cell function (Figure 2). The activation of the Toll-like receptor-Myeloid differentiation factor 88 (TLR-MyD88) signaling in CD8+ T cells enhances proliferation, cytotoxic function, and survival and make them resistant to MDSC-mediated suppression (Ciavattone et al., 2018)<sup>137</sup>. TLR8 signaling reverses tumor-induced T cell senescence by blocking cAMP production in tumor cells (Ye et al., 2014)<sup>138</sup>.

In human  $\gamma\delta$  T cells, TLR 3 and 7 agonists increase CD54 expression and the lysis of pancreatic adenocarcinoma cells (Shojaei et al., 2009)<sup>139</sup>.

In addition, TLR2, TLR5 and TLR8 agonists inhibit the suppressive activity of natural CD25(+)/CD4(+) regulatory T cells (Oberg et al., 2011)<sup>140</sup>.

## **4. Effects of TLR signaling on Cancer-associated fibroblasts (CAFs)**

Cancer-associated fibroblasts (CAFs) are the most abundant cell type within the active stroma of many cancer types (Tao et al., 2017)<sup>141</sup>. CAFs trigger pro-tumorigenic signals accompanied by disruption of the normal tissue architecture creating an optimal niche for tumors to progress (Alkasalias et al., 2018). They induce therapy resistance, epithelial-to-mesenchymal transition (EMT), and survival or stemness-related programs and metabolically reprogram tumor cells (Fiori et al., 2019)

<sup>142</sup>. CAFs secrete several cytokines and chemokines that are immunosuppressive (IL-6, TGF- $\beta$ , IL-1 $\beta$ , IL-10, IDO, and PGE2), recruit inflammatory cells (CXCL1, 2, 5, 6, 9, 10, 12, CCL2, 3, 5,7, 20, and 26), and are pro-angiogenic (VEGF, CXCL8, and FGFs) (Torres et al., 2013<sup>143</sup> ; De Boeck et al., 2013<sup>144</sup>).

Despite the fact that all 10 TLRs are constitutively expressed and functional (except TLR10) in fibroblasts (Yao et al., 2015)<sup>145</sup>, few studies investigate the impact of TLR stimulation in CAFs on tumor progression. TLR4 in CAFs is associated with a high rate of tumor recurrence and a shortened overall survival; particularly in colorectal cancer patients (Eiró et al., 2013)<sup>146</sup> (Figure 2).

In a cohort of luminal breast cancer patients, high TLR4 expression associates with more LC3II (a marker of autophagosomes) in CAFs and correlates with aggressive relapse rates and poor prognosis (Zhao et al., 2017)<sup>147</sup>. Intestinal mesenchymal cells (IMCs) and CAFs are activated by innate TLR4/MyD88-mediated signals that promote spontaneous intestinal tumorigenesis in mice. Similar TLR4/MyD88-regulated gene signatures also exist in human CAFs (Koliaraki et al., 2018)<sup>148</sup>.

On the contrary, breast carcinomas with high TLR9 expression in fibroblast-like cells are associated with low probability of metastasis (González-Reyes et al., 2010)<sup>149</sup>. The polysaccharide MPSSS triggers the TLR4-NF- $\kappa$ B pathway and reduces the inhibitory effect of prostate CAFs on CD4+ and CD8+ T cell proliferation (Xu et al., 2019)<sup>150</sup>.

**Table 2: Summary of the effect of TLR signaling in the TME**

Cell Component	Effect
Tumor Cells	<b>Protumoral (enhance proliferation, angiogenesis, metastasis, resistance to chemotherapy)</b>
Macrophages	<b>Controversial (antitumorigenic via M1 phenotype, or protumorigenic via M2 phenotype)</b>
DCs	<b>Mainly antitumoral (TLR2 signaling could be protumoral)</b>

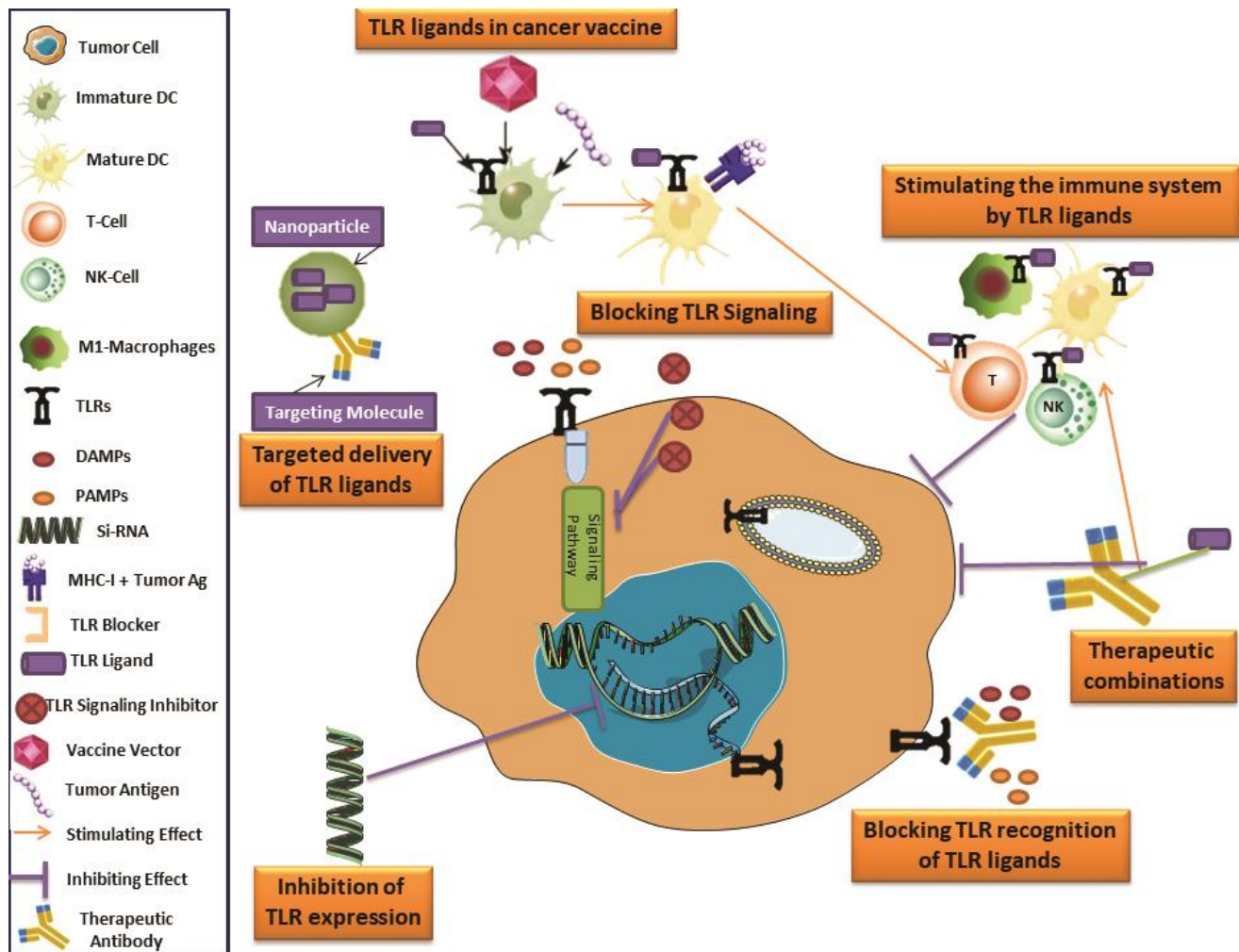
<b>MDSCs</b>	<b>Mainly protumoral</b>
<b>NK Cells</b>	<b>Antitumoral</b>
<b>T Cells</b>	<b>Antitumoral</b>
<b>CAFs</b>	<b>Mainly protumoral</b>

## **5. TLR-based treatment strategies in cancer therapy**

Based on the importance of TLR signaling in the TME, numerous studies and clinical trials have investigated the use of TLR ligands as potential treatments for cancer. Using TLR ligands to incite a powerful humoral and cellular immune response against tumors has seemed very promising. However, most TLR ligands used as single therapies to stimulate the immune system were not successful in preclinical studies or in clinical trials. The complex nature of the TME, the fact that TLR agonists can elicit protumoral effects in tumor cells, and the diverse consequences in immune and stromal cells can explain why the majority of activating TLR-based single therapies failed.

Various strategies either stimulating TLRs inciting their adjuvant effect or blocking TLR signaling have been tried with some showing promising outcomes. In addition to many preclinical studies, 10 clinical trials investigating the safety and therapeutic efficacy of TLR ligands or their adjuvant impact in cancer patients have been completed. However, the majority of these studies completed between 2016 and 2019 have not been published yet.

To date, only three TLR agonists are approved by FDA for use in humans: the bacillus Calmette-Guérin (BCG), monophosphoryl lipid A (MPL) and imiquimod. BCG is used during immunotherapy in non-invasive transitional cell carcinoma of the bladder, as an agonist for multiple TLRs; TLR2, TLR4, and TLR9. MPL (derived from the LPS of *Salmonella minnesota*) that signals through TLR2 and TLR4, is included in the formulation of Cervarix<sup>®</sup>, a vaccine against human papillomavirus-16 and -18 that are strongly associated with cervical carcinoma. Imiquimod (a synthetic TLR7 agonist) is routinely used for treatment of actinic keratosis, superficial basal cell carcinoma, and external genital warts (Vacchelli et al., 2012<sup>151</sup>; Smith et al., 2018<sup>152</sup>).



**Figure 3: TLR-based treatment strategies in cancer therapy.** An up-to-date illustration of 7 TLR-based treatment strategies used in preclinical models and clinical trials. NK cells: natural killer cells, DCs: dendritic cells, TLRs: toll-like receptors, PAMPs: pathogen-associated molecular patterns, DAMPs: damage-associated molecular patterns, MHC-I: major histocompatibility complex - class 1, Ag: Antigen.

### 5.1. TLR ligands as stimulators of the immune system

Some TLR ligands show a preferential stimulating effect of the immune system in some types of cancer. This could be explained by the relative abundance of the corresponding TLRs in immune cells compared to the absence/low expression or non-functionality of these receptors in tumor cells. However, further studies are required to explore the exact molecular mechanisms involved in this impact. TLR ligands in preclinical cancer models and clinical trials have shown diverse outcomes (Figure 3).

- Imiquimod 5% cream tested as a therapy for high-grade squamous intraepithelial lesions and anal cancer resulted in complete or partial response in 45% and 66% of patients, following 16 and 32 weeks of consequent treatment (van der Snoek et al., 2015)<sup>153</sup>. Imiquimod treatment recruits cutaneous effector T cells from the blood, stimulates IFN- $\gamma$ , granzyme and perforin production, lowers IL-10 and TGF- $\beta$  production and inhibits tonic anti-inflammatory signals within the tumor (Huang et al., 2009)<sup>154</sup>.
- OM-174, a lipid A analogue (region of LPS) that is considered as a TLR2/4 agonist, reduces tumor progression and prolongs survival in mice with subcutaneous B16 melanoma. These effects are mediated by an increase of the absolute numbers and the activation of natural killer (NK) cells and cytotoxic T lymphocyte (CTL) (D'Agostini et al., 2005)<sup>155</sup>. Additionally, in a Phase I study, OM-174 shows a good safety profile for its use in patients with refractory metastatic solid tumor (Isambert et al., 2013)<sup>156</sup>, and it is currently under development as a cancer immunotherapeutic agent (Phase Ib clinical trials). In a model of PROb colon cancer in rats, OM 174 induces complete regression in about 90% of cases. Since OM 174 is not directly toxic to tumor cells, the observed effect involves the host-mediated antitumor reaction (Onier, 1999)<sup>157</sup>.

- Immunomax, a plant-derived TLR4 agonist, eliminates micro-metastatic disease in the post-resection model of 4T1 mouse breast cancer. By stimulating tumoricidal NK cells, Immunomax prolonged overall survival and cured 31% of mice (Ghochikyan et al., 2014)<sup>158</sup>.
- SMP-105, a cell wall skeleton preparation of *M. bovis* BCG, has anti-tumoral effects in a model of subcutaneous murine NSCLC. By activating the NF- $\kappa$ B promoter in a TLR2-dependent manner, SMP-105 leads to a T-helper (Th) 1 type immune response and the proliferation of cytotoxic T lymphocytes (CTL) (Murata M., 2008)<sup>159</sup>.
- Intratumoral injection of acGM-1.8 - a modified polysaccharide that activates TLR2 - suppresses the growth of two murine tumors, S180 sarcoma and B16 melanoma, and demonstrates higher safety than four classical TLR agonists; LPS, MPLA (agonist of TLR4), Poly (I:C)(TLR3) and Pam3CSK4 (TLR1/2). The anti-tumor activity was mediated through a TLR2-mediated switch of macrophages from an M2, pro-tumor phenotype to an M1, anti-tumor phenotype (Feng et al., 2019)<sup>160</sup>.

## 5.2. Inhibition of TLR expression

Small interfering RNA (siRNA) to inhibit TLR expression, mainly on tumor cells, has been tried as a strategy for cancer therapy. Even though such therapy lacks the ability to specifically target tumor cells in vivo, few studies show encouraging results.

- Silencing TLR4 in human prostate cancer cells (PC3) using small interfering RNA (siRNA) results in a dramatic reduction of tumor cell viability, migration and invasion. In a mouse prostate cancer model, siRNA against TLR4 inhibited established tumor growth and survival (Hua et al., 2009)<sup>161</sup>. Similarly, in breast cancer cells and NSCLC cells, silencing TLR4 with siRNA inhibits proliferation and survival of tumor cells and drastically decreases inflammatory cytokine secretion, such as IL-6, TNF- $\alpha$  and IL-8 (Yang et al., 2010<sup>162</sup>; LI et al., 2014<sup>163</sup>).

- Knockdown of TLR2 by intratumoral injections of siRNA drastically reduces the size of human liver tumors transplanted in nude mice (Huang et al., 2012)<sup>164</sup>.

Although targeting TLR expression in tumor cells is beneficial, the lack of specificity and the difficulty to perform intratumoral injections for many tumor types hinders its use in humans.

### **5.3. Blocking the recognition of TLR ligands by TLRs**

Using antibodies to block the interaction between cell surface TLRs and their ligands is an effective strategy in some cancers (Figure 3).

- Activation of TLR2 with a yeast-derived ligand of TLR2, zymosan, promotes head and neck squamous cell carcinoma (HNSCC) organoid formation in an *ex vivo* model of tumor growth, while blockade with anti-TLR2 antibodies inhibits organoid formation. TLR2 blockade also inhibits growth of human xenografted HNSCC tumors in immunodeficient mice (Farnebo et al., 2015)<sup>165</sup>.
- In a mouse model of B16 melanoma, anti-TLR2 antibodies markedly reduce pulmonary metastases and increase the survival of mice by reversing the immunosuppressive microenvironment and restoring anti-tumor CD8+ T cells and M1 macrophages (Yang, 2009)<sup>166</sup>.
- In a model of prostate cancer, anti-TLR4 antibodies inhibit the pro-tumoral effects of LPS that include immune escape, tumor progression, and metastasis by inducing immunosuppressive TGFβ1 and the proangiogenic cytokine VEGF (Pei et al., 2008)<sup>167</sup>.
- Eritoran, that blocks TLR4, abrogates the enhanced migration of human esophageal cancer cells and colorectal cancer (CRC), and hepatic metastases in NSCLC induced by the administration of heat-inactivated *E. coli* and LPS ((Rousseau et al., 2013)<sup>168</sup>; (RY et al., 2011)<sup>169</sup>; (Chow et al., 2014)<sup>170</sup>).

### **5.4. Blocking TLR Signaling**

- TAK-242 (Resatoravid), a selective inhibitor of the TLR4-NF- $\kappa$ B signaling pathway, suppresses inflammatory mediators and inhibits growth, invasion and metastasis of hepatocellular carcinoma (HCC) (Yu et al., 2013)<sup>171</sup> (Figure 3).
- Luteolin, a suppressor of the TRIF signaling pathway, reduces the volume and weight of prostate tumors in a xenograft mouse model, by targeting angiogenesis, reducing cell viability and inducing apoptosis in cancer cells (Pratheeshkumar et al., 2012)<sup>172</sup>. Similarly, Luteolin decreases invasiveness, MMP secretion, and reverses IL-6-induced epithelial–mesenchymal transition in pancreatic cancer cell lines (Huang et al., 2015)<sup>173</sup>.
- Inhibiting TLR7 and TLR9 signaling using a specific antagonist (IRS-954) or chloroquine reduces hepatocellular carcinoma proliferation *in vitro* and inhibits tumor growth in a mouse xenograft model (Mohamed et al., 2014)<sup>174</sup>.

#### 5.5. TLR ligands in therapeutic combinations

Different combinations of TLR agonists and immune checkpoint inhibitors have been investigated in preclinical models and clinical trials (Figure 3).

- A combination of intratumoral injections of the TLR1/2 ligand, Pam3CSK4, and anti-CTLA-4 antibody enhances antitumor immune responses by mediating the depletion of regulatory T cells in a murine melanoma model (Sharma et al., 2019)<sup>175</sup>.
- In murine models of melanoma (B16.F10) and colon adenocarcinoma (MC38), a combination of R848-loaded  $\beta$ -cyclodextrin nanoparticles (CDNPs) and anti-PD-1 synergistically results in tumor shrinkage, a stable and homogenous anti-tumor response, and protects the mice from a tumor rechallenge by triggering anti-tumor memory (Rodell et al., 2018). In colorectal cancer (CRC), a combination of Taxol and R848 results in better control of tumor growth than single treatments and other combinations (Stier et al., 2013)<sup>176</sup>.



- Combining Imiquimod, a TLR7 agonist, with 1-Methyl-d-tryptophan, an IDO inhibitor, or with an iNOS inhibitor induces a Th1 response and slows the growth of established murine tumors in both thymoma (EG7) and colon carcinoma (C26)-bearing mice, while single treatments don't have any therapeutic effects ((Ito et al., 2015a)<sup>177</sup>; (Ito et al., 2015b)<sup>178</sup>).
- Combining IL-10 blockade with imiquimod results in long-term survival of mice with spontaneous breast tumors, while imiquimod alone doesn't show any efficacy (Lu et al., 2010)<sup>179</sup>.
- In a combined chemotherapy phase II clinical trial, patients with treatment-refractory breast cancer chest wall metastases treated with a TLR7 agonist (imiquimod), a TLR4 agonist and chemotherapy (plus albumin-bound paclitaxel) showed disease regression with an overall response rate of 72%, and 92% of adverse events were low grade (1 and 2). However, the responses were short-lived (4-28 weeks) (Salazar et al., 2017)<sup>180</sup>.
- A Phase II study examining the combination of an anti-CTLA-4 monoclonal antibody with the TLR9 agonist (SD-101) in patients with recurrent low-grade B-cell lymphoma, showed that the combination did not constitute a promising therapeutic option (Khodadoust et al., 2015)<sup>181</sup>.

#### **5.6. TLR ligands in cancer vaccine**

TLR ligands have been extensively explored as vaccine adjuvants in preclinical and clinical studies (Figure 3).

- In patients with high-risk myelodysplastic syndrome (MDS), a phase I study investigating an NY-ESO-1 vaccine containing (PolyI:C:LC), TLR3 agonist, showed the induction of an antigen-specific CD4+ and CD8+ T cell immune response associated with a detectable population of CD141<sup>Hi</sup> conventional dendritic cells (Griffiths et al., 2017)<sup>182</sup>.

- A vaccine of Glioma-associated antigen peptides and poly-IC:LC tested in a pilot study for pediatric recurrent low-grade gliomas was well tolerated but showed low immunological and clinical activity in 4 out of 14 kids (Pollack et al., 2016)<sup>183</sup>.
- The safety and tolerability of a DC-based vaccine in combination with (PolyIC:LC) tested in a Phase I study in patients with locally advanced unresectable pancreatic cancer, showed that, despite the good safety of the vaccine, the treatment elicited specific T-cell responses in only 3 of 8 patients, with a median overall survival of 7.7 months (Mehrotra et al., 2017)<sup>184</sup>.
- Oxaliplatin-based chemotherapy administered alone or combined with the poly(A:U), TLR3 agonist, failed to hamper the progression of murine subcutaneous melanoma (B16-OVA). However, a vaccine composed of the antigen (OVA) plus the adjuvant CpG-ODN, TLR9 agonist, given prior to oxaliplatin and poly(A:U) retarded tumor growth (Conforti et al., 2010)<sup>185</sup>.
- Targeted delivery of tumor antigen to DCs, in addition to adjuvants (Poly I:C and R848, TLR3 and 7/8 agonist), by encapsulating them in DC-targeting nanoparticles, enhances the maturation of DCs, the production of immune stimulatory cytokines and the antigen-specific activation of naive CD8+ T cells. Remarkably, such delivery reduces the serum cytokine storm and related toxicity that is associated with administration of soluble TLR ligands (Tacke et al., 2011)<sup>186</sup>. Using biodegradable, polymer microparticles, targeted delivery of triple adjuvant combination (TLR4/TLR7/TLR9) in murine tumor models result in increased antigen-specific antibody titer with an overall balanced Th1/Th2 response (Madan-Lala et al., 2017)<sup>187</sup>. However, evaluating the anti-tumor efficacy of these vaccines in tumor models is yet to come.

### **5.7. Targeted delivery of TLR ligands**

To avoid undesired effects of TLR ligands and to reduce acute toxicities that result from systemic diffusion of soluble TLR-based adjuvants, a new strategy that aims to deliver TLR ligands to the targeted cell type in the TME gives encouraging results (Figure 3).

- Targeted delivery of a 50 bp dsRNA TLR3 agonist (Riboxsol) conjugated to anti-Prostate Stem Cell Antigen (PSCA) single chain antibody - termed “Rapid Inducer of Cellular Inflammation and Apoptosis” (RICIA) – in PSCA-positive bladder cancer xenograft models (HT1376) induce a type I-interferon response and apoptosis of target cells. Although, treatment in immune-deficient mice did not induce adverse effects, tumor growth was only modestly inhibited as compared to controls (Schau et al., 2019)<sup>188</sup>.
- Loading  $\beta$ -cyclodextrin nanoparticles (CDNPs) - that exhibit affinity to macrophages - with R848, an agonist of TLR7/8, alters the tumor immune microenvironment in multiple murine tumor models towards an M1 phenotype, leading to controlled tumor growth (Rodell et al., 2018)<sup>189</sup>.

**Table 3: Summary of TLR-Based Treatments of Cancer in preclinical and clinical trials**

TLR-Based Strategy	Treatment	Cancer Type
Stimulation of the Immune System	Imiquimod	Skin cancers (BCC & SCC)
	OM-174	Melanoma & colon cancer
	Immunomax	Breast cancer
	SMP-105	NSCLC
	acGM-1.8	Sarcoma & melanoma
Inhibition of TLR Expression	siRNA for TLR4	Prostate & breast cancers
	siRNA for TLR2	Liver cancer

Blocking the recognition of TLR ligands by TLRs	anti-TLR2 antibodies	HNSCC & Melanoma
	anti-TLR4 antibodies	Prostate, esophageal & CRC
Blocking TLR Signaling	TAK-242 (Resatoravid)	HCC
	Luteolin	Prostate & pancreatic cancers
	IRS-954 or chloroquine	HCC
TLR Ligands in Therapeutic Combinations	Pam3CSK4 + anti-CTLA-4	Melanoma
	R848 + anti-PD-1	Colon cancer
	Imiquimod + IDO/iNOS inhibitors	Thymoma & colon cancer
	Imiquimod + IL-10 blockade	Breast
	Imiquimod + paclitaxel	Refractory breast cancer
	SD-101 + anti-CTLA-4	low-grade B-cell lymphoma
TLR ligands in Cancer Vaccine	NY-ESO-1 vaccine (PolyIC:LC)	MDS
	Glioma-asso. antigen (poly-IC:LC)	Pediatric gliomas
	DC-based vaccine (PolyIC:LC)	Advanced pancreatic cancer
	OVA vaccine (CpG-ODN)	Melanoma
Targeted delivery of TLR ligands	Riboxol conjugated to anti-PSCA	HT1376 bladder cancer cells
	CDNPs + R848	Multiple murine tumor models

## Future Perspectives

Despite advances in understanding the dual role of TLR signaling in the TME, many questions are yet to be answered. The difference between inflammation that induces tumor progression and inflammation that causes tumor regression is still unclear. Though both exogenous and endogenous TLR ligands are involved in TLR activation in tumor cells, their relative contribution to tumorigenesis remains unknown. In fact, despite the existence of natural TLR ligands, most experiments that investigate the role of TLRs in cancer use synthetic TLR ligands. While DAMPs released from dead tumor cells incite protumoral effects in tumor cells and diverse effects in immune cells, PAMPs from microbiota or exogenous pathogens (bacteria, virus, parasites) can also impact on the TME and tumor progression, especially in mucosal organs. Another intriguing question is why activation of different TLRs lead to different outcomes in the same tumor, even though most TLRs share the same signaling pathways and molecules. The answer could lie in the integration of signals of the different cells in the TME that can sense and respond to the ligands present. The outcome of activating TLR signaling in a given tumor depends on the nature of the cells that succeed to exploit it the most.

Considering that most TLR ligands have a anti-tumoral role when they activate cDCs and NK cells, and an protumoral role when they activate tumor cells, looking for TLR ligands that can preferentially activate DCs and NK cells, while sparing tumor cells is likely to be an effective strategy to enhance the anti-tumor response. Also, targeted delivery of TLR ligands to specific cell types using antibodies is a promising strategy to avoid off-target effects.

Tumor cells express more TLRs than their normal epithelial counterparts. In addition, some studies show that tumor cells can be more successful in sensing TLR ligands, than immune cells in the TME, considering the protumoral outcome of TLR agonist treatment.

The use of synthetic TLR agonists to explore the effect of TLR signaling in the TME impedes the comprehension of the role of the TLRs. Some TLR agonists are not specific to the TLR studied activating multiple TLRs. It is possible that synthetic TLR agonists have a tropism to TLRs expressed in particular

types of cells, which may not mimic the impact of natural DAMPs or PAMPs. This can explain the limited success of TLR agonists in clinical trials despite promising results in preclinical experiments.

TLR signaling can modulate the metabolic program in tumor cells, stromal cells and immune cells. It also regulates metabolites produced by tumor cells, such as cAMP and IDO, which are potent immune suppressors, thus maintaining the tumor-suppressive microenvironment. Identification of selective TLR agonists that specifically inhibit tumor metabolism and/or regulate tumor-derived metabolites is needed for better TLR-based tumor vaccines and therapy.

Since TLR polymorphisms can play important roles in tumorigenesis and influence outcomes of therapeutic interventions, analyzing TLR expression and determining polymorphisms in patients can not only be used as prognostic marker but also opens new avenues for the use TLR ligand-based therapies in personalized medicine.

TLR-mediated reprogramming of T-cell metabolism is a novel and feasible strategy for tumor immunotherapy. Developing effective strategies targeting TLR-mediated metabolic switches to appropriately activate macrophages and DCs in the TME is a promising future therapeutic approach.

Though TLR agonists as a single agent antitumor drug have yielded limited success in clinical trials, combining TLR-based cancer treatment with immune checkpoint inhibitors has given encouraging results supporting growing evidence that combination drugs are the future of cancer therapy.

## **Conclusion**

TLRs are widely expressed in tumor cells, stromal cells and tumor-infiltrating immune cells, are involved in the regulation of tumor pathogenesis and in antitumor immune responses. Increasing evidence strongly indicates that TLRs can be activated in various cells within the TME and can thereby modulate the microenvironment. Like the dragon in the fantasy TV series Game of Thrones, TLR signaling is a war changing weapon that can be exploited either by tumor cells or by immune cells, to control the TME.

A better understanding of the mechanistic effects of TLR signaling in different cells within the tumor-suppressive microenvironment will enable the development of novel strategies via TLR-mediated cancer therapies.

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